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Ciclo XXX

The neutrophil anti-tumoral response in cancer: role of ACKR2 and chemokine receptors

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ABSTRACT

Chemokines are key mediators of inflammation and are involved in both extrinsic and intrinsic pathway of cancer. Their main function is to induce leukocyte migration through the binding of specific seven transmembrane receptors. Beside canonical chemokine receptors, a smaller family of atypical chemokine receptors was described. ACKR2 binds with high affinity a broad panel of CC inflammatory chemokines mediating their internalization and intracellular degradation. Due to its chemokine scavenging activity, ACKR2 plays a protective role in chronic inflammation and in the extrinsic pathway of cancer.

The objective of my thesis was to investigate the role of ACKR2 in the intrinsic pathway of cancer using the NeuT (HER2) murine model of oncogene-driven breast cancer crossed with Ackr2$^{-/-}$ mice. In this model, we found that ACKR2 plays a dual and opposite role. It slows the primary tumor development while it promotes lung metastasis. We found the same phenotype about metastasis using the orthotopically transplanted 4T1 mammary carcinoma and melanoma B16F10 cell lines. We demonstrated that ACKR2 expression in the hematopoietic compartment acts as a negative regulator of the mobilization of neutrophils with anti-metastatic function.

In the last part of my thesis we also investigated the phenotype of circulating and tumor associated neutrophils (TANs) in glioma patients, a tumor context characterized by blood neutrophilia and a general immunosuppressive state. We found a higher grade of neutrophilia and an increased rate of immature neutrophils in glioblastoma patients compare to grade III glioma patients. Finally, we found that the relative abundance of circulating neutrophils on total leukocytes positively correlates with relative abundance of TANs.

Collectively taken, these results indicate that neutrophils are a heterogeneous population with both pro and anti-tumoral functions. Targeting of neutrophils in cancer context represent a potential therapeutic approach that limit their pro-tumoral role unleashing the anti-tumoral and anti-metastatic potential.

1 INTRODUCTION

1.1 Cancer-related inflammation

Since from the first observations made by Rudolf Virchow in 1863 in which he noticed leukocytes inside neoplastic tissue, it was suggest that cancer and inflammation are linked to each other [1]. From that moment, several evidence sustained a direct link between cancer and inflammation. Today, it is established that chronic inflammation supports carcinogenesis and conversely, cancer cells create an inflammatory microenvironment that sustain tumor growth [2, 3].

1.1.1 Two pathways link inflammation to cancer

The inflammatory process is described as a necessary but not sufficient cause of cancer. Several epidemiological studies indicate an association between chronic inflammation and organ-related cancer type indicating that patients with chronic inflammation are more susceptible to develop various cancer type [1, 4]. Nevertheless during all stages of cancer progression (from carcinogenesis to metastasis) it is registered an high amount of inflammatory mediators produced by cancer or stromal cells that lead to recruitment of pro-tumoral leukocytes that sustain tumor growth and spread [5]. The link between cancer and inflammation can be recapitulated in two pathways of cancer development: the extrinsic pathway and the intrinsic pathway. In the extrinsic pathway, a pre-existing chronic inflammation and the concomitant exposure to mutagen agents (for example smoke, ionic radiations, aromatic compounds) increase the risk to develop cancer. This observation is sustained by epidemiologic studies that indicate patients with chronic inflammation more susceptible to develop cancer [2] and experimental preclinical models in which mice exposed to chronic inflammatory and consequently mutagen agents are prone to develop cancer (Fig. 1.1) [6]. On the other hand, in the intrinsic pathway, after transformation cancer cells are able to produce or to induce the production of mediators of the inflammation such as cytokines, chemokines and prostaglandins. This

latter characteristic, belonging also to the extrinsic pathway, create an inflammatory microenvironment that enhance in a direct way tumor growth and metastasis spread and in an indirect way recruitment of corrupted leukocytes that sustain tumor growth and metastasis dissemination (Fig. 1.1) [2]. Finally, all these evidence indicates inflammation as a fundamental hallmark of tumor initiation, progression and spreading [3, 7].

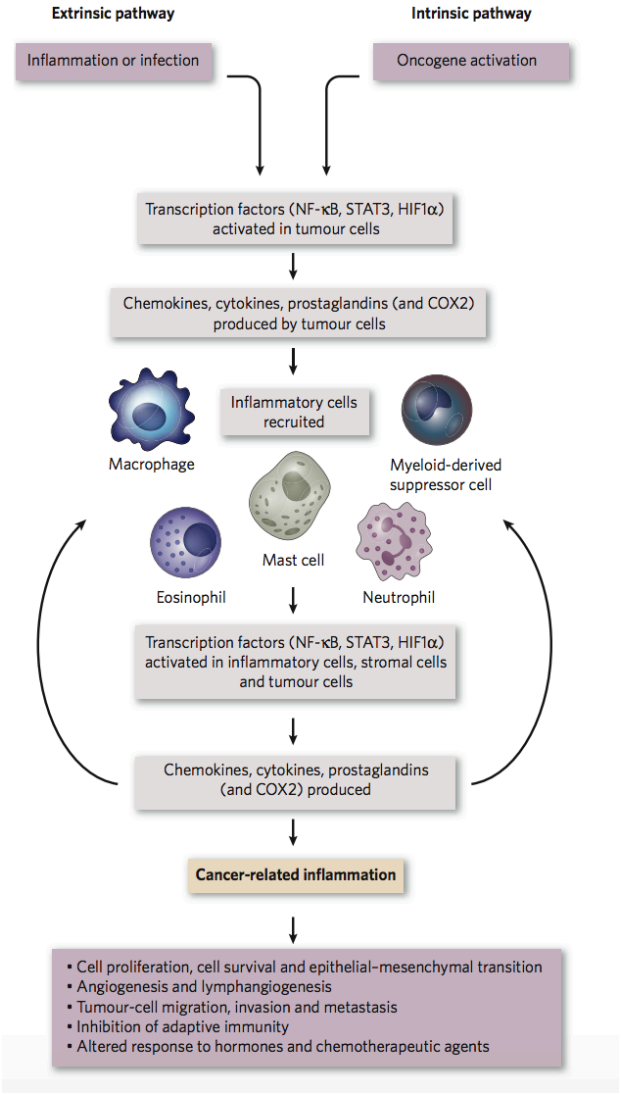


Figure 1.1: Two pathways link inflammation and cancer. In the extrinsic pathway, chronic inflammation increased cancer susceptibility after mutagen exposure. In the intrinsic pathway, mutated oncogene or tumor suppressor gene causes cell transformation. Both pathway converge in the production by cancer or stromal cells of mediators of inflammations [2].

1.1.2 Myeloid cells in cancer-related inflammation

As discussed above, mediators of inflammation are present in both pathway of cancer-related inflammation. One of the features exerted by mediators of inflammation is the recruitment of leukocytes that infiltrate the tumoral mass. Leukocytes are recruited from the blood stream to tumoral side by cytokine and chemokine produced by cancer cell and stromal cells. Both myeloid and lymphoid cells are involved in this process and play at the same time pro-tumoral and anti-tumoral functions depending on the concentration of polarizing cytokine. In detail, myeloid infiltrating cells include tumor associated macrophages (TAMs), tumor associated neutrophils (TANs) and monocyte-derived dendritic cells (mo-DC) [8]. Two categories belong to TAMs: tissue resident macrophages that are present in the neoplastic tissue before cell transformation and circulating monocytes that are recruited in the tissue and then differentiate to macrophages. TAMs differentiate into anti-tumoral phenotype (M1) and pro-tumoral phenotype (M2) depending on microenvironment cytokines. Macrophages are defined plastic cells based on their capacity to change their phenotype from M1 to M2 or vice versa [9]. The same paradigm was recently attributed to TANs. Neutrophils, as discussed in the related paragraph of this thesis, are described to polarize, as macrophage, in the tumor microenvironment to anti-tumoral (N1) and pro-tumoral (N2) phenotype. Otherwise, the concept of neutrophil plasticity is not completed elucidated at the moment [10]. For dendritic cell the polarization paradigm is not recognized, while it is well described their role as antigen presenting cells (APC) of danger associated molecule pattern (DAMP) released during cell death that enhance the adaptive immune anti-tumoral response [11].

1.1.3 Targeting cancer-related inflammation

Being inflammation a fundamental hallmark of cancer, its target represents an important therapeutic strategy [12]. Today, two main therapeutic strategies emerge as target of cancer-related inflammation.

The first strategy to target CRI is inhibition of the production of inflammatory mediators in the tumor microenvironment [13]. For example, genetic ablation of COX-1, COX-2 and prostaglandin E2 reduce inflammation and primary tumor growth

enhancing anti tumoral leucocytes activity [14]. The second attempt is to directly inhibit leukocyte infiltration of the tumors. The best example of this strategy is the use of antibodies blocking the chemokine CCL2, that is the main macrophage chemoattractant, that inhibits lung metastasis in breast cancer model [15].
Chemokines are mediators of inflammation involved in cancer and leukocytes biology, angiogenesis and other important process in cancer biology. In the following paragraph the role of chemokine as mediator of inflammation in cancer will be elucidated.

