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CXCL5-mediated accumulation of SiglecF^{high} neutrophils in lung tumor tissues impairs CD8 T cell responses and limits the efficacy of PD-L1 checkpoint blockade

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Abstract

Neutrophils are recruited in different type of cancer, including lung cancer, where they emerged as the most significant negative prognostic factor. Most experimental reports converge toward a pro-tumorigenic role of neutrophils via direct induction of cancer cell proliferation, promotion of metastasis, stimulation of angiogenesis and modulation of T cell responses. Nevertheless, in some cancer type and tumor stages, neutrophils exert anti-tumoral properties through direct killing of cancer cells and activation of T cell-dependent anti-tumor immunity. Thus, neutrophils in cancer display an enormous plasticity and heterogeneity which may be strongly influenced by the tissue and the constellation of immune modulating factors. Evaluate the complexity of neutrophils in tissue specific tumor microenvironment to unequivocal distinguish immunosuppressive neutrophils from other neutrophil subsets represent an important challenge in the field. In addition, understanding how these cells accumulate in tissues and how polarize to a pro- or anti-tumorigenic phenotype is crucial to develop successful cancer therapies.

A critical subset of neutrophils expressing high levels of the sialic-acid-binding protein SiglecF (neu-SiglecF^{high}) was identified in Kras$^{G12D/+}$; Trp53$^{-/-}$ (KP) mouse lung adenocarcinoma that correspond to a transcriptionally related human counterpart associated to negative outcomes. Neu-SiglecF^{high} are mature, long-lived, cells that display tumor promoting functions associated to angiogenesis, matrix remodeling and production of ROS. However, the mechanism of recruitment of neu-SiglecF^{high} in lung cancer and their impact on endogenous anti-tumor T cell responses are still unknown.

Here, we used a transplantable KP line to investigate the role of the C-X-C motif chemokine 5 (CXCL5) in recruitment and accumulation of neu-SiglecF^{high} in the microenvironment of lung tumors. By genome editing we abrogated the expression of CXCL5 in immunogenic KP cells (KP OVA KO^{CXCL5}) and we characterized neutrophils frequencies and T cell activation within lung tumor tissues. We observed a drastic decrease of Cxcl5 transcripts followed by a strong reduction of neu-SiglecF^{high} in KO^{CXCL5} tumors proving that the chemokine is a key player for their accumulation. Moreover, phenotypic and functional analysis of endogenous anti-cancer responses revealed a significant expansion of highly activated and cytotoxic tumor specific CD8^{+} T cells in tumor lacking neu-SiglecF^{high}. Immunofluorescence analysis of lung tissues shown tight CD8 T cell-neutrophils interactions, suggesting a contact-mediated mechanism of inhibition. Moreover, administration of antibodies to PD-L1 during challenge proved that neu-SiglecF^{high}, due to a high expression of PD-L1, hamper the full activity of checkpoint blockade. Thus, we infer that targeting the CXCL5-axis could be a viable improvement to existing immunotherapy.

1. Introduction

Cancer is one of the leading causes of death worldwide and the most common and aggressive is lung cancer. The emergence of lung cancer epidemic in the 20th century has been caused by cigarette smoking, the main risk factor for lung cancer development. Other factors include exposure to toxic substances (alcohol, air pollution), but also unhealthy diet, lack of physical activity and mutation of susceptibility genes. Screenings for patients with high risk for lung cancer showed reduction in mortality. In fact, timely detection of lung cancer could delay its progression and after the initial diagnosis, accurate staging is critical for determining appropriate therapy and achieve a good long-term survival. However, this finding is not echoed in practice. A majority of lung cancer cases are diagnosed in symptomatic individuals (e.g., cough, fatigue, chest pain, haemoptysis) that reduce the change to reach an effective tumor containment [1] [2].

Among different types of lung cancer, the non-small cell lung cancer (NSCLC) accounts for approximately 85% of all cases and is predominantly comprised of lung squamous cell carcinoma (LSCC) and adenocarcinoma (LADC). LSCC and LADC can be distinguished by their histopathology, biomarkers, gene expression patterns, genomic alteration, and response to therapies. The composition of immune microenvironment differs between LADC and LSCC and it is becoming important decipher the function of each cells populating the TME to better understand the role of them in tumor initiation and progression and to fully exploit the potential of the novel cancer treatment immunotherapies [3].

Depending on the stage, histology, genetic alterations and patient's condition, the treatment approaches in NSCLC usually include surgery, radiotherapy, chemotherapy, molecularly targeted therapy, or immunotherapy. Surgical approach is effective only in patients with early stages, whereas more advanced diseases are candidate for non-surgical treatment. Although chemotherapy is appropriate for many patients with lung cancer, there is a sense that the use of traditional approaches have reached a therapeutic plateau. Increased understanding of cancer biology has revealed pathways to be targeted for therapeutic purposes. These include mutated tyrosine kinase receptor (such as EGFR and ALK), vascular endothelial growth factor (VEGF), or altered PI3K/AKT/mTOR, RAS/MAPK and JAK-STAT pathways which regulate various cellular processes including cell cycle, apoptosis, protein synthesis, among others [4]. Nevertheless, while target therapy in NSCLC has provided disease control, the tumors often develop drug resistance. Discover other mechanisms of resistance and develop combinational therapies are essential to improve the treatment outcomes [5]. In the last few decades, immunotherapies, based on the idea to

stimulate the immune system to overcome the cancer progression, became an important part of treating different types of cancer, including lung cancer [6 7].

1.1. Cancer immunoediting and immunity cycle

In cancer, immunosurveillance is a term used to describe the processes by which cells of the immune system look for and recognise pre- or cancerous cells in the body. This concept was proposed in 1909, but only recently demonstrated with the development of gene targeting and transgenic mouse technologies [8]. The last fifteen years have seen a re-emergence of interest in cancer immunosurveillance and a broadening of this concept into one termed cancer immunoediting to include the ability of cancer to finally escape from immune system (Fig. 1.1). A deeper knowledge of the immune biology of cancer immunosurveillance and immunoediting will hopefully stimulate development of more effective immunotherapeutic approaches to control and/or eliminate human cancer.

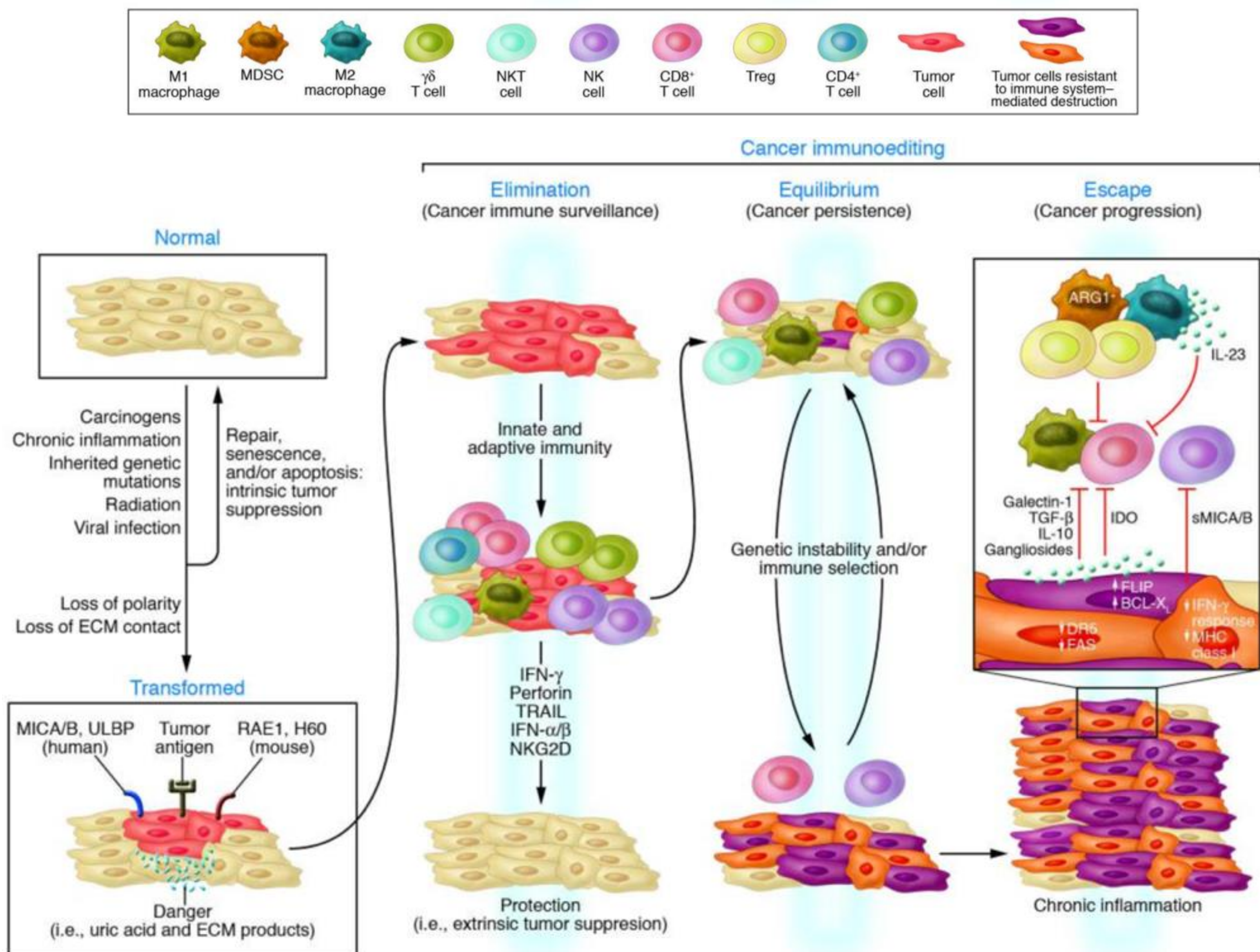

Figure 1.1. Cancer immunoediting. Normal cells that undergo different alterations can be recognized and eliminate by immune system. This is known as elimination phase (or immune surveillance) organized by innate and adaptive immune cell subsets that through the release of molecules (such as IFNs, Perforin, TRAIL among others) can eliminate nascent tumor cells. Tumor cells that survive to

immune elimination but are not able to overcome the immune system and become clinically evident characterized the equilibrium phase. However, cancer cells can escape the immune system because of the reduced immunogenicity and a large number of immunosuppressive mechanisms to shut down the antitumor immune response leading to the appearance of symptoms [9].

One of the main question concerns how the cells of the immunosurveillance network distinguish between a nascent or established tumor cells, from normal cells, which represent the first step of the cancer immunity cycle [8].

Tumor cells are genetically unstable, a feature that promotes accumulation of mutations and generation of neoantigens that can be recognized by the immune system in the context of major histocompatibility complex (MHC) molecules [10] [11] [12]. Since the first human tumor antigen was identified in 1991, many tumor antigens have been identified and segregated into five categories: 1) differentiation antigens, 2) mutational antigens (e.g. abnormal forms of p53), 3) overexpressed or amplified antigens, 4) cancer testis antigens and 5) viral antigens [13] [14] [15] [16] [17] [18] [19].

Thus, even at early stages of tumorigenesis, the cells that undergo transformation might express distinct tumor-specific markers and generate pro-inflammatory danger signals that initiate the elimination phase, first of cancer immunoediting process, in which both adaptive and innate immune cells play a critical role. During the tumor formation, effector cells such as macrophages, DCs, NK, NKT and T cells are recruited and activated by inflammatory cytokines released by tumor cells and stromal cells in the TME. The activated immune cells, therefore, start to produce molecules that mediate the killing of cancer cells (including IL-12, IFN-γ, Perforin, Fas-L and TNF-related apoptosis-inducing ligand-TRAIL) [20] [21] [22] [23] [24]. Moreover, several cell surface molecules expressed on tumor cells have been identified as ligands that bind receptors on immune cells inducing their activation. For example, the engagement of NKG2D receptor (expressed mostly on NK cell, NKT cells and $\gamma\delta$ T cells) with its ligands on cancer cells (which are induced in response to cellular stress, DNA damage or inflammatory milieu), leads to the release of granules containing perforin and granzyme that induce cell lysis and elimination of mutated tumor cells [25] [26]. Evidence shown that mice lacking NK cells develop spontaneous tumors supporting the important role of those cells in immunosurveillance [22].

Among immune cells, the professional antigen presenting cells (APCs) which include macrophages, B cells and dendritic cells (DCs), carry out an essential role in the elimination phase. The most specialized class of APC is composed by DCs which represent the interface between innate and adaptive immunity. DCs are able to recognize and present endogenous and exogenous tumor associated antigens (TAA) to T cells usually in lymphoid organs in the context of MHC molecules, resulting in priming and triggering of an effector T-cell response against cancer [27]. Antigens

presented on MHC class II molecules are recognized by $CD4^+$ T cells that acquire regulatory functions, instead antigens presented on MHC class I molecules are recognized by $CD8^+$ T cells with cytotoxic properties (CTLs). Once activated, T cells rapidly proliferate and migrate into tissues to carry out their effector responses. This process must be accompanied by signals such as proinflammatory cytokines that support the effective action of immune system in order to avoid antigen tolerance [28].

Among different subsets of CD4 T cells, the Thelper1 cells are the principal weapons against cancers which are able to orchestrate the beginning and maintenance of the adaptive immune response: not only these cells help the antibody responses B cell-mediated, but also mediate the activation and expansion of CD8 T cells and are necessary for establishment of effective CD8 T cell memory [29] [30] [31] [32]. Direct CD4 T cells cytotoxic activity on tumor cells presenting tumor associated antigens on MHCII molecules has been shown in some cancer patients [33]. However, as most tumor cells do not express MHCII molecules and $CD4^+$ T helper1 cells can promote the rejection of MHCII-negative tumors [34] [35], much of their action in cancer has been attributed to the production of cytokines such as IL-2, IL-12, TNF-α and IFN-γ associated with inhibition of tumor cell proliferation and activation of other immune cells including DCs, macrophages, NK cells and CD8 T cells [36] [37] . Nevertheless, activated $CD8^+$ T lymphocytes, thanks to their capacity to recognize tumor cells presenting TAA on MHC class I molecules and directly kill them via release of effector molecules (e.g., granzyme A/B, perforin) or induction of FasL-mediated apoptosis, play the prominent role in cancer [38] [39] [40]. CTLs also are able to release IFN-γ and TNF-α to induce cytotoxicity in cancer cells. Thus, the infiltration of tumors by cytotoxic $CD8^+$ T cells (hot tumor) is considered as beneficial for the survival of the patient and related to a better response to immunotherapy than CTL-low one (referred as cold tumor) [41] [42] [43].

The resultant killing of cancer cells induce the release of other tumor associated antigens that allow to restart the cancer immunity cycle (Fig. 1.2) [44] [45].

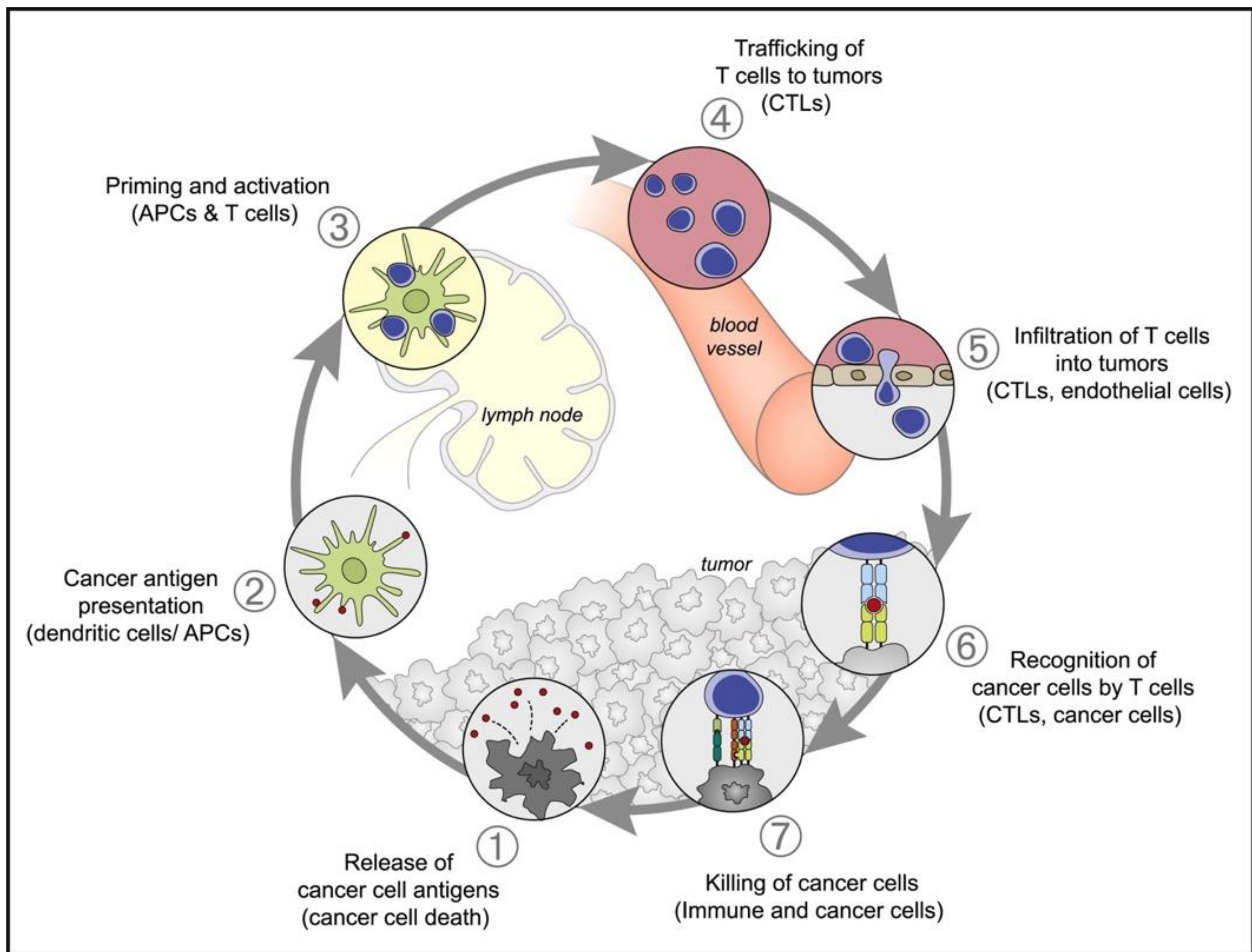

Figure 1.2 The cancer immunity cycle. A series of stepwise events leading the activation of T cell response against cancer cells is named cancer-immunity cycle. The first step (1) is characterized by the release of cancer cell antigens that are processed by antigen presenting cells (APCs) including dendritic cells (2). The migration of APCs into the lymph nodes allow the priming and activation of cytotoxic T cells (CTLs) (3) that acquire effector functions and the ability to reach the tumors site (4-5). Here, the CTLs are able to recognize antigen presented on major histocompatibility complex (MHC) class I molecules by cancer cells (6) and kill those cells (7) that induce the release of other tumor associated antigens to sustain the cycle [45].

However, the concept that a tumor can evolve to exist in the presence of an active immune system is well established and central to understand the tumor immunity. The phase where tumor cells, that have survived the elimination phase, are in equilibrium with the immune system, is refers as equilibrium phase. This state of dormancy is maintained by the immune system, which does not eliminate totally the tumor cells, but prevents tumors growing. This phase is thought to represent the longest of the three phases of tumor development. Indeed, cancer symptoms can occur many years after initial transformation. At some point, tumor cells can escape this period of equilibrium using two main strategies: avoid the immune recognition and induction of an immunosuppressive tumor microenvironment (TME). In fact, the first phase of elimination selects the proliferation of cancer cell variants that are less immunogenic and therefore less visible to immune detection. Lack of sufficient or suitable neoantigens leads to impaired formation of tumor-reactive T cells.

Moreover, even if a tumor expresses sufficient immunogenic antigens, immune detection depends on the capacity to present antigen in the context of a peptide-MHC complex. Tumors that lose MHCI expression or acquire defects in antigen presentation may escape immune-mediated elimination [46] [47] [48] [49]. Tumor-intrinsic mechanisms of immune evasion include epigenetic changes and activation of different oncogenic pathways that allow tumor cells to escape from immune surveillance by impair the anti-tumor immune response [50]. For example, loss of STK11/LKB1 in the setting of an oncogenic K-Ras mutation promotes production of IL-6 which decrease T cell infiltration and was associated with higher levels of T-cell exhaustion markers (PD-1, CTLA-4, and TIM-3) [51]. In addition, cancer cell-derive factors instigate an immune-tolerant TME by secretion of suppressive molecules (IL-10, TGF-β, prostaglandin E2 or VEGF), by expression of inhibitory checkpoint molecules (PD-L1, CTLA-4) and by induction of the recruitment of immune-suppressive subsets through the release of chemokine such as CCL2, CSF1, CCL5, CCL22, CXCL5, CXCL6 among others [52] [53]. Combined, these strategies result in a complex and efficient machinery for immune evasion [44].

Growing evidence showed that tumor-associated immune cells act in concert to both control and promote tumor formation [54] [55] [45]. It is true that, during the phase of elimination, NK cells exert a tumoricidal role. Moreover, through the secretion of CCL5 and XCL1 those promote the recruitment of cDCs to the TME, resulting in increased priming and activation of anti-tumor T cells stimulating the overall effector immune response. Additionally, the reciprocal interplay between NK cells, effector T cells and anti-tumor macrophages by the secretion of IFN-γ and TNF-α at the tumor site, boosts the differentiation of CTLs, increase macrophage phagocytosis, increase the recruitment of cytotoxic cMET^{+} neutrophils and enhances the cytotoxic ability of NK cells [56] [57]. However, once the tumors have escaped from initial tumoricidal immunity, the immune cells are influenced to acquire a pro-tumorigenic role and different strategies are used by cancer to tip the balance toward immune tolerance, with the TAMs, TANs and tumor-associated T regs as key orchestrators of this process [28]. The purpose of immunotherapy is to overcome the negative feedback mechanisms that lead to immune suppression and to sustain the cycle of immune system to achieve durable anti-cancer immune response leading to eradication of cancer. For the above reasons, fully understanding of which are the players in the TME and the process involved in the suppression of immune system is mandatory [45] [58].

1.2. The modern immunotherapies target dysfunctional T cells

Ten years ago, before the era of immune checkpoint inhibitors (ICIs), the solid tumor immunotherapy was based on cytokines such as interleukin-2 (IL-2) or alpha-interferon or cancer vaccines that were poorly effective and highly toxic [59]. The discovery that T cell activation requires co-stimulatory signals beside antigenic stimulation through the T cell receptor, and the identification of co-inhibitory signalling pathways that negatively regulate T cell activation, provided key insights into the complex network of interactions that control the balance between T cell activation and tolerance. In normal condition, the co-inhibitory pathways prevent aberrant or chronic activation of the immune system and maintain the immune homeostasis. However, these pathway can be used by cancer to damper anti-tumor T cell responses and promote immune escape [60 61]. Key players of co-inhibitory signalling are CTLA-4 and the PD-1/PD-L1 pathways [62]: the current FDA-approved immune check point blockade therapies target these molecules and are now use for the treatment of a broad range of tumor types including melanoma, non-small cell lung cancer, renal cell carcinoma and hepatocellular carcinoma (Fig. 1.3) [63].

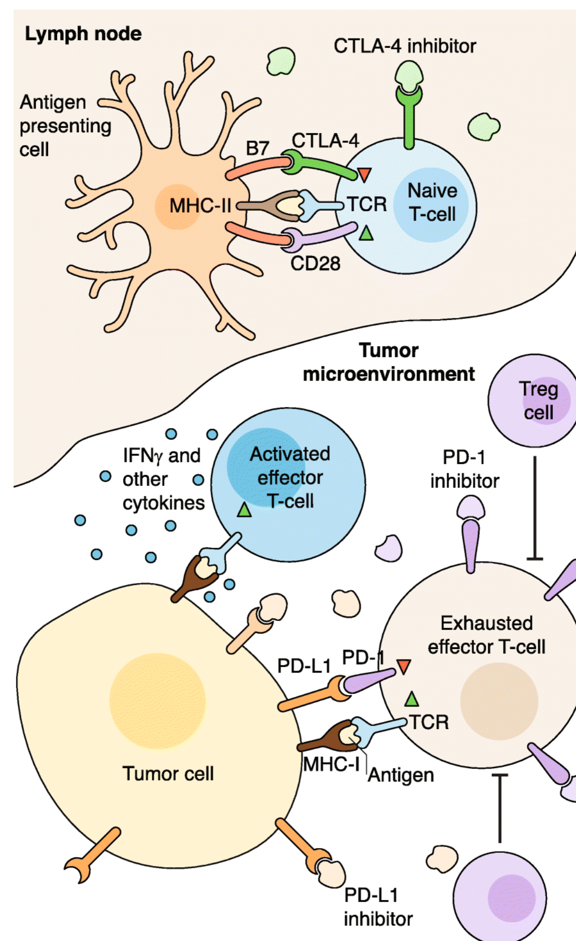

Figure 1.3 Immune checkpoint blockade. The binding between B7 ligands (CD80/CD86) expressed on antigen-presenting cells and CD28 receptor on naïve T cells leads to T cell amplification and immune response. Alternatively, binding of B7 ligands to CTLA-4

expressed on T cells induces suppression of their activity. PD-L1 molecule, often expressed on tumor cells, leads to the anergy of activated effector cells through the bind with its receptor PD-1 expressed on those cells. Pharmacological inhibition of immune checkpoints with monoclonal antibodies (CTLA-4 inhibitor or PD-1/PD-L1 inhibitor) restores antitumor activity and relieves immunosuppression [64].

In 2011, the first antibody blocking CTLA-4 (ipilimumab) was authorized, rapidly followed by the development of monoclonal antibodies targeting PD-1 (pembrolizumab and nivolumab) and PD-L1 (atezolizumab and durvalumab) [65]. Even if both CTLA-4 and PD-1/PD-L1 checkpoint inhibitors have resulted, when compared to conventional chemotherapies, in increased patient survival in a numbers of studies, including melanoma, renal cell carcinoma, squamous cell carcinoma and non-small cell lung cancer [7] [66] [67] [68], only a fraction of patients benefit from these therapies and severe immune-related adverse events were observed [69] [70] [66] [71] [72]. Thus, a full understanding of how checkpoint blockade works will be critical for widely effective anti-tumor responses.

CTLA-4 was the first negative regulator of T cell activation identified. Upon T cell activation, it is immediately upregulated to damp TCR signalling through competition with the costimulatory molecule CD28 for the B7 ligands (CD80 and CD86). The binding between CTLA-4 and B7 ligands leads to degradation of ligands needed for co-stimulation and secretion of other inhibitory signals which block T cell proliferation and induce anergy and tolerance in both sites of T cell priming (e.g., secondary lymphoid organs) and peripheral tissues [73] [74]. In addition, CTLA-4 can modulate T cell activation also through extracellular mechanisms often mediated by its expression on Treg cells [75]. Its expression in tumor lesions on infiltrating Treg or exhausted conventional T cells as well as tumor cells themselves has been associated with decreased survival in nasopharyngeal carcinoma and in non-small cell lung cancer [76] [77] and due to its negative role on T cell activation has become a target for immune checkpoint blockade.

The exact mechanism by which anti-CTLA-4 antibodies induce an anti-tumor response is unclear although seems that CTLA-4 blockade significantly decreases the competition for B7 co-stimulatory ligands promoting tumor rejection through an expansion of effector T cells within TME [78]. In addition, CTLA-4 blockade can induce the Treg depletion and consequently reduce Tregs-mediated suppression of immune response as observed in melanoma and other types of cancer [79] [80] [74]. Moreover, upon blockade of CTLA-4, antigens that are not normally sufficient to generate an effective T cell response may be allowed to emerge due to the broadening of TCR repertoire as effect of therapy [81].

The increased knowledge of negative T cell regulation pathways allowed to discover a second target for cancer immune blockade which is represent by PD-1/PD-L1 axis. In normal condition, this

pathway is useful to minimize tissue damage and maintain peripheral tolerance. The interaction between PD-1, that is expressed on a variety of immune cells, especially on T and B lymphocytes upon activation, as well as on suppressive Treg, and its ligands PD-L1 and PD-L2, directly regulates TCR signalling to attenuate T cell activity [82]. Upon ligation, PD-1 reduces glycolysis and simultaneously promotes fatty-acid oxidation and lipid catabolism inducing anergy [83]. The ligands for PD-1 are expressed on APCs and on different cells in nonlymphoid tissues in response to inflammatory cytokines (such as IFN-γ and TNF-α) or tumorigenic pathways, and have been also found on many different tumor types associated with tumor escape and poor prognosis [84 85 86 87]. To overcome this regulatory mechanism and promote an effective antitumor response, antibodies targeting this pathway have been developed. It has been shown that PD-1 blockade can principally induces reinvigoration of CD8 T cells by pharmacologically prevent PD-1 mediated attenuation of TCR signalling resulting in release of inflammatory cytokines and cytotoxic granules that lead to elimination of tumor cells [88]. In addition, blockade of PD-1 signalling axis can reverse the associated metabolic reprogramming to an extent, mediating T cell reinvigoration [89]. However, the precise mechanism of enhanced anti-tumor response PD-1 mediated is not fully understood. For example, although PD-1 blockade primarily leads to the expansion of CD8 T cells, it is known that CD4 T cells are required for effective responses to these immunotherapy [90]. Moreover, it has been demonstrated that also antibodies targeting PD-L1 can induce immune tumor rejection. The efficacy of these molecules, similarly to PD-1 blockade, is mainly due to the block of PD-1/PD-L1 interaction that extend the activation of T cells in the TME [63].

