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Targeting the aging of tumor-associated neutrophils in lung cancer

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ABSTRACT

Neutrophils, as the most abundant leukocytes in the human body, have a crucial role in innate immunity. While their mode of action during infections is well understood, their influence on various chronic diseases, including cancer, still requires further comprehension. High intra-tumoral neutrophil density correlates with poor prognosis in non-small cell lung cancer (NSCLC) patients. However, targeting this immune population is delicate, as it could result in the life-threatening risk of neutropenia, a condition that compromises the body's ability to combat infections.

Neutrophils are highly plastic cells and recent publications describe tumor-infiltrating neutrophils as a heterogeneous population, with different subgroups that can either prevent or support tumor growth. Here, I used a genetically engineered mouse model of NSCLC, the *Kras*$^{Frt\text{-}STOP\text{-}Frt\text{-}G12D/WT}$; *p53*$^{Frt/Frt}$ (KP), to study tumor-associated neutrophils (TANs). In this model, TANs possess tumor-supportive capabilities and exhibit a longer lifespan compared to circulating neutrophils. We discovered that TANs overexpress the anti-apoptotic protein B cell lymphoma extra-large (Bcl-xL) to survive in the tumor microenvironment. *In vitro*, we could recapitulate the enhanced neutrophil survival by incubating bone marrow-derived neutrophils with tumor cell-conditioned medium. In addition, we have found that tumor cell-derived granulocyte-macrophage colony-stimulating factor (GM-CSF) triggered Bcl-xL expression, leading to enhanced neutrophil survival through JAK/STAT signaling. Targeting of Bcl-xL with a specific BH3 mimetic, A-1331852, enabled the blocking of the GM-CSF-induced neutrophil survival without affecting their normal lifespan. *In vivo*, oral administration of A-1331852 to KP mice reduced TAN survival and abundance, resulting in decreased tumor growth without causing neutropenia. Additionally, we observed that granulocyte colony-stimulating factor (G-CSF), a drug used to combat neutropenia in chemotherapy patients, increased the proportion of young TANs and further improved the anti-tumor effect of Bcl-xL blockade.

Correlative human tumor data also point to the role of Bcl-xL in promoting pro-tumoral neutrophil survival. Taken together, these preclinical results open an avenue for developing safe therapeutic strategies to block pro-tumor TANs without compromising normal and essential neutrophil functions.

Keywords: non-small cell lung cancer, tumor-associated neutrophils, tumor cell-conditioned medium, survival, anti-apoptotic, Bcl-xL, GM-CSF, G-CSF, neutropenia

RÉSUMÉ

Les neutrophiles, en tant que leucocytes les plus abondants du corps humain, jouent un rôle crucial dans l'immunité innée. Bien que leur mode d'action lors d'infections est bien décrit, leur influence sur diverses maladies chroniques, y compris le cancer, nécessite encore une compréhension approfondie. Une densité élevée de neutrophiles intra-tumoraux est correlée à un prognositic défavorable chez les patients atteints de cancer du poumon non à petites cellules (CPNPC). Cependant, cibler cette population immunitaire est délicat, car cela pourrait entraîner un risque potentiellement mortel de neutropénie, une condition qui compromet la capacité du corps à lutter contre les infections.

Les neutrophiles sont des cellules très plastiques et des publications récentes décrivent les neutrophiles infiltrant les tumeurs comme une population hétérogène, avec différents sous-groupes qui peuvent soit empêcher, soit favoriser la croissance tumorale. Ici, j'ai utilisé un modèle de souris génétiquement modifié du CPNPC, le *Kras*$^{Frt\text{-}STOP\text{-}Frt\text{-}G12D/WT}$; *p53*$^{Frt/Frt}$ (KP), pour étudier les neutrophiles associés aux tumeurs. Dans ce modèle, ces neutrophiles présentent des capacités de soutien à la tumeur et ont une durée de vie plus longue par rapport aux neutrophiles circulant dans le sang. Nous avons découvert que les neutrophiles associés aux tumeurs sur-expriment la protéine anti-apoptotique B cell lymphoma extra-large (Bcl-xL) pour survivre dans le microenvironement tumoral. Nous avons découvert que les cellules tumorales sécrètent le facteur de stimulation des colonies de granulocytes et de macrophages (GM-CSF), ce qui déclenche l'expression de la protéine anti-apoptotique Bcl-xL et conduit à une survie accrue des neutrophiles dans les tumeurs par le biais de la signalisation JAK/STAT. En utilisant un inhibiteur spécifique de Bcl-xL, le A-1331852, nous avons pu bloquer la survie des neutrophiles induite par GM-CSF sans affecter leur durée de vie normale. L'administration orale de A-1331852 aux souris a réduit la survie et l'abondance des TAN, entraînant une diminution de la croissance tumorale sans causer de neutropénie. De plus, nous avons observé que le facteur de stimulation des colonies de granulocytes (G-CSF), un médicament utilisé pour combattre la neutropénie chez les patients sous chimiothérapie, a augmenté la proportion de jeunes TAN et amélioré davantage l'effet anti-tumoral du blocage de Bcl-xL.

Les données corrélatives obtenues sur les tumeurs humaines indiquent également le rôle de Bcl-xL dans la promotion de la survie des neutrophiles pro-tumoraux. Dans l'ensemble, ces résultats précliniques ouvrent la voie au développement de stratégies thérapeutiques sûres visant à bloquer les neutrophiles pro-tumoraux sans compromettre les fonctions normales et essentielles des neutrophiles.

Mots-clés : cancer du poumon non à petite cellules (CPNPC), neutrophiles associés aux tumeurs, survie, anti-apoptotique, Bcl-xL, GM-SF, G-CSF, neutropénie

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CONTENTS

ABBREVIATIONS

AH	Adenomatous hyperplasia
APC	Antigen-presenting cell
ARG1	Arginase 1
AT2	Alveolar type 2
BCL	B cell lymphoma
CAF	Cancer-associated fibroblast
CCSP	Club cell secretory protein
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte-associated protein 4
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
GEMM	Genetically engineered mouse models
GMP	Granulocyte monocyte progenitor cells
HDN	High density neutrophil
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cell
ICB	Immune checkpoint blockade
IFN-y	Interferon gamma
IL	Interleukin
KRAS	Kirsten rat sarcoma virus
LDN	Low-density neutrophil
LUAD	Lung adenocarcinoma
LUSC	Lung squamous cell carcinoma
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
MDSC	Myeloid-derived suppressor cells
NSCLC	Non-small cell lung cancer
NETs	neutrophil extracellular traps
PD-1	Programmed-cell death protein 1
PD-L1	Programmed death ligand 1
ROS	Reactive oxygen species
SCLC	Small cell lung cancer
TAA	Tumor-associated antigens
TAM	Tumor-associated macrophage
TAN	Tumor-associated neutrophil
TCR	T-cell receptor
TGF-b	Transforming growth factor beta
TNM	Tumor-node-metastasis
Th1	T helper 1
Th2	T helper 2
Treg	Regulatory T cells
TME	Tumor microenvironment
TNF a	Tumor necrosis factor-alpha
VEGF	Vascular endothelial growth factor

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1 INTRODUCTION

1.1 CANCER

The average adult human body is composed of around 30 trillion cells which closely collaborate to maintain health and balance [1]. Precise regulation of cell division and elimination of aged or defective cells is necessary to ensure that tissues and organs remain functional. For this aim, control mechanisms exist to inhibit any excessive proliferation. Yet, when disruptions in these regulatory mechanisms take place, it can result in uncontrolled cell division, leading to the emergence of "tumors", abnormal masses or lumps of tissue that have the potential to invade neighboring tissues and metastasize to other parts of the body, ultimately giving rise to the condition known as "cancer".

The earliest known evidence of human cancer dates back to approximately 1.7 million years ago. The fossil, an early human ancestor discovered in the prehistorical Swartkrans cave in South Africa, showed signs of osteosarcoma, a cancer arising from the cells forming the bone structure [2]. The specimen that suffered from it was young and the appearance of the disease could not be linked to any environmental factor. Nowadays, however, the prevalence of cancer has increased due to multiple factors such as an aging population and changes in lifestyle and in the environment we live in. While it is estimated that nearly half of cancers are caused by preventable factors such as cigarette smoking, alcohol consumption, poor diet and inactivity [3], there are also genetic and environmental factors, known and unknown, that participate in the complex development of the disease. Although a lot of progress has been made, much work remains in order to fully seize the complexity of cancer and find ways to counteract its growth. As the burden of cancer is expected to grow, extensive research is needed to provide solutions and hope to all patients who suffer or will suffer from it.

1.2 THE TUMOR MICROENVIRONMENT

The effectiveness of cancer's ability to spread and grow relies on multiple factors, which are famously referred to as the "hallmarks of cancer" [4]. Initially, most of these hallmarks were intrinsic features of cancer cells, such as the ability to continuously signal for proliferation, evade mechanisms that suppress growth, or elude cell death. However, with time, additional hallmarks related to the microenvironment surrounding the tumor cells have been identified. Indeed, tumors are not solely comprised of dividing cancer cells. The surrounding stroma, a structure that supports and enables communication between different cells of a tissue, undergoes substantial changes and plays an important part in the development of the disease. The tumor microenvironment (TME) is composed of a complex network of macromolecules like collagen called the extracellular matrix (ECM), and of a large variety of cells, including cancer-associated fibroblasts (CAFs), endothelial cells, mesenchymal stromal cells, and different tumor-infiltrating immune cells [5]. Cancer cells are recognized for their

ability to influence neighboring cells to support their growth and survival, both the immune and non-immune cells [6]. All these components collectively promote primary tumor growth, progression, metastasis, and even resistance to therapy [7]. Targeting of the TME has therefore become one of the new battlegrounds of cancer research and advances in the understanding of the tumor microenvironment have led to the development of new therapeutic options for cancer patients, such as immunotherapy, and will hopefully contribute to some more [8,9].

1.2.1 THE TUMOR IMMUNE MICROENVIRONMENT

The interplay between cancer cells and stromal cells is a crucial factor in tumor progression. Over time, the role of the tumor immune microenvironment (TIME) has been increasingly recognized as a pivotal element in this process. By the late 19th century, physicians had observed that concurrent infection and fever could lead to tumor regression in some patients. Notably, Dr. William Coley, a 19th-century physician, made a significant contribution by creating the first cancer vaccine using heat-killed bacteria, known as “Coley’s toxins”, and published many reports on tumor regression happening upon injection of this cocktail [10]. However, Paul Ehrlich was the first person to formulate the hypothesis that immune cells have a protective role against cancer by recognizing and eliminating aberrant cells early on, reasoning that otherwise, the incidence of cancer would be much higher [11]. It is now widely described that the immune system is a major component of tumor control, yet it is also clear that tumor cells establish different mechanisms to escape the destruction by the immune system, while at the same time, they can stimulate inflammation to promote their proliferation [12,13].

1.2.1.1 THE MYELOID CELL COMPARTMENT

The immune system is composed of two essential sets of leukocytes: innate and adaptive immune cells. Cells from innate immunity provide rapid protection against pathogens and set up the stage for the cells of adaptive immunity, which is fundamental to developing long-term protection. **Macrophages** are part of the innate immune subgroup and play a crucial role in tissue inflammation and systemic immunity. These ubiquitous cells have diverse functions, including phagocytosis of bacteria and apoptotic cells, the elimination of pathogens using reactive oxygen species (ROS), antigen presentation, stimulation of inflammation and coordination of tissue repair [14,15]. Tumor-associated macrophages (TAMs) are frequently among the most abundant immune cells found in tumors [16] and their high density often predicts a poor prognosis in different cancers [17–19]. TAMs exhibit a large variety of functions that can either promote or inhibit tumor growth and progression. This duality in their function is a reflection of their plasticity. On one hand, macrophages can adopt a “classically” activated M1 phenotype upon exposure to pro-inflammatory factors such as IFN-γ, TNF-α or LPS, displaying tumor-inhibitory functions. M1 macrophages were shown to block angiogenesis and secrete cytokines that stimulate antitumoral immunity, such as IL-6 or IL-12 [20]. On the other hand, M2 macrophages are “alternatively” activated upon exposure to certain cytokines such

as IL-4, IL-10 or IL-13 and are known to promote cancer progression. They secrete arginase I (ARG1), an enzyme that degrades the arginine that is necessary for T cell function, and release a variety of other factors that can suppress the immune response, such as IL-10 and TGF-β [21]. M2 macrophages also support tumor growth and metastasis by stimulating angiogenesis through the secretion of vascular endothelial growth factor (VEGF) and by secreting extracellular matrix-degrading enzymes that remodel the ECM [22,23].

Macrophages are highly plastic cells and can adopt multiple roles in cancer. This plasticity was initially shown *in vitro*, with macrophages switching from an M1 to an M2 phenotype depending on the factors they are exposed to [24]. This polarization is however merely limiting nowadays, as a greater diversity of macrophage phenotypes is observed *in vivo* thanks to new multi-omics technologies such as single-cell RNA sequencing. Some tumor-supporting TAMs do not strictly display an M2 phenotype, indicating multiple activation states based on the specific microenvironment they are exposed to [25,26]. A greater understanding of TAM diversity will enable a better characterization of their functions and the underlying pathways and will help define effective ways to target tumor-promoting macrophages [19,27].

Neutrophils are the most abundant type of leukocytes found in the human blood. They are short-lived cells specialized in the neutralization and killing of pathogens and are quickly recruited from the circulation into the inflamed or injured tissue [28,29]. For a long time, neutrophils were thought to be fully matured cells with minimal transcriptional activity and no ability to change. However, studies from the last decade have contradicted this notion and revealed a panoply of new functions and phenotypes in neutrophils, especially in the context of cancer, where they can exert both anti- and protumoral roles. As the main interest of this thesis project are tumor-associated neutrophils (TANs), a separate and more extensive introduction will follow.

Dendritic cells (DCs) hold an essential place in the immune system, serving as key orchestrators of both innate and adaptive immunity. These specialized antigen-presenting cells are adept at capturing, processing, and presenting antigens to activate T cells, thus bridging the gap between the innate and adaptive arms of the immune response [30].

In the context of cancer, dendritic cells (DCs) are essential components of the immune system with a crucial role in activating the anti-tumoral response. They capture and process tumor antigens and migrate to the lymph nodes to present those antigens to T cells. This presentation serves as a crucial step in activating T cells, particularly cytotoxic T cells (CD8+ T cells), which are responsible for recognizing and eliminating cancer cells. However, like for macrophages or neutrophils, DCs can be shaped by the tumor microenvironment and their function can be impaired in immunosuppressive conditions [31]. This may involve inhibiting the maturation of dendritic cells or inducing the generation of regulatory T cells, which suppress immune responses [32]

1.2.1.2 THE LYMPHOID CELL COMPARTMENT

The adaptive immune cells represent the second arm of our immune system. They include T and B lymphocytes, which create a pathogen-specific memory that provides long-lasting protection and enables a rapid immune response if re-infection with the same pathogen occurs later in life [33]. T cells are also able to recognize and kill cancer cells. The multistep process through which T cells become activated and eliminate tumor cells is called the cancer-immunity cycle [34]. Tumor cells' genetic instability promotes the accumulation of mutations, resulting in the creation of neoantigens that can be recognized by immune cells. Professional antigen-presenting cells (APCs), including dendritic cells and macrophages, can present these tumor-associated antigens (TAA) to T cells through the major histocompatibility complex (MHC) molecules, which bind to the surface T-cell receptors (TCRs) [35,36]. T cells are normally primed for a specific antigen by APCs in the lymph nodes and, once they become activated, they rapidly proliferate and migrate to tumors to perform the killing of cancer cells [37,38]. Tumor cell death eventually leads to the release of new antigens that can be taken up by APCs and presented to new T cells, thus fueling a new cycle of activated T cells against cancer cells [39] (Figure 1.1).

T cells are classified into two main subsets based on the expression of CD4 or CD8 surface molecules that play a crucial role in the activation of T cells through the interaction with the major histocompatibility complex (MHC) [40]. CD8+ T cells are referred to as cytotoxic T lymphocytes (CTLs). They can recognize tumor cells carrying TAA on MHC class I molecules on their surface and immediately kill them through the release of perforin and granzyme B [41,42]. Most of the CD8 T cells undergo apoptosis, but some remaining cells will differentiate into memory T cells that will be able to relaunch a quick response if needed [43]. The presence of CTLs in tumors is correlated with better overall survival in cancer patients and with a better chance of response to immunotherapy [44–46].

CD4+ T cells are composed of different subtypes which are involved in the modulation of the adaptive immune response. The T helper 1 (Th1) subtype promotes the antigen presentation through MHC of class II by APCs, stimulates the activation of CD8+ T cells and enables the generation of memory CD8 T cells [47,48].Th1 cells are activated upon exposure to IL-12 and can secrete diverse cytokines that are linked to immune cell activation such as TNF, IFN-y, IL-2 and IL-12 [49,50.] In some human cancers, infiltration of Th1 cells correlates with favorable patient prognosis, especially in breast and lung cancer [51,52]. T helper 2 (Th2) lymphocytes are involved in opposite roles. They are specialized in promoting the humoral response (antibody production by B cells) mainly in the context of parasite and bacterial infections, or in chronic inflammatory conditions such as asthma [53]. In cancer, they are shown to dampen the immune response through the secretion of IL-4, IL-5 and IL-13, which also promote tissue repair and fibrosis and contribute to tumor-supporting chronic inflammation [54,55]. Lastly, the regulatory T cells (Tregs) are a subtype of immunosuppressive T cells whose role is to prevent abnormal or

excessive immune responses. They can efficiently inhibit CTLs [56,57] and are associated with poor patient survival in cancer [58,59].

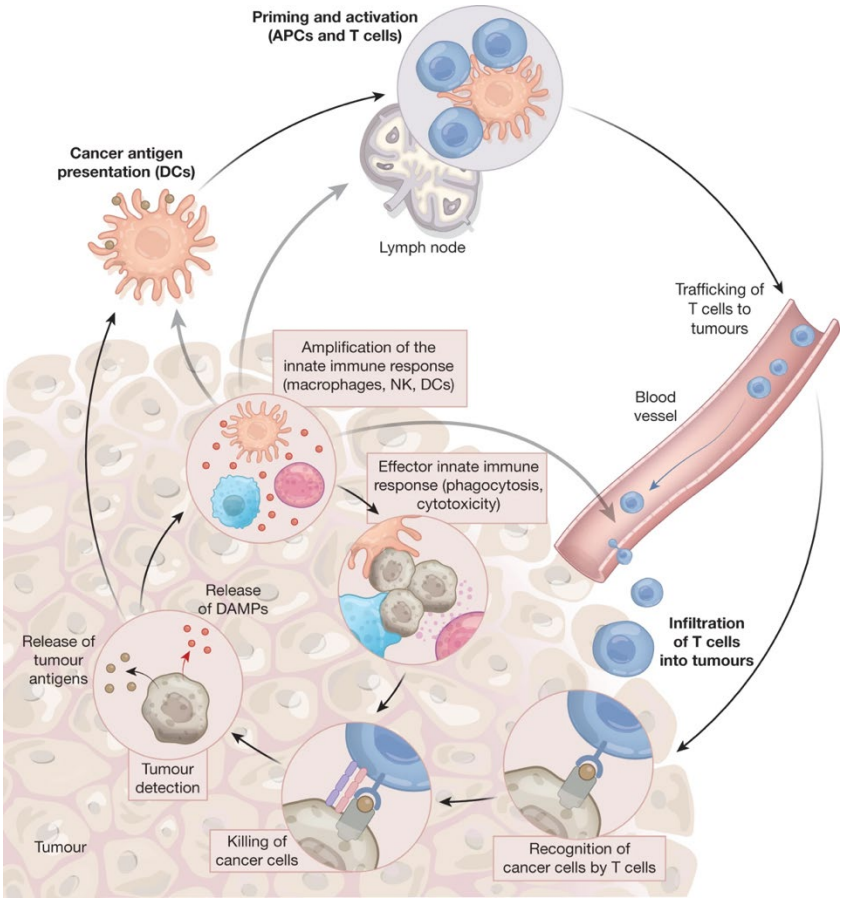


Figure 1.1 The cancer-immunity cycle

The cancer immunity cycle begins with the release of tumor antigens which are collected by antigen-presenting cells (APCs). APCs migrate to the lymph nodes and present the antigens to naïve T cells, which become activated. Activated cytotoxic T cells (CTLs) migrate to the tumor site, and recognize and eliminate the tumor cells. This destruction results in the release of additional tumor-associated antigens (TAAs), perpetuating the cycle. Scheme is taken from Demaria et al. (2019) [39]

1.2.1.3 THERAPEUTIC STRATEGIES INVOLVING CANCER IMMUNITY

Despite the existence of an elaborate immune system, cancer cells manage to counteract the immune system and prevail, a concept called immune evasion. In cancer, CD8+ T cells that are exposed to tumor antigens start to express checkpoint molecules at their surface over time. These inhibitory receptors represent a safety mechanism that ensures that the immune system does not overreact and cause collateral damage. Indeed, checkpoint inhibitors eventually lead to exhaustion and apoptosis of T cells [60]. However, these pathways are used by tumor cells to damper the response of T cells and thus escape anti-tumoral immunity [61–63].

The major immune checkpoints expressed by T cells are the programmed-cell death protein 1 (PD-1) and the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), which bind to PD-L1 and B7 ligands, respectively [63–67] (See Fig. 1.2). These ligands are expressed by various immune cells including dendritic cells, macrophages and even neutrophils [68]. Tumor cells also express these ligands, enabling them to inhibit T cells and escape the killing. PD-L1 expression in different tumor types has been associated with poor prognosis in certain cancers, including non-small cell lung cancer [69–71].

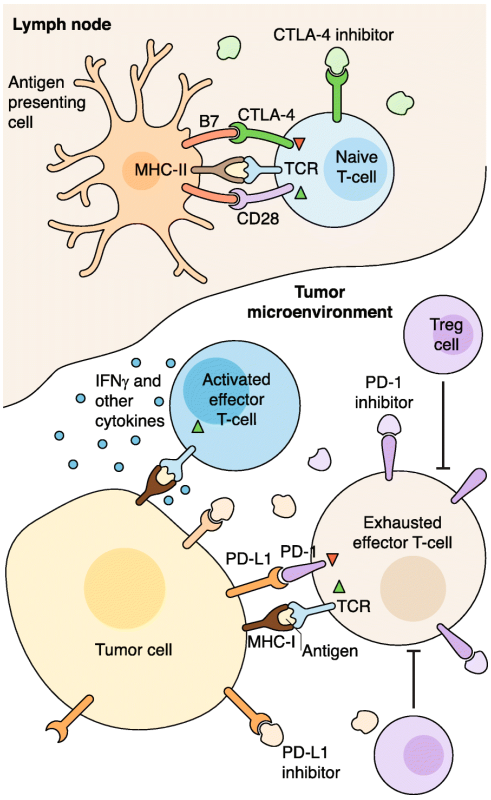


Figure 1.2 The Immune checkpoint blockade

The interaction between B7 ligands (CD80/CD86) on antigen-presenting cells and the CD28 receptor on naïve T cells provides a co-stimulatory signal and triggers T cell amplification. In contrast, the binding of B7 ligands to CTLA-4 expressed on T cells results in the suppression of their activity. The PD-L1 molecule, often found on tumor cells, induces anergy in activated effector cells by binding to its receptor PD-1 on those cells. The pharmacological inhibition of immune checkpoints using monoclonal antibodies, such as CTLA-4 inhibitors or PD-1/PD-L1 inhibitors, restores antitumor activity and alleviates immunosuppression. Scheme taken from Conway et al. (2018)

Major advances in the medical care of cancer patients come from the development of immunotherapies. Immune-checkpoint blockade (ICB) therapies are based on monoclonal antibodies binding to PD-1, CTLA-4 or their ligands, thus preventing the receptor-ligand binding responsible for T-cell exhaustion [72]. ICBs have demonstrated increased patient survival rates and sustained clinical response in multiple cancer types including lung cancer [73–76] . Nevertheless, these therapies are not universally effective, as some patients do not respond or develop acquired resistance to immunotherapies (reviewed in [77]). Both cell-autonomous and non-autonomous can cause tumor cell resistance to ICB [78–80]. Recruited myeloid cells for example (TAMs or TANs) can also curtail T cell functions through the secretion of immune-suppressive cytokines or by helping the tumors prevail through the secretion of angiogenic factors, altogether nullifying the effects of ICB therapy [81–84]. Considering this, new therapies that target the immunosuppressive tumor microenvironment are now considered for combination therapy with ICB [85,86]. Some of these approaches aim to selectively deplete subsets of pro-tumor myeloid cells or try to reprogram these cells toward anti-tumor functions [87]. Nevertheless, a better understanding of the heterogeneity of the components (both cellular and non-cellular) responsible for creating an immunosuppressive environment is needed in order to develop efficient strategies that can promote the efficacy of T-cell therapy in cancer.

1.3 LUNG CANCER

1.3.1 LUNG CANCER INCIDENCE AND MORTALITY

Although it is the second most diagnosed disease after breast and prostate cancer, lung cancer is still the worldwide leading cause of death due to cancer in both men and women. The Global Cancer Observatory reports that over 2.2 million people were diagnosed with lung cancer and that it accounted for 1.8 million deaths in 2020 [88]. These numbers are predicted to rise to more than 3.5 million new cases and 2.9 million deaths by 2040.

Tobacco smoke is composed of hundreds of compounds that may be harmful to human health and is the major cause of lung cancer [89]. Public health measures have helped reduce smoking habits and effectively decreased the incidence of the disease in high-income and developed countries [90,91]. Despite a decrease in smoking prevalence, the number of smokers worldwide is increasing, along with the global human population. Moreover, a high prevalence of smoking habit remains in low- and middle-income countries, especially in China, where more than half of men are regular smokers [90]. Besides smokers, lung cancer also impacts non or never-smokers[92]. In this case, the disease can be attributed to secondhand smoke, exposure to air pollution and environmental carcinogens such as radon or asbestos. Taken together, these observations explain why the number of new cases of lung cancer is predicted to grow.

The high lethality of lung cancer is due to several factors, the first one being the detection of the disease at an advanced stage, which mainly occurs when patients become symptomatic

[93]. In the USA, the Surveillance, Epidemiology and End Results program reported that for patients diagnosed with lung cancer between 2013 and 2019, the 5-year survival rate upon diagnosis was 19.4% in 2019, compared to 15.6% when assessed in 2011 with the previous years. This improvement in lung cancer survival is due both to a preventive approach that prompted a decrease in smoking and to the development of better therapeutic strategies based on targeted therapies and immunotherapy. Nevertheless, knowing that almost 60% of lung cancer cases are diagnosed at advanced stages [94], there is an important need for early diagnosis and efficient cancer therapies.

1.3.2 LUNG CANCER SUBTYPES

Lung cancer comprises several subtypes, which can first be histologically subdivided into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). SCLC represents around 15% of the total lung cancer cases and shows a significantly lower overall survival compared to NSCLC [95], which accounts for the other 85% of lung cancer cases [96,97]. NSCLC can then further be divided into lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC). These lung cancer subtypes differ histologically and genetically, but all are associated with high lethality and therapeutic choices are limited for both.

Besides the accurate diagnosis of lung cancer subtype, evaluation of the disease advancement is also essential for prognosis. Staging of lung cancer is based on the Union for International Cancer Control (UICC) TNM (tumor-node-metastasis) classification, which takes into consideration the appearance of the primary tumor and the presence or not of regional and distal lymph node metastasis and of distant metastasis [98]. The 5-year survival rate for lung cancer patients highly depends on the stage of the disease at diagnosis. Indeed, those diagnosed with localized disease had a 5-year survival rate of 62.8%, whereas this rate decreased to 34.8% for those diagnosed with regionally advanced disease and drastically to 8.2% for patients with advanced metastatic disease [99].

Cigarette smoke is responsible for almost all cases of SCLC and most NSCLC cases, but 10 to 20% of NSCLC occur in non-smokers [100]. These cases are mostly attributed to secondhand smoke and affect women predominantly [101]. For example, it has been shown that Japanese women, who are in vast majority non-smokers, have a significantly higher risk of developing lung adenocarcinoma if their husbands were smokers, and this risk increased with the number of cigarettes smoked by the husbands [102]. Due to irritants and carcinogens present in cigarette smoke, lung tumors present a high mutational burden [103]. In NSCLC, these carcinogens cause several mutations occurring in oncogenes and tumor suppressor genes. The most common genetic alterations are activating mutations in the epidermal growth factor receptor *EGFR* and the Kirsten rat sarcoma virus *KRAS*, followed by rearrangements in the *ALK* and *ROS1 proto-oncogenes*, *BRAF* mutations, *MET* amplifications, *HER2* mutations and *RET* gene fusions [104].

KRAS oncogenic mutations are present in about 25% of LUAD cases [105]. Almost all mutations that occur are found at the codons 12 and 13 in the exon 2 of the gene. Substitutions of the

amino acid glycine by cysteine (*KRAS* G12C) happen in about 45% of the cases and are primarily found in patients with a smoking history. The other common mutation is the *KRAS* G12D, where glycine is replaced by aspartic acid. This mutation is driving LUAD predominantly in patients who are non-smokers [106]. Moreover, mutations in the *p53* gene are found in 50% of the patients [107].

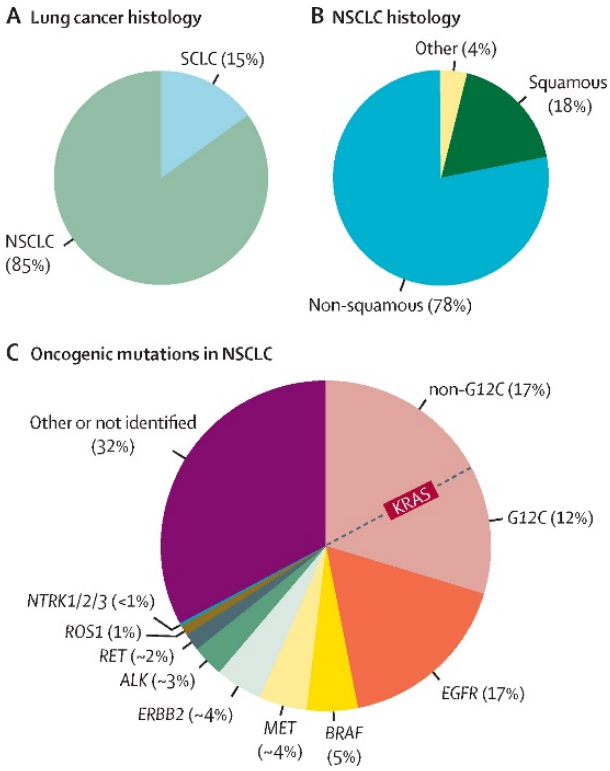


Figure 1.3 Lung cancer classification and NSCLC driver mutations

In (A) Subdivision of lung cancer into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). (B) Subivision of NSCLC into squamous and non-squamous histology (C) Common driver mutations in lung cancer and their frequency. Scheme is taken from Thai et al. (2021) [97]

1.3.3 NON-SMALL CELL LUNG CANCER (NSCLC)

The treatment plan for lung cancer patients takes into account several factors: the type of cancer, the advancement of the disease, the mutations present and the patient's general condition. All these factors will influence the therapeutic approach taken by clinicians, going from a curative goal for patients where the disease is diagnosed at an early stage and who are fit enough to support the treatments, to a more palliative approach for patients with advanced disease, where the aim is to prolong the overall survival while still maintaining a certain quality of life. It is interesting to note that smoking cessation after lung cancer diagnosis is significantly correlated with a better overall response to therapies and overall survival [108].

1.3.3.1 TREATMENT OPTIONS

1.3.3.1.1 SURGERY, CHEMOTHERAPY AND RADIOTHERAPY

For early-stage NSCLC, meaning for stages I and II, surgical resection is the standard of care [109]. In some cases, adjuvant chemotherapy is given after surgery [110111]. However, the 5-year recurrence-free survival (RFS) rate for stage I NSCLC is only around 80% after surgical resection [112]. Patients with stage III NSCLC have a heterogeneous disease and surgery can be considered in some cases, along with chemotherapy [110]. Recent advances in the fields of immune oncology and targeted therapies have revolutionized the treatment options of different cancers, including lung cancer (Fig.1.3). However, cytotoxic agents given as chemotherapy still remain a fundamental arm of lung cancer treatment, especially in patients that have progressed after targeted therapies or who do not respond to immunotherapy [113]. In the presence of an advanced disease and in the absence of targetable mutations, platinium-based chemotherapy (cisplatin, carboplatin) and pemetrexed, a cytostatic compound blocking DNA and RNA synthesis, are used as standard-of-care treatments [114].

Radiotherapy is commonly used in the treatment of NSCLC patients, with the aim of induce tumor shrinking. It can be used a primary treatment, especially for early-stage NSCLC, or in combination with surgery, chemotherapy, or immunotherapy depending on the stage and characteristics of the cancer. In more advanced stages of NSCLC, radiotherapy may be employed to alleviate symptoms, control tumor growth, or treat cancer that has spread to other areas [115,116] .

1.3.3.1.2 TARGETED THERAPIES

Enhanced comprehension of cancer biology has revealed molecular pathways that can be targeted for therapeutic purposes. In LUAD, the diagnostic procedure also includes molecular testing for treatable oncogenic mutations. This is not the case for SCLC [114]. Around 15% of Eurasian and 50% of Asian NSCLC patients show activating point mutations in the epidermal growth factor receptor (EGFR), whose activation sustains tumor cell proliferation. For these patients, targeted therapy against EGFR tyrosine kinase inhibitors (TKI) is available [117] (Fig. 1.3)

1.3.3.1.3 IMMUNOTHERAPIES

Immunotherapies have become part of the treatment plans for lung cancer patients. Despite the fact that these therapies have been life-changing for many[118,119], a substantial amount of patients either do not respond to the therapy or will develop resistance, as mentioned earlier. The selective pressure imposed by immune checkpoint inhibitors (ICIs) might drive the prevalence of non-immunogenic variants. In NSCLC, the resistance to anti-PD-1 was linked to the loss of neoantigens upon treatment [120]. Moreover, loss of human leukocyte antigen (HLA), a component of antigen-presenting processing and presentation, also leads to resistance to ICIs [121]. In NSCLC, loss of the HLA gene complex occurs in 40 % of the cases and it has been associated with resistance to immunotherapy [122]

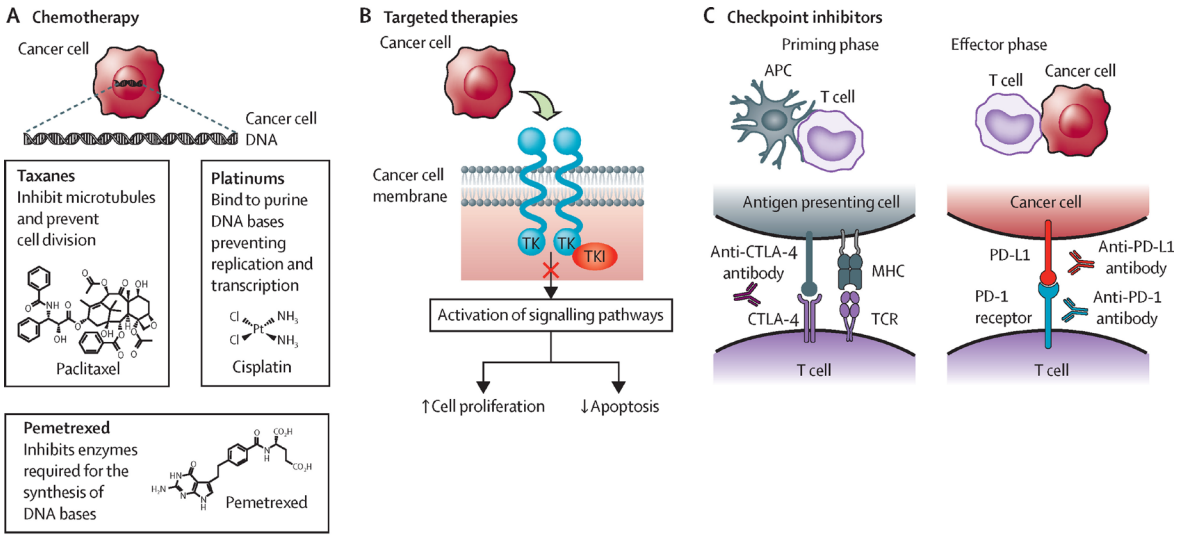


Figure 1.4 Pharmacological treatment options for lung cancer patients

In (A), general chemotherapeutic agents used to block cancer cell division. (B) Tyrosine kinase inhibitors (TKIs) inhibit the constitutive activation of kinase signaling and induces cell death. (C) immune checkpoint inhibitors that target PD-1, PD-L1 and CTLA-4 are also used in lung cancer patients. Scheme is taken from Thai et al. (2021) [97]

1.3.3.2 THE KP MOUSE AS A MODEL OF LUNG ADENOCARCINOMA

Effective cancer research depends on reliable models to study it. Initial mouse models of lung cancer were chemically induced with cigarette smoke or derived purified compounds which mainly gave rise to adenocarcinomas but also to some inconsistency through random mutagenic events [123,124]. Genetically engineered mouse models (GEMMs) allowed the introduction of genetic alterations in key oncogenes specific to lung cancer subtypes and prevented the variability occasioned by random mutagenesis. Futhermore, the generation of conditional alleles with tissue and time-specific activation enabled a better reconstitution of the human pathogenesis with spatiotemporal control of tumor initiation and reduced intra-animal variability.