1.2 Chemokines

Chemokines are a large family of chemotactic cytokines including more than 50 related molecules. The name "chemo-kines" derives from their ability to induce chemotaxis in responsive cells, that express dedicated chemokine receptors. Common features of chemokines, despite a low amino acid sequence homology, are four well-conserved cysteine residues, which form two disulphide bonds between the first and the third cysteine and between the second and the fourth. These bindings give to chemokines a conserved tertiary structure, with a disordered amino-terminus, three-stranded antiparallel β-sheets and a carboxy-terminal α-helix [16].
The chemokine system is described as redundant and promiscuous because it is composed by more that 50 ligands recognized by only 22 receptors (Fig. 1.2) [17, 18].

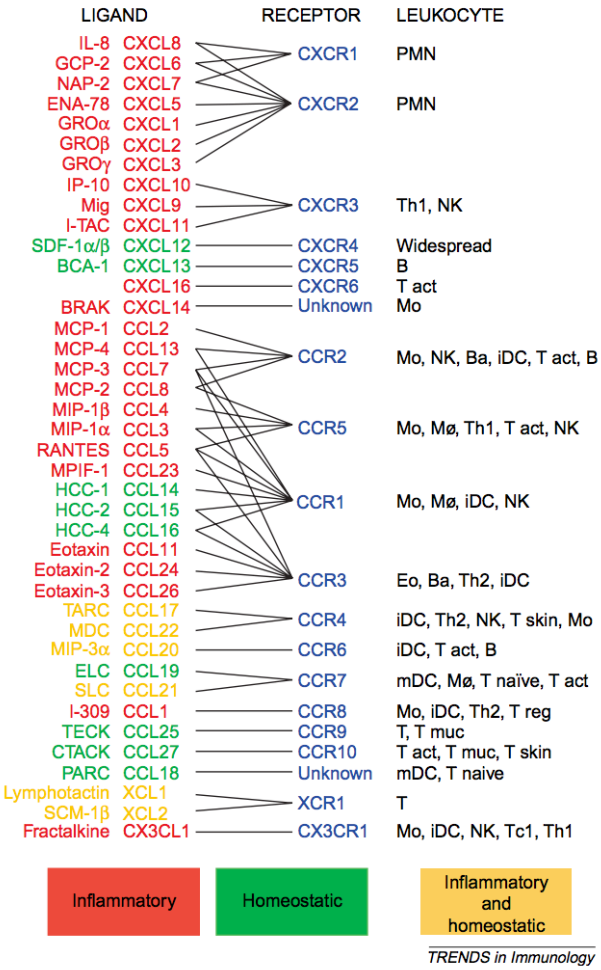


Figure 1.2: The chemokine system. Chemokines, their related receptors and expression on leukocytes [17]

1.2.1 Chemokine classification

Depending on the position of cysteine residues in their N-terminus, chemokines are divided in four subfamilies: CC, CXC, C and CX_3C [19, 20]. CC chemokines are the most represented group of chemokines and have the first two of the four cysteine residues in adjacent position while CXC chemokines have one amino acids between the first two cysteines (Fig. 1.3). CXC chemokines can be further classified in ERL- and ERL+ chemokines, depending on the presence of the ELR (Glu-Leu-Arg) motif. On the other hand, CX_3C chemokines have three amino acids separating the two cysteines and C chemokines have only two cysteines [18].

Moreover, chemokines are classified in homeostatic and inflammatory depending on the regulation of their expression. Homeostatic chemokines such as CCL19, CCL20 and CCL21 are constitutively produced and regulate leukocytes migration in healthy condition. On the other hand, inflammatory chemokines such as CC chemokines (e.g. CCL2, CCL3, CCL5) and ELR+ chemokines (e.g. CXCL8) are expressed during pathological condition and are secondary inflammatory mediators induced by pro-inflammatory mediators (e.g. IL-1 and TNF- α) [21].

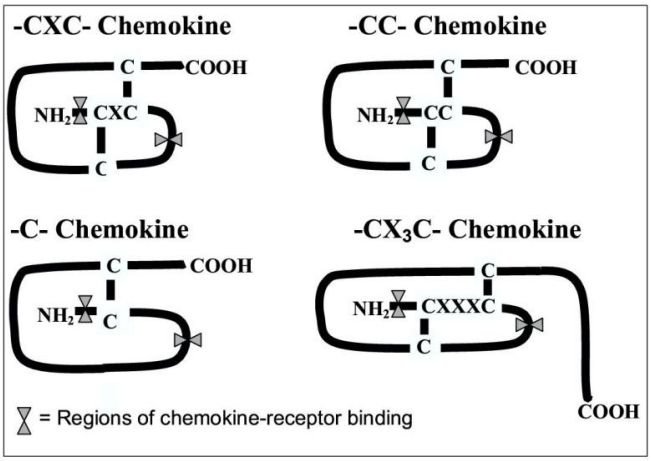


Figure 1.3: Classification of chemokines. Chemokine classification depends on the presence and the number of amino acids between the two first cysteines that form the two disulphure bounds with other cysteines in the sequence. Chemokine are classified in CC, CXC, C, CX_3C [22].

1.2.2 Chemokine receptors

Chemokines are soluble mediators acting on specific target cells that express related chemokine receptors. Chemokine receptors are classified according to the family of chemokines that bind in CXCR, CCR, CX_3CR, or XCR1. Chemokine receptors belong to the superfamily of 7-transmembrane receptors [16] coupled to hetero-trimeric GTP-binding proteins (G-protein) of the Gi type, sensitive to Bordetella pertussis toxin. Chemokine receptors have a highly-conserved structure constituted by a single peptide chain with three intracellular and extracellular loops, an external N-terminus domain essential for the specificity of ligand binding, and an intracellular carboxy-terminus tail. This latter domain, together with other motifs, such as the DRYLAIV (Asp–Arg–Tyr–Leu–Ala–Ile–Val) motive between the third transmembrane domain and the second intracellular loop, has a role in receptor signaling (Fig. 1.4). After chemokine binding, conformational changes occur and trigger intracellular signaling pathways promoting cell migration or activation [23].

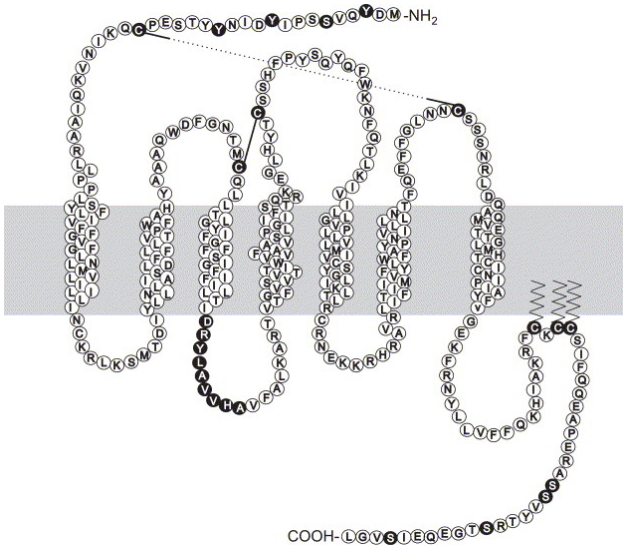


Fig 1.4: Structure of chemokine receptor. CCR5 as example of chemokine receptor structure [24]

1.2.3 Chemokines in cancer

Beyond the role of regulator of leukocytes mobilization, chemokines have a key role in many other functions: they regulate angiogenesis, fibrosis, cell proliferation and also cancer cell growth and dissemination. Chemokines are involved in both pathways that link inflammation to cancer. In the extrinsic pathway, chemokines are key mediators of chronic inflammation that increases the risk to develop cancer. In the intrinsic pathway, in which cancer raises from genetic events, chemokines expression is regulated by oncogenic pathways and transcription factors altered in cancer cells [25]. As discussed above, chemokines can act both on cancer and on stromal cells. Chemokines secreted at tumor site can recruit both immature cells and mature cells. Chemokines such as CCL2 and CXCL8 attract at tumor site monocytes and neutrophils that then differentiate to tumor associated macrophages (TAMs) and tumor associated neutrophils (TANs). TAMs and TANs have both pro-tumoral and anti-tumoral role depending on their polarization [8, 10]. Moreover, CCR4 ligands such as CCL17 and CCL22 have a role in the recruitment of Treg and Th2 lymphocytes that create an immunosuppressive microenvironment contributing to tumor growth [26]. On the other hand, ligand of CXCR3, CX_3CR1 and CXCR6 increased mobilization of NK cells and T lymphocytes, cells able to kill cancer cells through direct cytotoxic effect [25, 27]. Chemokines have a key role also in the angiogenetic process enhancing or inhibiting *de novo* vessel formation and vessel spread. Pro-angiogenetic effects are exerted by ELR+ CXC chemokines and CXCL12, ligand of CXCR2 and CXCR4, that promote angiogenesis and inhibit apoptosis of endothelial cells [27]. On the other hand, ELR- chemokines such as CXCL9, CXCL10, and CXCL11 bind CXCR3 expressing cells (Th1 lymphocytes and NK cells) and inhibit angiogenesis through the expression of type 1 cytokine [27-30]. A fundamental role of chemokines in the cancer context is the primary cancer spreading to secondary organs (metastasis). CXCR4 is the main receptor involved in this process. CXCR4 expression on cancer cells is up-regulated in many cancer type and CXCR4 positive cells have an increased migration to organs that express high level of the ligand CXCL12 such as lung, lymph node, bones and liver. Pharmacological block of the CXCL12/CXCR4 axis impairs metastatic seeding in different preclinical tumor models [31, 32]. Moreover, some tumors can express CCR7 and use it to migrate into lymph nodes because lymphatic

vessels express high amount of the ligand CCL21 [33]. Finally, chemokines have also a direct role in cell survival activating cancer cells via the PI3K-AKT-NF-kB and the MEK1/2 and Erk1/2 axis [34, 35].