A critical point is that PD-1 is a marker of activated T cells of which exhausted T cells are a subset. Exhausted T cells undergo transcriptional, epigenetic, and metabolic changes that limits T cell activity in presence of chronic antigen stimulation and acts to preserve T cell clones that would otherwise perish due to death cell signals in the TME. It was shown that many features of exhausted CD8 T cells are associated with differential expression of transcription factor including T-bet (T-box expressed in T cells) and EOMES (eomesodermin) [91]. Early effector CD8 T cells gradually increase T-bet expression to become terminally differentiated effectors, whereas memory CD8 T cell increase the expression of EOMES e downregulate T-bet expression. Moreover, in chronic stimulation as in tumors, the overexpression of EOMES has been linked to execution of exhaustion program rather than generation of memory CD8 T cells [92 93]. Thus, exhausted T cells are a heterogeneous population with altered or reduced functions and high levels of expression of inhibitory receptors (such as PD-1, TIM3, LAG3, CTLA-4 and TIGIT), that do not involve the complete absence of function. Exhausted

T cells can produce effector molecules, including inflammatory cytokines and granzyme, and exert some control over tumors [94 95]. Due to the role of co-inhibitor receptors in anergy of T cells, targeting these molecules has become a promising approach to treat tumors. However, evidence shown that, for example, PD-1 blockade may not be sufficient to functionally restore T cells once they reach a threshold level of exhaustion and the efficacy of these approach is still limited to the little knowledge of downstream signalling pathways that lead to the exhaustion program [96 97 98].

Nevertheless, immune checkpoint blockade of CTLA-4 and PD-1/PD-L1 axis removes inhibitory signals of T cell activation, which enables tumor-reactive T cells to overcome regulatory mechanisms and mount an effective antitumor response [99 100]. Consistent with the understanding that PD-1 axis and CTLA-4 attenuate T cell activation through different mechanisms, combination of PD-1/PD-L1 and CTLA-4 blockade improves therapeutic efficacy as compared with either monotherapy [101 102].

However, since ICI action mechanism relies on the inhibition of the physiological brake of immune activation, they often have off-target effects resulting in immune-mediated inflammation and autoimmune responses in diverse organs or tissues [103 104].

Numerous currently studies on predictive biomarkers (that are focused on immune cell infiltration, PD-L1 overexpression, neoantigen clonality, mutational landscape, miRNA, and others) will help to identify, in the future, responders from non-responders and to avoid unnecessary adverse effects. As an example, the current guidelines for NSCLC patients recommend to test for expression of PD-L1 before the use of PD-L1 inhibitors[105]. The biomarkers already approved, only marginally help to stratify responder and non-responder patients, underlying the need of further studies [106 107].

Besides improving patient's stratification, a second important goal is to improve efficacy of immune blockade treatments. The overall response rate to these agents is usually low and some patients who do have tumor regression initially, start losing that response over time supporting the existence of mechanisms of resistance to checkpoint inhibitors [108]. Therefore, a comprehensive exploration of the causes of immune drug resistance will help identify target to overcome limitations.

Several studies shown that both cell-autonomous (related on the inherent characteristics of tumors cells) and non-autonomous (mainly emanated from the tumor microenvironment) mechanisms can causes tumor resistance to ICIs [109].

The presence of neoantigens on mutated tumor cells boosts anti-tumor T cell response and improves treatment efficacy. Evidence suggests that these therapies are more effective for patients who already display effective anti-tumor immune processes prior to therapy [110 111 7]. Tumors with increased tumor infiltrating lymphocytes (TILs) exhibit improved immune-mediated elimination of

tumor cells and the presence of TILs in various malignancies can be used as potent predictive biomarker for response to ICIs [112]. However, the selective pressure caused by checkpoint inhibitor treatment may select for non-immunogenic variants that survive treatment and replace the initial population [113] [114] . For example, in NSCLC acquired drug resistance was associated to the loss of neoantigens upon PD-1 or combined PD-1/CTLA-4 treatments [115]. Acquired resistance may be caused also by tumor antigen presentation defects. For example, loss of antigen-presenting machinery components such as beta-2-microglobulin and human leukocyte antigen (HLA) is an important mechanism to avoid antigen processing and presentation by tumors and develop resistance to ICIs [116]. Loss of HLA gene is found in 40% of NSCLCs resulting in a loss of MHC-I-peptide presentation which has been associated with resistance to immunotherapy [53].

Moreover, epigenetic changes and abnormal activated carcinogenic pathways in tumor cells have been also associated with both innate and acquired resistance to ICIs. These include activation of Wnt/b-catenin signal that prevents the activation of de novo anti-tumor immune response [117], deletion of PTEN suppressor gene that increases the resistance of tumor cells to the cytotoxic effect, and activation of MAPK signalling that promotes the expression of immunosuppressive cytokine (IL-6 and IL-10) [118] [119]. In addition, mutation in key intermediate components of interferon signalling pathways (such as JAK/STAT and IFNGR) also leads to resistance to ICIs [109] [120] [114]. A disruption in tumor cell responses to IFN-γ signalling can prevents the induction of PD-L1 expression and thereby render its blockade ineffective [119].

Worth noticing, also tumor-extrinsic mechanisms, which involve stromal or immune cells or other systemic influences (host microbiota) acting in concert with cancer cells, can promote tumor growth and resistance to ICIs [121] [50]. High levels of immune suppressive cytokines (such as TGF-β, IL-6, TNF), angiogenic factor (such as VEGF and ANG2) or metabolites and recruited immune suppressive cells (e.g., TANs, TAMs, MDSCs and regulatory T cells) can nullify the impact of tumor-specific T cells and influence ICIs efficacy [121] [113]. As an example, PD-L1 themselves expresses on stromal cells adjacent to tumor could contribute to resistance especially in less immunogenic tumors [122] [123].

Collectively, these findings highlight that the success of immunotherapy relies on integration of all arms of the immune system and there is the striking need to overcome resistance by targeting putative mechanisms of immune evasion within the TME all in the effort to provide long-lasting disease control to more patients [124]. Many clinical trials are currently ongoing with novel immune-therapeutic agents (targeting LAG3, TIM3, TIGIT and VISTA), usually in combination with anti-CTLA-4 or anti-PD-1/PD-L1 antibodies [125].

However, it is only through an enhanced understanding of the tumor-immune interaction and its modulators (e.g., tumor microenvironment, tumor heterogeneity and patient microbiome) as well as discovering the mechanisms of clinical resistance to ICIs that it will be possible to design effective therapeutic combinations [126].

1.3. Mature and active dendritic cells as key subset to achieve elimination of cancer cells

Evidence clearly shown that many tumors express antigens that can be recognized by effector CD8 T cells leading to elimination of cancer cells. However, interactions between naïve T cells and professional antigen presenting cells (APCs) in the lymph nodes are crucial for initiating cell-mediated adaptive immune responses [127]. APCs are cells that can process a protein antigen, break it into peptides and present them in conjunction with MHC molecules on the cell surface to interact with appropriate T cell receptors (TCRs) in the lymphoid organs. Professional APCs includes macrophages, B cells and dendritic cells. Among them, dendritic cells are the most efficient in activating the immune response, having an important role in induction and maintenance of tolerance, but also in anticancer immune responses.

In normal conditions, DCs are in immature or semi-mature states in the periphery where they take up and process self-antigens to maintain self-tolerance. On the other hand, after recognition of exogenous and endogenous danger signals by pattern recognition receptor and sensor for danger molecules, DCs undergo full maturation. This process is characterized by a down-regulation of antigen capture activity, increased expression of MHC and co-stimulatory molecules and cytokines, and up-regulation of CCR7 receptor to migrate to lymph nodes where mature DCs can present the processed-antigen to naïve T cells, inducing their activation [128] [129]. Many subsets of DCs with specific functions and morphology and localization have been described including Langerhans cells, monocyte derived DCs, conventional (cDCs) and plasmacytoid DCs (pDCs) [130]. The most studied and relevant for anti-cancer responses are the conventional tissue-resident DCs which consist of two functionally specialized subsets: the type 1 cDCs (cDC1s) that excel in the priming and cross-presentation of cell-associated antigens to $CD8^+$ T cells, and type 2 cDCs (cDC2s) that are more potent at driving $CD4^+$ helper T cell responses [131]. Moreover, thanks to their unique ability to cross-present extracellular antigen on MHC class I to $CD8^+$ T cells, cDC1s are essential for initiation of anti-tumor responses [43].

Mature and active tumor-infiltrating cDCs (TIDCs) play a pivotal role in the TME and are mostly associated with better prognosis and survival in different type of cancer, including breast, lung, renal, gastric and ovarian cancers [132 133 134]. Not only DCs mediate the activation in lymph nodes and consequent migration of effector cells at the tumor site [135 136 137 138], evidence shown that they drive the expansion of tumor specific CTLs by directly presenting tumor-associated antigen to T cells in the TME [139 140 141]. Some data revealed that increased myeloid cell commitment to cross-presenting-DC lineage and activation of intra-tumoral DCs enhance the clinical response to checkpoint inhibitors [142 143]. Moreover, even if pDCs represent a small population, evidence shown this subset mediates tumor killing through the release of TRAIL and granzymes or by inducing activation of NK cells and cytotoxic T cells [144 145 146].

However, within the TME, cancer cells promote tumor growth, evade immune surveillance and confer resistance to immunotherapies by different ways which include impairment of DCs functions and their recruitment [147]. As an example, the secretion of prostaglandin E2 by cancer cells impairs the activation of tumor-associated NK cells and consequent NK cell-dependent DCs recruitment [148]. Moreover, despite the presence of DCs in TME and their potential in generating an anti-tumor response, TIDCs are often defective [149 150].

In the last few years, many tumor-derived mediators (e.g., VEGF, TGF-β, IL-10, M-CSF, IL-6, IL-2) have been identified as suppressor of dendritic cell functions. For example, vascular endothelial growth factor (VEGF) in the TME can impair both dendritic cell functions and maturation from hematopoietic precursors [151 152 153 154 132 155]. Also metabolites and physiological stimuli such as lactic acid can influence DC phenotype and functions [156]. The upregulation of microRNAs (such as miR-16-1, miR-22, miR-155) or transcription factors (such as STAT3) have been associated with induction of apoptotic signaling pathways [157] and impairment of DCs maturation, thereby blocking their responsiveness to local danger signals [158]. Moreover, immune suppressive molecules such as PD-1, PD-L1 and TIM3 expressed in the TME result in an impaired antigen presentation capacity of dendritic cells. Studies regarding different type of cancer including melanoma and ovarian cancer showed that blockade of these molecules can improve the ability of TIDCs to activate T cells [159 160 161 162].

Thus, defective dendritic cells are often characterized by an aberrant maturation, with insufficient expression of MHC class I and II and co-stimulatory molecules [163]. Therefore, immature, or not-fully mature DCs conditioned by the TME, results in tumor tolerance and expansion of T regulatory cells leading to a suppression of immune system. Moreover, defective TIDCs create an

immunosuppressive environment by induction of anergy of effector T cells through different mechanisms which include the production of inhibitory molecules and immunosuppressive cytokines such as arginase I (ARG1) and indoleamine 2,3-dioxygenase (IDO) impairing T cell proliferation and responsiveness to antigens presented by TIDCs [164] [165] [166] [167].

Alteration of DCs is an essential element of tumor-mediated immune suppression that leads to tumor immune escape, therefore, target the pathways that paralyze TIDCs appears to be a promising approach to improve cancer treatments [168]. Abnormal accumulation of lipids is one of the major mechanisms for DC disfunctions that could be mediated by PMN-MDSC [169] [170]. However, the precise molecular mechanisms that interfere with behaviors of DCs have not been fully elucidated and a second gap-in knowledge is the limited understanding of immune suppressive factors that target DCs in the TME, for these, further studies are needed.

1.4. Non-malignant cells in the TME as possible target for immunotherapy

As described before, leukocyte infiltration into tumor tissue and recognition of malignant cells is necessary for a successful immunosurveillance of tumors. Cytotoxic CD8 T cells and CD4 Thelper1 cells represent the most efficient subsets in this process, however, in the TME, different mechanisms organized by various population including cancer cells, immune cells and their associated cytokines and metabolites, induce T cells suppression, aspect that leads to cancer immune escape [171]. Therapies including immune checkpoint inhibitor that subvert the dysfunctional status of T cells already exist, however, due to their low effectiveness, the discovery of new target to disrupt the immune-inhibitory mechanisms will offer the possibility to restore antitumor immunity and eradicate cancer cells.

Variability in leukocytes infiltrate and their contradictory role in promotion or suppression of anti-cancer immune response have been described across different tumor types and is dependent on reciprocal interactions between the tumor and the host. Indeed, the tumor microenvironment is a specialized niche composed of tumor cells and the stroma, often characterized by hypoxia and vascular abnormalities that correlate with metabolic reprogramming of cancer cells (known as Warburg effect) [172] [173]. In solid tumors, the stroma is composed by the extracellular matrix (ECM) and stromal -nonmalignant- cells that include fibroblasts, endothelial cells, pericytes and infiltrating leukocytes. In normal conditions, the stroma forms a structure that allows the crosstalk between those cells, regulates the presence and the distribution of nutrients and waste products and it is a scaffold for resident inflammatory cells (mast cells, macrophages, immature dendritic cells)

monitoring for invading pathogens and mutated cells. However, the functional and structural characteristics of stroma undergo dramatic changes during progression of neoplasia, often characterized by chronic inflammation [174 175 176] due to the release of growth factor, pro-angiogenic mediators and cytokines, which regulate the leukocytes recruitment and shape their cellular fate [177 178 179].

Inflammatory immune cells are essential players of cancer-associated inflammation, which is present at different stages of tumorigenesis and is a critical hallmark of cancer. During the early stage of tumor development, the infiltration of leukocytes and the subsequent inflammation due to release of pro-inflammatory mediators can, in certain cases, eliminate tumor cells and prevent tumor development (immunosurveillance). For example, it has been recently demonstrated that some tumor lesions including ovarian cancer, breast cancer and non-small cell lung cancer, can display ectopic lymphoid-like structures called tertiary lymphoid structures (TLSs). The genesis of these structures is related to the local production of CXCL13 and IL-17 by lymphocytes or stromal cells and are associated with a favourable outcome. In fact, those structures contain T cells-rich zone with mature DCs juxtaposing a B cell, surrounded by plasma cells, thus representing a site for local presentation of neighbouring tumor antigens to T cells by DCs leading to an activation and proliferation of T and B cells and consequent long-lasting anti-tumor response [180 181 182 183].

However, it is currently accepted that the chronic inflammation and the consequent aberrant innate and adaptive immune response characterizing the established malignancies, contribute to immune escape and tumorigenesis by selecting aggressive clones, inducing immunosuppression and induction of cancer cell proliferation, angiogenesis and metastasis [184 185 186].

Beside DCs, T cells and B cells, other leukocytes have been found in the TME of most human cancer, including macrophages, MDSCs, and neutrophils. The latter, represent a frequent subset in different type of cancer, especially NSCLCs [187] and their role at the tumor site remain controversial and tissue context dependent. Thus, the composition of immune landscape is dependent on tumor type and constellation of immune-modulating factor in the tissue. How different immune cells are activated and recruited to the tumor site and how they interact with each other and with cancer cells are still not fully understood and crucial to fully exploit the development of new therapies [188 189].

Therefore, while most cancer research has focused on acquired mutations in oncogenes or tumor suppressor genes, and the most therapeutics are directed against tumor cells, it is now apparent that the non-malignant cells in the microenvironment evolve along with the tumor and provide

essential support for their malignant phenotype and can represent a target for new cancer therapies [190]. An overview on the most relevant immune suppressive subsets in cancer is described below.

1.5. Immune suppressive behaviour of CD4 T regulatory lymphocytes

Normal cells that undergo transformation becoming malignant can exhibit antigens on MHCI or MHCII molecules as targets for activated effector CD4 or CD8 T cells, respectively [191]. Although direct tumor cytotoxicity has been described, activated CD4 Thelper1 cells usually mediate anti-cancer response by release of cytokines (such as IL-2, IFN-γ and TNF-α) which promote activation of both innate and adaptive immune responses. However, other subsets of CD4 T cell exist in the TME, including Thelper2 cells, IL17 producing CD4 T cells and T regulatory cells (Tregs), often associated to an immune suppressive behaviour [192].

It has been demonstrated that $CD4^+$ T helper2 lymphocytes can promote tumor growth through the production of pro-tumorigenic cytokines such as IL-5 [193] [194], whereas, IL-17-producing $CD4^+$ T cells promote angiogenesis and tumor growth via release of the pro-inflammatory cytokine IL-17 [195] [196]. Overall, T regulatory cells are the most powerful inhibitors of anti-tumor immunity and the major barrier to successful immunotherapy. High infiltration of Tregs in tumors, and in particular high Tregs-CD8 T cells ratio, is associated with the shutdown of the immune system and with a poor prognosis in different type of cancer including melanoma, non-small-cell lung cancer, gastric, colorectal, breast and ovarian cancer [197] [198] [199] [200] [201]. Those cells accumulate in the TME in response to several mechanisms. Tregs can be recruited in the TME by chemokines (such as CCL1, CCL5, CCL28 and CCL17/22) produced by dysfunctional immune cells and tumor cells in the TME [202] [203] [204], or can expand in situ in response to cytokines such as IL10 where exhibit their strong immunosuppressive capacity by numerous cellular and humoral mechanisms. Through the secretion of inhibitory molecules (such as IL-10, TGF-β, IL-35, prostaglandin E2 and galectin-1) Tregs induce the apoptosis of target cells. Moreover, Tregs lead to immune suppression by the consumption of IL-2 and degradation of ATP which are necessary for proliferation and functionality of T cells [205] or by the expression of immune checkpoint molecule including CTLA-4, PD-1/PD-L1, TIM3, LAG-3 and TIGIT which inhibit priming and activation of T cells [42] [198]. Moreover, high level of circulating Tregs has been associated with higher risk of metastasis in patients with NSCLC, breast cancer and colorectal cancer [206] [207] [208]. In conclusion, considering the role of Tregs as key regulator of anti-tumor immune suppression, direct (targeting of molecules expressed by Tregs such as CD25, PD-1 and CTLA-4 or pathways that are crucial for Tregs survival and function) or indirect (targeting factors in the TME

such as VEGF or other immune-suppressive cells, MDSCs and TAMs, that may activate and/or recruit Tregs) suppression of Tregs represent a promising anticancer therapeutic strategy, although approaches to control these cells require further research [209 210] .

1.6. Tumor-associated macrophages (TAMs)

Another subset highly represented in TME is composed by macrophages which play a critical role in innate immunity and are responsible for defending the host against foreign pathogens. Those differentiate from circulating monocytes after extravasation into tissues. Here, macrophages are equipped to sense and respond to infections and tissue injuries. Even if there is evidence that TAMs contribute to the early elimination phases of nascent tumors orchestrated by T cells and interferons [211], high-grade tumor associated macrophages (TAMs) correlate with poor prognosis and reduced overall survival in different type of cancer, including NSCLC [212 213]. In established tumors, cytokines (such as IL-4 and IL-13) released from different immune cells (T cells, B cells, eosinophils and basophils) or signals originating from tumor cells (TGF-β, CSFs) and stromal cells (IL-1) elicit alternative activation of TAMs leading to tumor progression [52].

TAMs are recruited to the tumors by a range of chemokines including CCL2, VEGF, CCL5 and CSF1. Here, TAMs play a dominant role as orchestrator of cancer-related inflammation through their production of molecules such as IL-6, TNF-α among others that support tumor cell survival and proliferation [214 215]. Moreover, TAMs are able to promote the formation of metastasis by the release of growth factor (such as VEGF and EGF), extracellular matrix degrading enzymes (such as macrophages-derived metalloproteinases and cathepsins) that promote angiogenesis, remodelling of the ECM and epithelial-mesenchymal transition [150 216]. In addition, molecules secreted by TAMs such as IL-10, ROS, IDO, ARG1 and TGF-β, impair the activity of effector T cells and DCs maturation and promote the recruitment of Treg cells, leading to suppression of anti-tumor response [150 217 218]. Moreover, macrophages express ligands of PD-1 and CTLA-4 able to inhibit T cell cytotoxic functions and the activation of NK cells [219 220 221 28].

In conclusion, TAMs significantly contribute to tumor development by induction of angiogenesis, chronic inflammation and immune suppression and represent a target for cancer immunotherapy, however there are still massive issues and limits that need to be addressed that slow down the development of effective therapies [222].

1.7. Myeloid-derived suppressor cells (MDSCs)

Twenty years ago, a new population in cancer patients, named myeloid-derived suppressor cells (MDSCs) was described. These cells are an heterogeneous cellular population that comprises several maturation states of myeloid cells that expand during cancer and share the ability to suppress T cell responses, induce tumor angiogenesis, metastasis and drug resistance [223]. However, new scRNA seq data suggest revisiting the concept of tumor infiltrating myeloid cells and overcome the term MDSCs.

At steady-state, progeny of common precursors acquires specific markers and functions of circulating leucocytes and progressively lose their ability to self-renew (myelopoiesis). In pathological condition, emergency myelopoiesis is induced to provide cells to eliminate potential threats (including cancer cells). If these conditions resolve quickly, the balance of myeloid cells can be restored, however, in cancer, the chronic inflammation leads to an aberrant and sustained myelopoiesis. This results in the accumulation of immature myeloid cells able to induce immune dysfunction due to their pathological activation which are morphologically and phenotypically similar to neutrophils and monocytes (MDSCs) [224 225]. Indeed, in most types of cancers, MDSC consist of two main large groups of cells. The most frequent is termed polymorphonuclear (PMN-MDSC) which phenotypically and morphologically resemble neutrophils, the second, less frequent, be like to monocytes (M-MDSC) [226 227].

Overall, the ability to suppress anti-cancer responses is a distinctive characteristic of MDSCs. Evidence show that MDSCs expansion and activation are influenced by several factors that can be produce by cancer cells or by activated T cells and stromal cells (including VEGF, CSFs, TGF-β, IL-4, IL-13, IFN-γ among others) [228] and their main feature is the ability to suppress the immune system by different mechanisms including upregulation of arginase 1 (ARG1) and nitric oxide synthase (NOS) activities and release of reactive oxygen species (ROS) which suppress T cell activities. Recently, it has been shown that another mechanism of immune suppression MDSCs-mediated is the induction of Tregs through the production of cytokines or direct cell-cell contact [229 230 227]. Moreover, recent data suggest MPO-driven lipid peroxidation in PMN-MDSC could be a possible mechanism of inhibition of antigen cross-presentation by DCs [170]. Besides direct immune suppressive mechanisms, MDSCs can promote tumor progression by affecting the remodelling of the tumor microenvironment, and inducing tumor angiogenesis and metastases [231 232]. Thus, MDSCs are a critical element of the immune suppressive niche in cancer also related to resistance to immune check point inhibitors [233 234].

However, one of major unsolved issues is how to phenotypically distinguish MDSCs from neutrophils and monocytes in mice and human. Recently, a possible marker of identification of PMN-MDSC in humans has been identified and consist of lectin-type oxidized LDL receptor 1 (LOX-1), present on PMN-MDSCs but not on neutrophils. If confirmed in further studies, LOX-1 can be used for direct identification of PMN-MDSC in blood and in patients. On the contrary, the phenotypic distinction in mice is still challenging [235] and whether TAN activity can be attributed to MDSC remains a matter of debate [236]. Therefore, targeting of MDSCs could be promising for new cancer treatments and for this purpose a better characterization of this population is needed.

1.8. Heterogeneity of neutrophils in homeostatic conditions

Neutrophils are the most abundant circulating leukocyte, in human representing 50-70% of all leukocytes, and are the first responders to sites of infection and tissue damage. Those cells display an enormous functional plasticity and their primary function is to mediate host defense [237].

Neutrophils are produced in large numbers in the bone marrow and extramedullary tissues (including spleen) from the hematopoietic stem cells that differentiate into granulocyte-monocyte progenitors (GMPs). According to current paradigm that classify different stages of granulopoiesis on the basis of morphological features (cell size, nuclear condensation and granule content), a granulocyte-committed progenitor with proliferative properties deriving from GMPs, develops into post-mitotic immature neutrophils and, under control of the granulocyte colony-stimulating factor (G-CSF) and granulocytes-macrophage colony-stimulating factor (GM-CSF), into a mature neutrophil containing granules and secretory vesicles relevant for their functions [238] [239]. Recently, single-cells RNA sequencing (scRNA-seq) and mass cytometry by time-of-flight analysis (CyTOF) allowed to better understand the complete neutrophil-lineage hierarchy from GMPs to mature neutrophil. In particular, it has been identified in mouse and human, the so called early unipotent neutrophil progenitors (NePs) and pre-neutrophils (preNeus) as proliferative neutrophil-committed bone marrow-residing cells that are downstream of GMPs and may serve as a proliferative pool that can rapidly amplify neutrophils numbers on demand [240] [241] [242].

Maturation processes include changes in shape of nucleus and expression of various receptors. As an example, the CXCR4 is downregulated, while CXCR2 and TLR4 are upregulated. Indeed, under homeostatic conditions, the release of matured neutrophils is tightly controlled through CXCR4 and CXCR2 chemokine receptors signaling. The CXCL12 produced by osteoblasts and other bone marrow stromal cells binds CXCR4 expressed on neutrophils preventing their release into the circulation,

while ligands for CXCR2 such as CXCL1, CXCL2, CXCL5 and CXCL8 produced by endothelial cells outside the bone marrow drive their mobilization into the blood [243] [244]. In peripheral blood, neutrophils are short-lived cells which require a constant replenishment from the bone marrow precursors [245]. It has been showed that the lifespan of circulating neutrophils under steady-state conditions is up to 12.5 hours for mouse and 5.4 days for human and may be prolonged in an inflamed context [246]. Increased numbers of neutrophils are frequent in the peripheral blood of patients with various type of cancer, correlating with less favorable prognosis[247]. Blood-based inflammatory parameters, such neutrophils to lymphocyte ratio (NLR) has been reported to predict the prognosis in solid tumors. Interestingly, high NLR has been associated with a poor prognosis in lung cancer patients [248]. In absence of inflammation signals, neutrophils in the circulation rapidly undergo aging. This term is used to describe changes in phenotype and molecules expression from mature neutrophils that enter the circulation, to aged neutrophils. Thus, circulating neutrophils progressively lose their granule contents, NET-forming capacity and ability to migrate into inflamed tissues, limiting their tissue-damaging potential [249] [250]. Moreover, several studies showed that aged neutrophils downregulate the expression of CXCR2 and CD62L and upregulate CXCR4 driving their homing back to the bone marrow and consequent elimination through phagocytosis by macrophages. The aging process is regulated by gut microbiota and is controlled by neutrophils themselves through a cell-autonomous transcriptional program following circadian oscillation over time [250] [251]. Recently, scRNA-seq analysis, allowed to identify a subset of circulating neutrophil, observed also in tumors, characterized by the expression of interferon-stimulated genes that may represent a primed population to fight infections [252] [253].

Yet, neutrophils in the circulation could be mobilized to sites of inflammation or infection by a complex milieu of chemokine through a process named leukocytes adhesion cascade: glycoprotein ligands on neutrophils bind to adhesion receptors on activated endothelial cells nearby the affected site, leading to transmigration of neutrophils into peripheral tissues [254].

Once in the tissues, neutrophils are able to capture and destroy invading microorganisms through different mechanisms: 1) phagocytosis and intracellular degradation, 2) release of noxious substances including granule-derived compounds like antimicrobial peptides, reactive oxygen- (ROS) and nitric oxide- species (NOS) and 3) release of nuclear material in the form of extracellular fibrillary networks termed neutrophils extracellular traps (NETs) [247]. Finally, neutrophils undergo apoptosis and are cleared through phagocytosis by resident macrophages and dendritic cells [255].

As mentioned above, neutrophils maturation and release into the circulation is regulated predominantly by G-CSF that promotes the differentiation of hematopoietic precursors, induces the upregulation of CXCR2 and reduces the expression of CXCR4. The expression of G-CSF is in turn regulated through the axis IL-23/IL-17. Phagocytosis of apoptotic neutrophil triggers an anti-inflammatory response characterized by a reduction in IL-23 by macrophages and DCs leading to a downregulation of IL-17 levels produced by T lymphocytes and G-CSF production, finally reducing granulopoiesis [256 238 257]. Other cytokines have also been implicated in promotion of granulopoiesis including GM-CSF, M-CSF, and IL-6. The latter, has a special importance in emergency granulopoiesis in response to systemic infection [258 259].

An open question is whether tissue-specific neutrophil exist. With the exception of the brain and gonads, most tissues are actively infiltrated by neutrophils at steady state, at least in mice, supporting their homeostatic role within healthy tissues [260 261]. As an example, neutrophils found at the marginal zone of the spleen are able to induce somatic hypermutation and antibody production by B lymphocytes through the release of cytokines such as IL-21 [262]. In the lung, neutrophils have been found in the vascular lumen and in the interstitial space, kept in the tissue by a CXCR4-dependent mechanism that may facilitate rapid responses to microbial challenges [263]. In the lymph nodes, neutrophils expressing MHCII molecules have been found in proximity to T cell, suggesting a role of those in antigen presentation [264]. Moreover, neutrophils that express VEGFR1 are recruited to non-vascularized tissues under hypoxia conditions where they are able to promote angiogenesis and vascular repair [265 266]. Interestingly, this pro-angiogenic subset is similar to one observed in tumor supporting vascularization [267]. In addition, various subsets of neutrophils with distinct properties have been also detected in pathological conditions (such as cancer as described below) [268].

Although heterogeneity of neutrophils is now recognized, how different subsets of neutrophils are generated, recruited to tissues and the biological relevance of this remain under debate. So far there is no evidence for ontogenetically distinct neutrophils and their diversity may be the result of their high plasticity in response to environmental signals [269 270].

1.9. Tumor associated neutrophils (TANs) recruitment and polarization

It has long been known that neutrophils are present in different type of cancer including renal cell carcinoma (RCC), colorectal cancer, melanoma and lung cancer and their abundance in the TME is in general related to poor outcome [3 271 272 273]. High TANs infiltration is associated with a worse

response to the conventional chemotherapy and radiotherapy, but also with a poor response to more recent immune checkpoint inhibitors [274 275 276].

Neutrophils with different states of maturity and activation have been found in tumor conditions and understanding how neutrophils are mobilized from the bone marrow and influenced by tumor-derived signals is mandatory to explore their role in tumor progression [277].