As mentioned previously, the tumors of LUAD patients carry mutations in oncogenes and tumor suppressors, among which *KRAS* and *TP53* are the most prominent. Multiple mouse models therefore rely on the induction of oncogenic *Kras* for tumor initiation. However, as *Kras* is essential in mouse embryonic development [125], and as human lung cancer usually carries a single activating mutation in *KRAS*, these models rely on only one of the *Kras* alleles carrying the G12D oncogenic mutation. Addition of the Lox-STOP-Lox (LSL) cassette before the first exon allowed for a time-controlled activation of the $Kras^{LSL\text{-}G12D}$ allele, through the delivery of viral vectors carrying the Cre recombinase. Oncogenic Kras expression can be induced either in club cells, secretory cells in the lungs, or in alveolar type 2 (AT2) cells, both of which can give rise to lung adenocarcinoma. Cell-specific induction is mediated through the transduction with adenoviral vectors that carry the Cre recombinase under the control of Club cell secretory protein (CCSP/CC10) or surfactant protein C (SPC) promoters, which are expressed in club cells and AT2 cells, respectively [126]. These viral vectors can be delivered intranasally or instilled intratracheally and enable the synchronization of the tumor initiation, as well as the tumor burden, by adjusting the viral titer. Nevertheless, the $Kras^{LSL\text{-}G12D/WT}$ (K) mice showed limited tumor progression with the presence of lung adenomas but rarely advanced lung adenocarcinomas [127].

To overcome this, conditional loss of p53 was integrated into the mouse model with LoxP sites flanking the exons two to ten of the *Trp53* alleles. The $Kras^{LSL\text{-}G12D/WT}$; $Trp53^{Flox/Flox}$ (KP) mouse model exhibited a rapid tumor progression with large tumors that could recapitulate the human disease progression through the different tumor grades, going from the initial adenomatous hyperplasia (AH) to the most advanced adenocarcinoma [128,129]. Of note, the *Flp-Frt* system is also used to induce oncogenic Kras and p53 deletion. It functions similarly to the *Cre-lox* system and involves using Flippase (Flp) recombinase that will recognize the Flp recombinase target (Frt) sequences that can flank a genomic region of interest [130]. Therefore, the $Kras^{Frt\text{-}STOP\text{-}FRT\text{-}G12D}$; $Trp53^{Frt/Frt}$ is also used for the study of lung adenocarcinoma [131], leaving the possibility of using the *Cre-lox* system to conditionally activate or inactivate other genes of interest.

The KP mouse model closely recapitulates the major features of human disease and thus enabled significant advances in the understanding of cancer-cell intrinsic changes involved in tumor development. Nevertheless, it is worth mentioning that studying cancer immunity with this mouse model represents a challenge, as KP lung tumors are poorly immunogenic [132,133]. However, new mouse models have been developed to elicit the expression of neoantigens and stimulate anti-tumoral T cell responses, thereby providing a more effective solution to simulate and study of tumor immunology [134].

1.4 Neutrophils

1.4.1 Neutrophils in immunity and homeostasis

Neutrophils are the fastest cells to respond to damage, be it sterile or infection-driven. They are also the most abundant immune cell types in the human body. A study recently published calculated the number and mass of immune cells across organs in an average adult body and showed that neutrophils represent around 40% of the total number of leukocytes and 15% of their total weight (Sender et al., 2023). Interestingly, although neutrophils account for more than half of the immune cells in the blood, these circulating cells only represent about 20 % of the total neutrophils, the majority of which are found in the bone marrow, where they are produced in huge quantities (>10^{11} per day) [135]. Neutrophil differentiation and maturation within the bone marrow involves a series of distinct steps. Hematopoietic stem cells (HSCs) initiate the process by generating granulocyte-monocyte progenitor cells (GMP) [136,137]. These progenitors further differentiate into promyelocytes and myelocytes, forming clusters of actively dividing neutrophil progenitors. The next steps in the maturation pathways consist of nondividing progenitors transitioning from metamyelocyte to banded cell, and finally into mature neutrophils [138,139].

The role of neutrophils is to constitute a rapid and first barrier against pathogens and to instruct an efficient immune response. They can kill microorganisms in multiple ways. One of them is through neutrophil degranulation, which consists of the release of preformed cytosolic granules containing microbicidal components [140]. In addition, neutrophils are known for their capacity to generate superoxide, which is the basis for creating multiple reactive oxygen species (ROS) such as hydrogen peroxide (H_20_2) and Hypochlorous acid (HOCl), which they can use to kill microbes that they phagocytose [141]. Finally, they have the unique capacity of expulsing neutrophil extracellular traps (NETs), filaments of their DNA with proteins such as myeloperoxidase and elastase which can trap and kill bacteria [142].

1.4.2 Neutrophil phenotypic and functional heterogeneity in cancer

Neutrophils are often found in abundance in multiple cancers [143–146] and have been implicated in almost every aspect of cancer progression, through various tumor-promoting functions. However, they are also capable of anti-tumoral properties. In 2009, tumor-associated

neutrophil (TAN) plasticity was proposed, ranging from antitumor (N1) to protumor (N2) subsets, the same dichotomy applied to macrophages. This was due to the finding that TGF-β, expressed by tumor cells, was inducing a pro-tumorigenic phenotype in TANs in a mouse model of mesothelioma [147]. Indeed, TGF-β signaling blockade, using the TGF-β receptor inhibitor SM16, led to an increase in N1 neutrophils which displayed increased cytotoxicity against tumor cells and expressed immune-stimulatory cytokines that boosted CD8+ T cell response, altogether leading to a reduction in tumor growth in mice. In another study, myeloid cells isolated from the blood of patients with late-stage cancer expressed high levels of the TGF-β receptor II [148]. Deletion of myeloid-specific *Tgfbr2* inhibited metastasis formation in mouse models of spontaneous (injection of 4T1 breast cells into the mammary fat pad) and experimental (intravenous injection of murine 4T1, LLC lung, B16 melanoma, and MC26 colon cancer cell lines*)* mouse models of metastasis [148]. Alternatively, type I Interferon (IFN) signaling was shown to promote more N1-type neutrophils. In a mouse model, neutrophils from animals with a deficiency in this signaling exhibited an immature phenotype and were less efficient at tumor cell killing [149]. Accordingly, neutrophils extracted from melanoma patients could be reprogrammed towards a more N1 phenotype by incubation with IFN-α [149]. In the same sense, the metastatic burden in the lungs of mice lacking the receptor for IFN-α/β was increased compared to controls, due to high levels of Bv8 and MMP-9, molecules implicated in angiogenic and ECM remodeling, altogether indicating the importance of IFN signaling for neutrophil anti-tumoral functions [150].

Neutrophils are highly plastic cells and their diversity in phenotypes and functions in cancer reflects how they adapt in the different conditions imposed by the environment in which they evolve. Thanks to single-cell technologies, our understanding of neutrophil biology expanded and the distinction of multiple neutrophil subpopulations – or, rather, neutrophil states – beyond the simplistic and limiting N1/N2 dichotomy, opened up opportunities for possible specific targeting of tumor-supportive subsets, or modulation of neutrophils towards anti-tumor functions.

Before diving into the description of the tumor-supportive activities of neutrophils, it is perhaps important to mention the terms polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs) and granulocytic myeloid-derived suppressor cells (G-MDSCs). These terms are used in multiple publications to define a heterogeneous group of myeloid cells that accumulate in the blood of mice and humans with cancer and that show immune-suppressive capacities [151–153]. In these studies, neutrophils could be subdivided into low-density neutrophils (LDN) and high-density neutrophils (HDN) by centrifugation, as LDN showed lower granule content and an immature neutrophil phenotype [154,155]. LDNs accumulate with disease progression in lung cancer patients and correlate with poorer survival [154]. The observation of these low-density cells exhibiting mostly immune-suppressing functions encouraged the naming of this new category of myeloid suppressor cells. However, other subsets of neutrophils with tumor-

promoting functions were shown to express a mature phenotype. One example is the SiglecF-expressing TANs, a population of cells shown to promote lung tumor growth [156]. These TANs exhibited a mature phenotype with high nuclear segmentation and displayed prolonged survival within tumors [157]. This, and the fact that specific markers distinguishing MDSCs cells from neutrophils with immunosuppressive abilities have not been clearly established yet, we consider them as TANs with a certain phenotype in this project.

1.4.2.1 NEUTROPHIL RECRUITMENT TO TUMORS

At steady-state, the balance in the production, the differentiation and the maturation of neutrophils from the bone marrow is finely tuned and only fully differentiated neutrophils are released into the blood circulation. This balance can however be perturbed in pathological conditions and emergency granulopoiesis is triggered to produce additional cells to help control the disease, leading to the release of immature neutrophils in the process [158]. Once the infection is cleared, the balance can come back to its basal levels. Nevertheless, in chronic conditions such as cancer, sustained inflammation can foster myelopoiesis and increase the quantity of tumor-supportive neutrophils. Of note, the neutrophil to lymphocyte ratio (NLR) has been linked to poor prognosis in many cancers, including lung cancer [159–161]

Similar to inflamed tissues during infections, tumors release substances into the bloodstream that boost the production of granulocytes and increase the influx of neutrophils into the TME [162,163]. In this context, the secretion of **G-CSF** promotes the differentiation of hematopoietic cells into neutrophils and facilitates their entry into the bloodstream [164–167]. Additionally, G-CSF stimulates neutrophil migration toward tumors. In mouse models of lung and breast cancer, as well as in a spontaneous breast cancer metastasis model, Il-1β and IL-17 secreted by γδ T lymphocytes were shown to stimulate the secretion of G-CSF, which resulted in the recruitment of c-Kit+ tumor-infiltrating neutrophils [168,169], a marker associated to pro-metastatic myeloid cells [170,171] . In patients with cervical cancer, elevated G-CSF correlated with a shorter overall survival [172]. In a mouse model of this disease, G-CSF induced cisplatin resistance by recruiting MDSCs, which were shown to suppress the immune response and stimulate tumor angiogenesis through the secretion of Bv8 [172]. In addition, different subpopulations of 4T1 breast cancer cells were shown to seed to distinct secondary tissues [165], with some cancer cells seeding preferentially to the liver due to the secretion of G-CSF. In addition, in a mouse model of triple-negative breast cancer (TNBC), tumor cells mediated MDSC recruitment through the secretion of both G-CSF and GM-CSF [173]. **GM-CSF** stimulated neutrophil expansion in link with tumor progression in several studies [153,174,175], further confirming the induction of emergency granulopoiesis by cancer.

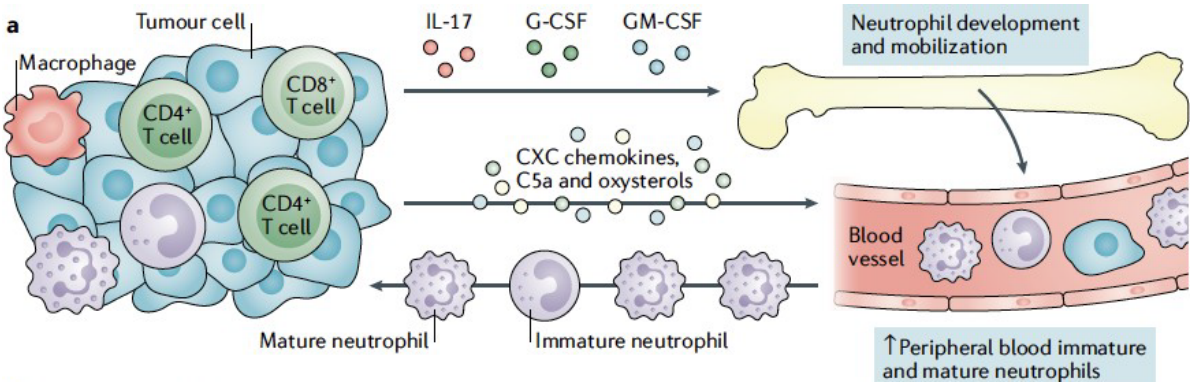


Figure 1.5 Neutrophil recruitment to tumors

Both mature and immature neutrophils can be mobilized from the bone marrow and attracted to the tumor through various mechanisms, including tumor-derived growth factors, cytokines, and chemokines such as Il-17or G-CSF. Figure taken from Jaillon et al., 2019

Besides growth factors, neutrophil trafficking is also mediated by several different cytokines. In a mouse model of colon cancer, CCL2 was shown to recruit immunosuppressive neutrophils [176]. The corresponding receptor, CXCR2, was expressed on TANs and stimulated their infiltration in KP mouse lung tumors, in response to SNAIL expression in tumor cells [177]. In an orthotopic mouse model of rhabdomyosarcoma, inhibiting the CXCR2-dependent neutrophil trafficking enhanced the efficacy of anti-PD1 blockade [178]. In breast cancer, the deletion of *Tp53* leads to increased secretion of WNT ligands, stimulating macrophages to secrete IL-1β aimed at neutrophil recruitment [179]

1.4.2.2 PRO-TUMORAL ROLES

Neutrophils are known for promoting cancer metastasis, where the formation of neutrophil extracellular traps (NETs) seems crucial for cancer progression [180,181]. Although NETs components can mediate tumor cell killing and limit cancer progression [182,183], most studies describe tumor-promoting roles. For example, it was recently shown that NET-DNA acts as a chemoattractant and promotes tumor cell metastasis to the liver [184]. Tumor cells could bind to NET-derived DNA through CCDC25, a transmembrane protein. This interaction modified the morphology of tumor cells and increased their invasive capacities. Along with this, patients with breast and colon cancer with metastases in the liver had significantly higher amounts of NETs in the metastases compared to the primary tumors and NET serum levels could serve as prognostic value for early-stage breast cancer [184].

TANs can also promote primary tumor outgrowth through different mechanisms. ROS production, for example, can cause tissue damage which can stimulate tumorigenesis[185]. Neutrophils can also secrete other factors that can induce DNA instability and provoke neoplasia, such as myeloperoxidase (MPO) [186]. One particular mechanism through which neutrophils sustain tumor progression is angiogenesis. TANs express proangiogenic factors

such as VEGF and Bv8, needed for the formation of new blood vessels to fuel cancer outgrowth [187–190].

Tumor-associated neutrophils also promote tumor progression through inhibition of anti-tumoral immunity, either through direct T-cell inhibition or by creating an immunosuppressive environment. Tumor cell-secreted GM-CSF, for example, promoted neutrophil survival and PD-L1 expression on their surface, leading to suppression of T cells in patients with gastric cancer [191]. TANs can also suppress T cell functions by secretion of arginase I (ARG1) which degrades the arginine found in the TME and is an important nutrient for T cells, thus hindering anti-tumor immunity [39,192,193]. TGF-β was shown to stimulate ARG1 expression in TANs in experimental mouse models of metastasis [148]. Moreover, high ARG1 expression was measured in MDSCs in the blood of NSCLC patients [194]. In addition, ROS production by neutrophils also led to the apoptosis or inhibition of the proliferation of T-cells [195,196].

TANs can also modulate their metabolism to support their immune-suppressive functions. Indeed, TANs stimulated by GM-CSF showed greater uptake of arachidonic acid by the upregulation of the fatty acid transport protein 2 (FATP2) and used it to generate prostaglandin E2, a mediator of immune suppression [197]. In patients with head and neck cancer and NSCLC, circulating PMN-MDSCs with overexpression of the lectin-type oxidized low-density lipoprotein (LDL) receptor-1 (LOX-1) correlated with poor patient survival [151]. In vitro, incubation of neutrophils with oxidized proteins polarized neutrophils into blocking T cell functions.

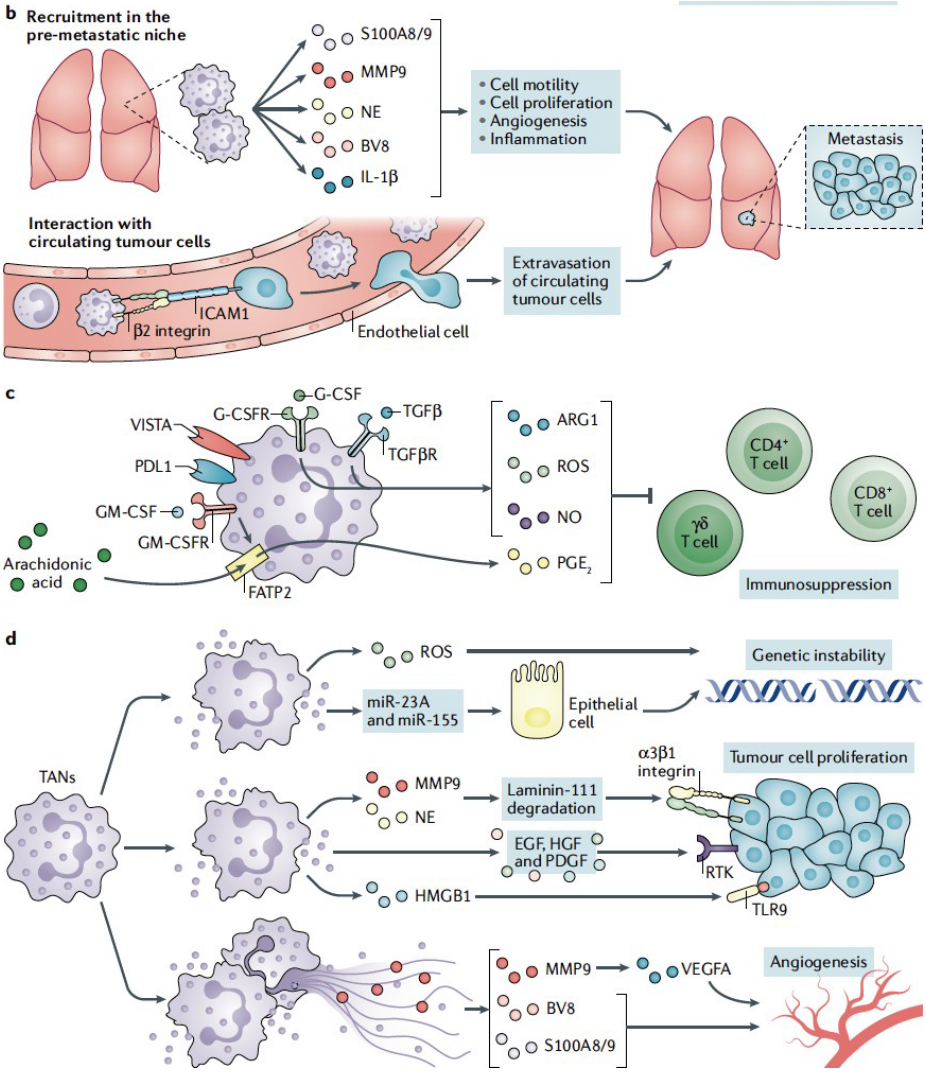


Figure 1.6 Neutrophil pro-tumoral roles

Neutrophils exert their influence on tumor growth through various mechanisms. (b) For instance, factors derived from neutrophils such as NE, BV8, and MMP9 contribute to cell proliferation and angiogenesis, fostering the formation of metastases. Additionally, circulating neutrophils play a role in promoting the extravasation of cancer cells, leading to the subsequent formation of metastases. (c) through the release of ARG1, ROS, NO, and the expression of immune checkpoint ligands, neutrophils hinder the activation of T cells, resulting in immune suppression. (d) neutrophils contribute to tumor initiation and progression by inducing genetic instability and supporting tumor cell proliferation and angiogenesis. Figure taken from Jaillon et al., 2019

1.4.2.3 ANTI-TUMORAL ROLES

As described, the pro-tumoral roles of neutrophils in cancer are diverse but often related to the induction of an immunosuppressive environment. Strikingly, a higher infiltration of neutrophils within tumors was associated with better prognoses in certain cancers such as colorectal cancer [198–200], and correlated with functional anti-tumor immunity, suggesting that neutrophils can, in some context, help establish an efficient tumor-killing by T cells. In early-stage lung cancer, TANs showed enhanced T cell stimulatory effect *in vitro* compared to blood-derived neutrophils [201]. Additionally, neutrophils were shown to develop antigen-presenting capacities and to stimulate CTLs against melanoma tumors [202]. A similar mode of action was described in HLA-DR+ neutrophils in early-stage lung cancer [203]. Neutrophils can also influence the behavior of other tumor-infiltrating immune cells. In hepatocellular cancer (HCC), TANs were shown to secrete CCL2 and CCL17, two cytokines that impacted macrophage and regulatory T-cell migration, leading to greater recruitment of these cells to the tumor [204].

Neutrophils possess several resources that endow them with tumor-killing ability. Elastase or cathepsin-G, both enzymes found in their granules, can provoke tumor cell death [205,206]. Neutrophils were also shown to produce nitric oxide (NO) that can induce tumor cell death [207]. Concretely, tumor-derived TNF stimulated the expression of the receptor tyrosine kinase MET in neutrophils, leading to enhanced neutrophil migration from endothelial cells into the tumor and to the production of inducible nitric oxide synthase (iNOS), necessary for NO generation. Specific deletion of *MET* in the immune compartment (achieved by transplanting C57BL/6 mice with *Met*-deficient bone marrow cells) led to increased tumor growth and metastasis of the subcutaneously injected murine Lewis lung carcinoma cells [207].

Use of catalase, an enzyme that mediates the decomposition of hydrogen peroxide (H_20_2), permitted to stop neutrophil tumoricidal activity [208]. Moreover, neutrophils can kill tumor cells through antibody-mediated trogoptosis, a type of necrotic cell death [209]. This process necessitates the recognition of the Fc region of the antibody (bound to cancer cells) by the FcγR expressed on the surface of neutrophils [209].

Nevertheless, some neutrophil-derived components seem to induce both pro- and anti-tumoral effects depending on the context. Cathepsin G for example, was upregulated in neutrophils, leading to increased IL-1b production by these cells and lung cancer promotion. ROS effect is puzzling as well and seems to be dependent on the condition. As previously mentioned, neutrophils have been shown to differentiate into APC-like cells with anti-tumoral properties, upon exposure to IFN-y and GM-CSF, in early NSCLC [201]. This was not the case in advanced disease, suggesting that neutrophils might exert anti-tumoral roles upon arrival to the tumor, before being polarized. Thus, a better understanding of the spatial but also temporal environment in which neutrophils evolve during cancer progression could help find targetable components that would prevent neutrophil polarization into tumor-promoting cells.

1.4.2.4 NEUTROPHILS IN LUNG CANCER

As for other cancers, a high neutrophil-to-lymphocyte (NLR) ratio also correlates with poor survival in lung cancer and an NLR greater than 2.5 was associated with lower progression-free survival in patients receiving immune checkpoint inhibitors [210–212], but the intra-tumoral presence of neutrophils is also prognostic. Indeed, the neutrophil transcript signature was identified as the strongest predictor of poor survival out of all other immune cells [213]. In this study, neutrophils were estimated to account for 2% of immune cells in NSCLC, based on transcript abundance, which is known to be low in these cells. However, since then, an extended flow-cytometry analysis revealed that neutrophils were the most abundant immune cell type in NSCLC, accounting for around 20% of immune cells [214] and their presence inversely correlated with CD8+ T cell and Th1 and Th17 cells. The Tregs were unaffected by the presence or not of neutrophils. Infiltration and high intra-tumoral density of neutrophils is correlated with a poor prognosis in patients and with treatment failure [177,214,215]. In the KP mouse model, neutrophils represent the major tumor-infiltrating cells as well [177]. Snail expression in tumor cells, a transcription factor involved in the epithelial-to-mesenchymal transition (EMT), together with TANs, promoted tumor progression in this model [177]. TANs were shown to alter angiogenesis and increase hypoxia, leading to the stabilization of Snail. In parallel, Snail-expressing tumor cells were shown to secrete a factor that induced Cxcl2 production by TANs, leading to greater neutrophil recruitment and infiltration, and thus fueling this vicious cycle. Moreover, deletion of the LKB1 tumor suppressor in lung tumors further increased the recruitment of tumor-promoting TANs [216].

Importantly, a study identified a subset of tumor-promoting TANs expressing high levels of the **SiglecF** marker on their surface, in the KP mouse model of LUAD [156]. These neutrophils expressed high levels of pro-angiogenic and immunosuppressive transcripts and were shown to have a direct impact on tumor growth when injected together with tumor cells in mice. Interestingly, it was also shown that the CXCL5 expressed by tumor cells was sufficient to promote TAN accumulation in both KP lung tumors and in tumors from a squamous cell lung cancer mouse model [217]. In addition, SiglecF+ TANs are present in even higher proportions in squamous tumors compared to adenocarcinomas [217]. A study published later recapitulated this finding in the KP mouse and showed that the deletion of Cxcl5, in addition to preventing the accumulation of TANs, enabled the infiltration of activated CD8+ T cells, which altogether overcame the resistance to PD-L1 checkpoint blockade [218].

A first step towards this was performed with single-cell RNA sequencing, which revealed the multiplicity of neutrophil populations in NSCLC patients and in an orthotopic lung cancer mouse model [219]. Five and six neutrophil populations were identified in human (hN1-hN5) and mouse (mN1-mN6) respectively, with conserved characteristics. Of interest, the hN5 subpopulation, characterized by high expression of peptidase inhibitor 3 (PI3), exhibited a similar transcriptional profile as the SiglecF+ TANs in mice. Of note, one human TAN subset,

displaying an important interferon response signature, did not have an analog among mouse TANs but among healthy tissue mouse neutrophils, suggesting that it did not manage to prevail in mouse tumor conditions but did so in humans.

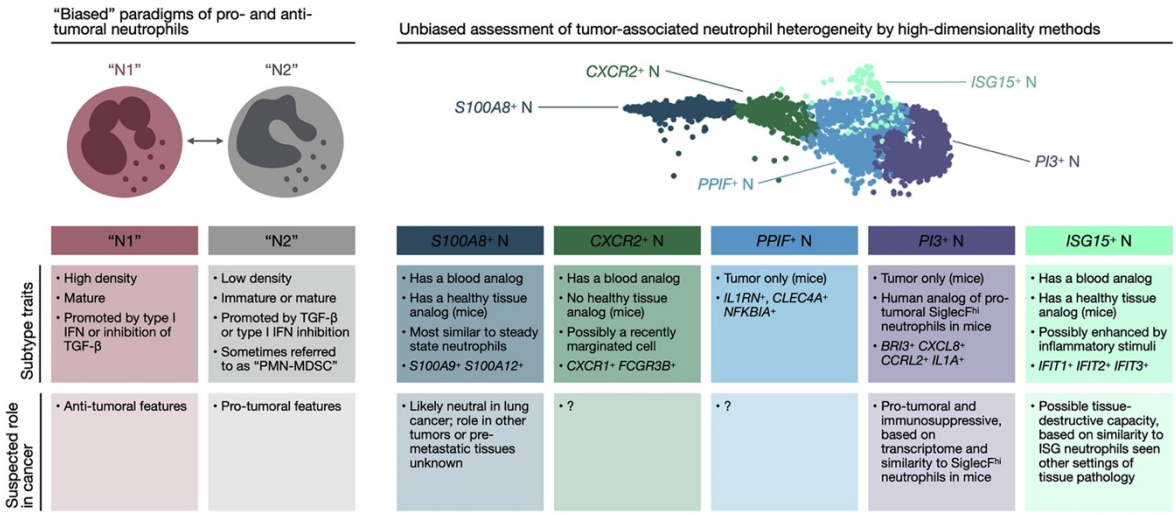


Figure 1.7 Tumor-associated neutrophil states

Neutrophils are capable of anti- and pro-tumoral properties and have been classified as N1 and N2 cells, respectively, due to their polarization in response to factors such as TGF-β or IFN-γ. However, this classification does not really apply to the diversity of neutrophils seen *in vivo*. High-resolution studies now suggest that neutrophils exist with different polarization states. One example is given by the study from Zilionis et al., (2019), for which the scheme on the right represents neutrophil states after unbiased clustering with single-cell RNA sequencing (human NSCLC). Figure taken from [220]

1.4.2.4.1 Neutrophils' prolonged survival in lung cancer

Neutrophils were long neglected in cancer research, possibly due to the belief that their short lifespan might not significantly affect chronic diseases like cancer. Assessing the lifespan of neutrophils in humans is challenging, but a recent study revealed that neutrophils, in homeostatic conditions, displayed different half-lives in some organs compared to blood [159]. In the absence of inflammation, however, neutrophils in the circulation progressively lose their capacity to create NETs or release their granule content as they become older [221]. These aged neutrophils then upregulate the expression of CXCR4, which drives their return to the bone marrow, where they will be eliminated by macrophages. This rapid turnover in the neutrophil pool is believed to prevent the deleterious effects neutrophils could have on the body.

In the KP mouse model, SiglecF+ TANs were shown to abnormally survive within lung tumors [157,222]. This subset of neutrophils are mature and non-proliferative cells, and were shown to rely on increased glycolytic metabolism to persist [222]. Indeed, deletion of the glucose transporter 1 (Glut1) in neutrophils led to reduced SiglecF+ TANs, increased neutrophil turnover and delayed tumor growth in these mice. Neutrophils therefore metabolically adapted

in order to survive in the tumor and this in turn sustained neutrophil polarization into tumor-supportive SiglecF+ cells.

There is no direct analog for SiglecF in humans, however, the conservation of tumor-associated neutrophil phenotypes across species will facilitate and enable the discovery of the mechanisms underlying neutrophil modulation in the TME. TANs extended lifespan, for example, could be triggered by different factors from the tumor microenvironment such as secreted factors (tumor-derived or not) or hypoxia. A better understanding of how neutrophil aging within tumors occurs could help find useful and targeted therapies to overcome their influence on tumor growth and resistance to therapy.

1.5 Apoptosis and cancer

1.5.1 Intrinsic apoptosis: an overview

Apoptosis is an evolutionarily conserved mechanism of cell death that maintains organismal homeostasis and function [223,224]. The process of apoptosis is very tidy. Once initiated, apoptotic cells undergo several morphological changes, starting from cell shrinkage and DNA condensation and fragmentation. This is followed by the disintegration of the organelles, causing the typical membrane blebbing. The final steps consist of the collapse of the cell unity and the formation of the apoptotic bodies, pieces of cellular fragments. These are eventually removed by macrophages through phagocytosis[225].

Apoptosis is initiated through intrinsic or extrinsic signaling pathways and can be activated by multiple factors. Both pathways end up in the activation of caspases, protease enzymes responsible for the degradation of essential cellular components. On one side, DNA damage, oncogene activation, metabolic stress and hypoxia can induce apoptosis through the intrinsic pathway, also known as the mitochondrial apoptotic pathway, which relies on the release of cytochrome c from the mitochondria into the cytosol. The presence of cytochrome c in the cytosol is considered a “point of no return”, as it then binds to the apoptotic protease-activating factor 1 (Apaf-1), a protein that assembles into a heptameric complex known as the apoptosome and which is responsible for the downstream cleavage and activation of caspases that will carry out the degradation of cellular components [226]. On the other side, the activation of the extrinsic pathway is mediated through the binding of death receptors by specific ligands from the tumor necrosis factor superfamily. This pathway results in the activation of caspases in the cytosol and the degradation of proteins essential for cell viability, therefore leading to cell death [227].

1.5.2 Bcl-2 family of proteins

Intrinsic apoptosis is tightly regulated by the Bcl-2 family of proteins. Bcl-2 was the first protein identified in the 1980s in the context of B-cell lymphoma after which it was named [228]. The other proteins discovered since then were integrated into the Bcl-2 family due to the presence

of one or more Bcl-2 homology (BH) domains, which are essential for their interaction. There are four domains in total, simply named BH1, BH2, BH3 and BH4. Proteins of the Bcl-2 family are categorized into one of three subgroups: anti-apoptotic Bcl-2 homologs, pro-apoptotic BH3-only "activator" proteins and downstream pro-apoptotic "effector" proteins[229]. Proteins of this family intricately interact with each other and the balance between pro and anti-apoptotic members dictates whether the cell will undergo apoptosis or not [230,231]. Initiation of apoptosis starts with the recruitment to the mitochondria of BH3-only proteins (composed of only the BH3 domain). Proteins from this category are Bim, Bid, Puma, Bmf, Hrk and Noxa. They can directly bind to Bak and Bax, which act as final pro-apoptotic inducers of apoptosis by oligomerizing and forming pores at the outer mitochondrial membrane (often referred to as the mitochondrial outer membrane permeabilization, MOMP), resulting in the release of cytochrome c [232]. To counteract this, anti-apoptotic (pro-survival) proteins can either bind to BH3-only proteins and block their activity, or directly bind to Bax or Bak and prevent the permeabilization of the mitochondrial membrane. This group is composed of the Bcl-2, Bcl-w, Bcl-xL, A1/Bfl-1 and myeloid leukemia cell differentiation protein (Mcl-1) proteins. Anti-apoptotic proteins are able to sequester the pro-apoptotic members using their BH3-binding pocket, a hydrophobic groove that can bind the BH3 motifs of pro-apoptotic proteins [233–235]. Whether a cell will undergo apoptosis depends on the quantity of pro- and anti-apoptotic proteins present.