1.3 Atypical chemokine receptors

Beyond canonical chemokine receptors, a new subfamily of chemokine receptors defined "atypical" has been recently identified and nomenclated [36]. Atypical chemokine receptors (ACKRs) have structural features similar to canonical chemokine receptors but have a modified DRYLAIV motif and after ligand binding, the receptors do not induce any G protein mediated signaling and cell mobilization. ACKRs bind with high affinity CC and CXC chemokines and their functional role is the regulation of chemokine availability. Their role is exerted scavenging, transporting or directly controlling signaling of other chemokine receptors [37]. Four members composed the ACKRs family: ACKR1 (previously nomenclated DARC), ACKR2 (D6), ACKR3 (CXCR7) and ACKR4 (CCX-CKR) (Fig. 1.5) [36, 38]. Our research group has focused the attention on ACKR2, a negative regulator of CC chemokines [38, 39].

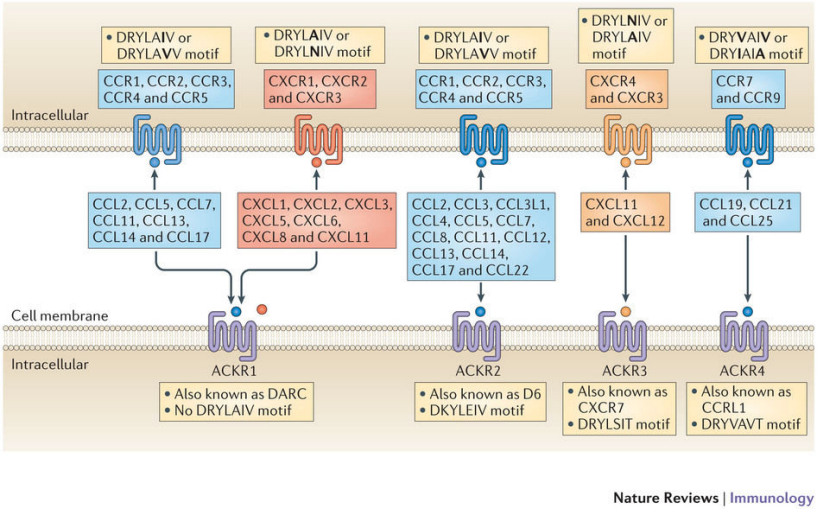


Figure 1.5: The atypical receptors family. Current and traditional nomenclature of atypical chemokine receptors, their ligand and canonical receptors which ACKRs act as agonist [37].

1.3.1 ACKR2/D6

ACKR2, previously called D6 or CCBP2, is a seven-transmembrane receptor that belongs to the atypical chemokine receptor family. ACKR2 binds a broad group of inflammatory CC chemokines (ligands of CCR1, CCR2, CCR3, CCR4 and CCR5), and leads to their internalization and degradation [39]. ACKR2 structure is different from canonical chemokine receptors for a modified DRYLAIV to a DKYLEIV motive that not allow binding with G-coupled protein [40]. For this reason, it was supposed that ACKR2 was a "silent" receptor not able to trigger any signaling pathway [41-43]. In basal conditions, ACKR2 is stored in endosomal vesicles and recycled continuously to the membrane [44, 45]. After ligand engagement, ACKR2 internalizes and targets chemokines to lysosomal degradation. At the same time, it activates β-arrestin-dependent signaling, which increases receptor recycling to plasma membrane localization to adapt its function in the control of chemokine extracellular concentrations [46, 47]. ACKR2 is described to be highly expressed by the syncytiotrophoblast layer in placenta, by lymphatic endothelial cells, and by some leukocyte populations, including innate-like B cells and alveolar macrophages [48-50]. ACKR2 role in physiology and pathology was demonstrated using gene-targeted mice in which ACKR2 expression is deleted.

1.3.2 ACKR2 role in physiology

ACKR2 has a role both in physiologic and pathologic conditions. In homeostasis, ACKR2 expressed by efferent lymphatic vessel regulates chemokine levels and prevents excessive leukocytes accumulation in the lymphatic system [51, 52]. It regulates lymphatic vessel density formation in ear, diaphragm and popliteal lymph nodes inhibiting the recruitment of CCR2 positive pro-lymphangiogenic macrophages [53]. The ACKR2 scavenger activity of CC inflammatory chemokines is also involved in the increased efficacy of the presentation of CCR7 ligand and promotion of DC migration [54]. Moreover, ACKR2 controls also the morphogenesis and the branching of mammary gland during development [55] and the traffic of inflammatory monocytes ($CD11b^{+}/Ly6C^{high}$) with immunosuppressive phenotype inhibiting egression from bone marrow [56].

1.3.3 ACKR2 role in inflammation and autoimmunity

ACKR2 emerges as negative regulator of the inflammation through its capacity to degrade CC inflammatory chemokines. In skin inflammation, ACKR2 exerts a protective role after phorbol ester skin painting and following injection of complete Freund's adjuvant (CFA) with a concomitant decrease of the inflammatory response [48, 57, 58]. Similar results were found in antimicrobial response to *Mycobacterium tuberculosis*. Ackr2$^{-/-}$ mice display reduced survival rate, increased number of leukocytes, increased production of pro-inflammatory cytokines such as TNF-α, IL-1β, INF-γ and chemokines such as CCL2, CCL3, CCL4 and CCL5 [59]. Being the syncytiotrophoblast one of the major site of expression of ACKR2, in a LPS-dependent fetal loss model, Ackr2$^{-/-}$ mice have an increased abort rate with concomitant increased levels of CC chemokine and leukocytes [60]. Contrasting results were published about gut inflammation. While Vetrano and colleagues found in Ackr2$^{-/-}$ mice an increased inflammation after administration of dextran sulfate sodium (DSS) [61], Bordon and colleagues found that Ackr2$^{-/-}$ mice are protected after DSS administration and this correlates with increased production of IL-17A by T cells [62]. Further experiments are needed to elucidate the role of ACKR2 in this context, taking in consideration animal house sanitary status and the intestinal flora.

Moreover, in a graft versus host disease (GVHD) Ackr2$^{-/-}$ mice have increased number of inflammatory monocytes with immunosuppressive activity and they are protected from disease development [56]. In human arthropathies, ACKR2 is upregulated in synovial tissue as a marker of inflammation [63]. Finally, ACKR2 was described to have a role in autoimmune diseases because it was found upregulated in psoriatic skin [64] and Ackr2$^{-/-}$ mice shown a faster disease progression in a psoriasis-induced model with increased cell autonomous neutrophil infiltration [65]. On the other hand, Ackr2$^{-/-}$ mice are protected in the development of autoimmune diabetes [66] and experimental autoimmune encephalomyelitis [67], but opposite results in this model are described by Hansell and colleagues in which they do not recapitulate phenotype found in the first study by the other group [68].

1.3.4 ACKR2 role in cancer

ACKR2 plays also a role in cancer through its ability to scavenge chemokines [49, 57, 62] [38]. ACKR2 role was elucidated using gene-targeted mice in different preclinical models. Firstly, ACKR2 was described to have a role in TPA/DMBA skin carcinogenesis in which ACKR2-deficient mice developed earlier and increased skin tumors [69]. Also in AOM/DSS model, Ackr2$^{-/-}$ mice increased colon cancer susceptibility confirming a protective role of ACKR2 in different organs [61]. On the other hand, ACKR2 did not affect the development of the DEN-induced hepatocellular carcinoma model, even if an increased macrophage infiltration was detected in gene-targeted mice [70]. Referring to colon cancer, ACKR2 expression in human colon adenocarcinoma samples is decreased compared with healthy tissues and correlates with an increased lymphatic vessel density and an increased production of CCL22 [71]. ACKR2 is described to be highly expressed in lymphatic endothelium [39] and it is implied in different vascular tumor including Kaposi's sarcoma spindle cells [54]. In this context, ACKR2 expressed by cancer cells have a protective role in tumor growth inhibiting inflammatory chemokines such as CCL2, CCL5, and CCL3 with a concomitant reduced macrophage infiltration and angiogenesis. Immunohistochemistry data reveal that in Kaposi's sarcoma patients ACKR2 is down-regulated through the oncogenic pathway KRas/BRaf/MEK/MAPK [72]. ACKR2 has a protective role also in breast cancer but the expression in healthy epithelial cells was not elucidated [73]. In human breast cancer samples ACKR2 expression is inversely correlated to lymph node metastasis and the clinical stage. ACKR2 over-expression in a human breast cancer cell line regulates inflammatory chemokines levels and tumor aggressiveness in vivo [74]. These data were confirmed by different groups: ACKR2 was found down-regulated during breast cancer progression, and concomitant down-regulation of ACKR1 and ACKR4 correlates with a worse pathology outcome confirming a protective role not only for ACKR2, but also for other atypical chemokine receptors involved in chemokines control [75, 76]. ACKR2 genetic polymorphisms are predictors for the clinical response in patients with breast cancer. One single nucleotide polymorphism in the coding sequence (rs2228468) increases scavenger activity of chemokines and is correlated with increased relapse-free survival [77, 78]. The protective role of ACKR2 was also described in cervical squamous cell

cancer and in gastric cancer in which immunohistochemistry expression of ACKR2, ACKR1, and ACKR4 was correlated with a better outcome of the disease [79, 80]. Another contest in which ACKR2 has a protective role is human lung cancer. The lung cancer cell line A549 over-expressing ACKR2 showed an inhibition of in vitro proliferation and in vivo tumor growth caused to enhanced scavenger activity by the receptor [81]. These results indicate that ACKR2 expression by cancer cells limits tumor growth through regulation of leukocytes recruitment. Limited and not well exhaustive data have explored the role of ACKR2 expressed by stromal tumor cells such as lymphatic vessel on carcinogenesis [39].