The presence of immature neutrophils in the circulation and in tissues is often the result of emergency hematopoiesis induced by tumors. As an example, emergency hematopoiesis has been linked to the overexpression of growth factors such as G-CSF observed in different type of cancer models such as breast cancer [278 279 280] and in human gastric and colon cancer [281]. Interestingly, immature neutrophils having immunosuppressive properties have been detected also in lung cancer [282 283]. Enhanced levels of G-CSF are induced by pro-inflammatory cytokines released by cancer cells themselves or tumor-associated stromal cells. In breast cancer model, IL-1β produced by macrophages in the TME elicited IL-17 expression from γδ T cells resulting in G-CSF production and neutrophils recruitment which facilitated immune suppression and pulmonary metastasis formation [279]. In addition, high levels of IL-17 have been found in breast cancer patients that was correlated with shorter disease-free survival and poor prognosis [284] and in human hepato-cellular carcinoma (HCC) where it promoted neutrophils recruitment [285]. Pro-inflammatory cytokines induce also neutrophils polarization. As an example, TGF-β is one of the known factors able to polarize different subsets of the immune system, including neutrophils [286]. It has been demonstrated that TGF-β within the TME induced the recruitment of TANs and promoted their polarization to pro-tumoral phenotype in mesothelioma [287]. Moreover, TGF-β-blockade promoted the migration and activation of neutrophils with anti-tumoral properties, which decreased tumor growth [287]. In addition, also TNFα has been associated with neutrophil recruitment and tumor progression in mouse colorectal cancer [288]. Yet, IL-6 produced by mesenchymal stem cells is crucial for the recruitment and activation of neutrophils in gastric cancer, enhancing their pro-tumoral polarization by activation of STAT3-ERK1/2 pathway [289]. On the other hand, evidence showed that other cytokines in tumors, such as type I interferon, are related to an anti-tumor polarization of TANs [290 267 291].

Moreover, neutrophils polarization can be attributed to tumor physiology. At early stage of tumorigenesis in lung and mesothelioma murine cancers, neutrophils showed higher cytotoxic activity toward tumor cells, whereas, in established tumors, TANs acquired a pro-tumorigenic phenotype [292]. Yet, factors released by tumor cells such as MIF (macrophage migration inhibitory

factor) [293] or hypoxia, modulated neutrophils lifespan resulting in increased neutrophil survival and polarization to pro-tumorigenic behavior [294]. Details regarding the lifespan of neutrophils in tumors remain to be fully elucidated. Interestingly, in a recent work it has been showed that the glucose metabolism upregulated in TANs in a model of lung cancer slowed down their turnover promoting the acquisition of a pro-tumorigenic behavior [295].

Other factors have been related with neutrophils recruitment in cancer. As an example, Wnt ligands produced by cancer cells in the TME stimulated the IL-1β production by tumor associated macrophages which resulted in immature neutrophils recruitment in breast cancer and subsequent metastasis formation [296]. Of note, commensal microbiota has been linked to the production of IL-1β and IL-23 by myeloid cells which in turn induced IL-17-producing T cells activation resulting in neutrophil infiltration and lung cancer development [297]. In addition, in melanoma, UV exposure triggered the release of high mobility group box 1 (HMGB1) promoting the recruitment and activation of neutrophils through toll-like receptor 4 (TLR4) signaling [298]. Yet, chronic exposure to crystalline silica, linked to inflammation and lung cancer initiation, has been related to neutrophils recruitment and enhanced tumor growth through mast cells- and macrophages- leukotriene (LTB4) production [299].

Also, chemokines (i.e., CXCL1, CXCL2, CXCL5, CXCL6 and CXCL8) often upregulated in the TME of different type of cancer, play a major role in neutrophil recruitment through the binding with chemokine receptors CXCR1 and CXCR2 highly expressed on those cells [300 301 302 303]. As an example, hypoxia has been showed to induce upregulation of Snail in lung cancer cells that accelerated disease progression and CXCL2 expression by neutrophil themselves leading to a cycle encompassing neutrophils and Snail to maintain a deleterious tumor microenvironment [304].

In this current work, we focused on the role of murine chemokine CXCL5 (which share the 80% of identity with CXCL6 in human [305]) in recruitment of a particular pro-tumorigenic subset of neutrophils in lung cancer (as described below). CXCL5 is involved in neutrophils recruitment in pulmonary infection and inflammation [306 307 308] and in lung cancer [3 304]. In colitis and colitis-associated colon cancer (CAC), over-expression of CXCL5 accelerated the recruitment of neutrophils through the CXCL5-CXCR2 axis, leading to cancer formation [309]. Moreover, the human homologous CXCL6 (also known as granulocytes chemotactic protein2, GCP-2) has been showed to induce neutrophils influx in pulmonary fibrosis in human [310]. In addition, up regulation of CXCL6 in small cell lung cancer correlated with tumor progression and poor prognosis [311 312 313] and pharmacological inhibition of CXCR2 decreased primary lung tumor growth and pancreatic cancer

metastasis in mice [314 315]. Interestingly, *in vivo* studies showed that KRAS signaling, often mutated in lung cancer, is able to regulate the expression of neutrophils-related cytokines such as CXCL8 and CXCL5 [316 3]. In addition, in human cancer, mutation of KRAS correlates with neutrophils recruitment raising the question of whether KRAS alone is sufficient to orchestrate neutrophils recruitment and polarization [317].

Thus, it has been well demonstrated that the tumor promotes the recruitment and polarization of neutrophils, however, the dynamics of neutrophils recruitment at the tumor site is not fully understood. Intravital multiphoton imaging showed that neutrophils infiltrate the tumor within 3 hours in a transplanted mouse model and persist for up to 3 days in the TME. Interestingly, the motility of peritumoral neutrophils increased when compared to intra-tumoral neutrophils, suggesting the presence of different states of polarization within the tumor itself [318]. Moreover, in early, but not late, NSCLC, IFNγ and GM-CSF have been showed to drive the differentiation of APC-like neutrophils with an anti-tumorigenic phenotype [319]. For this, in future will be important to demonstrate whether neutrophils are polarized before reaching the tumor site or TANs can mediate anti-tumor resistance at the initial phase of tumorigenesis, whereas, probably due to their high plasticity, during progression of cancer, acquire pro-tumorigenic phenotype contributing to tumor progression.

Thus, many factors have been associated with recruitment of neutrophils at the tumor site and with their polarization into phenotypically distinct pro- or anti-tumorigenic sub-populations. However, mechanisms of neutrophils polarization are still poorly defined and their role in tumor progression remains controversial and strongly dependent on the tumor type, the cellular microenvironment, and the constellation of immune modulating factors.

1.10. TANs in tumor initiation and progression

It is now well established that inflammation play an essential role in initiating tumorigenesis and neutrophils recruited in the TME represent a crucial component of this process. It has been shown that blocking neutrophils trafficking using CXCR2-deficient mice or antibody-mediated depletion of neutrophils prevented tumor formation [320 321 322]. In addition, neutrophil-released reactive oxygen species (ROS) can cause tissue damage which potentially favors tumorigenesis [323]. As an example, ROS produced by neutrophils recruited in hepatic parenchyma under inflammation have been associated with DNA damage which was sufficient to induce hepatocellular carcinoma [324]. Beside ROS, other genotoxic DNA substances produced by neutrophils including RNA, NOS, MMP-9, MPO

and TGF-β can induce DNA instability and neoplasia [325] [326] . Also, neutrophil-derived microparticles containing pro-inflammatory microRNAs, have been shown to promote DNA breaks and to induce lamin B1-dependent replication fork collapse and inhibition of homologous recombination leading to impaired colonic healing and neoplasia [327]. In addition, different cytokines including IL-8, IL1-β and IL-6 and growth factors such as EGF, HGF, PDGF, PGE2 released by neutrophils have been related to tumor initiation and progression [328] [329] [330] [331]. However, the role of neutrophils in cancer is still controversial and strongly dependent on the tumor type (Fig. 1.4).

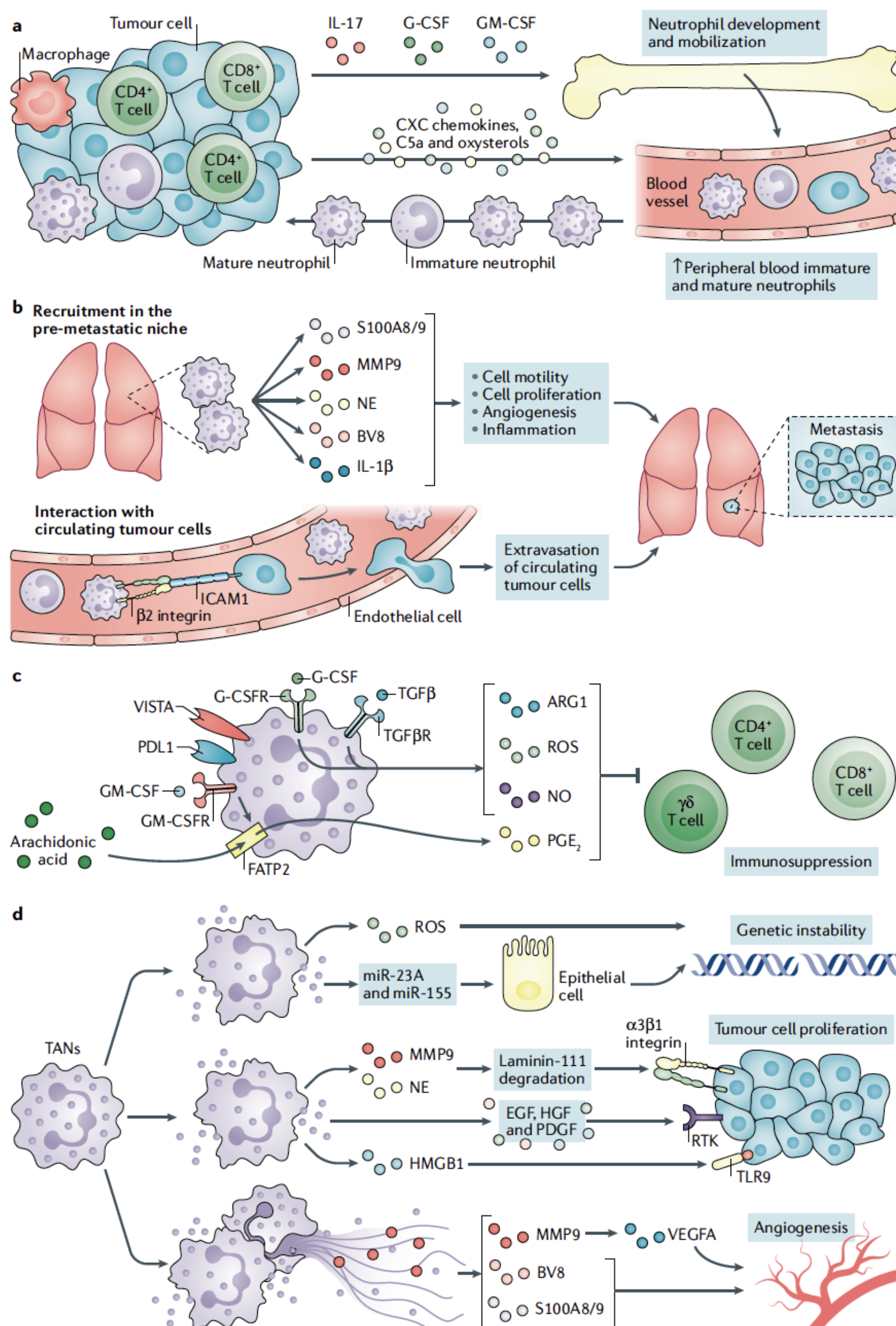

Figure 1.4. Pro-tumorigenic behavior of neutrophils. a) Neutrophils are recruited in the tumor through different mechanisms which include tumor-derived growth factor, cytokines, and chemokines. The latter has been associated with mobilization of neutrophils in

the pre-metastatic niche. B) neutrophils are able to promote tumor growth by different mechanisms. As an example, neutrophil-derived factors (NE, BV8, MMP9) promote cell proliferation and angiogenesis leading to metastasis formation. Moreover, circulating neutrophils promote the extravasation of cancer cells and consequent metastasis formation. c) Importantly, through the release of ARG1, ROS, NO and the expression of immune checkpoint ligands, neutrophils impair T cells activation, leading to immune suppression. d) Also, neutrophils induce tumor initiation and progression by inducing genetic instability and by promoting tumor cell proliferation and angiogenesis [302]*.*

One of the mechanisms used by neutrophils to promote tumor growth is through the induction of angiogenesis, a hallmark of malignant tumors [332 333]. In particular, it has been demonstrated that, in different mouse models (such as melanoma, pancreatic and squamous cell cancer), tumor-infiltrating neutrophils expressing proangiogenic factors such as VEGF and MMP9, were able to promote the formation of a new blood vessels required for tumors to acquire oxygen and nutrients essential for their growth [267 334 335]. Moreover, the neutrophil-derived Bv8 protein has been recently characterized as a regulator of angiogenesis implicated in the angiogenic switch during neoplasia [336]. Nevertheless, the mechanisms by which TANs induce tumor-angiogenesis are not fully elucidated. Evidence of angiogenesis mediated by neutrophils has been observed also in human. *In vitro* studies shown that, upon stimulation, human polymorphonuclear neutrophils (PMN) released pro-angiogenic factors (such as VEGF and IL8) which induced sprouting of capillary-like structure [337]. Interestingly, in human gastric cancer, neutrophils are highly represented and associated with MMP9 production which stimulated proangiogenic activity in gastric cancer cells [338]. In addition, myeloperoxidase (MPO) contained in NETs promoted a pro-angiogenic response in human pulmonary endothelial cells by activation of TLR4 signaling leading to proliferation and motility [339]. Importantly, NETs have been not only associated with tumor-angiogenesis, but also with extracellular matrix remodeling, proliferation of tumor cells, and consequent neutrophil-mediated invasiveness and metastasis [340]. As an example, murine metastatic breast cancer cells trigger NETs formation by neutrophils promoting metastasis and lung colonization. Using intravital imaging, NET-like structures have been observed around metastatic cancer cells in the lung of mice, and blockade of NET formation prevented lung metastasis [341]. Moreover, NETs were able to protect tumor cells from cytotoxicity mediated by CD8 T cells and natural killer cells by wrapping and coating tumor cells, favoring tumor growth [342]. In addition, the secretion of HMGB1 during NETosis in response to surgical stress promoted metastasis in resected liver in mouse and human. *In vitro* studies suggested that the activation of TLR9 pathway in cancer cells in response to HMGB1, promote their adhesion, proliferation, migration and invasion properties [343]. The neutrophil-dependent metastasis formation is also associated with other factors contained in the NETs. It has been observed that, in different mouse model (including breast and lung cancer models), NET-derived factors such as

MMP9, MMP8, Cathepsin G and NE, which modulate the extracellular matrix (ECM), were able to promote tumor cell proliferation and migration [344 341 345]. In particular, a study demonstrated that neutrophil-derived NE and Cathepsin G degraded the antitumorigenic factor thrombospondin-1 (Tsp-1) promoting lung metastasis [346].

Interestingly, several studies showed the ability of neutrophils to engage with circulating tumor cells promoting extravasation and subsequent metastasis formation [347 348 349 350]. Lastly, neutrophils participate to the constitution of the metastatic niche and to the acquisition of metastatic phenotype in certain tumor cell type [351]. Also, the atypical chemokine receptors (ACKRs) expressed on hematopoietic precursor and downregulated during myeloid differentiation may be responsible for neutrophil-mediated metastasis [352].

Beside induction of metastasis, several factors released by neutrophils in the TME have been associated with enhanced proliferation of tumor cells and consequent tumor growth [302]. As an example, neutrophil-derived leukotrienes promoted the expansion of a pool of breast cancer cells with high tumorigenic properties favoring lung colonization [353]. Moreover, elastase released by neutrophils (NE) was able to induce proliferation of cancer cells in a lung adenocarcinoma model and in human lung adenocarcinoma cell line. The mechanism of this effect was elucidated in great details and it involves access of NE to tumor cells endosomal compartment and degradation of insulin receptor substrate 1 (IRS1), promoting interaction between phosphatidylinositol 3-kinase (PI3K) and the mitogen platelet-derived growth factor receptor (PDGFR) [354 355]. The mitogenic activity of NE was also observed *in vitro* with human mammary epithelial cells through the activation of ERK signaling [356] and with esophageal cell line by inducing the release of growth factor such as PDGF, TGFα and VEGF [357]. Also, *in vitro* studies with human renal cell carcinoma cells showed that neutrophils promoted cancer cells proliferation by inducing up-regulation of androgen receptor signals [358].

Another known mechanism that neutrophils used to trigger tumor growth is the induction of epithelial to mesenchymal transition that enhance tumor cell migration and invasion ability. A recent study revealed that neutrophils in parenchyma of breast cancer patients were able to induce epithelial-mesenchymal transition (EMT) of breast cancer cells leading to metastasis formation and poor prognosis. Of note, neutrophils in the stroma of breast cancer were not associated with poor prognosis, underlying the importance of location-dictated interaction between tumor cells and neutrophils [359]. Moreover, in pancreatic ductal adenocarcinoma and lung adenocarcinoma patients,

neutrophils have been correlated with EMT most likely due to an elastase-mediated degradation of E-cadherin leading to a loss of cell-to-cell contact [360 361].

1.11. TANs as suppressor of anti-cancer immune responses

A further crucial way to promote tumor growth by TAN is via suppression of ongoing anti-cancer responses. Several factors released by neutrophils, including ROS, RNA and ARG have been associated with suppression of both innate and adaptive immune response [303 287]. In particular, TANs are mainly described as damper of T cell mediated anti-tumor responses. In a mouse model of breast cancer, in a glucose-limited environment, immature neutrophils engaged in oxidative mitochondrial metabolism leading to ROS production and nutrient consumption leading to suppression of T cell responses [362]. In addition, mammary tumor-induced IL-17-producing $\gamma\delta$ T cells were shown to drive systemic expansion and polarization of neutrophils to a pro-tumorigenic subpopulation characterized by iNOS overexpression and NO production which suppressed T cells responses and therefore promoted metastasis in distant organs [279]. Of note, NO produced by TAN has been shown to promote CD8 T cell apoptosis and consequent tumor-supportive environment [363].

Interestingly, pro-tumorigenic polarized neutrophils are able to produce arginase that in turn reduce CD8 T cell activation by limiting L-arginine availability in the TME [287]. Importantly, production of ARG1 by neutrophils impaired T cell responses also in human cancer, including renal cell carcinoma and NSCLC [364 365 366]. Moreover, recent evidence showed that neutrophil-derived MMP-9 induced activation of latent TGF-β and consequent suppression of tumor infiltrating T cells in colon cancer [367].

It has also been suggested that an altered lipid metabolism in neutrophils may induce immune suppression. Recently, a study revealed that neutrophils in different type of cancer in mouse and human, upregulated the fatty acid transport protein 2 (FATP2) under GM-CSF stimulation with consequent expression of prostaglandin E2 (PGE2), which is involved in suppression of anti-tumor CD8 T cell-mediated responses [368]. In addition, in patients with NSCLC and head and neck cancer, stress of the endoplasmic reticulum (ER) boosted the expression of the lectin-like oxidized LDL receptor 1 (LOX1) that triggered an altered lipid metabolism in neutrophils. Expression of LOX1 converted neutrophils into suppressive cells with higher expression of ROS and ARG1 having immunosuppressive activity [369].

Importantly, neutrophils can mediate the activation of immune checkpoints on T cells. It has been shown that, within hepatocellular carcinoma (HCC) and gastric cancer, cancer-mediated activation of STAT3 pathways in neutrophils prolonged their survival and increased expression of PD-L1 on their surface impairing T cell function through the PD-1/PD-L1 signaling pathway [370 371 372]. Beside PD-L1, other immune checkpoint regulators, including VISTA, are expressed on neutrophils. However, the role of VISTA in neutrophils and its impact on tumor immunity require further investigation [373]. Of note, in a model of melanoma, Fas-ligand expressed on TANs was able to induce cell apoptosis of tumor infiltrating lymphocytes that mediated resistance to cancer immunotherapy [374].

Although the crosstalk between neutrophils and T cells is the most studied, it has been reported as well that neutrophils can orchestrate the functions of other components of the immune system, in an immunosuppressive direction. Indeed, neutrophils are able to prevent the NK cell-mediated clearance of tumor cells [375]. In addition, it has been recently proven that neutrophils can promote metastasis by coating tumor cells with NETs, therefore preventing cytotoxic activity of CD8 T and NK cells [342]. Moreover, through the release of chemokines such as CCL2 and CCL17, neutrophils are able to recruit monocytes and T regulatory cells with pro-tumoral behavior at the tumor site [247]. In addition, evidence showed that MPO-driven lipids peroxidation and their accumulation in neutrophils impaired cross-presentation by DCs and pharmacological inhibition of MPO in combination with checkpoint blockade reduced tumor progression in different tumor models [376]. Finally, in ovarian cancer patients, tumor associated ascites prolonged neutrophils lifespan and induced ROS secretion that may inhibit T cell proliferation [377].

In conclusion, many evidence described neutrophils as an immunosuppressive and pro-tumorigenic subset. Some of the conclusions from these studies, however, require caution as the function of neutrophils was often determined *in vitro* using circulating or splenic neutrophils that do not correspond to tissue neutrophils, or *in vivo*, upon antibody-mediated depletion with the limitation inherent with this approach (Tab. 1). Therefore, consequent findings are challenging to interprets as they can be influenced by neutrophil survival, cytotoxicity and protocols used.

Title	Authors	Journal	Mechanism of suppression	Neu used to study mechanism	Tumor type
Polarization of Tumor-Associated Neutrophil (TAN) Phenotype by TGF-β: "N1" versus "N2" TAN	Z.G. Fridlender et al, 2010	Cancer Cell	Arginase production	TAN	Subcutaneus tumor induced with AB12, LKR or TC1 tumor cell lines or orthotopic lung cancer model
Renal Cell Carcinoma Are a Subpopulation of Activated Granulocytes	P.C. Rodriguez et al, 2010	Cancer Research	Arginase1 production suppress T cell proliferation and IFNγ production	Circulating human CD66b+ cells	Renal cell carcinoma
Population alterations of L-arginase- and inducible nitric oxide synthase-expressed CD11b+/CD14−/CD15+/CD33+ myeloid-derived suppressor cells and CD8+ T lymphocytes in patients with advanced-stage non-small cell lung cancer	C.Y. Liu et al, 2010	Journal of cancer research and clinical oncology	ARG1 and NO production	Circulatin human CD11b+/CD14-/CD15+/CD33+ cells	Non small cells lung cancer
IL17-producing γδ T cells and neutrophils conspire to promote breast cancer metastasis	S.B. Coffelt et al, 2015	Nature	NOS production	circulating	Breast cancer model
Lectin-type oxidized LDL receptor-1 distinguishes population of human polymorphonuclear myeloid-derived suppressor cells in cancer patients	T. Condamine et al, 2016	Science immunology	LOX1 overexpression under ER stress conditions led to ROS and ARG1 expression	Circulating human CD15+ cells	human NSCLC, head and neck, colon and multiple myeloma cancers
Tumour-activated neutrophils in gastric cancer foster immune suppression and disease progression through GM-CSF-PD-L1 pathway	T-T. Wang at al, 2017	Gut	PD-L1 expression induced by the activation of STAT3 pathway through tumor-derived GM-CSF	Human circulating neu and TAN	human gastric cancer
Reactive Neutrophil Responses Dependent on the Receptor Tyrosine Kinase c-MET Limit Cancer Immunotherapy	N. Glodde et al, 2017	Immunity	Suppression of T cell proliferation and IFNγ secretion	splenic and bone marrow derived	intracutaneously tumor induced with B16F1 cell line
Tumor-associated neutrophils induce apoptosis of non-activated CD8 T-cells in a TNFα and NO-dependent mechanism, promoting a tumor-supportive environment	J. Michaeli et al, 2017	OncoImmunology	Induction of CD8 T-apoptosis in a NO-dependent mechanism	TAN	subcutaneus tumor induced with AB12, LLC, LKR-M cell lines
Resistance to cancer immunotherapy mediated by apoptosis of tumor-infiltrating lymphocytes	J. Zhu et al, 2017	Nature Communications	Induction of tumor infiltrating lympocytes-apoptosis mediated by Fas-FasL pathway	TAN	Autochthonous TiRP melanoma model
Cancer-associated fibroblasts induce PDL1+ neutrophils through the IL6-STAT3 pathway that foster immune suppression in hepatocellular carcinoma	Y. Cheng et al, 2018	Cell death disease	PD-L1 expression induced by the activation of STAT3 pathway through IL6 produced by cancer associated fibroblast	Circulating human neutrophils (isolated by density gradient centrifugation)	human hepatocellular carcinoma (HCC)
Tumour-elicited neutrophils engage mitochondrial metabolism to circumvent nutrient limitations and maintain immune suppression	C.M. Rice at al, 2018	Nature Communications	ROS production and nutrient limitation	splenic and circulating immature cKit+	Breast cancer model
Fatty acid transport protein 2 reprograms neutrophils in cancer	F. Veglia et al, 2019	Nature	PGE2 production induced by the protein FATP2	murine splenic and TAN /human circulating CD15+ cells	subcutaneus tumor induced with EL4, LLC, CT26 cell lines or genetically engineered model of pancreatic cancer (KPC) or human head and neck, lung or breast cancer
Neutrophils suppress tumor-infiltrating T cells in colon cancer via matrix metalloproteinasemediated activation of TGFb	M. Germann et al., 2019	EMBO Molecular Medicine	neutrophil-derived MMP9 induces suppression of T cells TGF-mediated	TAN/ human circulating neutrophils	Inducible colon tumor model/human colon cancer

Table 1. Published work of T cell-suppressive behavior of neutrophils in tumors.

1.12. TANs with anti-tumorigenic properties

Even if most of the evidence showed that neutrophils are able to induce tumor progression, it has been proven that neutrophils can also mediate resistance against primary carcinogenesis in mouse and human. In fact, neutrophils are able to kill the cancer cells via 1) release of ROS, NO, TRAIL and TNF or 2) via contact-mediated mechanism or 3) by engaging in cooperative network with innate and adaptive lymphoid cells [378 379 57 380].

Regarding the crosstalk with other cells of immune system that promote anti-tumor response, it has been shown that neutrophils can release DC-maturing cytokines, such as TNF-α, or induce the maturation of mo-DCs by cell-cell contact-dependent mechanisms contributing to the launching of the adaptive immune response [381 382]. Moreover, neutrophils deficiency was associated to a selective impairment of type I polarization and IFN-γ production in a subset of unconventional CD4-CD8 T cells [383]. Also, in colorectal cancer, co-localization of neutrophils and CD8 T cells was associated with a better prognostic value [384].

Importantly, chemokines including CXCL10, CCL2, CCL3, CCL5, CXCL1 and CXCL2 released by neutrophils in the TME are able to recruit many immune subsets such as T cells and DCs, supporting anti-tumor immune responses [385 383 386 381]. Surprisingly, in early stage of lung cancer, a subset of

activated tumor infiltrated neutrophils (TANs) named “APC-like hybrid TANs” has been identified. This subset is characterized by an atypical expression of surface markers which belong to the professional APCs (CD86, CD14, HLA-DR, CCR7), which are able to amplify the anti-tumor T cell response and are absent in more advanced tumors [387 382].

Collectively, current data suggest that the significance of neutrophils and their functions in the circulation and in the neoplastic setting requires deeper clarification and may be strongly influenced by the tissue and the tumor context. Moreover, relate the complex plasticity of neutrophils to a specific microenvironment as well as patient prognosis and response to therapy represent an important challenge in the field. Lastly, there is no consensus nomenclature for the emerging complexity of neutrophils differentiation and activation states, and an unequivocal strategy to detect immunosuppressive neutrophils and other neutrophil subsets remains to be developed.

1.13. SiglecF^{high} neutrophil as tumor-promoting subset in lung cancer

TANs represent an important component of the niche of many murine and human tumors and understanding their complex diversity and plasticity in tissue specific tumor microenvironment will provide the basis to develop new therapeutic approaches. Usually, the presence of neutrophils in human tumors is assessed by immunohistochemistry and only recently a transcriptional signature based-identification has been introduced [272]. In particular, transcriptomic analysis and deconvolution of immune subsets in NSCLC showed a dominance of TAN that negatively correlates with T cell infiltration [388]. So far, a set of surface markers can be used to unequivocally identifies neutrophils which include CD11b/CD66b in human and CD11b/Ly6G in mice. However, the existing nomenclature (Fig. 1.5) is not sufficient to discriminate differently polarized subsets of neutrophils in tumor tissues and a more robust system to define this complex population is needed.

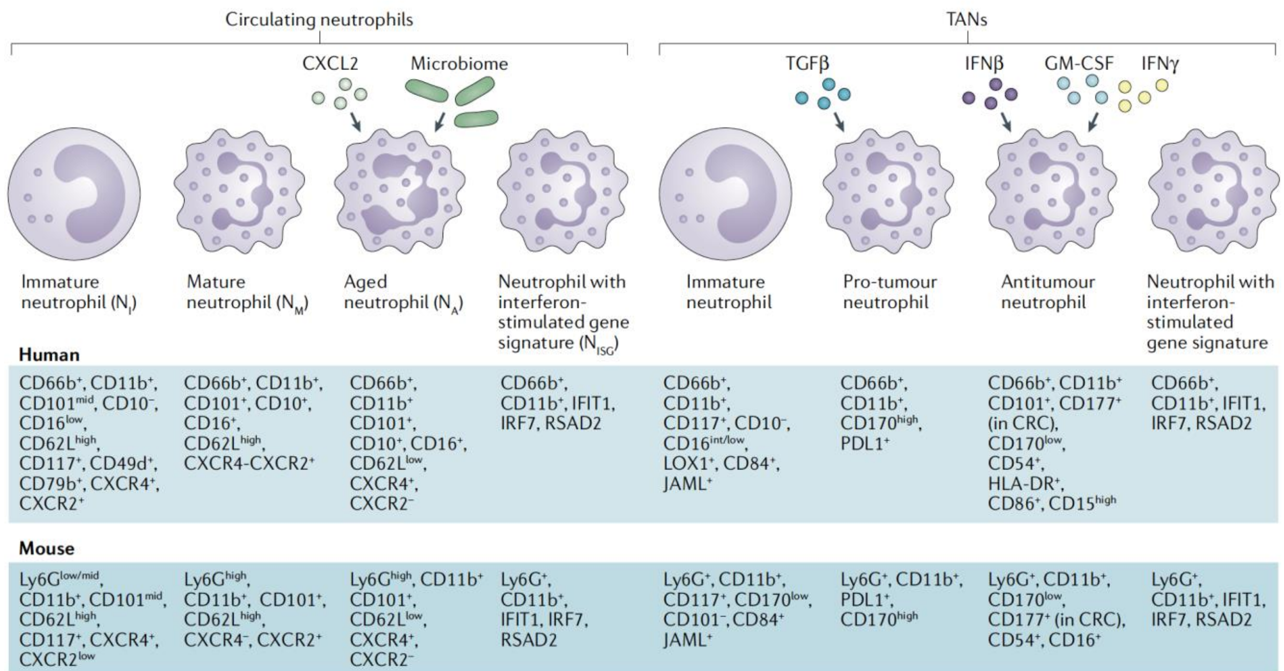

Figure 1.5. Nomenclature for neutrophil diversity in cancer. Set of surface molecules that identifies different subsets of neutrophils in human and mice. Additional molecules have been proposed to identify neutrophils in tumor contests which include PD-L1, LOX1, CD84 associated with tumor progression and CD101 and CD177 associated with tumor regression [302].

