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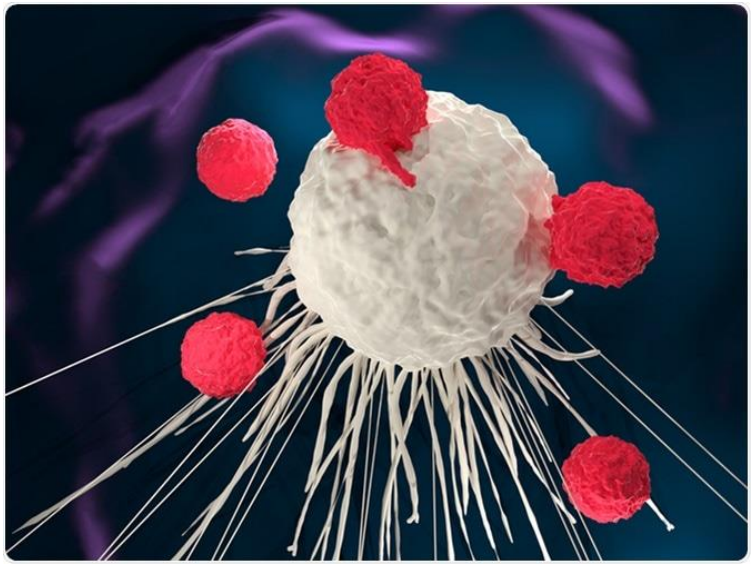
MSc Molecular Biomedicine

Mechanisms of Disease, Molecular and Cellular therapies and Bioinnovation

Delineation of the role of activin-A in T cell-mediated anti-tumor immunity in preclinical models of lung cancer



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Contents

Abstract

Lung cancer is the second most common cancer worldwide, with the highest cancer mortality. It is characterised by great heterogeneity and grouped by two types mainly; the small cell lung cancer (SCLC) and the non-small cell lung cancer (NSCLC), which comprises 85% of all cases. NSCLC exhibits a 5-year survival rate in approximately 15% of patients, with most of the patients presenting metastasis or disease recurrence despite treatment with cytotoxic chemotherapy regimens.

The **immune system** plays a crucial role in eliminating tumor cells. The adaptive immunity, comprising of T and B cells, has a specific immune response. An effective anti-tumor immune response is mainly determined by $CD4^+$ T helper cells that inhibit tumor cell proliferation and survival through the stimulation of cytotoxic $CD8^+$ T lymphocyte mediated cancer cell lysis, the release of effector cytokines and the activation of innate immune responses. However, tumor cells are able to avoid tumor immunosurveillance and often set an immunosuppressive environment, in a procedure called tumor immunoediting.

Activin-A is pleiotropic cytokine that regulates critical biological processes, such as, haematopoiesis, embryonic development, stem cell maintenance and pluripotency, tissue repair and fibrosis. Studies by our group and others have revealed that, activin-A exerts both pro- and anti-inflammatory effects depending on the site of expression, the cell activation status and the immunological context, a status that is being observed in lung cancer too.

Our **hypothesis** is that activin-A has an impact on patrolling T effector cells and as a result, plays a crucial factor in the regulation of lung cancer initiation and progression. The **aim** of our study is to decipher the *in vivo* effect of activin-A on T cell-mediated immune responses in lung cancer and to explore whether activin-A enhances anti-tumor human T cell immune responses in lung cancer patients.

To address our hypothesis regarding the mouse studies, we used CD4*Cre*ER^{T2}/*Acvr1b*^{Fx} mice and inoculated them with LLC cells. In brief, we disrupted *Avcr1b* expression on $CD4^+$ T cells, using tamoxifen. Tumor infiltrated leukocytes were obtained from lung cancer tissues of these mice, and were cultured upon antigen stimulation. $CD4^+$ T cells were isolated from the tumor infiltrating leukocyte fraction and analysed using FACS and ELISA. Pertinent to the human studies, we obtained primary human lung tumor tissue and adjacent lung healthy tissue

from NSCLC patients. Tumor infiltrating leukocytes (TILs), obtained from tissues, were polyclonally stimulated with anti-CD3/anti-CD28 in the presence or absence of recombinant activin-A. In some experiments, $CD4^+$ and $CD8^+$ T cells were isolated from TILs and cultured as mentioned above. These cells, as well as, their culture supernatants were further analysed using FACS, ELISA and LDH cytotoxicity assays.

Our studies revealed that disruption of activin-A's signalling on $CD4^+$ T cells accelerates lung tumor progression and decreases the overall survival of lung-tumor bearing mice (CD4ALK4 KO mice). Notably, $CD4^+$ T cells from CD4ALK4 KO mice produced less IFN-γ and TNF-α, concomitantly with increased levels of the immunoregulatory cytokine IL-10, compared to $CD4^+$ T cells from wild type mice, upon antigenic stimulation. In human lung tissues, activin-A unveils different effects on $CD4^+$ T cells that infiltrate the lung tumors, compared to $CD4^+$ T cells from the adjacent healthy lung tissue. In brief, $CD4^+$ T cells that infiltrated the lung tumors produced increased levels of the effector cytokines TNF-α and IL-2 and decreased levels of the immunosuppressive cytokine IL-10, whereas $CD4^+$ T cells from the adjacent health tissue produce less IFN-γ and IL-2. Moreover, activin-A-treated human lung tumor infiltrating $CD4^+$ T cells exhibited a less exhausted phenotype/profile and acquired a more immunostimulatory capacity towards autologous $CD4^+$ responder T cells. Lastly, $CD8^+$ T cells from the periphery supplemented with culture medium from act-A-$CD4^+$ T cell cultures were more prominent in killing the targeted primary lung cancer cells.

Taking together our data uncover activin-A as an important orchestrator of anti-tumor T cell responses. Importantly, this study will also set the bases for further exploitation of activin-A's utilization as a prognostic tool and a potential target for immunotherapy devoid of potentially harmful side effects, in the setting of human lung cancer.

1. Introduction

1.1 Lung Cancer

Cancer is an accumulation of diseases which are driven by genetic and epigenetic aberrations. Conventionally, when we refer to cancer pathogenesis, we mean the mutations having an impact on proto-oncogenes and tumor suppressors. Nonetheless, that has proven rather simplified, especially following the fact that host immune deregulation is now one of the hallmarks in cancer pathogenesis. Lung cancer is the second most common cancer with the highest cancer mortality rate between both sexes. There is significant lack of early detection methodologies and this leads to poor prognosis. As a result, there is an urgent need to find early screening tools or markers, as well as, improvising treatment strategies to target the disease, as soon as, possible. Consequently, it comes as no surprise that lung cancer is characterised as one of the most widely studied cancers in the field of immune–oncology (Saab et al., 2020). Lung cancer is a major health problem worldwide, with about 2.1 million people being diagnosed every year and 1.8 million dying. Lung cancer levels are rising globally, although, in some Western countries a decrease in rates among males is observed. According to the National Cancer Institute (NCI) lung cancer is estimated to cause 23.5% deaths compared to all other cancer deaths ("National Institutes of Health (NIH) - Turning Discovery into Health," n.d.). Additionally, the American Cancer Society predicts that 1,806,590 new cancer cases and 606,520 cancer deaths would occur in the United States by 2020 (Siegel, Miller, & Jemal, 2020).

1.1.1 Types of lung cancer

Lung cancer is characterized by great heterogeneity either clinically or pathologically. However, it is grouped by two types mainly; the non-small cell lung cancer (NSCLC), which represents about 85% of lung cancers and the small cell lung cancer (SCLC), which represents about 15% of lung cancers. Most lung cancer types are highly associated with tobacco smoking, while adenocarcinoma is also found in never-smoker patients. The classification of lung cancer is based on morphological, immunohistochemical (IHC) and molecular characteristics of the samples analysed. The determination of stage is based on the 8th edition of the TNM (TNM Classification of Malignant Tumors) Classification of Lung Cancer, developed and maintained by the Union for International Cancer Control (UICC). T describes the size of the original primary tumor and whether it has invaded in nearby tissue, N describes nearby (regional) lymph

nodes that are involved and M describes distant metastasis (spread of cancer from one part of the body to another). Tumor stage is extremely important for prognosis and treatment [(Rodriguez-Canales et al., 2004), (Pirker, 2020)]. Neuroendocrine features, characterized by microscopic morphology and immunohistochemistry, are indications of the high-grade SCLC and large-cell neuroendocrine tumors and of intermediate low-grade carcinoid tumors.

NSCLC can be easily distinguished histopathologically and clinically from SCLC (Bhattacharjee et al., 2001). Evidence, given by molecular profiling of the NSCLC, permits further classification of this cancer cell type into adenocarcinoma (ADC) and its variants, squamous cell carcinoma (SqCC) and large-cell lung carcinoma (LCLC) (Rodriguez-Canales et al., 2004). The majority of NSCLC is represented by ADC (38.5%), a malignant type of epithelial tumor with glandular differentiation and mucin production. Mucin can be easily detected by mucin staining using markers, such as, mucicarmin, pneumocyte marker expression like napsin A or thyroid transcription factor 1 (TTF1). ADC is typically found at the periphery of the lungs and can depict varied histological patterns. These patterns can be mixed in the same tumor consisting of lepidic, acinar, papillary, micropapillary and solid patterns, with the last two indicating a more aggressive phenotype (Rodriguez-Canales et al., 2004). Gene expression patterns that characterize adenocarcinoma include thyroid transcription factor (TTF1), associated with the regulation of surfactant gene expression, surfactant proteins B and C and pronapsin A, a protease involved in surfactant pro-protein processing. The latter are correlated very strongly with the expression of TTF1 and surfactant A1, a cluster of genes that comprise cyclin-dependent kinase inhibitor p16 and many other genes (Garber et al., 2001).

SqCC represents the 20% of all lung cancers and is mainly detected in a central location, arising in a main or lobar bronchus. As defined by the World Health Organization (WHO), SqCC is as a malignant epithelial tumor that either shows keratinization and/or intercellular bridges or expresses IHC markers of squamous cell differentiation. Keratinization is the major feature of this type of lung cancer, although poorly differentiated SqCC can have pseudoglandular appearance, a characteristic that can be confused with pseudosquamous features of poorly differentiated adenocarcinomas. SqCC identification by IHC includes markers of squamous cell differentiation, like p40 or 063 and cytokeratins 5/6. A discrete unit is the basaloid squamous cell carcinoma, a weakly differentiated malignant tumor with no morphological features of squamous cell differentiation that can easily be confused with SCLC. However, it is positive for immune

markers of squamous cell differentiation including p40, p63, and cytokeratins 5/6, whereas TTF-1 is negative.

LCLC represents only the 2.9% of all lung cancers. It is described as an NSCLC carcinoma, which does not show histological or IHC evidence of squamous cell, glandular, or small-cell differentiation. The diagnosis of LCLC requires extensive sampling of a surgical resected specimen after ruling out SqCC, ADC or SCLC, and therefore, it cannot be made on biopsies or cytology samples. There is also no detection of mucin by mucin staining. IHC evidence shows that LCLC may be positive to cytokeratins, but negative to TTF-1 and p40 (Rodriguez-Canales et al., 2004). SCLC in never or former light smokers is rare and is potentially a definite disease entity comprised of carcinomas that harbour oncogenic driver mutations and are morphologically and/or clinically mimicking SCLC.

SCLC tumors consist of small, round-shaped cells with a meagre cytoplasm, fine granular nuclear chromatin and recurrent nuclear shaping. The diagnosis is mainly based on histological characteristics. Nevertheless, morphologic similarities with other types of carcinomas, including large cell neuroendocrine carcinoma, basaloid squamous cell carcinoma, and “small round blue cell tumors” including lymphoma, poorly differentiated melanoma and sarcomas, may contribute to diagnostic errors and, as a result, the use of IHC to confirm the diagnosis is necessary. Some neuroendocrine (NE) markers, such as, NCAM (CD56), chromogranin A, synaptophysin and INSM1 are characteristically expressed in SCLC but are not exclusively specific. Recent studies suggest that SCLC can be heterogeneous, consisting of both NE and non-NE cells, which may need an alternative treatment approach. Recently, several researchers proposed that SCLC without expression of classical NE markers can be defined by differential expression of YAP1 and POU2F3, highlighting the biologic heterogeneity of this morphologic entity and suggesting a need for more comprehensive tumor profiling to better understand this heterogeneous disease (Ogino et al., 2020).

1.1.2 Molecular profile

During the past years, new molecular alterations have been identified in NSCLC including oncogenes and tumor suppressor genes, several of which could be used as novel predictive biomarkers or targets for cancer therapy. An illustration of the relative frequencies of molecular targets in NSCLC is shown in Fig. 1. The majority of molecular alterations are found

in genetic locus of the Epidermal Growth Factor Receptor (*EGFR*), reaching an overexpression percentage of 40-80% in NSCLC and other epithelial cancers. The encoded protein is a transmembrane glycoprotein which is a member of the protein kinase superfamily. Mutations in this gene elevate the kinase activity of *EGFR*, resulting in hyperactivation of downstream pro-survival signalling pathways. *EGFR* mutations are frequently found in tumors of female never-smokers with the adenocarcinoma subtype. However, *EGFR* mutations can also be found in patients with other clinicopathologic features, comprising of past and current smokers. Moreover, rearrangements in the genetic locus of Anaplastic lymphoma kinase (*ALK*) have been identified in nearly 3 to 7 % of lung tumors. The more common *ALK* fusion variants in the NSCLC include parts of it with the echinoderm microtubule-associated protein-like 4 (*EML4*) gene. *EML4-ALK* is an abnormal fusion gene that encodes a cytoplasmic chimeric protein with constitutive kinase activity. *EML4-ALK* fusions are more frequently found in younger patients who have never smoked or who have a history of light smoking and in patients with adenocarcinomas with acinar histology and with signet-ring cells.

Human Epidermal Growth Factor Receptor 2 (*HER2*) belongs to the EGFR family and has a significant role in cell growth, differentiation, and survival. *HER2* is a proto-oncogene that encodes a member of the epidermal growth factor (EGF) receptor family of receptor with tyrosine kinase activity. The HER2 protein has no ligand-binding domain of its own and as a result is unable to bind growth factors. Nevertheless, HER2 does bind forcefully to other ligand-bound EGF receptor family members to form a heterodimer, stabilizing ligand binding and increasing kinase-mediated activation of downstream signalling pathways. Activating mutations in the tyrosine kinase domain of HER2 have recently been stated in less than 5 % of NSCLC. ROS Proto-Oncogene 1 (*ROS*) is part of the tyrosine kinase insulin receptor gene family. It is a type I integral membrane protein and its tyrosine kinase activity may function as a growth or differentiation factor receptor. *ROS1* reorganisations result in constantly active fusion proteins and are reported in approximately 1–2 % of NSCLC. Ret Proto-Oncogene (*RET*) encodes a tyrosine kinase which is implicated in cell proliferation, migration and differentiation. *RET* rearrangements, most frequently, with the kinesin family member 5B (*KIF5B*) were found in approximately 1–2 % of NSCLC.

Neurotrophic tyrosine kinase receptor, type 1 (NTRK1), also called tropomyosin receptor kinase A (TrkA) or high-affinity nerve growth factor receptor, is a protein encoded by the gene

NTRK1. The role of NTRK1 is to control the cell growth and differentiation through the MAPK, phosphatidylinositol 3-kinase (PI3K) and PLC-c pathways when activated by the nerve growth factor (NGF) ligand. *NTRK1* fusions have been found in 3.3 % of cases with ADC histological type (Rodriguez-Canales et al., 2004). *MET* (member of the receptor tyrosine kinase family of proteins) gene encodes a receptor with tyrosine kinase activity. MET protein transduces signals from the extracellular matrix into the cytoplasm by binding to hepatocyte growth factor/HGF ligand. It regulates physiological processes, such as, proliferation, motility, invasion and survival. *MET* alterations in NSCLC adenocarcinoma fluctuates from 2 to 20 %. Kirsten Rat Sarcoma (*KRAS*) is another oncogene that encodes the KRAS protein and is involved in regulating cell division. KRAS is part of the RAS/MAPK signalling pathway, and it transmits signals from the outside of the cell to the cell's nucleus. Activating *KRAS* gene point mutations have been reported in approximately 15 to 25 % of patients with lung adenocarcinoma. B-Raf (*BRAF*) is also a proto-oncogene and encodes BRAF, a serine/threonine kinase that participates in transmitting chemical signals from the cell's outside to the cell's nucleus. The protein is part of the RAS/MAPK signalling pathway, an important molecular cascade that regulates vital cell functions, such as, proliferation, differentiation, migration and apoptosis. Mutations in the genetic locus of BRAF have been found in 1–4% of lung cancers and especially, in adenocarcinomas. Finally, there are other, less frequent, molecular alterations found in genes encoding Neuroblastoma RAS Viral (V-Ras), Oncogene Homolog (*NRAS*), v-AKT Murine Thymoma Viral Oncogene Homolog 1 (*AKT1*), Mitogen-Activated Protein Kinase 1 (*MAP2K1*) and Phosphatidylinositol-4,5-Bisphosphate 3-Kinase, Catalytic Subunit Alpha (*PIK3CA*) that are found in patients with lung cancer [(Collisson et al., 2014), (Rodriguez-Canales et al., 2004)].

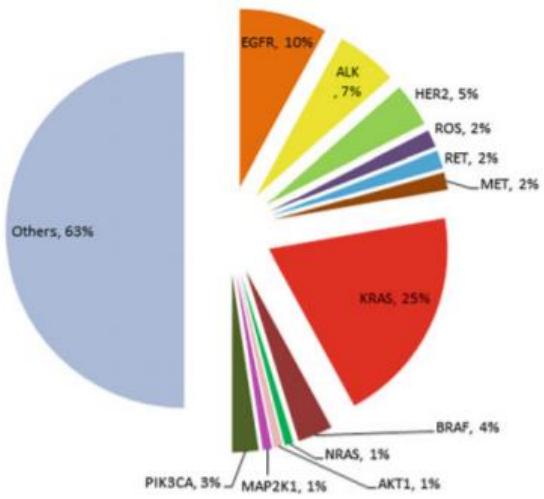

Fig. 1: *Representation of the relative frequency of the main molecular targets in NSCLC (Rodriguez-Canales et al., 2004).*

SCLC has a strong association with tobacco consumption. Nonetheless, among SCLC patients, 2-3% of them are never smokers, something that may be caused by molecular aberrations. There are several studies that try to reveal these molecular alterations and point out the exact responsible genes that may be used as potential targets for therapy, as SCLC is one of the most challenging cancers to be cured. Coexistent inactivation of retinoblastoma protein (*RB1*) and Tumor protein p53 (*TP53*) is nearly universal in SCLC (George et al., 2015). *RB1* codes the protein pRB, which acts as a tumor suppressor. It also interacts with other proteins to control cell survival, apoptosis and differentiation. *TP53* codes a protein called tumor protein p53, which is as a tumor suppressor. Other suspected driver mutations have also been detected in several genes including *PTEN*, *SLIT2*, *EPHA7*, *FGFR1*, *BRAF*, *KIT*, *PIK3CA*, *CREBBP*, *EP300* and *MLL* (Peifer et al. 2012, George et al. 2015). However, these driver mutations appear in low frequency and are not suitable for clinical targets. One study by Cardona et al. revealed that *EGFR*, *MET* and *SMAD4* (codes for SMAD4 protein which is part of the TGF-β signalling pathway) are more frequently mutated in never smokers. On the other hand, *RB1*, *CDKN2A* (cyclin dependent kinase inhibitor 2A) which codes tumor-suppressors proteins that also stop cell division in older cells and *CEBPA* (CCAAT enhancer binding protein alpha), a tumor-suppressor, are more frequent in smokers (Cardona et al. 2019). *NRAS*, together with *KRAS* and *HRAS* belongs to the RAS oncogene family and encodes a highly conserved small GTPase which regulates cell growth, proliferation and differentiation. *NRAS* is mutated in roughly 1% of lung cancers. Ogino et al. showed that other molecules, such as, *BRCA1*, a tumor-suppressor and *ATM* that codes the ATM serine/threonine kinase which controls the growth and division rate of cells are also implicated in lung cancer (Ogino et al., 2020).

1.1.3 Prognosis and Treatment

Tumor stage is important for prognosis and treatment. In general, 15-20% of patients have a survival rate of five years. Among patients with NSCLC, these rates reach 90% for stage 1A1 but rates get less than 10% for stage 4. Among patients with SCLC, the rates are about 30% for limited disease and below 10% for extensive disease. Treatment of patients with lung cancer is relied on multidisciplinary co-operation based on surgery, radiotherapy, systemic treatments (chemotherapy, targeted therapies and immune checkpoint inhibitors) and supportive care.

Treatment is determined by tumor characteristics, tumor stage and other factors related to patients (Pirker, 2020).

NSCLC

According to the American Cancer Society, the treatment options for NSCLC are based mainly on the stage of the cancer, but also on other factors, like a person's overall health and lung function.

Stage 0: NSCLC is restrained only in the inner layer of airways and has not been expanded deeper into the lung tissue or other areas. As a result, surgery alone is the cure, without the need of radiation therapy or chemotherapy.

Stage I: Treatment at this stage is again restricted to surgery, either by lobectomy; taking out the lobe of the lung with tumor or by segmentectomy; taking out a smaller piece of the lung. In some rare cases, additional chemotherapy or radiation therapy is added to lower the risk of recurrence.

Stage II: Healthy patients undergo surgery, either by lobectomy or sleeve resection or sometimes by removing the whole lung (pneumonectomy). Usually, surgery is followed by chemotherapy and radiation. Patients with serious medical problems may get only radiation therapy as their main therapy.

Stage IIIA: Treatment at this stage is a combination of chemotherapy, radiation therapy and/or surgery. Occasionally, people start with chemotherapy and radiation and only if it essential, they proceed to surgery. In cases that nothing of the above is tolerable by patients, immunotherapy with pembrolizumab (anti-PD1 humanized monoclonal antibody) may be defined as first treatment.