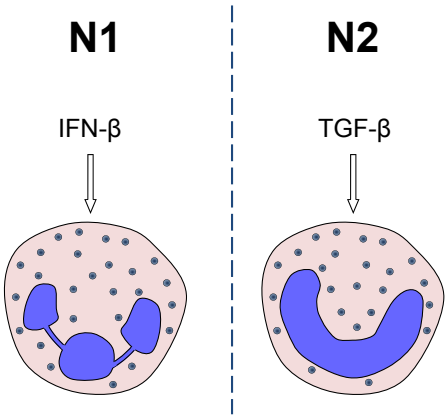
1.4 Neutrophils

Neutrophils are the most abundant circulating leukocytes in human (50 – 70% of neutrophils on total leukocytes) and have an estimated half-life of 7 hours in blood and 1 – 2 days in tissue. Neutrophils play a central function in recognition, phagocytosis and killing of pathogens through production of reactive oxygen species (ROS), degranulation of antimicrobial peptides and the formation of neutrophil extracellular traps (NETs) [82]. In addition to the role in the innate response against pathogens, neutrophils play a role also in the regulation and activation of the innate and adaptive immunity [83] and in several pathological diseases including cancer [82, 84]. In the cancer contest, neutrophils were considered neutral players for several years but in the last decade, neutrophils were described to have both pro-tumoral and anti-tumoral functions depending on the activation and polarization state [10, 85]. Clinical data indicate that circulating and infiltrating neutrophils have a predicting role about patients survival. Elevated neutrophil-to-lymphocytes ratio (NLR) is considered a poor prognostic indicator in various cancer type [86]. Moreover, a meta-analysis indicates neutrophils presence in tumor tissue associated with poor prognosis [87].

1.4.1 Neutrophil polarization in cancer

Neutrophils are involved in several pathological contexts including cancer [84]. Even if neutrophil infiltration constitutes an important portion of all infiltrating leukocytes

in many tumor types [87], only recently the neutrophils role in cancer was starting to be elucidated. It was demonstrated for the first time by Fridlender and colleagues in 1999 that neutrophils have a double role in cancer [85]. In this paper TANs were classified in two functional states: pro-inflammatory and anti-tumoral (N1) or anti-inflammatory and pro-tumoral (N2) [85]. Neutrophil polarization is due to cytokines present in the tumor microenvironment: IFN-β polarized TAN to N1 phenotype while TGF-β or the inhibition of IFN-β cause a polarization toward an N2 phenotype [88]. The main morphological feature of N1 neutrophils is the hypersegmented nucleus. N1 status is associated to production of pro-inflammatory cytokines (e.g. TNF-α) and ROS, expression of ICAM-1 and low levels of CD62L. From a functional point of view, N1 neutrophils are able to stimulate T cell responses and to kill cancer cells (Fig. 1.6). On the other hand, N2 neutrophils show a ring-like nucleus, they are negative for ICAM-1 and express at high level of CD62L; they exert their pro-tumoral role expressing MMP9 and VEGF (Fig. 1.6) [84, 89]. Little is known about neutrophils plasticity. It is supposed that TANs that infiltrated tumor in early stages have N1 phenotype and during tumor progression they are polarized toward N2 phenotype. In mice injected with Lewis Lung Carcinoma (LLC) and mesothelioma (AB12), TANs at early stages show N1 markers, produce high level of TNF-α and NO and have increased cytotoxic activity toward cancer cells, while in later stages neutrophils acquire pro-tumoral features [90]. The same feature was found in a model of breast cancer liver metastasis, in which TAN acquire N2 phenotype during progression and have pro-tumoral functions [91]. Also in human lung cancer TAN show in the early phase of the disease an activated phenotype (ICAM-1^{+}/$CD62L^{low}$), express a broad group of chemokine receptors (CCR5, CCR7, CXCR3, and CXCR4), produce pro-inflammatory cytokines and chemokines (CCL2, CXCL8, CCL3 and IL-6) and stimulate T cell proliferation through costimulatory molecules [92]. While at the moment the neutrophil polarization concept is supported by several evidences in many cancer type, it is not well elucidated if neutrophils phenotype changes during tumor progression or different neutrophil subsets are recruited during tumor growth [10]. In the next paragraph, neutrophil pro-tumoral and anti-tumoral activities will be discussed.



Adhesion molecules and receptors	ICAM-1 ^{pos} CD62L ^{low} MET Fas	ICAM-1 ^{neg} CD62L ?
Inflammatory mediators	TNF-α IL-12 ROS RNS	VEGF-A MMP9 Arg-1
Chemokines	CCL3 CXCL8 CCL9	CCL2 CCL5
Chemokine receptors	CCR2 CCR5 CXCR2 ?	CXCR4 CXCR2

Figure 1.6: Neutrophil polarization in cancer. N1 phenotype is induces by IFN-β activity and N2 phenotype is induces by TGF-β: N1 and N2 are differentiates by shape of the nucleus, surface markers, production of inflammatory mediators and chemokines and by the expression of chemokine receptors [10].

1.4.2 Neutrophil pro-tumoral role

Neutrophils in cancer can exert pro-tumoral and anti-tumoral functions [84]. Several data elucidate the mechanisms of their pro-tumoral functions. TANs are able to release enzymes contained in their granules such as neutrophil elastase (ELA2), neutrophils collagenase (MMP8) and neutrophils gelatinase B (MMP9) that allow tumor cells invasion by remodeling extracellular matrix (ECM) [93]. Neutrophils support tumor growth secreting cytokines and growth factors (EGF, TGF-β, PDGF, HGF, VEGF). In detail, hepatocyte growth factor (HGF) is described to promote invasion of human

pulmonary adenocarcinoma cells [94] and VEGF production is enhanced through the oncostatin M production [95, 96]. Moreover, granule enzymes are able to activate pro-angiogenic factors EGF, TGF-β, and PDGF from ECM [97]. ROS and reactive nitrogen species (RNS) are described to enhance tumorigenesis contributing to damage DNA and promote genetic instability [98]. ROS in the same time display anti-tumoral activity inhibiting metastatic seeding [99, 100] as discussed in the next paragraph. Direct promotion of cell proliferation is exerted by ELA2, prostaglandin E2 (PGE2) and leukotrienes [101, 102]. In particular, leukotrienes are implied in proliferation of metastasis initiating cells in different preclinical model of breast cancer [103]. Neutrophils dampen T cell cytotoxic activity through iNOS secretion after stimulation by IL-17 and G-CSF produced by γδ T cells [104]. As discussed above, neutrophils are able also to form NETs upon activation and cancer cells can be trapped in the net and increase their adhesion to hepatic and pulmonary microvasculature causing metastasis [105]. Moreover, neutrophils exert indirect tumor growth inhibiting anti-tumoral immune responses through production of arginase 1 (Arg-1) dampening T cell function upon CXCL8 stimulation [106], expressing programmed death-ligand 1 (PD-L1) [107] and producing CCL2 and other chemokine that enhance recruitment of other tumor-supporting leukocytes [108].

1.4.3 Neutrophil anti-tumoral role

Limited compared the pro-tumoral functions, but promising evidences indicate the neutrophils anti-tumoral role. As discussed above, ROS promote tissue damage and cell death but in same time, ROS have a direct cytotoxic effect against tumor cells [99, 100]. Neutrophils can inhibit the lung metastatic seeding in a preclinical breast cancer model trough the generation of hydrogen peroxide [109]. Another mechanism of cancer cell killing includes antibody-dependent cell-mediated cytotoxicity (ADCC) [110] and this process is important for anticancer monoclonal antibodies based therapies [111]. In addition, interferon-activated neutrophils are able to produce TNF-related apoptosis inducing ligand (TRAIL/APO2 ligand) with selective apoptotic activity against tumor cells [112, 113]. Neutrophils are also able to enhance Fas-mediated apoptosis [114]. MET, a proto-oncogene involved in cancer cell-cycle and

survival, is expressed by TANs after TNF-α stimulation. Its genetic ablation leads to decreased transmigration across endothelium and dampen nitric oxidase-dependent tumor cell killing that limit cancer cell growth [115]. Neutrophils are able to decrease uterine tumor development in pten$^{-/-}$ mice promoting tumor cell detachment [116]. Finally, neutrophils stimulate also adaptive immune response through the expression of costimulatory molecules. Neutrophil stimulate proliferation of CD4+ and CD8+ T cells through expression of OX-40L and 4-1BBL increasing their cytotoxic activity in early stage of human lung cancer [92]. Recently, a neutrophil subpopulation in lung cancer was discovered. It was defined as APC-like hybrid TANs due to the expression of costimulatory molecules and because it stimulates antigen non-specific T cell response [117]. Enhancing of anti-tumoral neutrophil functions is a potential immunotherapy approach that unleash neutrophils cancer cell killing ability.