In a recent work, R. Zilionis and colleagues, through a scRNA-seq analysis of immune population in NSCLC as well as in a transplantable orthotopic lung cancer model, were able to identify five subsets of neutrophils (hN1-hN5) in human and six in mice (mN1-mN6) with conserved aspects between the two species. They identified three conserved modules of neutrophil gene expression within mouse and human: 1) neutrophils that express canonical markers (hN1 and mN1), 2) tumor specific subsets that promote tumor growth in mice (hN5 and mN5) and 3) a subset characterized by the expression signature of type I interferon response (hN2 and mN2). In addition, the authors observed that the over-expression of subsets-specific genes in patients associated with the overall survival, opening an opportunity to expand the number of tumor-associated markers used to identify neutrophils. As an example, the hN5 marker phosphoinositide 3-kinase (PI3) negatively correlated with patient survival [253].

Importantly, in a previous work, C. Engblom and colleagues, were able to identify by flow cytometry two subsets of neutrophil in lung of mice which can be distinguished according to the expression levels of the sialic acid binding lg-like lectin F (SiglecF). They demonstrated that an increased bone-stromal activity in autochthonous lung adenocarcinoma model was able to affect distant tumor progression and Ocn$^+$ cells were required for tumor infiltration by a SiglecF^{high} neutrophils. Those neutrophils preferentially accumulate in murine lung cancer, while the SiglecF^{low} subset appeared in high numbers already in healthy lungs [389]. Interestingly, single cell transcriptomic data revealed that SiglecF^{high} neutrophils express gene associated with angiogenesis, extracellular matrix

remodeling and T cells suppression and correspond to mN4 and mN6 subsets that were indeed highly enriched in tumor. In contrast, SiglecF^{low} neutrophils (corresponding to mN1 to mN3 subsets) already exist in healthy tissue and were not associated to tumor growth [253 389 390]. Siglecs molecules are well characterized lectins important for tolerance induction, pathogen recognition and uptake, and regulation of cells activation. In particular, the SiglecF component was first associated with a suppressive role on eosinophils, but its function on other cells remains unclear [391].

In 2020, C. Pfirschke and colleagues, better characterized the SiglecF^{high} neutrophils populating transplantable murine lung adenocarcinoma defining those cells as bona-fide mature cells that differ from other myeloid cells expressing SiglecF (including eosinophils, macrophages and MDSCs). This population has not been found into the bone marrow, in the spleen and blood of lung-tumor bearing mice [392] but appeared in other districts such as heart and nasal mucosa during inflammation and tissue damage [393 394]. In addition, Pfirschke and colleagues demonstrated that neu-SiglecF^{high} are characterized by an increased lifespan (up to several days compare to hours of SiglecF^{low} counterpart) and therefore gradually accumulate in growing lung tumors [392]. Moreover, in 2021 P-B. Ancey and colleagues, demonstrated that in autochthonous model of lung cancer, neu-SiglecF^{high} undergo metabolic changes exhibiting several pro-tumor functions. They identified GLUT1 as key regulator of enhanced glucose metabolism and neutrophil survival which in turn promoted neutrophil tumor supportive behavior. In addition, loss of GLUT1 accelerated neutrophils turnover and reduced the presence of neu-SiglecF^{high} in tumors leading to decrease of tumor growth and better responses to radiotherapy [295].

Thus, neutrophils are an essential part of the lung cancer microenvironment and those expressing SiglecF may represent a target for new therapeutic approaches. Generally, to decipher whether neutrophils are involved or not in pathological events, anti-neutrophils antibodies (that target surface markers such as Ly6G or Gr1 which are not able to discriminate different subsets of neutrophils) are widely used to deplete these cells *in vivo*. However, the available antibodies suffer some limitations and are partially effective because of continuous recruitment from the bone marrow [395].

Because of the lack of a valid approach to interfere with neu-SiglecF^{high} recruitment *in vivo*, understanding how this population interact with different subsets in the TME and how influence the microenvironment of lung cancer remain to be elucidated. In addition, even if present evidence suggests that tumor derived circulating factors (such as sRAGE) could promote the activation of bone marrow-resident cells that support accumulation of cancer-promoting neu-SiglecF^{high} at the

tumor site [389], the origin of this class of neutrophils and the mechanism of their recruitment in lung cancer remain elusive. Moreover, the crosstalk with lung tissue specific anti-cancer T cell responses has not been investigated. In conclusion, neutrophils expressing SiglecF are a subset of neutrophils populating tumor bearing lungs which exhibit pro-tumorigenic behavior *in vivo.* A better characterization of this population may open possibilities for development of new specific therapeutic approaches for lung cancer without affecting other neutrophil subsets that are essential for the host defense.

2. Aim

Neutrophils have emerged as a key immune suppressive population in lung cancer. Their diversity and complexity in lung cancer tissue have become apparent with the identification of one specific subset of long-lived neu-SiglecF^{high} being preferentially enriched in adenocarcinoma. Evidence shown that the accumulation of neutrophils expressing SiglecF in lung cancer is mediated by the activation of bone marrow-resident cells and this subset expressed gene associated to tumor progression. However, the tumor secreted factors that determine their accumulation in lung cancer tissues are still elusive as well as their impact on endogenous anti-cancer T cell responses.

The goal of this thesis was first to identify cancer derived factors involved in recruitment and accumulation of neu-SiglecF^{high} in lung tumors. Upon identification of the chemokine CXCL5 as the major pathway, the next objectives have been 1) to interfere with expression of the chemokine by KP cancer cells, which was achieved by Crispr/Cas9 genome editing approach and 2) to analyze the implication of CXCL5 depletion in lung cancer microenvironment by multiparametric analysis by flow cytometry used to assess the accumulation of neutrophils and other immune cell subsets in CXCL5-null KP tumors. Last, having established the system I have evaluated the impact of neu-SiglecF^{high} on anti-tumoral T cell activation and on efficacy of PD-L1 immunotherapy.

3. Materials and methods

3.1. Mice and *in vivo* treatment

C57BL/6 mice were purchased from Envigo Laboratories, whereas the OT-I (C57BL/6-Tg(TcraTcrb)1100Mjb/J) strain was purchased from Jackson Laboratories. Animals were maintained in sterile isolators at the ICGEB animal Bio-experimentation facility. Ethical and experimental procedures were reviewed and approved by the Italian Ministry of Health (approval number 1133/2020-PR, issued on 12/11/2020).

To establish the adenocarcinoma tumor models C57BL/6 WT mice at 8-10wks of age, were intravenously injected with $7x10^4$ non immunogenic KP cells or with $2x10^5$ immunogenic variants (KP OVA, WT or KO^{CXCL5}, $KO^{CXCL5\ (lenti\text{-}CXCL5)}$ or $KO^{CXCL5\ (lenti\text{-}Vec)}$). Tumor bearing mice were sacrificed at initial (9 days upon tumor induction) or at more advanced stage of tumorigenesis (18 days upon induction), otherwise indicated, to perform downstream analysis.

In some experiments, mice challenged with KP OVA cells were treated every 3 days, starting from the 6th day to 12th day, with anti-Ly6G (InVivo Plus, clone 1A8, BioXcell) antibody or isotype control (Rat IgG2a isotype control, clone 2A3, BioXcell) at the concentration of 200µg/100µL intraperitoneally to induce *in vivo* neutrophils depletion. For these experiments, mice were sacrificed at day 13th.

In one experiment, mice were challenged with KP OVA WT or KO^{CXCL5} cells and treated starting from the 3rd day to 12th day, every 3 days, with antibody to PD-L1 (InVivoMab, clone 10F.9G2, BioXcell) or isotype control (InVivoMab, rat IgG2b isotype control, clone LTF-2, BioXcell) at the concentration of 200µg/100µL intraperitoneally to assess sensibility to immune checkpoint blockade therapy. In this setting, mice were sacrificed at day 18th.

For adoptive transfer of tumor specific T cells, CD8 T cells were isolated from single cell suspension from lymph nodes of OT-I mice at 8-10 wks of age using CD8a+ T cell isolation Kit (Miltenyi) following the manufacturer's instructions. The isolated OTI-$CD8^+$ T cells were labelled with CFSE (thermoFisher) and $2x10^6$ OTI^+CFSE^+ T cells were intravenously injected into mice carrying WT or KO^{CXCL5} tumors induced 9 days before. Mice were sacrificed 2 days after adoptive transfer to assess proliferation and IFN-γ production in OTI T cells.

3.2. Cell lines

Transplantable KP cell line (KP1233) has been generated from lung tumors of C57BL/6 KP mice (K-ras$^{LSLG12D/+}$;p53$^{fl/fl}$ mice) and was kindly provided by Dr. Tyler Jacks (Massachusetts Institute of Technology, Cambridge, USA) in July 2015.

To obtain an immunogenic cell line (KP OVA), KP cells were transduced with a lentivirus carrying the expression vector Pdual-liOVAha-PuroR, encoding ovalbumin (OVA) protein (kindly provided by David Murrugarren, CIB, Navarra). After 2 days of antibiotic selection with puromycin, cells were subcloned and single cell clones were tested for HA-OVA expression by intracellular staining by flow cytometry. One clone with intermediate OVA expression was selected as KP OVA cell line.

To generate KO^{CXCL5} a specific guide for CXCL5 gene (for 5'-caccgCTGCCGCAGCATCTAGCTGA-3' rev 5'-aaacTCAGCTAGATGCTGCGGCAGc-3') was designed using the online tool "Broad Institute web portal" to minimize potential off-target effects. The pZac2.1-U6sgRNA-CMV-ZsGreen plasmid was digested with bbsI restriction enzymes to allow cloning of the guide under the control of U6 promoter. This plasmid was transiently transfected, together with a second plasmid (pSpCas9(BB)-(PX458)) coding for Cas9 protein, in KP OVA cells by lipofectamine 3000 (Invitrogen) following manufacturer's instructions. 24hrs after the transfection, the ZsGreen^{+} cells were purified using FACS cell sorting (FACS Aria II BD bioscience) and subcloned. Single clones were tested for CXCL5 expression by qRT-PCR and by ELISA kit (abcam). Plasmids were kindly provided by dr. Mauro Giacca, ICGEB, Trieste.

To generate KO$^{CXCL5\ (lenti\text{-}CXCL5)}$ cells, KO^{CXCL5} cells were transduced with a lentivirus carrying the expression vector pLVX-IRES-G418-CXCL5, encoding CXCL5. As control, KO^{CXCL5} cells were also transduced with lentivirus carrying empty pLVX-IRES-G418 vector. After 2 days of antibiotic selection with G418, cells were tested for CXCL5 production by qRT-PCR and ELISA kit (abcam).

All cell lines were maintained in DMEM media (containing 1g/L of glucose) supplemented with 10% fetal bovine serum (FBS, Euroclone) and Gentamicin (50µg/mL, Gibco) and routinely tested for mycoplasma contamination. Cells were expanded to passage 3 and stored in aliquots in liquid nitrogen. For tumor induction, cells were cultured less than five passages.

3.3. Lentivirus production and cell infection

The expression plasmid and relative packaging with envelope (Table 2) were co-transfected into HEK293T cells using Lipofectamine 3000 (Invitrogen) following manufacturer's instructions. Forty-eight hours after transfection, the supernatant containing virus particles was collected and filtered

with a 0,45μm filter (millex-HV). Concomitantly, 9×10^6 KP or KO^{CXCL5} cells were plated in a 6-well plate (Falcon). Once attached, supernatant containing the virus was added to the cells which were subsequently infected through spinoculation at 800g for 60 min in the presence of polybrene (6μg/mL, Invitrogen). The day after, the virus was removed and supernatant replaced with 2mL of fresh medium supplemented with the respective antibiotic to select the transduced cells.

Lentiviral expression	**Packaging**	**Envelope**	**Bacterial resistance**	**Cell resistance**
pLVX-IRES-G418-CXCL5	sPAX2	p-CMV-VSV-G	Ampicillin	G418
Pdual-lOVAha	sPAX2	p-CMV-VSV-G	Ampicillin	Puromycin

Table 2. Summary of lentivirus plasmid.

In order to produce the lentiviral expression pLVX-IRES-G418-CXCL5 plasmid, the pLVX-IRES-G418 vector (kindly provided by dr. Serena Zacchigna, ICGEB, Trieste) was digested with NotI and XhoI restriction enzymes to allow the insertion of the coding sequence (CD) of Cxcl5 (https://www.ncbi.nlm.nih.gov/nuccore/NM_009141.3) under the control of CMV promoter. The CD of Cxcl5 was isolated from pGi-CXCL5 plasmid through NotI and XhoI restriction enzyme-mediated excision and DNA isolation from agarose gel by GFX PCR DNA AND GEL BAND purification kit (GE Healthcare) following manufacturer's instructions. The AAV plasmid pGi-CXCL5, containing the CD of Cxcl5 was kindly provided by dr. Mauro Giacca, ICGEB, Trieste.

3.4. *In vitro* cell line growth rate

To determine the growth rate of cells *in vitro*, 3×10^3 cells were cultured in a flat p96-well (Falcon) and fixed at different time points (day 1, day 2 and day 3 of culture) in 1%PFA for 2 hrs. Cells were washed with PBS and stained with crystal violet dye following manufacturer's instructions to assess cells viability. Upon dye solubilization by 1%SDS the OD at 595nm, proportional to vital and proliferated cells, was measured by microplate reader (iMark, Biorad).

3.5. Tissue preparation and cell isolation

Normal or tumor-bearing lungs were harvested after PBS lung circulatory perfusion, mechanically cut into small pieces and digested with 0,1 % Collagenase type 2 (265U/mL; Worthington) and DNase I (250U/mL; Thermo scientific) at 37°C for 30'. Collagenase was stopped by EDTA 10mM and the cell suspension was filtered using 70μm cell strainer (Corning). Blood cells in the suspension

were lysate by ACK (Gibco) solution. The obtained single cell suspension was stained for flow cytometry analysis.

In one experiment, single cell suspension obtained from healthy or tumor bearing lungs was stained with CD45-A647 (30-F11, Biolegend) antibody and $CD45^{+ \text{ or } -}$ populations were sorted by FACS Aria II (BD Biosciences) for qRT-PCR analysis.

Other tissues were collected from healthy or tumor bearing mice including blood (collected through subclavian vein puncture), spleen, lymph nodes (mediastinal and inguinal) and bone marrow. To avoid coagulation, EDTA (0,5M, Gibco) was added to blood samples. Single cell suspension from spleen and lymph nodes was obtained through filtration with a 70 µm and 40 µm cell strainer respectively. Bone marrow single cell suspension was obtained through flushing with 0,5mL-26G syringe (Terumo) from leg bones (femur and tibia). Therefore, red cells were lysate in all samples by ACK solution and the obtained single cell suspension was stained for flow cytometry analysis.

3.6. Flow cytometry

Antibodies used are listed in table 3. Viability of cells was assessed by staining with LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Life Technologies). FcR binding sites were blocked by using αCD16/CD32. Myeloid cell subsets were identified as showed in Figure 4.2a by multiparametric-based analysis which include classification based on physical parameters (SSC-A and FSC-H), dead cell and doublets exclusion, and expression of extracellular markers as following: cDCs ($CD45^{+}CD11c^{+}MHCII^{hi}SiglecF^{-}$), AM ($CD45^{+}CD11b^{-}CD11c^{+}SiglecF^{+}$), TAM ($CD45^{+}SiglecF^{-}Ly6C^{-}Ly6G^{-}MHCII^{+}CD64^{+}$), neu ($CD45^{+}CD11b^{+}Ly6G^{+}$), neu-$SiglecF^{high}$ ($CD45^{+}CD11b^{+}Ly6G^{+}SiglecF^{+}$), neu-$SiglecF^{low}$ ($CD45^{+}CD11b^{+}Ly6G^{+}SiglecF^{-}$), neu-$SiglecF^{high \text{ or } low}$ expressing CXCR2 ($CXCR2^{+}$), neu-$SiglecF^{high \text{ or } low}$ expressing PD-L1 ($PD\text{-}L1^{+}$), eosinophils ($CD45^{+}CD11b^{+}SiglecF^{+}Ly6G^{-}$), monocytes ($CD45^{+}CD11b^{+}Ly6C^{+}Ly6G^{-}$), NK cells ($CD45^{+}CD19^{-}CD3^{-}B220^{-}NK1.1^{+}$), B cells ($CD45^{+}CD3^{-}CD19^{+}B220^{+}$), CD4+ T cells ($CD45^{+}CD3^{+}CD4^{+}$), CD8+ T cells ($CD45^{+}CD3^{+}CD8^{+}$), activated T cells ($PD1^{+}LAG3^{+}TIM3^{+}$), memory T cells ($CD62l^{-}CD44^{+}$). Where indicated, absolute cell count was analyzed by adding TrueCount Beads (Biolegend) to the samples following manufacturer's instructions.

To identify OVA specific CD8 T cells ($CD45^{+}CD3^{+}CD8^{+}pentamers^{+}$) lung cell suspensions were stained with MHC-I-OVA pentamers tool (SIINFEKL/H-2Kb Pro5, Proimmune) following manufacturer's instruction.

For intracellular detection of IFNγ, single cell suspensions from lung or mLN were stimulated with OVA peptide (SIINFEKL) 2µM 37°C for 4 hrs in the presence of Golgi Stop (monensin, BD Biosciences)

to allow accumulation of cytokines within Golgi apparatus. Upon extracellular staining, cells were fixed and permeabilized using Cytofix/Cytoperm solution (BD Biosciences) following manufacturer's instructions, and then stained with IFNγ-PE antibody.

To identify effector EOMES$^-$Tbet$^+$ CD8$^+$ T cells, upon extracellular staining labelling CD45$^+$CD3$^+$CD8$^+$ T cells, single cell suspensions from lung and mLN were fixed and permeabilized using Foxp3/transcription factor staining buffer set (ThermoFisher) following manufacturer's instructions and stained with anti-mouse EOMES and anti-mouse T-bet antibodies.

To measure OVA expression, different cell lines were fixed and permeabilized using Cytofix/Cytoperm solution (BD Biosciences) following manufacturer's instructions, stained with rat-antibody against HA bonded to OVA protein and with αRAT-IgG-AF488 (Invitrogen). Samples were acquired with FACS Celesta (BD Biosciences) and analyzed with FlowJo software (Tree Star, Inc.).

Antibody	Fluorophore	Clone	Brand
CD45	APCCy7	30-F11	Biolegend
CD3	PerCPCy5.5	145-2C11	Biolegend
CD3	FITC	145-2C11	Biolegend
CD4	BV785	GK1.5	Biolegend
CD8	APC	53-6.7	Biolegend
CD62l	BV650	MEL-14	Biolegend
CD44	PE	IM7	Biolegend
PD-1	EF450	J43	eBioscience
TIM3	PerCPCy5.5	RMT3-23	Biolegend
LAG3	BV650	C9B7W	Biolegend
IFN-γ	PE	MG1.2	Biolegend
EOMES	A647	W17001A	Biolegend
Tbet	BV421	4B10	Biolegend
SIINFEKL/H-2Kb Pro5	PE		ProImmune
CD11b	BV421	M1/70	BD Bioscience
SiglecF	PerCPEF710	IRNM44N	eBioscience
SiglecF	BB515	E50-2440	BD Bioscience
CD11c	BV786	N418	Biolegend

CD11c	APC	N418	Biolegend
MHC-II	APCR700	M5/114.15.2	Biolegend
CD86	PE	B7-2	BD Bioscience
PD-L1	PerCPeF710	MIH5	eBioscience
CD64	BV605	X54-5/7.1	Biolegend
Ly6C	AF488	HK1.4	eBioscience
Ly6C	BV570	HK1.4	Biolegend
Ly6G	AF488	1A8	Biolegend
Ly6G	PE	1A8	Biolegend
CXCR2	PerCPCy5.5	SA044G4	Biolegend
B220	APC	C363-16A	Biolegend
CD19	PE	1D3	BD Bioscience
NK1.1	Biotin	PK136	Biolegend
Streptavidin	PE-Cy7		Biolegend
L&D	BV510		Life Technologies
CD16/CD32		93	Biolegend
HA		3F10	Roche

Table 3. list of used antibodies

3.7. Real time PCR

Normal or tumor bearing lung were mechanically dissociated and RNA was extracted from dissociated normal or tumor bearing lung tissues or cells by using Trizol reagent (ThermoFisher Scientific), according to manufacturer's instruction. cDNA was synthesized using SuperscriptII (ThermoFisher) and real-time PCR for gene expression was performed using SsoFast EvaGreen Supermix (Biorad) with specific primers: Gapdh For (AGAAGGTGGTGAAGCAGGCAT) Rev (CGAAGGTGGAAGAGTGGGAGT), Cxcl5 For (GCT GCC CCT TCC TCA GTC AT) Rev (CAC CGT AGG GCA CTG TGG AC).

Thermal cycle conditions were the following: 95°C for 3 minutes, 43 cycles of 95°C for 10 seconds, and 60°C for 30 seconds. Each sample was analyzed in triplicates. Cxcl5 expression in cell lines was obtained by normalizing target genes to Gapdh used as reference gene (ΔC(t)). Expression corresponding to $2^{\Delta C(t)}$ ($2^{C(tCxcl5)-C(tGapdh)}$, where CT is the signal of the PCR product which exceeds the background signal) was showed. CXCL5 expression in tissues was obtain by normalizing the ΔC(t) of

tumor bearing lung to ΔC(t) of normal lung (ΔΔC(t)). The relative abundance corresponding to $2^{\Delta\Delta C(t)}$ was showed.

In one experiment, gene expression profiling of inflammatory cytokines and receptors of normal and KP-OVA tumor bearing lungs was performed by custom RT^2 Profiler PCR Array (Qiagen, cat. 330221) following manufacturer's instructions.

3.8. CXCL5 detection by ELISA

$3x10^6$ cells/200uL were incubated at steady state with medium 5 hrs and CXCL5 production in the supernatant was detected by mouse CXCL5 ELISA kit (GCP-2, abcam), following manufacturer's instructions.

3.9. Immunohistochemistry

To assess tumor burden, lung tissues were harvested previous PBS lung circulatory perfusion, fixed in formaldehyde 10% and paraffine embedded following standard procedure. Consecutive sections of 8μm were dewaxed and rehydrated and stained with the H&E using (Bio-Optica, Milano Spa). Limonene was used as mounting media. The area of tumor nodules was quantified manually over consecutive sections and averaged (3 sections/sample). The area occupied by tumor nodules was expressed as a function of the total lung lobe area. Automatic thresholding and measurements were performed using Ilastik or imageJ software, respectively.

To identify $CD8^+$ T cells or neutrophils infiltrating lung tumors or proliferating cells within nodules, sections of paraffine embedded lung tissue were dewaxed and rehydrated and treated with antigen-retrieval solution (Vector laboratories) for 20 min at 120°C. Slides were treated for 10 minutes in H_2O_2 to block endogenous alkaline phosphatase. After blocking in 10% goat serum in 0.1% Tween20 for 30 minutes, slides were incubated overnight at 4°C in a humidified chamber with specific antibody diluted in PBS 0,1%Tween20: anti-mouse CD8 (4SM15, Invitrogen cat 14-0808-82), or anti-mouse Ly6G (1A8, BD Pharmingen, cat 551459), or anti-mouse Ki67 (D3B5, Cell Signaling, cat 12202s). Detection was performed using the ImmPRESS polymer detection system (Vector Laboratories), according to manufacturer's Instructions. Slices were therefore stained with the hematoxylin to manually identify the tumor area (for proliferating cells eosin staining was performed instead). Limonene (Electron Microscopy Science) was used as mounting medium. Automatic thresholding and measurements were performed using Ilastik or imageJ software, respectively. Images were acquired by Leica microscope using 10x, 20x or 40x objectives.

3.10. Immunofluorescence staining

To identify neu-SiglecF^{high} cells within nodules of KP OVA tumors, lungs tissues were harvested previous lung perfusion via trachea with 1% paraformaldehyde (PFA), fixed in 4% PFA and embedded in a frozen tissue matrix following standard procedure. Sections of 5μm were dried 15' at room temperature (RT) and permeabilized for 15' in 0,5% Triton. After blocking in 5% mouse serum in 1%BSA/0,1%NP-40 for 30', slides were incubated overnight at 4°C in a humidified chamber with specific fluorophore-conjugated antibody diluted in PBS 1%BSA/0,1%NP-40: anti-mouse Ly6G-PE and anti-mouse SiglecF-BB515 (listed in table 3). Nucleus were labelled by Hoechst and Mowiol 4-88 (Sigma) was used as mounting medium. Images were acquired with LSM880 META reverse microscope with a 40x objective.

To assess spatial distribution of neutrophils and CD8 T cells lung tissues bearing KP OVA tumors were harvested previous PBS lung circulatory perfusion, fixed in formaldehyde 10% and paraffine embedded following standard procedure. Consecutive sections of 8μm were dewaxed and rehydrated and treated with antigen-retrieval solution (Vector laboratories) for 20 min at 120°C. After blocking in 10% goat serum in 0.1% Tween20 for 30 minutes, slides were incubated overnight at 4°C in a humidified chamber with rat anti-mouse CD8 (4SM15, Invitrogen) and rabbit anti-mouse Ly6G (E6Z1T, Cell Signaling) antibodies diluted in PBS 0,1%Tween20 followed by specific secondary antibodies. Nucleus were finally labelled by Hoechst and Mowiol 4-88 was used as mounting medium.

Images were acquired with C1 Nikon reverse microscope with a 20x objective. Automatic thresholding was performed by Ilastik. ImageJ software was used to quantify CD8 T cells and neu and to measure nodule's area. Nodules having an area <0,09 mm^2 were classified as small, the ones having an area ranging from 0,091 to 0,2 mm^2 as medium and those with an area >0,2 mm^2 as large nodules.

3.11. Statistic

Primary data were collected in Microsoft Excel and statistical analysis were performed by using Graphpad Prism 8 software. Values reported in figures are expressed as the standard error of the mean, unless otherwise indicated. For comparison between two or more groups with normally distributed datasets 2-tailed Student's T test, multiple T test, one-way ANOVA or 2-way ANOVA were used as appropriate. For the comparison of matched groups, we used Wilcoxon test. The non-parametric Kruskal-Wallis test with Dunn's multiple comparison was performed to compare 3 or

more unmatched groups. p values > 0.05 were considered not significant, p values ≤ 0.05 were considered significant: * p≤0.05, ** p≤0.01, *** p ≤0.001, **** p ≤0.0001.

4. Results

4.1. Orthotopic KP tumors are dominated by SiglecF high neutrophils

To study the role of neutrophils in lung tissue during progression of primary lung tumors, we established a transplantable model of lung adenocarcinoma using a cell line derived from primary KP tumors (Kras$^{G12D/+}$; Trp53$^{-/-}$) [396] that gives rise to lesions similar to human non-small cell lung adenocarcinoma in terms of genetic mutations and histopathological features [397]. We generated an OVA expressing variant of the original line by lentiviral transduction of the model antigen ovalbumin (OVA-HA) to allow tracking of T cell responses against a defined tumor antigen in an immunogenic model. The transduced cells were selected under puromycin resistance and subsequently subcloned (Fig. 4.1a). Single clones were therefore tested for OVA expression by intracellular staining by flow cytometry using an antibody against the hemagglutinin (HA) tag. We tested different clones and we selected, a clone (A4) which showed an intermediate expression of OVA. This selected clone is referred to KP-OVA cell line hereafter and it was used to induce immunogenic lung tumor lesions *in vivo* (Fig.4.1b-c).