Stage IIIB: NSCLC has spread to the nearest lymph nodes or even to important structures of the chest. As surgery is not the most effective treatment, chemotherapy and radiation therapy once more, are the additional methods. If cancer insists after chemoradiation, the immunotherapy drug durvalumab could be given for holding the cancer stable.

Stage IV: NSCLC at this stage is widely spread when diagnosed and is difficult to cure. Treatment options are restricted to the spread site, the number of tumors and the overall health state of the patient. Patients with good overall health state can be treated, but not cured, by surgery, chemotherapy, targeted therapy, immunotherapy and radiation therapy. If cancer has

spread to one other site, surgery, radiation therapy or chemotherapy are the treatment solutions, depending on the spread site. On the contrary, if cancer has spread widely, targeted therapy is the first treatment. Targeted therapy depends on the mutated genes of the cancer cells, such as, *EGFR*, *ALK*, *ROS1*, or *BRAF* genes. More specifically, the treatments are:

- for *ALK* gene mutation, an AKL inhibitor
- for *EGFR* gene mutation, anti-EGFR drugs
- for *ROS1* gene mutation, drugs like crizotinib or ceritinib
- for *BRAF* gene mutation, a combination of the targeted drugs dabrafenib (Tafinlar) and trametinib (Mekinist)
- for NTRK gene mutation, larotrectinib or entrectinib drugs
- for high levels of PD-L1 protein tested, combined pembrolizumab (Keytruda) or atezolizumab (Tecentriq) with chemotherapy, or pembrolizumab alone

For most other cancers that have spread, chemotherapy is usually at least part of the main treatment. Sometimes it might be used in combination with other types of drugs ("Treatment Choices for Non-Small Cell Lung Cancer, by Stage," n.d.):

- The immunotherapy drug pembrolizumab (Keytruda) might be used along with chemotherapy.
- For people with non-squamous cell NSCLC, the targeted drug bevacizumab (Avastin) might be given along with chemotherapy. Some people with squamous cell cancer might still be given bevacizumab, as long as the tumor is not close to large blood vessels in the center of the chest.
- The immunotherapy drug atezolizumab (Tecentriq) might be used along with bevacizumab and chemotherapy in people with non-squamous cell type of NSCLC.
- An option for people with squamous cell NSCLC is to get chemotherapy along with the targeted drug necitumumab (Portrazza).

SCLC

Small cell lung cancer is very responsive to chemotherapy and radiotherapy. Despite initial responses though, relapse is common and most patients in the end give in to the disease (Cooper & Spiro, 2006). For SCLC, a five-year survival rate of 4-5% is estimated and the common therapy treatment over the past decades is the platinum doublet chemotherapy (Ogino et

al., 2020). Patients with advanced stage SCLC are treated with platinum, coupled with the drug etoposide in combination with an immune checkpoint inhibitor. This alteration from chemotherapy to chemoimmuntherapy is based on results from two phase 3 clinical trials where patients with advanced stage SCLC exhibited increased overall survival when they received chemotherapy plus atezolizumab or durvalumab compared to chemotherapy alone. Still, patients with limited stage SCLC receive as first therapy cisplatin along with etoposide and thoracic radiotherapy. Patients are also considered for prophylactic cranial irradiation. If the cancer progresses, topotecan is considered as the standard therapy (Pirker, 2020).

1.2 The immune system in malignancies

The role of the immune system is to recognise, control and eliminate malignant cells, either microbes or mutated self-cells, while maintaining homeostasis for normal tissue. This is achieved by components of innate and adaptive immune system, which interact with each other. First, components of the innate immune system are activated following antigen exposure, produce non-specific cytolytic molecules and orchestrate the activation of the adaptive immune system, composed of T and B cells, which is a specific immune response [(Liu & Zeng, 2012), (Carbone et al., 2015)].

The up-to-date model proposes that activation of an adaptive host defence, mediated by the components of the immune system, relies on “three-signal activation” between a T cell and an antigen presenting cell (APC). The “signal one” interaction takes place between a CD8 or CD4 molecule on the surface of the cytotoxic and helper T cells, respectively, and the non-peptide binding regions on the major histocompatibility complex proteins (MHC) class I or II molecule, correspondingly. The T-cell receptor (TCR) also recognises the antigen presented on the APC’s MHC molecule. The second signal includes the binding of CD28, the classical co-stimulatory molecule on T cells, to either CD80 or CD86 on APCs. This signal then activates the third signal, which is the production of an effector cytokine by the APC. The three signals permit full activation of T cells and promote their clonal expansion. The three-signal is also the underlying mechanism for our growing knowledge of tumor immune biology, immune evasion, and the clinical highlights in cancer immunotherapy (Hanahan & Weinberg, 2011).

Tumor immunosurveillance is defined by the ability of immune cells to recognise and destroy cancerous cells (Aranda et al., 2018). Regardless of tumor immunosurveillance, tumors

find ways to develop in the presence of an operative immune system, in a dynamic process called immunoediting. The tumor immunoediting procedure is divided into three faces, elimination, equilibrium, and escape (Fig. 2). The elimination phase is the same as described in tumor immunosurveillance. The elimination phase can be complete, so all tumor cells are eradicated, or incomplete, when only a portion of tumor cells is eliminated. In the case of partial tumor elimination a temporary state of equilibrium can be developed between the immune system and the growing tumor. Throughout this period, tumor cells either remain dormant or continue to expand, acquiring further changes (such as, DNA mutations or changes in gene expression), that can moderate the tumor-specific antigens. While this process continues, the immune system utilizes a selective pressure by eliminating susceptible tumor clones where possible and this pressure is enough to control tumor progression. However, if the immune response is still unable to eliminate the tumor entirely, the process results in the selection of tumor cell variants that are able to resist, avoid, or suppress the antitumor immune response, resulting in the escape phase. During the escape phase the immune system is no longer able to control tumor growth, and a gradually developing tumor results (Swann & Smyth, 2007).

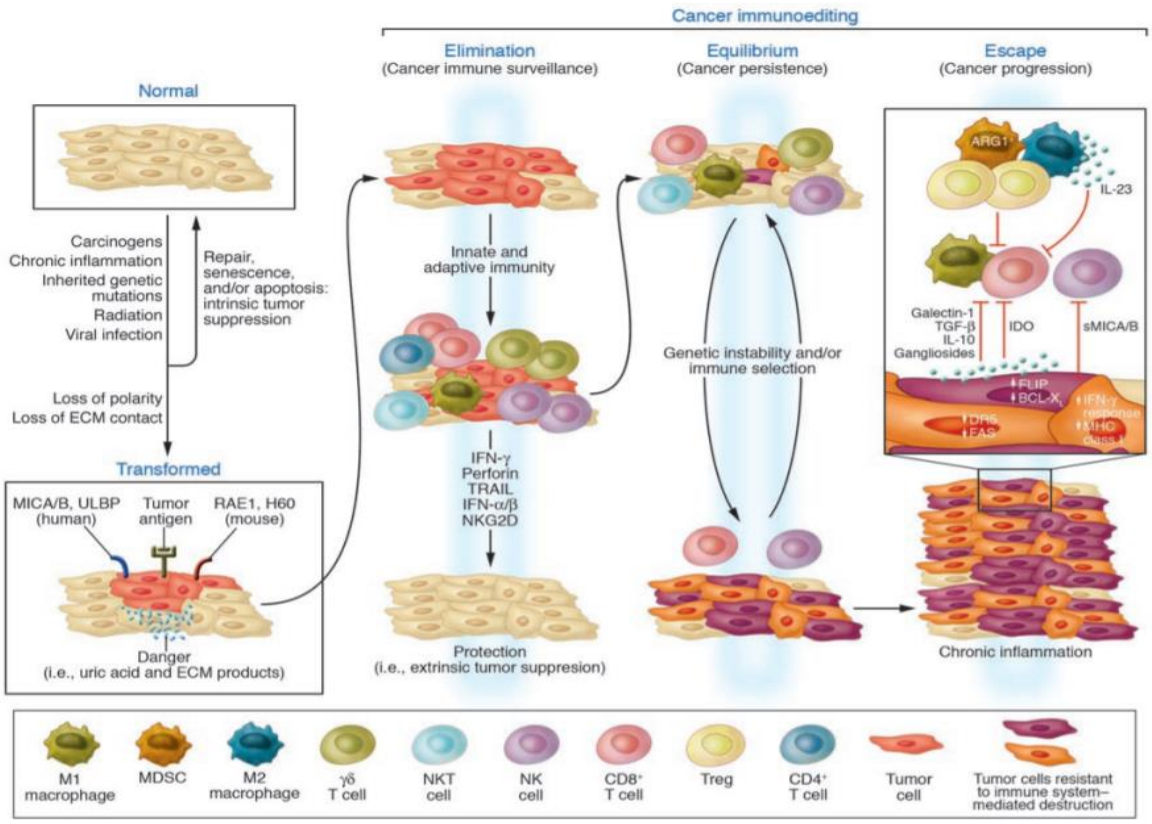

Fig. 2: *Cancer immunoediting is considered a process of 3 phases: elimination, or cancer immune surveillance; equilibrium, a phase of tumor dormancy where tumor cells and immunity enter into a dynamic equilibrium that keeps tumor expansion in check; and escape, where tumor cells emerge that either display reduced immunogenicity or engage a large number of possible immunosuppressive mechanisms to attenuate antitumor immune responses leading to the appearance of progressively growing tumors (Swann & Smyth, 2007).*

Tumor immunosurveillance is mediated by both the innate and adaptive immune system, although the latter plays a more prominent role (Klingemann, 2014), due to the importance of cancer-unique antigens. One such group is known as tumor associated antigens (TAA), which can be found in healthy cells, but are also overexpressed in cancer cells [(Y. Li et al., 2018), (Barfoed et al., 2000)] (CD19, PRAME, MAGE, ERBB2, p53, and L2A5) [(Barfoed et al., 2000), (Kessler et al., 2001), (Wang et al., 2012), (Ilyas & Yang, 2015), (Lian et al., 2018), (Weir et al., 2014), (C. Guo et al., 2013), (Vansteenkiste et al., 2016), (Butts et al., 2014), (Le et al., 2015), (Oliveres et al., 2018)]. Another group of antigens, called tumor specific antigens (TSA), are unique to tumors and are a result of accumulation of mutations in a tumor cell line [(Y. Li et al., 2018), (Kelderman & Kvistborg, 2016)]. Neoantigens are a special category of this

group and are present on MHC molecules (Sivanandam et al., 2019) (Fig. 3). It is important to mention that neoantigens are unique to the patient and not to the tumor itself. As a result, tumor neoantigens are appropriate targets for personalizing cancer vaccines and adoptive cell therapy (ACT) [(Lee et al., 2018), (Smits & Sentman, 2016)]).

1.3 Immune Checkpoints

Immune checkpoints consist of receptors on the surface of activated T cells that can change the ability of the immune system to recognize malignant cells [(Wei et al., 2018), (Shi et al., 2019)]. Their role is to guarantee immune homeostasis and self-tolerance in normal conditions, but they also contribute to tolerance against tumor cells, by blocking the immune activation [(X. Li et al., 2018), (Jung & Choi, 2013)]. In malignancy, clinical inhibition of these checkpoints, blocks their inhibitory action and re-activates the immune system to eliminate tumor cells. Such immune checkpoints are LAG-3, TIM3, TIGIT, as well as programmed cell-death receptor 1 (PD-1) and cytotoxic T lymphocyte associated protein- 4 (CTLA-4).

How tumor cells mediate the inhibition on T cell activation and thus on anti-tumor immunity, can be described for CTLA-4, a B7 ligand and inhibitory homolog of CD28 (Ott et al., 2017) (Fig. 3). After antigen presentation, CTLA-4 is upregulated [(Ott et al., 2017), (Wei et al., 2018)]. It competes with CD28 for binding to CD80 (B7-1 ligand) or CD86 (B7-2 ligand), and thereby lessens the T cell-mediated response, thus the malignant cells avoid the immune destruction [(X. Li et al., 2018), (Ott et al., 2017)]. Under physiological situations, CTLA-4's expression on regulatory T cells (T regs) plays an important role in immune tolerance, a regulatory mechanism that prevents the formation of self-reactive T cells, which can lead to autoimmune diseases in the host (Knickelbein et al., 2008).

PD-1 engagement with its ligands (PD-L1 or PD-L2) represents a main immune checkpoint axis that controls self-tolerance and contributes to the maintenance of immune homeostasis (Sharma et al., 2019), [(Okazaki & Honjo, 2007)]. PD-1 is often expressed on the surface of activated T cells, macrophages, B cells, and NK cells. Its expression is upregulated in response to chronic antigen exposure as seen in cancer (Fig. 3). This upregulation indicates immune adaptation to chronic stimulation and therefore leads to the decrease of the immune response (Simon & Labarriere, 2018). Furthermore, it has been shown that PD-1 expression is elevated upon the secretion of type I and type II IFNs from the tumor stroma [(Alsaab et al.,

2017), (Shi et al., 2016)]. PD-1's engagement with its ligand PD-L1, found on tumor cells, TILs, APC, endothelial, and epithelial cells, further lessens the apoptotic pathway, and induces anergy, as well as, T cell depletion [(X. Li et al., 2018), (Buchbinder & Desai, 2016), (Pico de Coaña et al., 2015), (O'Neill & Pearce, 2016), (Zhu et al., 2017), (Meyers et al., 2018)]. The expression of PD-L2, another ligand for PD-1, is limited to dendritic cells (DCs) and macrophages [(X. Li et al., 2018), (O'Neill & Pearce, 2016)]. Accumulating knowledge suggests that lung tumors overexpress the immunosuppressive protein, PD-L1, and inhibiting this pathway has led to durable benefit in a subset of advanced-stage NSCLC patients [(Meyers et al., 2018), (Azuma et al., 2014)].

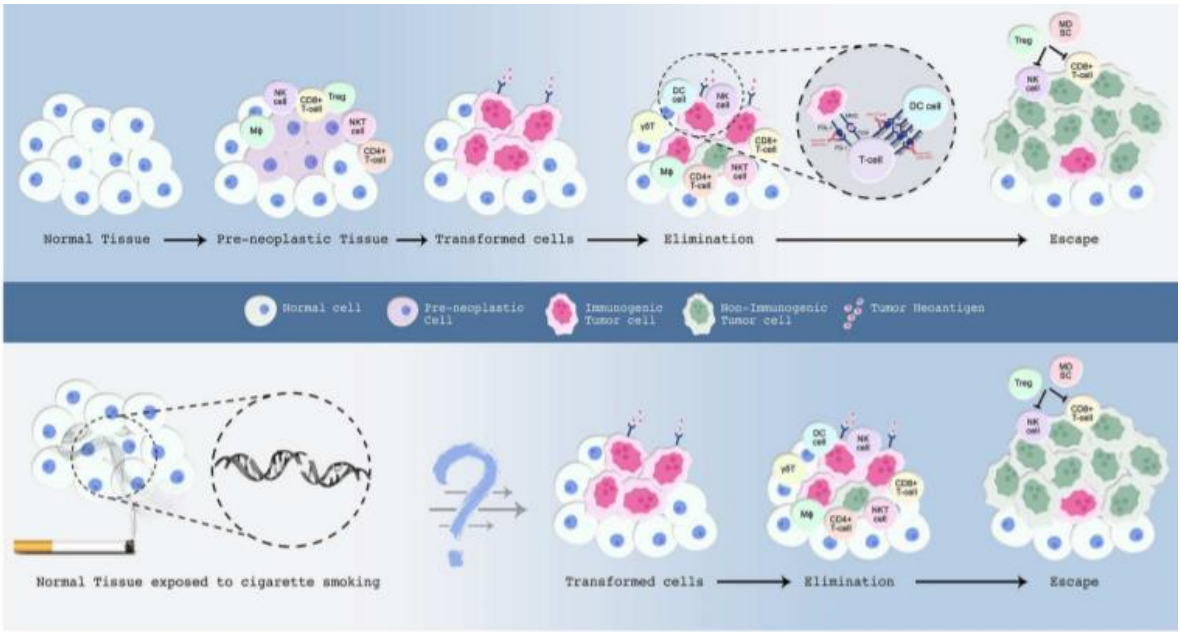

Fig. 3: *A proposed model for the malignant transformation of normal tissue with emphasis on the immune microenvironment. The events underlying this process are explained in the text. Normal cells are in blue; preneoplastic cells in violet; transformed cells in pink; and malignant cells in green. Upper panel: Normal cells accumulate somatic mutations in driver genes leading to the formation of premalignant cells. Those preneoplastic cells attract the immune system, wherein cells from both the innate and adaptive immune system infiltrate the tissue. Certain tumor cells evolve several mechanisms to evade host immune-mediated surveillance and destruction. Clinical inhibition of immune-checkpoints blocks checkpoint inhibitory action and re-activates the immune system to launch an attack on tumor cells. Lower panel: Smoking induces an extensive mutational repertoire leading to the formation of transformed cells. Many of the immune molecules and cells that participate in the elimination phase*

have been characterized, but future work is required to determine their exact sequence of action. In addition, further studies are warranted to understand the sequence of events that render a subset of smokers more prone to develop lung cancer compared to others who do not develop this malignancy throughout their lifetime (Saab et al., 2020).

1.4 Immunotherapies targeting lung cancer

There has been a progression in conventional and targeted therapies across different types of cancer. However, lung cancer has still low survival rate, mostly because of the advanced stage at the point of diagnosis. US Food and Drug Administration's (FDA) has approved several immunotherapies, some of which are first-line therapies, and are described below (Saab et al., 2020).

1.4.1 Adoptive Cell Therapy

Adoptive cell therapy (ACT) is a "living" treatment because the administered cells can proliferate *in vivo* and maintain their antitumor effector functions. In ACT, large numbers of antitumor lymphocytes (up to 10^{11}) can be readily grown *in vitro* and selected for high-avidity recognition of the tumor, as well as, for the effect or functions needed to cause cancer relapse. *In vitro* activation allows such cells to be released from the inhibitory factors existing *in vivo* (Rosenberg & Restifo, 2015). This is based on the fact that proliferation of tumor-reactive lymphocytes is made possible when carried out away from the suppressive effects of the tumor microenvironment (TME). Most of all lymphocytes used are tumor infiltrating lymphocytes (TILs) which infiltrate the TME, detect cancer antigens and release pro-inflammatory cytokines as a response. TILs extracted from surgically resected tumors are cultured *ex vivo*, screened for the best antitumor activity and expanded before reinfusion (Saab et al., 2020). The ability of TILs to trigger tumor regression is enhanced by administering chemotherapy to patients prior to TIL infusion, which eliminates immune cells that mediate tolerance, while increasing the levels of homeostatic cytokines like IL-15, IL-17, and perhaps IL-21 (Cohen & Vokes, 2001).

1.4.2 Adoptively Transferred Tumor Reactive T Cells

Adoptive transfer of TILs that are genetically engineered to respond to tumor-specific mutational epitopes is also used for the enhancement of anti-tumor immunity (Rosenberg & Restifo, 2015). In more details, peripheral T cells are engineered to express TCRs against

specific tumor neo-antigens (tumor specific antigens) in highly mutated tumors, such as, melanoma and lung cancer [(Carreno et al., 2015), (Kreiter et al., 2015), (Linnemann et al., 2015), (Lu et al., 2014), (Ott et al., 2017), (Robbins et al., 2013), (Nienke van Rooij et al., 2013)]. Many potential TCR targets are currently being investigated, as part of the engineered T cell treatments in clinical trials for lung cancer, including NSCLC (NCT03778814) (Thomas et al., 2018). A main limitation in the mechanisms of TCR engineered TILs, is the MHC recognition, as in many tumors there is a down-regulation of the expression of MHC molecules. In order to surpass this, chimeric antigen receptors (CAR) have been discovered (Gauthier & Yakoub-Agha, 2017). CARs are artificial receptors that boost T-cell antitumor effector activity (Zeltsman et al., 2017). A major disadvantage for solid tumors is the complex nature of the solid TME that restricts the efficacy of CAR-T cell therapy and makes it difficult in finding ideal target-antigens that are highly and selectively expressed on cancer cells, but are less or not expressed on normal cells. Moreover, except from TILs, natural killer (NK) cells have been recently introduced as the new CAR-engineered cells. There are significant advantages that CAR-NK cells possess over CAR-T cells, reported in several pre-clinical reports, and those are: decreased risk of overexpansion in patients, release of safer cytokines thereby reducing/eliminating the risk of life the threatening cytokine release syndrome (CRS) (the most known and severe side effect of CAR-T therapy) and avoiding the need for tumor-specific surface receptors and autologous production (Klingemann, 2014).

1.4.3 Cancer Vaccines

Cancer vaccines target tumor-specific or tumor-associated entities and enhance T cell, or B cell-mediated antitumor response, urging the microenvironment to attack the tumor. Tumor cell vaccines could be either autologous, that is produced from the patient's tumor cells, or allogeneic, that is derived from human tumor cell lines. The vaccines are sorted into different categories, depending on what they target, such as, cell-based vaccines (whole tumor vaccines), genetic vaccines (DNA vaccines), protein vaccines, bacterial vaccines, small molecule vaccines, and DC-based vaccines (Weir et al., 2014). Several tumor vaccines are now being assessed in clinical trials for lung tumors that target mostly antigens or proteins, specific or highly abundant in lung cancer. Some of these are 5T4, CEA, mesothelin, survivin, NY-ESO-1, telomerase, WT1, EGFR pathway proteins, along with personalized neoantigens and tumor-associated antigens (TAA) [(Oliveres et al., 2018), (Maemondo et al., 2010), (Grah et al., 2014)].