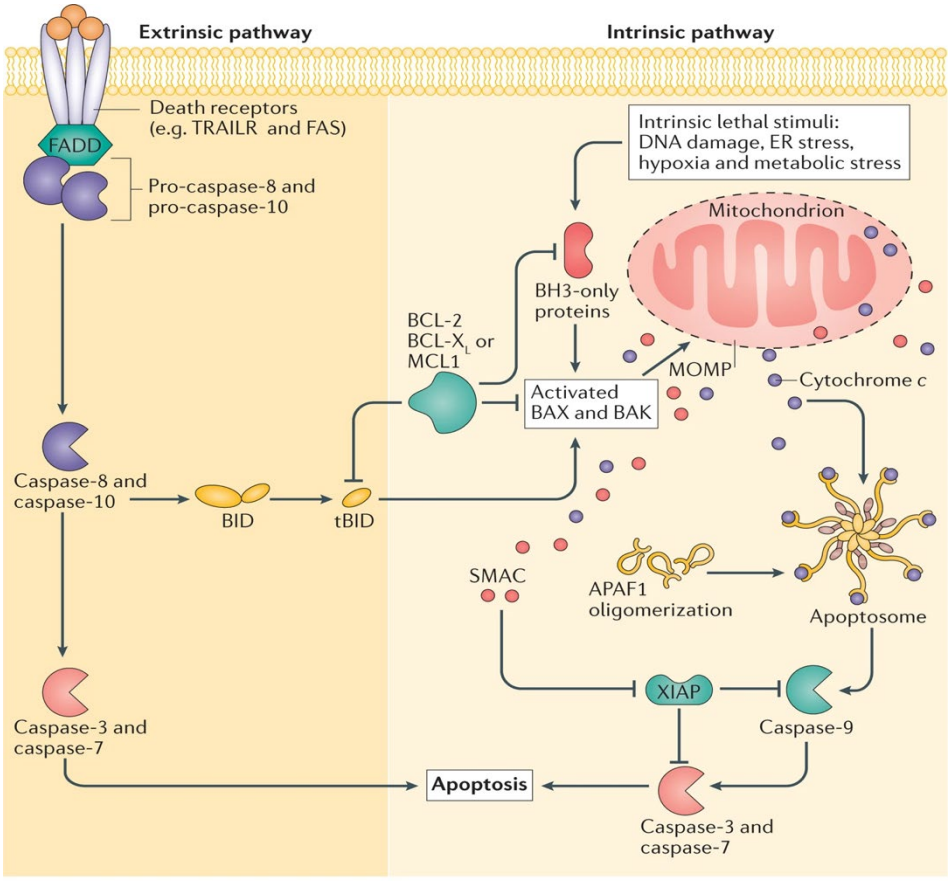


Figure 1.8 Intrinsic and extrinsic apoptotic pathways

In the extrinsic apoptotic pathway, death receptors like TRAIL receptor (TRAILR) and FAS activate initiator caspases when bound to their ligands. Active caspase-8 and caspase-10 then trigger apoptosis by cleaving and activating effector caspase-3 and caspase-7. The intrinsic pathway, on the other hand, involves mitochondrial outer membrane permeabilization (MOMP). Cell stresses activate BH3-only proteins, leading to BAX and BAK dimerization and initiation of MOMP. Anti-apoptotic BCL-2 family proteins counteract this process. Following MOMP, proteins like SMAC and cytochrome c are released into the cytosol. Cytochrome c triggers apoptosome assembly with APAF1, activating caspase-9, which, in turn, activates caspase-3 and caspase-7, ultimately leading to apoptosis. Additionally, caspase-8 cleaves the BH3-only protein BID, allowing crosstalk between the extrinsic and intrinsic apoptotic pathways

1.5.3 BCL-2 FAMILY IN CANCER

Cancer growth does not only happen through the hijacking of cellular proliferation pathways. It also relies on the ability of cancer cells to escape cell death. Resisting cell death was among the first six hallmarks of cancer to be described in 2000 by Douglas Hanahan and Robert Weinberg [236]. While the Nomenclature Committee on Cell Death has now described 13 different mechanisms of cell death [237], impaired apoptosis remains one of the most studied ones in the cancer field. As the tumor grows in size, multiple factors can push tumor cells into apoptosis: lack of nutrients and oxygen, loss of cell-to-cell contact and restrained access to locally secreted cytokines [238–240]. Cancer cells can modulate the members of the Bcl-2 family in order to survive the environmental stress and boost their expansion. Bcl-2, Bcl-xL and Mcl-1

are genes commonly found to be over-expressed in leukemias and lymphomas [241–245]. Over-expression is also observed and is in some cases associated with poor prognosis in various solid cancers [246,247]. High levels of Bcl-2 were detected in glioma, small and non-small cell lung cancer [248–253]. Bcl-xL was shown to support the progression of breast cancer, colon cancer and glioma [254–256]. Mcl-1 is found to be expressed at high levels in cancers from multiple different tissues such as the breast, lung, liver, prostate and ovary [257]. Besides an increase in anti-apoptotic members, cancer cells also aberrantly decrease the levels of the pro-apoptotic members of the Bcl-2 family. Low expression of Noxa and Bim were detected in colon and small-cell lung cancers, for example [258].

Of note, the cellular stress induced by chemotherapy or radiotherapy also triggers tumor cell apoptosis [259,260]. While cancer cell death is a sign of response to therapy, the stress imposed on tumors often leads to the establishment of resistant cells which can persist within the tissue or disseminate, thus participating in the relapse of the disease [261]. Indeed, the overexpression of the anti-apoptotic Bcl-2 family members and the downregulation of pro-apoptotic ones have been shown to support tumor cell survival upon treatment [231,262]. Blocking Bcl-xL, for example, prevents adenoma outgrowth in a mouse model of colorectal cancer and potentiates the efficacy of chemotherapy [255]. This can be explained by the fact that chemotherapy is sometimes not sufficient to induce cell death but can prime cells for apoptosis. The "readiness" for apoptosis can be measured by BH3 profiling, a functional assay where cells are exposed to pro-apoptotic BH3 peptides and their dependence on specific proteins for survival is assessed (rf). This assay enables to define the dependence of tumor cells on specific anti-apopotitc proteins of the Bcl-2 family. The administration of inhibitors targeting the anti-apoptotic members is therefore interesting as it can push the primed cells towards apoptosis. These compounds are interesting options to have in combination therapies to prevent the outburst of resistant cancer cells and potentially enhance the efficacy of individual treatments.

1.5.4 Targeting of anti-apoptotic proteins with BH3 mimetics

The identification of the BH3 binding pocket on the surface of anti-apoptotic Bcl-2 proteins has paved the way for promising therapeutic strategies [235,263–265]. Indeed, as mentioned earlier, this hydrophobic groove serves as a docking site for the BH3 domains of other proteins. Over the years, small molecules were discovered with the capacity to bind this pocket, mimicking the action of BH3-only proteins and therefore earning the name of BH3 mimetics. Of note, several small molecules were discovered but only the ones that exhibit a strong binding activity for a certain anti-apoptotic protein and mediate apoptosis through Bax/Bak mitochondrial permeabilization are considered BH3 mimetics [266,267].

ABT-263/navitoclax was the initial BH3 mimetic tested in clinical trials. This orally available inhibitor exhibits strong binding, in the nanomolar range, to Bcl-2, Bcl-xL and Bcl-w [268]

proteins. Preclinical work demonstrated its efficacy in mouse models of acute lymphoblastic leukemia and small-cell lung cancer [269]. Moreover, navitoclax was shown to act in synergy with several commonly used chemotherapy drugs [270], particularly docetaxel [271]. However, when tested as monotherapy in patients with hematologic malignancies or solid tumors, navitoclax displayed dose-limited toxicity due to Bcl-xL inhibition, which is highly expressed by platelet cells, resulting in thrombocytopenia in all of the patients [272]. Preliminary results of phase I and phase II clinical trials have demonstrated only a limited role for navitoclax in the treatment of advanced solid tumors as monotherapy [273–275] and new studies are trying to assess the combination with other therapies [276,277] .

The on-target side effect of navitoclax causing thrombocytopenia prompted the generation of a Bcl-2-specific inhibitor, ABT-199/venetoclax [278]. The overall clinical tolerance by patients was good and the drug was rapidly approved by the U.S: Food and Drug Administration (FDA). Venetoclax is now the first line of treatment for acute myeloid leukemia (AML) and refractory chronic lymphocytic leukemia (CLL) [279,280] Bcl-2 inhibition with ABT-199 can render cells from triple-negative breast cancer (TNBC) sensitive to doxorubicin [281].

1.5.5 Cell death regulation in neutrophils

The balance in pro and anti-apoptotic proteins of the Bcl-2 family was shown to be important in modulating the lifespan of mature neutrophils [282,283]. Neutrophils express the proapoptotic members under physiological conditions [283–285]. This is less clear when considering the anti-apoptotic molecules. Bcl-2 and Bcl-W are not detected at protein levels in mature neutrophils in peripheral human blood and mouse neutrophils [284,286]. Mcl-1 was shown to participate in the delayed neutrophil apoptosis in sepsis [287]. Whereas neutrophils from mice lacking the A1/Bfl-1-a isoform showed prolonged survival when incubated with pro-inflammatory molecules [288], neutrophils extracted from *A1-1a, b and d alleles* knock-out mice showed no difference in survival compared to wild-type neutrophils [289]. Delayed apoptosis and up-regulation of A1 was however observed in human neutrophils upon the same stimulatory conditions [288]. While the expression of Bcl-xL in neutrophils remained controversial for a long time, recent publications show induction of Bcl-xL in conditions where neutrophil apoptosis is delayed [290], in sepsis for example [287,291]. The aim of this work is therefore to dissect the molecular mechanism of neutrophil-delayed apoptosis in lung cancer and propose a chemical way to counteract it.

1.6 Aims of the thesis

Previous research from our group has shown that TANs in the KP mouse model rely on high glycolysis to prevail in the tumor microenvironment and differentiate into SiglecF-expressing TANs, a subset that promotes tumor growth. Intriguingly, it was shown that these cells can survive substantially longer within the tumor microenvironment. However, how this phenomenon takes place was not described.

The goal of this thesis was to identify the mechanisms underlying the extended neutrophil survival in tumors and find a way to block it. Since SiglecF+ TANs are only found within tumors, we thought that the extended survival of these cells is triggered by the tumor microenvironment. We hypothesized that a tumor-secreted factor might enable it. We therefore decided to incubate healthy bone marrow-derived neutrophils with the conditioned medium (or supernatant as we will call it further) of tumor-derived cell lines. With this in vitro assay, we discovered that the SN stimulated neutrophil survival and led to the expression of Bcl-xL, an anti-apoptotic protein. With this observation in hand, the aims of the thesis were the following:

Aim 1: Identify specific factors secreted by tumor cells that contribute to the extended survival of neutrophils and determine which pathways mediate Bcl-xL expression.

Aim 2: Investigate the role of Bcl-xL in neutrophil survival *in vitro* and *in vivo*. I first compared the expression of Bcl-xL between TANs and healthy neutrophils. Then, I monitored if Bcl-xL blockade, using A-1331852, a Bcl-xL-specific inhibitor, decreased aged SiglecF+ TANs in lung tumors from KP mice after both short-term and long-term treatment.

Aim 3: Explore the effects of long-term Bcl-xL blockade on lung tumor growth and identify potential combination therapies to prevent cancer growth. I monitored the growth of lung tumors in KP mice treated with A-1331852. As neutrophils can compromise the success of immunotherapies, I assessed if pre-treatment with Bcl-xL blockade can enhance the efficacy of anti-PD-1 therapy. In addition, I tried to try to shift the balance between aged and young neutrophils in tumors, by recruiting more young neutrophils, with potential anti-tumor capacities, to the tumors using G-CSF injections.

2 MATERIALS AND METHODS

This section is adapted from the published article: Bodac et al. (2023). Bcl-xL targeting eliminates ageing tumor-promoting neutrophils and inhibits lung tumor growth, *EMBO Molecular Medicine*

2.1 MOUSE EXPERIMENTATION

2.1.1 MOUSE MODEL

$Kras^{Frt\text{-}STOP\text{-}Frt\text{-}G12D/WT}$;$Tp53^{Frt/Frt}$ (KP) mice were generously provided by D.G. Kirsch from Duke University Medical Center. These mice were generated by crossing $Kras^{Frt\text{-}STOP\text{-}Frt\text{-}G12D/WT}$ (RRID: IMSR_JAX:008653) [292] and $Tp53^{Frt/Frt}$ (RRID: IMSR_JAX:017767) [131] mice and bred in a mixed 129-C57BL/6 background.

2.1.2 TUMOR INITIATION AND FOLLOW-UP

To activate the oncogenic KrasG12D and delete Tp53 and initiate tumor formation in lung epithelial cells, KP mice were administered intratracheally 10^7 plaque-forming units (PFU) per mouse of a commercially available adenoviral CMV-Flp vector (Ad5CMVFlpo), obtained from the University of Iowa, from the Viral Vector Core Facility), as described by DuPage et al 2009 [129]

Practically, mice between 12 and 16 weeks of age were anesthetized by a single dose of Domitor (Medetomidine hydrochloride) and Ketasol (ketamine, Ketasol 100). The anesthetics were adjusted according to the mouse weight in order to inject 1 mg/kg Domitor and and 33 mg/kg Ketasol. Administration of the viral load was then done with a catheter inserted in the trachea, which was localized with a light bulb placed close to the mouse throat. After the intratracheal delivery of the virus, Antisedan (Atipamezole hydrochloride, the Medetomidine antidote), was injected subcutaneously at a dose of 2.5 mg/kg to wake the mice up. Tumor initiation is expected to happen approximately 14 weeks after the viral administration. To monitor the initiation and the progression of tumors, lung images were captured using an X-Ray microtomography machine (µCT) (Quantum FX; PerkinElmer) with a voxel size of 50 µm, using retrospective respiratory gating. The volume of individual tumors was obtained using Osirix MD (Pixmeo, RRID:SCR_013618) and following the protocol described here: bio-protocol.org/prep390. During the imaging procedure, mice were subjected to isoflurane anesthesia and maintained in this anesthetized state throughout the entire scanning process.

2.1.3 MOUSE TREATMENT MODALITIES

The Bcl-xL inhibitor A-1331852 (Catalog #: HY-19741, MedChemExpress) was administered at a dosage of 25 mg/kg. The dual Bcl-2 and Bcl-xL inhibitor Navitoclax (ABT-263, Catalog #: HY-10087, MedChemExpress) was given at a concentration of 100 mg/kg, and the Bcl-2-specific inhibitor Venetoclax (ABT-199, Catalog #: HY-15531, MedChemExpress) was administered at a dose of 50 mg/kg. All these compounds, intended for *in vivo* treatments,

were prepared in a solution consisting of 10% DMSO, 40% PEG-300, 5% Tween-80, and 45% saline, and they were orally administered on a daily basis. For the BrdU assay, a solution containing 2 mg of freshly prepared BrdU (Merck, Catalog #: 10280879001) in 100 µL of PBS was administered via intraperitoneal injection. Regarding immunotherapy treatment, anti-mouse PD-1 (clone 29F.1A12, Bio X Cell, Catalog #: BE0273, RRID:AB_2687796) was intraperitoneally injected at a dosage of 200 µg per mouse three times a week for a duration of 2 weeks. For G-CSF treatment, intended to stimulate bone marrow production of neutrophils and release into the blood flow, recombinant mouse G-CSF (PeproTech, Catalog #: 250-05) was administered via intraperitoneal injection daily at a dose of 10 µg per day for a period of 3 weeks. To deplete neutrophils, a double antibody approach was employed, involving the injection of anti-Ly6G antibody (clone 1A8, Catalog #: #BP0075-1), followed by anti-rat Kappa immunoglobulin (clone MAR 18.5, Catalog #: #BE0122), following a previously published method [293]. The antibodies and corresponding isotype controls (#BP0290 and #BP0089) were purchased from BioXCell.

2.1.4 Broncho-alveolar lavage fluid (BALF) collection

Broncho-alveolar lavage fluid (BALF) collection protocol was adapted from this published protocol [294]. Briefly, mice were euthanized and the front of the thoracic cage was cut and removed, making the lungs and the trachea visible. The trachea was then seized and maintained with tweezers and 1mL of PBS containing 100 µM of ethylenediaminetetraacetic acid (EDTA) was injected into the lungs through the trachea, with the use of a syringe. The BALF was then retrieved by aspirating the liquid back into the syringe

2.1.5 Tissue preparation and isolation

2.1.5.1 Lung tumor isolation and conditioned medium preparation

KP tumors were dissociated into single-cell suspensions as described in a protocol published by our group [177]. Briefly, individual tumors were isolated and digested by mechanical dissociation using the GentleMACS tissue dissociator (Miltenyi) coupled with enzymatic digestion using DNase I (0.02 mg/ml) and collagenase (1 mg/mL), resuspended in DMEM without FBS.

Single-cell suspensions obtained from dissociated lung tumors were filtered through 40µm filters and resuspended in complete DMEM (10% FBS and 1% PenStrep). The cells were plated at a density of 10^6 cells/mL. After 24h of incubation, the conditioned medium (CM) or supernatant (SN) was collected and passed through 0.22 µm filters. The supernatants were kept at -80°C until use. For the SN produced from SV2 cells, 106 cells/mL were cultured in DMEM and SN was retrieved and filtered after 24h as well.

2.1.5.2 Neutrophil isolation

Neutrophils from different tissues were isolated using anti-Ly6G MicroBeads UltraPure magnetic beads (clone REA526, Miltenyi Biotec, 130-120-337) according to the manufacturer's instructions. For bone marrow neutrophils, bone marrow was collected from femurs and tibia by flushing of the bone with PBS and filtered through a 40 µm filter. For healthy lung neutrophils, lungs from healthy mice were prepared the same way as lung tumors.

2.2 HUMAN NEUTROPHIL ISOLATION

Peripheral blood neutrophils were isolated from whole blood samples of healthy donors. Neutrophils were isolated from the blood using a Polymorphprep (Progen, Cat# 1895) gradient and the remaining red blood cells were lysed with ACK-buffer. The purity was assessed to be >95% with Quick-Fix staining of CytoSpinned samples. The viability of neutrophils incubated with A549 cell line supernatant was assessed by flow cytometry using the LIVE/DEADTM fixable blue dead stain kit (Invitrogen, Cat#: L23105). The viability of human neutrophils upon treatment with A-1331852 was determined using PrestoBlue.

2.3 CELL LINES

2.3.1 ORIGIN OF CELL LINES AND CULTURE CONDITIONS

The human cell line A549 (RRID:CVCL_0023) was purchased from ATCC and cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS). The murine cell lines SV2 and T5 were generated in our laboratory from a single KrasLox-STOP-Lox-G12D/WT; p53Flox/Flox and a KrasFrt-STOP-Frt-G12D/WT; p53Frt/Frt lung tumor, from a male and a female mouse, respectively. Briefly, tumors were digested into single-cell suspensions and cultured for 25 passages before experimentation in DMEM supplemented with 10 % FBS and 1% PenStrep. Cells were cultured at 37°C with 5% CO2. Mycoplasma tests were performed to ensure mycoplasma-free cell cultures.

2.3.2 CELL VIABILITY ASSAY

SV2 lung tumor cells were seeded at 3'000 cells per well in 96-well plates and treated with A-1331852, diluted in ten-fold steps (range). The cell viability was measured using PrestoBlue (ThermoFisher, Cat#: A13261) after 48h and 72h of incubation, following the manufacturer's protocol. PrestoBlue measures the metabolic activity of cells as an indicator of cell viability. Briefly, cell culture medium was removed from the 96 well plate and 100 µL per well of medium with 10% PrestoBlue reagent was added to the cells. After 1h of incubation at 37°C, fluorescence was read and viability plotted. To test the proliferative capacity of tumor cells, a clonogenic assay was performed by incubating 100, 200 or 400 single SV2 cells into 6-well plates with increasing doses of A-1331852 and letting them grow for one week. Incubation with crystal violet solution enabled to stain for the colonies formed, which were then counted manually.

2.4 FLOW CYTOMETRY

2.4.1 TUMOR IMMUNE MICROENVIRONMENT ASSESSMENT

Single-cell suspensions obtained from tumors with mechanical and enzymatic digestion were resuspended in FACS buffer (2%FCS and 2 mmol/L EDTA) with FcR-block (anti-CD16/32, BioLegend) and stained with antibodies (Table 1), in darkness and for 20 minutes on ice. For intracellular staining of Bcl-xL, cells were fixed in BD Cytofix/Cytoperm (BD #554714) for 30 minutes on ice, then washed and incubated with Permeabilization buffer (Invitrogen #00–8333-56). For quantitative assessment of neutrophils in tumors, 15 µL of counting beads (CountBrightTM, Invitrogen, Cat#: C36950) were added to the sample before acquisition. Acquisitions were performed with the full-spectrum analyzer Cytek Aurora (Cytek Biosciences). For the experiments, acquisitions were performed using the LSRII SORP (Becton Dickinson), a 5-laser and 18-detector analyzer at the EPFL Flow Cytometry Core Facility. Data analysis was performed using FlowJo (FlowJo LLC ©). Flow cytometry gating strategy is indicated in Figure 2.1.

Antibody	Source	Reference (Cat#)
Bcl-xL-PE	Abcam	ab208747
SiglecF-PeVio 615	Miltenyi BioTec	130-112-172
CD45-Pacific orange	ThermoFisher scientific	MCD4530
CD45-PerCP	Miltenyi BioTec	130-102-785
Ly6G-PE	BioLegend	127607
Ly6G-PerCP-Cy5.5	BioLegend	127615
Ly6C-PerCP	BioLegend	128027
CD11b-BV711	BioLegend	101242
CD11c-BV421	BioLegend	117330
F4/80-BV605	BioLegend	123133
CD3-PE-Cy5.5	eBioscience	35-0031-82
CD4-AF700	BioLegend	100536
CD8a-BV570	BioLegend	100740
FoxP3-PE-eFluor 610	eBioscience	61-5773-82
B220-PE-Cy5	BioLegend	103209
Nk1.1-BV650	BioLegend	108736
PD-1-PE-Cy7	BioLegend	329917
PD-L1-BV785	BioLegend	124331
EpCAM-APC	eBioscience	15-5791-82
Ki67-FITC	BD Pharmingen	556026
BrdU-APC	BioLegend	339808

Table 1 Antibodies used for flow cytometry

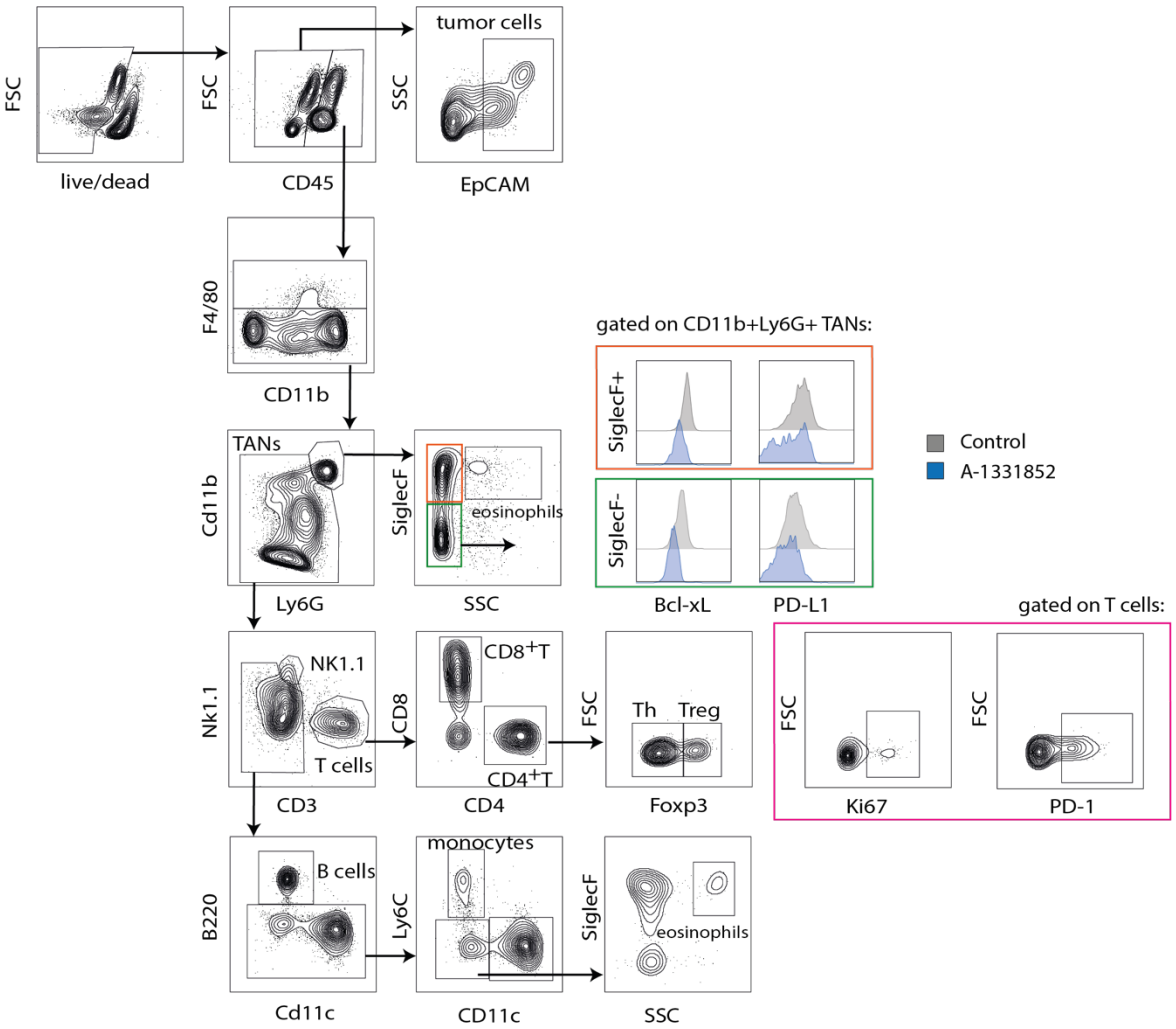


Figure 2.1 Gating strategy used for flow cytometry in this work

2.4.2 Neutrophil survival, apoptosis, SiglecF and Bcl-xL expression assessment

For the neutrophil survival assay, freshly isolated bone marrow neutrophils (10^5 cells) were incubated in 96-well plates and in 200 µL of tumor or SV2-cell-derived SN for 24h. The viable cells were measured by flow cytometry using the LIVE/DEADTM fixable blue dead stain kit (Invitrogen, Cat#: L23105) staining for dead cell exclusion. Neutralization of GM-CSF was done by pre-incubation of the SN with 10 ng/mL of anti-GM-CSF (PreproTech, Cat#500-P65) before adding it to the neutrophils. For the survival assay with GM-CSF, neutrophils were incubated with 10 ng/mL of recombined murine GM-CSF (PreproTech, Cat#315-03). For the experiments related to pathways inhibitors, Stattic (STAT3 inhibitor, MedChemExpress, Cat#: HY-13818), Ruxolitinib (Jak1/2 inhibitor, MedChemExpress, Cat#: HY-50856), MLN120B (IKKß, MedChemExpress, Cat#: HY-15473) and LY294002 (PI3K inhibitor, MedChemExpress, Cat#: HY-10108) were resuspended in DMSO and diluted at the concentrations indicated in the figure legends.

Clear assessment of neutrophil apoptosis was performed by double staining with Annexin V (FITC) and 7-AAD (detection kit, BioLegend, #640922) and analyzed by flow-cytometry.

Briefly, 100’000 neutrophils were resuspended in 100 µL of Annexin V binding buffer and 5 µL of Annexin V-FITC and 5 µL of 7-AAD were added. The samples were kept in the dark for 15 min at room temperature and analyzed by flow cytometry witin one hour. Additionally, cleaved-caspase 3 (Asp175, Cell Signalling, rabbit mAb #9664) was detected by Western blot. TNF (Preprotech: Cat#: 315-01A) was used at 5 ng/mL as a positive control for neutrophil apoptosis.

TANs were isolated from single-cell tumor suspensions using the anti-Ly6G MicroBeads UltraPure magnetic beads. Isolated TANs were stained with anti-SiglecF-Pe-Vio615 antibody (Cat#: 130-112-172) for 15 minutes on ice, before incubation, to specifically determine the effect of A-1331852 on SiglecF+ and SiglecF- TANs.

2.5 HISTOLOGY

2.5.1 IMMUNOHISTOCHEMITRY

As previously described, after the dissection of tumor-bearing mice, lungs with tumors were isolated and the lobes were separated. The tissues were then fixed with 3.7% formaldehyde solution (Sigma) overnight and then paraffin-embedded. Blocks of tissues were cut into sections of 4 µm of thickness and let to dry overnight at room temperature or 1h at 37°C. For immunohistochemistry (IHC), slides were dewaxed, and antigen retrieval was performed in a solution of 10 mM sodium citrate. Blocking was performed with 1% bovine serum albumin (BSA). The slides were then stained with primary antibodies overnight at 4°C, washed and incubated with secondary antibodies for 1h at room temperature. The antibodies used for IHC are the following: anti-Ki67 (ThermoFisher, MA5-14520), anti-cleaved-caspase 3 [Asp175] (Cell signaling, #9661) and anti-pan cytokeratin (Novusbio, NBP600-579). After washing, slides were incubated for 40 minutes with anti-rabbit Immpress horseradish peroxidase (HRP, Vector Laboratories, RRID:AB_2336529). The positive cells were then revealed using 3-3’-Diaminobenzidine (DAB) substrate. Harris hematoxylin counterstain of the nuclei was subsequentially performed.

2.5.2 IMMUNOFLUORESCENCE

2.5.2.1 MOUSE LUNG TUMORS

For immunofluorescence, after dewaxing and antigen retrieval, slides were blocked with 1% BSA and incubated with the following primary antibodies: anti-Bcl-xL (Abcam, ab32370) and anti-MPO (R&D SYSTEMS, Cat#: AF3667). Secondary antibodies used were anti-rabbit Alexa 488 (ThermoFisher Scientific A-21206) and anti-goat Alexa 568 (ThermoFisher Scientific A-11057). To assess the infiltration of different T cell subpopulations in tumors, multiplexing (4plex) immunofluorescence was performed by the histology facility using the fully automated Ventana Discovery ULTRA (Roche Diagnostics) and with the Ventana solutions. Briefly, paraffin sections were dewaxed, rehydrated and incubated sequentially with the following

antibodies: anti-pan-cytokeratin (Novusbio, NBP600-579), anti-CD8 (Dako, M7103), anti-CD4 (Invitrogen Cat#: 14-0042-82) and anti-Foxp3 (Invitrogen Cat#: 14-5773). Antibodies were incubated for one hour at 37°C and incubated with anti-rabbit ImmPRESSTM HRP (Vector Laboratories, Cat#: MP-7401). They were then sequentially revealed with the following TSA kits: the FAM (Roche Diagnostics, 07988150001), Red 610 (Roche Diagnostics, 07988176001), Rhodamine-6G (Roche Diagnostics, 07988168001) and Cyanine 5 (Roche Diagnostics, 07551215001) and counterstained with DAPI for nuclear staining.

For both IHC and immunofluorescence, tissue slides were scanned with the VS120-SL Olympus slide scanner at 20X magnification and quantification of positive cells on IHCs was performed using the QuPath software.

2.5.2.2 PATIENT TISSUES

Formalin-fixed and paraffin-embedded tissue sections obtained from patients with lung cancer were deparaffinized, rehydrated, and were subjected to antigen retrieval in EnVision FLEX Target Retrieval High pH Solution (Tris/EDTA, pH9.0; Agilent) at 95 °C for 20 minutes using PT Link (Agilent Technologies, Santa Clara, CA, US). The sections were then permeabilized with 0.1 % Triton X-100 in PBS for 15 minutes and blocked with 5% V/V donkey serum (Jackson ImmunoResearch, 017-000-121). The primary antibodies, anti-MPO (R&D SYSTEMS, AF3667) and anti-BCL-XL (Abcam, ab32370), were added for overnight incubation at 4°C. The primary antibodies were washed with washing solution 0.05 % Tween-20 in PBS followed by incubation with secondary antibodies, Alexa Fluor 488-conjugated Donkey anti-Goat (Jackson ImmunoResearch, 705-545-003) and Cy3-conjugated Donkey anti-Rabbit (Jackson ImmunoResearch, 711-165-152) diluted in donkey serum, for 1 h at room temperature. The secondary antibodies were washed and counterstained with DAPI for nuclear inspection. The slides were scanned using Axioscan7 (Carl Zeiss SA, Oberkochen, Germany) and double-positive cells were analyzed using QuPath's (v 0.3.2) classifier.

2.6 RNA AND PROTEIN

2.6.1 RNA EXTRACTION AND REAL-TIME PCR

TRIzol Reagent (Invitrogen, 15596018) was utilized to extract the total RNA following the manufacturer's instructions. The High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, 4368814) was employed to synthesize cDNAs from 1 µg total RNA. Real-time PCR was carried out using 5 ng of cDNA with the Taqman universal PCR master mix (ThermoFisher, 4324018) and the following Taqman probes: Bcl2l1: Mm00437783_m1; Bcl2a1: Mm03646861_mH; Bcl2: Mm00477631_m1; Mcl-1: Mm01257351_g1 SiglecF: Ifit3: Mm01704846_s1; Ifnb1: Mm00439552_s1; Irf3: Mm00516784_m1; Dxd58: Mm01216853_m1 and the gene expression level normalization was done with Rpl30: Mm01611464_g1 or with or with Hprt: Mm00446968_m1.

2.6.2 Protein extraction and Western blot

Protein extracts from isolated neutrophils were obtained through lysis and sonication of the cells in RIPA buffer (20 mmol/L Tris pH8, 50 mmol/L NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol/L Na3VO4, protease inhibitor cocktail (Roche, 11836145001). Protein quantification was done using the Bradford assay. The following antibodies were used: anti-Bcl-xL (Abcam, ab32370) and y-tubulin (Invitrogen, Cat#MA1-850).

2.6.3 GM-CSF detection by ELISA

GM-CSF presence in the supernatant derived from digested tumors was measured with the enzyme-linked immunosorbent assay ELISA MAX™ Standard Set Mouse GM-CSF (432201, Biolegend), following the manufacturer's protocol.

2.7 Statistics

2.7.1 Data analysis

The figure legends contain information on the statistical details of the experiments, including the number of repeats performed and the statistical tests used. All results are shown as mean ± SD unless stated otherwise. The normality of distribution was tested using the Kolmogorov-Smirnov Test. Multiple comparisons of normally distributed samples were carried out using ANOVA, and Student t-tests were used for two independent samples. For non-parametrically distributed samples, the Kruskal-Wallis ANOVA with the Bonferroni correction for multiple comparisons, and Mann-Whitney U-Test for two independent samples. Statistical significance is indicated as in the figure legends. Statistical analysis was performed using Prism 9 software.

2.7.2 Biofinformatics

2.7.2.1 TCGA data analysis

Five public transcriptome datasets (Table 2) have been combined to assess the overall survival of LUAD patients. To ensure data consistency and allow meaningful comparisons, background subtraction and normalization were performed on each dataset using the robust multi-array average (RMA) method from the affy package [295]. In addition, to address potential batch effects that might arise due to differences in data collection and processing protocols, we employed the ComBat method from the sva package [296]. This method utilizes an empirical Bayes framework to adjust for known batch effects, harmonizing the combined dataset. The median expression of CSF2 was then used to stratify patients into high or low groups and their survival probability was compared using a log-rank test.

	Number of samples used (total patients)	Age (median, range)	Gender	Stage	Dataset Name	Institution	Source	Reference
Lung-0 Dataset	82 (82)	61 (35-79)	F (36) / M (46)	I (56) / II (26)	DFCI	Dana-Farber Cancer Institute	Director's Challenge Consortium	Nat Med 14, 822–827 (2008)
	79 (79)	68 (36-87)	F (39) / M (40)	I (41) / II (20) / 3 (15) / NA (3)	HLM	Moffitt Cancer Center	Director's Challenge Consortium	Nat Med 14, 822–827 (2008)
	28 (90)	63 (38-75)	F (13) / M (15)	NA (28)	JBR	Ontario Cancer Institute	Zhu *et al.*	J Clin Oncol. 2010 Oct 10; 28(29): 4417–4424
	177 (178)	65 (33-86)	F (77) / M (100)	I (116) / II (29) / III (32)	MI	University of Michigan	Director's Challenge Consortium	Nat Med 14, 822–827 (2008)
	104 (104)	65 (38-82)	F (67) / M (37)	I (63) / II (20) / III (21)	MSKCC	Memorial Sloan-Kettering Cancer Center	Director's Challenge Consortium	Nat Med 14, 822–827 (2008)
Lung-0 Dataset (total)	470 (533)	65 (33-87)	F (232) / M (238)	I (276) / II (95) / III (68) / NA (31)				

Table 2 Data used for human transcriptomics analyses

2.7.2.2 HUMAN TANS SINGLE-CELL SEQUENCING

UMAPS and filtered normalized counts for human TANs showing anti-apoptotic proteins gene expression were retrieved from:

https://singlecell.broadinstitute.org/single_cell/study/SCP739/single-cell-transcriptomics-of-human-and-mouse-lung-cancers-reveals-conserved-myeloid-populations-across-individuals-and-species#study-summary (human_gene_expression_matrix.tsv), linked to the study of Zilionis et al. 2019 [219]. Counts were summed per patient and per tumor neutrophil population (N1-5) and the mean expression of BCL2L1 is shown for each population.