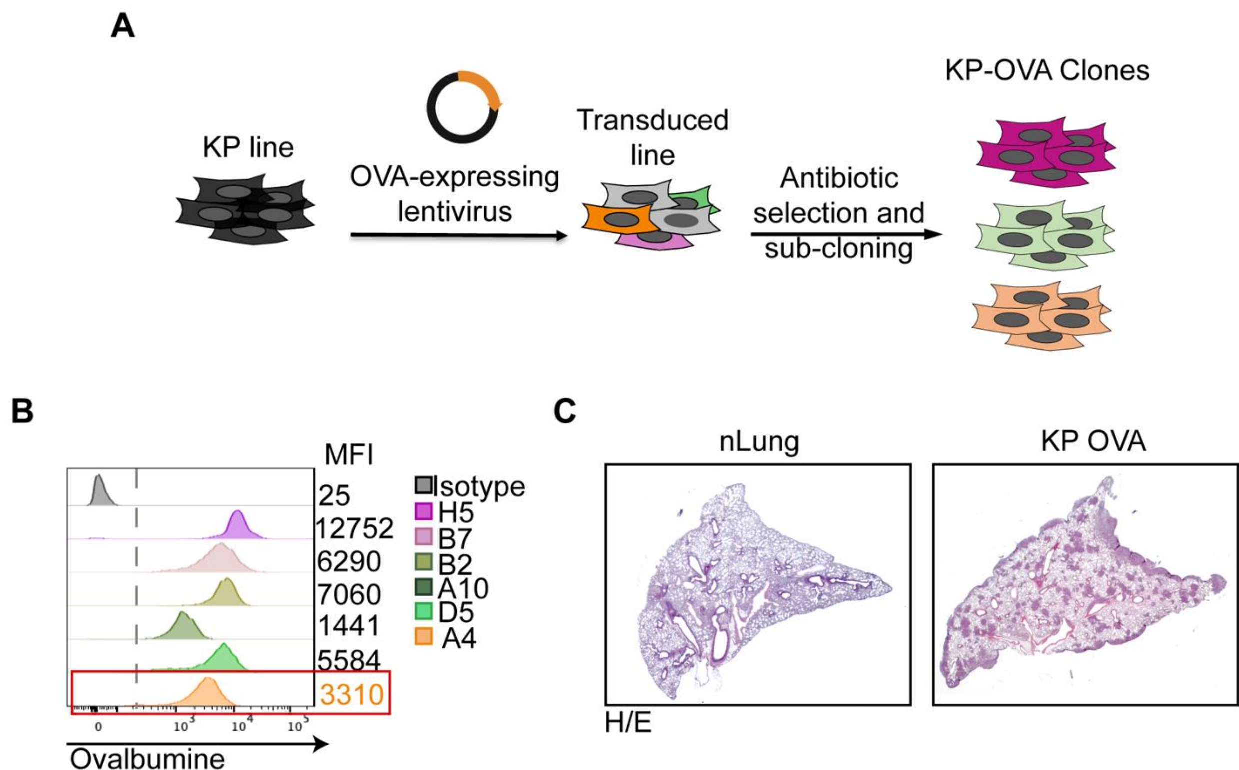

Figure 4.1 Generation of KP-OVA cell line. A) KP cell line was transduced with OVA-expressing lentivirus. The transduced cell line was selected under puromycin resistance and sub-cloned. B) Single clones were analyzed for OVA expression by intracellular flow cytometry staining and mean fluorescence intensity (MFI) of OVA was plotted nearby each corresponding histogram. C) The "A4" clone were injected intravenously in WT mice and 20 days upon challenge the tumor growth were analyzed by H/E staining of paraffine embedded lung tissues.

KP and KP-OVA tumors were intravenously induced and lung tissues were harvested when nodules were clearly visible (15-20 days after challenge). To phenotype the immune infiltrate of KP lung tumors we set up a multiparametric-based gating strategy to identify several immune subsets (neutrophils, NK cells, eosinophils, alveolar macrophages, B cells, T cells and DCs) as illustrated in Fig.4.2a. In particular we noted that the KP model is characterized of an abundant myeloid infiltration dominated by Ly6G$^+$ CD11b$^+$ neutrophils (neu). The immune landscape of KP-OVA tumors is similar to KP tumors and is characterized by an even higher infiltration of neu. In both models we observed a slight decrease in B cells and CD4 T cells compare to normal lung (nLung) and as expected, an increment in CD8 T cells in the immunogenic KP-OVA model (Fig.4.2b).

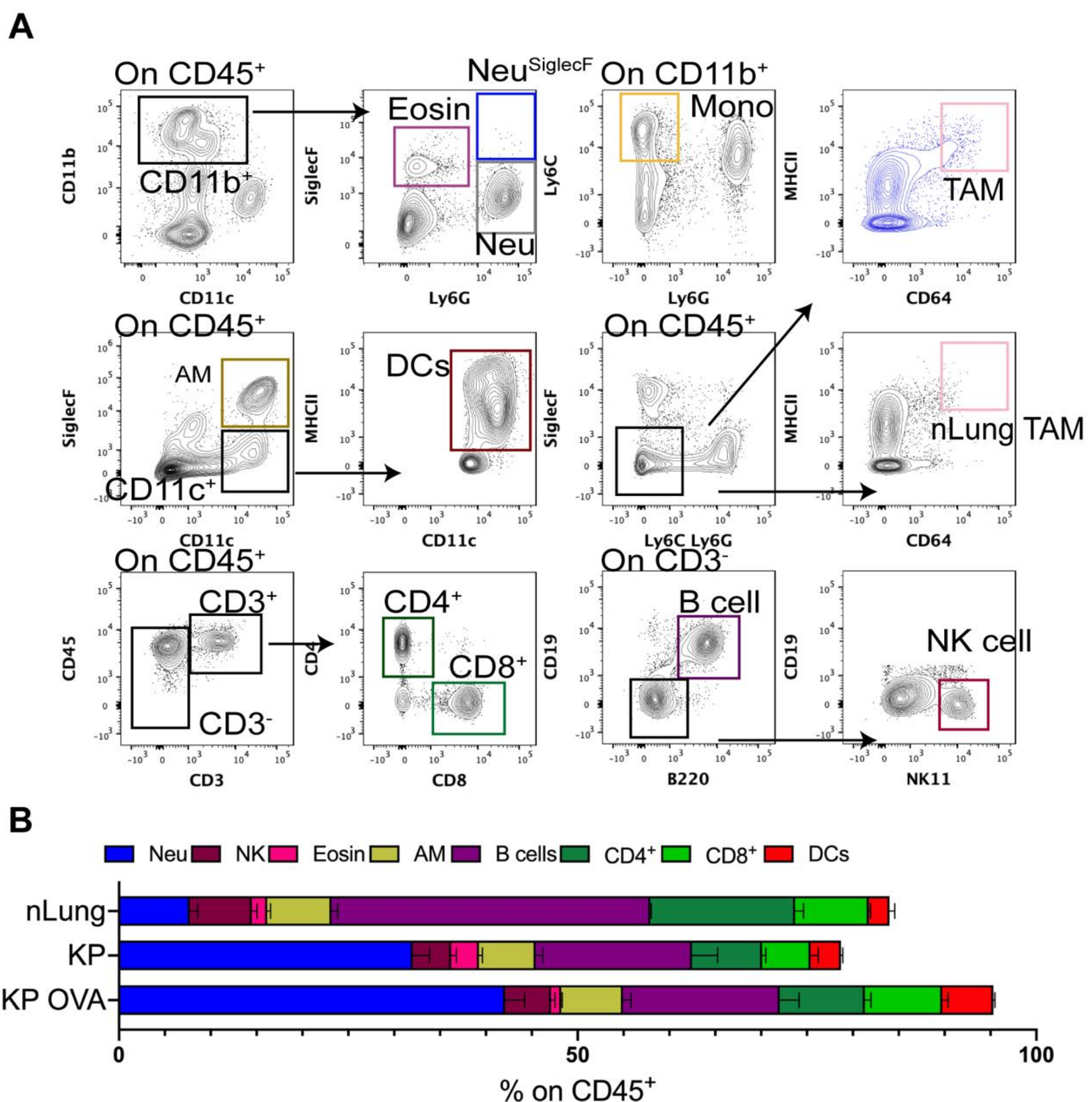

Figure 4.2 Immune infiltrate of KP or KP-OVA tumors. ***A)*** *Gating strategy used to identify by flow cytometry different myeloid populations in normal lungs and established KP or KP-OVA tumors.* ***B)*** *mice were challenged with KP and KP-OVA cells and 20 days after induction normal and tumor bearing lungs were harvested to analyze the immune landscape by flow cytometry. Quantification of relative abundance of each subset expressed as a fraction of CD45$^+$ cells. Data are mean±SEM of 3-4 mice each group.*

To further phenotype tumor infiltrating neu, we analyzed by flow cytometry the expression of the marker SiglecF which was recently shown to identify a population of long-lived mature neu in lung

tumors [392]. In line with recent evidence [389 392] we found that up to 70% of neu in KP tumors expressed the lectin SiglecF (neu-SiglecF^{high}), whereas neu in normal lung showed low SiglecF expression (neu-SiglecF^{low}). Of note, Neu-SiglecF^{high} were present in similar proportion in immunogenic KP-OVA tumors, demonstrating that their accumulation occurs also in the presence of an ongoing response against tumor antigens (Fig.4.3).

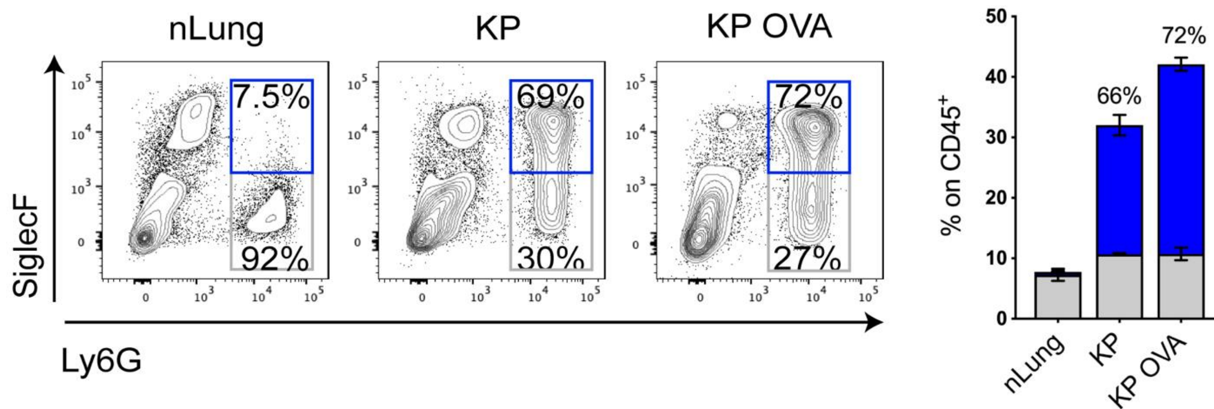

Figure 4.3 Representative dot plot and quantification of relative abundance of SiglecF $^{high/low}$ neutrophils expressed as a fraction of CD45^{+} cells. Mice were inoculated with KP or KP-OVA cells and neutrophils subsets were analysed after 18 days by flow cytometry in normal or tumor bearing lungs. Frequencies represent the mean±SEM of three mice each group. Also, frequencies of SiglecF $^{high/low}$ neutrophils expressed on total CD11b^{+}Ly6G^{+} were showed as % in the graph.

Having confirmed the presence of neu-SiglecF^{high} in immunogenic tumors we performed the remaining experiments using the KP-OVA model.

We next asked whether neu-SiglecF^{high} are present systemically or in lymphoid organ of tumor challenged mice. To this aim we analyzed the spleen, the bone marrow (BM), the mediastinal lymph node (mLN) draining the lung, other lymph nodes (such as the inguinal LN) and the blood of KP OVA tumor bearing mice by flow cytometry.

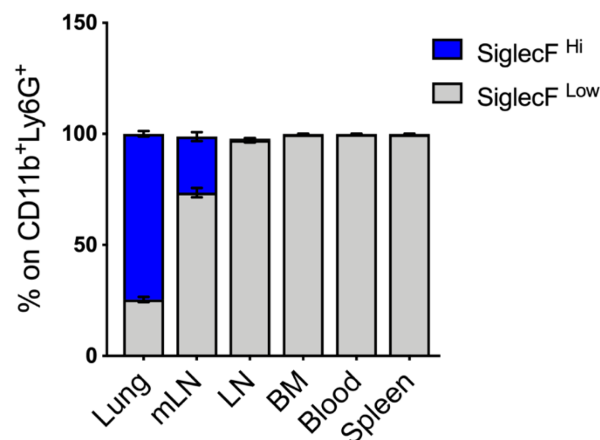

Figure 4.4 Expression of the lectin SiglecF on systemic or lymphoid-derived neutrophils. Tumors were induced with KP-OVA cell line and 15 days after challenge the expression of SiglecF on neutrophils from lung, BM, blood, spleen, and lymph nodes (lung draining mLN and inguinal LNs) of tumor bearing mice were analyzed by flow cytometry. Quantification of relative abundance of neutrophils SiglecF$^{high\ or\ low}$ expressed on total CD11b^{+}Ly6G^{+} was showed in the graph. Data represents the mean±SEM of 4 mice each group.

According to previous data, neu-SiglecF^{high} represented the 70% of total neutrophils in lungs. Of note, we were able to detect the SiglecF expressing subset also in mLN where represented the 20% of total CD11b^{+}Ly6G^{+} cells. However, those cells were absent in all the other tested compartments (Fig.4.4), indicating that neu-SiglecFhigh populated specifically lung tumor tissues and connected lymph nodes.

To visualize the distribution of neu within lung tumor tissues we performed IHC and tissue immune fluorescence (IF). Labeling with Ly6G antibody of paraffine embedded KP OVA tumor bearing lung showed that the accumulation of neu in lung tumors occurred only inside the nodule (Fig.4.5a). In addition, by labelling of cryo-tissues with both SiglecF and Ly6G antibodies we confirmed by IF the presence of neu-SiglecF^{+} infiltrating nodules of KP OVA tumors (Fig.4.5b).

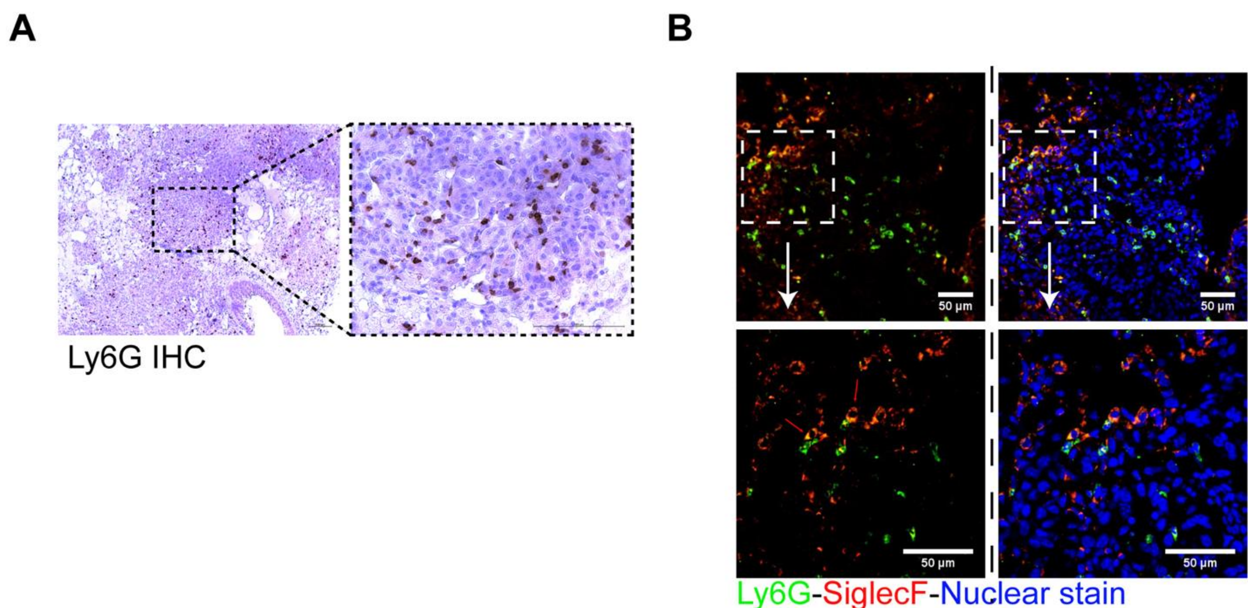

Figure 4.5 Neu accumulate in nodules of KP-OVA tumors. Mice were challenged with KP OVA cells and after 9 days tumor bearing lungs were analyzed by IHC and IF. A) Representative 10x section of paraffine embedded KP OVA tumor bearing lung labelled with Ly6G antibody identifying neutrophils (brown dots) and haematoxylin used to identify the tumor area (left). On the right the 40x magnification of a nodule was showed. B) the upper sections are representative IF analysis of a nodule of cryo-lung tumor tissues labelled with Ly6G (green) and SiglecF (red). The lower parts are a 60x magnification of a nodule. In the images on the right DAPI (blue), used to identify the tumor area within the tissues, was also showed.

4.2. CXCL5 is highly expressed in KP tumors

To explore the mechanism controlling accumulation of neu-SiglecF^{high} in lung tumors, we profiled normal and tumor lung tissues using a gene array of chemokines and their receptors. As shown in Fig.4.6a, tumor tissue displayed 20-fold more Cxcl5 and 10-fold more Cxcl9 than normal tissues, besides a slight induction of other chemokines implicated in monocytes or neu recruitment. Cxcl5 over-expression was confirm also by RT-PCR in KP and KP-OVA tumor bearing lung (Fig.4.6b).

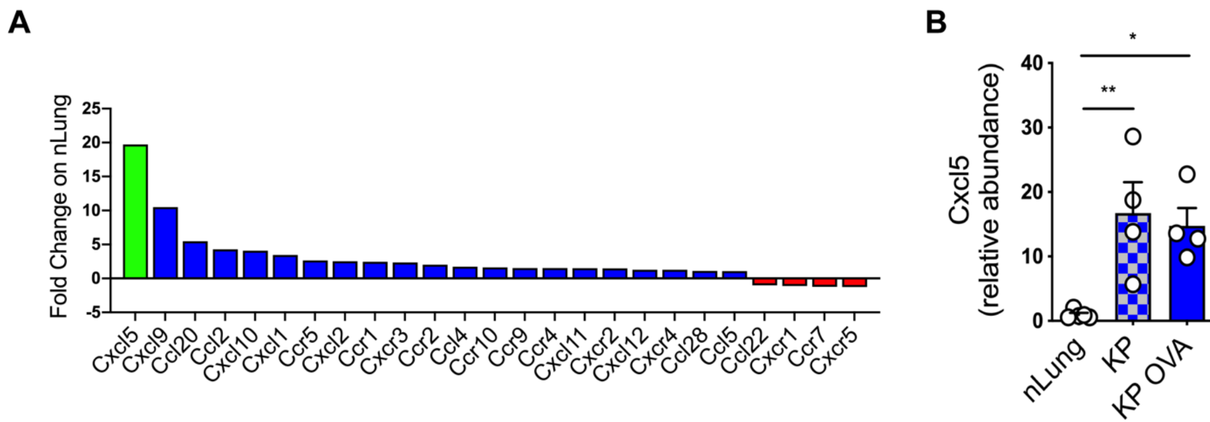

Figure 4.6 Cxcl5 is over-expressed in both KP and KP-OVA tumors. ***A)*** *mice were challenged with KP-OVA cells and after 20 days normal and tumor bearing lungs were harvested to analyze transcriptional profiles by Qiagen gene array. The graph shows differential chemokines and receptors gene expression in established KP-OVA tumors compared to nLung.* ***B)*** *Tumors were induced by KP or KP-OVA cells in WT mice and 20 days after challenge Cxcl5 relative abundance was measured by real-time PCR in normal and tumor bearing lungs. Data represent the mean±SEM of 4-5 independent RNA extraction. Significance was determined by one-way anova with *p≤0.05, **p≤0.01.*

CXCL5 binding to its receptor CXCR2 has been previously associated with neu recruitment in inflammation and cancer [307 306 308 309]. Therefore, we measured by flow cytometry the expression of CXCR2 on tumor infiltrating neutrophils and we observed that it was higher on neu-SiglecF^{high} infiltrating KP-OVA tumors as compared to SiglecF^{low} counterpart (Fig.4.7).

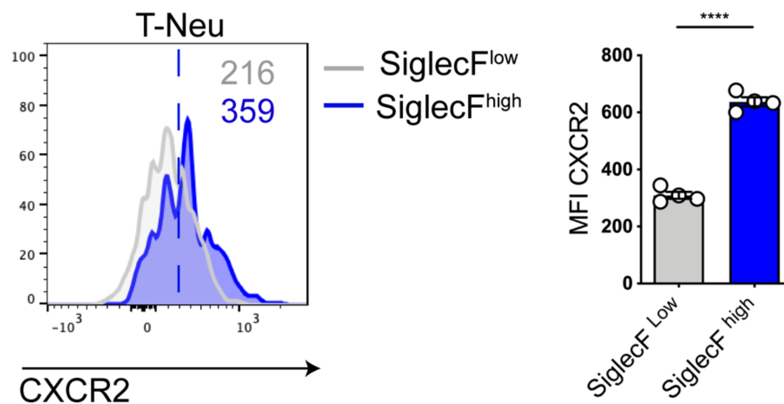

*Figure 4.7 Neu-SiglecF^{high} express high level of CXCR2 receptor. Mice were challenged with KP-OVA cells and after 18 days tumor bearing lungs were harvested and CXCR2 expression in neu-SiglecF$^{high/low}$ measured by flow cytometry. Representative histogram with corresponding median fluorescence intensity (MFI) of CXCR2 expression in neu-SiglecF$^{high/low}$ was showed. The relative quantification represents the mean±SEM of 4 mice. Significance was determined by t-test with ***p≤0.001.*

To identify the source of CXCL5 production in lung tumor tissues, we isolated by FACS cell sorting the myeloid (CD45^{+}) and not-myeloid (CD45^{-}) fractions from normal and tumor-bearing lungs and Cxcl5 expression in those fractions was assessed by qRT-PCR. The CD45^{-} fraction isolated from tumor tissues, which include stromal and tumor cells, had the highest expression of the chemokine, suggesting that cancer cells themselves may be responsible for CXCL5 production in lung cancer (Fig.4.8a). Worth noticing, the KP OVA cell line *in vitro* at steady state, unlike others tumor cells (e.g., B16 melanoma and ID8 ovarian cell line), expressed higher amount of Cxcl5 compared to nLung

tissues. Importantly, Lewis lung carcinoma cells (LLC) did not express high amount of Cxcl5 at steady state, in line with previous reports documenting that elevated Cxcl5 expression is linked to Kras mutation [3 51], absent in these cells (Fig.4.8b). We conclude that increased Cxcl5 transcripts in KP lung tumor tissues is determined mostly by secretion of CXCL5 by KP tumor cells.

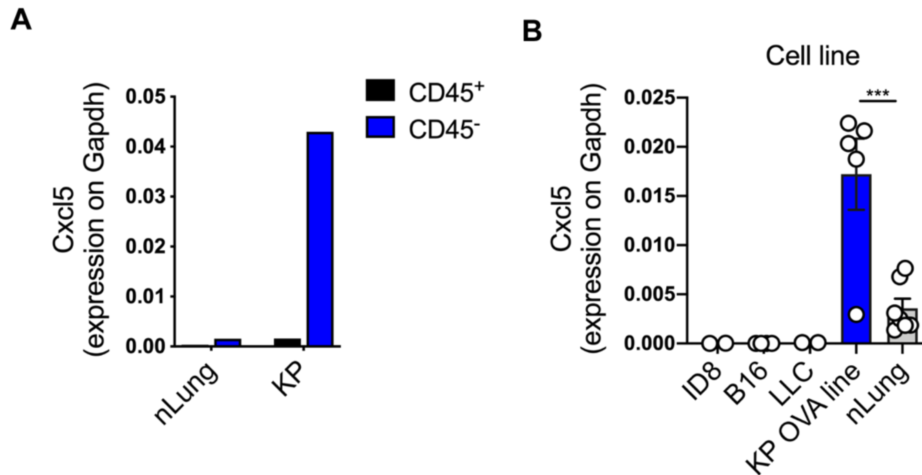

*Figure 4.8 CXCL5 is mainly produced by tumor cells. **A)** Mice were inoculated with KP cells and after 18 days CD45$^{+\ or\ -}$ were isolated by cell sorting from normal or tumor bearing lungs and Cxcl5 expression in those fractions was analyzed by qRT-PCR. Data represent the relative expression of Cxcl5 on Gapdh. **B)** Cxcl5 expression was analyzed by qRT-PCR in different cell lines at steady state and in normal lungs. The relative expression of Cxcl5 on Gapdh was showed. Data represent the mean±SEM of 2-7 independent RNA extraction. Significance was determined by one-way anova with **p≤0.01.*

4.3. Ly6G antibody-mediated depletion of neu is poorly effective in lung tumors

To explore the role of neu-SiglecF^{high} in shaping anti-tumoral immune responses, we first tested the widely applied depletion strategy with antibodies to Ly6G, the specific marker of neu. Mice were challenged orthotopically with KP OVA cells and αLy6G antibodies were administered starting from the 6th day to the 12th day, every 3 days, to induce the *in vivo* complement-mediated elimination of neu. Mice were therefore sacrificed at day 13th to perform downstream analysis (Fig.4.9a)

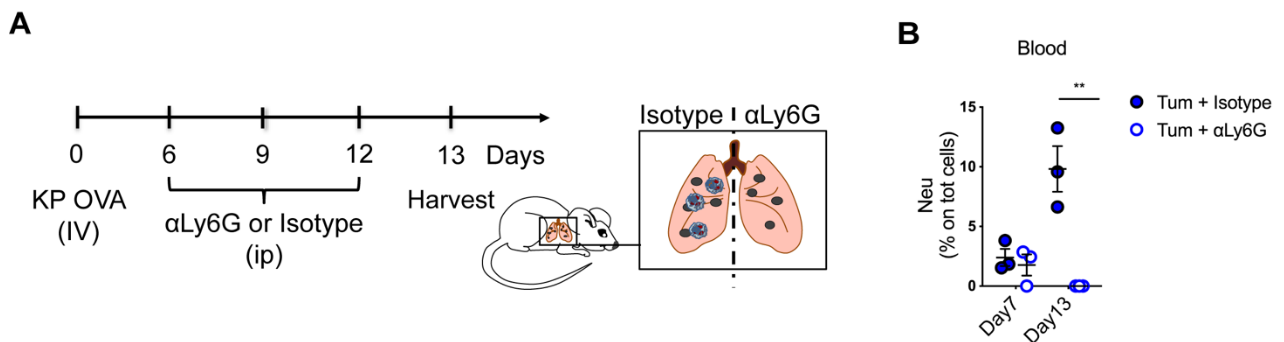

*Figure 4.9 Scheme of in vivo antibody-mediated depletion of neu. Mice were challenged with KP-OVA cells and treated every 3 days, starting from the 6th day to 12th day, with αLy6G or isotype to in vivo deplete neutrophils. Mice were sacrificed at day 13th and frequencies of neutrophils analysed in blood and lung tissues by flow cytometry. **B)** Frequencies of neutrophils CD11b^{+}Ly6G^{+} expressed as % of total cells were analysed by flow cytometry in blood of challenged mice at days 7th and 13th. Data represent the mean±SEM of 3 mice/group. Significance was determined by 2-way anova with ***p≤0.001.*

This approach efficiently depleted circulating neu that instead increased over time in blood of isotype-challenged mice (Fig.4.9b). We therefore analyzed by flow cytometry and IHC the presence of neutrophils in the lungs of challenged mice. In line with other evidence which shown the poorly effectiveness of antibody-mediated neutrophils depletion in tissues [395], frequencies of neu in αLy6G-tumor bearing lungs, analyzed by flow cytometry, slightly decreased as compared to isotype treated mice (Fig.4.10a). In addition, Ly6G labelling of paraffine embedded lung tumors revealed that neu (brown dots) still persisted within nodules upon αLy6G treatment (Fig.4.10b).

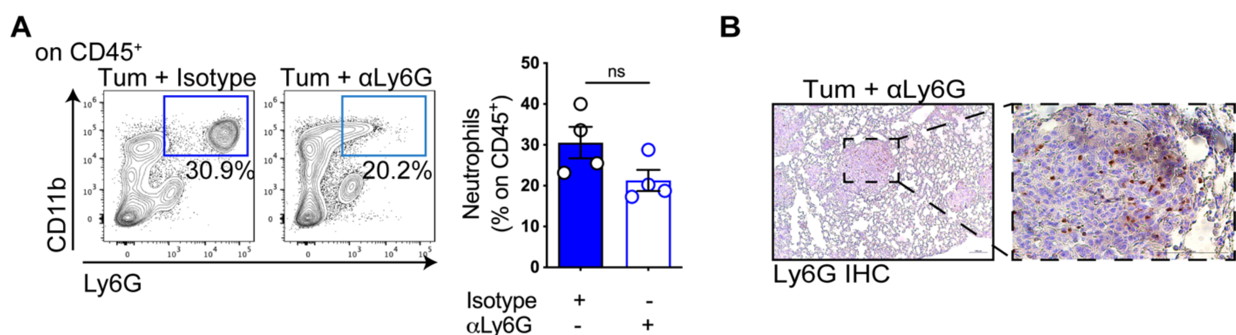

*Figure 4.10 Neutrophil-depletion is poorly effective in tumor bearing lungs. Mice were challenged with KP OVA cells and treated every 3 days, starting from the 6th day to 12th day, with αLy6G or isotype. Lungs were harvested at day 13th and frequencies of neutrophils analysed by flow cytometry and IHC. **A**) Representative dot plot and frequencies of neutrophils expressed as % of $CD11b^+Ly6G^+$ cells on $CD45^+$ cells in isotype- or Ly6G-treated tumor bearing lungs. Frequencies represent the mean±SEM of 4 mice/group. **B)** paraffine embedded lung tumors were labelled with Ly6G identifying neutrophils (brown dots). Representative 10x section (left) and 40x magnification of nodule (right) were showed.*

Of note, in αLy6G-tumor bearing lungs, neutrophils showed a decreased expression of Ly6G as compared to isotype treated challenged mice. This may be due to the phenomenon of antigen masking, whereby an antibody used *in vivo* will prevent the binding of the same antibody if used to detect the target cells, resulting in low sensitivity and false negative measures. Moreover, evidence showed that neutrophils continuously recruited from bone marrow had lower Ly6G membrane expression that also reduced targets for anti-Ly6G antibody [395].

Thus, we confirmed that the widely used approach of antibody-mediated depletion is not an effective way to deplete tumor infiltrating neutrophils in our model, stressing the need to search for a selective and specific way to target tumor infiltrating neu.

4.4. Editing of KP OVA cells by Crispr/Cas9

Based on findings presented in Figures 4.6-4.8, we next decided to delete the expression of CXCL5 by Crispr/Cas9-based genome editing of KP-OVA cells to interfere with recruitment of the neu-SiglecF^{high} subset.

To this aim, by using the online tool "Broad Institute web portal", we designed a sgRNA-guide specific for Cxcl5 gene that we cloned in a vector expressing the ZsGreen protein (sgCXCL5-ZsGreen vector). In order to avoid the constitutive expression of Cas9 protein which would arise from lentiviral transduction, this vector was transiently transfected, together with a second vector expressing the Cas9 protein (Cas9-vector), into KP OVA cell line. Therefore, after 24 hours the ZsGreen^{+} cells were isolated by FACS cell sorting and subcloned (Fig.4.11).