1.4.4 Oncolytic Viruses

Oncolytic viruses (OV) are DNA or RNA viruses that are able to replicate and kill cancer cells in a targeted and specific way, especially since they lack virulence against non-malignant cells [(Bonaventura et al., 2019), (Cattaneo et al., 2008)]. Oncolytic viruses have been tested in pre-clinical and clinical trials by intratumoral administration of viral particles delivered locally to the tumor (Garofalo et al., 2018). Cancer cells infected with OVs self-destruct attract immune cells, such as, TILs or neutrophils leading to the production of inflammatory mediators, consequently eliminating the main tumor and potentially other tumors within the body. OVs are also able to induce viral infection cascades known to elicit a type 1 interferon response thereby stimulating cytokine release, cancer cell lysis, and apoptosis [(Dyer et al., 2019), (Musella et al., 2017)]. The first OV that was approved by the FDA was Imlygic, a herpes simplex virus I modified to preferentially kill cancer cells, which was approved in 2015 for the treatment of advanced melanoma [(Sivanandam et al., 2019), (Pol et al., 2016)].

1.4.5 Targeted antibodies

Targeted antibodies are antibodies modified to identify particular cancer cell antigens. Along with cancer-specific monoclonal antibodies (mAb), two effective modifications, antibody-drug conjugates (ADCs) and bi-specific T cell-engaging antibodies (BiTEs) are being evaluated as anti-cancer immunotherapies [(Yu & Liu, 2019), (Smits & Sentman, 2016)]. ADCs are highly efficient constructs of tumor-specific mAbs equipped with anti-cancer drugs which are effective once internalized by a tumor cell (Yu & Liu, 2019). BiTEs provide boosted efficacy and safety by concurrently binding a cancer cell antigen and the CD3 of T cells, thus leading the host immunity toward a T cell–driven cytotoxic antitumor immune response (Smits & Sentman, 2016). Antibody targets under assessment in lung cancer clinical trials include: cMET, DLL/Notch, FGF/FGF-R, HER2, mesothelin, PDGFR-alpha, TROP2, and lastly EGFR and VEGF/VEGFR, whose respective targeting antibodies necitumumab and bevacizumab, have been approved for a subsets of patients with advanced NSCLC, including as a first-line therapy [(Corraliza-Gorjón et al., 2017), (Hellström et al., 2007), (Okamoto et al., 2011), (Smith et al., 2014)].

1.4.6 Immune Checkpoint Blockers

Up until now, six immune checkpoint blockers (ICBs) have been FDA approved for the treatment of liquid malignancies and some solid tumors. Two of the most extensively studied ICBs include monoclonal Abs (mAbs) that block the immune checkpoint inhibitors PD-1 or CTLA-4. Normally, the role of these immune checkpoint blockades is to inhibit proteins that suppress the immune system. By blocking these inhibitors, the endogenous antitumor immunity thereby is strengthening. When type I and II IFNs are secreted by the tumor stroma, the PD-1 expression elevates. Consequently, PD-1 engages with its ligand PD-L1, found on tumor cells, TILs, APC, endothelial and epithelial cells and this binding further reduces the apoptotic pathway. It also induces anergy and depletion of T cells. T cell anergy is a tolerogenic mechanism in which the lymphocyte is intrinsically functionally inactivated following an antigen encounter, but remains alive for an extended period of time in a hyporesponsive state. Accumulating knowledge suggests that lung tumors overexpress the immunosuppressive protein PD-L1 and inhibition of this pathway has led to durable benefit in a subset of advanced-stage NSCLC patients. For example, the two best-studied ICBs, anti-CTLA-4 and anti-PD-1 mAbs, mediate distinct yet complementary antitumor responses despite both having a suppressive effect on T cells (Saab et al., 2020).

Immune check point inhibitors (ICIs) are a fundamental part of the systemic therapy for metastatic NSCLC, in both first-line and later-line stages (Cohen & Vokes, 2001). In 2019, the FDA approved pembrolizumab as first-line treatment of patients with stage III NSCLC, PD-L1-positive and not able to undergo a surgery or chemo-radiation treatment. Furthermore, nivolumab, an anti-PD1 ICB treatment, was approved in 2018 for metastatic SCLC patients whose tumors progressed after treatment with platinum containing chemotherapy and at least one other systemic therapy. Other PD-1/PD-L1 pathway-targeting ICBs approved for specific subsets of NSCLC and SCLC cancer patients include atezolizumab including as a first-line therapy, durvalumab (this agent represents a major improvement in treatment of stage III NSCLC), and nivolumab (advanced NSCLC and a subset of metastatic SCLC as described above). Advanced studies revealed that clinical efficacy of anti-PD-1 treatment was also achieved in a subset of lung cancer patients, among which 15–40% have low or no tumor-specific PD-L1 expression. The efficacy levels of targeting the PD-1/PD-L1 axis across multiple tumor types and among patients with the same type of cancer depends on various factors, such as, gender, driver

mutations, genomic instability (like translocations in ALK, KRAS, EGFRI in lung cancer patients) and the degree of tumor metastases (Saab et al., 2020).

1.4.7 Combination ICB

Despite the fact that most of the patients treated with single-agent ICB show promising long-time control of disease, sometimes they end up with patient relapse or tumor resistance to ICB, as they are unable to activate an antitumor immune response [(Tumeh et al., 2014), (Ribas & Wolchok, 2018)]. As a result, there is the possibility of combining two or more immunotherapies in order to achieve synergistic effects, which may hold more advantages in terms of patients' survival, in comparison to immunotherapy in some cancer types [(Pico de Coaña et al., 2015), (Chae et al., 2018), (Curran et al., 2010), (Postow et al., 2015)]. The two most studied ICBs for example, anti-CTLA-4 and anti-PD-1 mAbs, could serve complementary antitumor responses, despite the fact that both have a suppressive effect on T cells [(Buchbinder & Desai, 2016), (Baumeister et al., 2016), (Fife & Bluestone, 2008), (Sage et al., 2013)]. One major advantage that combination therapy offers is the enhancement of TILs, which in turn leads to an increase in effector cytokines that change the tumor microenvironment towards an immuno-active situation [(Curran et al., 2010), (Duraiswamy et al., 2013)]. However, although some combinations were showing less toxicities compared to single-agents, other immune-related adverse effects were being addressed (Jiang et al., 2018). Combination ICB has a potential to shift immunologically cold tumors into hot tumors and consequently it is worth being further investigated. Supplementary approaches involve ICB combined with other immunotherapies (ACT, OVs, cancer vaccines, mAbs), epigenetic modifiers, targeted therapies, or conventional therapies (e.g., radiotherapy and chemotherapy, such as, FDA-approved atezolizumab and chemotherapy for SCLC) [(Marshall & Djamgoz, 2018), (Z. S. Guo & Bartlett, 2014), (Haanen, 2017)].

1.5 Activin-A

1.5.1 Structure

Activins are pluripotent cytokines that belong to the transforming growth factor-β (TGF-β) superfamily which consists of more than 45 members, including TGFβs, activins, NODAL, bone morphogenetic proteins (BMPs), growth and differentiation factors (GDFs) and the anti-Müllerian hormone (AMH) (Wakefield & Hill, 2013). Activins are found either as homodimers or heterodimers, consisting of βA or/and βB subunits connected with disulfide

bonds. Therefore, there are three functional isoforms of activins: activin-A (βAβA), activinB (βBβB) and activinAB (βAβB) (Xia & Schneyer, 2009). All β subunits have as basic characteristic the presence of a cysteine knot in their carboxyl-terminus. This cysteine knot is a folding domain including nine conserved cysteines, which are crucial for the stabilization and dimerization of the ligands (Mason et al., 1996). Specifically, cysteine number six is important for the dimerization, whereas the other eight create intramolecular disulphide bonds. The three-dimensional structure of activins is defined by this bond. Almost every human tissue has transcripts of the βA and βB subunits and displays high expression in the reproductive system. On the contrary, the βC and βE subunits are mainly expressed in the liver [(Woodruff et al., 1998), (Morianos et al., 2019)].

Activin-A is the most studied protein among the family of activins. It is a cytokine of approximately 25 kDa and was originally discovered as a gonadal protein that stimulates the biosynthesis and secretion of the follicle-stimulating hormone from the pituitary (Hedger & De Kretser, 2013). Activin-A is highly conserved among vertebrates, in a homology rate up to 95% between species (Kariyawasam et al., 2011). Its role is to regulate basic biological processes, such as, haematopoiesis, embryonic development, stem cell maintenance and pluripotency, tissue repair and fibrosis (Kariyawasam et al., 2011), (W. Chen & Ten Dijke, 2016). Its importance in developmental processes becomes evident as mice that lack the βA subunit die within 24 h after birth due to severe craniofacial defects, while mice that lack the βB subunit display abnormal development and reduced reproductive capacity (Matzuk et al., 1995). Activin-A is expressed and secreted by a plethora of cell subsets of the immune system, such as, macrophages, monocytes, dendritic cells, B and T cells, Natural Killer cells and neutrophils, in both humans and mice. Its role is very important in the microbial (bacteria, parasites and viruses) host defence and its effects on immune cells are variable and dependent on the cell type, activation state and the status of the immune responses. A growing body of evidence underlies the important role that activin-A plays in the pathophysiology of several diseases, such as, allergic asthma, multiple sclerosis and cancer (Morianos et al., 2019).

1.5.2 Signalling

Activin-A signals through two type I and two type II receptors which, upon ligand binding, assemble the final receptor complex (Namwanje & Brown, 2016). Type I receptors

include activin receptor type 1A (or Activin receptor Like Kinase2, ALK2), Activin receptor type 1B (or ALK4) and Activin receptor type 1C (or ALK7) (Fig. 4). Activin-A has a ‘’preference’’ for ALK4 binding, whereas it has less affinity for ALK2 and ALK7. The type II receptors are the Activin receptor type IIA (ActRIIA) and Activin receptor type IIB (ActRIIB) and are signified by constitutively-active serine/threonine kinase activity (Pangas & Woodruff, 2000). Strikingly, the Acvr2b gene (encoding for ActRIIB) produces four alternatively spliced transcripts that exhibit discrete binding affinities for activin-A. Interestingly, crystallography studies of the activin-A- ActRIIB receptor complex revealed that activin-A shows a different binding pattern in comparison with the rest of the TGF-β superfamily members. When activin-A is bound to its type II receptors, two type I receptors are recruited and become phosphorylated by the type II receptors. This leads to the activation of their kinase activity. Subsequently, type I receptors phosphorylate the intracellular mothers against decapentaplegic homolog (SMAD) 2 and SMAD3 signalling proteins at their carboxyl-terminal SSXS motif, which then form a complex with co-SMAD4, translocate to the nucleus and activate or silence the expression of gene-targets (Massagué & Chen, 2000) (Fig. 4). SMAD6 and SMAD7 are the major inhibitory SMAD (I-SMAD) proteins that prevent SMAD4 binding to the SMAD2/3 complex and disrupt their transcriptional activity in response to activin-A (Namwanje & Brown, 2016).

Activin-A has the ability to activate alternative, non-canonical, intracellular signalling pathways, comprising of the p38 mitogen activated protein kinase (MAPK), extracellular signal-regulated kinases1/2 (ERK1/2) and c-Jun N-terminal kinases (JNKs) which affect cell migration and differentiation [(Xia & Schneyer, 2009), (Namwanje & Brown, 2016)]. Additionally, activin-A, can activate the canonical Wnt signalling pathway, through SMAD2 activation (Tsuchida et al., 2009) (Fig. 4). Remarkably, SMAD proteins contain a connecting domain, comprised of regulatory sites. Among these, there are sites for ERK and calcium-regulated kinases. SMAD nuclear translocation and transcriptional activity are inhibited by phosphorylation at this area, suggesting a complicated regulation of activin-A signal transduction that is characterized by spatiotemporal and cell type dependence (Massagué & Chen, 2000).

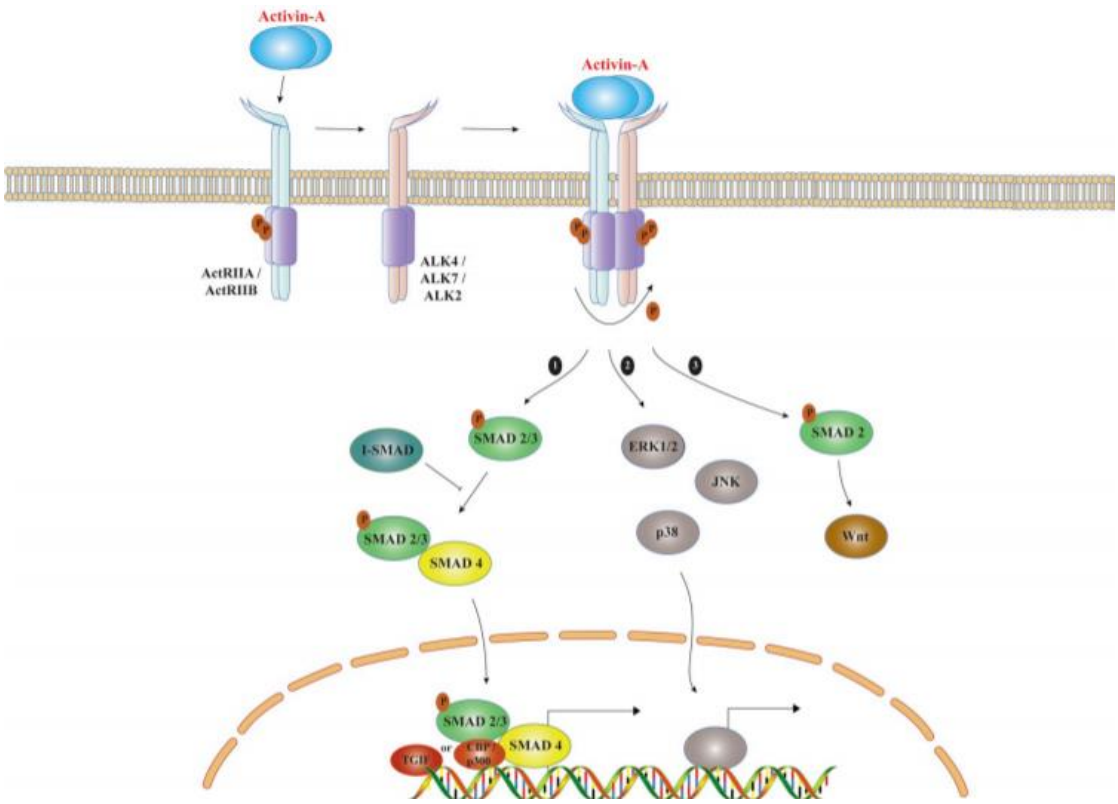

Fig. 4: *The signaling pathways of activin-A. Activin-A signals through a heterotetrameric receptor complex. Initially, activin-A binds to the constitutively active ActRII (ActRIIA or ActRIIB). This interaction leads to the recruitment and phosphorylation of the ActRI (ALK4, ALK7 or ALK2). Activated ActRI phosphorylate the intracellular mediators SMAD2/3, which then form a complex with SMAD4 and translocate to the nucleus, where they regulate the transcription of target genes in cooperation with transcription co-factors (e.g CBP/p300 or transforming growth interacting factor (TGIF). In the non-canonical activin-A signal transduction pathway, ActRI activates the ERK, p38 and/or JNK kinases which in turn regulate the transcription of target genes. In addition, activin-A, through SMAD2 activation, can act as a co-activator of the canonical Wnt signaling pathway (Morianos et al., 2019).*

1.5.3 Regulation

Considering the vital roles of activin-A in several biological processes, its mode of action is highly regulated by a great number of molecules, both at the extracellular and intracellular level. Follistatin (FS) represents the main inhibitor of activin-A as it binds to activin-A with high affinity and neutralizes its functions by preventing activin-A's interaction with its type II receptors (Fig. 5). Alternative splicing results in the creation of two isoforms of FS; the FS288 isoform binds heparin sulphate proteoglycans with high affinity and is considered as a local

regulator of activin-A functions, while the FS315 isoform neutralizes circulating activin-A. FS does not inhibit the effects of activin-A only as it neutralizes the functions of all activins, some BMPs and myostatin. Inhibins represent soluble heterodimeric proteins and contain the inhibin α subunit and the activin βA (inhibin A, αβA), or βB subunit (inhibinB, αβB). Inhibins compete for binding to the type II receptors but can also bind straightforward to activins with variable affinities (Fig. 5). Beta glycan is another protein that binds inhibins and makes their affinity for ActRII stronger, leading to the inhibition of the interaction between activins and the type II receptors (Fig. 5). The pseudoreceptor BMP and activin membrane-bound inhibitor homolog (BAMBI) restricts activin-A's signalling by interacting with the type I receptors and inhibiting the formation of the receptor signalling complex. Moreover, overexpression of Cripto, the co-receptor for nodal ligands inhibits activin-A's signalling, while enhancing nodal signalling by binding to nodal and activin-A receptors (Harrison et al., 2005) (Fig. 5). As mentioned above, activin-A functions are also controlled at the intracellular level. More specifically, the inhibitory I-SMADs bind to the type I receptors and diminish the recruitment and phosphorylation of receptor-regulated SMADs (R-SMADs) (Fig. 5). I-SMADs also promote the binding of the SMAD ubiquitin regulatory factors 1 and 2 to activin-A receptors provoking their ubiquitin-dependent degradation [(Afrakhte et al., 1998), (Morianos et al., 2019)].

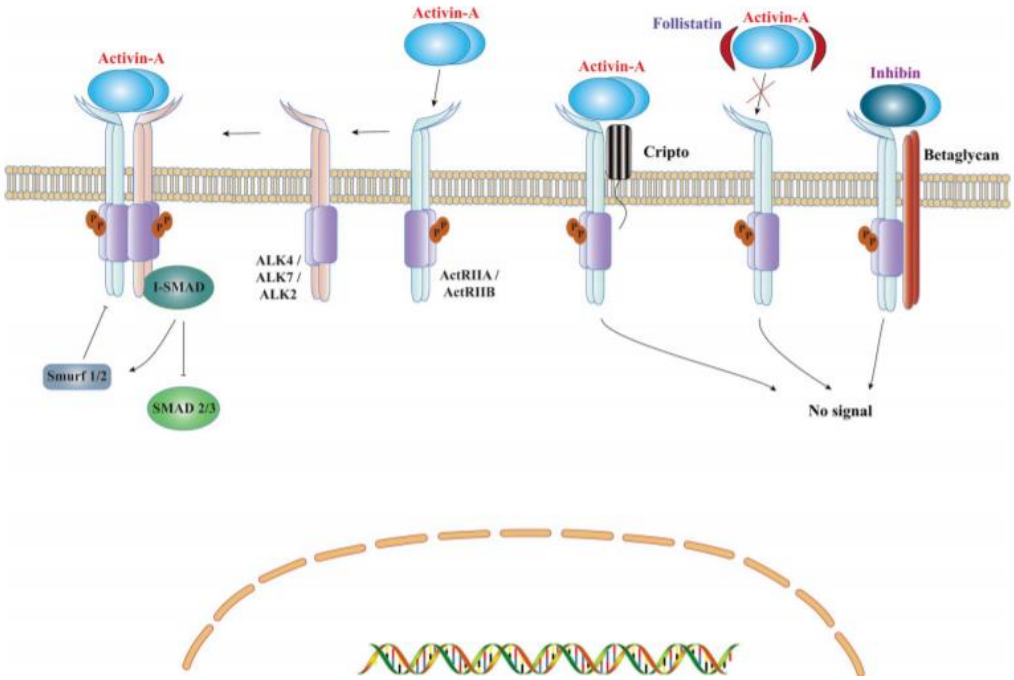

Fig. 5: *Regulation of activin-A functions. Activin-A signals through a heterotetrameric receptor complex. Initially, activin-A binds to the constitutively active ActRII (ActRIIA or ActRIIB). This interaction leads to the recruitment and phosphorylation of the ActRI (ALK4, ALK7 or ALK2). Inhibitory SMADs (I-SMADs), SMAD-6 and SMAD-7, are upregulated upon activin-A binding to its receptors and bind to the activated ActRI, inhibiting signal propagation intracellularly. Follistatin (FS) is one of the major inhibitors of activin-A which neutralizes its actions by binding with high affinity to activin-A and preventing its interaction with its type II receptors. Inhibins compete for binding to the type II receptors but can also bind directly to activins with variable affinities. Betaglycan binds inhibins and enhances their affinity for ActRII, leading to the inhibition of the interaction between activins and the type II receptors. Furthermore, Cripto the co-receptor for nodal ligands, inhibits activin-A signaling by binding to activin-A receptors (Morianos et al., 2019).*

1.5.4 Activin-A and cancer

Activin-A's role in the different aspects of cancer development and progression is cancer-type dependent. In a non-melanoma skin cancer model, where tumors were generated by topical treatment with 7,12-dimethylbenz[*a*]anthracene (DMBA) and 12-O-tetradecanoylphorbol 13-acetat (TPA), activin-A inhibited responses mediated by dendritic epidermal γδ T cells (DETCs), cells that provide protection against the development of skin tumors in mice (Ferrarini et al., 2002). Particularly, activin-A suppressed the proliferation of DETCs and transgenic animals that overexpress activin-A in keratinocytes showed decreased frequencies of DETCs upon DMBA/TPA-induced skin carcinogenesis (Fig. 6). Concurrently, the frequency of $CD4^+Foxp3^+$ Treg cells infiltrating the epidermis was enhanced in activin-A-overexpressing animals developing skin cancer, whereas the percentages of activated $CD86^+$ Langerhans cells remained unchanged (Antsiferova et al., 2011) (Fig. 6).

In another model of skin cancer, caused by the human papillomavirus (HPV) 8, heightened levels of activin-A induced a pro-tumorigenic program on macrophages which consequently enabled the progression of skin carcinogenesis (Antsiferova et al., 2017). In this model, γδ T cell-infiltration was restrained in the ear epidermis of mice overexpressing activin-A in keratinocytes, resulting in increased tumor development. However, $CD4^+$ T effector cells and $CD4^+Foxp3^+$ Treg cells accumulated in the ear epidermis (Fig. 6). Moreover, activin-A overexpression promoted the accumulation of $CD206^+$ macrophages in the skin, independently of HPV8 expression and tumor induction, due to enhanced migration of CCR2+ monocytes towards increased activin A concentrations (Fig. 6). Gene expression analyses further revealed

that skin macrophages expressed *Acvr1, Acvr1b, Acvr2a* and *Acvr2b* and responded to activin-A by exhibiting the signature of M1 type macrophages. Still, *Arg1* (encoding arginase 1), a characteristic M2 marker, was also upregulated in these macrophages (Weber et al., 2016). The depletion of macrophages in HPV8-infected, activin-A-overexpressing mice resulted in a significant delay in the spontaneous formation of skin tumors, signifying that activin-A pro-tumorigenic effects were predominantly mediated by the induction of tumor-promoting macrophages (Antsiferova et al., 2017).