3 RESULTS

This section is adapted from the publication based on my doctoral research project:

Bodac A, Mayet A, Rana S, Pascual J, Bowler A, Roh V, Fournier N, Craciun L, Demetter P, Radtke F and Meylan E (2023): Bcl-xL targeting eliminates ageing tumor-promoting neutrophils and inhibits lung tumor growth. *EMBO Molecular Medicine 16: 158-184*

3.1 ANTI-APOPTOTIC BCL-XL IS INDUCED IN TUMOR-ASSOCIATED NEUTROPHILS (TANS)

As the longevity of neutrophils extends within the tumor mass in the KP mouse model of LUAD, we investigated genes within our previously generated bulk RNA sequencing data [222] that could modulate survival or apoptosis. We found that the apoptosis gene set was downregulated in tumor-associated neutrophils (TANs) compared with healthy lung neutrophils (HLNs) suggesting that the tumor microenvironment stimulates the escape of TANs from apoptosis (Fig. 3.1A). Proteins of the Bcl-2 family regulate the intrinsic apoptotic pathway and are separated into pro and anti-apoptotic proteins. Among apoptosis gene regulators, *Bcl2l1*, coding for the anti-apoptotic protein Bcl-xL, was upregulated in TANs compared to HLNs (Fig. 3.1B). It was also the case for *Bcl2a1b*, coding for Bfl-1. *Bcl-2* however, was not detected in our data and *Mcl-1* was more expressed in HLNs than TANs. We also measured the expression levels of these genes by real-time PCR analysis and determined that the expression of *Bcl2l1*, but not that of the other Bcl-2 family members, was significantly increased in TANs compared to HLNs (Fig. 3.1C).

In order to gain a better understanding of the expression of these anti-apoptotic genes in TANs, we took advantage of the publicly available single-cell RNA sequencing performed on an orthotopic mouse model of lung cancer, where six distinct neutrophil subsets (N1-N6) were identified [219] . The N4 subset was of particular interest to us, as it was characterized by elevated *SiglecF* expression, the marker of tumor-promoting neutrophils that we aim to block. Interestingly, this subgroup of TANs also displayed a pronounced upregulation of the *Bcl2l1* and *Bcl2a1* genes when compared with the other subsets. *Mcl-1* was highly expressed in most subsets, particularly in N1 and, in contrast, *Bcl-2* was almost not expressed in any subset (Fig. 3.1D).

Taken together, we hypothesized that TANs escape apoptosis by upregulating anti-apoptotic members of the Bcl-2 family, supporting them in the differentiation into tumor-supportive cells. We therefore aimed to selectively inhibit key anti-apoptotic proteins, namely Bcl-xL and Bfl-1. The targeting of Bfl-1 is challenging as there is no specific and potent inhibitor of this protein yet [297]. This is not the case for Bcl-xL however, for which a BH3 mimetic with strong binding affinity was developed [298]. We therefore decided to investigate the importance of Bcl-xL in TANs, with the idea of potentially using this drug to specifically target TANs *in vivo*, without depleting other neutrophil populations. Immunofluorescence staining showed colocalization of

MPO, a marker of neutrophils, and Bcl-xL in KP tumors, validating the expression of the protein in TANs *in vivo* (Fig. 3.1E)

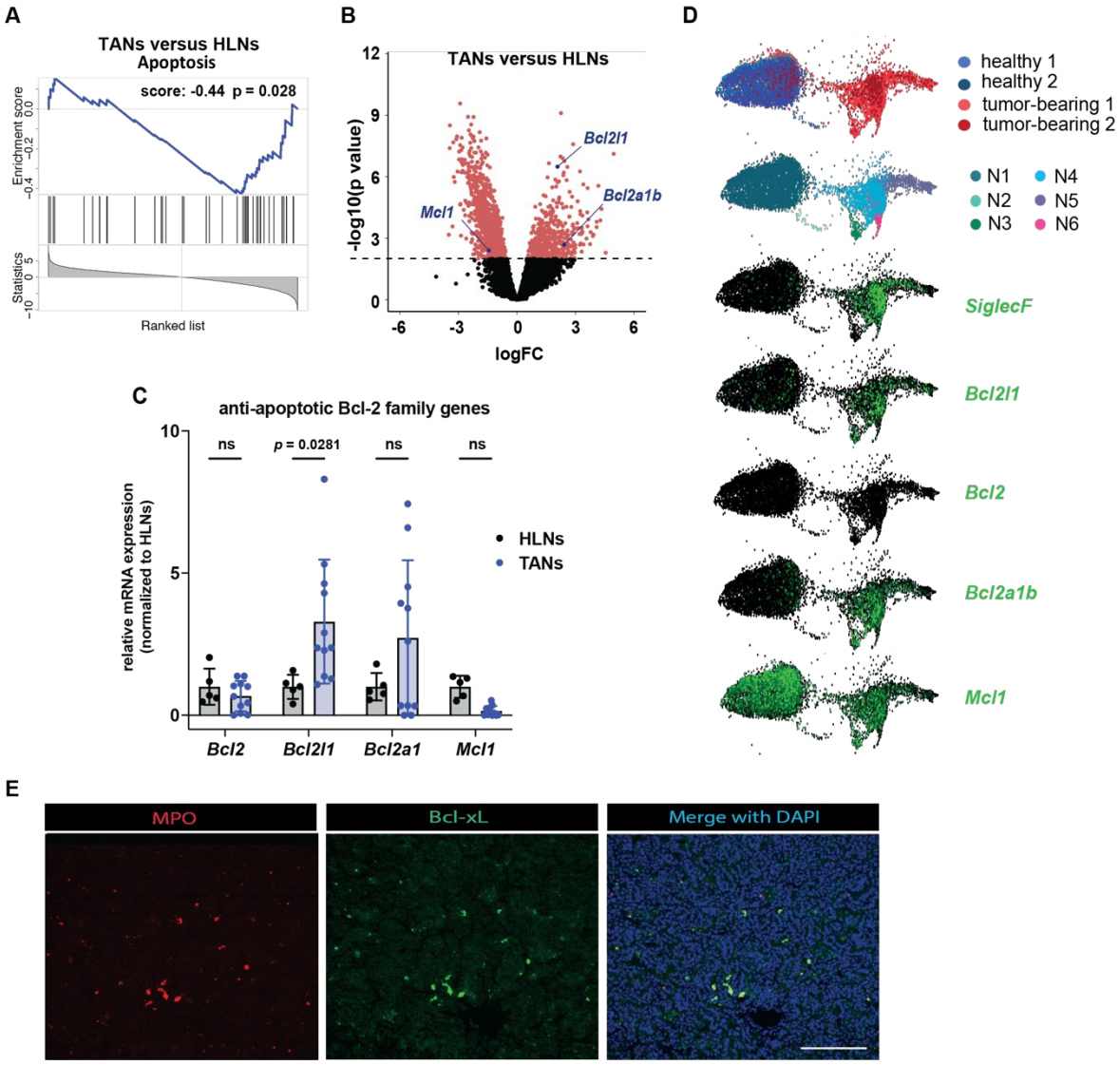


Figure 3.1 Tumor-associated neutrophils (TANs) exhibit high anti-apoptotic Bcl-xL (*Bcl2l1*) gene expression

(A) Gene set enrichment analysis (GSEA) showing downregulation of the apoptosis pathway in TANs compared to HLNs **(B)** Volcano plot showing differentially expressed genes (DE) in TANs versus HLNs. Genes with an adjusted p-value superior to 0.01 (-log10(adj. p-value > 2) were highlighted in light pink, and the anti-apoptotic genes Bcl2l1, Bcl2a1b and Mcl1 are highlighted in dark blue **(C)** Real-time PCR showing *Bcl2*, *Bcl2l1*, *Bcl2a1* (Bfl-1) and *Mcl1* gene expression in TANs (n=11 biological replicates) normalized to expression in HLNs (n=5). *Rpl30* was used as a reference gene. Significance was obtained with two-way ANOVA with Sidàk's multiple comparisons. *p ≤ 0.05 and ns, non-significant. **(D)** Representation of neutrophil subsets in naïve and tumor-bearing mice from the available single-cell transcriptomics. *SiglecF*, *Bcl2l1*, *Bcl2*, *Bcl2a1b* and *Mcl1* expressions are highlighted in green. **(E)** Immunofluorescence staining of neutrophils (MPO) and Bcl-xL in tumors of KP mice. Scale bar: 100 µm

To determine whether Bcl-xL expression is triggered in neutrophils upon arrival in the tumor mass or before, we compared Bcl-xL levels from healthy or tumor-bearing mice, in neutrophils

extracted from the bone marrow (BMN), peripheral blood (PBN), lung (HLN) or tumors (TAN), using flow cytometry. All neutrophil populations expressed Bcl-xL, but the levels were elevated in HLNs and the highest in TANs, suggesting tissue-specific induction of Bcl-xL (Fig. 3.2A-C).

In addition, we wanted to explore if SiglecF^{+} TANs, which were shown to be long-lived cells compared to their SiglecF^{-} counterparts [157] were expressing higher levels of the anti-apoptotic protein in autochthonous KP tumors. Using flow cytometry, we measured higher Bcl-xL levels in SiglecF^{+} TANs compared to their SiglecF^{-} counterparts (Fig. 3.2D), suggesting that Bcl-xL supports TAN differentiation toward pro-tumorigenic SiglecF+ cells.

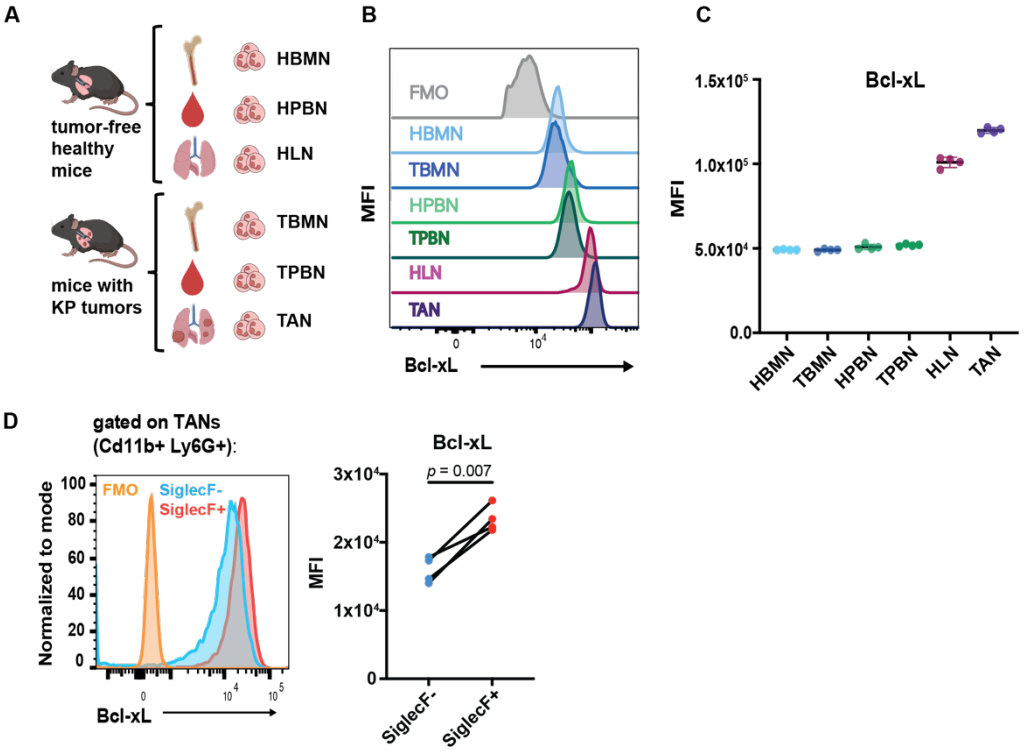


Figure 3.2 Tumor-associated neutrophils (TANs) overexpress the anti-apoptotic protein Bcl-xL

(A) Scheme describing the different neutrophil populations analyzed. For healthy naïve mice: bone marrow-derived neutrophils (HBMN), peripheral blood-derived neutrophils (HPBN), lung neutrophils (HLN). For tumor-bearing mice: bone marrow-derived neutrophils (TBMN), peripheral blood-derived neutrophils (TPBN) and tumor-associated neutrophils (TAN). (B) Representative flow cytometry histograms showing Bcl-xL levels in different neutrophil populations in naïve mice and mice with KP tumors. (C) Representative flow cytometry histograms showing Bcl-xL levels in SiglecF– and SiglecF+ TANs and corresponding MFI quantification (n=4). (D) Representative flow cytometry histograms showing Bcl-xL levels in SiglecF– and SiglecF+ TANs and corresponding MFI quantification (from n=4 tumors). Results are shown as mean ± S.D. and statistical analysis was performed using paired t-test (D).

We hypothesized that the tumor cell secretome triggers the extended survival of TANs. To test this, we incubated BMNs with conditioned medium from KP tumors or from SV2, a cell line derived from a *Kras*$^{Lox\text{-}STOP\text{-}Lox\text{-}G12D/WT}$; *p53*$^{Flox/Flox}$ tumor (see scheme Fig. 3.3A). We measured

by flow cytometry the percentage of neutrophils alive after 24h of incubation and determined that tumor (or SV2)-derived supernatant enhanced neutrophil survival by two-fold compared to neutrophils cultured with medium only (Fig. 3.3B and 3.3C). Real-time PCR analysis further revealed an increased Bcl2l1 gene expression in BMNs incubated with tumor-cell supernatant (Fig. 3.3D), which was confirmed at the protein level by Western blot (Fig. 3.3E)

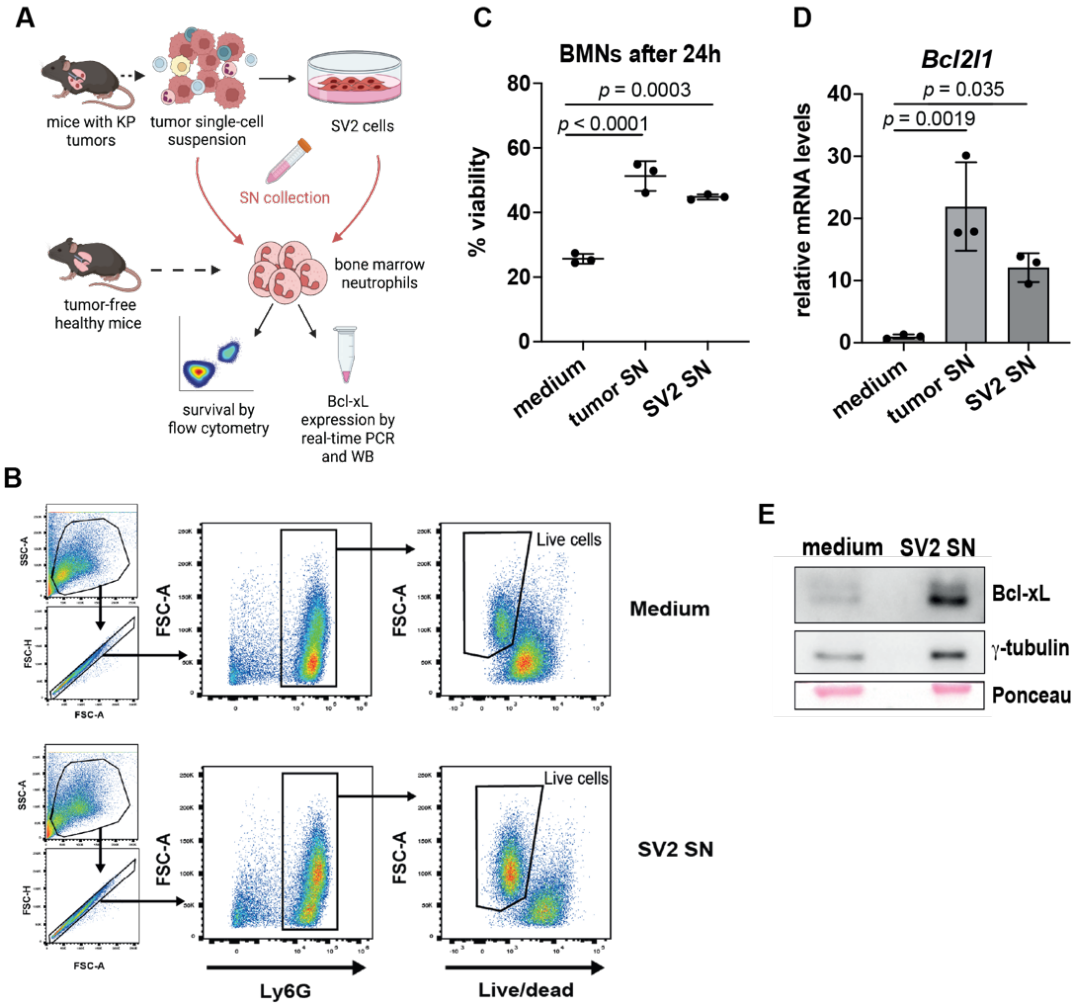


Figure 3.3 Tumor-cell supernatant mediates bone marrow neutrophils' survival and Bcl-xL expression. **(A)** Scheme representing the experimental setup for the neutrophil survival assay. **(B)** Representative flow cytometry gating strategy of alive bone marrow-extracted neutrophils after 24h of incubation with medium or SV2 SN **(C)** Percentage of viable neutrophils after 24h in medium, tumor-derived supernatant (SN) or SV2 cell-derived SN (n=3 biological replicates). **(D)** Real-time PCR analysis of *Bcl2l1* in BMNs upon incubation with medium or supernatant from tumors or from SV2 cells. **(E)** Bcl-xL expression in BMNs after 24h incubation in medium or SV2 SN. Data are shown as mean ± S.D. of n=3 mice of a representative experiment reproduced three times. *In vitro* assays were performed at least three times. Statistical significance was determined by ordinary one-way ANOVA with Dunnett's multiple comparisons test.

3.2 TUMOR CELLS INDUCE BCL-XL EXPRESSION IN NEUTROPHILS VIA GM-CSF-MEDIATED JAK/STAT SIGNALING

To determine which tumor-cell-secreted molecules could be responsible for inducing neutrophil survival in vitro, we took an unbiased approach by comparing the conditioned medium of healthy lung cells and the conditioned medium of tumor cells on a multiplexed enzyme-linked immunosorbent assay (ELISA). The signal was however weak, and we concluded that the cytokines and growth factors are probably secreted at relatively low concentrations. We therefore opted for a candidate-based approach and sought the available single-cell transcriptomics data for molecules known to impact neutrophil function. It is known that cancer cells can skew hematopoiesis toward increased myeloid cell production by releasing factors such as G-CSF and GM-CSF [164,299]. We therefore looked at the levels of expression of the receptors for both these growth factors and we saw a predominant G-CSF receptor (*Csf3r*) expression in neutrophils from healthy mice (Fig. 3.4A). In contrast, the N4 SiglecF+ TAN subset showed a lower *Csf3r* expression with an increased expression of *Csf2rb*, which encodes one of the chains of the heterodimeric GM-CSF receptor (GM-CSFR); the gene encoding the other chain, *Csf2ra*, was expressed in all neutrophil subsets (Fig. 3.4A). By Real-time PCR analysis, we confirmed a trend towards upregulated *Csf2rb* gene expression in TANs compared to healthy neutrophils (Fig. 3.4B).

It is known that tumor cells can secrete GM-CSF to promote the egress of neutrophils from bone marrow. We therefore measured the levels of GM-CSF from the blood of KP mice but did not detect any in all the mice tested (n=10, data not shown), suggesting that GM-CSF secretion happens and remains locally situated in the lungs. We therefore measured if GM-CSF was present in the bronchoalveolar lavage fluid (BALF) of healthy and tumor-bearing mice. We detected none or trace amounts of it in the BALF of healthy individuals. In parallel, three out of four tumor-bearing mice showed elevated levels of this growth factor in the BALF (Fig. 3.4C).

To confirm our interest in this growth factor, we investigated the relationship between GM-CSF and SiglecF by quantifying GM-CSF from single KP tumors with ELISA. We determined a positive correlation between GM-CSF and the absolute numbers of total TANs (R^2 = 0.42), which was weaker and stronger when considering SiglecF$^-$ (R^2=0.39) and SiglecF+ TANs (R^2 = 0.47), respectively (Fig. 3.4D).

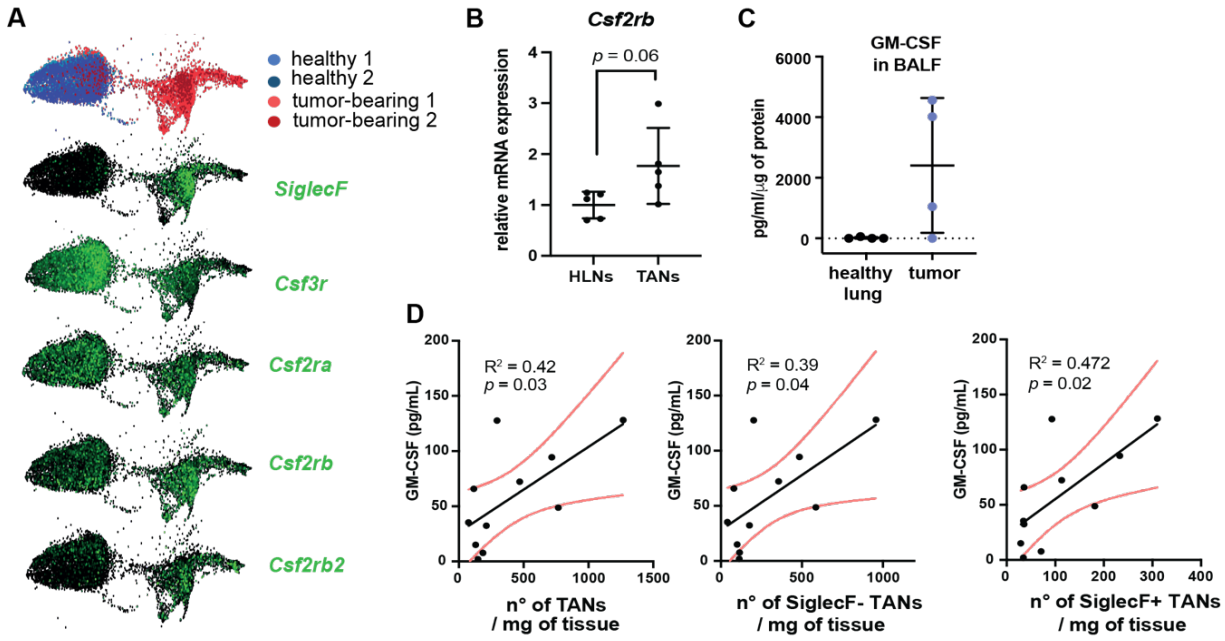
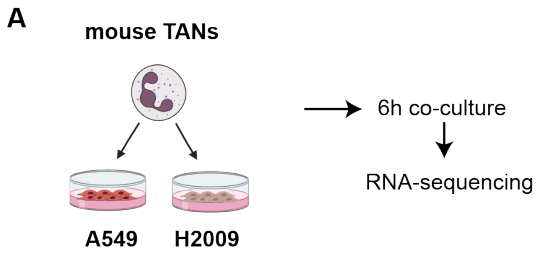


Figure 3.4 GM-CSF presence correlates with pro-tumoral neutrophil phenotype.

(A) Gene set enrichment analysis (GSEA) showing downregulation of the apoptosis pathway in TANs compared to HLNs. **(B)** Real-time PCR showing *Csf2rb* gene expression in TANs, normalized to expression in HLNs (n=5 biological replicates in each group). **(C)** GM-CSF concentration measured in the bronchoalveolar lavage fluid (BALF) from healthy (n=4) and tumor-bearing mice (n=4). **(F)** Pearson correlation analysis between the frequency of TANs (gated on CD45+CD11b+Ly6G+ cells), SiglecF^{-} TANs and SiglecF+ TANs and GM-CSF levels in individual tumors (n=11). Data are shown as mean ± S.D. For **(B)**, significance was determined with Mann-Whitney test.

Interestingly, when incubating mouse TANs with two human lung cancer cell lines, A549 and H2009, we observed an increased expression of *CXCL5* in the former, a cytokine shown to recruit neutrophils to lungs and sustain tumor-promoting SiglecF^{+} TANs [218] and *CSF2* (coding for GM-CSF) expression in the latter, suggesting that tumor cells in contact with TANs upregulate chemokines and growth factors that can recruit additional neutrophils and promote their transition into tumor-supportive cells (Fig. 3.5A). Moreover, in human LUAD, CSF2 expression correlated with poorer overall survival (Fig. 3.5B). Altogether, these data suggest that local GM-CSF secretion, possibly by tumor cells, mediates TAN polarization toward SiglecF+, long-lived and tumor-supportive cells *in vivo*.



TANs_A549_vs_A549_only

gfold_ sign	Gene Symbol	Gene name	Gene description	GFOLD_0.01
up	ZNF28	ENSG00000198538	zinc finger protein 28	8.29383
up	MUC4	ENSG00000145113	mucin 4, cell surface associated	7.87969
up	DDX43	ENSG00000080007	DEAD-box helicase 43	7.84919
up	THEMIS2	ENSG00000130775	thymocyte selection associated family member 2	7.81760
up	CSF2	ENSG00000164400	colony stimulating factor 2	7.41990
up	LRRC61	ENSG00000127399	leucine rich repeat containing 61	7.39715
up	ICAM1	ENSG00000090339	intercellular adhesion molecule 1	7.33246
up	ZNF702P	ENSG00000242779	zinc finger protein 702, pseudogene	7.16572
up	FUT3	ENSG00000171124	fucosyltransferase 3 (Lewis blood group)	7.13715
up	DNAH2	ENSG00000183914	dynein axonemal heavy chain 2	7.12976

TANs_H2009_vs_H2009_only

gfold_ sign	Gene Symbol	Gene name	Gene description	GFOLD_0.01
up	MUC5AC	ENSG00000215182	mucin 5AC, oligomeric mucus/gel-forming	12.27670
up	FGB	ENSG00000171564	fibrinogen beta chain	11.87500
up	CXCL5	ENSG00000163735	C-X-C motif chemokine ligand 5	11.39780
up	ALDH1A1	ENSG00000165092	aldehyde dehydrogenase 1 family member A1	11.23410
up	FGG	ENSG00000171557	fibrinogen gamma chain	11.10340
up	CLU	ENSG00000120885	clusterin	11.03440
up	SPP1	ENSG00000118785	secreted phosphoprotein 1	10.90550
up	AKR1C2	ENSG00000151632	aldo-keto reductase family 1 member C2	10.78790
up	RHCG	ENSG00000140519	Rh family C glycoprotein	10.57570
up	SRGN	ENSG00000122862	serglycin	10.51290

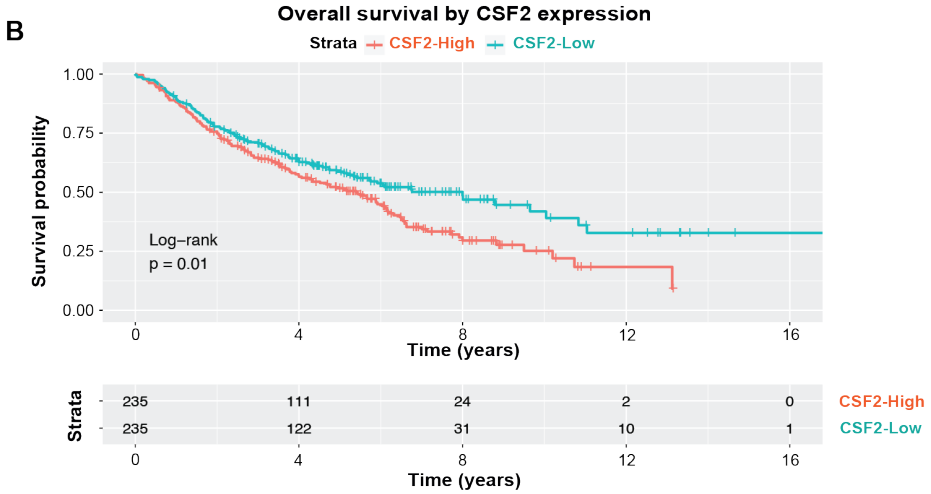


Figure 3.5 Tumor cells incubated with TANs upregulate CXCL5 and GM-CSF gene expression and *CSF2* expression correlates with poor overall survival in LUAD patients

(A) Mouse TANs were cocultured with the human lung-tumor cell lines A549 and H2009 for 6h. The tables show the top 10 genes upregulated in each cell line when incubated with TANs versus alone. Arrows point at *CSF2* and *CXCL5*. **(B)** Kaplan–Meier curves for overall survival and p-value of pairwise differences between groups with high or low *CSF2* expression from the combined LUAD transcriptome dataset.

During inflammation, the death of neutrophils can be delayed in response to several signals, such as growth factors, cytokines or danger-associated motifs [300,301] with different signaling pathways reported. To determine which pathway is involved in our context, we decided to incubate freshly isolated BMNs with SV2 supernatant and increasing doses of selected pathway inhibitors including ruxolitinib (JAK1/2 inhibitor), stattic (STAT3 inhibitor), MLN120B (IKKβinhibitor) and Ly294002 (PI3K inhibitor). After 24h of incubation, each of stattic and ruxolitinib inhibited neutrophil survival, while the other two compounds had no effect (Fig. 3.6A). In the same conditions, both stattic and ruxolitinib repressed Bcl-xL in a dose-dependent manner (Fig. 3.6B). These results altogether show that the tumor supernatant expands neutrophil survival and induces Bcl-xL expression through the JAK-STAT3 pathway. Of note, stattic did not diminish basal neutrophil survival, confirming that JAK-STAT signaling is selectively triggered when neutrophils are incubated with a tumor cell-derived supernatant (Fig. 3.6 C).

This observation was interesting, as GM-CSF is a known activator of the JAK-STAT3 pathway [302]. Thus, we hypothesized that GM-CSF sustains neutrophils in the lung tumor microenvironment and supports their transition into SiglecF+ cells. First, we tested if GM-CSF was able to induce Bcl-xL expression. We cultured BMNs with 10 ng/mL of GM-CSF for 24h, which increased their survival to a similar extent as the tumor cell supernatant did (Fig. 3.6C). Importantly, both were prevented upon the addition of 5 μM stattic, indicating GM-CSF-induced neutrophil survival is mediated by the JAK-STAT signaling pathway (Fig. 3.6C). Western blot analysis confirmed the upregulation of Bcl-xL in BMNs incubated with GM-CSF, which was abrogated by stattic (Fig. 3.6D). Finally, neutralization with anti-GM-CSF counteracted the tumor supernatant-dependent increased neutrophil viability (Fig. 3.6C).

Simultaneously, we wanted to measure if GM-CSF derived from the supernatant could induce SiglecF$^+$ expression in cultured bone marrow neutrophils. After 24h of incubation, both the tumor cell supernatant and GM-CSF induced cell surface SiglecF expression in BMNs, which was diminished with increasing doses of stattic (Fig. 3.6E).

Together, these data position GM-CSF as a major contributor for neutrophil survival mediated by a supernatant of *in vitro* cultured lung tumor cells.

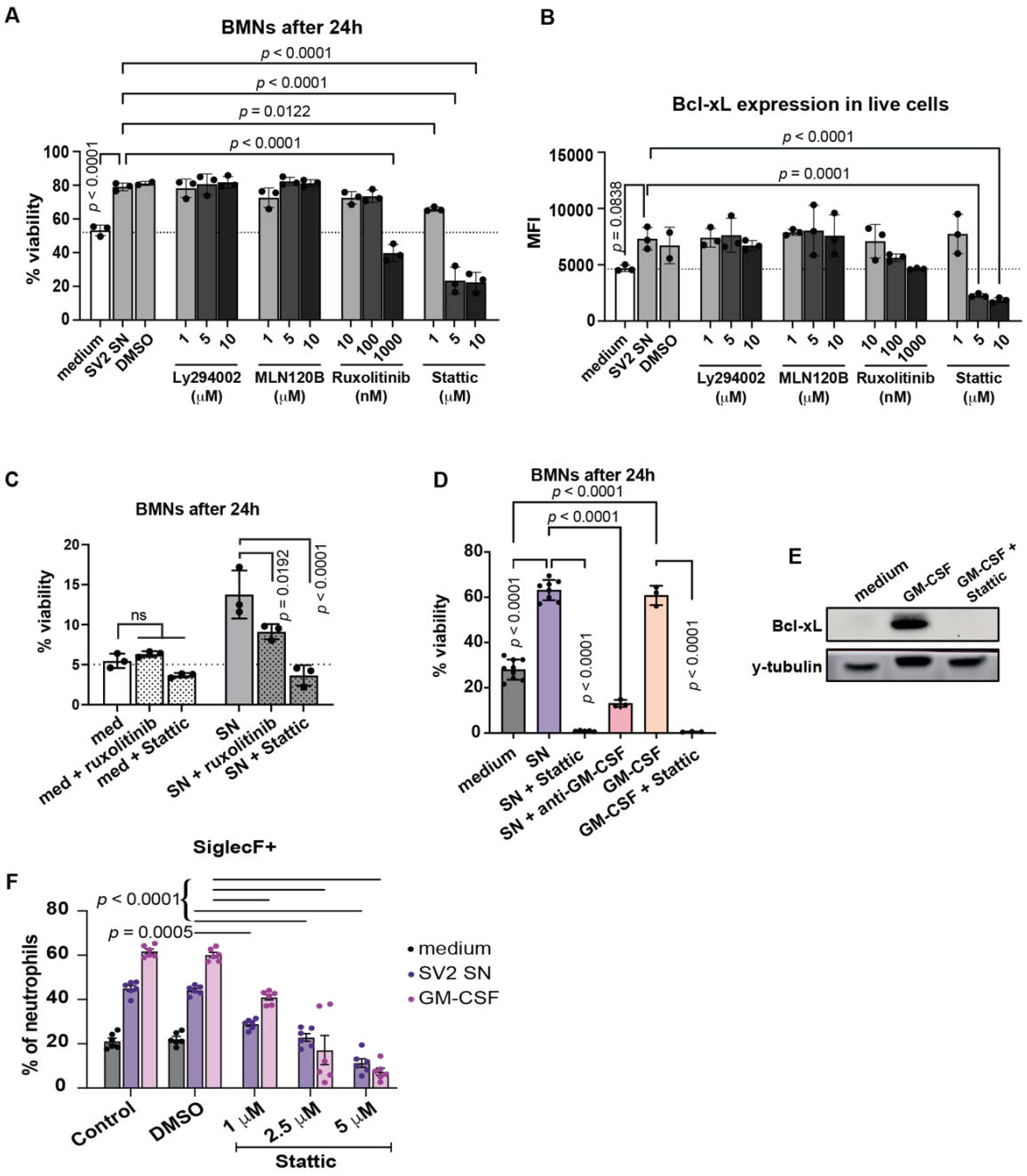


Figure 3.6 Bcl-xL is induced by GM-CSF-mediated JAK-STAT signaling

(A) Percentage of viable bone marrow neutrophils (BMN) after 24h incubation with medium, SV2 cell supernatant (SN), DMSO as control or SN with the indicated doses of Ly294002 (PI3K inhibitor), MLN120B (IKKb inhibitor), ruxolitinib (JAK1/2 inhibitor) or stattic (STAT3 inhibitor). The mean basal neutrophil survival (incubated with medium) is indicated with a dashed line. **(B)** Mean fluorescence intensity (MFI) of Bcl-xL expression in neutrophils from **(A)**. The mean basal intensity is indicated with a dashed line. **(C)** BMN viability was measured by flow cytometry 24h after incubation with indicated doses of ruxolitinib (1 mM) or stattic (5 mM) in medium or SV2 supernatant, with BMNs extracted from n=3 mice. Data are shown as mean ± S.D. **(D)** Percentage of viable BMNs after 24h incubation with SN or GM-CSF (10ng/mL) and with or without stattic (5 μM), and SN with GM-CSF neutralizing antibody **(E)** Western blot analysis of Bcl-xL from BMNs incubated with GM-CSF (10 ng/mL) and stattic (5 μM) for 24h. **(F)** SiglecF surface protein expression measured in BMNs (n=6 biological replicates) incubated with SV2 SN or GM-CSF and with or without stattic at the indicated concentrations for 24h. This experiment was performed by Dr. Sarika Rana, a post-doc from our

group Data are shown as mean ± S.D. Significance was determined using ordinary one-way ANOVA with Dunett's **(A-B)** and Tukey's **(C-D)** multiple comparison tests. For **(F)**, two-way ANOVA with Tukey's multiple comparisons test was performed.