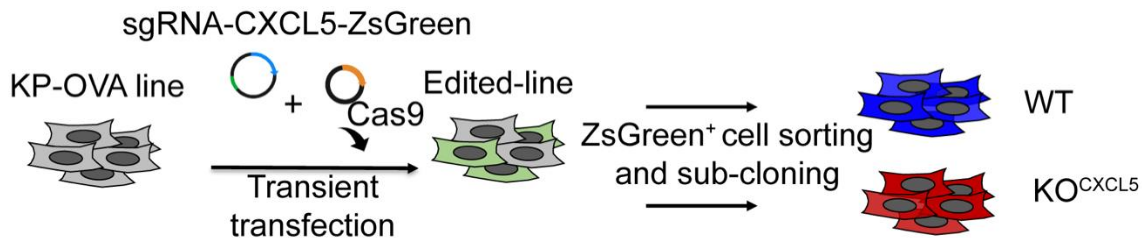

Figure 4.11 Generation of deficient CXCL5 KP-OVA cell line. Scheme of the experimental procedure. KP-OVA cell line was transiently transfected with CAS9 and sgCXCL5-ZsGreen^{+} vectors. ZsGreen^{+} cells were sorted and subcloned to identify by ELISA WT or KO^{CXCL5} clones.

Upon subcloning of edited cells, we measured by ELISA the production of CXCL5 to identify both WT and KO^{CXCL5} clones. For the screening, edited cells were incubated in medium and the supernatant collected to test chemokine production by ELISA. We selected two clones with null expression of the chemokine and two clones that showed expression comparable to the original cell line (Fig.4.12a). Finally, we pooled the selected WT or KO^{CXCL5} clones in 1:1 ratio to reduce clone-related specific effects. Therefore, the resultant KP-OVA WT and KO^{CXCL5} cells were incubated in medium and the CXCL5 production in the supernatant validated by ELISA (Fig.4.12b).

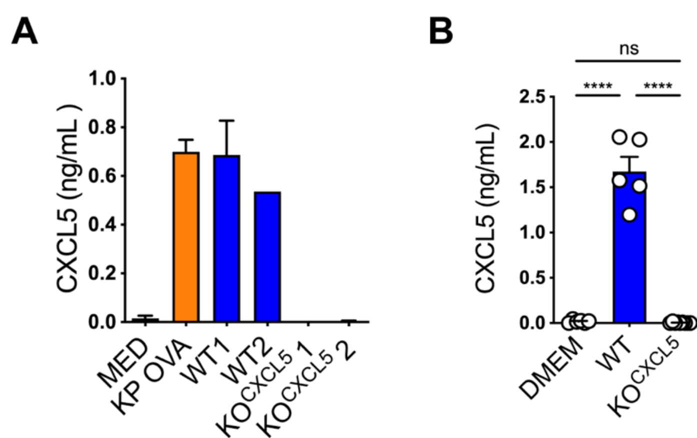

*Figure 4.12 CXCL5 protein quantification. **A)** Single clones or **B)** the pooled WT or KO^{CXCL5} clones, were incubated 5 hrs at 37°C and CXCL5 production were measured in the supernatant by ELISA Data of panel A) represents the mean±SEM of 1 or 2 independent measurements. Data of panel B) represents the mean±SEM of 5 independent measurements. Significance was determined by a one-way anova with ****$p \leq 0.0001$.*

To control whether WT and KO^{CXCL5} cells maintained a similar OVA expression downstream the editing process, we performed an intracellular staining by flow cytometry using an antibody against the HA tag. FACS analysis showed a similar OVA expression in WT cells, expressed as median fluorescence intensity, as compared to KO^{CXCL5} (Fig.4.13a). Furthermore, we assessed the growth rate of the clones *in vitro*. To this goal, equal number of cells were cultured and fixed at different time points (at day 0, at day 1 and at day 2 of culture) and a crystal violet-based assay was used to quantify cell proliferation that was similar between WT and KO^{CXCL5} cells (Fig.4.13b).

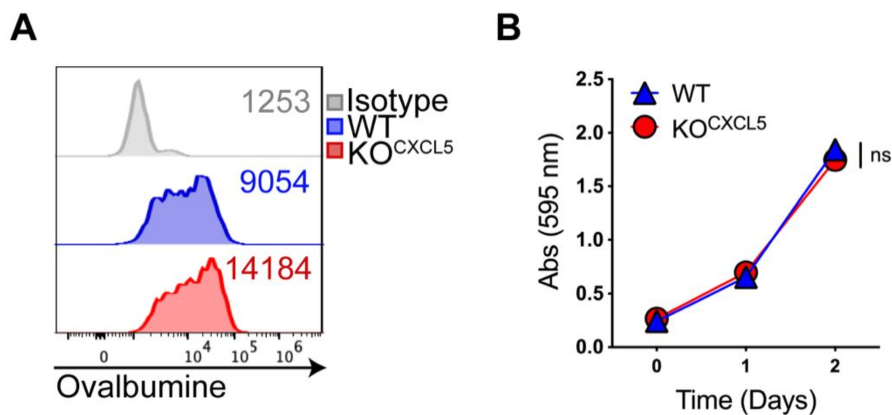

*Figure 4.13 In vitro validation of WT or deficient CXCL5 KP-OVA cell line. **A**) Representative histogram of OVA expression measured by flow cytometry in KP-OVA WT or KO^{CXCL5} cells. The median of fluorescent intensity was plotted nearby the corresponding histogram. **B)** In-vitro growth rate of KP-OVA WT or KO^{CXCL5} cells. $3x10^4$ cells were plate in a P96-well, fixed at day 0, day 1 and day 2 and the absorbance at 595nm upon the solubilization of the crystal violet dye was measured to determine the growth rate of cells. Data represent the mean±SEM of 4 independent measurements. Significance was determined by a two-way anova with ns $p > 0.05$.*

4.5. CXCL5 expressed by cancer cells regulate accumulation of neu-$SiglecF^{high}$

Once validated *in vitro*, WT and KO^{CXCL5} cells were injected into C57BL/6 mice to induce orthotopic lung cancer formation and Cxcl5 expression in tissues was assess by qRT-PCR, at two consecutive time points: at initial and at more advanced stage of tumorigenesis (Fig4.14a). Cxcl5 transcripts, as expected, were increased in WT tumor bearing lungs as compared to normal tissues, at both time points. In contrast, lungs of mice challenged with KO^{CXCL5} cells showed a minor increase in Cxcl5 expression, even at later time point, confirming that the chemokine is produced primarily by cancer cells and not by other stromal cells conditioned by the tumor environmental factors (Fig.4.14b).

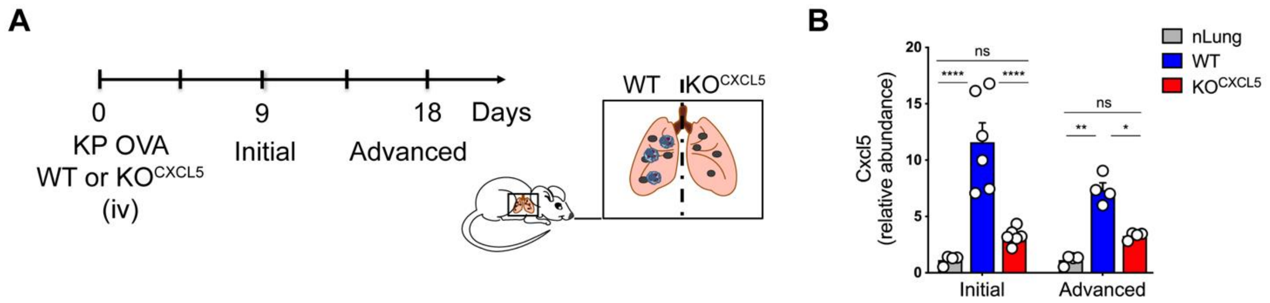

*Figure 4.14 Challenging with WT or KO^{CXCL5} cells. **A-B)** Experimental scheme. Mice were intravenous injected with KP-OVA WT or KO^{CXCL5} cells and sacrificed at initial (9 days) or advanced (18 days) time points to analyse Cxcl5 expression in lungs. **B)** Cxcl5 relative abundance on Gapdh measured by qRT-PCR from normal or tumor bearing lung at initial and at advanced time points. Data represent the mean±SEM of 4-6 independent RNA extraction. Significance was determined by 2-way anova with *p≤0.05, **p≤0.01, ****p≤0.0001.*

We next examined by flow cytometry how lack of cancer-derive CXCL5 impact on recruitment of the two subsets of neutrophils populating lung cancer. We observed that frequencies of neu-SiglecF^{low} were similar between WT or KO^{CXCL5} tumor lungs as compared to nLung, at both initial and at more advanced time points. In addition, in line with data in Fig.4.3, neu-SiglecF^{high} were highly represented in WT tumors and increased over time with tumor progression. Interestingly, tumors induced by KO^{CXCL5} cells did not show any increase in neu-SiglecF^{high} at early time points and showed only a modest increased in more advanced tumors (Fig.4.15).

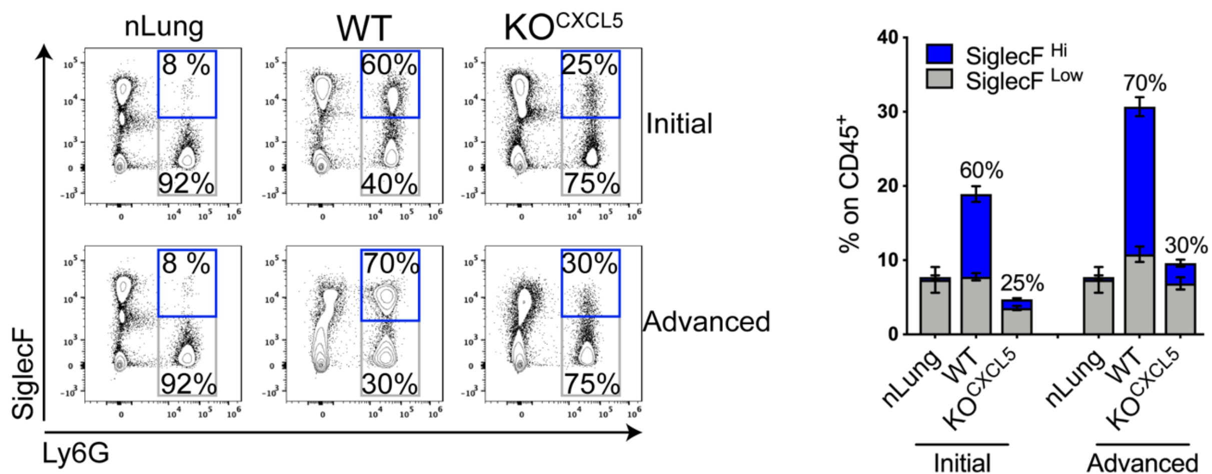

Figure 4.15 Cxcl5-null tumor lungs fail to accumulate neu-SiglecF high. Mice were intravenous injected with KP-OVA WT or KO^{CXCL5} cells and at initial and more advanced time point neu infiltrating normal and tumor bearing lungs were analysed by flow cytometry. Representative dot plot and quantification of relative abundance of SiglecF $^{high/low}$ neutrophils expressed as a fraction of CD45^{+} cells. Also, frequencies of SiglecF $^{high/low}$ neutrophils expressed on total CD11b^{+}Ly6G^{+} were showed as % in the graph. Data represent the mean±SEM of 2 independent experiment, 3-4 mice each group.

Furthermore, we performed IHC analysis by labelling paraffine embedded WT or KO^{CXCL5} lung tumor tissues with neu-specific Ly6G antibody (brown dots). In line with flow cytometry data, neu were detected within nodules of WT growing tumors. By contrast, IHC showed the lack of Ly6G labelling within tumor nodules of KO^{CXCL5} challenged mice, both at initial stages and in more advanced tumors

(Fig.4.16), confirming that lack of tumor-derived CXCL5 chemokine is sufficient to block the accumulation of neu in our model.

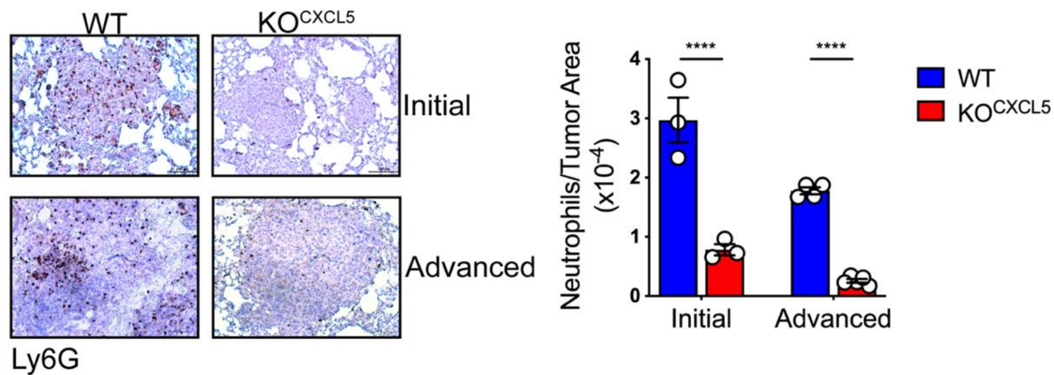

*Figure 4.16 IHC confirmed lack of neu in KO^{CXCL5} tumors. 20x representative sections and relative quantification of αLy6G staining identifying neutrophils (brown dots) in WT (left) or KO^{CXCL5} (right) tumors at day 9 (upper sections) and at day 18 (lower sections). Frequencies, expressed as neutrophils within nodules identified by haematoxylin, represent the mean±SEM of 2-3 sections/mouse, 3 mice each group. Significance was determined by a two-way anova with ****$p\leq0.0001$.*

To explore whether the deletion of CXCL5 in lung cancer cells affects the recruitment of other immune subsets expressing CXCR2 receptor (such as macrophages [398 399 400]), we used the gating strategy showed in Fig.4.2a to analyse by flow cytometry the immune landscape in KO^{CXCL5} tumor bearing lungs. Importantly, beside the significant reduction of neutrophils in KO tumors as compared to WT, we found no other statistically significant differences in immune composition between WT and KO tumors (Fig.4.17a). Moreover, to assess whether the lack of CXCL5 expression by tumor cells impacts on myelopoiesis in lymphoid organs or on circulating neutrophils, we analysed by flow cytometry frequencies of neutrophils in BM, spleen and blood of KO^{CXCL5} tumor bearing mice. We observed that frequencies of neutrophils increased in BM of KO^{CXCL5} bearing mice as compared to WT, however no statistically significant differences were observed in the spleen nor in blood of tumor bearing mice (Fig.4.17b-c).

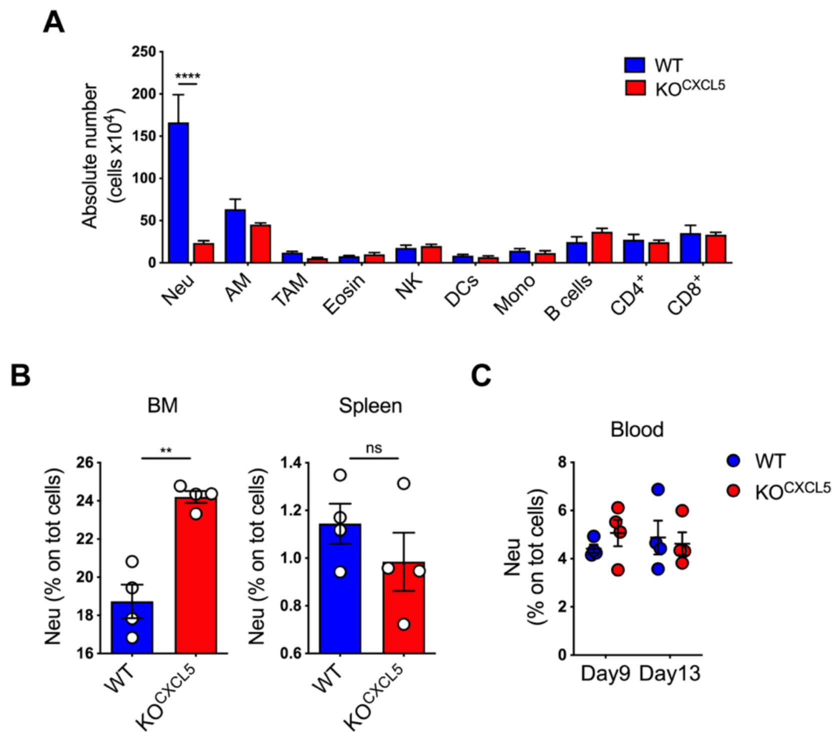

Figure 4.17 Lack of tumor-derived CXCL5 selectively affect the neu-SiglecFhigh recruitment in tumor bearing lung. ***A-C)*** *mice were challenged with WT or KO^{CXCL5} cells and 15 days after tumor induction mice were sacrificed to perform downstream analysis*. ***A)*** *Absolute numbers of different myeloid subsets were measured by counting beads by flow cytometry. Data represent the mean±SEM of 3-4 mice/group. Significance was determined by 2-way anova with ****p≤0.0001.* ***B)*** *frequencies of neu expressed as % of total cells were analysed in bone marrow and spleen by flow cytometry. Frequencies represent the mean±SEM of 3 mice/group. Significance was determined by one-way anova with **p≤0.01.* ***C)*** *frequencies of circulating neu were analysed at 2 time points. Data represent the mean±SEM of 5 mice/group.*

4.6. Re-expression of CXCL5 restore accumulation of neu-SiglecF^{high} in lung tumors

To ascertain that lack of neu accumulation in KO^{CXCL5} tumors is causally linked to the chemokine expression and does not depend on a clone specific effect, we restored CXCL5 expression in KP-OVA KO^{CXCL5} cells by lentiviral transduction. To this aim, we cloned the Cxcl5 coding sequence downstream of a CMV promoter in a lentiviral vector (lenti-vec). We produced lentiviral particles expressing CXCL5 (lenti-CXCL5) and we transduced the KP-OVA KO^{CXCL5} cells. In addition, the empty lenti-vec was used as negative control for lentiviral transduction. The transduced cells were selected under G418 resistance and the resultant KO$^{CXCL5\ (lenti\text{-}CXCL5)\ or\ (lenti\text{-}vec)}$ validated *in vitro* for CXCL5 expression (Fig.4.18).

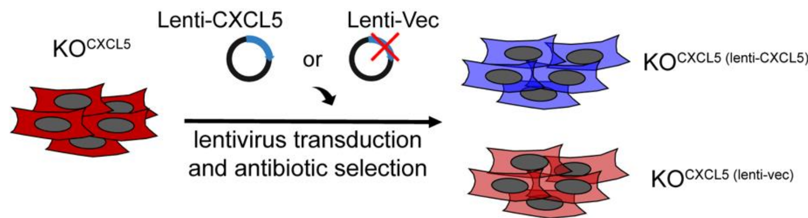

Figure 4.18 Re-expression of CXCL5 in KO tumor cells by lentiviral transduction. KP-OVA KO^{CXCL5} cells were transduced with CXCL5-expressing (lenti-CXCL5) or ctrl lentivirus (empty lenti-vec). Transduced cells were selected under G418 resistance and validated in vitro and in vivo.

Thus, transduced KO^{CXCL5} cells were incubated at steady state with medium and the production of CXCL5 were assessed in the supernatant by ELISA. Furthermore, the results were compared with data of Figure 4.12b. We observed that the production of the chemokine was higher in KO$^{CXCL5(lenti-CXCL5)}$ as compared to WT cell line. Conversely, KO^{CXCL5} and KO$^{CXCL5\ (lenti-vec)}$ showed a null expression of CXCL5 (Fig4.19a). Moreover, we performed an intracellular staining by flow cytometry against HA-OVA to compare OVA expression in the transduced cells that was similar between the KP-OVA KO$^{CXCL5(lenti-vec)}$ and the re-expressing CXCL5 counterpart as showed in Figure 4.19b. Also, growth rate in vitro was analysed as in figure 4.13b and resulted to be equal between control and CXCL5 expressing cells (Fig.4.19c).

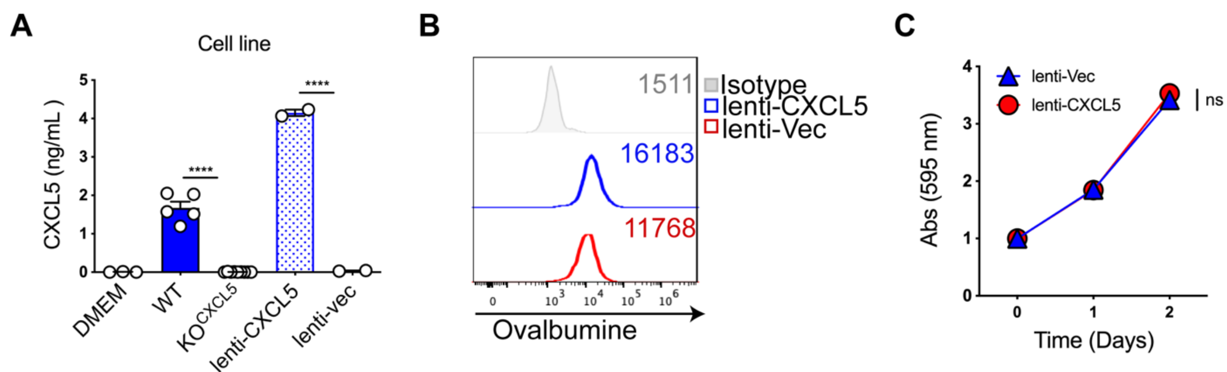

*Figure 4.19 In vitro validation of CXCL5 re-expressing cells. **A)** The production of CXCL5 chemokine were measured by ELISA from the supernatant of cells after 5hrs of incubation. Data represent the mean±SEM of 2-5 independent measurements. Significance was determined by a two-way anova with ****p≤0.0001. **B)** Representative histogram of OVA expression measured by intracellular staining by flow cytometry in KP-OVA KO$^{CXCL5(lenti-CXCL5)}$ or KO$^{CXCL5(lenti-vec)}$ cells. The corresponding median of fluorescent intensity was plotted nearby each histogram. **C)** In-vitro growth curve of KO$^{CXCL5(lenti-CXCL5)\ or\ (lenti-Vec)}$ cells. 3x10^{4} cells were plate in a P96-well and fixed at 3 different time points. Cells were incubated with crystal violet dye and upon its solubilization the absorbance at 595nm was measured. Data represents the mean±SEM of 4 independent measurements. Significance was determined by a two-way anova with ns $p > 0.05$.*

Thus, upon *in vitro* validation, we used the KO$^{CXCL5\ (lenti-CXCL5)}$ and KO$^{CXCL5\ (lenti-vec)}$ cells to induce orthotopic lung cancer formation and Cxcl5 expression in lung tissues upon challenge was measured by qRT-PCR. As shown in Figure 4.20a, in line with higher basal level observed *in vitro*, the expression of Cxcl5 was rescued in KO$^{CXCL5(lenti-CXCL5)}$ as compared to KO^{CXCL5} and was even higher than in WT tumors. As expected, lungs challenged with KO$^{CXCL5(lenti-Vec)}$ had similar expression to KO^{CXCL5} tumors.

Therefore, we analysed by flow cytometry the presence of the two subsets of neutrophils within lung tissues upon challenge with the CXCL5 re-expressing cells. Of note, accumulation of neu-SiglecF^{high} was restored in the TME of KO$^{CXCL5(lenti-CXCL5)}$ tumors, but not in control KO$^{CXCL5(lenti-Vec)}$. In addition, congruent with higher expression of CXCL5 showed in Figure 4.20a, in the setting of KO$^{CXCL5(lenti-CXCL5)}$ tumors, we noted a higher fraction of neu-SiglecF^{low} than in WT tumors, indicating that the chemokine alone is sufficient to induce neu influx. In conclusion, CXCL5 produced by KP tumor cells is the dominant factor inducing accumulation of neu in lung cancer and Cxcl5 deletion selectively and efficiently abrogates enrichment of neu-SiglecF^{high} at the tumor site.

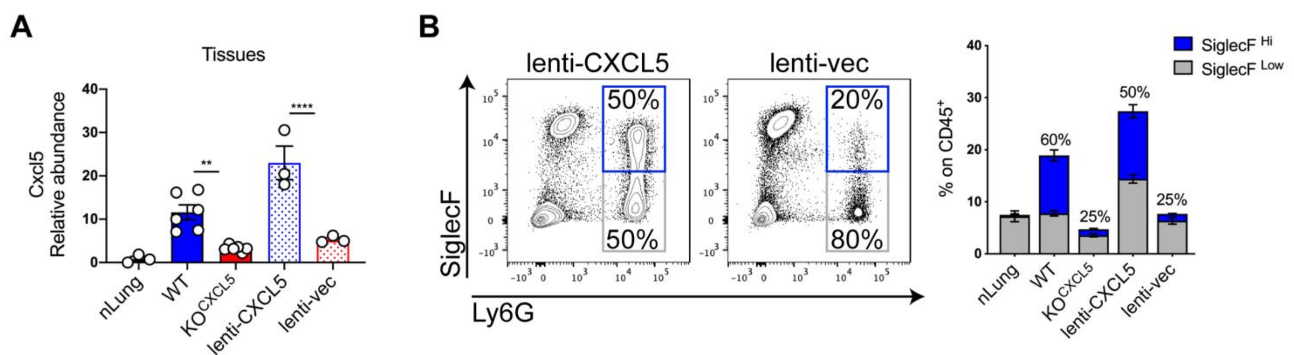

Figure 4.20 Recruitment of neu-SiglecF^{high} is restored in the TME of re-expressing KO^{CXCL5} cells. Mice were challenged with KP-OVA KO$^{CXCL5 (lenti-CXCL5) or (lenti-Vec)}$ and after 9 days lungs were analyzed. Data from previous experiments of WT and KO^{CXCL5} tumor bearing lung at initial stage were plotted together. ***A)*** *relative abundance of Cxcl5 on Gapdh was measured by qRT-PCR from normal or tumor bearing lungs. Data represent the mean±SEM of 3-6 independent RNA extraction. Significance was determined by 2-way anova with **p≤0.01, ****p≤0.0001.* ***B)*** *Representative dot plot and relative abundance of SiglecF $^{high/low}$ neutrophils expressed as a fraction of CD45^{+} cells analysed by flow cytometry. Also, frequencies of SiglecF $^{high/low}$ neutrophils expressed on total CD11b^{+}Ly6G^{+} were showed as % in the graph. Data represent the mean±SEM of one or two independent experiment, 3-4 mice each group.*

4.7. Absence of neu-SiglecF^{high} lead to an expansion of tumor specific CD8 T cells

Multiple reports documented T cell suppression by neutrophils using, in most cases, circulating or splenic neutrophils (refs in Tab.1). In a few studies, the impact of tissues infiltrating neu on T cell responses was investigated by Gr-1 or Ly6G mediated depletion, with the inherent limitations of these approaches [353 395].

Presently, the functional significance of neu-SiglecF^{high} populating KP tumors on endogenous anti-tumoral T cells remains elusive. To address this question, we took advantage of the KO^{CXCL5} model to analyse T cell responses to KP-OVA tumors in a neu-SiglecF^{high} competent or depleted tumor microenvironment. Considering their most relevant role in anti-cancer immune response and immunosurveillance [38 42 43], we focused the following analysis on early stages of tumorigenesis.

Flow cytometry data did not show any differences in the frequencies of total CD8^{+} T cells within WT and KO^{CXCL5} lung tumors (Fig.4.21a). However, flow cytometry analysis using MHC-I-OVA pentamers to label OVA-specific CD8 T cells revealed that 7% of CD8 T cells infiltrating WT lung tumors are OVA

specific and that this fraction is 3-fold higher in KO^{CXCL5} tumor bearing lung, suggesting that absence of neu-SiglecF^{high} permit a higher expansion of tumor specific CD8 T cells within tumor tissues (Fig.4.21b).

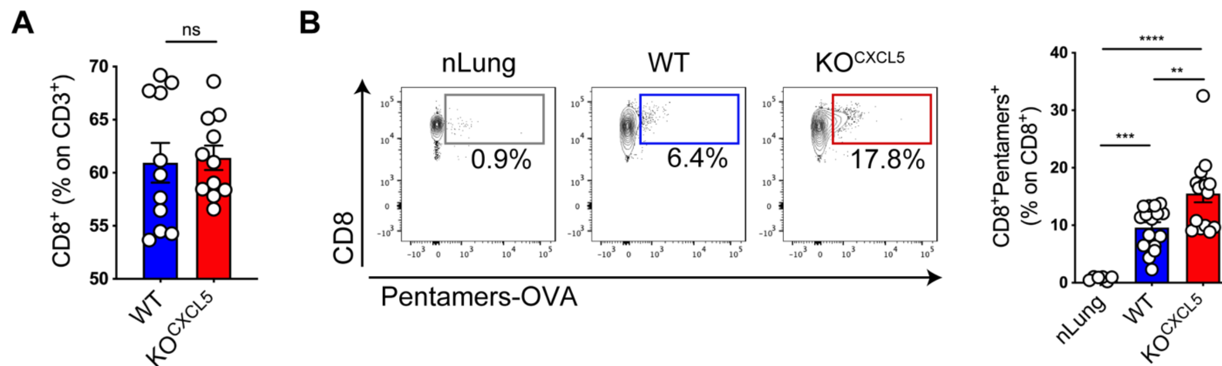

Figure 4.21 tumor specific CD8 T cells expansion within KO^{CXCL5} tumors. ***A-B)*** *Mice were challenged with WT or KO^{CXCL5} cells and frequencies of endogenous T cells were analysed by flow cytometry 9 days after induction in tumor bearing lung.* ***A)*** *Frequencies of endogenous CD8 T cells expressed as % of CD3$^+$ cells were showed. Data represent the mean±SEM of 3 independent experiments with 3-4 mice each group. Significance was determined by unpaired t-test with ns p>0.05.* ***B)*** *Frequencies of OVA specific CD8 T cells expressed as % of total CD8 T cells from healthy or tumor bearing lung were analyzed by MHCI-OVA pentamers by flow cytometry. Data represent the mean±SEM of 4 independent experiments with 4 mice/group. Significance was determined by one-way anova t test with **p≤0.01, ***p≤0.001, ****p≤0.0001.*

To corroborate this hypothesis, we labelled paraffine embedded WT or KO^{CXCL5} tumor bearing tissues with CD8 antibody to visualize the spatial distribution of CD8$^+$ T cells (brown dots) within tissues. Despite FACS analysis did not show increased in overall frequencies (Fig.4.21a), IHC analysis showed that CD8$^+$ T cells were enriched within nodules of KO^{CXCL5} tumors as compared to WT (Fig.4.22).