In a mouse melanoma model induced by the intradermal injection of the B16F1 melanoma cell line, lentiviral-induced activin-A overexpression by melanoma cells reduced tumor infiltration by $CD8^+$ cytotoxic T-lymphocytes (Fig. 6). Furthermore, the TCGA Skin Cutaneous Melanoma database showed that, among melanoma patients those with the highest levels of activin-A, exhibited reduced infiltration of cells of the lymphocytic and the myeloid lineage (Donovan et al., 2017). In another study, using the B16 mouse melanoma model, blocking the effects of activin-A either through the use of a neutralizing antibody or disrupting its signalling through ALK7 decreased the frequencies of $CD4^+Foxp3^+$ Treg cells infiltrating the tumors, concurrent with increased tumor infiltration by IFNγ-producing $CD8^+$ and $CD4^+$ T cells and slower melanoma tumor growth (Ni et al., 2018). In sharp contrast, in other studies using the B16 mouse model of melanoma and the Lewis Lung Carcinoma (LLC) model of lung cancer, *in vivo* administration of DCs, previously exposed to activin-A, enhanced tumor-specific cytotoxic $CD8^+$ T cell responses, inhibited tumor growth and prolonged the overall survival of mice (Fig. 6). These effects were mediated by the expression of the cytokines BAFF and APRIL by activin-A-treated DCs, as knockdown of these genes deteriorated DC anti-tumor potential (Shurin et al., 2016).

In the context of pancreatic ductal adenocarcinoma, *in vitro* studies demonstrated that cancer stem cells secreted activin-A which, in turn, induced the expression of the anti-microbial peptide LL-37 by human M1-polarized, monocyte-derived macrophages (Fig. 6). In particular, when M1-polarized macrophages were treated with conditioned media obtained from cancer stem cells, they were polarized towards a M2 cell phenotype, accompanied by increased LL-37 mRNA and protein levels, dependent on activin-A signalling. Remarkably, LL-37 secreted by macrophages sustained pancreatic ductal adenocarcinoma growth, establishing a pro-tumorigenic

link between cancer stem cells and macrophages that was partly mediated by activin-A (Sainz et al., 2015).

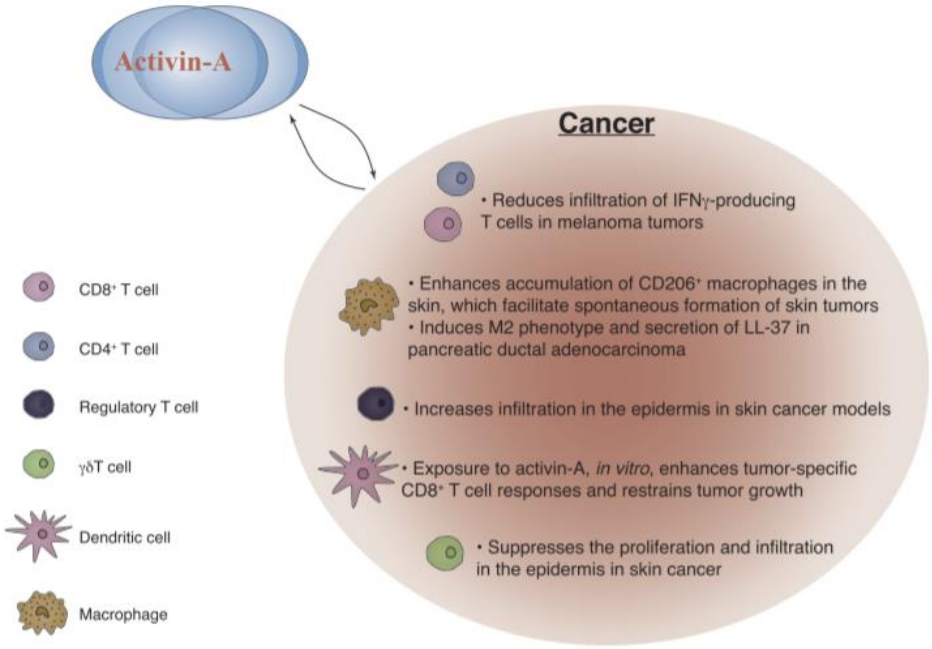

Fig. 6: *Pro and anti -tumorigenic functions of activin-A in cancer. Activin-A exerts both pro-tumorigenic functions, promoting immunosuppressive activities of macrophages and Treg cells and anti-tumorigenic effects boosting CD4+ and CD8+ T effector responses and DC antigen presenting functions. These divergent effects of activin-A depend on the type of cancer and the context of the immune response and are summarized in the figure (Morianos et al., 2019).*

1.5.5 Activin-A and lung cancer

Activin-A has also a dual role in lung cancer as in many other forms of cancer mentioned above, independently of the lung cancer type. Several human and mouse studies have revealed both pro- and anti- tumorigenic properties of activin-A in the development and progression of lung cancer. As a result, there is no standard implication of activin-A in lung cancer, although scientists are trying to assess its different roles in each type of lung cancer and evaluate its potential biomarker activity.

Notably, lung cancer patients with high levels of circulating activin-A displayed decreased overall survival, independently of the tumour stage. These patients also exhibited higher prevalence of low muscularity and skeletal mass density, properties that are also linked to decrease overall survival and poor prognosis (Loumaye et al., 2017). In line with the

aforementioned studies, Hoda et al., showed that in patients with lung adenocarcinoma (LADC), higher serum activin-A levels were linked to more advanced disease stage, organ metastases and poor prognosis. The authors also proposed that activin-A could be used as a biomarker for identifying LADC patients with metastatic disease (Hoda et al., 2016). Furthermore, another study revealed that *INHBA* mRNA and protein levels are overexpressed in primary lung adenocarcinoma (AD) compared to normal lung tissue. Kaplan-Meier analysis unravelled that the level of *INHBA* mRNA expressed by primary ADs inversely correlates with patient survival in stage I disease. Interestingly, activin-A administration induced the proliferation of the adenocarcinoma cell lines H460 and SKLU1 *in vitro*, an effect that was abolished upon follistatin treatment and INHBA-targeting siRNA treatment of AD cell lines (Seder et al., 2009). This finding is highly correlated with a very interesting study which displayed *INHBA* as a significantly mutated gene in lung adenocarcinoma (Ding et al., 2009).

Moreover, Wamsley and colleagues reported that TGFb/TNF-induced epithelial–mesenchymal transition (EMT) activates NF-kB to upregulate INHBA/Activin, which is necessary to maintain cancer-initiating cells' (CICs) properties and promote NSCLC metastasis. They also declared that the stimulation of EMT in A549 cells could promote the development of CICs, which are highly metastatic and, without the need for hypodermic tumor growth [(Meuwissen & Berns, 2005), (Cai et al., 2008)]. The same group also reported that A549.C and A549.A spheroid cultures treated *in vitro* with cytokines, when injected subcutaneously into nude mice led to the formation of lung surface metastases. However, the knockdown of activin-A in these A549.C and A549.A spheroid cultures, decreased their metastatic ability (Wamsley et al., 2015). In another study, whole-genome synthetic lethal small interfering RNA (siRNA) screen in A549 cells was performed, and the researchers observed that activin-A and the associated ligand growth differentiation factor 11 (GDF11) activate the TGFβ-activated kinase 1 (TAK1), a SMAD-independent element of TGFβ signalling known to be a significant regulator of cell death. They also showed that activin-A's signalling was responsible for the resistance to platinum chemotherapy observed in lung adenocarcinoma's patients. The aforementioned findings were further supported by the fact that inactivation of activin-A's signalling using a small molecule inhibitor or the endogenous activin/GDF11-binding protein follistatin (FST) overcame innate platinum resistance *in vivo*. This finding is also important as it has the potential to improve the safety and efficacy of chemotherapy in lung cancer patients (Marini et al., 2018).

Kalli et al. showed in their study that activin-A signalling may promote tumorigenesis, migration and lung metastasis of basal-like breast cancer (BLBC), by inducing directly or indirectly interleukin 13 receptor alpha 2 (IL13Rα2) expression, found overexpressed in BLCB and linked to poor distant metastasis-free survival (DMFS). They used previously published gene expression microarray data from the non-tumorigenic MCF10A (MI) mammary epithelial cell line and a meta-analysis of breast cancer patients and revealed that INHBA ablation downregulates IL13Rα2 expression in metastatic breast cancer cells, while treatment with activin-A in non-metastatic cells enhances its expression levels. They also showed that activin-A stimulated Smad2 phosphorylation and that activin-A-mediated upregulation of IL13Rα2 was Smad2-dependent. Their data revealed that knocking down INHBA levels in breast cancer cells delayed primary tumor growth, suppressed migration *in vitro* and inhibited the formation of lung metastases *in vivo* (Kalli et al., 2019). In support of the studies mentioned above, another group showed that IL-6, G-CSF and activin-A released by murine stromal fibroblasts trigger lung carcinoma cells' dedifferentiation and cancer stem cells (CSC) formation (Rodrigues et al., 2018).

Nevertheless, another set of studies suggest that activin-A has anti-tumorigenic effects. Specifically, Chen and colleagues showed that follistatin was significantly increased in the serum of patients with LADC and its levels positively correlated with disease stage and the differentiation of cancer cells. The authors also showed that activin A inhibited the proliferation of lung adenocarcinoma A549 cells and also induced their apoptosis (F. Chen et al., 2014). In line with the above, one more group demonstrated that activin-A-mediated A549 cells' apoptosis was associated with the up-regulation of the pro-apoptic molecules Bax, Bad and Bcl-Xs and down-regulation of the anti-apoptotic factor Bcl-2. In addition, activin-A administration increased the expression of its type II receptors, activated ERK signalling and caspase 3 in A549 cells. These results clearly show that the induction of apoptosis by activin-A involves multiple cellular and molecular pathways and strongly suggest that pro- and anti-apoptotic Bcl-2 family proteins and caspase 3 participate in activin A-induced apoptotic process in A549 cells. On the other hand, activin-A treatment had little effect on primary human small airway epithelial cells (SAECs) (Wang et al., 2009).

An additional anti-cancer action of activin-A is that it elicits SMAD2 and ERK1/2 pathways in dendritic cells (DC) expressing type I and II activin receptors and upregulates the

production of the TNFa family cytokines BAFF and APRIL. Consequently, BAFF and APRIL, upregulate the proliferation and survival of T cells. Activin-A significantly increased the ability of DC to inhibit tumor growth in melanoma (B16) and lung carcinoma (LLCs) models. The inhibition of tumor growth by activin-A-treated-DCs was linked with animal survival and generation of tumor-specific IFNg-producing T cells (Shurin et al., 2016).

2. Hypothesis and Aims

Our **hypothesis** is that activin-A restrains the initiation and propagation of lung cancer through the enhancement of T cell-driven anti-tumor responses.

The aim of this thesis is to elucidate the effects of activin-A in the development of lung cancer by studying its role on $CD4^+$ T cells, using a well-established mouse model of this particular cancer. In a translational approach we will further explore the role of activin-A in the enhancement of anti-tumor immune responses in human lung cancer patients.

Objective 1: To decipher the *in vivo* effect of activin-A on T cell-mediated immune responses in lung cancer. To address this, we will disrupt activin-A's signalling specifically on $CD4^+$ T cells using an inducible model of $CD4^+$ T cell-specific knockout of the ALK4 receptor ($CD4^{ERt2/Cre}$/$Acvr1b^{fl/fl}$). These mice will be subsequently inoculated with Lewis Lung Carcinoma (LLC) cells to induce primary lung tumors.

Objective 2: To explore whether activin-A enhances anti-tumor human T cell immune responses in lung cancer patients. To address this, we will obtain lung cancer specimens from patients with NSCLC, isolate tumor infiltrating $CD4^+$ T cells and stimulate them polyclonally with antibodies against CD3/CD28 in the presence or absence of activin-A.

3. Materials and Methods

3.1 Human Subjects

Primary human lung tumor tissue, as well as part of the adjacent healthy lung tissue, were obtained from NSCLC patients who fulfilled the criteria to undergo surgery for therapeutic purposes at the thoracic surgical clinics of Athens Chest Hospital "Sotiria" (*n* =30-40). Written informed consent was obtained from all patients, and the study protocol was approved by the Institutional Review Board of the Athens Chest Hospital "Sotiria".

3.2 Mice

C57BL/6 and CD4*Cre*ERT2 mice (in C57BL/6 background) were purchased from The Jackson Laboratory. *Acvr1b*^{Fx} mice (in C57BL/6 background) were kindly provided by Dr P. Bertolino (Inserm - Cancer Research Centre Lyon). *CD4*$^{-/-}$ mice (C57BL/6 background) were purchased from The Jackson Laboratory and kindly provided by Dr K. Karalis (BRFAA). All mice were maintained at BRFAA's animal facility. For our experiments, we used 8-12 weeks old male mice (*n*=25-30/group). Mice from different experimental groups, including knockout and their WT counterparts, were housed in the same cage to limit the impact of inter-individual microbiota on the variability of the experimental outcome. Animal handling and procedures are in accordance with US National Institutes of Health Statement of Compliance (Assurance) with Standards for Humane Care and Use of Laboratory Animals (#A5736-01) and with the European Union Directive 86/609/EEC on protection of animals used for experimental purposes.

3.3 Cell lines

The ovalbumin (OVA) expressing Lewis Lung Carcinoma (LLC-OVA) cell line was kindly provided by Dr M. Tsoumakidou (BSRC Al. Fleming). The cell line was negative for Mycoplasma spp., tested by PCR. Lewis Lung Carcinoma cells (LLC) are immunologically compatible with C57BL/6 mice and are known to metastasize to lungs and form solid tumors when injected into their tail vein. LLC cell line was maintained 1-2 weeks ahead of injection in DMEM culture medium at 37°C. We administrated $5*10^5$ cells/150 μl PBS LLC cells (day 0) to 8-10 weeks old mice intravenously (*n*=25-30/group). LLC cells colonize the lungs and lead to the formation of pulmonary tumors. After 20-21 days, mice were sacrificed.

3.4 *In-vivo* disruption of activin-A's signalling in LLC lung tumor model

CD4*Cre*ER^{T2} mice were crossed to *Acvr1b*^{Fx} mice to generate CD4*Cre*ER^{T2}/*Acvr1b*^{Fx} mice, where administration of tamoxifen induces the expression of Cre recombinase under the control of the mouse *Cd4* promoter. Disruption of *Avcr1b* expression on CD4^{+} T cells was induced right before LLC cell inoculation in order to rule out any previously established effect of endogenous activin-A on CD4^{+} T cells. For this, 3 mg/mouse/day of tamoxifen (Sigma Aldrich) were administrated daily to 8-10 weeks old CD4$^{ERt2/Cre}$/ Acvr1b$^{fl/fl}$ mice (*n*=25-30/group) by intraperitoneal injection for 5 consecutive days (days -4 – 0 & 7). Between days 18-23 the animals were euthanized and cells were collected from the lungs and mediastinal lymph nodes for downstream analysis.

3.5 Murine cell cultures

Lung tissues from mice bearing either LLC lung tumors were collected and minced. Single-cell suspensions were prepared by passing the minced tissues through 70μm cell strainers. Cells were resuspended in 40% percoll, laid over equal volume of 80% percoll and centrifuged at 600g for 25min without deceleration. The tumor infiltrating leukocyte cell fraction was collected at the interface between 40% and 80% discontinuous percoll gradient. Activation of tumor infiltrating T cells from LLC-OVA-tumor bearing mice was induced with the addition of 50μg/ml OVA in the presence of recombinant activin-A (50ng/ml, R&D Systems) or PBS for 3-5 days.

For the measurement of proliferation, CD4^{+} T cells were isolated from the tumor infiltrating leukocyte fraction using commercially available kits (Biolegend), labelled with CellTrace CFSE (1μm) and cultured in the presence of 50μg/ml OVA and mitomycin-C-treated (Sigma Aldrich) splenic APCs for 4 days. Culture media (CM) were collected and stored at -80^{o}C for further experimentation and CD4^{+} T cell proliferation was measured via flow cytometry.

3.6 Human cell cultures

Primary human lung tumor tissue and adjacent lung healthy tissue were obtained from NSCLC patients undergoing surgical resection. Tissues were minced and incubated with 0.14 WÃ¼nsch units/ml Liberase TL (Sigma Aldrich) and 0.1mg/ml DNase I (Sigma Aldrich) in 37^{o}C for 1h. Single-cell suspensions from lung cancer and healthy tissues were prepared by passing the minced tissues through 70μm cell strainers and tumor infiltrating leukocytes versus the tumor

cell fraction were separated using percoll density-gradient centrifugation (GE Healthcare Bio-Sciences AB). In brief, cells were resuspended in 40% percoll, laid over equal volume of 80% percoll and centrifuged at 600g for 25min without deceleration. Tumor or healthy lung infiltrating T cells were activated with 2μg/ml soluble anti-human CD3 (OKT3; Biolegend) and 2μg/ml anti-human CD28 (CD28.2; Biolegend) in the presence of recombinant activin-A (50ng/ml, R&D Systems) or PBS for 3-5 days.

Activin-A or PBS-treated $CD4^+$ T cells were isolated using commercially available kits (ThermoFischer Scientific; Dynabeads® CD4 Positive Isolation Kit) and further stimulated with soluble anti-human CD3/CD28 (2μg/ml both) for 3-5 days. In brief, Dynabeads® were mixed with the prepared sample in a tube. The Dynabeads® were incubated with the cells of interest for 20 minutes and afterwards the bead-bound cells were separated by a magnet. The positively isolated cells were washed and the appropriate amount of DETACHaBEAD® was added so as for the cells to be released from the beads.

Autologous $CD8^+$ T cells were isolated from Peripheral blood mononuclear cells (PBMCs) using commercially available kits (Biolegend; MojoSort™ Human CD8 T Cell Isolation Kit) and cultured overnight with recombinant human IL-2 (100 U/ml). In brief, non $CD8^+$ T cells were depleted by incubating the sample with the biotin antibody cocktail followed by incubation with magnetic Streptavidin Nanobeads. The magnetically labelled fraction was retained by the use of a magnetic separator. The untouched $CD8^+$ T cells were collected by decanting the liquid in a clean tube. Culture media (CM) were collected and stored at -80^{o}C for further experimentation.

For the *in vitro* suppression assays, activin-A or PBS-treated $CD4^+$ T cells isolated from primary lung tumors (effectors) were labelled with CellTracker Red CMTPX (Invitrogen) (1μm, 15 minutes, 37°C) and co-cultured with CellTrace (Invitrogen) CFSE-labelled autologous $CD4^+$ responder T cells isolated from the peripheral blood (5μm in DMSO, 15 minutes, 37°C) at a ratio 2:1 (responders:effectors). $CD4^+$ T cells were stimulated with soluble anti-human CD3 (2μg/ml; OKT3; Biolegend) and soluble anti-human CD28 (2μg/ml; CD28.2; Biolegend) and the proliferation of responder cells was assessed on days 3-5 via flow cytometry.

For cytotoxicity assays, autologous $CD8^+$ T cells isolated from PBMCs, were pre-treated for 14-16h with 100U/ml recombinant IL-2 (Peprotech) in the presence or absence of CM from $CD4^+$ T cell cultures, as described above. In parallel, single cell suspensions collected after the

mechanical and enzymatic digestion of primary lung tumors, were subjected to negative selection of cancer cells ($CD45^-$ cells) by depleting the $CD45^+$ cell fraction, using commercially available kit (Biolegend). The isolation is based on the biotin antibody cocktail followed by incubation with magnetic Streptavidin Nanobeads. The magnetically labelled fraction is retained by the use of a magnetic separator. The untouched $CD45^-$ cells are collected by decanting the liquid in a clean tube. Primary lung cancer cells were seeded in different densities, in order to facilitate cell adherence. The following day, human $CD8^+$ T cells were co-cultured with primary lung cancer cells at different ratios (5:1-40:1) for 4-6h and $CD8^+$ T cell mediated cytotoxicity was measured using the LDH Cytotoxicity Detection Kit (Takara), as long as using the NucView® 488 Caspase-3 Assay Kit (Biotium). The LDH Cytotoxicity Detection Kit uses LDH, a stable cytoplasmic enzyme that is present in large amounts in most cells. LDH is released into the cell culture supernatant during cytoplasmic membrane damage and can be easily measured using standard reagents. The NucView® 488 Caspase-3 Assay Kit provides a convenient tool for profiling apoptotic cell population based on caspase-3/7 activity using flow cytometry.

3.7 Lung histology

Paraffin-embedded sections (4μm) were stained with hematoxylin & eosin (H&E) to evaluate lung infiltration. Hematoxylin stains nucleic acids and eosin stains proteins nonspecifically. In a typical tissue, nuclei are stained blue, whereas the cytoplasm and extracellular matrix have varying degrees of pink staining.

3.8 ELISA

The levels of the cytokine and chemokine release of human and mouse IL-10, IL-2, IFN-γ and TNF-α, (R&D systems) were evaluated by the enzyme-linked immunosorbent assay (ELISA) kits. For the evaluation, we used serum of patients and mice, mouse lungs homogenates, as well as supernatants taken by all different kinds of cell cultures. The results were then analysed in GraphPad Prism 6 program.