1.4.4 Neutrophil subpopulations

Neutrophils were considered for many years a homogeneous population among granulocytes, but several evidences indicate the presence of neutrophil subsets in inflammation and in cancer both in mice and human. Circulating neutrophils in healthy mice are mainly Ly6G^{+}/CD62^{high}/ICAM-1^{neg} but during inflammation it was observed a fraction of circulating neutrophils that upregulated ICAM-1 and shed CD62L [118]. CD62L (also known as L-selectin) is a cell adhesion molecule normally expressed by neutrophils and lymphocyte subsets [119]. CD62L^{low} neutrophils were associated with an aged phenotype with higher expression of CXCR4, TLR4, CD11b, CD49 and ICAM-1 exhibiting an increased αMβ2/Mac1 integrin activation and NET formation in inflammatory conditions [120]. In homeostatic conditions, also human circulating neutrophils are described as a unique population labelled as CD16^{high}/CD62L^{high}, but in inflammatory conditions other two populations were described: CD16^{dim}/CD62L^{high} that are freshly released from bone marrow or young neutrophils and CD16^{high}/CD62L^{low} that are older and have increased anti-microbial function and produced higher amount of ROS [121]. CD16^{high}/CD62L^{low} neutrophil subpopulation was further described to upregulate CD11b and CD11c with improved suppression of T cell proliferation [122, 123]. In human and mouse during inflammation, a neutrophil

subpopulation was also described for its ability to reverse transmigrate from tissue to blood. These neutrophils are called reverse transmigrated neutrophils (rTEM) and showed activated phenotype in terms of expression ICAM-1, ROS production and low level of CXCR1 [124]. rTEM role is not completely elucidated because they can potentially spread the inflammation to distal organs form the site of inflammation [125], but in a zebrafish model rTEM are described dampening inflammation [126]. Another neutrophil subset was described in humans and mice for its ability to produce pro-angiogenic factors, in particular VEGF-A, the expression of VEGFR1, CD49d (VLA4), high level of MMP9 and CXCR4 [127]. CD10, normally expressed by neutrophils, has been indicated as marker of mature neutrophils. CD10 negative neutrophils have an activated phenotype, they able to stimulate T cell proliferation and IFN-γ production [128]. As discussed above, a subpopulation of APC-like hybrid TANs has been recently described in lung cancer but it is not known if they are CD10 negative [117]. Finally, in cancer patients and tumor-bearing mice neutrophil subsets can be identified on the basis of their density. Indeed, Sagiv et colleagues identified immature low-density neutrophils (LDNs) and mature high-density neutrophils (HDNs). LDNs and HDNs, in addition to be different in terms of density and shape, have different functional roles: HDNs have increased cancer cell killing activity while LDNs have an immunosuppressive role resembling features of granulocytes – myeloid derived suppressor cells (G-MDSC) [129]. Collectively taken, redundant and not officially adopted nomenclature of neutrophil subsets create a confused classification. Further efforts will be necessary to better describe neutrophil subsets linking functional activity and markers expression in order to create a unique nomenclature. This will be useful to describe neutrophil heterogeneity among different diseases underlying common features that summarize neutrophil biology.

1.4.5 Chemokines in neutrophil polarization

As discussed above chemokines and chemokine receptors are mediators of the inflammation and play a key role in physiological and pathological contexts including cancer. Neutrophils express both chemokines and chemokine receptors as regulators of fundamental biological and functional processes including mobilization,

transmigration and cell activation [130, 131]. In this paragraph, it will be discussed the role of CXC and CC chemokines in neutrophil biology in cancer.

1.4.6 CXC chemokines and related receptors

Neutrophils express high level of CXCR1 and CXCR2 and, after binding with their ligands, they induce a strong migrating effect [131]. In cancer, agonist of CXCR1 and CXCR2 such as CXCL8, CXCL5 and CXCL6, are produced by cancer cells causing neutrophils mobilization and infiltration into tumor mass. At the same time, neutrophils are able to produce and release chemokines, for example CXCL2, that contribute to sustain tumor infiltration by neutrophils. The most abundant chemokine produced by tumoral cells is CXCL8; it is over-expressed by many tumors type such as colon, lung, prostate, ovarian carcinoma and melanoma [132]. CXCL8 is not only associated with promotion of inflammation and neovascularization [133], but also with neutrophil-dependent cell mutation that leads to tumorigenesis [134]. On the same way, CXCL5 is described to be involved in tumor progression and metastasis in several cancer types [135, 136]. CXCL5 was found to recruit pro-tumoral neutrophils in preclinical models of hepatocellular carcinoma (HCC) [137] and intrahepatic cholangiocarcinoma (ICC) [138]. Also in human, CXCL5 expression correlates with poor prognosis in HCC and ICC patients [138]. Furthermore, CXCL6, CXCL2 and CXCL1 are involved in neutrophil recruitment to tumor. CXCL6 induce neutrophil infiltration sustaining tumor angiogenesis [139] and tumor growth in melanoma [140]. CXCL1 and CXCL2 were found to recruit neutrophils expressing S100A8 and S100A9 that increased cancer cell survival and resistance to chemotherapy [141]. In line with these results, genetic deletion of CXCR2 inhibited neutrophil recruitment into the tumor, inhibited tumor growth and reduced angiogenesis in B16F10 and MCA205 tumor models [142]. On the contrary, the role of CXCR2 in metastasis is not fully elucidated. In renal cell carcinoma (RCC) CXCR2 ligands CXCL5 and CXCL8 produced by cancer cell are able to recruit anti-metastatic neutrophils that inhibit cancer cell seeding [143]. It is not known if CXCR2, beside allowing recruitment of circulating neutrophils, is differentially expressed by neutrophil subpopulations and has a role in their functional activities. Treatment with IFN-β, a N1 polarizing cytokine, does not modulate CXCR2 expression in murine neutrophils [142], while in

N1 TANs in lung cancer patients CXCR2 is down regulated [92]. Moreover, CXCR1 is downregulated in rTEM [124] during inflammation but no evidence indicates chemokine receptors modulation on rTEM in cancer. Neutrophils express also CXCR4 and its ligand CXCL12 (also known as SDF-1) that negatively regulate neutrophils release from BM. Hematopoietic progenitors express high levels of CXCR4 and it is downregulated during maturation. Circulating neutrophils express low levels of CXCR4 and it is further downregulated by a panel of cytokine including IFN-γ, IFN-α, granulocyte-macrophage colony-stimulating factor (GM-CSF) and G-CSF [144]. CXCR4 is upregulated in senescent neutrophils and promote their recalling to BM [145]. In the tumor context, CXCR4 was found upregulated in a subset of circulating neutrophils promoting angiogenesis and tumor progression [127]. CXCR4 is also inhibited by IFN-β suggesting its expression as N2 marker [89]. Pharmacological inhibition of CXCR4 through administration of AMD3100 (also known as Plerixafor), increased anti-tumoral immune response in murine models of hepatocellular carcinoma inhibiting immunosuppressive microenvironment [146]. However, being CXCR4 highly expressed also by tumor cells, it is difficult to extrapolate its role in neutrophil function and polarization.

1.4.7 CC chemokines and related receptors

Circulating neutrophils from healthy individuals express low level of CC chemokine receptors and for this reason their role in neutrophil biology is not fully understood [147, 148]. For example, the role of CCR2 in neutrophils is emerging only recently. Neutrophils express lower CCR2 levels compared to monocytes, but Fujimura and colleagues found that CCR2 is important for neutrophil mobilization from BM [149] and it is responsible for neutrophil accumulation in a model of rheumatoid arthritis (RA) [150]. Upregulation of CCR1, CCR2, CCR3, CCR5 enhances the respiratory burst for bacterial killing [147] and CCR2-depend ROS generation has an anti-metastatic role through direct cancer cell killing [109]. On the other hand, neutrophils producing the CCR2 ligand CCL2, enhances the recruitment of CCR2 positive leukocytes that can have a pro-tumoral role [108]. Neutrophil CCR5 expression is also associated to enhanced recruitment of myeloid cells with immunosuppressive role that

sustain tumor growth [151]. Collectively taken, CC chemokine receptors can be considered markers of activated neutrophils or N1 phenotype and could be potential target to enhance anti-tumor responses. On the other hand, expression of CC chemokine receptor ligands are markers of a N2 phenotype. This address a complex role for CC chemokines and their receptors. While CC chemokine receptors can trigger anti-tumoral function of neutrophils, their ligand could at the same time enhance recruitment of other pro-tumoral leukocytes (Fig. 1.6) [10].

1.5 Breast cancer

Breast cancer represents the second common female tumor and counts about 25% of all tumor types. The estimated incidence rate is 19.4 per 100,000 people in East Africa and 89.7 per 100,000 in West Europe (WHO, 2015) [152]. Breast cancer includes a wide range of tumor of the breast with different anatomical and molecular characteristics [153]. Histological classification divides breast cancer in "in situ" and invasive (infiltrating) carcinoma. In situ carcinoma can be sub-classified in ductal or lobular carcinoma depending on tissue of origin. Invasive carcinoma is further sub-divided in tubular, ductal lobular, invasive lobular, infiltrating ductal, mucinous and medullary [154]. Breast cancer heterogeneity encloses also differential expression of molecular marker belonging to hormone receptors and oncosuppressor genes. Tumors are classified depending on the expression of estrogen receptor (ER), progesterone receptor (PR), ErbB2 (Her2/neu), epidermal growth factor receptor (EGFR) and p53. Based on molecular expression, breast cancer is subdivided as: basal like (that includes triple negative breast cancer, ER, PR and Her2 negative, ErbB2^{+}), normal breast cancer, luminal subtype A, luminal subtype B (further subdivided in Her2 positive and negative) and claudin-low (Fig. 1.7) [154]. Pharmacological therapy based on hormone receptors expression represents an efficacious therapy strategy being molecular heterogeneity an important feature within cancer with the same histological classification that predicts clinical outcome [155]. As discussed below, immune system plays a central role in breast cancer and immunotherapy emerges as a concrete therapeutic approach in the treatment of this pathology [156-158].