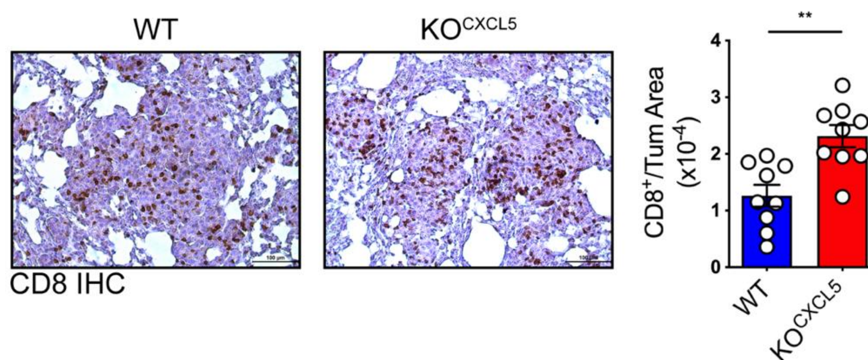

*Figure 4.22 CD8 T cells accumulate in nodules of KO^{CXCL5} tumors. Mice were challenged with WT or KO^{CXCL5} cells and 9 days after induction tumor bearing lungs were harvested to analyze CD8 T cell distribution by IHC. Thus, paraffine embedded WT or KO^{CXCL5} tumor bearing lung tissues were labelled with CD8 antibody (brown dots). Quantification of CD8 T cells expressed as ratio of CD8 and tumor area measured by hematoxylin staining represent the mean±SEM of 3 independent experiments with 2-3 mice each group. Significance was determined by an unpaired t test with **p≤0.01.*

4.8. CD8 T cells in KO^{CXCL5} tumors are highly activated and cytotoxic

To begin assessing the functionality of tumor infiltrating CD8 T cells in the two conditions, we started by testing expression of surface activation markers on CD8 T cells by flow cytometry. It has been

shown that CD8 T cells co-expressing PD-1, LAG3 and TIM3 include a group of highly activated and functional cells [95]. As showed in Figure 4.23a, WT tumors were characterized by an expansion of up to 15% of triple positive CD8 T cells compared to nLung that was major in lungs of mice bearing KO^{CXCL5} tumors. Furthermore, we measured by flow cytometry the frequencies of $CD62L^-$ $CD44^+$ CD8 T cells, defined as effector memory T cells, in tumor bearing lungs. We observed an increase of this population in WT tumors as compared to nLung with the highest representation in KO^{CXCL5} tumors (Fig.4.23b). Lastly, we noticed an enrichment, that occurred only in KO tumors, of $EOMES^-$ $T\text{-}bet^+$ CD8 T cells, which have been recently demonstrated to represent activated CD8 T cells that did not run the exhaustion program [93] (Fig.4.23c). Overall, these data showed that CD8 T cells are more active in a neu-$SiglecF^{high}$ depleted lung tumor microenvironment as compared to CD8 T cells in $SiglecF^{high}$ competent counterpart. To directly test the cytotoxic functions of CD8 T cells we next performed *ex-vivo* restimulation and intracellular staining to measure IFN-γ production.

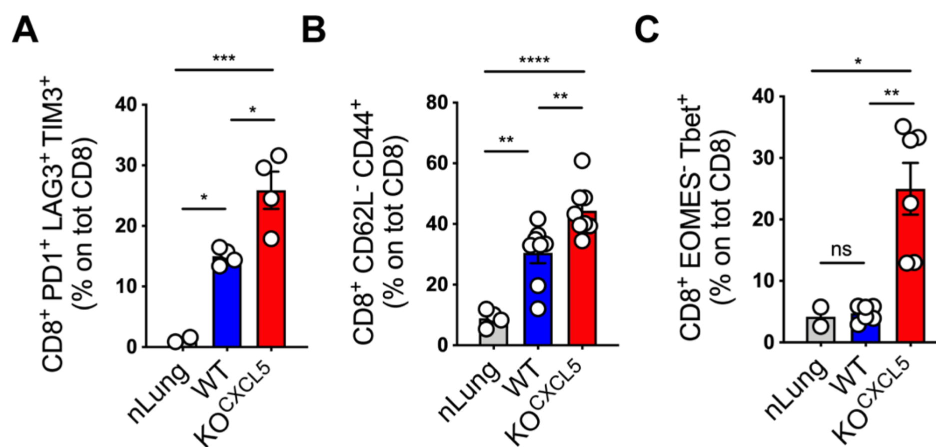

Figure 4.23 CD8 T cells activation in WT or KO^{CXCL5} tumors. ***A-C)*** *Mice were inoculated with KP-OVA WT or KO^{CXCL5} cells and 9 days after challenge markers of activation/memory in CD8 T cells were analyzed by flow cytometry from normal or tumor bearing lungs.* ***A)*** *frequencies of triple positive (PD1+LAG3+TIM3+) CD8 T cells* ***B)*** *frequencies of effector memory (CD62l-CD44+) CD8 T cells and* ***C)*** *frequencies of effector EOMES-Tbet+ CD8 T cells, expressed as % of total CD8 T cells, were analyzed by flow cytometry. Data represent mean±SEM of 4-8 mice/group. Significance was determined by one-way anova with *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001.*

We detected up to 5% of IFN-γ producing CD8 T cells in lung tissues bearing WT KP-OVA cells indicating activity of tumor infiltrating CD8 T cells. Remarkably, the fraction of IFN-γ producing CD8 was twice as much in lungs of animal carrying KO^{CXCL5} tumors (Fig.4.24), indicating that CD8 T cells infiltrating lung tumors are more active and cytotoxic in absence of neu-$SiglecF^{high}$.

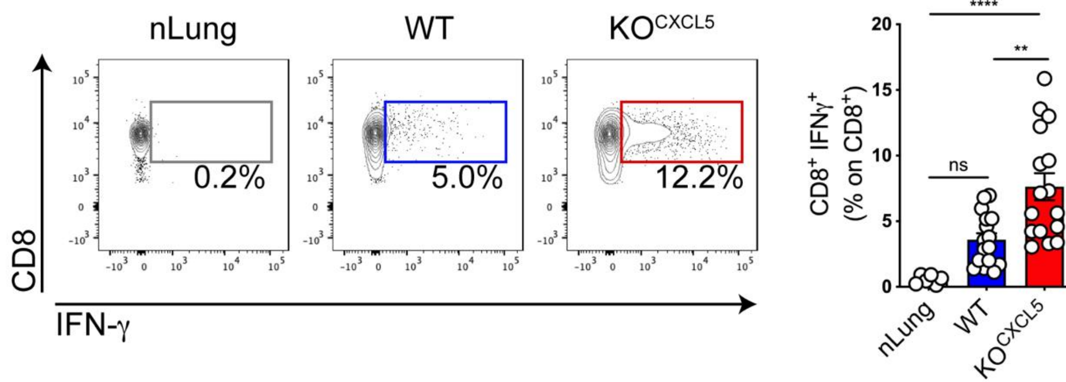

*Figure 4.24 CD8 T cells produce higher level of IFN-γ in KO^{CXCL5} tumors. Representative dot plot and quantification of ex-vivo restimulation of endogenous CD8 T cells. Mice were injected with KP-OVA WT or KO^{CXCL5} and 9 days after challenge T cells from healthy or tumor bearing lung were re-stimulated ex-vivo 4hrs with SIINFEKL in presence of Golgi Stop and the IFN-γ production measured by intracellular staining by flow cytometry. Data represent mean±SEM of 4 independent experiments with 4 mice each group. Significance was determined by one-way anova with **p≤0.01, ****p≤0.0001.*

To additionally demonstrate the causal link between the higher activation of CD8 T cells and the absence of neu-SiglecF^{high} in lung tumor microenvironment, we performed the adoptive transfer (AT) of naïve OVA-specific CD8 T cells, isolated from OTI mice labelled with cell trace CFSE dye, into WT or KO^{CXCL5} tumor bearing mice. Two days after the AT we analyzed by flow cytometry the dilution of CFSE, corresponding to the proliferation of OTI CFSE^{+} T cells *in vivo*. As showed in Figure 4.25, the fraction of proliferated-OTI T cells was similar in WT and KO^{CXCL5} tumors.

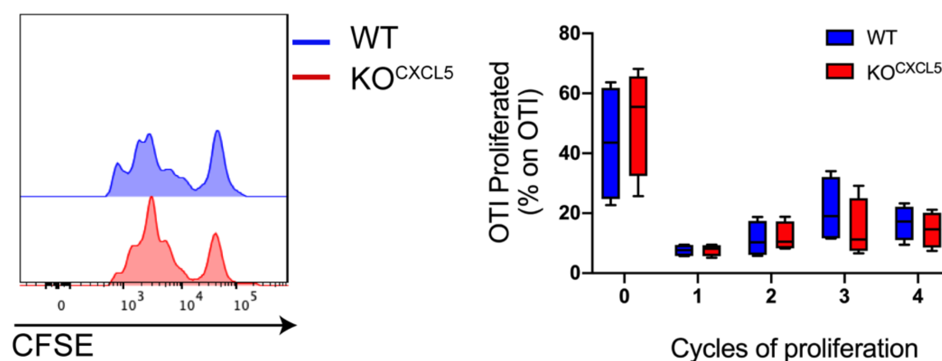

Figure 4.25 In vivo proliferation of transferred OVA specific CD8 T cells. Mice were challenged with WT or KO^{CXCL5} cells and 9 days after tumor induction, naïve OVA specific OTI CFSE^{+} T cells were adoptively transferred into tumor bearing mice and their proliferation was analyzed two days later by CFSE dilution by flow cytometry. Representative histogram of CFSE dilution and frequencies of proliferated OTI T cells for each cycle of proliferation expressed as % of total OTI were showed. Data represent the mean±SEM of 4 mice each group. Significance was determined by two-way anova.

However, by *ex-vivo* restimulation of OT-I T cells performed as described before, we observed a 2-fold higher frequency of IFN-γ producing OTI T cells in KO^{CXCL5} tumors as compared to WT, suggesting that exogenous tumor specific T cells transferred into a neu rich environment are induced to proliferate but their effector functions are suppressed by the presence of neu-SiglecF^{high} (Fig.4.26).

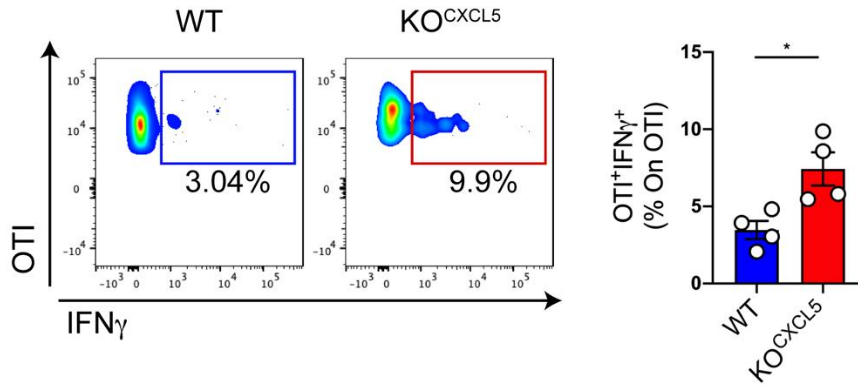

*Figure 4.26 Adoptively transferred OTI T cells produce higher level of IFN-γ in KO^CXCL5 tumors. Representative dot plot and quantification of ex-vivo restimulation of adoptively transferred OTI T cells. Mice were injected with KP-OVA WT or KO^CXCL5 and 9 days after challenge naïve CD8 T cells isolated from OTI mice were adoptively transferred (AT) in tumor bearing mice. Two days after AT OTI T cells were re-stimulated ex-vivo 4hrs with SIINFEKL in presence of Golgi Stop and the IFN-γ production measured by intracellular staining by flow cytometry. Data represent mean±SEM of 4 mice each group. Significance was determined by unpaired t-test with *p≤0.05.*

To furthermore underscore the importance of an appropriate system to assess neu-T cells crosstalk, we performed assays of CD8 T cell activation in the contest of a Ly6G-mediated neu depletion during challenge with KP OVA cells (Fig.4.8a).

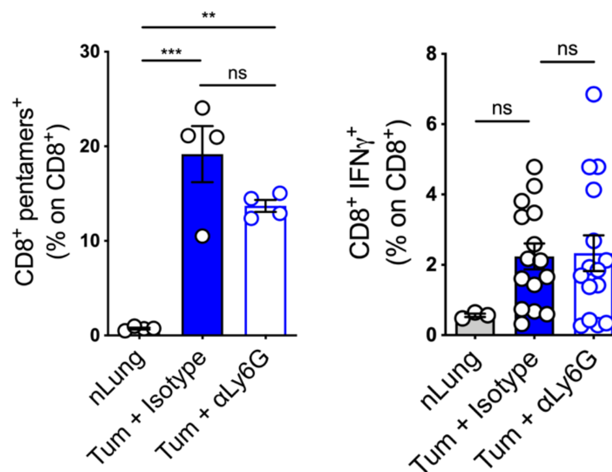

*Figure 4.27 Mice were challenged with KP-OVA cells and starting from the 6th to 12th day treated every 3 days with αLy6G or isotype to in vivo deplete neutrophils. Lungs were harvested at day 13th and frequencies of tumor specific CD8 T cells analysed by MHCI-OVA pentamers (left) and IFN-γ producing CD8 T cells upon ex-vivo restimulation with SIINFEKL and Golgi stop (right) were analysed by flow cytometry in normal or tumor bearing lungs. Frequencies expressed as % of total CD8 T cells represent the mean±SEM of 1 or 3 experiments with 4-5 mice each group. Significance was determined by one-way anova with ns p>0.05, **p≤0.01, ***p≤0.001.*

Opposite to the rescue of T cell responses observed in KO^CXCL5 tumors, neither the expansion of endogenous tumor specific CD8 T cells measured by MHC-I-OVA pentamers by flow cytometry (left), nor the increase of IFN-γ producing CD8 T cells upon *ex-vivo* restimulation (right) was observed in Ly6G-mediated neu depleted tumor bearing lung compare to KP-OVA tumors (Fig. 4.27), underling the relevance of KO^CXCL5 model.

4.9. Increased number of effector memory CD8 T cells in mLN draining KO tumors

Given that we found a small fraction of neu-SiglecF^{high} in lymph nodes draining KP OVA lung tumors (Fig.4.4), we decided to analyze by flow cytometry the activation of CD8 T cell in mLN draining WT or KO^{CXCL5} tumor bearing lungs. We observed that activated triple positive PD1^{+}LAG3^{+}TIM3^{+} CD8 T cells are not represented in mLN (Fig.4.28a). On the other hand, consistent with higher activation of CD8 T cells observed in lungs bearing KO tumors, we observed a significative increase of effector memory CD8 T cells in mLN draining neu-SiglecF^{high} deficient tumors as compared to mLN draining WT tumors in which instead the fraction of CD62l^{-}CD44^{+} CD8 T cells was similar to nLung (Fig.4.28b). However, beside an increase if compared to mLN draining nLung, FACS analysis did not show any differences between fractions of effector EOMES^{-}T-bet^{+} CD8 T in mLN draining WT and KO^{CXCL5} tumors (Fig.4.28c), nor between frequencies of IFN-γ producing CD8 T cells upon *ex-vivo* restimulation (Fig.4.28d), suggesting that neu-SiglecF^{high} in mLN are not sufficient to impair CD8 T cells activation.

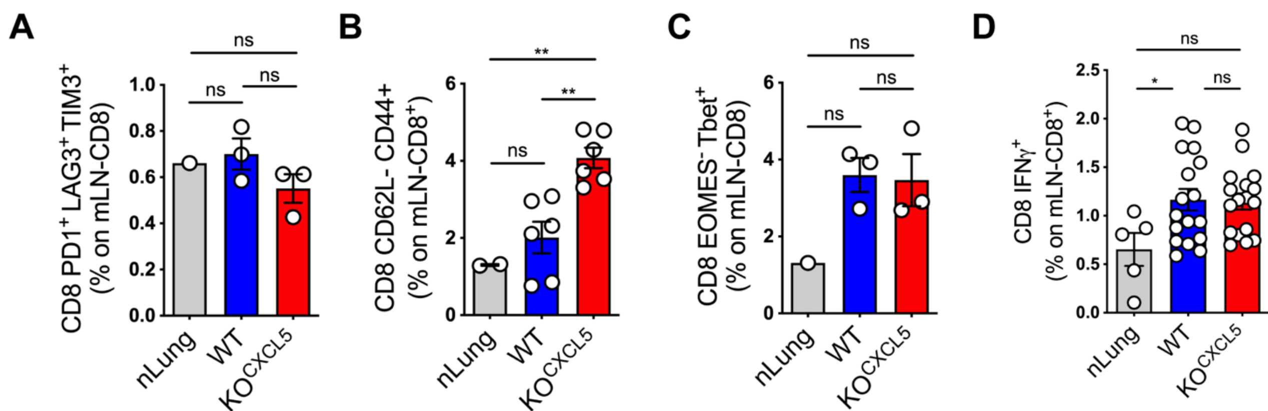

Figure 4.28 Effector memory CD8 T cells increased in LNs draining KO^{CXCL5} tumors. ***A-D)*** *Mice were challenged with KP-OVA WT or KO^{CXCL5} and T cell were analyzed by flow cytometry in LNs draining normal or tumor bearing lung (mLN).* ***A)*** *frequencies of triple positive (PD1+LAG3+TIM3+) CD8 T cells* ***B)*** *frequencies of effector memory (CD62l-CD44+) CD8 T cells and* ***C)*** *frequencies of effector EOMES-Tbet+ CD8 T cells, expressed as % of total CD8 T cells, were analyzed by flow cytometry. Frequencies represent the mean±SEM of 3-6 mice each group. Significance was determined by one-way anova with ns p>0.05, **p≤0.01.* ***D)*** *T cells from mLN of healthy or tumor bearing mice were re-stimulated ex-vivo 4hrs with SIINFEKL in presence of Golgi stop and the IFN-γ production measured by intracellular staining by flow cytometry. Data represent the mean±SEM of 5 independent experiments with 3-4 mice each group. Significance was determined by one-way anova with ns p>0.05, *p≤0.05.*

4.10. CD4 T cells activation is not affected by neu-SiglecF^{high}

Beside the key role of CD8 T cells in cancer, also CD4 T cells can contribute to elimination phase of tumors [31 30]. To assess whether the neu-SiglecF^{high} impact on CD4 T cells functions, we analysed the expression of activation markers on CD4 T cells in WT or KO^{CXCL5} models by flow cytometry, both in lungs and draining lymph nodes (Fig. 4.29). FACS analysis showed that, WT and KO^{CXCL5} tumors were characterized by an increase of triple positive PD1^{+} LAG3^{+} TIM3^{+} CD4 T cells, even if in KO tumors it

was not statistically significant (Fig.4.29a-left). In line with data on CD8 T cells (Fig.4.28a), this population was not represented in draining lymph nodes (Fig.4.29b-left). In addition, we analysed by flow cytometry the frequencies of effector memory CD4 T cells in tumor bearing lungs. We noted that $CD62l^{-}CD44^{+}$ CD4 T cells expanded at the same rate in WT and KO^{CXCL5} tumor bearing lungs as compared to nLung (Fig.4.29a-right), and no differences were observed in draining lymph nodes (Fig.4.29b-right). Overall, these data suggest that neu-$SiglecF^{high}$ do not significantly impact on the activation of CD4 T cell compartment.

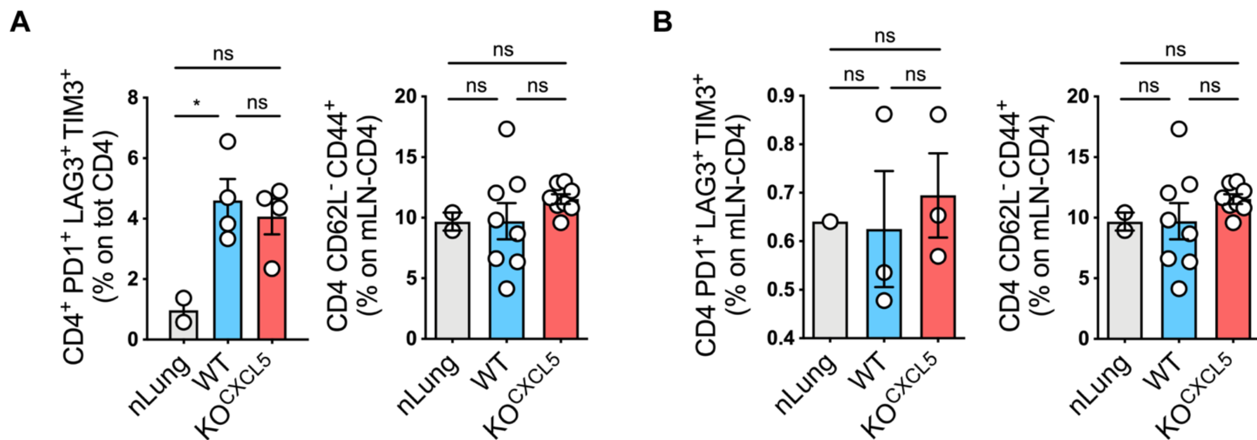

*Figure 4.29 CD4 T cell are not affected by neu-$SiglecF^{high}$. **A-B)** Mice were challenged with KP-OVA WT or KO^{CXCL5} and 9 days after induction CD4 T cell were analyzed by flow cytometry in normal and tumor bearing lungs and corresponding draining lymph nodes. **A)** frequencies of triple positive (PD1+LAG3+TIM3+) CD4 T cells (left) and frequencies of effector memory (CD62l-CD44+) CD4 T cells (right) expressed as % of total CD4 T cells were analyzed by flow cytometry in normal or tumor bearing lungs. Frequencies represent mean±SEM of 1 or 2 experiments with 4 mice/group. Significance was determined by one-way anova with ns $p>0.05$, *$p\leq0.05$, ***$p\leq0.001$. **B)** frequencies of triple positive (PD1+LAG3+TIM3+) CD4 T cells (left) and frequencies of effector memory (CD62l-CD44+) CD4 T cells (right) expressed as % of total CD4 T cells were analyzed by flow cytometry in lymph nodes draining normal or tumor bearing lungs. Frequencies represent the mean±SEM of 3-8 mice each group. Significance was determined by one-way anova with ns $p>0.05$.*

4.11. Neu-$SiglecF^{high}$ may reduce CD8 T cell activation by a contact dependent mechanism

Inhibition of T cell functions by neu infiltrating tumors has been attributed to several pathways such as production of ROS, T cell anti-proliferative molecules, nutrient deprivation (Tab1). However, the mechanism underlying the T cell inhibitory potential of neu-$SiglecF^{high}$ is not known.

We first explored whether neu-$SiglecF^{high}$ may impact on antigen presentation by cDC1s, a key subset to initiate T cell responses. We analysed by flow cytometry the maturation state of cDC1 in WT and KO^{CXCL5} models. Mature DCs upregulate the expression of molecules such as MHCII and CD86 acting as co-stimulatory molecules for T cells activation. FACS analysis showed that MHCII expression slightly increased on cDC1 from KO^{CXCL5} tumors as compared to WT, however the expression of CD86 molecule was similar between cDC1 from the two models (Fig.4.30). These data

suggest that at earlier time point of tumorigenesis when effects on CD8 T cells are already visible, cDC1 compartment is only marginally affected.

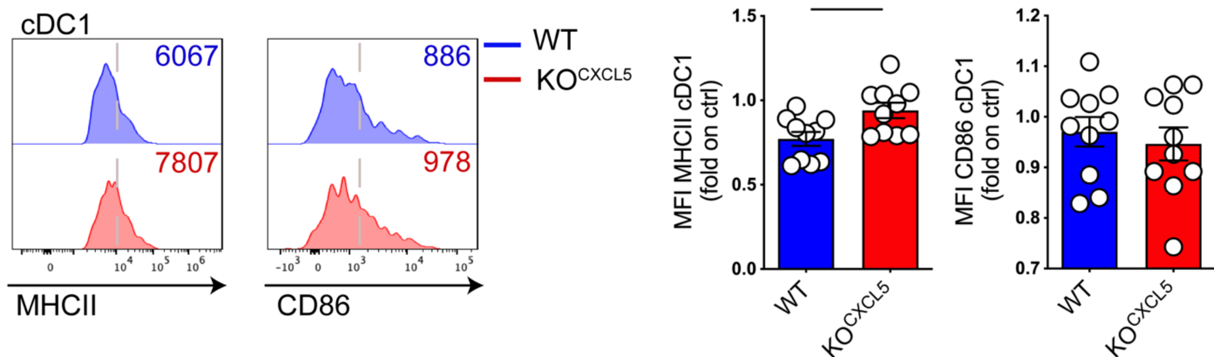

*Figure 4.30 Maturation of cDC1 was similar between WT and KO^{CXCL5} tumors. Mice were challenged with WT or KO^{CXCL5} cells and 9 days after induction the expression of MHCII and CD86 were analyzed by flow cytometry on cDC1 from tumor bearing lungs. Representative histogram with corresponding median fluorescence intensity (MFI) were showed. Quantifications expressed as MFI fold on nLung represent the mean±SEM of 2 experiments with 5 mice each group. Significance was determined by unpaired t-test with *p≤0.05.*

As a second approach to understand mechanism of neu-SiglecF^{high} mediated T cell suppression, we labelled paraffine embedded KP OVA tumors with both CD8 and Ly6G antibodies to study the relative spatial distribution of the two population within tumor nodules. We found nodules of different area at the initial stage of tumorigenesis tested. Interestingly, small nodules were heavily infiltrated by CD8 T cells and contained few neutrophils whereas nodules of intermediate size contained equal proportions of CD8 T cells and neutrophils. In contrast, large nodules were heavily enriched in neutrophils and depleted in CD8 T cells. In addition, we often observed CD8 T cell surrounded by neutrophils (Fig.4.31). Overall, these data suggest that neutrophils accumulating in lung tumor nodules, may directly impair CD8 T cell activation in a cell contact dependent manner or through a physically exclusion of CD8 T cells.

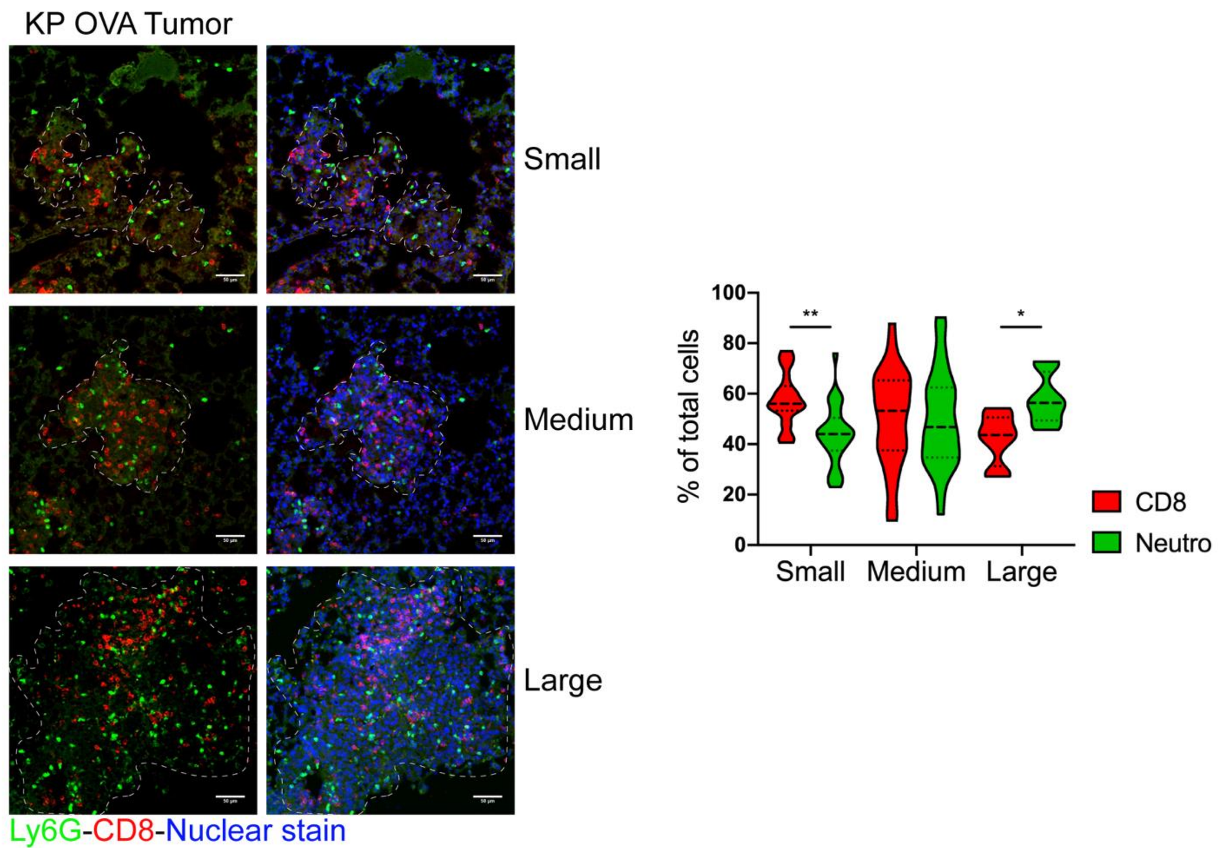

*Figure 4.31 large nodules are mostly populated by neutrophils in KP OVA tumors. Mice were challenged with KP OVA cells and 9 days after induction tumor bearing lungs were harvested to analyze neutrophils and CD8 T cell distribution by IF. Thus, paraffine embedded KP OVA tumor tissues were labelled with Ly6G and CD8 antibodies. Nodules were classified based on area and inside each nodule neutrophils and CD8 T cells were quantified. Data represent the mean±SEM of 2 independent experiments with 2 mice each experiment. Significance was determined by two-way anova with *p≤0.05, **p≤0.01.*

4.12. Neu-SiglecF^{high} contribute to tumor growth and limit PD-L1 blockade efficiency

To evaluate how lack of neu-SiglecF^{high} in the TME impact the tumor growth, we measured by H/E the tumor burden in paraffine embedded lungs after challenge with WT or KO^{CXCL5} cells, at two different time points. At initial time point, in line with higher activation of CD8 T cells described in previous chapters (4.7 and 4.8), tumor burden was reduced in KO^{CXCL5} tumors as compared to WT, however, this advantage was lost at more advanced stage of tumorigenesis. To corroborate this result, we performed IHC analysis by labelling the proliferating Ki67^{+} cells (brown dots) within nodules of paraffine embedded WT or KO^{CXCL5} tumor tissues. As expected, the proliferating cells were reduced in KO tumors when compared to WT at the initial time point. However, the difference was lost at later time point (Fig.4.32). Overall, these data suggest that tumor containment at initial time point of tumorigenesis in depleted neu-SiglecF^{high} tumors, is bypassed at later stages.