3.9 Flow-Cytometry analysis

The composition of activin-A- or PBS-treated tumor-infiltrating leukocytes was analyzed by flow cytometry. Cells were stained with fluorescently-labelled antibodies to mouse CD45, CD3, CD4, CD8 (ThermoFischer Scientific), PD-1, CTLA-4, LAG-3, Tim-3, perforin, granzyme-b

(Biolegend) and to human CD45, CD69 (Thermo Scientific), PD-1, CTLA-4 and LAG-3 (Biolegend). For intracellular cytokine staining, cells were re-stimulated with 10ng/ml PMA (Sigma-Aldrich), 250ng/ml ionomycin (Sigma-Aldrich) and 1μl/ml medium Golgi-Stop (brefeldin) (BD Biosciences) and stained with antibodies against mouse or human IL-10 and IFN-γ (eBiosciences), according to the manufacturer's instructions. Foxp3 staining was performed according to the manufacturer's instructions (eBiosciences). FACS acquisition was performed with the cytometer Cytomics FC500 (Beckman Coulter) and the cytometer BD FACSAria II (Becton Dickinson) and the data were analyzed using the FlowJo X software 8.7 (Tree Star, Inc).

3.10 Statistical analysis

The number of independent experiments, biological replicates and donors analyzed are stated in each figure legend. Graph Pad Prism 7 (Graph Pad Software Inc.) was used for all statistical analyses. All data are presented as mean ± SEM. The comparison of survival curves was performed using Gehan-Breslow-Wilcoxon test. To calculate differences between groups, we used Student's t test, the Mann-Whitney test and the Wilcoxon matched-pairs signed rank test as appropriate. We considered any difference with a p value of 0.05 or less to be statistically significant ($*p \leq 0.05$, $**p \leq 0.01$ and $***p \leq 0.001$).

4. Results

4.1 Administration of activin-A *in vivo* restrains the development of lung tumors

The first part of our work was to examine the *in vivo* role of activin-A in the establishment of T-cell mediated anti-tumor immune responses in the airways using the syngeneic Lewis Lung Carcinoma (LLC) mouse lung cancer model. To investigate this, LLC cells were inoculated intravenously to C57BL/6 mice (day 0). Mice were sacrificed between days 18-22 (Fig. 7A). Several studies have shown that activin-A's expression is increased in the serum and biopsies of lung cancer patients. In accordance with the human studies, we also observed that activin-A was elevated in the serum and lung homogenates of LLC-bearing mice compared to healthy mice (Fig. 7B).

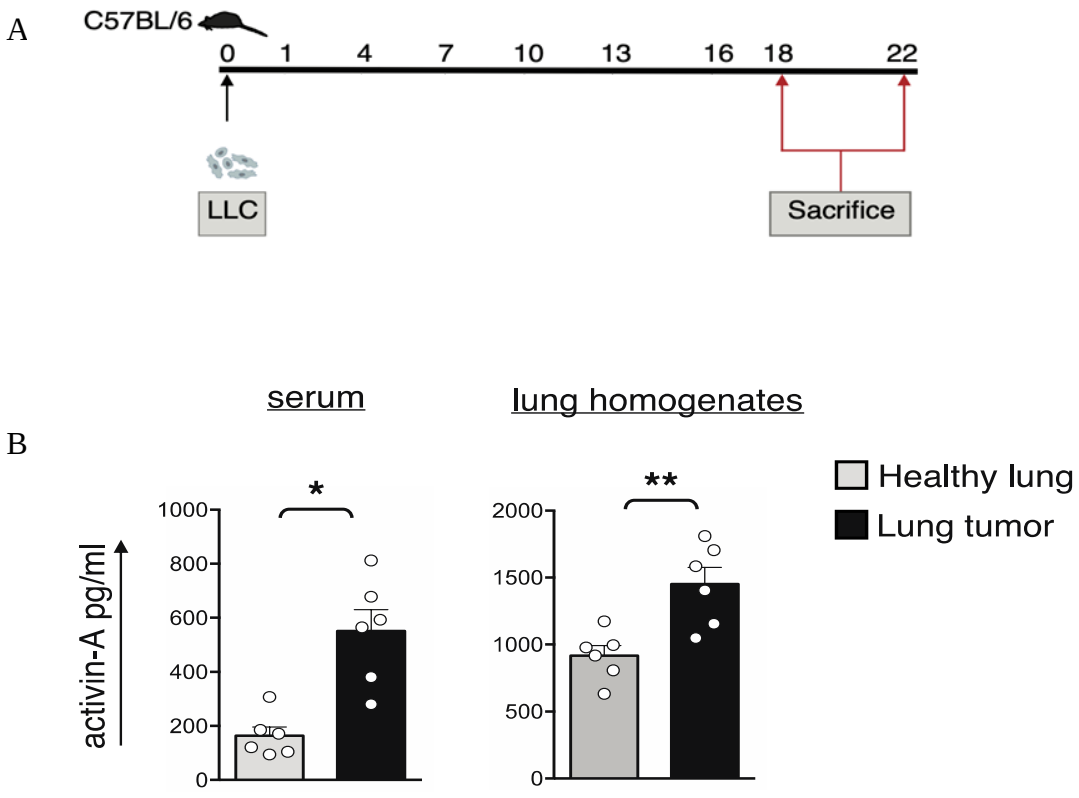

Fig. 7: *(A) Experimental protocol utilized.* (B) *Activin-A levels in serum and lung homogenates from healthy and LLC tumor-bearing mice. Data are mean ± SEM of triplicate wells and are representative of 6 independent experiments. Data are representative of 4-7 independent experiments. Statistical significance was obtained by unpaired Student's t-test; *P < 0.05 and **P < 0.01.*

4.2 Disruption of activin-A's signalling on $CD4^+$ T cells accelerates lung tumor progression

Since activin-A was overexpressed during lung tumor formation in the LLC model, we subsequently examined the effects of endogenous activin-A specifically on $CD4^+$ T cells. For this purpose, we used an inducible model of $CD4^+$ T cell-specific knockout of ALK4, activin-A's major type I receptor (CD4*Cre*$ER^{T2+/-}$/*Acvr1b*$^{fx/fx}$, hereafter CD4/ALK4-KO). In brief, CD4*Cre*ER^{T2} mice were crossed to *Acvr1b*^{fx} mice to generate CD4*Cre*ER^{T2}/*Acvr1b*^{fx} mice. We administrated $5*10^5$ cells/150 μl PBS LLC cells (day 0) to 8-10 weeks old CD4/ALK4-KO mice and their wild-type littermates (CD4*Cre*$ER^{T2-/-}$/*Acvr1b*$^{fx/fx}$, hereafter WT) intravenously in order to induce pulmonary tumors. Tamoxifen was administrated daily by intraperitoneal injection for 5 consecutive days (days -4 – 0 & 7), right before LLC cell inoculation. Between days 18-23 mice were euthanized (Fig. 8A).

We first examined the overall survival between CD4/ALK4-KO and WT mice. Notably, LLC-inoculated CD4/ALK4-KO mice exhibited decreased overall survival compared to WT mice, as shown in Fig. 8B. In order to further evaluate disease onset and progression, paraffin-embedded sections (4μm) of the lungs of CD4/ALK4-KO and WT mice were stained with hematoxylin & eosin (H&E) and microscopic observations exhibited accelerated formation of peribronchial and perivascular tumors in WT mice, compared to CD4/ALK4-KO (Fig. 8C). Moreover, macroscopic observations revealed increased formation of superficial lung tumors, and as a result heightened lung weights in CD4/ALK4-KO, compared to WT mice (Fig. 8D).

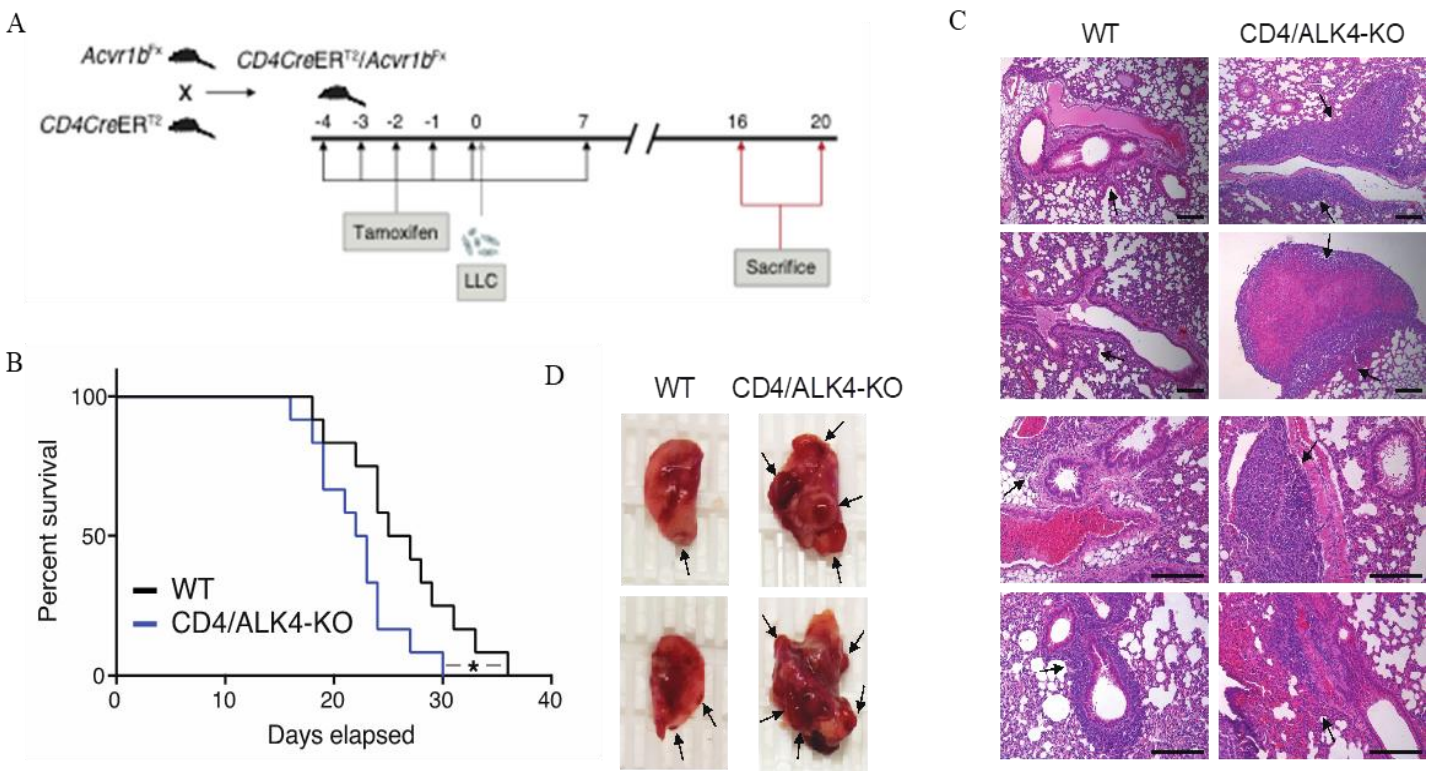

Fig. 8: Disruption of activin-A's signalling on CD4⁺ T cells accelerates lung tumor progression. *(A) Experimental protocol utilized. (B) Survival plot of WT and CD4/ALK4-KO LLC-tumor bearing mice (n=12). (C) Representative macroscopic images of lung lobes. (D) Representative photomicrographs of H&E-stained lung sections (scale bars: 100μm).*

4.3 Disruption of activin-A's signalling on CD4⁺ T cells does not influence the infiltration of leukocytes in the airways of mice

We next assessed the infiltration of leukocytes in the airways of CD4/ALK4-KO mice, compared to their WT littermates. For this purpose, we performed FACS staining for $CD45^+$ leukocytes, $CD3^+CD8^+$ and $CD3^+CD4^+$ T cells infiltrating the lung tumors of both LLC-bearing mice groups (WT and CD4/ALK4-KO). Disruption of activin-A's signalling on $CD4^+$ T cells had no impact on either $CD45^+$ total leukocytes nor $CD3^+CD8^+$ and $CD3^+CD4^+$ T cells infiltrating the lung tumors of LLC-bearing mice of both groups (WT and CD4/ALK4-KO) (Fig. 9A-C).

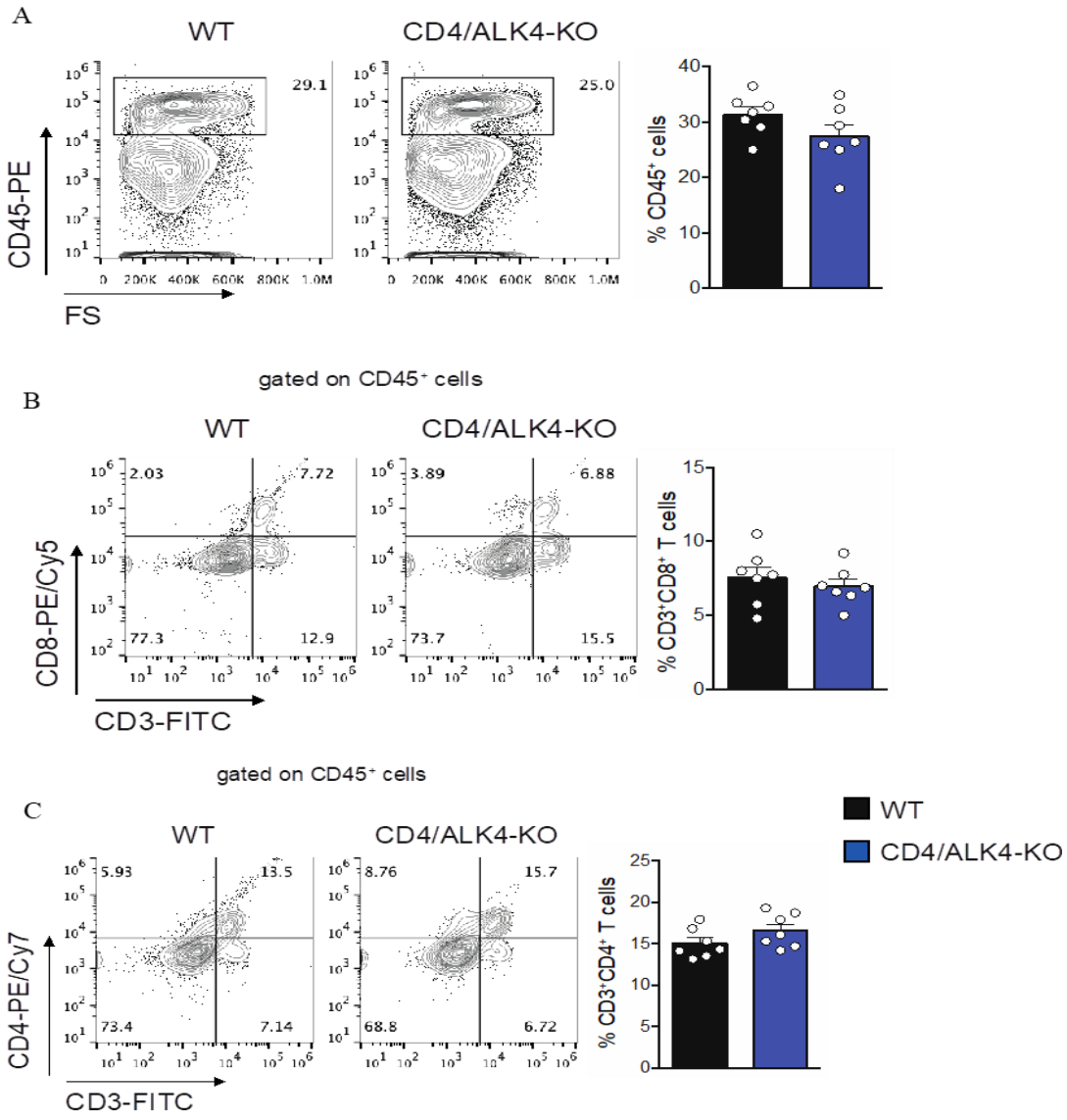

Fig. 9: Lung tumor cells' infiltration. *(A) Representative flow cytometry plots (left) and cumulative percentages of $CD45^+$ lung tumor infiltrating cells (right). (B) Representative flow cytometry plots (left) and cumulative percentages of $CD3^+CD8^+$ T cells among $CD45^+$ lung tumor infiltrating cells (right). (C) Representative flow cytometry plots (left) and percentages of $CD3^+CD4^+$ T cells among $CD45^+$ lung tumor infiltrating cells (right). Data are representative of 4-7 independent experiments. Statistical significance was obtained by unpaired Student's t-test; *P < 0.05 and **P < 0.01.*

4.4 Disruption of activin-A's signalling on $CD4^+$ T cells alters the profile of infiltrating $CD4^+$ T cells

Despite the fact that there was no alteration in the cells infiltrating the lung tumors of LLC-bearing mice in both groups (WT and CD4/ALK4-KO), we observed that $CD4^+$ T cells isolated from the TILs of CD4/ALK4-KO mice produced significantly less IFN-γ and TNF-α concomitantly with increased levels of IL-10 upon *ex vivo* antigenic stimulation, compared to $CD4^+$ T cells from WT mice (Fig. 10A). These findings were further strengthened by the fact that $CD4^+$ T cells from lung tumors of CD4/ALK4-KO mice demonstrated an exhausted profile, exemplified by higher levels of PD-1, CTLA-4 and LAG-3 compared to WT mice (Fig. 10B). In addition, FACS analysis revealed significantly increased percentages of Foxp3-expressing regulatory T cells concomitantly with fewer IFN-γ-expressing $CD4^+$ T cells infiltrating the lung tumors of CD4/ALK4-KO mice compared to WT mice (Fig. 10C and 10D). Importantly, in order to examine the proliferation capacity of $CD4^+$ T cells, we used CFSE analysis and observed that $CD4^+$ T cells from CD4/ALK4-KO mice appeared to be less capable to respond to antigenic stimulation in comparison to $CD4^+$ T cells from WT mice (Fig. 10E).

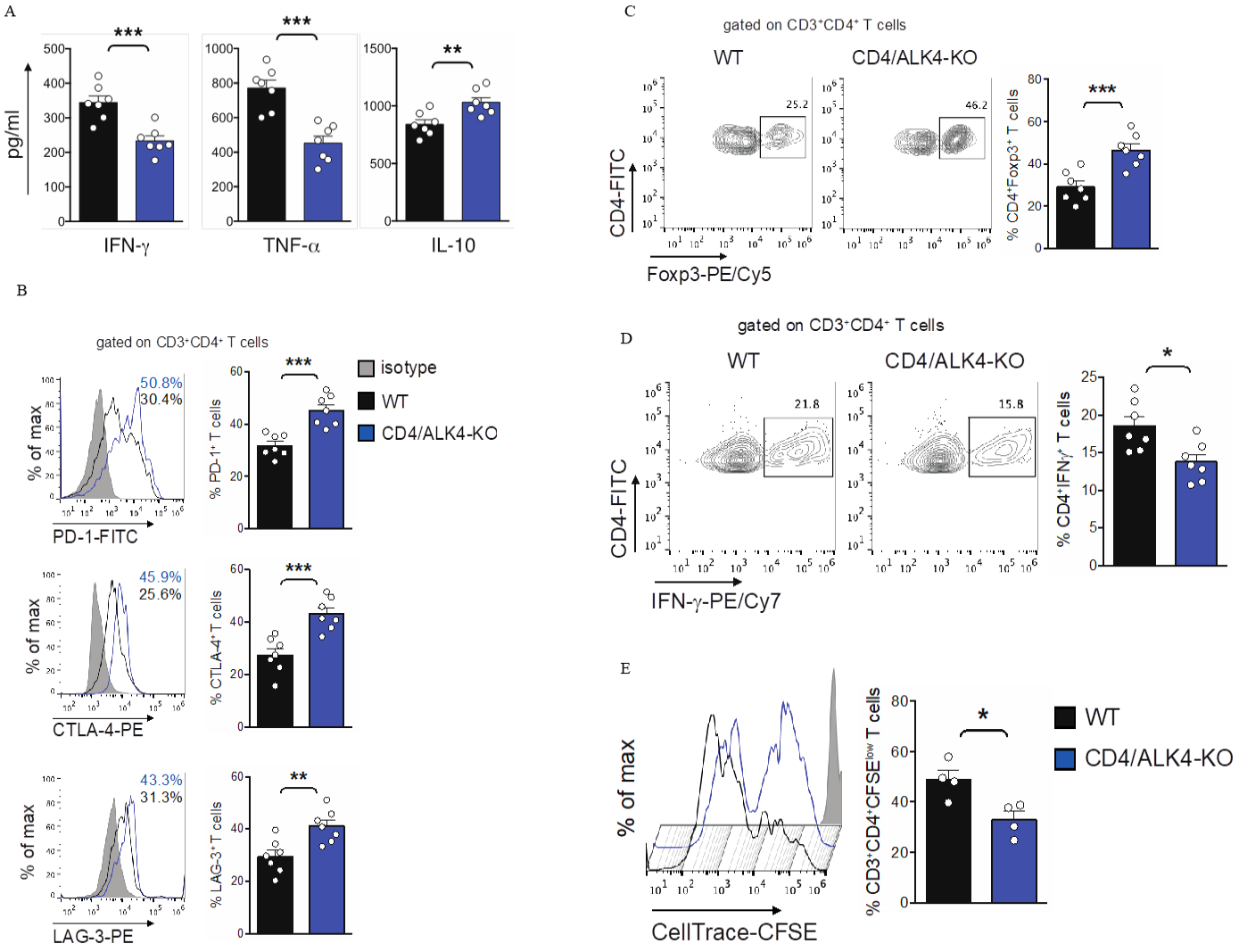

Fig. 10: Disruption of activin-A's signalling on CD4⁺ T cells alters the profile of infiltrating CD4⁺ T cells. *(A) Cytokine levels in supernatants of lung tumor infiltrating leukocyte cultures. Data are mean ± SEM of triplicate wells and are representative of 5 independent experiments. (B) Representative flow cytometry plots (left) and cumulative percentages of PD-1, CTLA-4 or LAG-3-expressing cells among CD3⁺CD4⁺ T cells infiltrating lung tumors (right). (C) Representative flow cytometry plots (left) and cumulative percentages of Foxp3-expressing cells among CD3⁺CD4⁺ T cells infiltrating lung tumors (right). (D) Representative flow cytometry plots (left) and cumulative percentages of IFN-γ-expressing cells among CD3⁺CD4⁺ T cells infiltrating lung tumors (right). (E) Representative flow cytometry plot (left) and cumulative percentages of CD3⁺CD4⁺CFSE low T cells isolated from lung tumors (right). Data are mean ± SEM and are representative of 7 (A-D) and 4 (E) independent experiments. Statistical significance was obtained by Log-rank (Mantel-Cox) test and unpaired Student's t-test; *P < 0.05, **P < 0.01 and ***P < 0.001*

4.5 Activin-A unveils different effects on human T cells that infiltrate the lung tumors

A major limitation in the use of preclinical mouse models in most types of cancer is that they fail to recapitulate the complexity of the malignancy, as well as, the immune contexture within the tumor microenvironment. For this, we next explored the potential anti-tumorigenic effects of activin-A in patients with NSCLC. For this purpose, we obtained primary human lung tumor tissues, as well as part of the adjacent healthy lung tissue of NSCLC patients. Initially, lung tumor infiltrated leukocytes (hereafter TILs) and healthy lung leukocytes were isolated and cultured under polyclonal stimulation (anti-human CD3 and anti-human CD28) in the presence of activin-A or PBS. Elevated levels of TNF-α and IL-2 were detected concomitantly with decreased secretion of IL-10 after 3-days of culture in the supernatants of TILs treated *ex vivo* with activin-A compared to PBS, while there was no difference on the secretion of IFN-γ between the two groups (Fig. 11A). Interestingly, supernatants from healthy lung leukocytes exhibited reduced levels of IFN-γ and IL-2 upon *ex vivo* activin-A administration, but had no differences on TNF-α and IL-10 levels.