3.3 BCL-XL BLOCKADE IMPAIRS NEUTROPHIL AGING IN VITRO AND IN VIVO

Having established the link between Bcl-xL induction and neutrophil ageing, we decided to investigate if Bcl-xL blockade could prevent neutrophil survival *in vitro* and reduce TANs' lifespan *in vivo*. For this, we selected two well-known BH3 mimetics that have undergone clinical testing: Navitoclax, a Bcl-2/Bcl-xL dual inhibitor [269,278] and Venetoclax a Bcl-2-specific inhibitor [278,298] (See Fig. 3.7A). We also chose A-1331852, a BH3 mimetic that has been developed more recently and exhibits a robust binding affinity to Bcl-xL, along with high selectivity in inhibiting it [298.] Of note, A-1331852 has a stronger affinity for Bcl-xL than Navitoclax and is comparatively more potent to impair the growth of Bcl-xL-dependent tumor cells [303]

After incubating BMNs with SV2 cell-derived supernatant together with increasing doses of each inhibitor, we noticed that A-1331852 diminished neutrophil survival even at the lowest dose of 0.1 nM. In contrast, Navitoclax and Venetoclax only partially reduced neutrophil survival at the highest dose (100 nM) (Fig. 3.7B), showing that Bcl-xL but not Bcl-2 mediates neutrophil survival in tumor supernatant conditions. Importantly, A-1331852 did not affect neutrophils cultured in normal medium (Fig. 3.7B), suggesting a window of opportunity for targeting aberrantly aging TANs while sparing normal neutrophils.

We then wanted to test if Bcl-xL, and not Bcl-2, supports neutrophil survival *in vivo* as well. To separate TANs based on their age, we injected mice with BrdU, a thymidine analog that incorporates into the DNA of proliferating cells, including neutrophil progenitors in the bone marrow. As mature neutrophils are post-mitotic, BrdU will remain detectable in these cells until they die. Knowing that TANs can survive for more than 6 days within the lung tumor microenvironment [222], we chose to inject KP mice with BrdU and to start the treatment the following day with either Venetoclax or Navitoclax for 6 days (See Fig.3.7C). Endpoint analysis revealed no difference in total TAN and SiglecF+ TAN prevalence upon each treatment. However, we observed a significant reduction in SiglecF+BrdU+ TANs, i.e. in 6.5 days old TANs from mice treated with Navitoclax, compared to the control and Venetoclax-treated groups (Fig. 3.7D). These results suggest a contribution for Bcl-xL but not Bcl-2 in sustaining neutrophil survival within tumors.

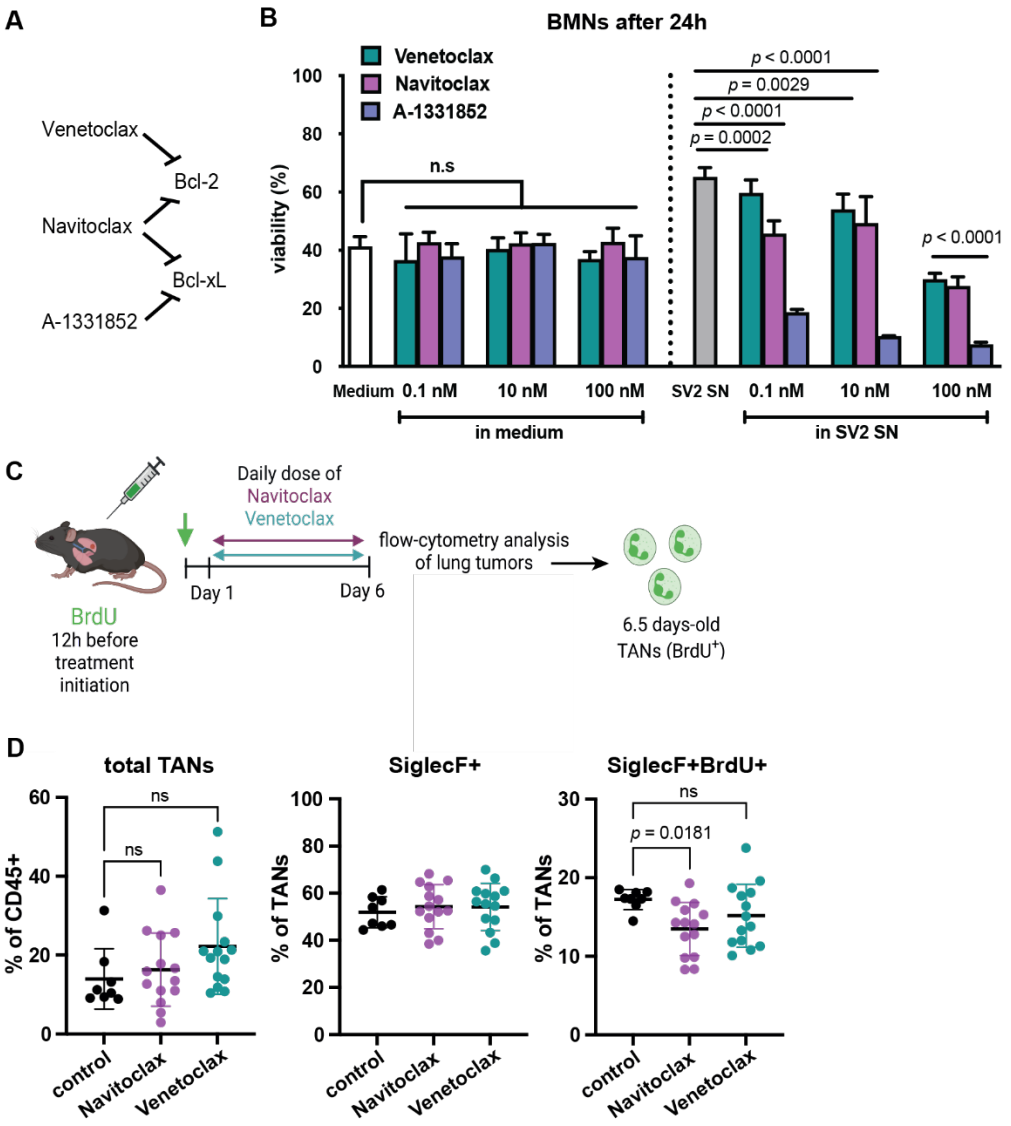


Figure 3.7 Bcl-xL-blockade with Navitoclax reduces neutrophil survival *in vitro* and *in vivo*

(A) Scheme representing BH3-mimetics inhibition specificity. **(B)** Viability (%) of BMNs incubated with 0.1, 10 and 100 nM of Venetoclax, Navitoclax or A-1331852 in medium or SV2 SN for 24h. **(C)** Scheme showing the experimental design and **(D)** Percentages of neutrophils, SiglecF$^+$ and SiglecF$^+$BrdU$^+$ 6-days-old TANs in control KP mice (n=7 tumors), mice treated with Navitoclax (n=12 tumors) or with Venetoclax (n=14). For **(B)**, conditions with drugs in the medium were analyzed compared to the medium-only condition, and drugs in SV2 SN were compared to the SV2 SN condition and significance was determined by ordinary one-way ANOVA with Dunnett's multiple comparisons test. For **(F)**, total TANs were analyzed by Kruskal-Wallis with Dunn's multiple comparisons test. SiglecF+BrdU+ TANs were analyzed by ordinary one-way ANOVA and Dunn's multiple comparisons test.

We then wanted to assess the sensitivity of TANs to Bcl-xL inhibition *in vitro*. For this, we isolated neutrophils from KP lung tumors using positive Ly6G magnetic cell sorting and incubated them with the same doses of A-1331852 as for BMNs, in normal or in tumor cell supernatant. As opposed to BMNs, whose survival remained unaffected by the drug (Fig. 3.8A), A-1331852 induced TAN cell death already in basal conditions, demonstrating that Bcl-xL induction in TANs renders them vulnerable to its blockade (Fig. 3.8B). Interestingly, despite the low percentage of viable cells after 20h of incubation, TANs survival was increased upon SN addition (Fig. 3.8B). Finally, to directly compare the cytotoxic activity of Bcl-xL blockade on TAN subsets, we incubated freshly isolated TANs with an anti-SiglecF antibody for 15 minutes to mark them and then incubated the cells with A-1331852 for 6 hours. This experiment demonstrated a specific killing of SiglecF+ TANs (Fig. 3.8C), highlighting the vulnerability of this tumor-supportive subset in comparison to other TANs.

Since Bcl-xL is a known anti-apoptotic protein, we decided to confirm if Bcl-xL upregulation sustains neutrophil survival by preventing apoptosis, or if it is implicated in another cell survival mechanism. Therefore, instead of staining for dead cells, which does not discriminate between the different types of cell deaths, we performed an annexin-V and 7-AAD apoptosis assay. Tumor necrosis factor (TNF) was used as a positive control for inducing neutrophil apoptosis. After 24 hours of incubation, we noted a decrease in early apoptotic neutrophils (Annexin-V^+ cells) when incubated with the supernatant, compared with cells incubated with medium only (Fig. 3.8D) indicating that the supernatant-induced Bcl-xL expression is extending neutrophil survival by preventing the cells from undergoing apoptosis. The increased percentage of apoptotic cells upon the addition of A-1331852 further confirmed the anti-apoptotic role of Bcl-xL in this setting (Fig. 3.8E). Along with this, we detected low levels of cleaved caspase-3 in the condition with the SN, which was abrogated by the addition of the Bcl-xL inhibitor, therefore further confirming that neutrophils survive longer in the tumor SN thanks to the blocking of the apoptotic cascade by Bcl-xL (Fig. 3.8F). Moreover, Bcl-xL seemed to have the same role of delaying apoptosis in TANs. Indeed, TANs remained sensitive to A-1331852 when incubated Altogether, these results indicate that Bcl-xL supports TANs survival by inhibition of cell apoptosis. with the tumor SN but this effect was lost upon pre-incubation with Z-VAD-FMK, a pan-caspase inhibitor (Fig. 3.8G). Altogether, these results indicate that Bcl-xL supports TANs survival by inhibition of cell apoptosis.

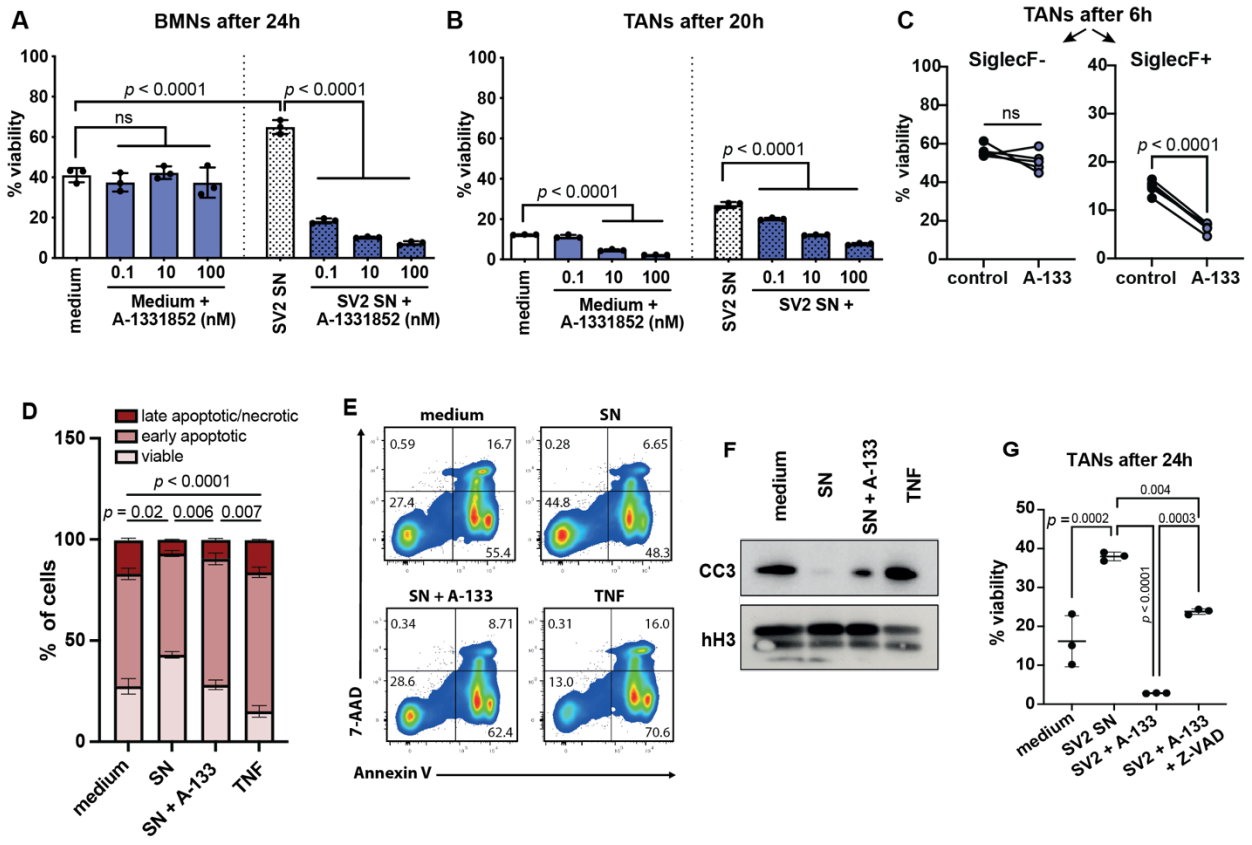


Figure 3.8 Bcl-xL-specific blockade with A-1331852 reduces neutrophils survival *in vitro*

(A-B) Bone marrow **(A)** and TAN **(B)** viability (% of live cells out of total) after 24h in vitro culture in medium only or with increasing doses of A-1331852. **(C)** SiglecF^{-} and SiglecF+ TAN viability (% of live cells out of total) after 6h in vitro culture in medium only or with 50 nM of A-1331852. TANs were obtained from n=5 tumors. **(D)** percentage of viable (AnnexinV^{-}7-AAD^{-}), early (AnnexinV^{+}7-AAD^{-}), and late apoptotic (AnnexinV^{+}7-AAD^{+}) BMNs incubated with medium or SV2 SN with or without A-1331852. TNF (5 ng/mL) was used as control to induce neutrophil apoptosis. **(E)** representative Annexin-V and 7-AAD flow cytometry staining for **(D)**. **(G)** Western blot showing cleaved-caspase 3 in the conditions shown in **(D)**. **(F)** Percentage of surviving TANs after 24h, with SV2, SV2 + A-1331852 with or without preliminary incubation with the pan-caspase inhibitor z-VAD-FMK (20 mM). TANs are from n=3 tumors. Data are shown as mean ± S.D.For **(A**, **B** and **G)**, significance was determined by ordinary one-way ANOVA with Tukey's multiple comparisons test. For **(C)**, significance was based on a paired t-test. For **(D)**, significance was based on two-way ANOVA with Tukey's multiple comparisons test. ns, non-significant.

To confirm the importance of Bcl-xL in TAN survival, we injected tumor-bearing mice with a single dose of BrdU and started A-1331852 treatment the next morning for a duration of 8 days before sacrifice (See scheme Fig. 3.9A). Flow cytometry analysis revealed that there was no difference in total TAN prevalence (Fig. 3.9B). However, qualitative differences in TAN subsets occurred: SiglecF+ TANs were reduced in treated mice compared to controls, which was attributed to a very significant decrease of the 8.5 days old, SiglecF+BrdU+ cells (Fig. 3.9C). The loss of this old TAN subset was accompanied by a gain of younger, SiglecF$^-$ BrdU- TANs, highlighting an old-to-young TAN shift upon short-term A-1331852 treatment. Correspondingly, Siglef expression, measured by real-time PCR analysis from total TANs, was reduced upon Bcl-xL blockade (Fig. 3.10).

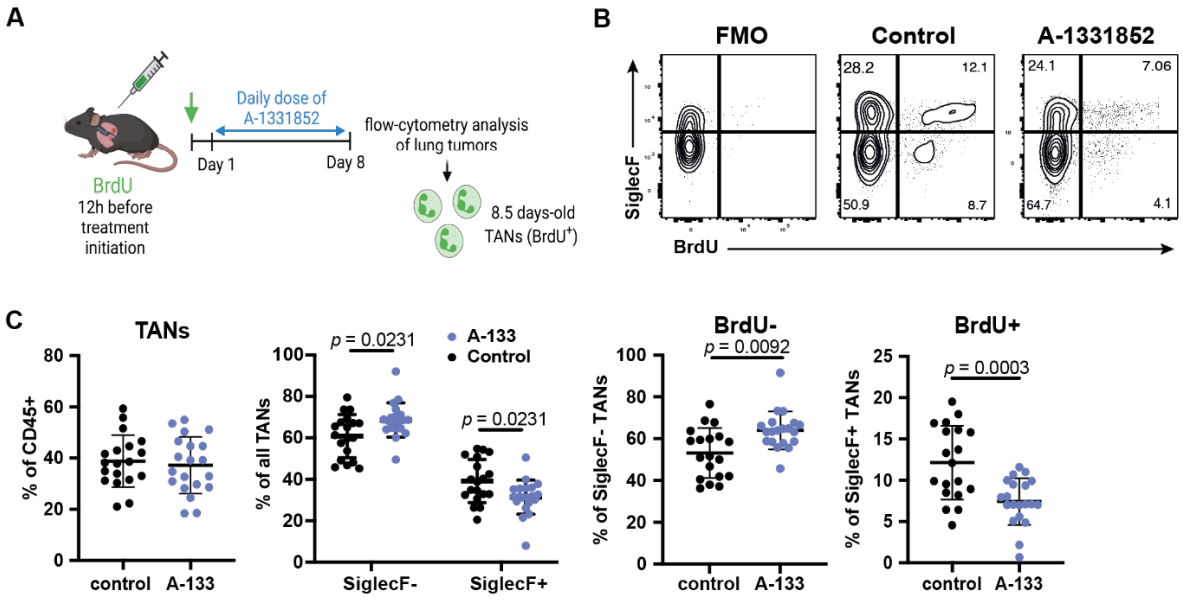


Figure 3.9 Bcl-xL blockade with A-1331852 reduces the prevalence of aged TANs *in vivo*

(A) Scheme describing the experimental design for tracking aging neutrophils with BrdU. **(B)** representative flow-cytometry plots showing the prevalence of aged 8.5 days-old TANs (BrdU$^+$) in control and A-1331852-treated mice. **(C)** Prevalence of total, SiglecF$^+$, SiglecF$^-$, BrdU$^-$ SiglecF$^-$ and BrdU$^+$SiglecF$^+$ TANs. Each data point represents a single tumor. For control group, n=18 tumors from 3 mice and n=20 tumors from 3 A-1331852-treated mice. Data are shown as mean ± S.D. Significance was based on a two-tailed Student's t-test

Recently, a TAN subset distinct from SiglecF^{high} cells and marked by an interferon-stimulated gene (ISG) signature was identified as essential for the successful response to anti-CD40 in orthotopic Kras$^{Lox\text{-}STOP\text{-}Lox\text{-}G12D/WT}$; p53$^{Flox/Flox}$ tumors [304]. In contrast to Siglecf, A-1331852 did not diminish the expression of interferon-related genes and ISGs by TANs; it even increased Cxcl10 and Ddx58 (Fig. 3.10), suggesting that Bcl-xL blockade selectively eliminates SiglecF^{high} neutrophils and preserves ISG-expressing TANs.

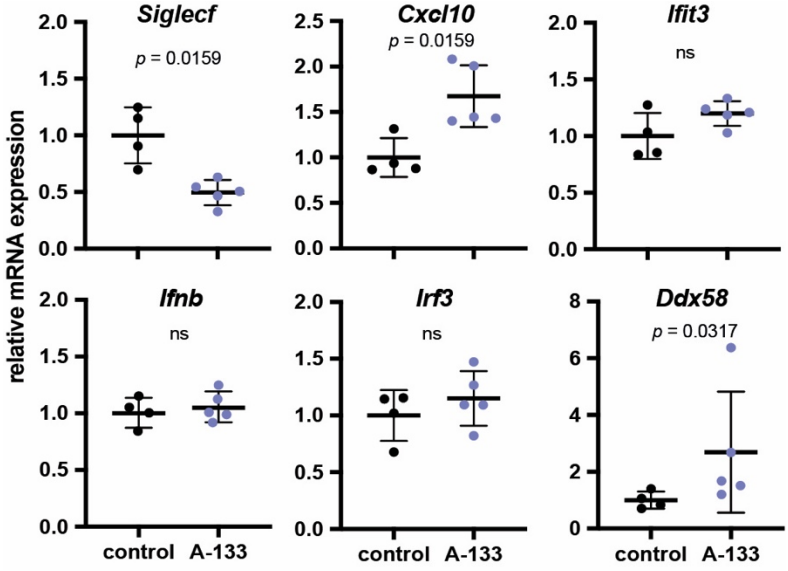


Figure 3.10 TANs with interferon-stimulated gene (ISG) signature are maintained after treatment with A-1331852

Real-time PCR analysis of expression of the indicated genes in TANs extracted from n=4 control or n=5 A-1331852 treated tumors. Significance was based on the Mann-Whitney test. ns, non significant

3.4 BCL-XL BLOCKADE MODIFIES THE TUMOR MICROENVIRONMENT

To interrogate the consequences of Bcl-xL inhibition on the lung tumor immune microenvironment globally, we treated mice with A-1331852 for two weeks and performed a full spectrum flow cytometry analysis of tumors (Fig. 3.11A and see the gating strategy in Fig. 2A from the Methods section). Our data revealed trends toward fewer neutrophils and more CD3+ T cells upon treatment (Fig. 3.11B). We observed an almost significant increase in CD8+ cytotoxic T cells (p = 0.07), a significant increase in CD4+ T helper cells and Foxp3+ regulatory T cells (Fig. 3.11C), but no change in PD-1 expression by any of these subpopulations (Fig. 3.11D).

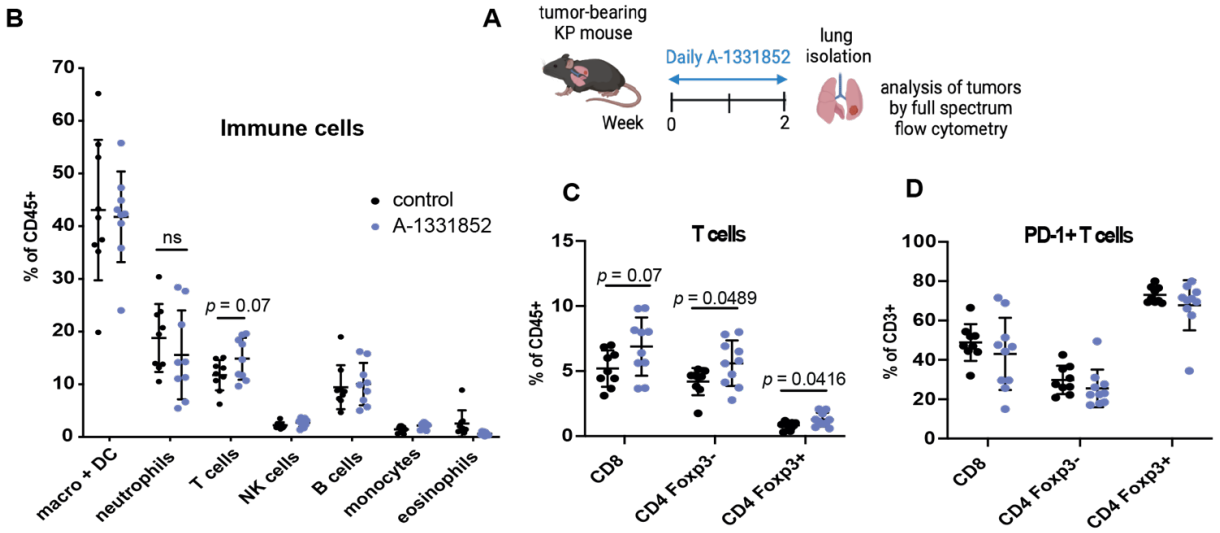


Figure 3.11 Two weeks treatment with A-1331852 increases T cell population in tumors

(A) scheme describing the experimental setup. **(B)** Percentage of the different immune cell populations in KP tumors in mice treated for 2 weeks with A-1331852. **(C)** Percentage of the different T cells among immune cells **(D)** Percentage of PD-1-expressing T cells treated and non-treated tumors. Each data point corresponds to one tumor. n=8 tumors from 2 control mice were analyzed and n=9 tumors from 3 A-1331852-treated mice. Data are shown as mean ± S.D. Significance was based on a two-tailed Student's t-test

Looking more in depth at the neutrophil population, we found that SiglecF+ TANs were significantly decreased (Fig. 3.12B), which was accompanied by a decrease in PD-L1-expressing TANs, the majority of them being SiglecF+ (Fig. 3.12C). Of note, Bcl-xL expression, which was higher in SiglecF+ than in SiglecF$^-$ TANs, significantly diminished in each subset after treatment (Fig. 3.12D). This suggests that SiglecF- TANs, which we have shown previously to express Bcl-xL (Fig 3.2D), are also impacted by the treatment, despite the SiglecF- pool of neutrophils being maintained. Thus, short and long-term Bcl-xL inhibition *in vivo* counteracts the aging of TANs and increases the proportion of tumor-infiltrating T lymphocytes.

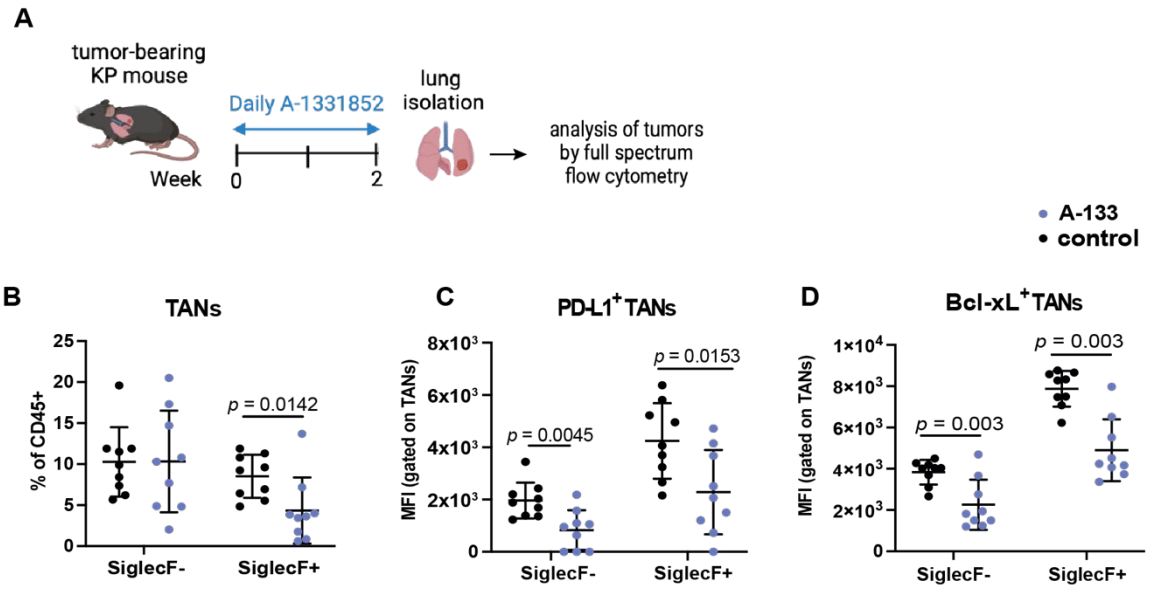


Figure 3.12 Two weeks treatment with A-1331852 reduces the proportions of SiglecF+ and PD-L1+ TANs

A) scheme representing the experimental setup **(B)** Percentage of SiglecF$^-$ and SiglecF+ TANs in KP tumors in mice treated for 2 weeks with A-1331852. **(C-D)** Mean fluorescence intensity showing **(C)** PD-L1 and **(D)** Bcl-xL expressions in SiglecF$^-$ and SiglecF+ TANs. Each data point corresponds to one tumor. n=8 tumors from 2 control mice were analyzed and n=9 tumors from 3 A-1331852-treated mice. Data are shown as mean ± S.D. Significance was based on a two-tailed Student's t-test. ns, non-significant.

3.5 A-133 TREATMENT IMPACTS TUMOR GROWTH

We next sought to evaluate the effect of Bcl-xL inhibition on tumor development when used as monotherapy. We treated KP tumor-bearing mice for an extended period of three weeks with A-1331852 and performed micro-computed tomography (μCT) imaging of the lungs before treatment initiation and every week thereafter (see scheme Fig. 3.13A). Bcl-xL blockade significantly delayed tumor growth after two weeks of treatment (Fig. 3.13B). On average, tumors in the control group doubled their size after two weeks, whereas tumors in treated mice were on average 1.3 times bigger compared to their size before treatment. Importantly, the anti-tumor response was maintained one week later (Fig. 3.13B and C). Staining by immunohistochemistry revealed a reduced number of tumor cells positive for Ki67 in treated mice, aligning with the decreased growth measured by μCT (Fig. 3.13D).

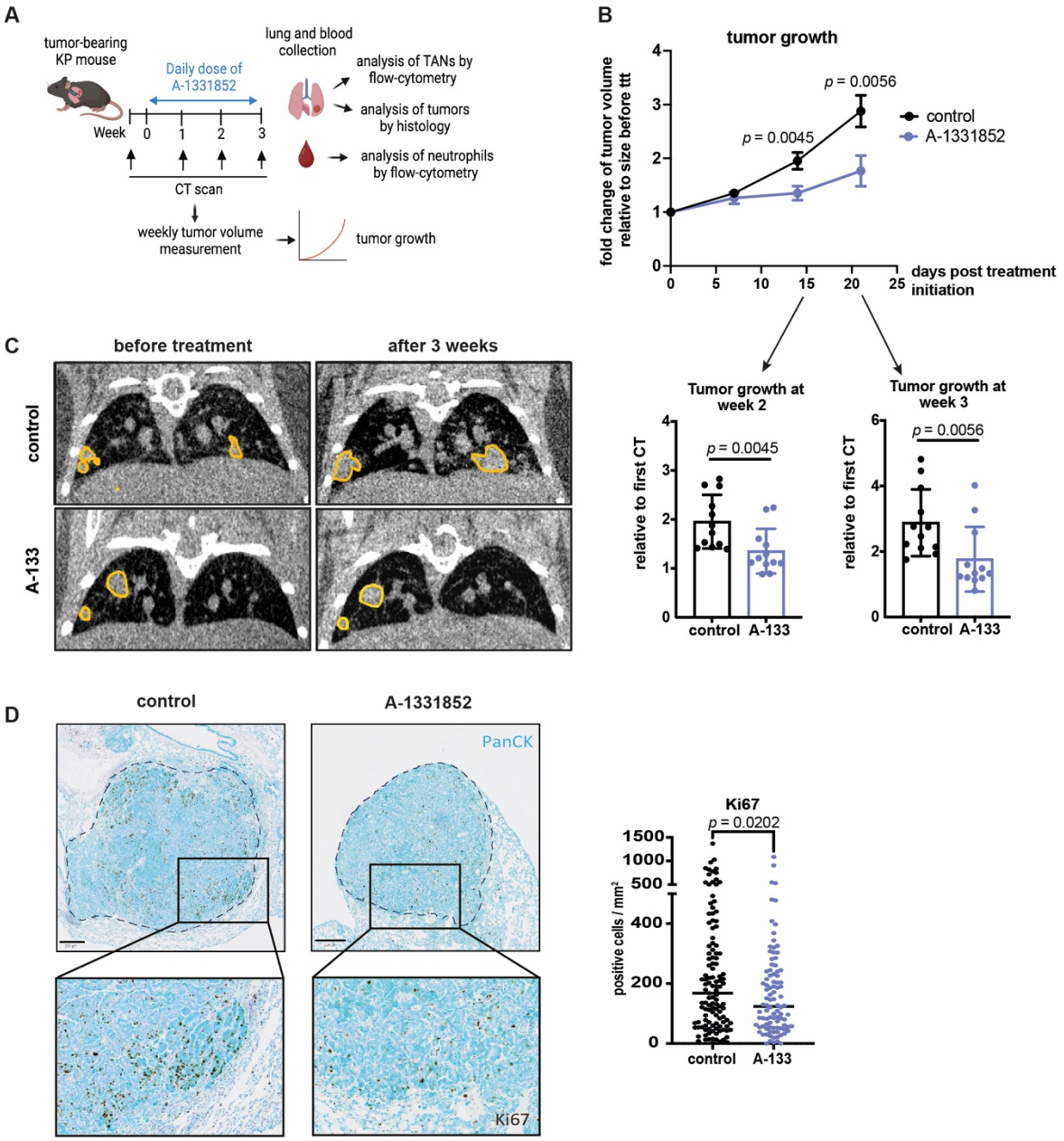


Figure 3.13 Bcl-xL blockade reduces tumor growth *in vivo*

(A) Scheme showing the experimental design. Tumor-bearing mice were treated daily for 3 weeks. For these mice and for controls, tumors were measured one day before treatment initiation and then once weekly until the endpoint. **(B)** Evolution of tumor volume over 3 weeks in control mice or A-1331852-treated mice and dot plots showing the number of tumors analyzed (n=12 tumors per group). Data show the ratio of tumor volumes relative to the initial volume size before treatment. **(C)** Examples of µCT scans of KP lungs with highlights of tumors in yellow, before treatment initiation and 3 weeks post-treatment. **(D)** Representative IHC images showing pan-cytokeratin and Ki67 staining and dot plot showing the quantification of $Ki67^+$ cells. Measurements are reported as the number of positive cells per mm^2 of lesion area. Each dot represents a single lesion analyzed. Scale bars: 100 mm. Data are shown as mean ± S.D., except for **(D)**, where the median is shown. Significance was determined by the Mann-Whitney test for **(B)** and unpaired t-test for **(D)**.

When looking at intra-tumoral neutrophils, we measured a reduced proportion of total TANs, as well as significantly decreased SiglecF+ TAN and PD-L1+ TAN percentages (Sig. 3.14A) and absolute numbers (Fig. 3.14.B) in treated compared to control tumors. Because long-term A-1331852 reduced the total number of TANs, we wondered if Bcl-xL blockade targets the circulating neutrophil pool and thus reduces the number of neutrophils recruited to the tumors. Contrary to our expectations, A-1331852 induced a significant increase in blood neutrophils (Fig. 3.14C), suggesting that long-term Bcl-xL blockade impacts granulopoiesis or neutrophil egress from the bone marrow into the circulation.