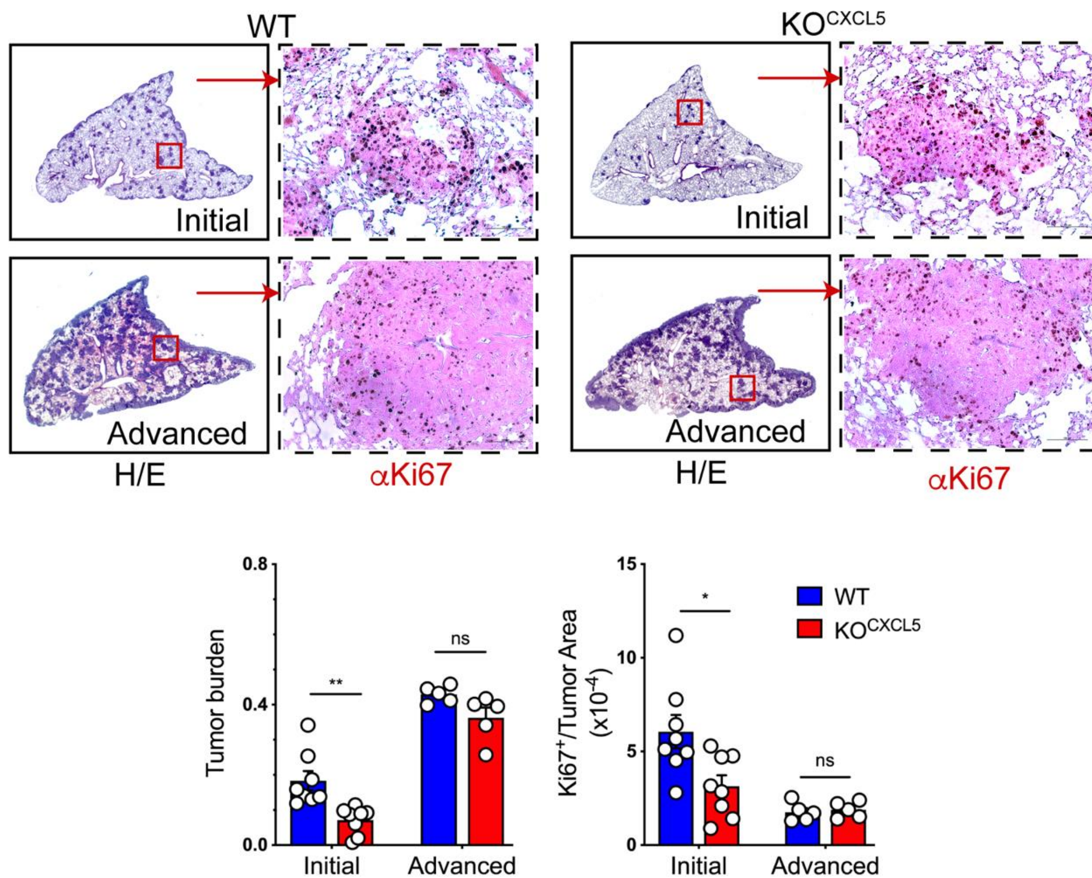

*Figure 4.32 Tumor growth was reduced in KO^{CXCL5} tumors. Mice were challenged with KP-OVA WT or KO^{CXCL5} cells and 9 or 18 days later lungs were paraffine embedded to perform downstream analysis by IHC. Representative sections of tumor burden (left) and representative 20x sections of proliferating Ki67^{+} cells (right-brown dots) were shown. Tumor burden was expresses as ratio of tumor area measured by H/E staining and total area of lobe. Proliferating cells were expressed as ratio of Ki67^{+} cells (brown dots) counted within nodules identified by eosin staining. Data represent the mean±SEM of 1 or 2 experiments with 4-5 mice each group. Significance was determined by one-way anova with ns $p>0.05$, *$p \leq 0.05$, **$p \leq 0.01$.*

Expression of inhibitory ligands on tumor cells and tumor infiltrating myeloid cells is a key obstacle to spontaneous T cell responses to tumor antigens [60 61]. To assess whether neu-SiglecF^{high} contribute to increase the burden of inhibitory signals in the TME, we measured by flow cytometry the expression of PD-L1 on neutrophils infiltrating KP OVA tumors. Of note, the expression of PD-L1 on tumor-derived neu-SiglecF^{low} was similar to neutrophils from nLung. By contrast, PD-L1 expression was significatively increased in SiglecF^{high} counterpart (Fig.4.33a). Also, due to the lack of accumulation of neu-SiglecF^{high} in KO^{CXCL5} model, the frequencies of PD-L1 expressing neu was consistently low as compared to WT tumors (Fig.4.33b). Therefore, we reasoned that blocking of neu-SiglecF^{high} infiltration combined to check-point inhibitors could enhance rejection of tumors even at later time point.

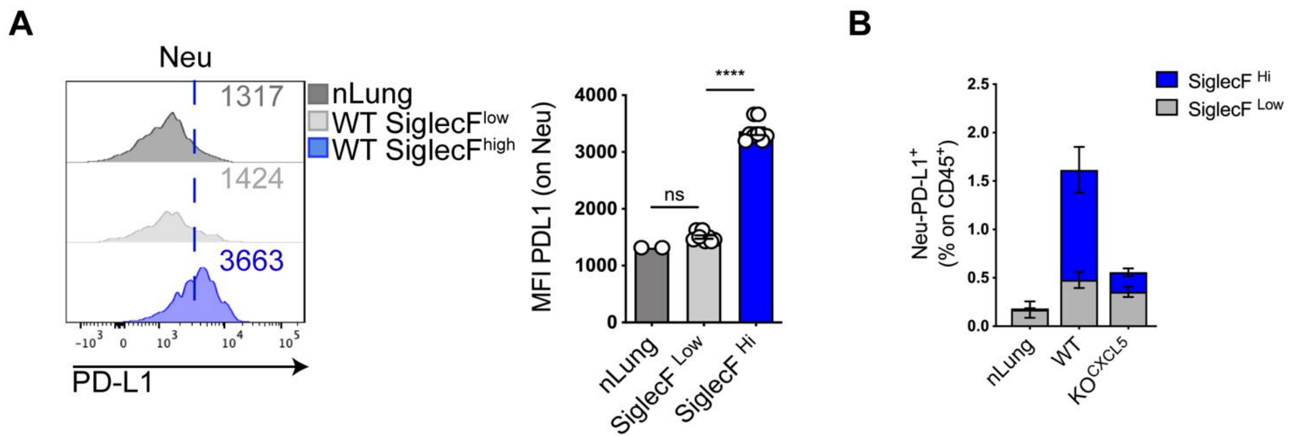

*Figure 4.33 High expression of PD-L1 on neu-SiglecF^{high}. **A)** Mice were challenged with WT tumor cells and 15 days after induction PD-L1 expression on neutrophils infiltrating normal or lung tumors were analyzed by flow cytometry. Representative histograms of PD-L1 with corresponding median fluorescence intensity were shown. Relative quantification represents the mean±SEM of 2 experiments with 4 mice each group. Significance was determined by one-way anova with ns $p>0.05$, ****$p\leq0.0001$. **B)** Mice were challenged with WT or KO^{CXCL5} tumor cells and after 15 days frequencies of neu expressing PD-L1 as % of CD45^{+} cells in normal or tumor bearing lungs were analyzed by flow cytometry.*

To assess this question, we administered blockade to PD-L1 in mice upon challenge with WT or KO^{CXCL5} cells as depicted in the scheme (Fig.4.34), therefore we analysed the tumor burden at advanced stage of tumor by H/E staining of paraffine embedded lung tumor tissues.

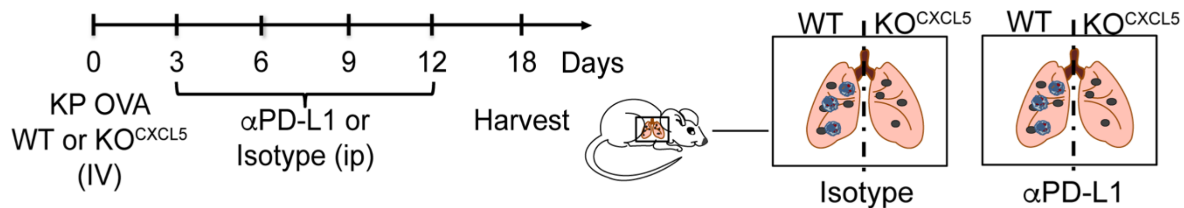

Figure 4.34 Scheme of PD-L1 blockade. Mice were challenged with WT or KO^{CXCL5} and blockade to PD-L1 was administered every 3 days from 3rd to 12th day. At 18th day mice were sacrificed and lung of treated tumor bearing mice were paraffine embedded to analyze the tumor growth by H/E analysis.

Treatment with isotype used as control showed that, as expected, the tumor containment in depleted neu-SiglecF^{high} tumors was bypassed at later stages and no differences in tumor growth were observed as compared to WT tumors. By opposite, PD-L1 blockade led to a strong reduction of tumor burden in mice carrying KO^{CXCL5} tumors, whereas had a marginal effect on the growth of WT tumors. In conclusion, these results show that neu-SiglecF^{high} in lung cancer limit the efficacy of PD-L1 blockade (Fig.4.35).

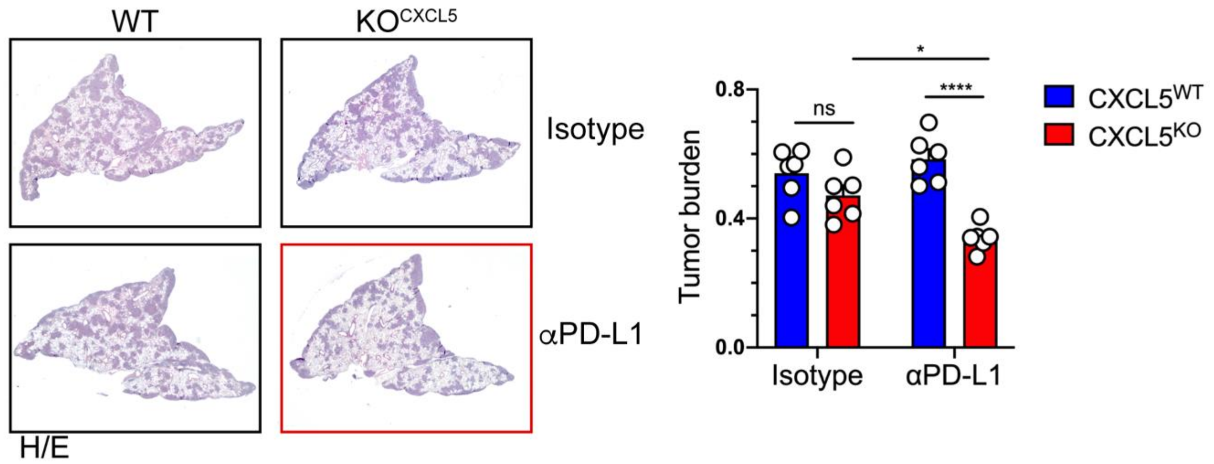

*Figure 4.35 PD-L1 blockade is more effective in KO^{CXCL5} tumors. Mice were challenged with WT or KO^{CXCL5} cells and blockade of PD-L1 was administered as depicted in Figure 4.34. Representative sections and relative quantification of tumor burden from paraffine embedded tumor bearing lung treated with isotype or PD-L1 blockade were shown. Tumor burden was expressed as ratio of tumor area measured by H/E analysis and total area of lobe. Data represent the mean±SEM of 2 experiments with 3 mice each group. Significance was determined by two-way anova with ns $p>0.05$, *$p\leq0.05$, ****$p\leq0.0001$.*

5. Discussion

Neutrophils represent an important component of the tumor microenvironment in different type of cancers, including lung cancer and are mostly associated to bad prognosis. Several studies demonstrated that neutrophils play an essential role in tumorigenesis and progression via induction of genetic instability [324 325], stimulation of angiogenesis [332 334 336], promotion of metastasis [279 353 296], enhancement of cancer cells proliferation [327 354 355] and modulation of immune system [368 274 362 287]. Nevertheless, it has been proven that neutrophils mediate resistance against primary carcinogenesis in some cancer type and tumor stages through direct killing of cancer cells and activation of T cell-dependent anti-tumor immunity [57 383 387]. Many factors are involved in their recruitment and polarization into phenotypically distinct pro- or anti-tumorigenic sub-populations. However, mechanisms behind these processes are still elusive and strongly dependent on the tumor type, the cellular microenvironment and immune modulating factors in the TME. Thus, assess the complexity of neutrophils in a specific microenvironment will provide the basis to develop new therapeutic approaches.

Recently, scRNA-seq analysis allowed to identify different subsets of neutrophils in human NSCLC as well as in orthotopic lung adenocarcinoma model providing insight into lung tumor associated neutrophils heterogeneity [253 401]. A critical subset of neutrophils expressing high levels of the sialic-acid-binding protein SiglecF (neu-SiglecF^{high}) was identified in Kras$^{G12D/+}$; Trp53$^{-/-}$ (KP) mouse lung adenocarcinoma that correspond to a transcriptionally related human counterpart associated to negative outcomes [389].

Neu-SiglecF^{high} have been identified as bona-fide, mature and long-lived population associated to angiogenesis, extracellular matrix remodeling and T cell suppression that specifically accumulated in growing lung tumors [392 295]. However, whether neutrophils are polarized within tumors or before reaching the tumor site, how neu-SiglecF^{high} accumulation in lung tumors occur and their role on anti-cancer immune response are mostly unknown. In this manuscript, we used a transplantable KP cell line to assess the role of CXCL5 chemokine in neutrophils recruitment and explore their role in the lung tumor microenvironment.

In line with above evidences, by a deeper characterization of the immune landscape of KP tumors by flow cytometry, we observed that neutrophils SiglecF^{high} are absent in normal lung, whereas represented the 70% of total neutrophils in lung tumors. Neu-SiglecF^{high} also accumulated in the presence of an ongoing response against tumor antigens giving us a model to study their role on anti-cancer immune response. By contrast, neu-SiglecF^{low} counterpart already populate healthy

lungs and were not associated to tumor promotion [253]. Even if the role of lectin SiglecF on neutrophils has not been addressed, SiglecF was first associated with a suppressive role on eosinophils [391], overall suggesting that SiglecF could represent a marker to specifically identify pro-tumorigenic tumor-associated neutrophils in lung cancer. We also observed, by flow cytometry analysis, a decrease in B cells and CD4 T cells in both immunogenic and not immunogenic tumor bearing lungs as compared to normal lung, an observation that deserves to be further investigated in the future considering the role of those subsets in adaptive anti-tumor immunity.

In line with previous work [392], we were not able to identify by flow cytometry neutrophils expressing SiglecF neither in lymphoid organs (spleen, bone marrow and peripheric lymph nodes) nor into the circulation. By contrast, up to 20% of neu in lung draining lymph node were SiglecF^{high}. Further studies to assess how neu-SiglecF^{high} migrate into mLN and whether they could promote distant metastasis of lung cancer are required.

In several reports, the role of neutrophils in shaping anti-tumoral immune response was assessed using *ex-vivo* assays based on circulating or splenic neutrophils, or by their antibodies-mediated *in vivo* depletion. Here, by flow cytometry and IHC analysis, we found that the αLy6G-mediated depletion of neutrophils was effective only on circulating neutrophils but was poorly efficient in depleting tumor infiltrating one. This result is in line with recent evidences by other group [395] and suggest cautions in interpretation of past results, especially concerning the role of tissue infiltrating neutrophil. This result also highlights the need to develop alternative ways to specifically target neutrophils in tissues.

To explore the mechanism controlling accumulation of neu-SiglecF^{high} in lung tumors, we profiled normal and tumor lung tissues using a gene array of chemokines and receptors. We observed that lung tumors are characterized by a huge over expression of the chemokine CXCL5, data confirmed also by qRT-PCR in both KP and its immunogenic counterpart. FACS analysis showed also that neu-SiglecF^{high} expressed higher levels of CXCL5-receptor, CXCR2, as compared to SiglecF low counterpart, axis which has been already linked to neutrophils recruitment in inflammation and cancer [309 304 306]. In addition, from analysis of gene expression on CD45^{+} and CD45^{-} populations isolated from normal and tumor bearing lungs, we pointed out that the chemokine is produced mainly by the not myeloid population in cancer which is composed by stromal and tumor cells. Taking into consideration that most of lung cancer arise from epithelial cells which have been demonstrated to be the main source of CXCL5 in tuberculosis infection [306], these data together suggest that tumor cells themselves may promote the recruitment of neutrophils expressing SiglecF

in lung tissues through CXCL5 production. To assess this hypothesis, we first analyzed the Cxcl5 expression in several cell lines and unlike other tumor cells (including ovarian and melanoma cell lines) KP OVA cells did express high amount of the chemokine at steady state. Importantly, Lewis lung carcinoma cells (LLC), which is wild-type for Kras, did not express Cxcl5 at steady state, suggesting that, in line with previous reports [3 316 317], CXCL5 mediated neu-SiglecF^{high} accumulation could be a peculiarity of lung tumors carrying Kras mutation.

Thus, following data of gene expression, we generated by Crispr/Cas9 technology a null CXCL5 KP OVA cell line (KP OVA KO^{CXCL5}). Those cells were firstly validated *in vitro* to exclude editing-related clone specific effects, thereafter we injected them to induce lung tumors *in vivo*. By qRT-PCR of tumor tissues, we observed that in KO^{CXCL5} tumors Cxcl5 transcript levels were lower as compared to WT tumors, even at later time point, confirming that the chemokine is produced mainly by cancer cells and not by other stromal cells conditioned by the tumor microenvironment. Impressively, flow cytometry and IHC analysis showed that KO^{CXCL5} tumor failed to accumulate neu-SiglecF^{high} in lung tissues at initial stage of tumorigenesis and showed only a minor increased in more advanced tumors. This result demonstrated that lack of tumor-derived CXCL5 chemokine is sufficient to block accumulation of neutrophils expressing SiglecF in our model, without affect SiglecF low counterpart, normally present in lungs.

Importantly, flow cytometry analysis also revealed that the lack of CXCL5 expression by cancer cells did not affect the recruitment of other immune cell subsets in lung tumors, including those expressing CXCR2 such as macrophages [398 399 400]. In addition, flow cytometry data showed that lack of CXCL5 expression by tumor cells did not impact on frequencies of circulating and splenic neutrophils. Whereas it resulted in higher neutrophils content within bone marrow, likely reflecting less mobilization than in WT tumor. It is also possible that the over-expression of IL-6 in tumors lacking neu-SiglecF^{high} (data not shown) could contribute to emergencies myelopoiesis [289] and increased frequencies of neutrophils observed in bone marrow of KO^{CXCL5} tumor bearing mice. However, further studies to assess the correlation between IL-6 overexpression and lack of tumor-derived CXCL5 expression are needed.

Importantly, tumors induced by CXCL5-restored KO^{CXCL5} cells showed an increase of Cxcl5 transcripts that was even higher as compared to WT tumors. We speculate that the strong promotor upstream from the Cxcl5 sequence in vector used for lentiviral production, may explain the higher expression of the chemokine in CXCL5-restored KO^{CXCL5} cells observed at steady state that led to inflated expression in tissues. Additionally, flow cytometry analysis showed that neu-SiglecF^{high} influx was

restored in $KO^{CXCL5\ (lenti\text{-}CXCL5)}$ tumors, demonstrating that their recruitment is causally linked to the chemokine expression.

An open question is whether neutrophils are polarized within tumors or before reaching the tumor site. It has been shown that circulating lung tumor-derived molecules (such as sRAGE) are able to promote the activation of bone marrow-resident cells and consequent accumulation of neu-$SiglecF^{high}$ in lungs [389], which suggest that polarization of neutrophils may occur before reaching the tumor site. On the other hand, evidences suggesting that TANs acquire a pro-tumorigenic phenotype within tissues along with tumor progression also exist. First, neutrophils with different states of polarization have been observed within the tumor itself [318] [319]. Moreover, it has been shown that tumor derived factors promote tissue accumulation of neutrophils with metabolic alteration and increased lifespan. The same authors also shown that acceleration of neutrophil turnover in lung tumors reduced frequencies of TANs expressing SiglecF which led to reduced tumor growth and augmented efficacy of radiotherapy [295].

In the present work, congruent with the higher expression of CXCL5 at steady state and tissues, we observed a higher fraction of neu-$SiglecF^{low}$ in $KO^{CXCL5\ (lenti\text{-}CXCL5)}$ tumors as compared to WT, indicating that the chemokine alone is sufficient to induce neutrophils influx. In addition, we speculate that the higher fraction of neu-$SiglecF^{low}$ in $KO^{CXCL5\ (lenti\text{-}CXCL5)}$ tumors may reflect accelerated recruitment/turnover in tissues which is sufficient to counteract their pro-tumorigenic polarization in tissues. Nevertheless, future studies should address this open question by deciphering the exact mechanisms involved in neutrophils polarization, specifically in lung cancer.

All together these data proved that CXCL5 produced by KP cells is the dominant factor inducing accumulation of neutrophils in lung cancer and Cxcl5 deletion selectively and efficiently abrogates enrichment of neu-$SiglecF^{high}$ at the tumor site, without affecting SiglecF low counterpart, providing a model to study their role on anti-cancer T cell response. In addition, ongoing bioinformatic analysis in our group will help to understand the relevance of the human homologous CXCL6 axis in human lung cancer.

Flow cytometry analysis of WT and KO^{CXCL5} tumor bearing lung did not show any differences in frequencies of infiltrating CD8 T cells, however, lungs of animal carrying KO tumors were characterized by a higher expansion of endogenous tumor specific CD8 T cells as compared to WT. In addition, through a deeper characterization of CD8 T cells by flow cytometry, which include identification of activated cells co-expressing PD1/TIM3/LAG3 markers [95], effector memory $CD62l^-$ $CD44^+$ cells and effector $EOMES^-$ $T\text{-}bet^+$ cells [93], we proved that CD8 T cells in KO^{CXCL5} tumors were

more activated and functional as compared to CD8 T cells in WT tumors, suggesting that neu-SiglecF^{high} may be responsible for suppression of CD8 T cells-mediated anti-cancer responses. Furthermore, by *ex-vivo* restimulation with OVA peptide of endogenous or transferred tumor specific T cells, we showed that the fraction of cytotoxic IFNγ-producing CD8 T cells importantly increased in KO^{CXCL5} tumors as compared to WT, corroborating the suppressive role of neutrophils expressing SiglecF on CD8 T cells.

Despite the presence of neu-SiglecF^{high} within mLN of tumor bearing mice, we did not observe any differences in activation of CD8 T cells nor in IFNγ production between mLNs draining WT and KO^{CXCL5} tumors, however, consistently with higher activation of CD8 T cells observed in KO tumor bearing lungs, we noted an increase of effector memory CD62l$^-$CD44$^+$ CD8$^+$ T cells in mLNs draining tumors lacking neu-SiglecF^{high} as compared to WT.

In the setting of antibody-mediated neutrophils depletion, we observed any differences neither in frequencies of tumor specific CD8 T cells, nor in fraction of cytotoxic IFNγ-producing CD8 T cells in lung tumors, underlaying the importance of an appropriate system to assess neu-T cell crosstalk.

Considering the emerging role of CD4 T cells in anti-cancer responses, we took advantages from our model to analyze the impact of neutrophils expressing SiglecF on CD4 T cell compartment by flow cytometry. Fractions of activated CD4 T cells co-expressing PD1/TIM3/LAG3 and effector memory CD62l$^-$CD44$^+$ CD4 T cells were similarly represented in lungs and draining lymph nodes of WT and KO^{CXCL5} tumor bearing mice, suggesting that neu-SiglecF^{high} do not significantly affect CD4 T cell compartment.

It has been showed that neutrophils are able to produce ROS [362, 279], anti-proliferative molecules [287] and other factors responsible for T cell suppression [367, 368, 303], however, tissue and subset specific T cell inhibitory mechanisms remain poorly defined.

DCs, thanks to their ability to recognize tumor associated antigens and present processed-antigens to naïve T cells, inducing their activation, play an essential role in cancers [129]. In particular, the most prominent role is carried out by cDC1s which, upon maturation, are able to cross-present extracellular antigens on MHCI molecules directly to CD8 T cells [131, 43]. Looking for a mechanism of T cell suppression, firstly, we used KO^{CXCL5} model to assess the impact of neutrophils expressing SiglecF on cDC1c. Flow cytometry analysis showed a slight increase in maturation, characterized by enhanced MHCII, but not CD86 expression, of cDC1 deriving from KO^{CXCL5} tumors as compared to WT. Thus, at initial time point of tumorigenesis, when we performed the experiments on cDC1s and when the suppression of CD8 T cells was clearly visible, cDC1 compartment is only marginally

affected by the presence of neu-SiglecF^{high}. Nevertheless, additional experiments to assess whether neutrophils expressing SiglecF impair cDC1 functions at more later time point are needed.

As a second approach to understand mechanism of neu-SiglecF^{high} mediated T cell suppression, we performed IF analysis of paraffine embedded KP OVA tumor bearing lungs to study the relative spatial distribution of neutrophils and CD8 T cells within tumor nodules.

We pointed out that depending on the area of nodules, an inverse relation exists between the two populations. We observed that in small nodules, sparsely infiltrated by neutrophils, CD8 T cells represent a major fraction. By contrast, in large nodules neutrophils accumulated and were found often in touch with few CD8 T cells remaining, suggesting that neutrophils expressing SiglecF infiltrating tumor tissues may directly impair CD8 T cells activation by cell contact dependent manner or through their physically exclusion. However, further studies to identify the exact mechanisms of T cells suppression neu-SiglecF^{high} mediated are required.

We expected that the higher activation of CD8 T cells in a neu-SiglecF^{high} depleted lung tumors may results in tumor containment as corroborated by IHC analysis of paraffine embedded tumor tissues which shown that both tumor burden and frequencies of proliferating cells within nodules were initially reduced in KO^{CXCL5} tumors as compared to WT. Nevertheless, previous works demonstrated that neutrophils-derived factors (leukotrienes, NE and growth factors) are able to promote tumor cells proliferation [353] [354] [355] [358]. In this setting, we cannot exclude direct effects of neutrophils expressing SiglecF on tumor cells and experiment of *in vivo* depletion of CD8 T cells are needed to establish a causal link between CD8 T cell suppression neu-SiglecF^{high} mediated and tumor growth. Furthermore, IHC analysis showed that the tumor containment was bypassed at later time point, suggesting that tumors implement other suppressive mechanisms to avoid immune control.

Tumor-intrinsic mechanisms of immune evasion include downregulation of MHCI expression and defects in antigen presentation [46] [48], activation of oncogenic pathways [51], secretion of immunosuppressive molecules (such as IL10, TGF-β, PGE2) and expression of inhibitory checkpoint molecules (such as PD-L1 and CTLA-4) [52] which combined result in a complex machinery for impairment of the anti-tumor response. In addition, growing evidence showed that different immune cell subsets including Tregs [75] [198], TAMs [219] [220] and TANs [370] [371] [372] express high level of immune checkpoint inhibitors which increase the burden of T cell inhibitory ligands.

Of note, we observed that the expression of PD-L1 on tumor-derived neu-SiglecF^{low} was similar to neutrophils from nLung. By contrast, PD-L1 expression was significatively increased in SiglecF^{high}

counterpart, corroborating the hypothesis that SiglecF could represent a marker to specifically identify pro-tumorigenic tumor-associated neutrophils in lung cancer.
Furthermore, to assess whether the lower burden of T cell inhibitory ligands in tumors lacking neu-SiglecF^{high} may increase sensibility to checkpoint blockade, we administered antibodies to PD-L1 upon challenge with WT and KO^{CXCL5} cells and we measured the tumor growth at advanced stage of tumorigenesis. Impressively, the treatment with immune checkpoint inhibitor was strongly effective in mice carrying KO tumors, while having a marginal effect in WT tumors, showing that neu-SiglecF^{high} in lung cancer limit the efficacy of PD-L1 blockade and their depletion combined with modern immunotherapy could represent a viable solution to counteract immune evasion.
In conclusion, we demonstrated that CXCL5 produced by lung cancer cells carrying Kras mutation is a key regulator of accumulation of neutrophils expressing SiglecF in lung tissues. We provide a model that allow to study the crosstalk between T cells and specific subsets of neutrophils within lung tumors, overcoming issues related to studies which use not-tissue neutrophils with different localization and characteristics. We demonstrated that only neutrophils expressing SiglecF, and not SiglecF^{low} counterpart already present in normal lungs, contribute to T cell suppression and limit the efficacy of PD-L1 checkpoint blockade. Even if the exact molecular mechanisms of T cells suppression neutrophils-mediated required further investigation, we hypothesized that neutrophils may act in a contact-dependent manner or through exclusion of CD8 T cells from nodules of lung tumors. All together, these data suggest that targeting the CXCL5-axis could be a viable improvement to existing immunotherapy which could lead to achievement of a durable and wide effective response in patients.

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