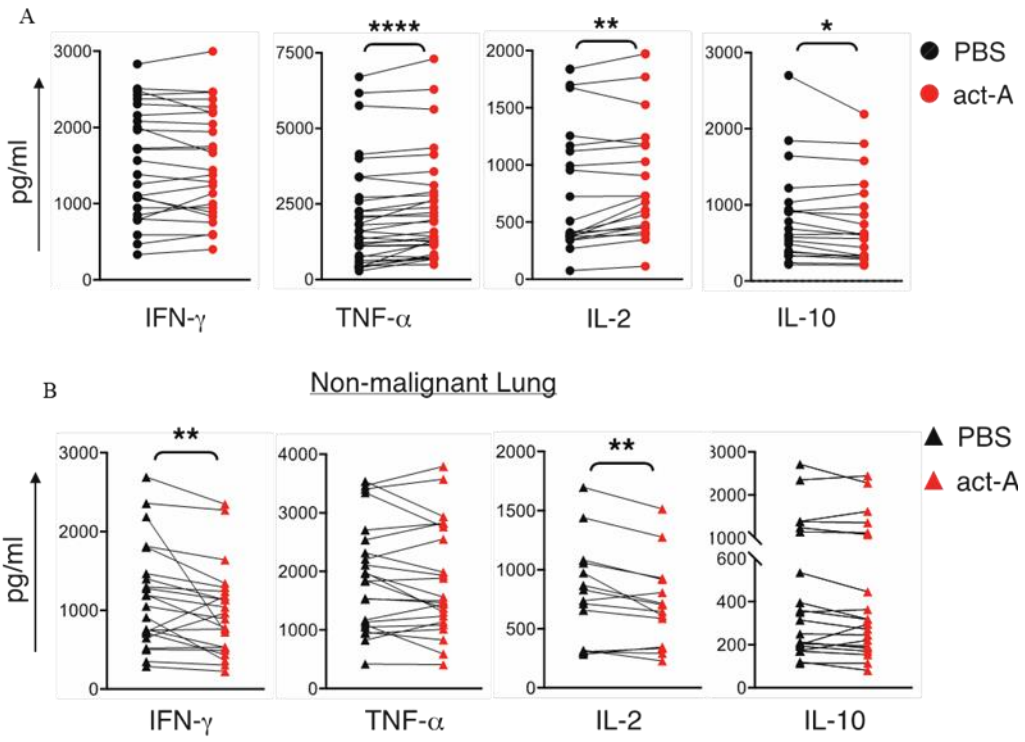

Fig. 11: Activin-A unveils different effects on T cells that infiltrate the lung tumors *(A) Primary lung tumor infiltrating leukocytes were isolated from lung cancer patients and stimulated with antibodies against CD3/CD28 in the presence of activin-A or PBS. Cytokine levels in culture supernatants of PBS- or activin-A-treated lung tumor*

*infiltrating leukocytes (n= 20-25 donors). Data are mean ± SEM of triplicate wells. (B) Primary lung infiltrating leukocytes were isolated from, adjacent to tumors, healthy tissues form lung cancer patients and stimulated with antibodies against CD3/CD28 in the presence of activin-A or PBS. Cytokine levels in culture supernatants of PBS- or activin-A-treated lung tumor infiltrating leukocytes (n= 20-26 donors). Data are mean ± SEM of triplicate wells. Statistical significance was obtained by Wilcoxon matched-pairs signed rank test; *P < 0.05 and **P < 0.01.*

4.6 Activin-A-treated human lung tumor infiltrating CD4$^+$ T cells exhibit a less exhausted phenotype/profile

Next, we characterized the profile of human CD4$^+$ T cells infiltrating the lung tumors, upon *ex vivo* activin-A administration. Notably, we observed that activin-A-treated CD4$^+$ T cells (act-A-CD4$^+$ T cells) displayed heightened expression of the activation marker CD69 along with reduced expression of the immune checkpoint inhibitory molecules PD-1, CTLA-4 and LAG-3 compared to PBS-treated CD4$^+$ T cells (PBS-CD4$^+$ T cells) (Fig. 12A). In line with the aforementioned results, act-A-CD4$^+$ T cells demonstrated reduced expression of the transcription factor Foxp3 (Fig. 12B).

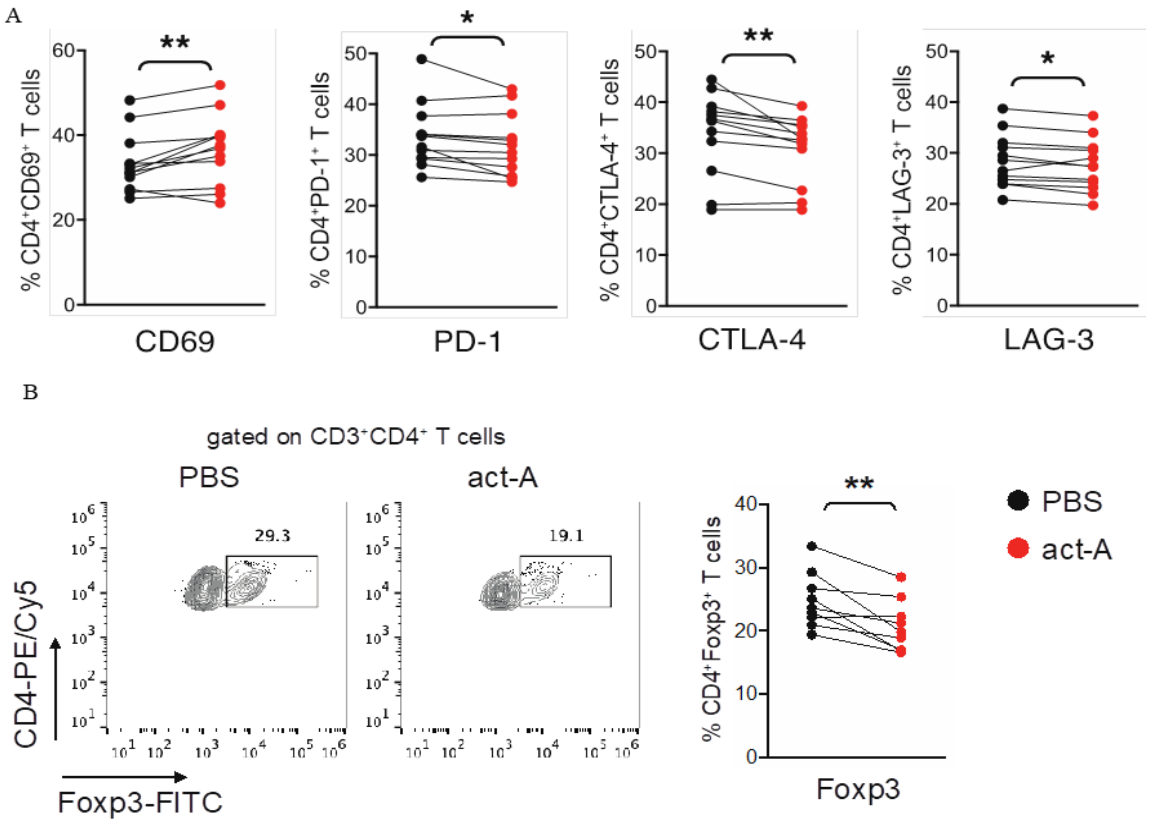

Fig. 12: Activin-A-treated human lung tumor infiltrating CD4⁺ T cells exhibit a less exhausted phenotype/profile. *(A) Cumulative percentages of CD69, PD-1, CTLA-4 or LAG-3-expressing cells among human lung tumor infiltrating CD4⁺ T cells cultured as in Fig.5A (n= 12 donors). (B) Representative flow cytometry plots (left) and cumulative percentages (right) of Foxp3-expressing cells among human lung tumor infiltrating CD4⁺ T cells cultured as in Fig.11A (n= 9 donors). Statistical significance was obtained by Wilcoxon matched-pairs signed rank test; *P < 0.05 and **P < 0.01.*

4.7 Activin-A-treated human lung tumor infiltrating CD4⁺ T cells display enhanced immunostimulatory capacity

Despite the fact that act-A-CD4⁺ T cells seemed to have acquired a more activated phenotype compared to PBS-CD4⁺ T cells, both populations exhibited similar capacity to proliferate upon *ex vivo* polyclonal stimulation (Fig. 13A). Next, in an attempt to evaluate the immunostimulatory capacity of act-A-CD4⁺ T cells in comparison to PBS-CD4⁺ T cells, we cultured them along with autologous CD4⁺ T cells obtained from the periphery (PB CD4⁺ T cells). Notably, we observed that CD4⁺ T responder cells cultured along with act-A-CD4⁺ T cells demonstrated increased proliferative capacity compared to those cultured with PBS-CD4⁺ T cells (Fig. 13B). This observation was complemented by the detection of heightened levels of IFN-γ and lower levels of IL-10 in culture supernatants (Fig. 13C).

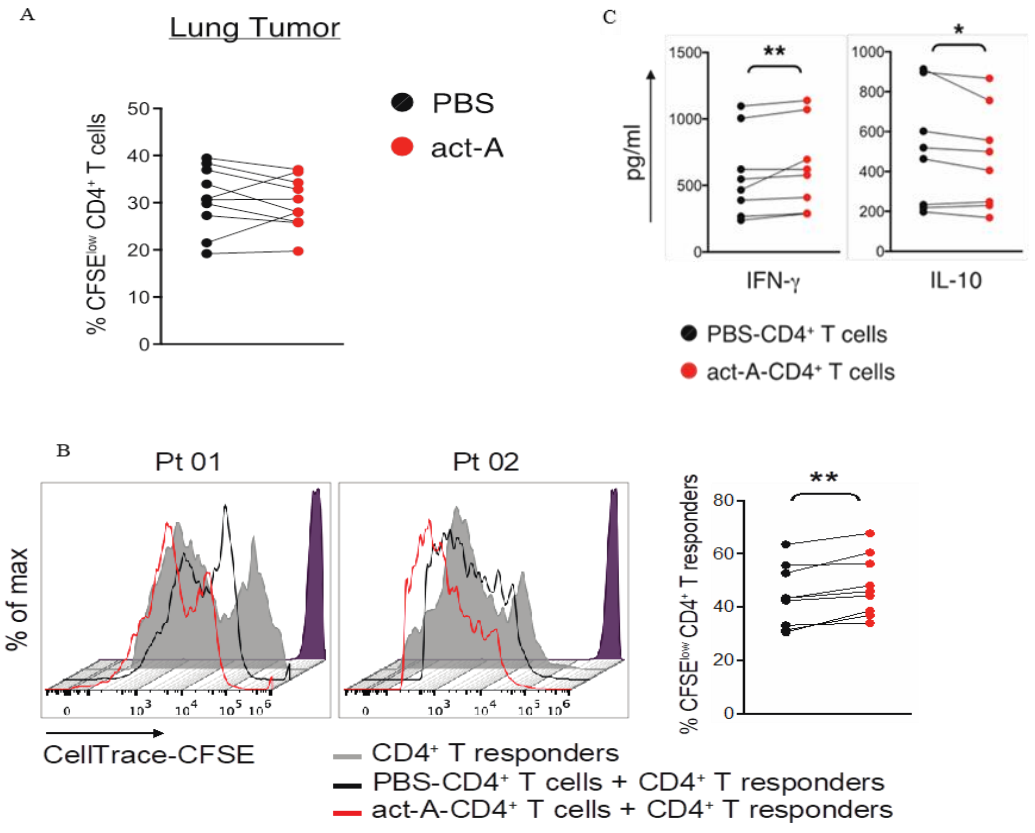

Fig. 13: Immunostimulatory capacity of CD4$^+$ T cells. *(A) PBS- or activin-A-treated human lung tumor infiltrating CD4$^+$ T cells were stimulated as in Fig.5A and labelled with CellTrace-CFSE. Cumulative data of CD4$^+$ T cell proliferation, measured by flow cytometry are depicted (n= 9 donors). (B) PBS- or activin-A-treated human lung tumor infiltrating CD4$^+$ T cells, generated as in Fig.6A, were CellTracker Red-labelled and co-cultured with CellTrace-CFSE-labelled autologous peripheral blood CD4$^+$ T responders. Representative flow cytometry plots showing T responder cell proliferation (left) and cumulative data (right) (n= 9 donors). (C) Cytokine levels in co-culture supernatants as described in Fig.7B (n= 8 donors). Data are mean ± SEM of triplicate wells. Statistical significance was obtained by Wilcoxon matched-pairs signed rank test; *P < 0.05 and **P < 0.01.*

4.8 CD8$^+$ T cells from the periphery supplemented with CM from act-A-CD4$^+$ T cell cultures were more prominent in killing the targeted primary lung cancer cells

Having observed that act-A-CD4$^+$ T cells were able to enhance the proliferation of autologous PB CD4$^+$ T cells compared to PBS-CD4$^+$ T cells, we sought to investigate whether these cells could also directly affect the cytotoxic capacity of autologous PB CD8$^+$ T cells. For this purpose, we used LDH cytotoxicity assays, where CM from act-A- or PBS-CD4$^+$ T cell cultures was supplemented overnight into CD8$^+$ T cell cultures from the periphery of the same patient (PB CD8$^+$ T cell cultures). Afterwards, they were co-cultured with autologous CD45$^-$ lung cancer cells. Notably, PB CD8$^+$ T cells supplemented with CM from act-A-CD4$^+$ T cell cultures were more prominent in killing the targeted primary lung cancer cells, compared to PB CD8$^+$ T cells supplemented with CM from PBS-CD4$^+$ T cell cultures (Fig. 14A). These results were further supported by NucView assay, using FACS analysis (Fig. 14B).

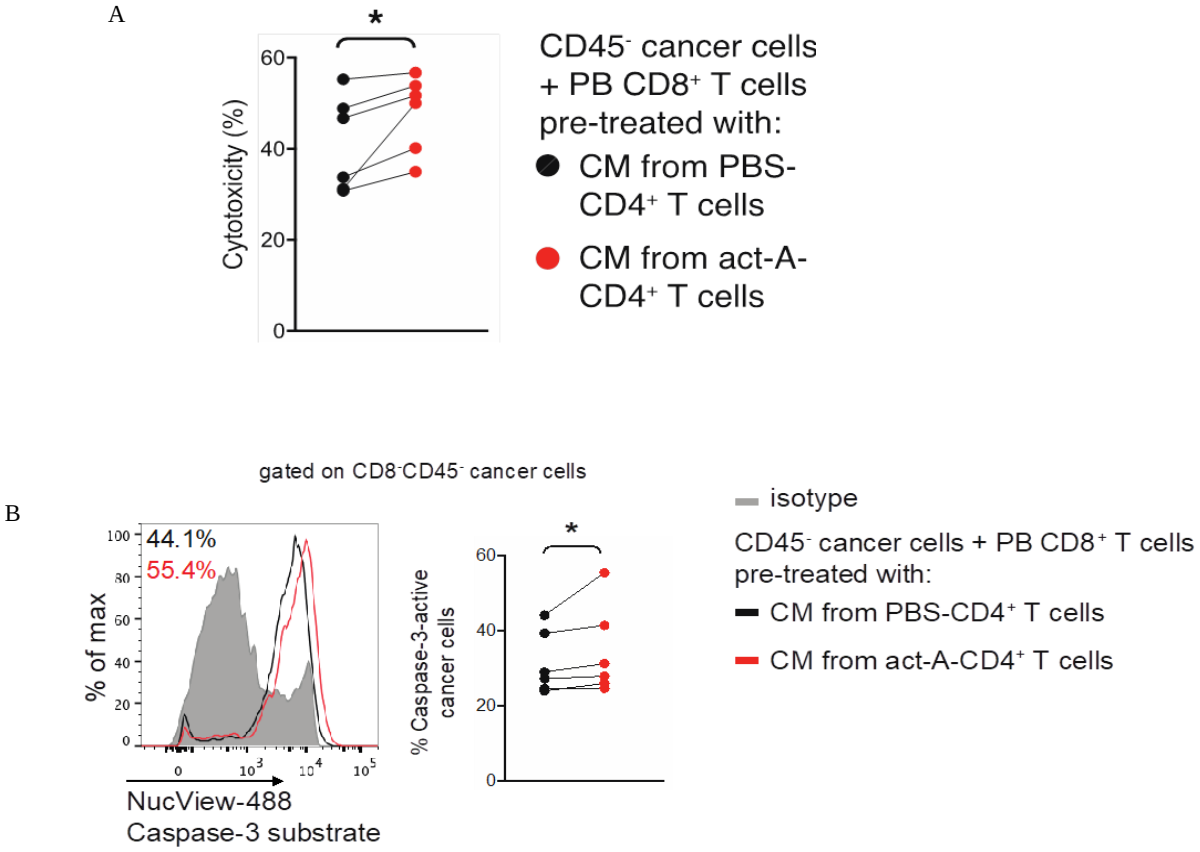

Fig. 14: *(A) CD45- lung cancer cells were co-cultured with autologous PB CD8+ T cells pre-treated with CM from act-A- or PBS-CD4+ T cell cultures. Cumulative data of cytotoxicity, assessed by LDH measurement in cell culture supernatants (n=6). (B) CD45- lung cancer cells were co-cultured with autologous PB CD8+ T cells pre-treated with CM from act-A- or PBS-CD4+ T cell cultures. Representative flow cytometry plot (left) and cumulative percentages (right) depicting caspase-3 activity of CD45- lung cancer cells (n=6). Cumulative data of cytotoxicity, assessed by LDH measurement in cell culture supernatants (n=6). Statistical significance was obtained by Wilcoxon matched-pairs signed rank test; *P < 0.05 and **P < 0.01.*

5. Discussion

Lung cancer is currently the leading cause of cancer in western societies with an approximate 5-year survival rate of 15%. It is characterized by high mortality rates and is responsible for nearly 20% of cancer-related deaths. During the last decade, translational research advances have achieved major breakthroughs in the understanding, diagnosis and management of lung cancer, particularly for the more common NSCLC. Unfortunately, the majority of lung cancer patients are diagnosed with advanced disease (stage III/IV), and for these patients, conventional treatment options including surgery, chemotherapy, and radiation are unlikely to result in cure. Therefore, there is an unmet need for the development of new and effective therapeutic interventions that can lead to durable tumor regression in lung cancer patients.

A principal pathogenetic process in cancer is that transformed cells proliferate incessantly and often exhibit low immunogenicity, thus managing to escape immune surveillance. Although, lung cancer was once thought of as a type of cancer that was poorly recognized by the immune system, recent trials have emerged it as a plausible target of immune-based therapies. Current breakthroughs in cancer immunotherapy have focused on blocking the function of T cell inhibitory receptors, known as immune checkpoints (Lesokhin et al., 2015). However, immune checkpoint therapy can lead to severe side effects, such as toxicities, and aberrant immune cell activation, inducing autoimmunity (Michot et al., 2016). Moreover, there is a significant cohort of lung cancer patients that remain unresponsive to immune checkpoint therapies. Thus, the imperative need to identify novel immune pathways that can be targeted more specifically has turned the attention of the scientific community towards attractive alternatives. Indeed, certain lung cancer patients have benefited from immunotherapies targeting the checkpoint inhibitors CTLA-4 and PD-1 and experienced durable remissions and prolonged survival. Nevertheless, novel immunotherapies have been also associated with serious adverse effects of inducing overt immune cell activation and autoimmunity. Additionally, a significant number of patients fails to respond to checkpoint immunotherapies.

Our group has extensively studied the role of activin-A in the regulation of T cell-mediated immune responses generated in lung airways, during inflammatory conditions. Activin-A signalling is initiated through binding to ActRII/IIB, which recruits ALK4, its major type I

receptor, forming a signal transducing heterodimer (X. Li et al., 2018). Pertinent to cancer, there is a growing body of evidence emphasizing the contrasting context- and cell type-dependent effects of activin-A in cancer pathogenesis. In addition to its direct inhibitory effects on cancer cells, activin-A restrains tumorigenesis by altering the tumor microenvironment. Studies by our group and others have shown that activin-A inhibits VEGF-induced human pulmonary endothelial cell proliferation *in vitro* and enhances the expression of the anti-angiogenic factors, VEGFR1 and IL-18 (Kaneda et al., 2011; Samitas et al., 2016). However, the precise role for activin-A on the development and enhancement of anti-tumor immune responses, in the context of lung cancer, remains elusive.