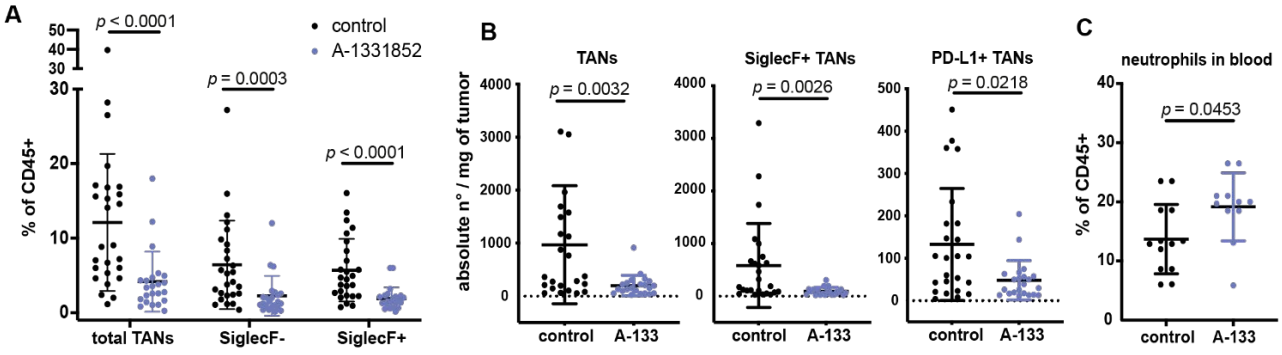


Figure 3.14 Three weeks Bcl-xL blockade reduces TANs without inducing neutropenia

(A) Percentage of total TANs, SiglecF^{-} and SiglecF^{+} TANs out of CD45^{+} cells in control (n=24 tumors) and A-1331852-treated mice (n=22 tumors analyzed). **(B)** Absolute numbers are shown for total TANs, SiglecF^{+} TANs and PD-L1^{+} TANs per tumor. **(C)** Percentage of neutrophils in blood in control (n=13) and treated mice (n=11). Data are shown as mean ± S.D. Significance was determined by the Mann-Whitney test.

As the 3-week treatment had a significant impact on all TANs, we tested whether selectivity toward SiglecF+ cells could be restored by introducing a pause in treatment. Specifically, instead of daily administration of A-1331852, we opted for a 5-day ON / 2-day OFF regimen for the same total duration (Fig. 3.15A). Notably, intermittent treatment with A-1331852 did not alter total TAN abundance and SiglecF- TANs became the major subset with almost 80% of total TANs, while SiglecF+ TANs were very significantly reduced (Fig.3.15B). These results suggest that intermittent Bcl-xL blockade can deplete SiglecF+ TANs specifically and spare SiglecF- counterparts (which exhibit lower levels of Bcl-xL) which could be beneficial for tumor control, as we have seen that TANs maintain interferon-stimulated gene expression after short term A-1331852 treatment (Fig. 3.10).

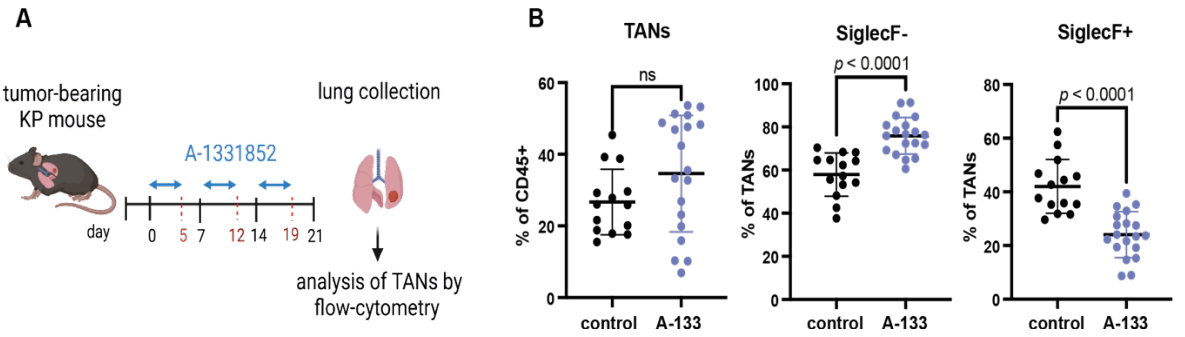


Figure 3.15 Intermittent A-1331852 treatment enables selective SiglecF+ TANs targeting

(A) Scheme describing the treatment regimen and experimental setup. Mice were treated with A-1331852 for 5 days then with two days break, for a duration of three weeks. Lung tumors were then isolated and the TAN population was analyzed by flow cytometry. **(B)** Graphs showing the percentages of total TANs, SiglecF^{-} and SiglecF+ TANs. n=14 tumors were analyzed for control mice (vehicle treated) and n=19 tumors from A-133-treated mice. Data shown are mean ± S.D. Significance was determined with a Mann-Whitney test for total TANs and two-tailed t-test for SiglecF^{-} and SiglecF+ percentages. ns, non-significant.

Although our results demonstrate that ageing TANs are particularly sensitive to Bcl-xL blockade, the tumor growth delay could be due to the action of A-1331852 on other cell types including tumor cells. In human NSCLC cell lines, Navitoclax and A-1331852 were not cytotoxic as single agents but augmented the cell death response to chemotherapies [305,306] . However, A-1331852 was reported to reduce tumor growth in a subcutaneous xenograft model of NSCLC [307]. We first wondered if KP lung tumors express Bcl-xL and found that, although this seemed to be the case, the expression was highly heterogeneous (Fig. 3.16A). To test if Bcl-xL blockade could inhibit tumor cell growth, we took the tumoral fraction remaining after TANs isolation (Ly6G- cells) and incubated them overnight with A-1331852. We could observe that these cells were not sensitive to Bcl-xL blockade (Fig. 3.16B). In addition, to test if A-1331852 affects tumor cell proliferation or viability, we used two Kras$^{Lox\text{-}STOP\text{-}Lox\text{-}G12D/WT}$;p53$^{Flox/Flox}$ tumor-derived cell lines, SV2 and T5, and incubated them with increasing concentrations of the compound. Cell viability measurements after 48 and 72h demonstrated that the cells were resistant even to high doses (Fig. 3.16C). Moreover, in a clonogenic assay performed on SV2 cells, the number of colonies did not vary at any drug concentration used compared to controls (Fig. 3.16D). These data favor the hypothesis that Bcl-xL blockade, at least when used as a single agent, does not directly impact lung tumor epithelial cells.

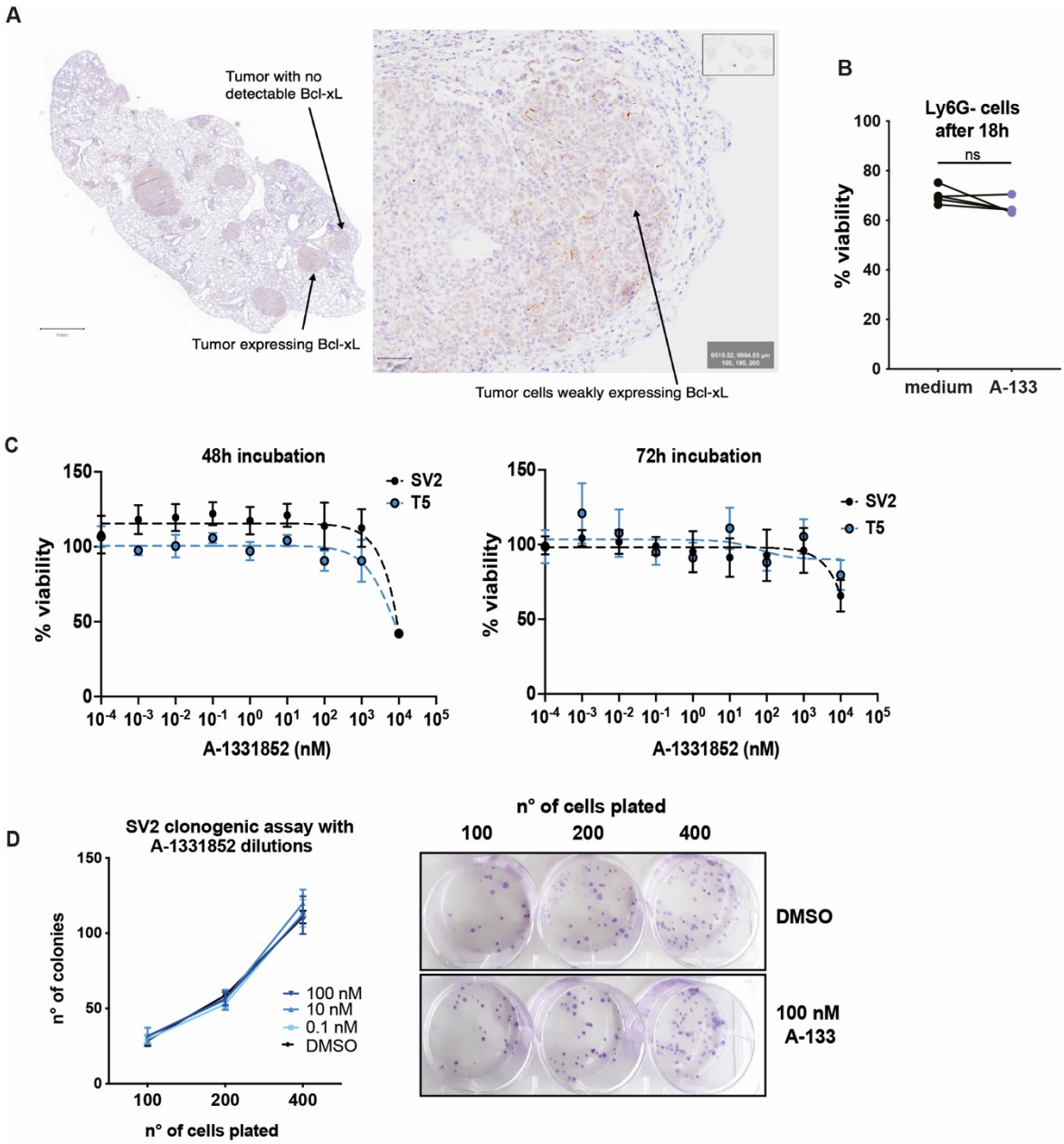


Figure 3.16 Bcl-xL blockade does not affect the viability of lung tumor cells

(A) Immunohistochemistry staining showing heterogeneous Bcl-xL expression in KP lung tumors. (B) Percentage of viable Ly6G- cells after 18h of incubation with 10 nM of A-1331852 (n=5 tumors). **(C)** Viability of SV2 and T5 cell lines, measured with PrestoBlue after 48 and 72h of incubation with serial dilutions of A-1331852. **(D)** Clonogenic assay performed with 100, 200, 400 single SV2 cells incubated with 0.1, 10 or 100 nM of A-1331852. Data are shown as mean ± S.D. For **(B)**, significance was determined based on a paired t-test.

To assess if part of the effects of Bcl-xL blockade on tumor progression are nevertheless neutrophil-independent, we initiated a regimen of neutrophil depletion using daily anti-Ly6G + anti-rat antibodies [293] two days before A-1331852 treatment initiation and continued the combination for two weeks. Neutrophil depletion alone showed a trend toward decreased tumor growth as analyzed by μCT (Fig. 3.17A). While A-1331852-treated mice confirmed the tumor growth delay, this effect was partly lost upon combined A-1331852 and neutrophil

depletion (Fig. 3.17A). Of note, anti-Ly6G + anti-rat antibodies effectively diminished the abundance of blood neutrophils over these 2 weeks (Fig. 3.17B) but only partially decreased that of TANs (Fig. 3.17C), suggesting that the importance of TANs in these conditions is underestimated. In addition, the proportion of SiglecF+ TANs was only decreased in the A-133-treated condition (Fig. 3.17D). Together, these results indicate that the effect of A-1331852 on tumor growth is at least partly mediated through TAN targeting.

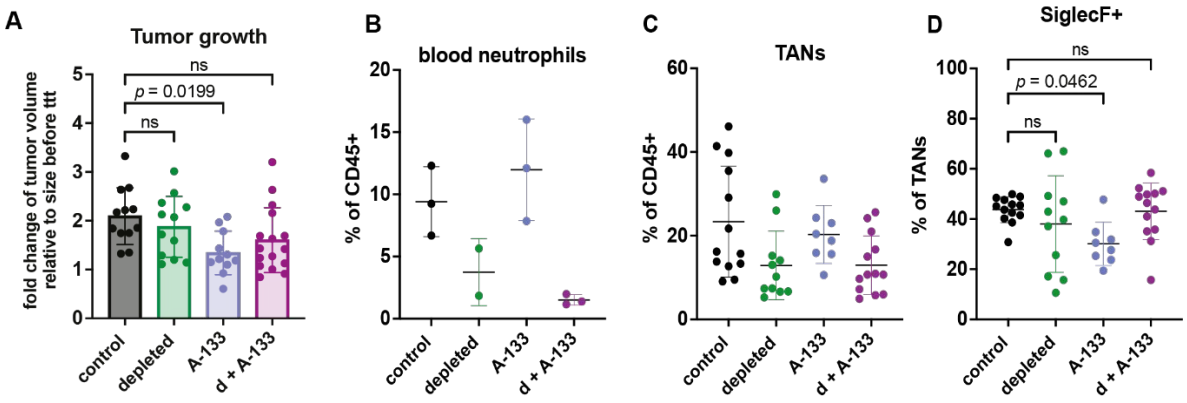


Figure 3.17 Neutrophil depletion prior A-1331852-treatement reduces Bcl-xL blockade efficacy

(A) Plots showing the growth of single tumors in control (n=12), A-1331852-treated (n=11), neutrophil-depleted (n=12) and neutrophil-depleted in combination with A-1331852 (d + A-133, n=16) KP mice. **(B)** Blood neutrophil levels are also shown for n=3 in control mice, A-1331852-treated and depletion with A-1331852, and n=2 for neutrophil-depleted only mice. **(C)** TAN proportions out of total CD45$^+$and **(D)** SiglecF$^+$ cells out of total TANs are shown for single tumors for control (n=13), A-1331852-treated (n=8), neutrophil-depleted (n=10) and neutrophil-depleted in combination with A-1331852 (n=13). Data information: Data are shown as mean ± S.D. For **(A)**, ordinary one-way ANOVA with Tukey's multiple comparisons test was performed for the tumor growth and Kruskal-Wallis with Dunn's multiple comparisons test was performed for the other panels. ns, non-significant.

3.6 G-CSF AUGMENTS THE ANTI-TUMORAL EFFECT OF BCL-XL-BLOCKADE

Cancer patients are often administered G-CSF as a recombinant protein to counteract chemotherapy-induced neutropenia. As anti-tumoral properties have also been shown in TANs [304] , we wanted to test if this clinical agent could be used in conjunction with TAN-targeting A-1331852, reasoning that (i) it could act against A-1331852 by increasing neutrophil production and their infiltration into tumors, augmenting the total pool of TANs, or (ii) it could potentiate the effects of A-1331852, by providing younger neutrophils while Bcl-xL blockade selectively removes older TANs, together shifting the balance toward young (and possibly anti-tumor) TANs.

To discriminate between these two possibilities, we combined A-1331852 treatment with G-CSF injections for three weeks and measured tumor volumes with μCT before treatment initiation and three weeks later and injected BrdU two days before sacrifice to determine the percentage of freshly recruited neutrophils to the tumors (Fig. 3.18A). As reported previously (see Fig. 3.13B), Bcl-xL blockade diminished tumor growth significantly. Although G-CSF alone showed only a trend toward decreased tumor growth, it accentuated the anti-tumor response of A-1331852, with 25% tumors (6 out of 24) that regressed after 3 weeks, compared to only 1 out of 26 tumors in the single A-1331852 treatment group (Fig. 3.18B). 48h before sacrifice, we injected mice with BrdU and measured by flow cytometry the proportions of BrdU+ TANs, representing newly recruited cells. In control and A-1331852-treated mice, there were almost no BrdU+ neutrophils within the tumors, whereas they represented around 10% TANs when G-CSF was used, indicating that G-CSF stimulated neutrophil accumulation in tumors as intended and that A-1331852 did not prevent it (Fig.3.18D). In addition, we observed that G-CSF did not diminish the efficacy of A-1331852, as SiglecF+ TANs remained decreased in the combination group compared to control mice (Fig. 3.18E). These findings collectively suggest that effective tumor control may be attained by shifting the balance in favor of young, anti-tumoral TANs over their old/aged pro-tumoral counterparts.

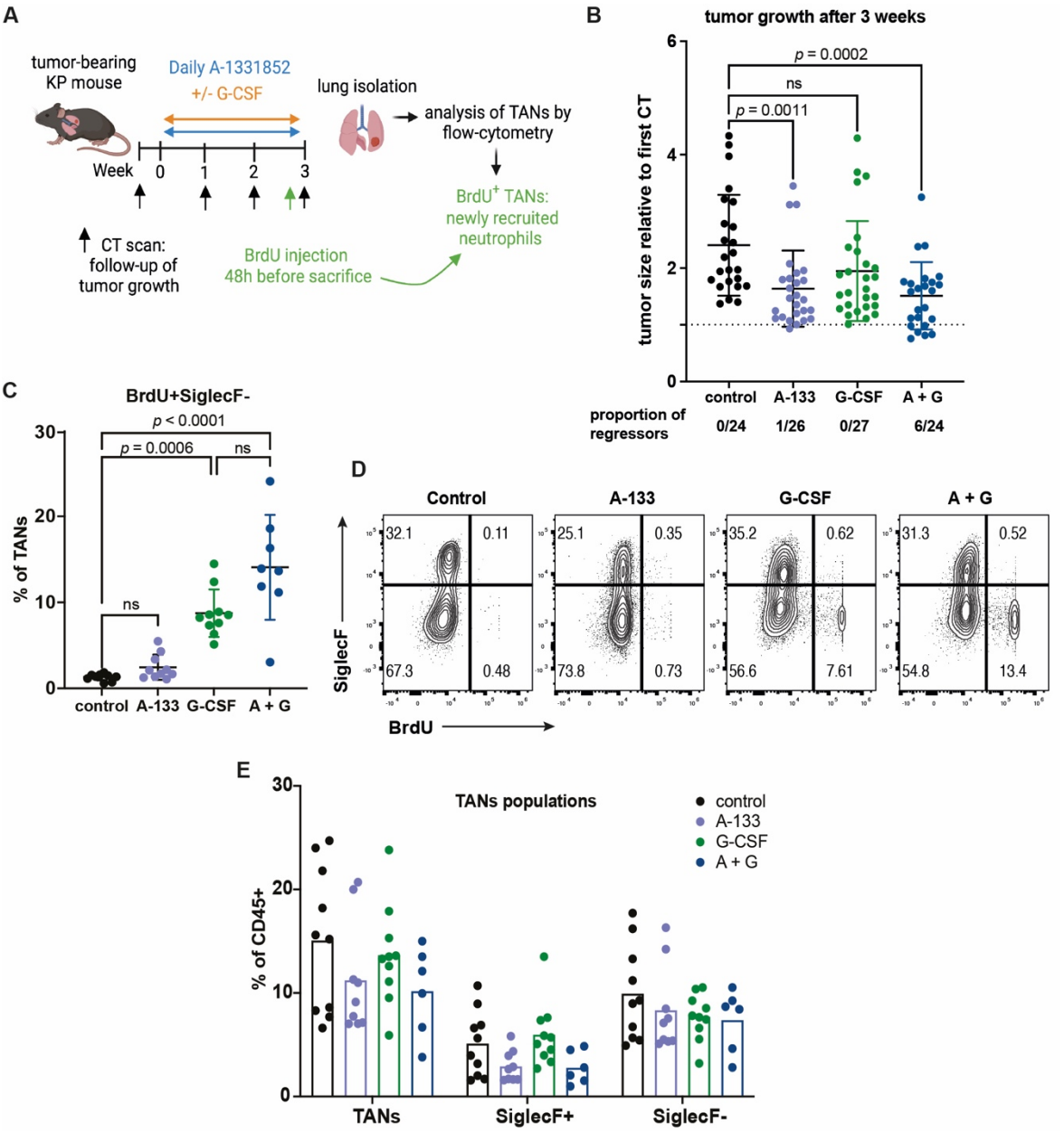


Figure 3.18 G-CSF administration potentiates the anti-tumor effect of Bcl-xL blockade

(A) Scheme representing the treatment strategy. Control, A-1331852-treated, G-CSF, G-CSF + A-1331852-treated mice were treated daily, and tumor growth was measured by mCT once weekly for three weeks. n=3 mice/group. BrdU injection 48h before sacrifice enabled to track neutrophils that are newly recruited to the tumors. (**B**) Tumor growth was measured after three weeks, relative to the tumor volume size measured before treatment. Each data point represents a single tumor and the proportion of regressing tumors is shown below each group. n=24 tumors analyzed for control and A-1331852 + G-CSF (A + G) groups, n=26 for A-1331852 (A-133) and n=27 for G-CSF-treated mice. **(C)** Percentage of $BrdU^+$ newly recruited TANs (gated on $CD11b^+$ $Ly6G^+$ $SiglecF^-$ cells) and **(D)** representative FACS plots showing the recruitment of $BrdU^+$ neutrophils to the tumors in the indicated treatment conditions. n=10 tumors analyzed for control, A-1331852 and G-CSF and n=8 tumors for A-1331852 + G-CSF. Data are shown as mean ± S.D. **(E)** Percentages of total TANs, SiglecF+ and SiglecF- TANs in tumors shown in **(D).** For **(B)** and **(C)**, significance was determined by the Kruskal-Wallis test followed by Dunn's multiple comparisons test. ns, non-significant.

3.7 BCL-XL-BLOCKADE DOES NOT IMPROVE THE EFFICACY OF ANTI-PD-1 IMMUNOTHERAPY

TAN prevalence often correlates with decreased T-cell infiltration and lower efficacy of immunotherapies [308]. Our data obtained from the analysis of the tumor immune microenvironment after two weeks of treatment with A-1331852 showed an increase in CD8+ T cells (Fig. 3. 11B) suggesting that immune checkpoint blockade could be tested in combination with Bcl-xL inhibition. Also, we have observed that around half of tumor-infiltrating CD8+ T cells expressed PD-1 on their surface, therefore we decided to test if A-1331852 would sensitize tumors to anti-PD-1, which was shown to be inefficient in this model [309].

We decided to initiate the experiment with one week of treatment with A-1331852, leaving time for neutrophil depletion and hopefully concomitant CD8+ T cell infiltration, before initiating anti-PD-1 treatment in combination for two following weeks (Fig. 3.19A). Combination treatment revealed a trend toward increased infiltration of CD8 T cells, but showed no improvement compared to A-1331852 alone (Fig. 3.19B). Moreover, the anti-tumoral effect of A-1331852 was abrogated with the addition of anti-PD-1 (Fig. 3.19C). We decided to analyze T cell infiltration by immunofluorescence and observed that T cells did not re-localize from the tumor periphery to the tumor interior upon A-1331852-treatment (Fig. 3.19D) Therefore, Bcl-xL blockade does not sensitize tumors to anti-PD-1 in this experimental system but displays increased anti-tumor efficacy when used in combination with a clinical anti-neutropenic agent.

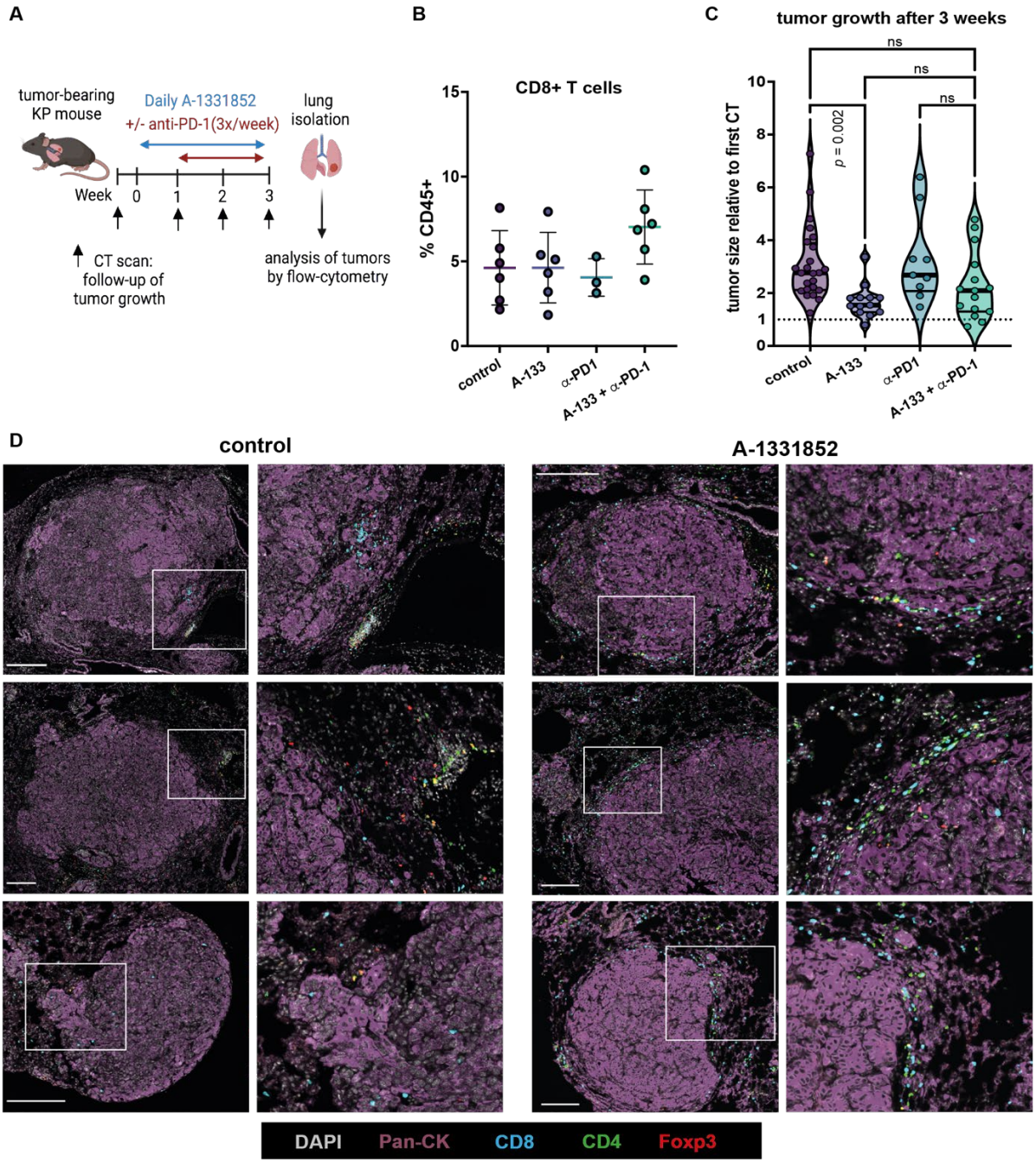


Figure 3.19 Bcl-xL blockade does not sensitize KP tumors to anti-PD1

(A) Scheme showing the experimental design. Tumor-bearing mice were treated with A-133 (n=3) for three weeks, with or without anti-PD-1 (n=3) during the last two weeks. **(B)** Percentage of CD8+ T cells among CD45+ cells in tumors of control (n=6), A-1331852 (A-133, n=6), anti-PD1 (n=3) or A-1331852 (A-133) + anti-PD1 (n=6). **(C)** Tumor growth relative to the first tumor volume measured and after three weeks. Data are shown as mean ± S.D. Each data point represents a single tumor analyzed. For **(C)**, significance was determined by the Kruskal-Wallis test followed by Dunn's multiple comparisons test. ns, non-significant. **(D)** Representative examples and magnified views of multiplex-immunofluorescence of tumors from control or A-1331852-treated mice. Scale bars: 200 mm.

3.8 TANs overexpress Bcl-xL in human LUAD

At some point, we wanted to assess the value of our results in the human disease. For this, we first visualized single-cell RNA sequencing data obtained from patients with NSCLC [219]. While only a few circulating neutrophils exhibited *BCL2L1* expression, it was enriched in the N5 population of neutrophils (Fig. 3.20A), a TAN subset specifically associated with poor prognosis [22]. In comparison, *BCL2* was almost not expressed in any of the subsets, while *BCL2A1* and *MCL1* were strongly expressed, especially in N4/N5 and in N1, respectively (Fig. 3.20A).

To monitor Bcl-xL protein, we performed immunofluorescence staining of human LUAD tissue samples with anti-myeloperoxidase (MPO) to identify neutrophils and with anti-Bcl-xL. By quantifying and comparing Bcl-xL expression levels in MPO-positive cells in tumors and tumor-adjacent non-cancerous tissue, we demonstrated that TANs express significantly more Bcl-xL compared to peritumoral neutrophils (Fig. 3.20B).

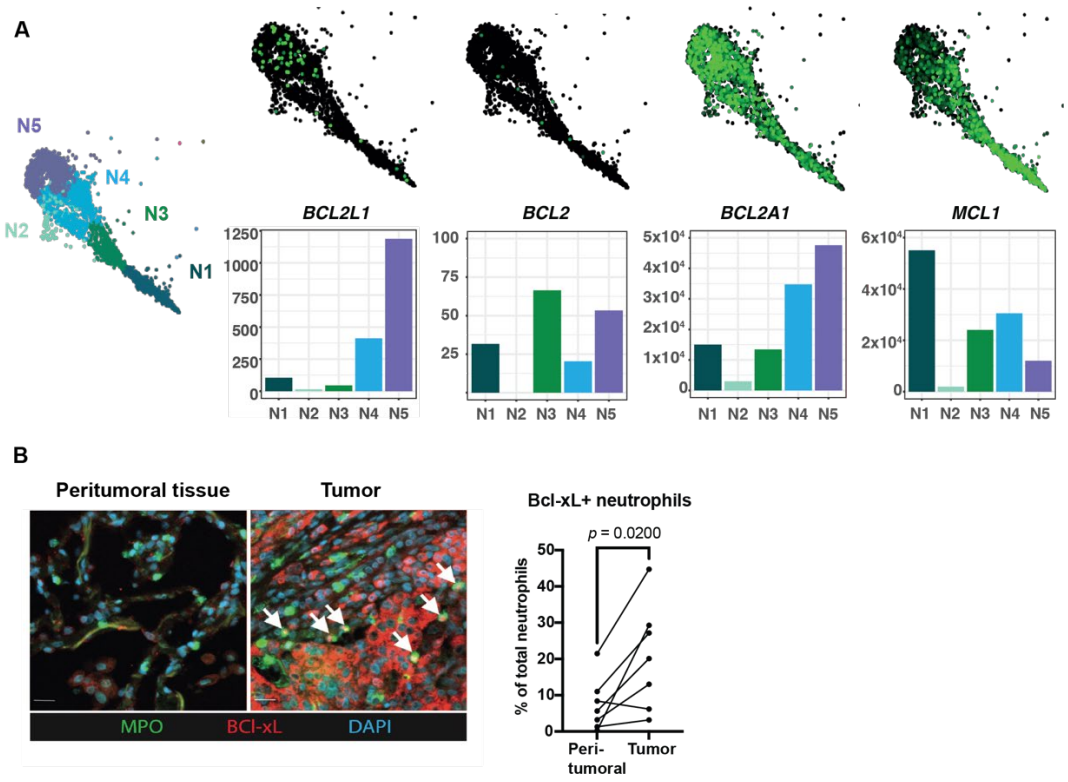


Figure 3.20 Human neutrophils upregulate Bcl-xL in tumors

(A) Representative images of the available single-cell transcriptomics showing the 5 TAN subsets from patients with lung cancer. *BCL2L1*, *BCL2*, *BCL2A1* and *MCL1* expression are shown per subset. **(B)** Representative example of TANs (MPO^+ cells) and Bcl-xL co-immunostaining in 7 LUAD patient samples and quantification of the percentage of Bcl-xL^+ neutrophils in matched tumor and peritumoral tissue. Scale bars: 20 mm. This analysis was perfomed by Adbullah Mayet, a PhD student from our lab. For **(B)**, significance was determined with a paired t-test.

In addition, we aimed to test if the extended survival due to the tumor cell supernatant observed in mouse neutrophils could also be replicated with human cells. We performed the similar assay with human neutrophils isolated form the blood of healthy donors and noted that human neutrophil survival was augmented after 24h of incubation with a supernatant prepared from the A549 human lung tumor cell line (Fig. 3.21A). This survival was accompanied by significantly-induced Bcl-xL expression, as observed by flow-cytometry (Fig. 3.21B)

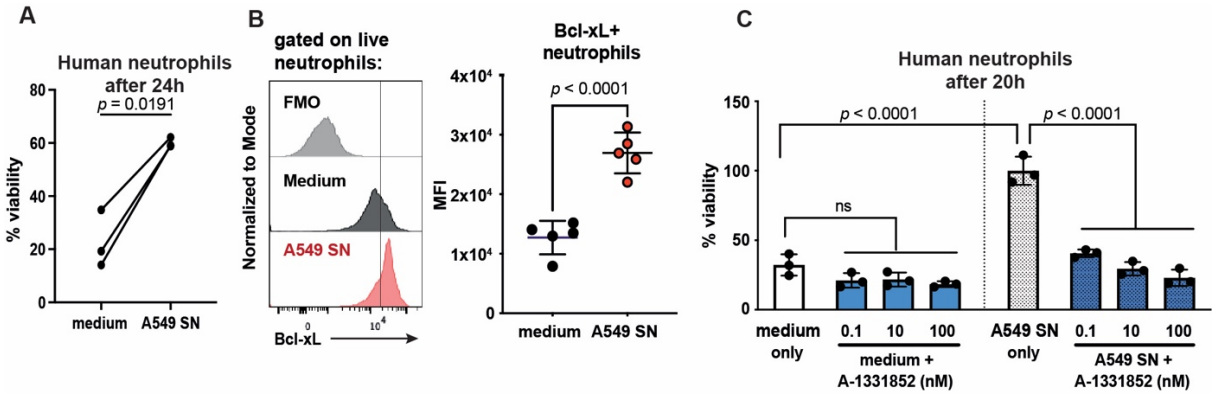


Figure 3.21 Human neutrophils survive longer and upregulate Bcl-xL with tumor cell-conditioned medium

(A) Percentage of live healthy blood donor-derived neutrophils after 24h of incubation with A549 tumor cell line supernatant. **(B)** Representative flow cytometry and quantification showing Bcl-xL upregulation in human blood-derived neutrophils upon incubation with tumor cell line supernatant after 24h. n=5 donors **(C)** Viability of human neutrophils with A549 SN and increasing doses of A-1331852 *in vitro* after 18h of incubation. Data are shown as mean ± S.D. For **(A)** and **(B)**, significance was determined with a paired t-test. For **(C)**, significance was determined with a one-way ANOVA followed by Šídák's multiple comparisons test. ns, non-significant.

Importantly, we noticed that A-1331852-mediated Bcl-xL blockade efficiently prevented the supernatant-induced neutrophil survival, without interfering with basal neutrophil survival (Fig. 3.21 C), similarly to what we had observed with mouse neutrophils. Altogether, these findings support our mouse data and provide a path forward to consider Bcl-xL-dependent TAN targeting clinically.