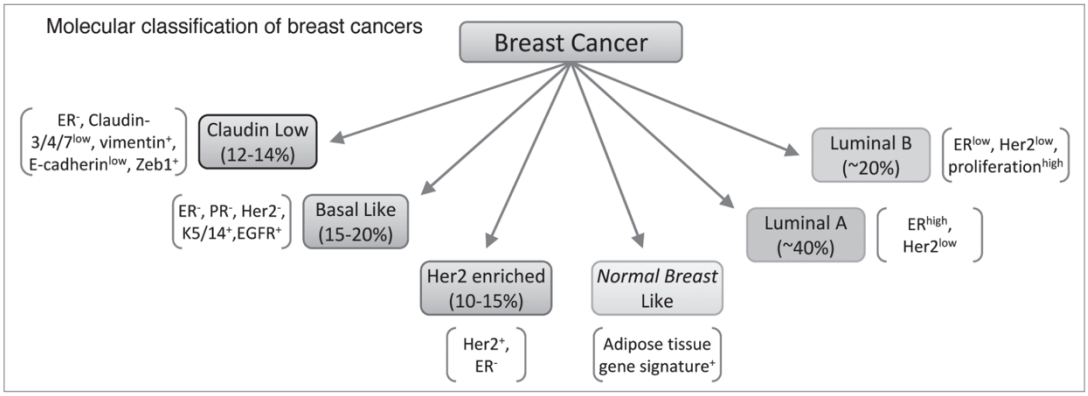


Figure 1.7: Molecular classification of breast cancer [154].

1.5.1 The immune system in breast cancer

Immune system plays a crucial role in breast cancer biology [159]. Leukocytes infiltrate breast cancer since the first phases of the pathology and co-evolve with disease progression [156]. In the initial phase, leukocytes are able to fight against tumor cells, but mechanism of immune escape and immunosuppression exerted by cancer cells cause a switch in leukocytes activity that limit their cancer cell killing activity, sustain primary tumor growth and contribute to metastasis spread [156]. Both innate and adaptive cells infiltrate tumor tissue and exert a role in cancer biology. Referring to innate immunity, macrophages have a paradigmatic role in disease progression [160]. Early phases of the disease are associated to the anti-tumoral M1 macrophage phenotype, while established tumors are associated to pro-tumoral and pro-metastatic M2 phenotype. Targeting of TAM represents a valid therapeutic option to prevent M2 and M2-like polarization that promote tumor growth [161, 162]. Mast cells were observed infiltrating breast cancer and are implied directly and indirectly in angiogenesis [163] while eosinophils were not found infiltrating the tumoral mass [164]. Referring to adaptive immunity, the presence of tumor infiltrating lymphocytes (TILs) is correlated with negative axillary nodal status, smaller tumor size, and lower grade [165]. Among TILs there are both pro and anti-tumoral cells. The positive effects on disease progression have to be attributed to CD4+ Th1 Tcells and CD8+ T cells. On the other hand, CD4+ Th2 T cell and CD4+ regulatory cells (T reg) have a pro-tumoral role and suppress immune response and lead to tumor growth [156]. Breast cancer can be considered as a good example of cancer immunoediting. Cancer cells

downregulate MHC-I molecule and at the same time overexpress check point inhibitors such as PD-L1 that cause evasion and dampening of the immune system, respectively [156]. Many immunotherapeutic strategies are proposed to enhance immune response against breast cancer. The most used strategy is focused on monoclonal antibody (for example trastuzumab that impairs HER2 signaling) and immune check point inhibitors alone or in combination with surgery ablation, radiotherapy, chemotherapy or hormonal therapy. Promising but more technical challenge approach, such as adoptive T cell therapy and vaccines, are also under clinical evaluation [157, 166].

1.5.2 Neutrophils in breast cancer

Neutrophils exert both pro-tumoral and anti-tumoral functions as described above depending on the tumor microenvironment [10]. In breast cancer context, several evidence indicates a key role of neutrophils in primary tumor growth and metastasis dissemination. In patients, high NLR is associated to poor overall survival (OS) and disease-free survival (DFS) [167, 168]. Among the different cancer subtype, high NLR is associated to reduced OS in luminal A subtype [167] and in ER and HER2 negative tumors [168]. Using Polyoma Middle T (PyMT) model it was demonstrated that neutrophilia is caused by G-CSF production by cancer cells [169]. In breast cancer patients there is a circulating neutrophil subpopulation displaying an immunosuppressive phenotype that sustain tumor growth [129]. Collectively taken, both increased number and altered phenotype of circulating neutrophils correlate with pathology outcome. Several evidence indicates a key role of neutrophils in breast cancer metastasis exerting both anti-metastatic and pro-metastatic role [170]. The anti-metastatic role was described by Granot and colleagues. They found that the secretion of cytokines and chemokines such as CCL2 by cancer cells directly activates neutrophils to kill tumor cells by ROS production [109]. MET was described to enhance neutrophil extravasation with anti-tumoral and anti-metastatic activities [115]. On the contrary, many evidence indicates a pro-metastatic role of neutrophils. Coeffelt and colleagues have observed that the production of iNOS by neutrophils, induced by IL-17 secreted by γδ T cells, inhibits CD8 T cells activity [104].

Neutrophils can have a direct effect of metastasis acting on the pre-metastatic niche [103] trapping cancer cells through the formation of NET [171]. Mechanisms of recruiting pro-metastatic neutrophils are IL-5 and GM-CSF in obesity conditions [172], IL-16 [173]. and TNF-α that recruits CXCR2+ neutrophils [174]. Neutrophils interact with other immune cells involved in metastasis control such as NK cells. In 4T1 model, neutrophils are described to inhibit NK cells and promote tissue remodeling thus facilitating metastasis seeding [175]. Collectively taken, immune cells and in particular neutrophils, play a key role in breast cancer biology. Targeting of neutrophils represents a potential approach to limit disease progression in particular metastasis spread.

1.6 Glioma

Glioma represents about 80% of malignant brain tumors with an estimated annual incidence of 6.6 per 100,000 individuals in the USA. The traditional classification divided glioma into four major histological classes (grades I–IV), taking count of their microscopic characteristics (for example cytological atypia, anaplasia, mitotic activity, microvascular proliferation, and necrosis) and clinical behavior. In 2016 World Health Organization (WHO) revised Classification of tumors of the central nervous system (CNS) [176]. The new classification also dived tumors in: astrocytomas (WHO grade I–IV), oligodendrogliomas (WHO grade II–III) and mixed oligoastrocytomas (WHO grade II–III) depending on their putative cells of origin. Grade IV glioma or Glioblastoma (GBM) have the higher incidence among primary brain tumor and have a lifespan from 12.2 to 18.2 months with a 5-year survivor rate less than 5%. GMB classification takes also advantage of molecular classification: IDH mutations, 1p and 19q co-deletion, methylation status [177]. Lower-grade gliomas (WHO grade II–III) are about 30% of all glioma and less aggressive and the clinical behavior is not predictable based on their histologic class [176].

GBM care protocol is not changed in the last 10 years and consists of maximal tumor rejection followed by radiotherapy and chemotherapy with Temozolomide [178]. Lower-grade glioma therapeutic protocol consists of surgical rejection, clinical monitoring, radiation alone or in concomitant with chemotherapy. Therapy protocol

depend on histologic class, grade and molecular features (IDH mutations, 1p and 19q co-deletion). New therapeutical strategies were tested in these years to try to prolong patients survival, but clinical trials based on blocking angiogenesis [179] and targeting dysregulated cell signaling pathways [180] failed to increase survival.
In the last 5 years, many successful data in the battle against cancer come from immunotherapy [181-183]. Due to the fact that in glioma there is a strong immunosuppressive microenvironment, unleashing the immune response against cancer could be a potential efficacious treatment also for this tumor [184].