Preliminary studies by our group revealed, for the first time to our knowledge, that administration of activin-A *in vivo* in lung tumor-bearing mice leads to a striking regression in lung tumor formation. In line with the observations from lung cancer patients where activin-A is increased in the serum, we also observed, heightened levels of activin-A in the serum and lung homogenates of lung-tumor bearing mice in comparison to tumor-free mice. Considering that activin-A restrains the development of lung cancer, we sought to decipher the *in vivo* effect of endogenous activin-A on T cell-mediated immune responses in lung cancer. Disruption of activin-A's signalling had no impact on the either $CD45^+$ total leukocytes nor $CD3^+CD8^+$ and $CD3^+CD4^+$ T cells infiltrating the lung tumors of LLC-bearing mice. Nevertheless, disruption of activin-A's signalling, specifically on $CD4^+$ T cells, restrained the development of effective anti-tumor immune responses, as $CD4^+$ T cells isolated from the lung tumors of CD4/ALKA-KO mice secreted significantly less IFN-γ and TNF-α, but increased levels of the immunosuppressive cytokine IL-10 upon *ex vivo* antigenic stimulation, compared to $CD4^+$ T cells from WT mice. Through the disruption of activin-A's signalling on $CD4^+$ T cells we observed deteriorated T cell-mediated anti-tumor responses and augmented lung tumor growth *in vivo*. Moreover, $CD4^+$ T cells from lung tumors of CD4/ALKA-KO mice exhibited an exhausted profile exemplified by higher levels of PD-1, CTLA-4 and LAG-3 compared to WT mice, in addition to increased percentages of Foxp3-expressing regulatory T cells and IFN-γ-expressing $CD4^+$ T cells infiltrating the lung tumors of CD4/ALKA-KO mice compared to WT mice. Importantly, $CD4^+$ T cells from CD4/ALKA-KO mice appeared to be less capable to respond to antigenic stimulation in comparison to $CD4^+$ T cells from WT mice.

We further explored whether *ex vivo* administration of activin-A could enhance anti-tumor human T cell immune responses. For this purpose, we obtained primary lung cancer specimens, as well as part of the adjacent healthy lung tissue from NSCLC patients, isolated tumor infiltrated $CD4^+$ T cells and cultured them in the presence or absence of activin-A. We observed elevated levels of the effector cytokine TNF-α and decreased secretion of IL-10 in culture supernatants of TILs treated *ex vivo* with activin-A compared to PBS, while there was no difference on the secretion of IFN-γ between the two groups. Interestingly, culture supernatants from healthy lung leukocytes exhibited reduced levels of IFN-γ upon *ex vivo* activin-A administration and no differences in TNF-α and IL-10 levels, indicating that activin-A unveils different effects on T cells that infiltrate the lung tumors compared to those found in the adjacent healthy lung tissue.

We next sought to characterize the profile of human $CD4^+$ T cells infiltrating the lung tumors, upon *ex vivo* activin-A administration. We observed that act-A-$CD4^+$ T cells displayed heightened expression of the activation marker CD69 and reduced expression of the immune checkpoint inhibitory molecules PD-1, CTLA-4 and LAG-3 compared to PBS-$CD4^+$ T cells. In line with the aforementioned results, act-A-$CD4^+$ T cells demonstrated reduced expression of the transcription factor Foxp3, suggesting that activin-A renders human $CD4^+$ T cells that infiltrate the lung tumors with a less exhausted/suppressive phenotype. Despite the fact that act-A-$CD4^+$ T cells seemed to have acquired a more activated phenotype compared to PBS-$CD4^+$ T cells, both populations exhibited similar capacity to proliferate upon *ex vivo* polyclonal stimulation.

Next, in order to evaluate the immunostimulatory capacity of act-A-$CD4^+$ T cells in comparison to PBS-$CD4^+$ T cells, we cultured them along with autologous $CD4^+$ T cells obtained from the periphery (PB CD4+ T cells). Notably, we observed that $CD4^+$ T responder cells cultured along with act-A-$CD4^+$ T cells demonstrated increased proliferation, compared to those cultured with PBS-$CD4^+$ T cells. This observation was accompanied by the detection of heightened levels of IFN-γ and lower levels of the immunosuppressive cytokine IL-10 in these culture supernatants. Subsequently, we explored whether these cells could also directly affect the cytotoxic capacity of autologous PB $CD8^+$ T cells. For this purpose, CM from act-A- or PBS-$CD4^+$ T cell cultures were supplemented overnight into PB $CD8^+$ T cell cultures which were subsequently co-cultured with autologous $CD45^-$ lung cancer cells. Notably, PB CD8+ T cells supplemented with CM from act-A-$CD4^+$ T cell cultures were more prominent in killing the

targeted primary lung cancer cells, compared to PB $CD8^+$ T cells supplemented with CM from PBS-$CD4^+$ T cell cultures. To conclude, in a translational approach, we demonstrate that *ex vivo* activin-A administration drives human lung tumor infiltrating $CD4^+$ T cells towards a less exhausted phenotype and endows them with enhanced immunostimulatory capacity towards autologous $CD4^+$ and $CD8^+$ T cells, mimicking its anti-tumorigenic effects observed in our mouse models.

There is an imperative need to identify novel and more effective cancer immunotherapies that can boost specific aspects of the T cell response, without the adverse risk of inducing overt immune cell activation and autoimmunity. Taking together, our data uncover activin-A as an important orchestrator of anti-tumor T cell responses. Importantly, this study will also set the bases for further exploitation of activin-A's utilization as a prognostic tool and a potential target for immunotherapy devoid of potentially harmful side effects, in the setting of human lung cancer. We hope that these findings will shed new light into the mechanisms involved in the regulation of T cell responses in the tumor microenvironment, advance our understanding of the disease and enhance our alternatives towards novel immunotherapeutic approaches.

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References

Afrakhte, M., Morén, A., Jossan, S., Itoh, S., Sampath, K., Westermark, B., … Ten Dijke, P. (1998). Induction of inhibitory Smad6 and Smad7 mRNA by TGF-β family members. *Biochemical and Biophysical Research Communications*, *249*(2), 505–511. https://doi.org/10.1006/bbrc.1998.9170

Alsaab, H. O., Sau, S., Alzhrani, R., Tatiparti, K., Bhise, K., Kashaw, S. K., & Iyer, A. K. (2017). PD-1 and PD-L1 checkpoint signaling inhibition for cancer immunotherapy: mechanism, combinations, and clinical outcome. *Frontiers in Pharmacology*, *8*(AUG), 1–15. https://doi.org/10.3389/fphar.2017.00561

Antsiferova, M., Huber, M., Meyer, M., Piwko-Czuchra, A., Ramadan, T., MacLeod, A. S., … Werner, S. (2011). Activin enhances skin tumourigenesis and malignant progression by inducing a pro-tumourigenic immune cell response. *Nature Communications*, *2*(1), 510–576. https://doi.org/10.1038/ncomms1585

Antsiferova, M., Piwko-Czuchra, A., Cangkrama, M., Wietecha, M., Sahin, D., Birkner, K., … Werner, S. (2017). Activin promotes skin carcinogenesis by attraction and reprogramming of macrophages. *EMBO Molecular Medicine*, *9*(1), 27–45. https://doi.org/10.15252/emmm.201606493

Aranda, F., Chaba, K., Bloy, N., Garcia, P., Bordenave, C., Martins, I., … Senovilla, L. (2018). Immune effectors responsible for the elimination of hyperploid cancer cells. *OncoImmunology*, *7*(8), 1–9. https://doi.org/10.1080/2162402X.2018.1463947

Azuma, K., Ota, K., Kawahara, A., Hattori, S., Iwama, E., Harada, T., … Okamoto, I. (2014). Association of PD-L1 overexpression with activating EGFR mutations in surgically resected nonsmall-cell lung cancer. *Annals of Oncology*, *25*(10), 1935–1940. https://doi.org/10.1093/annonc/mdu242

Barfoed, A. M., Petersen, T. R., Kirkin, A. F., Thor Straten, P., Claesson, M. H., & Zeuthen, J. (2000). Cytotoxic T-lymphocyte clones, established by stimulation with the HLA- A2

binding p5365-73 wild type peptide loaded on dendritic cells in vitro, specifically recognize and lyse HLA-A2 tumour cells overexpressing the p53 protein. *Scandinavian Journal of Immunology*, *51*(2), 128–133. https://doi.org/10.1046/j.1365-3083.2000.00668.x

Baumeister, S. H., Freeman, G. J., Dranoff, G., & Sharpe, A. H. (2016). Coinhibitory Pathways in Immunotherapy for Cancer. *Annual Review of Immunology*, *34*(February), 539–573. https://doi.org/10.1146/annurev-immunol-032414-112049

Bhattacharjee, A., Richards, W. G., Staunton, J., Li, C., Monti, S., Vasa, P., … Meyerson, M. (2001). Classification of human lung carcinomas by mRNA expression profiling reveals distinct adenocarcinoma subclasses. https://doi.org/10.1073/pnas.191502998

Bonaventura, P., Shekarian, T., Alcazer, V., Valladeau-Guilemond, J., Valsesia-Wittmann, S., Amigorena, S., … Depil, S. (2019). Cold tumors: A therapeutic challenge for immunotherapy. *Frontiers in Immunology*, *10*(FEB). https://doi.org/10.3389/fimmu.2019.00168

Buchbinder, E. I., & Desai, A. (2016). CTLA-4 and PD-1 pathways similarities, differences, and implications of their inhibition. *American Journal of Clinical Oncology: Cancer Clinical Trials*, *39*(1), 98–106. https://doi.org/10.1097/COC.0000000000000239

Butts, C., Socinski, M. A., Mitchell, P. L., Thatcher, N., Havel, L., Krzakowski, M., … Shepherd, F. A. (2014). Tecemotide (L-BLP25) versus placebo after chemoradiotherapy for stage III non-small-cell lung cancer (START): A randomised, double-blind, phase 3 trial. *The Lancet Oncology*, *15*(1), 59–68. https://doi.org/10.1016/S1470-2045(13)70510-2

Cai, K. X., Lai, Y. T., Leung, C., Tam, P. K. H., Xu, R., & Mai, H. S. (2008). Suppression of lung tumor growth and metastasis in mice by adeno-associated virus-mediated expression of vasostatin. *Clinical Cancer Research*, *14*(3), 939–949. https://doi.org/10.1158/1078-0432.CCR-07-1930

Carbone, D. P., Gandara, D. R., Antonia, S. J., Zielinski, C., & Paz-Ares, L. (2015). Non-small-cell lung cancer: Role of the immune system and potential for immunotherapy. *Journal of Thoracic Oncology*, *10*(7), 974–984. https://doi.org/10.1097/JTO.0000000000000551

Carreno, B. M., Magrini, V., Becker-Hapak, M., Kaabinejadian, S., Hundal, J., Petti, A. A., … Linette, G. P. (2015). A dendritic cell vaccine increases the breadth and diversity of

melanoma neoantigen-specific T cells. *Science*, *348*(6236), 803–808. https://doi.org/10.1126/science.aaa3828

Cattaneo, R., Miest, T., Shashkova, E. V., & Barry, M. A. (2008). Reprogrammed viruses as cancer therapeutics: Targeted, armed and shielded. *Nature Reviews Microbiology*, *6*(7), 529–540. https://doi.org/10.1038/nrmicro1927

Chae, Y. K., Arya, A., Iams, W., Cruz, M. R., Chandra, S., Choi, J., & Giles, F. (2018). Current landscape and future of dual anti-CTLA4 and PD-1/PD-L1 blockade immunotherapy in cancer; lessons learned from clinical trials with melanoma and non-small cell lung cancer (NSCLC). *Journal for ImmunoTherapy of Cancer*, *6*(1). https://doi.org/10.1186/s40425-018-0349-3

Chen, F., Ren, P., Feng, Y., Liu, H., Sun, Y., Liu, Z., … Cui, X. (2014). Follistatin is a novel biomarker for lung adenocarcinoma in humans. *PLoS ONE*, *9*(10), 1–8. https://doi.org/10.1371/journal.pone.0111398

Chen, W., & Ten Dijke, P. (2016). Immunoregulation by members of the TGFβ superfamily. *Nature Reviews Immunology*, *16*(12), 723–740. https://doi.org/10.1038/nri.2016.112

Cohen, E. E., & Vokes, E. E. (2001). Locally advanced non-small cell lung cancer. https://doi.org/10.1007/s11864-001-0014-8

Collisson, E. A., Campbell, J. D., Brooks, A. N., Berger, A. H., Lee, W., Chmielecki, J., … Cheney, R. (2014). Comprehensive molecular profiling of lung adenocarcinoma: The cancer genome atlas research network. https://doi.org/10.1038/nature13385

Cooper, S., & Spiro, S. G. (2006). Small cell lung cancer: Treatment review. https://doi.org/10.1111/j.1440-1843.2006.00850.x

Corraliza-Gorjón, I., Somovilla-Crespo, B., Santamaria, S., Garcia-Sanz, J. A., & Kremer, L. (2017). New strategies using antibody combinations to increase cancer treatment effectiveness. *Frontiers in Immunology*, *8*(DEC). https://doi.org/10.3389/fimmu.2017.01804

Curran, M. A., Montalvo, W., Yagita, H., & Allison, J. P. (2010). PD-1 and CTLA-4 combination blockade expands infiltrating T cells and reduces regulatory T and myeloid cells within B16 melanoma tumors. *Proceedings of the National Academy of Sciences of the*

United States of America, *107*(9), 4275–4280. https://doi.org/10.1073/pnas.0915174107

Ding, L., Getz, G., Wheeler, D. A., Mardis, E. R., Mclellan, M. D., Sougnez, C., … Eric, S. (2009). *NIH Public Access*. *455*(7216), 1069–1075. https://doi.org/10.1038/nature07423.Somatic

Donovan, P., Dubey, O. A., Kallioinen, S., Rogers, K. W., Muehlethaler, K., Müller, P., … Constam, D. B. (2017). Paracrine Activin-A Signaling Promotes Melanoma Growth and Metastasis through Immune Evasion. *Journal of Investigative Dermatology*, *137*(12), 2578–2587. https://doi.org/10.1016/j.jid.2017.07.845

Dunn, G. P., Bruce, A. T., Ikeda, H., Old, L. J., & Schreiber, R. D. (2002). Cancer immunoediting: From immunosurveillance to tumor escape. *Nature Immunology*, *3*(11), 991–998. https://doi.org/10.1038/ni1102-991

Duraiswamy, J., Kaluza, K. M., Freeman, G. J., & Coukos, G. (2013). Dual blockade of PD-1 and CTLA-4 combined with tumor vaccine effectively restores T-cell rejection function in tumors. *Cancer Research*, *73*(12), 3591–3603. https://doi.org/10.1158/0008-5472.CAN-12-4100

Dyer, A., Baugh, R., Chia, S. L., Frost, S., Iris, Jacobus, E. J., … Lei, J. (2019). Turning cold tumours hot: oncolytic virotherapy gets up close and personal with other therapeutics at the 11th Oncolytic Virus Conference. *Cancer Gene Therapy*, *26*(3–4), 59–73. https://doi.org/10.1038/s41417-018-0042-1

Ferrarini, M., Ferrero, E., Dagna, L., Poggi, A., & Zocchi, M. R. (2002). Human γδ T cells: A nonredundant system in the immune-surveillance against cancer. *Trends in Immunology*, *23*(1), 14–18. https://doi.org/10.1016/S1471-4906(01)02110-X

Fife, B. T., & Bluestone, J. A. (2008). Control of peripheral T-cell tolerance and autoimmunity via the CTLA-4 and PD-1 pathways. *Immunological Reviews*, *224*(1), 166–182. https://doi.org/10.1111/j.1600-065X.2008.00662.x

Garber, M. E., Troyanskaya, O. G., Schluens, K., Petersen, S., Thaesler, Z., Pacyna-Gengelbach, M., … Petersen, I. (2001). Diversity of gene expression in adenocarcinoma of the lung. https://doi.org/10.1073/pnas.241500798

Garofalo, M., Villa, A., Rizzi, N., Kuryk, L., Mazzaferro, V., & Ciana, P. (2018). Systemic

administration and targeted delivery of immunogenic oncolytic adenovirus encapsulated in extracellular vesicles for cancer therapies. *Viruses*, *10*(10). https://doi.org/10.3390/v10100558

Gauthier, J., & Yakoub-Agha, I. (2017). Chimeric antigen-receptor T-cell therapy for hematological malignancies and solid tumors: Clinical data to date, current limitations and perspectives. *Current Research in Translational Medicine*, *65*(3), 93–102. https://doi.org/10.1016/j.retram.2017.08.003

George, J., Lim, J. S., Jang, S. J., Cun, Y., Ozretia, L., Kong, G., … Thomas, R. K. (2015). Comprehensive genomic profiles of small cell lung cancer. https://doi.org/10.1038/nature14664

Grah, J. J., Katalinic, D., Juretic, A., Santek, F., & Samarzija, M. (2014). Clinical significance of immunohistochemical expression of cancer/testis tumor-associated antigens (MAGE-A1, MAGE-A3/4, NY-ESO-1) in patients with non-small cell lung cancer. *Tumori*, *100*(1), 60–68. https://doi.org/10.1177/1430.15817

Guo, C., Manjili, M. H., Subjeck, J. R., Sarkar, D., Fisher, P. B., & Wang, X. Y. (2013). Therapeutic cancer vaccines. Past, present, and future. In *Advances in Cancer Research* (1st ed., Vol. 119). https://doi.org/10.1016/B978-0-12-407190-2.00007-1

Guo, Z. S., & Bartlett, D. L. (2014). Oncolytic viruses as platform for multimodal cancer therapeutics: A promising land. *Cancer Gene Therapy*, *21*(7), 261–263. https://doi.org/10.1038/cgt.2014.31

Haanen, J. B. A. G. (2017). Converting Cold into Hot Tumors by Combining Immunotherapies. *Cell*, *170*(6), 1055–1056. https://doi.org/10.1016/j.cell.2017.08.031

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: The next generation. *Cell*, *144*(5), 646–674. https://doi.org/10.1016/j.cell.2011.02.013

Harrison, C. A., Gray, P. C., Vale, W. W., & Robertson, D. M. (2005). Antagonists of activin signaling: Mechanisms and potential biological applications. *Trends in Endocrinology and Metabolism*, *16*(2), 73–78. https://doi.org/10.1016/j.tem.2005.01.003

Hedger, M. P., & De Kretser, D. M. (2013). The activins and their binding protein, follistatin-Diagnostic and therapeutic targets in inflammatory disease and fibrosis. *Cytokine and*

Growth Factor Reviews, *24*(3), 285–295. https://doi.org/10.1016/j.cytogfr.2013.03.003

Hellström, M., Phng, L. K., Hofmann, J. J., Wallgard, E., Coultas, L., Lindblom, P., … Betsholtz, C. (2007). Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis. *Nature*, *445*(7129), 776–780. https://doi.org/10.1038/nature05571

Hoda, M. A., Rozsas, A., Lang, E., Klikovits, T., Lohinai, Z., Torok, S., … Laszlo, V. (2016). High circulating activin A level is associated with tumor progression and predicts poor prognosis in lung adenocarcinoma. *Oncotarget*, *7*(12), 13388–13399. https://doi.org/10.18632/oncotarget.7796

Ilyas, S., & Yang, J. C. (2015). Landscape of Tumor Antigens in T Cell Immunotherapy. *The Journal of Immunology*, *195*(11), 5117–5122. https://doi.org/10.4049/jimmunol.1501657

Jiang, P., Gu, S., Pan, D., Fu, J., Sahu, A., Hu, X., … Liu, X. S. (2018). Signatures of T cell dysfunction and exclusion predict cancer immunotherapy response. *Nature Medicine*, *24*(10), 1550–1558. https://doi.org/10.1038/s41591-018-0136-1

Jung, K., & Choi, I. (2013). Emerging Co-signaling Networks in T Cell Immune Regulation. *Immune Network*, *13*(5), 184. https://doi.org/10.4110/in.2013.13.5.184

Kalli, M., Mpekris, F., Wong, C. K., Panagi, M., Ozturk, S., Thiagalingam, S., … Papageorgis, P. (2019). Activin a signaling regulates IL13Rα2 expression to promote breast cancer metastasis. *Frontiers in Oncology*, *9*(FEB), 1–12. https://doi.org/10.3389/fonc.2019.00032

Kaneda, H., Arao, T., Matsumoto, K., De Velasco, M. A., Tamura, D., Aomatsu, K., … Nishio, K. (2011). Activin A inhibits vascular endothelial cell growth and suppresses tumour angiogenesis in gastric cancer. *British Journal of Cancer*, *105*(8), 1210–1217. https://doi.org/10.1038/bjc.2011.348

Kariyawasam, H. H., Semitekolou, M., Robinson, D. S., & Xanthou, G. (2011). Activin-A: A novel critical regulator of allergic asthma. *Clinical and Experimental Allergy*, *41*(11), 1505–1514. https://doi.org/10.1111/j.1365-2222.2011.03784.x

Kelderman, S., & Kvistborg, P. (2016). Tumor antigens in human cancer control. *Biochimica et Biophysica Acta - Reviews on Cancer*, *1865*(1), 83–89. https://doi.org/10.1016/j.bbcan.2015.10.004

Kessler, J. H., Beekman, N. J., Bres-Vloemans, S. A., Verdijk, P., Van Veelen, P. A., Kloosterman-Joosten, A. M., … Melief, C. J. M. (2001). Efficient identification of novel HLA-A*0201-presented cytotoxic T lymphocyte epitopes in the widely expressed tumor antigen PRAME by proteasome-mediated digestion analysis. *Journal of Experimental Medicine*, *193*(1), 73–88. https://doi.org/10.1084/jem.193.1.73