4 DISCUSSION

4.1 GM-CSF-INDUCED BCL-XL SUPPORTS NEUTROPHIL SURVIVAL AND POLARIZATION IN LUNG CANCER

In the KP mouse model, an important subset of neutrophils expressing the sialic-acid-binding protein SiglecF display tumor-promoting functions and are long-lived cells [157]. However, the mechanisms underlying their extended survival are not clear. To investigate this, I utilized conditioned medium derived from tumor cell lines to enhance neutrophil viability *in vitro*. Several studies have performed similar assays and obtained comparable results. For example, the conditioned medium of head and neck cancer cells triggered a p38-MAPK-dependent survival in human neutrophils [310]. In another study, neutrophils that were cultured with supernatants obtained from different solid tumor cells exhibited prolonged survival and even tumor-promoting phenotypes [311]. While it is unknown whether these supernatants induce neutrophil survival through upregulation of Bcl-xL, a study by Safaa et al. (2020) [191,290] demonstrated that tumor-secreted vesicles, containing peptides of the a2 V-ATPase, induced Bcl-xL expression and enhance the survival of human neutrophils. This, in conjunction with my findings, suggests that tumor cell-induced Bcl-xL expression in neutrophils might be a common response to the secreted factors of tumor cells across different cancer types and suggests the neutrophil survival is modulated in different cancer conditions.

In this research project, the results reveal that prolonged survival of neutrophils is at least partly enabled through GM-CSF-induced Bcl-xL expression, mediated by JAK/STAT signaling. Importantly, GM-CSF induced the expression of SiglecF on bone marrow-derived neutrophils *in vitro*. Within the scope of this project, more work is required to decipher whether GM-CSF directly promotes the polarization of neutrophils into SiglecF+ cells, or whether it only sustains neutrophil viability, which is needed for polarization to occur. Additionally, although the abundance of GM-CSF in tumors correlates positively with the prevalence of SiglecF+ TANs, additional experiments, such as the administration of depleting antibodies against GM-CSF, could shed the light on the impact of GM-CSF on neutrophil aging and phenotype *in vivo*.

Previous studies have shown that GM-CSF not only delays neutrophil apoptosis but also influences their transition into immunosuppressive cells. Consistent with my findings, tumor cell-secreted GM-CSF promoted neutrophil survival *in vitro* through the induction of the JAK/STAT3 pathway in gastric cancer [191]. In addition, GM-CSF induced PD-L1 expression by neutrophils, leading to the suppression of co-cultured T cells. In the scope of this project, I tried to investigate whether GM-CSF or the tumor cell supernatant could trigger PD-L1 expression in healthy BMNs. However, I did not detect PD-L1 expression by Real-time PCR. The induction of immunosuppressive neutrophils by tumor-secreted GM-CSF was also described in other cancer types. For instance, breast tumor-derived GM-CSF stimulated

neutrophil expression of arginase-1 [312]. In metastatic head and neck cancer, GM-CSF, through JAK/STAT3 activation, induced an immunosuppressive phenotype in neutrophils [313]. *In vivo*, the activation of oncogenic KrasG12D was demonstrated to enhance GM-CSF secretion by pancreatic ductal cells, resulting in the attraction of myeloid cells with CD8 T-cells suppressive properties, ultimately leading to cancer growth [314]. Further research on pancreatic adenocarcinoma revealed that tumor-secreted GM-CSF induced the expansion of c-Kit+ lineage- splenocytes and their transformation into immunosuppressive myeloid cells, which, in turn, stimulated tumor cell growth [299]. Collectively, the results obtained in this project and the mentioned studies demonstrate that GM-CSF derived from various tumor cells can not only boost neutrophil survival but also induce neutrophil transition into tumor-promoting cells.

In addition, we inferred that GM-CSF stimulates neutrophil survival and transition into SiglecF+ cells locally within KP lung tumors. This is based on the fact that GM-CSF could not be detected in blood samples of mice with tumors and was only detectable in the bronchoalveolar lavage fluid (BALF) of these mice. In addition, the quantity of GM-CSF in single tumors positively correlated with the prevalence of SiglecF+ TANs in the same tumor. This observation aligns with the fact that SiglecF+ TANs were exclusively found within tumors and not in healthy lungs or in spleen, blood and bone marrow [157,315], indicating that the transition into this state occurs locally within the tumors. In line with this, SiglecF+ neutrophils were observed in the injured kidney in a mouse model of renal fibrosis and were shown to arise from locally present cells upon the secretion of TGF-β and GM-CSF by damaged epithelial cells [315.] Although the study did not mention whether SiglecF+ neutrophils exhibited longer survival, the neutrophils that accumulated in the injured kidney over time displayed hypersegmented nuclei, indicating that they were aged cells. This study, together with my observations, indicate that neutrophil polarization by GM-CSF occurs locally within injured and lung tumor tissue, respectively. Curiously, while Bcl-xL expression remains low in bone marrow neutrophils, it increases in neutrophils found in healthy lungs, suggesting that neutrophil survival is mediated in a tissue-specific manner by GM-CSF [159]. Nevertheless, GM-CSF was not detectable in the BALF of healthy mice, suggesting that other factors might contribute to neutrophil survival, which GM-CSF might then enhance in the cancer context.

The reproduction of *in vitro* neutrophil survival upon the addition of the tumor cell-line conditioned medium with human neutrophils is very encouraging, as it suggests that a similar underlying mechanism is happening in human tumors. Throughout this PhD project, although neutrophil survival upon GM-CSF was demonstrated in mice, it would have been interesting to evaluate whether the tumor cell-line supernatant similarly induces human neutrophil survival through the GM-CSF-JAK/STAT pathway. The RNA sequencing revealing increased GM-CSF gene expression in the human lung cancer cell line H2009 following incubation with mouse KP TANs further suggests that the same survival mechanism mediated by GM-CSF is conserved in the human disease as well.

Although numerous studies describe the development of tumor-promoting neutrophils upon exposure to GM-CSF, the role of this cytokine in cancer remains ambiguous, as it has been shown to have anti-tumoral properties in several other studies (reviewed in [316]). A systematic review of clinical data on Sargramostim, an FDA-approved yeast-derived recombinant human GM-CSF, revealed its capacity to accelerate hematologic recovery during infection and to act as an immune-modulator in different cancer types, by boosting anti-tumor immunity [317]. Sargramostim enhanced the generation of dendritic cells from mononuclear precursors, which then stimulated the anti-tumor immunity in metastatic breast and endometrial cancers, and in relapsing ovarian and cervical cancers [318]. Administration of hGM-CSF also delayed the time to disease progression in prostate adenocarcinoma [319] and increased the progression-free and overall survival of patients with metastatic colorectal cancer [320]. In the context of lung cancer, GM-CSF administration enhanced the abscopal effect of radiation therapy in a subset of patients [321]. Nevertheless, the clinical relevance of GM-CSF in lung cancer remains complex, as increased serum levels of GM-CSF are markers of poor prognosis in patients with NSCLC [322] which correlates to our findings where high *CSF2* (coding for GM-CSF) expression in patients with LUAD indicated poor prognosis as well. Furthermore, GM-CSF expression enhanced lung cancer cells' metastatic capacity [323]- In summary, it seems that GM-CSF can influence tumor progression in different ways and should be considered with care, especially when administered to neutropenic patients to restore their neutrophil levels. Further research should help differentiate the impact of GM-CSF in the tumor-microenvironment to guide more suited therapeutic interventions.

4.2 Targeting Bcl-xL with A-1331852 to prevent neutrophil aging

In this project, I showed that neutrophil aging *in vitro* and *in vivo* can be blocked with the use of A-1331852, a specific Bcl-xL inhibitor. Importantly, the basal viability of neutrophils was not affected *in vitro* and long-term treatment with A-1331852 did not induce neutropenia in treated KP mice. In contrast, A-1331852 even increased the percentage of neutrophils in the blood. A-1331852 administration to healthy non-tumor-bearing mice also provoked greater proportions of circulating neutrophils. This phenomenon was also observed in rats treated with the same inhibitor for five days, indicating that Bcl-xL-blockade increases neutrophil mobilization [307]. In addition, A-1331852 did not suppress ex vivo granulopoiesis in isolated human bone marrow cells in the same study, thus indicating that Bcl-xL targeting would not be causing neutropenia in clinics. This project therefore shows a potential for specific targeting of pro-tumoral neutrophils and the sparing of healthy neutrophils based on their age difference.

Ideally, it would have been encouraging to test if Bcl-xL blockade with A-1331852 would impact the survival of TANs extracted from lung cancer patient samples and whether the autologous circulating blood neutrophils would be insensitive to the treatment. The *in vitro* data obtained with blood neutrophils from healthy donors supports this possibility. Specifically,

human neutrophil survival was prevented by A-1331852 only in the condition where cells are incubated with tumor-cell supernatant, indicating that human neutrophils also upregulate Bcl-xL induction upon exposure to tumor cell-derived factors. Furthermore, the percentage of Bcl-xL-expressing neutrophils was significantly higher within the tumors compared to the peripheral healthy tissue, suggesting that neutrophil polarization through Bcl-xL-enhanced survival occurs locally within tumors. Unfortunately, there is currently no way to estimate the aging of intra-tumoral TANs in lung cancer patients. Nevertheless, we can assume that the same mechanism is happening in humans as in mice. Indeed, the human and mouse neutrophil states share some conserved gene expression, among which human N5 highly resembles the mouse N5 state, which includes SiglecF^{high} neutrophils [219]. The Bcl-xL gene expression that we observed in the human N5 subset in this project therefore suggests that these cells are also long-lived and mature neutrophils. Notably, the human N5 subset was strongly associated with poor patient survival [219], suggesting that Bcl-xL blockade with A-1331852 might prevent tumor growth by eliminating these tumor-promoting cells. In addition, Bcl-xL gene expression was almost nonexistent in human blood neutrophils, indicating that A-1331852 treatment would not induce neutropenia if given to patients.

Targeting neutrophil aging might be beneficial in other cancer types. Indeed, TANs that I isolated from mouse models of lung, colon, cervix and pancreatic cancer showed increased Bcl-xL expression compared to control neutrophils (data not shown). This implies that the prolonged survival of TANs observed in KP tumors is a characteristic share across multiple cancer types, despite the presence of distinct microenvironments. Research performed in mouse model of lung squamous cell carcinoma indicates that this might be the case, as TANs were shown to be recruited by CXCL5 and around 40% of them were SiglecF+ [217], suggests an increased lifespan in this model as well. Interestingly, SiglecF-expressing neutrophils have also been reported in conditions other than cancer, indicating that some common baseline mechanisms promoting neutrophil survival and phenotypic modulation might exist between different tissues and conditions. In cardiac arrest for instance, an accumulation of long-lived SiglecF^{high} neutrophils was observed in the injured cardiac tissue starting from day one after the myocardial infarct was induced in mice [324]. These neutrophils were enriched for genes associated with Myc and NF-κB signaling, which contributed to the cells's survival according to the authors. This indicates that shared baseline mechanisms across different tissues and conditions promote neutrophil survival and phenotypic modulation.

Interestingly, a study conducted on rheumatoid arthritis provided insight into the potential clinical translation of A-1331852 for targeting enhanced neutrophil survival [325]. In this study, neutrophils sorted from inflamed joints of arthritic mice survived longer and exhibited high levels of Bcl-xL compared to blood neutrophils. They were also sensitive to A-1331852, whereas blood neutrophils remained unaffected, indicating the possibility of specific targeting of inflammatory neutrophils only. Of note, Bcl-xL induction was shown to be dependent on

GM-CSF. Treatment of mice with A-13311852 diminished the number of neutrophils in the joint and halted the progression of the disease. Furthermore, *ex vivo* analysis of human neutrophils extracted from patients with inflamed joints showed a decrease in neutrophil viability upon culture with A-1331852, but not with Mcl-1 or Bcl-2 inhibitors. In addition, the authors tested the Bcl-xL blockade in a mouse model of airway inflammation and showed that it preferentially depletes neutrophils from the lungs, leaving the blood neutrophils unaffected [325]. This shows that enhanced Bcl-xL expression in neutrophils can impact the development of chronic diseases, making A-1331852 an interesting therapeutic option in such cases.

It is perhaps worth mentioning that Bfl-1, another anti-apoptotic member, was also expressed in TANs based on our gene expression studies, suggesting that this protein also contributes to the extended survival of neutrophils in these conditions. Yet, my *in vitro* assays demonstrated that neutrophil survival, when exposed to A-1331852, reverted to basal levels, indicating that Bf-l1 did not compensate for the loss of Bcl-xL. Moreover, long-term treatment (3 weeks) with A-1331852 diminished the total number of TANs, suggesting that there was no rescue of neutrophil survival by other anti-apoptotic proteins. In agreement with this observation, a study previously mentioned showed that neutrophil survival was triggered by GM-CSF and could only be abrogated by Bcl-xL blockade, even though GM-CSF also induced Bfl-1 expression [325]. Therefore, we can suppose that Bcl-xL alone is essential for maintaining neutrophil survival in KP mouse lung tumors.

4.3 BH3 MIMETICS AS POTENTIAL THERAPEUTIC STRATEGIES FOR CANCER

BH3 mimetics were designed to induce tumor cell death, resulting in the clinical approval of Venetoclax for several hematological malignancies [279]. The results obtained in this project support the repurposing of Bcl-xL-specific BH3 mimetics against tumor-supportive neutrophils however, Bcl-xL inhibition could also inhibit tumor growth directly. Indeed, BH3 mimetics were previously considered as therapeutic options for treating lung cancer. Bcl-xL is widely expressed in both SCLC and NSCLC cells [326,327] and is associated with resistance to chemotherapy [328].

In this project, neutrophil depletion prior to A-1331852 treatment resulted in a regain of tumor growth, indicating that the reduced tumor growth observed upon Bcl-xL blockade is at least partly mediated through neutrophils. *In vitro*, Bcl-xL blockade with A-1331852 did not affect tumor cell lines' survival and growth. This is not surprising, as Navitoclax and A-1331852 only sensitized non-small cell lung cancer (NSCLC) cell lines to apoptosis when these were treated with chemotherapeutic agents [305,329]. Moreover, Navitoclax worked best when combined with etoposide, suggesting the possibility that A-1331852 would also increase the chemotherapeutic-mediated tumor cell death if combined with this particular drug. Things can however be different in vivo. Indeed, A-1331852 has been shown to delay tumor growth in vivo in subcutaneous xenografts of NSCLC and SCLC cell lines in immunodeficient mice, and

this effect was further increased when combined with docetaxel, a cytotoxic agent that stabilizes tubulin and induces cell cycle arrest [307]. Therefore, the combined administration of a Bcl-xL inhibitor and chemotherapy could have a direct tumor cell-killing effect and an indirect inhibitory effect of tumor growth through targeting TANs.

While Navitoclax had shown promise in cancer treatment for solid tumors in mice, among which xenografts of NSCLC and SCLC cells [271,330], its clinical application was restrained due to the on-target effect on platelets (thrombocytes) which rely on Bcl-xL [331], and the resulting thrombocytopenia [332]. Since then, proteolysis-targeting chimeras (PROTACs), such as DT2216, have been developed to prevent the uptake of Bcl-xL-specific inhibitors by platelets [333,334]. DT2216 binds Bcl-xL and promotes its degradation by the proteasome through Von Hippel-Lindau (VHL) E3 ligase. Since platelets express minimal levels of VHL, they are preserved. These products indeed demonstrated an efficient reduction in platelet depletion, suggesting that they could be a safe option to use in clinics. These PROTACs also demonstrated efficiency in blocking tumor growth. Indeed, targeting of Bcl-xL with DT2216 and Mcl-1 with AZD8005 was shown to synergistically inhibit small cell lung cancer growth [335]. This co-targeting enabled the killing of SCLC cells that were dependent on both anti-apoptotic proteins, while sparing normal cells, and resulted in reduced growth of patient-derived xenografts and in the *Rb1/p53/p130* genetically engineered mouse model of SCLC, without causing thrombocytopenia. Since neutrophils were also shown to express the VHL protein [336], it would be interesting to assess whether TANs still express it and if Bcl-xL targeting could be achieved with the DT2216 PROTAC.

4.4 Recruitment of young neutrophils as anti-tumoral therapy

Growing evidence suggests that neutrophils play a dual role in cancer, acting both as pro and anti-tumoral cells. In early-stage human lung cancer, neutrophils have been found to enhance the tumor-killing abilities of T cells [201]and *in vitro* studies have shown that neutrophils from healthy blood donors possess tumor-cell-killing abilities [337]. Recently, studies have described a neutrophil response leading to successful tumor control in lung, colon [304], and melanoma mouse models following immunotherapy [338].

In the scope of this project, one of our goals was to shift the balance from old/aged TANs towards young TANs, which possess potentially anti-tumoral properties. To achieve this, we supplemented A-1331852 treatment with G-CSF injections to stimulate the production and release of neutrophils from the bone marrow [339]. We observed that G-CSF led to a marked reduction of tumor growth in mice treated with A-1331852 and even led to regression in some tumors compared with A-1331852 treatment alone, which indicates an additional anti-tumoral capacity of young TANs. This is interesting because G-CSF is administered to patients undergoing chemotherapy as a first-line treatment where neutropenia is a common side effect [340], suggesting that this treatment could potentiate the effect of Bcl-xL blockade in patients.

Furthermore, concurrent administration of G-CSF with radiotherapy was shown to enhance neutrophil tumor-killing ability in mice [341]. This is interesting, as previous research from our lab has shown that neutrophil-specific loss of Glut1 accelerated neutrophil turnover, reduced SiglecF+ TANs prevalence and augmented the efficacy of radiotherapy [222]. Because A-1331852 together with G-CSF leads to a greater neutrophil turnover, I anticipate that this combination could potentiate the response to radiotherapy as well.

4.5 Bcl-xL-blockade does not potentiate anti-PD-1 therapy

In multiple types of cancer, neutrophils play a crucial role in creating an immunosuppressive environment. They achieve this by expressing PD-L1 or releasing Arginase I, both of which hinder the normal functioning of T cells. In NSCLC, neutrophil infiltration inversely correlates with T-cell infiltration [177,214], indicating that neutrophils mediate T-cell exclusion from the tumors. In the KP mouse model, neutrophil depletion using anti-Gr1 reverted the immune exclusion and enabled anti-PD-1 treatment efficacy [177]. We, therefore, thought that depletion of TANs upon Bcl-xL blockade might also potentiate anti-PD-1 treatment. However, A-1331852 treatment failed to potentiate the efficacy of anti-PD-1 therapy. This could be due to several reasons.

KP lung tumors have been described as poorly immunogenic, explaining their unresponsiveness to checkpoint inhibitors [309]. Although we observed an increase in T cells in the flow cytometry analysis upon A-1331852 treatment, multiplexing analysis of T-cell populations by histology revealed that T cells remained excluded from the tumor mass and resided mainly in the tumor periphery. They were also found in tertiary lymphoid structures in the lungs of both untreated and A-1331852-treated mice, as it was previously described in this model [132,342], altogether indicating that they cannot efficiently fight cancer outgrowth.

Moreover, it has been shown that CD8 T cells fail to be efficiently activated before arriving to the tumor, despite the fact that they proliferate [343]. Single-cell RNA sequencing of an orthotopic NSCLC mouse model revealed unconventional differentiation of CD8 T cells, lacking typical effector or exhausted gene expression. Similar findings were observed in CD8 T cells from NSCLC patients. The full differentiation of CD8T cells and resulting control of KP tumors was enabled through the administration of IL-2 and IL-12. Additionally, a lung-specific mechanism involving Th1-like Tregs blocking cytotoxic T cells in mediastinal lymph nodes was identified. This unique Treg subset, induced by IFN-γ, inhibited dendritic cell signaling essential for efficient CD8 T cell differentiation. The lung-specific effect was attributed to higher IFN-γ levels in mediastinal lymph nodes, possibly influenced by the lung microbiota, as this effect was only observed in mice where KP tumors were implanted into the lungs, and not subcutaneously in the flank of animals.

Taken together, this research might suggest efficient combination therapies that would involve antibiotics. It would be interesting to deepen our understanding of the lung microbiota, as it was already shown to modulate lung-specific immunity in viral infection and through IFN signaling [344]. Importantly, commensal microbiota was shown to promote lung cancer development in the KP mouse model, by driving the proliferation of γδ T cells, responsible for increased inflammation and tumor cell proliferation [345]. Altogether, these studies show multiple mechanisms and limitations limiting the development of an efficient adaptive immune response to lung cancer and which are not necessarily linked to neutrophils.

5 CONCLUSIONS AND PERSPECTIVES

Tumor-associated neutrophils contribute to the progression of lung cancer. However, neutrophil targeting in humans is difficult due to the high risk of neutropenia. Extensive research and advanced technologies enabled to uncover the incredible plasticity of neutrophils in cancer and gave insight into the various conditions that these cells have to adapt to. While the diverse neutrophil states might seem like adding complexity to our research, they actually constitute a precious opportunity for specific targeting. In this project, I discovered that Bcl-xL upregulation permitted the extended survival of tumor-associated neutrophils in tumors, which is necessary for their transition into SiglecF+ tumor-promoting cells. With the use of a BH3 mimetic drug targeting Bcl-xL, I was able to specifically deplete aged SiglecF+ TANs, resulting in reduced lung tumor growth. At the same time, the healthy pool of blood neutrophils was unaffected by Bcl-xL blockade and exhibited anti-tumoral properties which could be enhanced through increased G-CSF-mediated recruitment of these cells to the tumors. This current work suggests a safe approach to selectively target pro-tumoral TANs while keeping the healthy pool of neutrophils, which can further participate in limiting tumor outgrowth.

In the future, it would be interesting to further develop our knowledge of neutrophil aging and work on the translation of our research into the clinics. Some of the aims would be to:

- **Evaluate the expression of Bcl-xL and other Bcl-2 family of proteins in human TANs** across various human cancer types and investigate their sensitivity to specific BH3 mimetics, providing insight into potential therapeutic targets
- **Test DT2216 sensitivity in TANs for clinical translation**. Investigate *in vitro* and *in vivo* if TANs are sensitive to Bcl-xL-blockade with this inhibitor, which preserves platelets and therefore could be more easily translated into the clinics.
- **Explore synergistic combination therapies**. Bcl-xL-blockade might be tested in combination with other treatment options such as chemotherapy, radiotherapy or immunotherapy, potentially enhancing the overall therapeutic outcomes

In a broader picture, future research should focus on a deeper understanding of neutrophil plasticity in cancer. A better knowledge of the mechanisms underlying neutrophil

transformation will certainly help refine possible targeted therapies while minimizing potential side effects. These targeting strategies will hopefully constitute additional therapeutic options for cancer patients and perhaps enhance the efficacy of other cancer therapies.

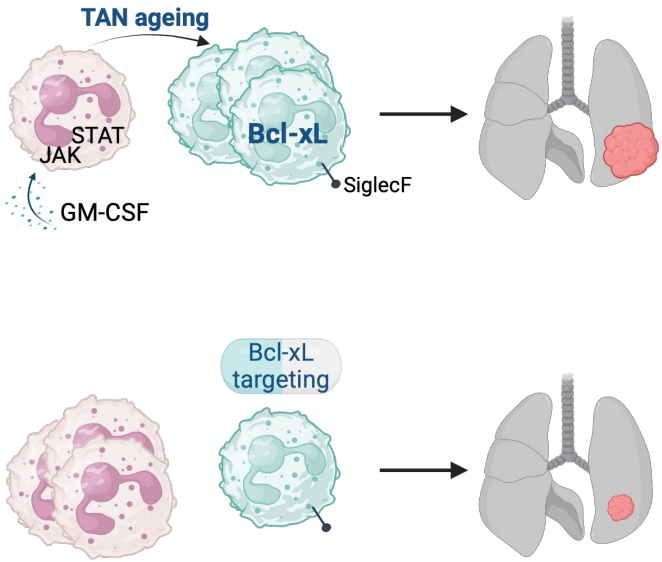


Figure 5.1 : Graphical abstract of the project

TAN aging is supported by Bcl-xL expression, induced by GM-CSF through JAK/STAT signaling. Targeting of Bcl-xL with a specific inhibitor leads to a decrease of intra-tumoral TANs and to a reduction of lung tumor growth.

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7 APPENDIX : NEUTROPHIL METABOLISM IN THE CANCER CONTEXT

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Review

Neutrophil metabolism in the cancer context

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ABSTRACT

Neutrophils are critical innate immune cells for the host anti-bacterial defense. Throughout their lifecycle, neutrophils are exposed to different microenvironments and modulate their metabolism to survive and sustain their functions. Although tumor cell metabolism has been intensively investigated, how neutrophil metabolism is affected in cancer remains largely to be discovered. Neutrophils are described as mainly glycolytic cells. However, distinct tumor-associated neutrophil (TAN) states may co-exist in tumors and adapt their metabolism to exert different or even opposing activities ranging from tumor cell killing to tumor support. In this review, we gather evidence about the metabolic mechanisms that underly TANs' pro- or anti-tumoral functions in cancer. We first discuss how tumor-secreted factors and the heterogenous tumor microenvironment can have a strong impact on TAN metabolism. We then describe alternative metabolic pathways used by TANs to exert their functions in cancer, from basic glycolysis to more recently-recognized but less understood metabolic shifts toward mitochondrial oxidative metabolism, lipid and amino acid metabolism and even autophagy. Last, we discuss promising strategies targeting neutrophil metabolism to combat cancer.

1. Introduction

Neutrophils are part of the innate immunity and represent the most abundant type of circulating leukocytes in blood. They are specialized in the neutralization and killing of microorganisms at sites of infection, with very fast recruitment and reaction. For a long time, neutrophils were considered as fully differentiated cells with little transcriptional activity and no plasticity. However, studies in the last decade have challenged this notion, revealing a panoply of new roles and phenotypes, particularly in cancer. Neutrophils are often found in abundance in mouse and human tumors [1–4] and modulate tumor progression in many ways. Although anti-tumor functions of neutrophils have been described in cancer, most studies report tumor-supportive roles. Neutrophils can promote angiogenesis [5] and directly increase the outgrowth and seeding capacity of cancer cells. They also secrete nitric oxide (NO) and reactive oxygen species (ROS) that inhibit T cell-mediated anti-tumor immunity [6,7].

Neutrophils require a lot of energy and fast metabolic adaptation to sustain their effector functions. Although the study of immunometabolism has recently gained interest in cancer research [8,9], emphasis has been placed on how metabolism alters T cell functions, while neutrophil metabolism remains poorly investigated. More broadly, both cancer

Abbreviations: Acetyl-CoA, acetyl-coenzyme-A; ARG, arginine; CMP, common myeloid progenitor; DNI, delta neutrophil index; ER, endoplasmic reticulum; FAO, fatty acid β-oxidation; FATP, fatty acid transport protein; G6P, glucose-6-phosphate; G6PC3, glucose-6-phosphatase-β; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; GLUT, glucose transporter; GLR, glucose-to-lymphocyte ratio; GMP, granulocyte-monocyte progenitor; HDN, high-density neutrophil; HIF-1α, hypoxia-inducible factor 1-alpha; HSC, hematopoietic stem cell; iNOS, inducible nitric oxide synthase; LDL, low-density lipoprotein; LDN, low-density neutrophil; LOX, lectin-type oxidized LDL receptor; MDSC, myeloid-derived suppressor cell; MMP, matrix metalloproteinase; NADH, nicotinamide adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; NE, neutrophil elastase; NET, neutrophil extracellular trap; NO, nitric oxide; NOX, NADPH oxidase; NSCLC, non-small cell lung cancer; OSM, oncostatin M; OXPHOS, oxidative phosphorylation; PET, positron emission tomography; PMN, polymorphonuclear leukocyte; PPP, pentose phosphate pathway; ROS, reactive oxygen species; TAN, tumor-associated neutrophil; TCA, tricarboxylicacid; TME, tumor-microenvironment.

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vironment (TME), through direct metabolic reprogramming [10] or through competition for nutrients [11]. With this review, we aim to discuss the current scientific evidence of neutrophil metabolic adaptations in cancer and to highlight future perspectives for preventing their detrimental role.

2. Metabolic changes in the neutrophil life cycle

2.1. Granulopoiesis and basal metabolism

Throughout their life cycle, neutrophils primarily utilize glucose for survival and function (Fig. 1A). However, they can take-up different nutrients and modulate their metabolism to survive and sustain their functions in varying and challenging conditions. Here, we summarize the major metabolic pathways engaged in the neutrophil life cycle.

It is estimated that 10^{11} neutrophils arise daily from the granulocyte-monocyte progenitor cells (GMP) in the bone marrow. Their stepwise differentiation is mediated by a high transcriptional activity and is supported by sequential metabolic shifts. In the beginning, hematopoietic stem cells (HSC) that give rise to the common myeloid progenitors (CMP) largely rely on glycolysis [12], whereas CMPs switch to a stronger mitochondrial respiration [12,13], before glycolysis becomes again the predominant metabolic process in differentiated neutrophils. Glycolysis form of ATP. Glucose is internalized through two types of glucose transporters: facilitated diffusion glucose transporters (GLUT) and sodium-glucose linked transporters (SGLTs) [14]. In the cytosol, the first step of glycolysis consists in the phosphorylation of glucose by hexokinase to form glucose-6-phosphate (G6P). Of note, intracellular hexokinase localization can vary when neutrophils pass from a resting to an activated phenotype, which could be important for their optimal activation [15]. G6P is metabolized further, finally yielding two molecules of pyruvate, two ATPs and two reduced forms of nicotinamide adenine dinucleotide (NADH) per glucose molecule. In anaerobic conditions, pyruvate remains in the cytosol and is converted to lactate, generating NAD^+, which fuels back the glycolytic process [16,17]. This process is termed anaerobic glycolysis. In aerobic conditions, eucaryotic cells usually engage the Krebs/Tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS). In that case, pyruvate is converted to acetyl-coenzyme-A (Acetyl-CoA) before entering the mitochondria. Altogether, this process results in the complete oxidation of glucose into CO_2 and generates large quantities of ATP. Although neutrophils do not seem to rely on this pathway for basal bioenergetics, it has been shown that a shift towards an oxidative metabolism and ensuing ATP are necessary for neutrophil chemotaxis [18,19].

In physiological states, G6P can also be a substrate for the pentose phosphate pathway (PPP) to produce nicotinamide adenine

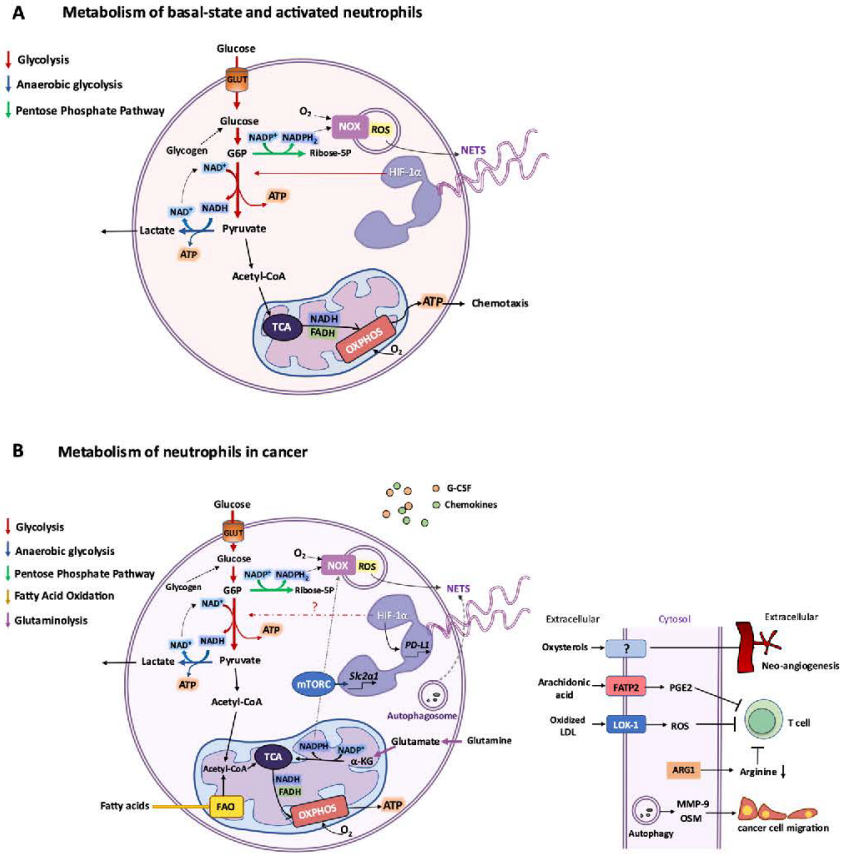

Fig. 1. Metabolic pathways active in neutrophils. (**A**) basal-state and activated neutrophils and (**B**) neutrophils in the tumor context are represented. Glycolysis consists in the degradation of glucose into pyruvate, generating ATP. In anaerobic conditions, pyruvate is converted into lactate and the NAD^+ that is produced is reused to fuel the glycolytic process. When oxygen is present, pyruvate is converted to acetyl-CoA, which is used in the Krebs/TCA cycle to produce metabolic intermediates NADPH and FADH, and ATP through the OXPHOS. The PPP uses G6P to generate NADPH, an important mediator of the NOX-dependent ROS formation. ROS can trigger NET formation. Glycogen also serves as a source of glucose when the demand is high. Under hypoxic conditions triggered by infection or inflammation, HIF-1α induces the expression of glycolytic enzymes. It is not yet clear if this is the case in TANs. Free fatty acids are transported to the mitochondria and undergo FAO to produce acetyl-CoA for the TCA cycle. Glutaminolysis also supports the TCA cycle through the production of α-ketoglutarate (α-KG), an intermediate of acetyl-CoA degradation. NADPH created from the degradation of α-KG can support NOX-dependent ROS production in low glucose conditions. mTORC signaling induces the upregulation of *Slc2a1*, encoding GLUT1 transporter [10]. *Lower right*: autophagy can promote the production of matrix-metalloproteinase 9 (MMP-9) and oncostatin M (OSM), responsible for tumor migration [123]. It is also linked to NET formation [124]. Oxysterols recruit TANs that support tumor angiogenesis [109]. Arachidonic uptake *via* the FATP2 transporter results in T cell inhibition through creation of PGE2 [100]. Oxidized LDL binding to LOX-1 stimulates neutrophil ROS production and subsequent T cell inhibition [101]. TANs secrete Arginase 1 (ARG1), which degrades arginine in the TME, an important T-cell nutrient [116].

dinucleotide phosphate (NADPH). NADPH serves as a cofactor and electron donor for the NADPH oxidase (NOX), which mediates the creation of superoxide and oxygen radicals [20]. These radicals are further transmuted into different ROS and trigger neutrophil extracellular trap (NET) formation [21]. Phorbol myristate acetate-stimulated neutrophils express higher GLUT1 and increase glycolysis to produce NETs [22]. Hence, an enhanced glucose metabolism through each of the PPP and glycolysis appears important for NET formation and release.

Because neutrophils possess only few mitochondria, do these organelles really contribute to energy production? For a long time, their role was mostly attributed to neutrophil survival through the maintenance of the mitochondrial membrane potential [17]. Further, the use of respiratory chain complex-specific inhibitors showed that mitochondria do not contribute to basal ATP production in neutrophils, and that complex III utilizes electrons obtained from glycolysis through the glycerol-3-phosphate shuttle [23]. Because NAD^+ is reduced during glycolysis, NADH needs to be reoxidized to provide for new NAD^+ and maintain the pathway active. The glycerol-3-phosphate shuttle thus provides a way for neutrophils to maintain both the glycolytic pathway and a redox balance necessary for cell survival. Finally, a metabolic reprogramming toward increased fatty acid catabolism occurs during granulopoiesis, whereby free fatty acids are liberated from lipid droplets in an autophagy-dependent manner. This enhances fatty acid β-oxidation (FAO), generating acetyl-CoA needed for mitochondrial respiration and normal neutrophil differentiation [24].