1.6.1 The immune system in glioma

Traditionally CNS was described as an immune-privileged site due to the presence of the Blood-Brain Barrier (BBB) and the absence of leukocytes and lymphatic vessels. These was demonstrated with preclinical models of tissue engraftment in the brain that lack of the rejection due to immune response. However, it is becoming clear that the CNS actively interact with immune cells also in presence of an intact BBB [185, 186]. Moreover, Louvenau et al demonstrated the presence of lymphatic vessels within the dural sinuses. These vessels are able to transport leukocytes between cerebrospinal fluid and deep cervical lymphatic vessels [187]. In glioma context, disruption of BBB cause an important interaction between glioma cells and the immune system and the establishment of an immunosuppressive microenvironment that dampen cell-mediated immunity (Fig. 1.7) [188]. Immunosuppression is caused by glioma cells that over-express molecules with immunosuppressive function such as E2, TGF-β, indoleamine 2,3-dioxygenase (IDO), and IL-10. These molecules cause recruitment of T regulatory and myeloid derived suppressor cells to the tumor site that dampen APC presentation activity and T cell cytotoxic activity. Other mechanisms of immunosuppression occur in glioma; glioma cells downregulate their MHC-I complex provoking tumor escape and express PD-L1, ligand of PD-1 that inhibit MHC signaling limiting PD-1^+ cells cytotoxic activity [189-191]. Blocking of PD-1 is considered a potential pharmacological effect to enhance T cell cytotoxic activity against cancer cells. In glioma preclinical models, the survival rate is increased after administration of α-PD-1 in combination with radiotherapy [192]. Concomitant treatment with α-CTLA-4 and

α-PD-1 shows further increase in survival rate with increased number of activated $CD8^+$ and NK cells and the decrease immunosuppressive cells in the lesion [193]. Moreover, concomitant pharmacological blocking of CTLA-4, PD-1 and IDO increased survival rate in other glioblastoma models confirming previous data. Blocking of CTLA-4 alone, and CTLA-4 and administration of IL-12 provoked an increase of tumor infiltrating effector T cell and decreased T regulatory infiltration [194-196]. Several clinical trials are ongoing to test the efficacy of checkpoint inhibitors in glioma patients. In addition other strategies to improve the immune anti-tumoral response including vaccination with tumor-specific, tumor-associated peptides, infusion of primed autologous APCs and adoptive transfer of engineered T cell are under evaluation [197]. Also, myeloid cells in glioma are potential targets to improve immune response because TAMs are described as the main infiltrating leukocytes in glioma. Pharmacological inhibition of CSF-1R (CD115) that blocks monocyte maturation and differentiation toward an M2-like phenotype that sustains tumor growth, increases survival rate in a GMB mouse model [198, 199]. Neutrophils are able to infiltrate glioma lesion [200, 201] and could have both pro-tumoral and anti-tumoral functions.

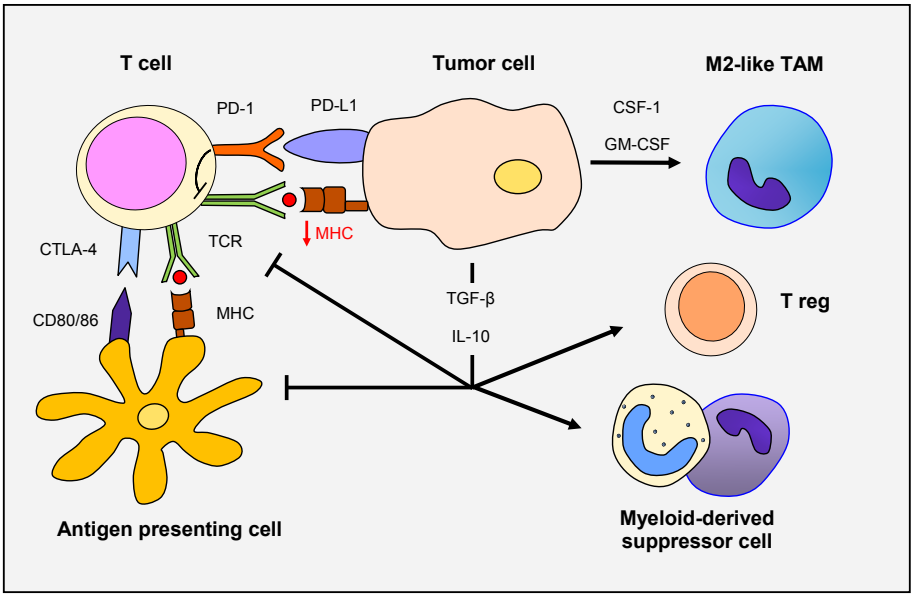


Figure 1.8: The immune response in glioma. Glioma cells secrete TGF-β and IL-10 promoting an immunosuppressive microenvironment that inhibit immune response against tumor cells [188].

1.6.2 Neutrophils in glioma

In glioma context, neutrophil role is not completed elucidated [188, 199]. Evidence coming from patients indicates an alteration of circulating neutrophils and the presence of TANs into brain lesion. It is reported that glioma patients have a strong neutrophila [202] due to massive production of G-CSF by the tumor [203, 204]. In glioma patients the neutrophil-to-lymphocytes ratio (NLR), that indicates neutrophilia normalized on lymphocytes count [86], is predictive of their outcome. Glioma patients with an NLR higher than 4 develop a worse outcome independently that NLR is measured before therapy [205, 206], after second surgery [207] and after treatment with temozolomide and radiotherapy [208]. On the other hand, NLR minor than 4 is associated to favorable outcome of the disease only in glioblastoma with wild type gene IDH1 that is one the most frequently mutated gene in GBM [209]. The upregulation of CD11b expression in neutrophils of GBM patients is an early sign of tumor progression [210]. Neutrophils participate in tumor growth maintaining an immunosuppressive microenvironment through the production of arginase I [211]. Bevacizumab treatment (anti-VEGF-A) is

more efficacious in patients with higher baseline neutrophil count and the TGCA database indicates that *Csf3* expression is correlated with VEGF-A dependent angiogenesis (Fig. 1.9) [212]. Neutrophils can infiltrate also brain tumor lesion [200]. NRL is correlated with infiltrating neutrophils [213] and there is a positive correlation between glioma grade and anti-VEGF-A efficacy in GBM [200, 214]. Neutrophils are recruited to tumor site by CXCL8 produced after FasL triggering by glioma cells [215, 216]. Neutrophils exert their immunosuppressive role in the lesion also when they are attracted by macrophage migration inhibitory factor (MIF) produced by cancer cells [217], inducing proliferation of cancer cells through the secretion of S100A4 [214] and contributing to the resistance to anti-angiogenic therapy (Fig. 1.9) [218]. Preclinical data confirm the pro-tumoral role of neutrophils. Neutrophil depletion in a glioma model through the administration of anti-Ly6G prolong mice overall survival [219]. Finally, IDH-1 mutant glioma showed a longer survival rate compared to wild type IDH-1 with a reduced neutrophil infiltration [220].

Preclinical glioma models are valid approach to study immune system in glioma but there is an important limitation due to the inverted leukocytes formula in mice (10 – 25% of neutrophil on total leukocytes) compared to human (50 – 70% of neutrophil on total leukocytes). Preclinical data could underestimate the role of neutrophils in humans and necessitate a better characterization of circulating and infiltrating neutrophils in human samples [188].

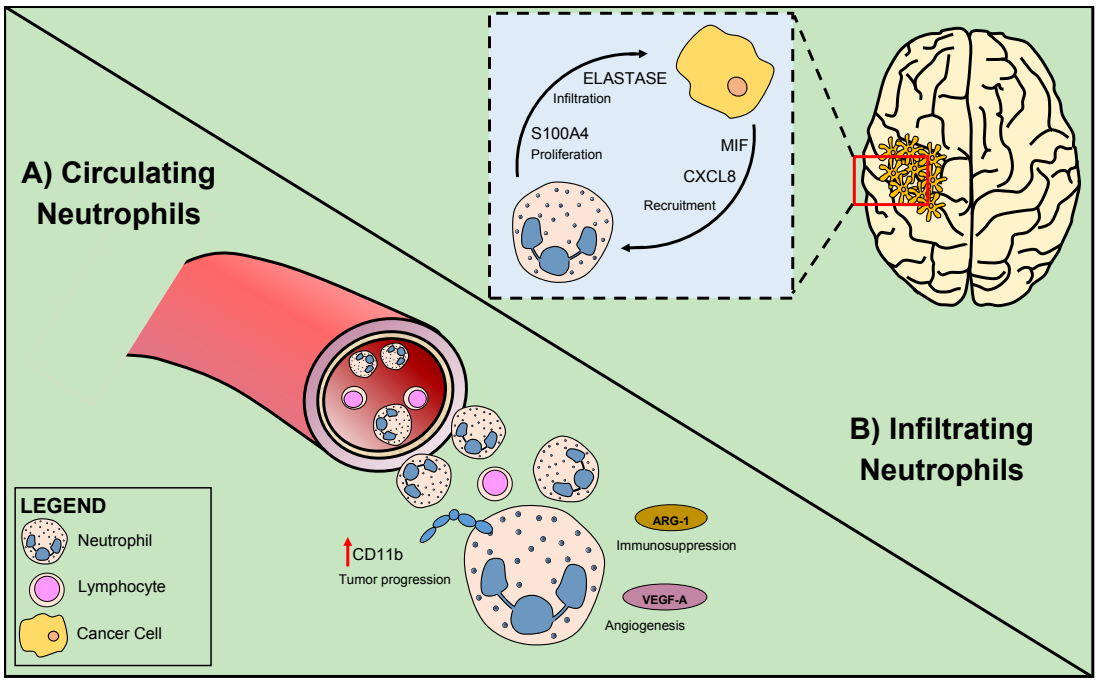


Figure 1.9: Neutrophils in gliomas. A) Circulating neutrophils upregulate CD11b as early sign of tumor progression, express Arg-1 and VEGF-A that sustain angiogenesis. B) Infiltrating neutrophils are recruited to the tumor site through MIF and CXCL8 secretion by cancer cells and produce S100A4 that regulate cancer cell proliferation and elastase that promote cancer cell infiltration in healthy tissue [188].