Klingemann, H. (2014). Are natural killer cells superior CAR drivers? *OncoImmunology*, *3*(4), 37–41. https://doi.org/10.4161/onci.28147

Knickelbein, J. E., Khanna, K. M., Yee, M. B., Baty, C. J., Kinchington, P. R., & Hendricks, R. L. (2008). CTLA-4 Control over Foxp3+ Regulatory T cell function. *Science*, *322*(5899), 268–271. https://doi.org/10.1126/science.1164164

Kochenderfer, J. N., Wilson, W. H., Janik, J. E., Dudley, M. E., Stetler-Stevenson, M., Feldman, S. A., … Rosenberg, S. A. (2010). Eradication of B-lineage cells and regression of lymphoma in a patient treated with autologous T cells genetically engineered to recognize CD19. *Blood*, *116*(20), 4099–4102. https://doi.org/10.1182/blood-2010-04-281931

Kreiter, S., Vormehr, M., Van De Roemer, N., Diken, M., Löwer, M., Diekmann, J., … Sahin, U. (2015). Mutant MHC class II epitopes drive therapeutic immune responses to cancer. *Nature*, *520*(7549), 692–696. https://doi.org/10.1038/nature14426

Le, D. T., Wang-Gillam, A., Picozzi, V., Greten, T. F., Crocenzi, T., Springett, G., … Jaffee, E. M. (2015). Safety and survival with GVAX pancreas prime and Listeria monocytogenes-expressing mesothelin (CRS-207) boost vaccines for metastatic pancreatic cancer. *Journal of Clinical Oncology*, *33*(12), 1325–1333. https://doi.org/10.1200/JCO.2014.57.4244

Lee, C. H., Yelensky, R., Jooss, K., & Chan, T. A. (2018). Update on Tumor Neoantigens and Their Utility: Why It Is Good to Be Different. *Trends in Immunology*, *39*(7), 536–548. https://doi.org/10.1016/j.it.2018.04.005

Lesokhin, A. M., Callahan, M. K., Postow, M. A., & Wolchok, J. D. (2015). On being less tolerant: Enhanced cancer immunosurveillance enabled by targeting checkpoints and agonists of T cell activation. *Science Translational Medicine*, *7*(280), 1–12. https://doi.org/10.1126/scitranslmed.3010274

Li, X., Shao, C., Shi, Y., & Han, W. (2018). Lessons learned from the blockade of immune

checkpoints in cancer immunotherapy Ahmed Tarhini; Timothy Burns; Rahul Parikh; Guarvel Goel; Annie im. *Journal of Hematology and Oncology*, *11*(1), 1–26. https://doi.org/10.1186/s13045-018-0578-4

Li, Y., Hermanson, D. L., Moriarity, B. S., & Kaufman, D. S. (2018). Human iPSC-Derived Natural Killer Cells Engineered with Chimeric Antigen Receptors Enhance Anti-tumor Activity. *Cell Stem Cell*, *23*(2), 181-192.e5. https://doi.org/10.1016/j.stem.2018.06.002

Lian, Y., Meng, L., Ding, P., & Sang, M. (2018). Epigenetic regulation of MAGE family in human cancer progression-DNA methylation, histone modification, and non-coding RNAs. *Clinical Epigenetics*, *10*(1), 1–11. https://doi.org/10.1186/s13148-018-0550-8

Linnemann, C., Van Buuren, M. M., Bies, L., Verdegaal, E. M. E., Schotte, R., Calis, J. J. A., … Schumacher, T. N. M. (2015). High-throughput epitope discovery reveals frequent recognition of neo-antigens by CD4+ T cells in human melanoma. *Nature Medicine*, *21*(1), 81–85. https://doi.org/10.1038/nm.3773

Liu, Y., & Zeng, G. (2012). Cancer and innate immune system interactions: Translational potentials for cancer immunotherapy. *Journal of Immunotherapy*, *35*(4), 299–308. https://doi.org/10.1097/CJI.0b013e3182518e83

Loumaye, A., de Barsy, M., Nachit, M., Lause, P., van Maanen, A., Trefois, P., … Thissen, J. P. (2017). Circulating Activin A predicts survival in cancer patients. *Journal of Cachexia, Sarcopenia and Muscle*, *8*(5), 768–777. https://doi.org/10.1002/jcsm.12209

Lu, Y. C., Yao, X., Crystal, J. S., Li, Y. F., El-Gamil, M., Gross, C., … Robbins, P. F. (2014). Efficient identification of mutated cancer antigens recognized by T cells associated with durable tumor regressions. *Clinical Cancer Research*, *20*(13), 3401–3410. https://doi.org/10.1158/1078-0432.CCR-14-0433

Maemondo, M., Inoue, A., Kobayashi, K., Sugawara, S., Oizumi, S., Isobe, H., … Nukiwa, T. (2010). Gefitinib or chemotherapy for non-small-cell lung cancer with mutated EGFR. *New England Journal of Medicine*, *362*(25), 2380–2388. https://doi.org/10.1056/NEJMoa0909530

Marini, K. D., Croucher, D. R., McCloy, R. A., Vaghjiani, V., Gonzalez-Rajal, A., Hastings, J. F., … Neil Watkins, D. (2018). Inhibition of activin signaling in lung adenocarcinoma

increases the therapeutic index of platinum chemotherapy. *Science Translational Medicine*, *10*(451). https://doi.org/10.1126/scitranslmed.aat3504

Marshall, H. T., & Djamgoz, M. B. A. (2018). Immuno-oncology: Emerging targets and combination therapies. *Frontiers in Oncology*, *8*(AUG), 1–29. https://doi.org/10.3389/fonc.2018.00315

Mason, J., Farnworth, P. G., & Sullivan, J. (1996). *Characterization and Determination of the Biological Activities of Noncleavable High Molecular Weight Forms of lnhibin A and Activin A*. (September), 1055–1065.

Massagué, J., & Chen, Y. G. (2000). Controlling TGF-β signaling. *Genes and Development*, *14*(6), 627–644. https://doi.org/10.1101/gad.14.6.627

Matzuk, M. M., Kumar, T. R., & Bradley, A. (1995). Different phenotypes for mice deficient in either activins or activin receptor type II. *Nature*, Vol. 374, pp. 356–360. https://doi.org/10.1038/374356a0

Meuwissen, R., & Berns, A. (2005). Mouse models for human lung cancer. *Genes and Development*, *19*(6), 643–664. https://doi.org/10.1101/gad.1284505

Meyers, D. E., Bryan, P. M., Banerji, S., & Morris, D. G. (2018). Targeting the PD-1/PD-L1 axis for the treatment of non-small-cell lung cancer. *Current Oncology*, *25*(4), e324–e334. https://doi.org/10.3747/co.25.3976

Michot, J. M., Bigenwald, C., Champiat, S., Collins, M., Carbonnel, F., Postel-Vinay, S., … Lambotte, O. (2016). Immune-related adverse events with immune checkpoint blockade: A comprehensive review. *European Journal of Cancer*, *54*, 139–148. https://doi.org/10.1016/j.ejca.2015.11.016

Morianos, I., Papadopoulou, G., Semitekolou, M., & Xanthou, G. (2019). Activin-A in the regulation of immunity in health and disease. *Journal of Autoimmunity*, *104*(July), 102314. https://doi.org/10.1016/j.jaut.2019.102314

Musella, M., Manic, G., De Maria, R., Vitale, I., & Sistigu, A. (2017). Type-I-interferons in infection and cancer: Unanticipated dynamics with therapeutic implications. *OncoImmunology*, *6*(5). https://doi.org/10.1080/2162402X.2017.1314424

Namwanje, M., & Brown, C. W. (2016). Activins and inhibins: Roles in development, physiology, and disease. *Cold Spring Harbor Perspectives in Biology*, *8*(7). https://doi.org/10.1101/cshperspect.a021881

National Institutes of Health (NIH) - Turning Discovery into Health. (n.d.). Retrieved from https://www.nih.gov/about-nih/what-we-do/nih-almanac/national-cancer-institute-nci

Ni, X., Tao, J., Barbi, J., Chen, Q., Park, B. V., Li, Z., … Pan, F. (2018). YAP is essential for treg-mediated suppression of antitumor immunity. *Cancer Discovery*, *8*(8), 1026–1043. https://doi.org/10.1158/2159-8290.CD-17-1124

Nienke van Rooij, Marit M. van Buuren, Daisy Philips, Arno Velds, Mireille Toebes, Bianca Heemskerk, and L. J. A. van D. (2013). J OURNAL OF C LINICAL O NCOLOGY Tumor Exome Analysis Reveals. *JCO Diagnosis in Oncology*, *31*(32), 439–442. https://doi.org/10.1200/JCO.2012.47.7521

O'Neill, L. A. J., & Pearce, E. J. (2016). Immunometabolism governs dendritic cell and macrophage function. *Journal of Experimental Medicine*, *213*(1), 15–23. https://doi.org/10.1084/jem.20151570

Ogino, A., Choi, J., Lin, M., Wilkens, M. K., Calles, A., Xu, M., … Jänne, P. A. (2020). Genomic and pathological heterogeneity in clinically diagnosed small cell lung cancer in never/light smokers identifies therapeutically targetable alterations. https://doi.org/10.1002/1878-0261.12673

Okamoto, I., Takeda, Nishimura, & Nakagawa. (2011). Nimotuzumab, a novel monoclonal antibody to the epidermal growth factor receptor, in the treatment of non-small cell lung cancer. *Lung Cancer: Targets and Therapy*, 59. https://doi.org/10.2147/lctt.s16440

Okazaki, T., & Honjo, T. (2007). PD-1 and PD-1 ligands: From discovery to clinical application. *International Immunology*, *19*(7), 813–824. https://doi.org/10.1093/intimm/dxm057

Oliveres, H., Caglevic, C., Passiglia, F., Taverna, S., Smits, E., & Rolfo, C. (2018). Vaccine and immune cell therapy in non-small cell lung cancer. *Journal of Thoracic Disease*, *10*(Suppl 13), S1602–S1614. https://doi.org/10.21037/jtd.2018.05.134

Ott, P. A., Hu, Z., Keskin, D. B., Shukla, S. A., Sun, J., Bozym, D. J., … Wu, C. J. (2017). An immunogenic personal neoantigen vaccine for patients with melanoma. *Nature*, *547*(7662),

217–221. https://doi.org/10.1038/nature22991

Pangas, S. A., & Woodruff, T. K. (2000). Activin signal transduction pathways. *Trends in Endocrinology and Metabolism*, *11*(8), 309–314. https://doi.org/10.1016/S1043-2760(00)00294-0

Pico de Coaña, Y., Choudhury, A., & Kiessling, R. (2015). Checkpoint blockade for cancer therapy: Revitalizing a suppressed immune system. *Trends in Molecular Medicine*, *21*(8), 482–491. https://doi.org/10.1016/j.molmed.2015.05.005

Pirker, R. (2020). Conquering lung cancer: current status and prospects for the future. https://doi.org/10.1016/j.pulmoe.2020.02.005

Pol, J., Kroemer, G., & Galluzzi, L. (2016). First oncolytic virus approved for melanoma immunotherapy. *OncoImmunology*, *5*(1), e1115641. https://doi.org/10.1080/2162402x.2015.1115641

Postow, M. A., Chesney, J., Pavlick, A. C., Robert, C., Grossmann, K., McDermott, D., … Hodi, F. S. (2015). Nivolumab and ipilimumab versus ipilimumab in untreated melanoma. *New England Journal of Medicine*, *372*(21), 2006–2017. https://doi.org/10.1056/NEJMoa1414428

Ribas, A., & Wolchok, J. D. (2018). Cancer immunotherapy using checkpoint blockade. *Science*, *359*(6382), 1350–1355. https://doi.org/10.1126/science.aar4060

Robbins, P. F., Lu, Y. C., El-Gamil, M., Li, Y. F., Gross, C., Gartner, J., … Rosenberg, S. A. (2013). Mining exomic sequencing data to identify mutated antigens recognized by adoptively transferred tumor-reactive T cells. *Nature Medicine*, *19*(6), 747–752. https://doi.org/10.1038/nm.3161

Rodrigues, C. F. D., Serrano, E., Patrício, M. I., Val, M. M., Albuquerque, P., Fonseca, J., … Alpoim, M. C. (2018). Stroma-derived IL-6, G-CSF and Activin-A mediated dedifferentiation of lung carcinoma cells into cancer stem cells. *Scientific Reports*, *8*(1), 1–12. https://doi.org/10.1038/s41598-018-29947-w

Rodriguez-Canales, J., Parra-Cuentas, E., & Wistuba, I. I. (2004). Lung cancer. https://doi.org/10.1007/978-3-319-40389-2

Rosenberg, S. A., & Restifo, N. P. (2015). Adoptive cell transfer as personalized immunotherapy for human cancer. https://doi.org/10.1126/science.aaa4967

Saab, S., Zalzale, H., Rahal, Z., Khalifeh, Y., Sinjab, A., & Kadara, H. (2020). Insights Into Lung Cancer Immune-Based Biology, Prevention, and Treatment. https://doi.org/10.3389/fimmu.2020.00159

Sage, P. T., Francisco, L. M., Carman, C. V., & Sharpe, A. H. (2013). The receptor PD-1 controls follicular regulatory T cells in the lymph nodes and blood. *Nature Immunology*, *14*(2), 152–161. https://doi.org/10.1038/ni.2496

Sainz, B., Alcala, S., Garcia, E., Sanchez-Ripoll, Y., Azevedo, M. M., Cioffi, M., … Heeschen, C. (2015). Microenvironmental hCAP-18/LL-37 promotes pancreatic ductal adenocarcinoma by activating its cancer stem cell compartment. *Gut*, *64*(12), 1921–1935. https://doi.org/10.1136/gutjnl-2014-308935

Samitas, K., Poulos, N., Semitekolou, M., Morianos, I., Tousa, S., Economidou, E., … Gaga, M. (2016). Activin-A is overexpressed in severe asthma and is implicated in angiogenic processes. *European Respiratory Journal*, *47*(3), 769–782. https://doi.org/10.1183/13993003.00437-2015

Seder, C. W., Hnrtojo, W., Lin, L., Silvers, A. L., Wang, Z., Thomas, D. G., … Beer, D. G. (2009). Upregulated INHBA expression may promote cell proliferation and is associated with poor survival IN lung adenocarcinoma1 www.neoplasia.com. *Neoplasia*, *11*(4), 388–396. https://doi.org/10.1593/neo.81582

Sharma, A., Subudhi, S. K., Blando, J., Scutti, J., Vence, L., Wargo, J., … Sharma, P. (2019). Anti-CTLA-4 immunotherapy does not deplete Foxp3 þ regulatory T cells (Tregs) in human cancers. *Clinical Cancer Research*, *25*(4), 1233–1238. https://doi.org/10.1158/1078-0432.CCR-18-0762

Shi, L. Z., Fu, T., Guan, B., Chen, J., Blando, J. M., Allison, J. P., … Sharma, P. (2016). Interdependent IL-7 and IFN-γ signalling in T-cell controls tumour eradication by combined α-CTLA-4+α-PD-1 therapy. *Nature Communications*, *7*. https://doi.org/10.1038/ncomms12335

Shi, L. Z., Goswami, S., Fu, T., Guan, B., Chen, J., Xiong, L., … Sharma, P. (2019). Blockade of

CTLA-4 and PD-1 enhances adoptive t-cell therapy efficacy in an ICOS-mediated manner. *Cancer Immunology Research*, *7*(11), 1803–1812. https://doi.org/10.1158/2326-6066.CIR-18-0873

Shurin, M. R., Ma, Y., Keskinov, A. A., Zhao, R., Lokshin, A., Agassandian, M., & Shurin, G. V. (2016). BAFF and April from activina- Treated dendritic cells upregulate the antitumor efficacy of dendritic cells in vivo. *Cancer Research*, *76*(17), 4959–4969. https://doi.org/10.1158/0008-5472.CAN-15-2668

Siegel, R. L., Miller, K. D., & Jemal, A. (2020). Cancer statistics, 2020. https://doi.org/10.3322/caac.21590

Simon, S., & Labarriere, N. (2018). PD-1 expression on tumor-specific T cells: Friend or foe for immunotherapy? *OncoImmunology*, *7*(1). https://doi.org/10.1080/2162402X.2017.1364828

Sivanandam, V., LaRocca, C. J., Chen, N. G., Fong, Y., & Warner, S. G. (2019). Oncolytic Viruses and Immune Checkpoint Inhibition: The Best of Both Worlds. *Molecular Therapy - Oncolytics*, *13*(June), 93–106. https://doi.org/10.1016/j.omto.2019.04.003

Smith, D. C., Eisenberg, P. D., Manikhas, G., Chugh, R., Gubens, M. A., Stagg, R. J., … Sikic, B. (2014). A phase I dose escalation and expansion study of the anticancer stem cell agent demcizumab (Anti-DLL4) in patients with previously treated solid tumors. *Clinical Cancer Research*, *20*(24), 6295–6303. https://doi.org/10.1158/1078-0432.CCR-14-1373

Smits, N. C., & Sentman, C. L. (2016). *Bispeci fi c T-Cell Engagers (BiTEs) as Treatment of B-Cell Lymphoma*. *34*(10).

Swann, J. B., & Smyth, M. J. (2007). Review series Immune surveillance of tumors. *The Journal of Clinical Investigation*, *117*(5), 1137–1146. https://doi.org/10.1172/JCI31405.antigens

Thomas, R., Al-Khadairi, G., Roelands, J., Hendrickx, W., Dermime, S., Bedognetti, D., & Decock, J. (2018). NY-ESO-1 based immunotherapy of cancer: Current perspectives. *Frontiers in Immunology*, *9*(MAY). https://doi.org/10.3389/fimmu.2018.00947

Treatment Choices for Non-Small Cell Lung Cancer, by Stage. (n.d.). Retrieved from https://www.cancer.org/cancer/lung-cancer/treating-non-small-cell/by-stage.html

Tsuchida, K., Nakatani, M., Hitachi, K., Uezumi, A., Sunada, Y., Ageta, H., & Inokuchi, K.

(2009). Activin signaling as an emerging target for therapeutic interventions. *Cell Communication and Signaling, 7*, 1–11. https://doi.org/10.1186/1478-811X-7-15

Tumeh, P. C., Harview, C. L., Yearley, J. H., Shintaku, I. P., Taylor, E. J. M., Robert, L., … Ribas, A. (2014). PD-1 blockade induces responses by inhibiting adaptive immune resistance. *Nature*, *515*(7528), 568–571. https://doi.org/10.1038/nature13954

Vansteenkiste, J. F., Cho, B. C., Vanakesa, T., De Pas, T., Zielinski, M., Kim, M. S., … Altorki, N. (2016). Efficacy of the MAGE-A3 cancer immunotherapeutic as adjuvant therapy in patients with resected MAGE-A3-positive non-small-cell lung cancer (MAGRIT): a randomised, double-blind, placebo-controlled, phase 3 trial. *The Lancet Oncology*, *17*(6), 822–835. https://doi.org/10.1016/S1470-2045(16)00099-1

Wamsley, J. J., Kumar, M., Allison, D. F., Clift, S. H., Holzknecht, C. M., Szymura, S. J., … Mayo, M. W. (2015). Activin upregulation by NF-κB is required to maintain mesenchymal features of cancer stem-like cells in non-small cell lung cancer. *Cancer Research*, *75*(2), 426–435. https://doi.org/10.1158/0008-5472.CAN-13-2702

Wang, K., Wei, G., & Liu, D. (2012). CD19: a biomarker for B cell development, lymphoma diagnosis and therapy. *Experimental Hematology & Oncology*, *1*(1), 36. https://doi.org/10.1186/2162-3619-1-36

Weber, C., Telerman, S. B., Reimer, A. S., Sequeira, I., Liakath-Ali, K., Arwert, E. N., & Watt, F. M. (2016). Macrophage infiltration and alternative activation during wound healing promote MEK1-induced skin carcinogenesis. *Cancer Research*, *76*(4), 805–817. https://doi.org/10.1158/0008-5472.CAN-14-3676

Wei, S. C., Duffy, C. R., & Allison, J. P. (2018). Fundamental mechanisms of immune checkpoint blockade therapy. *Cancer Discovery*, *8*(9), 1069–1086. https://doi.org/10.1158/2159-8290.CD-18-0367

Weir, C., Hudson, A. L., Moon, E., Ross, A., Alexander, M., Peters, L., … Howell, V. M. (2014). Streptavidin: a novel immunostimulant for the selection and delivery of autologous and syngeneic tumor vaccines. *Cancer Immunology Research*, *2*(5), 469–479. https://doi.org/10.1158/2326-6066.CIR-13-0157

Woodruff, T. K. (1998). Regulation of cellular and system function by activin. *Biochemical*

Pharmacology, *55*(7), 953–963. https://doi.org/10.1016/S0006-2952(97)00477-2

Xia, Y., & Schneyer, A. L. (2009). The biology of activin: Recent advances in structure, regulation and function. *Journal of Endocrinology*, *202*(1), 1–12. https://doi.org/10.1677/JOE-08-0549

Yu, B., & Liu, D. (2019). Antibody-drug conjugates in clinical trials for lymphoid malignancies and multiple myeloma. *Journal of Hematology and Oncology*, *12*(1), 1–17. https://doi.org/10.1186/s13045-019-0786-6

Zeltsman, M., Dozier, J., McGee, E., Ngai, D., & Adusumilli, P. S. (2017). CAR T-cell therapy for lung cancer and malignant pleural mesothelioma. *Translational Research*, *187*, 1–10. https://doi.org/10.1016/j.trsl.2017.04.004

Zhu, J., Powis De Tenbossche, C. G., Cané, S., Colau, D., Van Baren, N., Lurquin, C., … Van Den Eynde, B. J. (2017). Resistance to cancer immunotherapy mediated by apoptosis of tumor-infiltrating lymphocytes. *Nature Communications*, *8*(1). https://doi.org/10.1038/s41467-017-00784-1