3. Metabolic modulation of neutrophil functions in cancer

3.1. Metabolic plasticity of TANs

In 2009, Fridlender and colleagues opened up the path to neutrophil heterogeneity in cancer, demonstrating that TAN phenotypes can range from anti-tumoral (N1) to pro-tumoral (N2), with TGF-β favoring the accumulation of N2 [25]. While this initial picture was enlightening, today a more complex one emerges, where neutrophil functional diversity and plasticity in cancer might be greater than initially shown [26–28]. A direct consequence of this is the diversity in the terminology used to describe neutrophil populations: immunosuppressive neutrophils are sometimes referred to as granulocytic-myeloid derived suppressor cells (G-MDSCs) or polymorphonuclear-myeloid derived suppressor cells (PMN-MDSCs), with a common denominator being Ly6G expression on their surface in the mouse. In this review, we try to use the terms from the original papers.

As neutrophils are believed to be fully differentiated upon maturation, proofs that their functions and phenotypes can be modulated in the cancer context offer an opportunity to characterize the mechanisms enabling neutrophils to adopt tumor-supportive functions and how to prevent them. Below, we highlight recent findings that help us to underscore the implication of cellular metabolism in the plasticity of neutrophils in cancer.

3.2. Secreted factors that influence granulopoiesis and neutrophil trafficking to the tumor

Different signals regulate granulopoiesis, the main one being granulocyte colony-stimulating factor (G-CSF) [29]. Besides differentiation, G-CSF plays a role in multiple neutrophil functions. In glucose-6-phosphatase-β (*G6pc3*)-deficient mice, which are impaired in forming glucose from glycogen stocks, administration of G-CSF enhances glucose uptake and the levels of G6P, lactate and ATP in neutrophils, improving altogether their survival and functionality such as ROS formation and chemotaxis [30].

Comparably to inflamed tissues during infection, tumors secrete factors into the circulation that can enhance granulopoiesis and augment infiltration of neutrophils into the TME [31,32]. Here, G-CSF secretion favors hematopoietic cell differentiation toward the neutrophil lineage and enhances the exit of neutrophils into the circulation [33]. Additionally, G-CSF stimulates neutrophil homing to tumors. In patients with cervical cancer, high G-CSF expression correlated with shorter overall survival, whereas G-CSF induced resistance to cisplatin through the recruitment of MDSCs in a mouse model of this disease [34]. These cells participate in tumor resistance through immune suppression, but also in part through the secretion of Bv8, a potent factor for neutrophil-dependent tumor vascularization [35]. In subcutaneous and orthotopic mouse models of lung and breast cancer, respectively, and in a spontaneous metastasis model of breast cancer, G-CSF secretion was stimulated by IL-1β and γδ T lymphocyte-derived IL-17 [36,37]. Neutralization of G-CSF or IL-17 led to a decrease of $c\text{-}Kit^+$ tumor-infiltrating neutrophils [37], a marker of pro-metastatic myeloid cells [38,39]. Moreover, it has been shown that different subpopulations of 4T1 breast cancer cells seed to distinct secondary tissues, with G-CSF being necessary for immature neutrophil recruitment and liver metastasis [40]. Authors showed that conditioned medium from liver metastatic cells contains more G-CSF, CXCL1 and CCL2 compared to the lung metastatic cell-derived medium, which could explain the enhanced recruitment of low-density neutrophils to the liver metastases.

Other important secreted factors are CCL2, which recruits immunosuppressive neutrophils in mouse models of colon cancer [41], and CXCL2, whose expression increases in TANs, fostering their infiltration in response to SNAIL expression in tumor cells from a mouse model of lung adenocarcinoma [42]. In an orthotopic mouse model of rhabdomyosarcoma, inhibition of CXCR2-dependent neutrophil trafficking enhanced the effect of anti-PD1 blockade [43]. In human gastric cancer, tumor cell-secreted granulocyte-macrophage colony stimulating factor (GM-CSF) fosters neutrophil survival *in vitro*, leading to programmed death-ligand 1 (PD-L1) expression on their surface and suppression of T cell immune responses [44]. In mouse models of triple negative breast cancer, glycolysis inhibition in tumor cells reduced their production of both GM-CSF and G-CSF, diminishing MDSC recruitment. Mechanistically, glycolysis inhibition resulted in AMPK, ULK1 and autophagy activation, which attenuated the expression of LAP, a specific CEBPB isoform regulating GM-CSF and G-CSF expression [45].

While many studies show a skewing of neutrophils towards a pro-tumoral phenotype within the TME, a higher neutrophil infiltration within tumors has also been associated with better prognosis in some cancers [46–48]. Accordingly, anti-tumor functions for neutrophils have been demonstrated in mouse models. As striking example, the cytotoxic activity of neutrophils in tumors was supported by their expression of the receptor tyrosine kinase MET, whose deletion in the immune compartment only (achieved by transplantation of C57BL/6 *Met*-deficient bone marrow cells into lethally-irradiated C57BL/6 wild-type mice) enhanced tumor growth and metastasis of subcutaneously-injected murine Lewis lung carcinoma cells. MET induction, which was stimulated by tumor-derived TNF, was critical for neutrophil transmigration from the endothelium to the tumor and for production of inducible nitric oxide synthase (iNOS) and nitric oxide (NO) that triggers tumor cell killing [49]. Another study showed that TGF-β secreted by tumor cells can stimulate the production of NO in myeloid cells. In patients with late-stage cancers, myeloid $CD15^+$, $CD33^+$ and $CD34^+$ cells isolated from the blood expressed high levels of TGF-β receptor II [50] and myeloid-specific *Tgfbr2* deletion from mouse models of spontaneous (injection of 4T1 breast cells into the mammary fat pad) and experimental (intravenous injection of murine 4T1, LLC lung, B16 melanoma and MC26 colon cancer cell lines) metastasis led to a significant inhibition of metastasis formation. While these studies together provide evidence that tumor-secreted factors can remodel neutrophil functions, how cytokines and chemokines induce metabolic changes in the process of granulopoiesis or in mature neutrophils still largely remains to be explored.

4. Metabolic shifts occurring in the TME

4.1. Hypoxia

The rapid growth of solid tumors and accompanying abnormal blood vessel organization can result in defective oxygen supply to the tumor center. The effects of hypoxia on tumor cells are diverse but mostly tumor-supporting, encompassing clonal selection of treatment-resistant cells, inhibition of apoptosis and transition to a malignant phenotype (reviewed in [51]). Additionally, hypoxia is important in diverse inflammatory conditions and modulates the innate immune cell response and adaptation, particularly in macrophages and neutrophils [52,53].

Both human and mouse neutrophils depend on the hypoxia-inducible factor, HIF-1α transcriptional regulator for their adaptation to hypoxic stress. Under low oxygen conditions, HIF-1α stimulates a glycolytic response through the induction of glycolytic enzymes and activates NF-κB signaling to support neutrophil survival [54]. In hypoxic conditions, HIF-1α stabilization in neutrophils was augmented by ROS produced through the glycerol-3-phosphate shuttle, the latter maintaining the mitochondrial membrane potential through increased glycolysis [55]. Although there is no described implication of the glycerol-3-phosphate shuttle in TANs, loss of the mitochondrial membrane potential initiates cell death, indicating that this pathway may sustain neutrophil survival in the cancer context [17,56]. HIF-1α can also promote the expression of PD-L1 on MDSCs, inhibiting T cell activation [57]. An elegant study using *Pten*-deficient autochthonous mouse models of uterine cancer showed that animals housed in hyperoxia underwent tumor reoxygenation. This change of the tumor microenvironment was accompanied by a decreased neutrophil recruitment due to reduced tumor cell-secreted CXCL5, and ultimately tumor cell death. In hyperoxia, tumor control depended on an infiltration by a specific subset of neutrophils marked by increased NOX2-derived ROS and matrix metalloproteinase 9 (MMP9) production, which were cytotoxic to tumor cells in a T cell-independent manner [58]. Moreover, the authors proposed that the inhibition of tumor growth in hyperoxia might be due to a blocking effect on neutrophil elastase (NE), often linked to tumor proliferation. They also described that infiltrating TANs in normoxia were expressing SiglecF on their surface, hyperoxia diminishing the number of SiglecF^{+} neutrophils. This is interesting, as tumor-promoting SiglecF-expressing TANs had been reported in a *Kras*$^{Lox\text{-}STOP\text{-}Lox\text{-}G12D/WT}$; *Tp53*$^{Flox/Flox}$ mouse model of lung cancer [59].

4.2. Glycolysis as major source of energy

In the presence of oxygen, some cell types including cancer cells increase their glycolytic flux and preferentially engage in glycolysis and lactate production, although this yields less ATP compared to OXPHOS. This process is known as the Warburg effect or the aerobic glycolysis [60,61]. Since his seminal contributions, countless studies accumulated data about aerobic glycolysis used for many aspects of cancer development such as tumor cell proliferation, vessel sprouting, reprogramming of the TME and regulation of protein translation [62]. These findings can be relevant to understand the scope of neutrophil metabolism in cancer. As previously said, myeloid cells mostly rely on glycolysis to fuel their functions, as this process can be more promptly and rapidly activated when the need for energy is high [63]. In the 1950's already, neutrophils were shown to mainly engage in anaerobic glycolysis for ATP production [64], which goes in line with these cells containing few mitochondria and consuming little oxygen [65]. Higher glucose uptake in presence of oxygen in neutrophils would therefore reflect a Warburg effect for fast energy production, or for increased engagement of the PPP to generate NADPH, essential for ROS production including H_20_2 [66]. Human blood neutrophils stimulated to undergo both NOX-dependent and independent NETosis displayed increased lactate dehydrogenase (LDH) activity and pyruvate kinase type M2 (PKM2) dimerization, both processes described in cancer cells to promote the Warburg effect [67,68]. In future studies, it would thus be interesting to delineate precisely which factors can stimulate neutrophil glycolysis. In macrophages for example, a shift toward aerobic glycolysis occurs in response to tumor-derived lactic acid [69].

^{18}F-fluorodeoxyglucose positron emission tomography (PET) scanning elegantly demonstrated that myeloid cells in the TME from different mouse models have a higher glucose uptake capacity compared to tumor cells and T cells on a per-cell basis [10]. This important notion contrasts with a more common view that cancer cells are the main contributors of the enhanced glucose uptake in tumors. Mechanistically, mTORC1 signaling promotes glucose uptake by myeloid cells through the upregulation of glycolysis-related genes including *Slc2a1*, encoding for the GLUT1 transporter, *Hk2* and *Hk3*, encoding hexokinases. In contrast, tumor cells showed a higher glutamine and lipid consumption. Conversely, mouse treatment with V9302, an inhibitor of glutamine transport, increased glucose uptake in all cells from the TME. The authors thus suggested that glutamine metabolism limits the use of glucose *in vivo*, challenging the concept of cancer cell-immune cell competition for glucose [11,70].

In a mouse model of lung adenocarcinoma, TANs were characterized by stronger GLUT1 expression, enhanced glucose uptake and glycolysis compared to healthy lung neutrophils. Neutrophil-specific *Slc2a1* deletion diminished TAN survival and tumor growth while increasing the response to radiotherapy, demonstrating that increased glucose usage endows TANs with tumor-supporting functions [71].

In recent years, aerobic glycolysis became functionally implicated to the capacity of innate immune cells to develop an unspecific memory after infection, termed "trained immunity" or "innate immune memory" [72,73]. Neutrophils from mice pre-treated with β-glucan, an agonist of trained immunity, were able, upon adoptive transfer to naïve recipient mice, to inhibit tumor growth in a subcutaneous model [74]. This effect was mediated by NADPH oxidase and ROS production in trained neutrophils. Importantly, trained immunity was shown to take place in the bone marrow, where hematopoietic progenitor cells upregulated the expression of genes involved in glycolysis and in cholesterol biosynthesis, important to increase the β-glucan-dependent myelopoiesis in the bone marrow [75].

In early tumor-bearing mice, bone marrow neutrophils exhibited higher facility to migrate compared to neutrophils from healthy mice [76]. These cells showed increased glycolysis and ATP production, without expressing an immunosuppressive phenotype, appearing later as the tumors progressed.

Interestingly, hyperglycemic tumor-bearing mice showed an inhibition of primary tumor growth but increased metastasis formation [77]. Neutrophils in hyperglycemic mice exhibited the same functional capacities as in control mice, but they were present in lower amounts in the circulation because of decreased G-CSF secretion. The authors stipulated that lack of G-CSF prevents the recruitment of anti-tumor neutrophils, which contrasts with studies showing a correlation between high neutrophil presence in the blood and poor prognosis [78–80].

4.3. The pentose phosphate pathway, divergent branch of glycolysis

4.3.1. ROS and NO production

As previously described, the PPP is a parallel metabolic pathway to glycolysis, diverging at the level of glucose-6-phosphate and generating NADPH important for ROS production. ROS constitute one of the main defense mechanisms used by neutrophils against pathogens and, in cancer, the oxidative burst created by neutrophils is responsible for T cell apoptosis and inhibition of proliferation [81,82] (Fig. 1B). In a mouse model of breast cancer, G-CSF induced ROS production in neutrophils, reprogramming them towards an immunosuppressive phenotype [83]. A study from 2017 showed that TANs trigger apoptosis of non-activated CD8 T cells *in vitro*, through the production of NO. Specifically, TANs isolated from subcutaneous tumors (from mouse mesothelioma or lung cancer cell lines) cocultured with CD8 T cells extracted

from the spleens of the same mice induced a strong CD8 T cell death [81]. Of note, other metabolic pathways can trigger neutrophil ROS and NETs. This is discussed in the following chapters.

4.3.2. NET formation

NETs are expulsed sticky meshes composed of neutrophils' decondensed chromatin and cytoplasmic proteins that capture bacteria and microbial factors, killing pathogens and preventing their dissemination [84]. In cancer, NETs have been shown to have anti- or pro-tumor roles. NET components can kill tumors directly and prevent tumor growth and metastasis [85,86]. In mouse models, NET formation has been tightly linked to cancer metastasis [87,88]. Recently, Yang and colleagues showed that NET-DNA acts as a chemoattractant for tumor cells to form metastasis in the liver [89]. They found that in patients with breast or colon cancers, liver metastasis contained high amounts of NETs compared to primary tumors and that NETs present in the serum could serve as a prognostic tool for metastasis formation in early-stage breast cancer patients. In addition, they identified a transmembrane protein on the cell surface, CCDC25, able to detect and bind NET-derived DNA. This interaction modified the tumor cell's shape and improved not only their proliferation but also their invasive capacity. It is therefore important to understand how metabolism supports neutrophil capacity to produce NETs. Neutrophils upregulate glycolysis and shift intermediates toward the PPP to induce NET formation [21,22]. When incubated with 2-deoxyglucose, an inhibitor of glucose uptake and phosphorylation, NET formation is suppressed in neutrophils *in vitro*, suggesting that NET formation relies on glycolysis [22]. Moreover, it has been shown that tumor-secreted G-CSF stimulates NETosis [90]. Treatment of 4T1 tumor-bearing mice with a G-CSF neutralizing antibody reduced the capacity of peripheral blood neutrophils to form NETs *ex vivo*. Interestingly, it has also been observed that immature and low-density neutrophils can utilize fatty acid oxidation and glutamine catabolism for NET formation in mice bearing liver metastases from 4T1 breast cancer cells [40]. We will describe this further below.

4.4. Mitochondria, OXPHOS, energy production and apoptosis

Neutrophils in the blood of tumor-bearing mice and cancer patients can be subdivided into two groups by centrifugation: low-density neutrophils (LDN) and high-density neutrophils (HDN). A recent study described a subtype of immature low-density neutrophils (iLDNs) that, under nutrient-deprived conditions, shifted their metabolism towards OXPHOS for ATP production [40]. These neutrophils showed a globally higher bioenergetic capacity and promoted metastasis of breast cancer to the liver through NET formation. When compared to HDNs in glucose-limiting conditions, iLDNs showed greater capacity to undergo NETosis. A similar upregulation of OXPHOS was also observed in activated splenic neutrophils in a mouse model of breast cancer where 4T1 cells are injected into the mammary fat pad [91]. Notably, in normal glucose conditions, iLDNs perform glycolysis rather than fully metabolizing glucose through OXPHOS, suggesting that these cells are not entitled to a defined pathway but can flexibly modulate their energy metabolism according to the environment. These cells exhibited higher mitochondrial content and OXPHOS capacity. LDNs have been shown to expand in cancer patients and in diverse mouse models of cancer [92,93]. In lung cancer patients, LDNs accumulate along disease progression and correlate with poorer overall survival of patients with late stage tumors [94].

Neutrophils have been left aside for a long time in cancer research, probably because of the idea that their short lifespan may limit their impact on long chronic diseases such as cancer. Recent studies however demonstrated that neutrophils can have an extended survival within the tumor mass. SiglecF-expressing TANs from a mouse model of lung adenocarcinoma were shown to persist in the TME for several days, a substantial increase compared to neutrophils in other tissues [95]. Although the mechanisms accounting for the induced TAN survival are not yet elucidated, G-CSF could be important, as it was shown to promote survival of neutrophils by inhibiting mitochondria-dependent caspase-3 activation [96]. GLUT1-mediated glucose uptake was implicated in their survival, too, as neutrophil-specific *Slc2a1*-deletion reduced the proportion of old, SiglecF$^+$ TANs [71].

In a mouse model of cervical cancer, the presence of MDSCs was not only due to increased recruitment, but to inhibition of apoptosis through G-CSF-mediated STAT3 activation [34]. Interestingly, STAT3 was also shown to induce immunosuppressive neutrophil survival during chronic viral infections [97]. This was due to downstream kinase PIM1, responsible for mitochondrial fitness. These neutrophils had high mitochondrial mass, linked to increased oxidative metabolism, and inhibited CD8 T cells through ROS production. Treatment with a PIM1 kinase inhibitor resulted in mitochondrial fragmentation, neutrophil death and a concomitant increased CD8 T cell function, suggesting that mitochondria are supporting the metabolic fitness and immunosuppressive functions of neutrophils.

4.5. Lipid metabolism

As we have discussed above, neutrophils can oxidize fatty acids to compensate for the lack of glucose in the TME and sustain their functions, notably to support ROS production. As an illustration, in 4T1 breast tumor-bearing mice, a subset of immature c-Kit$^+$ neutrophils used FAO and mitochondrial oxidative metabolism to maintain sufficient NADPH supply, necessary for ROS production and subsequent T cell inhibition in a glucose limited environment [91].

In another study where 4T1 cells metastasize to the liver with the help of immature LDNs, lipid staining revealed increased levels in these cells compared to normal HDNs. The authors suggested that either these immature neutrophils rely on this pathway for energy production, or that early release of immature neutrophils from the bone marrow may impair FAO to occur, an important catabolic process for neutrophil differentiation [24]. In several mouse models of cancer, FAO was upregulated in TANs, which displayed higher mitochondrial mass and oxidative metabolism. This adaptation enables TANs to efficiently impair the T cell response [98].

Several studies described lipid metabolism as a way for TANs to survive in glucose-limited environments [98,99]. However, a recent article has shown that TANs, in addition to using FAO to maintain their cellular energetics, use lipids differently to support tumor growth. Specifically, stimulation of PMN-MDSCs by GM-CSF upregulated the fatty acid transport protein 2 (FATP2) receptor through STAT5 activation, enabling the uptake of arachidonic acid [100], a precursor of prostaglandin E2 that mediates immune suppression. Pharmacological inhibition of FATP2 reduced tumor growth and potentiated the effects of immune checkpoint blockade.

Besides fatty acids, neutrophils can also uptake and use lipoproteins. In patients with head and neck cancer and non-small cell lung cancer (NSCLC), it has been shown that PMN-MDSCs overexpress the lectin-type oxidized low-density lipoprotein (LDL) receptor-1 (LOX-1) that binds oxidized lipoproteins. Importantly, the number of circulating neutrophils expressing this receptor correlates with poor patient survival [101]. The incorporation of oxidized lipoproteins supports the immunosuppressive phenotype of neutrophils *in vitro*, blocking T cell functions more efficiently compared to neutrophils that do not express LOX-1. Interestingly, tumor cells experiencing endoplasmic reticulum (ER) stress can trigger LOX-1 expression in neutrophils [102], a result that was obtained by exposing healthy human neutrophils to conditioned medium from squamous carcinoma cells treated with ER stress inducers. Such reprogrammed neutrophils were more prompt to inhibit T cell proliferation *in vitro*. Although it is not yet clear how lipoproteins influence neutrophil functions, in endothelial cells LOX-1 triggering leads to the activation of the NADPH oxidase [103]. By analogy, it is therefore conceivable that the use of lipoproteins by TANs sustains immune-suppressive ROS production. Another use of lipids by

neutrophils might be to produce NETs. In mouse models of atherosclerosis, cholesterol uptake by neutrophils activates the inflammasome and NETs, which participate in the formation of plaque erosion and thrombosis (reviewed in [104]).

An interesting insight into how tumor growth is supported through modulations of TAN metabolism was also provided with a zebrafish model of $Kras^{G12V}$-induced liver cancer [105]. Transcriptomic analyses of TANs from male and female zebrafish revealed an activation of liver X receptor/retinoid X receptor (LXR/RXR), a heterodimer of nuclear hormone receptors able to sense intracellular lipid changes and to modulate cholesterol transport and fatty acid metabolism [106]. In this model, neutrophil motility diminished after entering the tumor [107]. Impaired TAN movements within the tumor mass warrants further investigations, as the total time spent within the TME may favor TAN reprogramming toward tumor promotion. It is known that some tumors show abnormally increased synthesis of cholesterol and downstream metabolites that stimulate cancer cell proliferation and drug resistance, and that can suppress tumor immunity (reviewed in [108]). In the case of neutrophil modulation in cancer, oxysterols (cholesterol-derived metabolites) but not cholesterol were shown to stimulate the recruitment of $CXCR2^{+}$ neutrophils to the tumors through direct binding and activation, which favored neoangiogenesis and immune suppression [109]. A pro-metastatic action of oxysterols *via* neutrophils was also reported in mice injected intravenously with murine breast cancer cell lines and fed with a high-fat diet [110]. Such studies may help explain the link between obesity and reduced survival in breast cancer patients [111].

4.6. Amino acid metabolism

In mouse models of LPS-induced acute lung injury, airway neutrophils subjected to both hypoxic and hypoglycemic conditions were shown to take up and catabolize extracellular proteins resulting from lung damage and to use the derived glutamine to support their energy requirements [112]. Additionally, LPS-treated neutrophils increased glutamine consumption [113]. Glutaminolysis can be engaged in neutrophils when glucose supply is insufficient. Glutamine is first converted into glutamate, which is metabolized to alpha-ketoglutarate in the mitochondria, an intermediary metabolite used in the Krebs cycle for OXPHOS-driven energy production. Recently, in mice injected intraperitoneally with ID8, a murine ovarian cancer cell line, TANs were shown to use glutaminolysis as a major fuel for OXPHOS to support their immunosuppressive roles [114]. Furthermore, glutaminolysis offers an alternative to the PPP for NADPH production needed for ROS generation, as exemplified in glutamine-exposed human neutrophils [115]. Interestingly, NET formation in immature LDNs relied on glutamate and proline catabolism in glucose- and amino acid-deprived conditions [40], since the addition of each amino acid to such medium rescued NET formation.

It is not clear if neutrophils can use other amino acids to sustain their energetic demands. Nevertheless, they produce enzymes capable to degrade arginine for tumor support and other than for self-usage. Indeed, TANs secrete arginase 1 (ARG1) in the TME [116], which degrades and reduces the amount of L-arginine, an important nutrient for T cell function, thus fostering suppression of anti-tumor immunity [117–120]. In the blood from NSCLC patients, MDSCs expressed high levels of ARG1 [121]. In experimental mouse models of metastasis where breast, lung, melanoma or colon cancer cell lines were injected intravenously, TGF-β-stimulated TANs expressed higher levels of iNOS and ARG1 and were responsible for limited T cell-based anti-cancer immune responses [50].

In contrast, a recent study showed that ARG1 secreted by neutrophils can induce ER stress-mediated apoptosis in cancer cells [122], introducing an anti-tumoral role for a protein considered to prevail in immunosuppressive granulocytes.

4.7. Autophagy

Autophagy is a survival process activated when nutrient stress is elevated and consists in the "digestion" of the cell's own organelles. This process fuels the transition from GMPs to immature neutrophils, by generating ATP through mitochondrial metabolism. Deletion of *Atg7*, a major gene regulating autophagy, results in suppression of OXPHOS and higher glycolysis. This eventually leads to an impairment in neutrophil generation. Thus, during neutrophil development, autophagy triggers lipolysis, which in turn generates free fatty acids that are used for mitochondrial respiration [24].

In human hepatocellular carcinoma, neutrophils upregulate autophagy to maintain functional mitochondria and to survive in the TME. In these cells, autophagy also induced the production of MMP-9 and oncostatin M (OSM), which stimulated the migration of cancer cells [123]. Upon inhibition of autophagy, TANs isolated from mouse models of pancreatic ductal adenocarcinoma showed a decreased capacity to form NETs [124]. In acute myeloid leukemia, high ATPase inhibitory factor (IF1) expression, a protein responsible for ATP synthase regulation, was associated with a poor prognosis [125]. While IF1 deficiency reduced the number of circulating neutrophils, IF1 triggered neutrophil autophagy and their infiltration to the colon mucosa in a mouse model of colitis.

5. Implication of neutrophil metabolism in the clinics

5.1. Targeting neutrophil metabolism in the TME

While the neutrophil-to-lymphocyte ratio (NLR) is often associated with poor prognosis [80], a blood glucose-to-lymphocyte ratio (GLR) was a marker of poor prognosis in patients with inoperable pancreatic cancer [126]. In addition, immature neutrophils may have prognostic value. The delta neutrophil index (DNI) measures the percentage of neutrophils from the blood that have an immature phenotype, as first described in the cases of sepsis [127,128]. Recently, two clinical trials aimed to interrogate the prognostic value of DNI in patients with thyroid malignancies (NCT04425512) and in patients with breast cancer metastasis (NCT04729647).

Because of their immense daily production, manipulating neutrophils in cancer patients will most likely be challenging. Moreover, therapies targeting all neutrophils would put patients in life-threatening conditions due reduced innate immunity. Understanding neutrophil heterogeneity in cancer thus represents an opportunity to formulate therapies aimed to selectively target tumor-supportive neutrophils. Although our understanding of neutrophil metabolic plasticity in cancer may help for such treatment perspectives, targeting cellular metabolism may affect many other cells, too. However, targeting metabolic pathways that are prevalent and shared by tumor-supportive TANs and tumor cells might offer some therapeutic window. In this regard, targeting glutamine metabolism may become an interesting therapeutic option, as both tumor cells and TANs need this amino acid. Treating mice bearing subcutaneously-injected 4T1 breast tumors with JHU083, an orally active glutaminase antagonist, resulted in decreased G-CSF and MDSC recruitment and triggered apoptosis of both intra-tumor and circulating MDSCs [129]. In addition, the same drug induced T cell activation and survival, and blocked glycolytic and oxidative metabolism in cancer cells from several syngeneic tumor models [130].

Neutrophil autophagy could also be considered as a potential target for clinical application [131]. In psoriasis, treatment with a topical inhibitor of the autophagy inducer ULK1 resulted in decreased neutrophil infiltration within the skin [132].

Modulating or inhibiting metabolic reprogramming of neutrophils in tumors to suppress tumor-supportive subsets is of interest but currently complex, as metabolic plasticity of TANs may counteract the effectiveness of such approaches. An insight may come from the studies of AML, where neutrophil progenitors behave as cancer cells and modulate their

metabolism in ways that promote their survival and expansion. Metabolic targeting of these cells was suggested as potential treatment [133], which could be considered for other cancer contexts, provided that immature neutrophils observed in solid cancers and metastases share metabolic characteristics with myeloid cancer cells.

One particularly interesting and promising way to promote anti-tumor neutrophils is through the trained immunity that we described above. This was pioneered by the work from Priem and colleagues who developed nanobiologics that shifted myelopoiesis toward the generation of anti-tumor myeloid cells, which potentiated the effect of immune checkpoint blockade in a B16F10 mouse melanoma model *in vivo* [134].

5.2. Targeting neutrophil recruitment to tumors

Unless we identify efficient strategies to deplete pro-tumor TANs or to shift their functions toward anti-tumor activity in tumors directly, the best way to suppress tumor-supportive neutrophils within the TME is to block their recruitment to the tumor. As we mentioned above, many factors can stimulate granulopoiesis and neutrophil egress from the bone marrow, including G-CSF. However, modulating systemic neutrophil levels can be life-threatening in case of neutropenia [135], which is a risk that is already present in patients who undergo chemotherapy as first line treatment [136,137] and where G-CSF is administered for neutrophil recovery.

In the clinics, several inhibitors are being tested to block immune-suppressive neutrophil factors such as ARG1, NO and ROS [138]. ARG1 blockade could be a promising way to restimulate anti-tumor immunity; while the incubation of T cells with human PMN supernatant led to suppression of T cell proliferation, addition of arginase inhibitors resulted in an highly augmented T cell proliferation and cytotoxicity [139]. Taken together, these inhibitors could potentiate the restauration of an effective anti-tumor immunity in combination with existing immunotherapies [138].

6. Conclusions

While neutrophil research in cancer is a relatively new field of investigations, much newer is our current understanding of the metabolic fluctuations of neutrophils in cancer and how they impact their functions. Adding to the complexity to identify the main metabolic pathways induced or repressed in TANs, factors or nutrients secreted by cancer cells or other cells of the TME may vary between cancer types and thus influence neutrophil metabolism in a cancer type-specific manner (Table 1). Accordingly, neutrophils transcriptionally and functionally adapt in different tissues [140].

Although TANs are highly committed to glycolysis, they can also circumvent it when needed and upregulate other metabolic pathways (Fig. 1). How these adaptations occur, especially at the global gene expression level, remains unclear. An element of answer could reside in the function of supramolecular organizing centers (SMOCs), such as the inflammasome, which mediates inflammatory responses both in a transcription-dependent and independent manner. Another example of SMOC is the myddosome, downstream of Toll-like receptor stimulation, which rapidly stimulates glycolysis upon downstream TBK1 activation, without actual NF-κB activation [141].

Although cancer immunometabolism has been more intensively investigated for other cells of the TME such as T lymphocytes, with this review we hoped to highlight its growing importance and recognition in neutrophils. Many research questions remain: what are the metabolic changes that promote tumor support by TANs? Can we hijack TAN metabolism to convert tumor-supportive neutrophils into anti-tumor neutrophils? Are functionally-distinct TAN subsets controlled by different metabolic pathways? Undoubtedly, much research is needed to precisely understand the influence of neutrophil metabolism on their function in cancer.

In conclusion, neutrophils exhibit a great capacity to modulate their metabolism through the use of multiple nutrients in the TME. Yet, little is known about the exact mechanisms through which these adaptations take place, such as what triggers them and what downstream pathways are involved. In the future, we hope that a better understanding of neutrophil metabolic plasticity will lead to possible targeted therapies either to block tumor-supportive cells or to convert them into anti-tumor ones.

Table 1
Comparison of neutrophil metabolic pathways reported in different cancer types in mouse models or in humans. The arrows represent increased activities or expression. BMN: bone marrow neutrophils. PMN-MDSCs: polymorphonuclear myeloid-derived suppressor cells. FAO: fatty acid β-oxidation, OXPHOS: oxidative phosphorylation.

Cancer type	Metabolic pathways used by neutrophils	
	Mouse	Human
Lung	GLUT1 ↑, glycolysis, survival ↑ in TANs [71] Glycolysis, OXPHOS and ↑ATP in BMN in early-stage cancers [76] FAO [99] FATP2 ↑ for PGE2 synthesis [100]	LOX^+ PMN-MDSCs, ↑ ROS, ARG1 and ER stress [101] Lipid uptake ↑ and FATP2 ↑ in blood and tumor PMN-MDSCs [100]
Breast	OXPHOS, glutaminolysis, NETs ↑ in iLDNs [40] $c\text{-}kit^+$ neutrophils utilize FAO-OXPHOS for ROS [91]	FAO in blood and tumor MDSCs [98] Lipid uptake ↑ and FATP2 ↑ in blood and tumor PMN-MDSCs [100]
Colorectal	FAO [98] FATP2 ↑ for PGE2 synthesis [100]	FAO in blood and tumor MDSCs [98]
Ovarian	Glutaminolysis, OXPHOS [114]	
Endometrial	Hypoxia prevents ROS production [58]	
Renal		FAO in blood and tumor MDSCs [98]
Hepatocellular		Autophagy, survival ↑ of TANs [123]
Prostate	BMN increased glycolysis, OXPHOS, ATP [76]	
Pancreatic	BMN increased glycolysis, OXPHOS, ATP [76] Autophagy linked to ↑ NETs [124] FATP2 ↑ for PGE2 synthesis [100] Trained neutrophils ↑ ROS [76]	Autophagy, ↑ NETs [124]
Melanoma	BMN increased glycolysis, OXPHOS, ATP [76] Fatty acid uptake by FATP2 [100]	
Head and neck		LOX^+ PMN-MDSCs, ↑ ROS, ARG1 and ER stress [101] Lipid uptake ↑ and FATP2 ↑ in blood and tumor PMN-MDSCs [100]

Authors' contributions

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Data availability

Data will be made available on request.

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8 CURRICULUM VITAE

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EDUCATION

2019 – now **Ecole Polytechnique Fédérale de Lausanne (EPFL)**
PhD program Molecular Life Sciences
Swiss Institute for Experimental Cancer Research (ISREC) Group of Prof. Freddy Radtke
In co-supervision with Prof. Etienne Meylan, from the Institut Jules Bordet, Bruxelles
PhD thesis: "*Targeting tumor-associated neutrophil aging in lung cancer*"

2017 – 2019 **Université de Lausanne (UNIL)**
2 years Master of Science (MSc) in Medical Biology
Specialization: Immunology and Cancer
Master thesis under the supervision of Prof. Fabienne Tacchini-Cottier
Master thesis: "Deciphering the role of IL-33 in neutrophils in the context of cutaneous leishmaniasis"

2014 – 2017 **Université de Lausanne (UNIL)**
Bachelor of Science (BSc) in Biology

PROFESSIONAL EXPERIENCE

July 2017 Research training at the department of Infectious diseases, Pierre-Yves Bochud lab CHUV, Lausanne

Feb 2015 Research training at the department of Toxicology and Pharmacology, Prof. Edith Hummler lab, CHUV, Lausanne

2015-2016 Mentor for Bachelor students, UNIL

PUBLICATIONS

Bodac A, Mayet A, Rana S, Pascual J, Bowler A, Roh V, Fournier N, Craciun L, Demetter P, Radtke F and Meylan E (2023): Bcl-xL targeting eliminates ageing tumor-promoting neutrophils and inhibits lung tumor growth. *EMBO Molecular Medicine 16: 158-184*

Bodac A, Meylan E (2021) Neutrophil metabolism in the cancer context. *Seminars in Immunology* 57: 101583

CONFERENCE PRESENTATIONS

06 / 2023 Stem Cell retreat 2023
Bern,, Switzerland ***oral presentation***

05 / 2023 EPFL Initiative for Cancer Science Engineering (EICSE) retreat
Lausanne,, Switzerland *poster presentation*

09 / 2022 U-CR seminars at ULB
Remote ***oral presentation***

06 / 2022 Stem Cell retreat 2022
Puidoux, Switzerland *poster presentation*

11 / 2021 10th Faculty and Staff annual retreat of the Lausanne Research Community
Lausanne, Switzerland *poster presentation*

05 / 2021 EPFL Initiative for Cancer Science Engineering (EICSE) retreat
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LANGUAGES

French (native) – **English** (C1) – **Croatian** (native) – **German** (B2)

Awards

2023 Second place in the EPFL finale of the competition of “Ma thèse en 180 secondes”