2 AIM OF THE THESIS

The general aim of this PhD thesis was to investigate the role of the atypical chemokine receptor ACKR2, a scavenger for inflammatory CC chemokines, in cancer.

In particular, being ACKR2 protective in chronic inflammation and in the extrinsic pathway of cancer, the first objective was to assess the role of ACKR2 in the intrinsic pathway of cancer development, using both the HER2 oncogene-driven breast cancer model NeuT and orthotopical and intravenous injection of the breast cancer 4T1 and melanoma B16F10 cell lines. The objective was to determine if ACKR2 has a role in primary tumor growth and lung metastasis development elucidating the role of innate immune cells, monocytes and neutrophils.

The second objective was to study by flow cytometry the phenotype of circulating and infiltrating monocytes and neutrophils in high grade glioma patients, a tumor characterized by neutrophilia and immunosuppression, in order to understand the prognostic role of these cells.

3 MATERIAL AND METHODS

3.1 ACKR2 study material and methods

3.1.1 Cell lines

4T1 and 4T1-66cl4 cells (kindly provided by Dr Claudia Chiodoni, Department of Experimental Oncology and Molecular Medicine, Istituto Nazionale dei Tumori, Milano, Italy) were grown in DMEM (Lonza) supplemented with 10% FBS (Sigma), 1% penicillin/streptomycin (Lonza), 1% L-glutamine (Lonza), 1% sodium pyruvate (Lonza), 1% Hepes (Lonza). B16-F10, kindly provided by Massimiliano Mazzone (Vesalius Research Center, Leuven, Belgium), were grown in DMEM (Lonza) supplemented with 10% FBS (Sigma), 1% penicillin/streptomycin (Lonza), 1% L-glutamine (Lonza). 4T1-luc from PerkinElmer were grown in RPMI 1640 (Lonza), 10% FBS (Sigma), 1% penicillin/streptomycin (Lonza), 1% L-glutamine (Lonza), 1% sodium pyruvate (Lonza), 5.4 g/l glucose (Sigma).

3.1.2 Animals

Ackr2$^{-/-}$ mice were maintained on pure Balb/c and C57BL/6J genetic background. Balb/c WT and Ackr2$^{-/-}$ mice were crossed with NeuT mice (kindly donated by Professor Federica Cavallo, University of Turin, Italy). WT and WT CD45.1 mice were obtained from Charles River Laboratories (Calco, Italy) or were cohoused littermates. All colonies were housed and bred in the SPF animal facility at Humanitas Clinical and Research Center in individually ventilated cages. Mice used for experiments were 8 to 12 weeks old. Procedures involving animals handling and care were conformed to protocols approved by the Humanitas Clinical and Research Center (Rozzano, Milan, Italy) in compliance with national (4D.L. N.116, G.U., suppl. 40, 18-2-1992) and international law and policies (EEC Council Directive 2010/63/EU, OJ L 276/33, 22-09-2010; National Institutes of Health Guide for the Care and Use of Laboratory Animals, US National Research Council, 2011). The study was approved

by the Italian Ministry of Health (approval n. 88/2013-B, issued on the 08/04/2013). All efforts were made to minimize the number of animals used and their suffering.

3.1.3 Tissue collection

Blood was collected from the retro-orbital plexus and by cardiac puncture as described [221]. Briefly, blood was collected in 2KD-EDTA spray coated tubes (BD Bioscience), washed in FACS buffer ($PBS^{-/-}$, 1% BSA, 0.05 % sodium azide), red blood cells were lysed, washed again and cells were stained as indicated. Lungs were instilled with PBS for FACS analysis or 10% neutral buffer formalin for histological analysis. For FACS analysis, lungs were minced, digested for 45 min in 1 mg/ml collagenase IV (Sigma) in $PBS^{-/-}$ (Sigma), filtered with 70 μm cell strainer. Red blood cells were lysed and cells stained as indicated. BM was collected by femurs. Bones were harvested, cleaned, flushed and filtered with 70 μm cell strainer. Red blood cells were lysed and cells stained as indicated below.

3.1.4 Tumor models

Tumor volume was assessed with caliper using the formula: (Length x Width x Width)/2. Tumor take in NeuT model was determinate by palpation as number of mammary tumors per mouse. For 4T1 and 4T1-66cl4 models 5 x 10^5 cells were injected in the mammary fat pad of Balb/c mice. For lung metastasis evaluation in NeuT, 4T1 and 4T1-66cl4 model, lungs were instilled and fixed for 24 h with 4% neutral buffered formalin, routinely processed for paraffin embedding, sectioned at 4 μm thickness, and stained with hematoxylin and eosin. Sections were evaluated in a blinded fashion under a light microscope. Lung metastasis in NeuT, 4T1 and 4T1-66cl4 models were classified according to their size into: small (<30 neoplastic cells), medium (30–300 neoplastic cells), and large (>300 neoplastic cells). A total metastatic score was then calculated for each lung as follows: number of small metastases*1 + number of medium metastases*3 + number of large metastasis*5. Representative images were acquired with Slide Scanner VS120 dotSlide (Olympus) and analyzed with ImageJ. The melanoma cell line B16F10 (2 x 10^5 cells) was injected i.v. in

C57BL/6 mice and metastases were macroscopically counted as dark nodules on the lung surface. For all the models, metastatic ratio was calculated as ratio of metastasis in Ackr2$^{-/-}$ or depleted mice compared to indicated control mice. For macrophages depletion, mice were treated with 100 μg of α-CD115 antibody (clone AFS98, Bioxcell) the day before 2 x 10^5 B16-F10 injection and every two days for the entire duration of the experiment. For neutrophils depletion, mice were treated with 200 μg of α-Ly6G antibody (clone 1A8, Bioxcell) the day before 2 x 10^5 B16-F10 injection and with 100 μg every three days for the entire duration of the experiment. For B cell depletion, mice were treated with 250 μg of α-CD20 (clone 5D2, Genentech Inc.) three days before 2 x 10^5 B16-F10 injection. For adoptive transfer experiments, neutrophils were isolated from WT and Ackr2$^{-/-}$ BM using the Mouse Neutrophil Isolation Kit (Miltenyi Biotec) and an autoMACS Pro separator (Miltenyi Biotec). Cell purity was assessed by flow cytometry (CD45, CD11b, Ly6G) and used only if neutrophils were ≥ 95% on CD45$^+$ cells. For B16F10 model, recipient WT mice were injected i.v. with 5 x 10^6 WT or Ackr2$^{-/-}$ neutrophils every 3 days for the entire duration of the experiment. For adoptive transfer experiment, recipient CD45.1 mice were injected i.v. with 2 x 10^6 CD45.2 WT or Ackr2$^{-/-}$ neutrophils 15 minutes before CCL3L1 (R&D) injection and after 1 hour lung and blood were collected and leukocytes counted by flow cytometry.

3.1.5 Immunohistochemistry

Serial 4 μm formalin-fixed and paraffin-embedded lung sections were deparaffinized and underwent heat-induced epitope retrieval with pressure cooker. Endogenous peroxidase activity was blocked by incubating sections in 3% H_2O_2 for 15 min. Slides were rinsed and treated with Rodent Block M (Biocare Medical) for 30 min to reduce nonspecific background staining and then incubated for 1 h at room temperature with Ly6G antibody (1:200; clone 1A8; BD Bioscience), Sections were incubated for 30 min with Rat on Mouse HRP-Polymer kit (Biocare Medical). The immunoreaction was visualized with 3,3'-diaminobenzidine (Peroxidase DAB Substrate Kit, Vector Laboratories) substrate and sections were counterstained with Mayer's haematoxylin. Negative immunohistochemical controls for each sample were prepared by replacing

the primary antibody with normal serum. Positive control sections were included in each immunolabeling assay. Tissues were dehydrated with ethanol, mounted with Eukitt and acquired with an Olympus BX61 virtual slide scanning system using Cell^F software (Olympus). In each section 10 independent field of view were acquired. To evaluate the extent of granulocytes infiltration in the lung parenchyma, the percentage of Ly6G-positive area was analyzed with Image-Pro Analyzer 7.0 (Media Cybernetics) software. Representative images were generated using the ImageJ analysis program (http://rsb.info.nih.gov/ij/).

3.1.6 Flow cytometry analysis

Flow cytometry analysis were performed as previously described [221]. To exclude death cells from analysis, cells were stained with Violet dead cell stain kit (Thermo Fisher). Single cell suspension was stained with antibodies listed in table 3.1 and related isotype. All antibodies were purchased from BD Bioscience, BioLegend, eBioscience or AbD Serotec. Flow cytometry data were acquired using a FACSCanto II (BD Bioscience) and LSR Fortessa (BD Bioscience) and data were analyzed with FACS Diva (BD Bioscience) and representative images were generated with FlowJo Software (Tree Star). To analyze ROS production, neutrophils were stained with 5 μM CellROX Deep Red Reagent (Thermo Fisher) for 20 min at 37°C in RPMI 1% FBS. Staining was blocked on ice, red blood cells were lysed, and neutrophils analyzed by flow cytometry within 2 h from the staining. The absolute number was determined by using TruCount beads (BD Biosciences) according to the manufacturer's instructions. Cell sorting was performed using a FACSAria III (BD Bioscience).

3.1.7 Leukocyte mobilization

Mice were injected i.p. with 3 μg CCL3L1 (R&D) and after 1 hour blood was collected and leukocytes counted by flow cytometry.

3.1.8 Generation of BM chimeras

Recipient mice received gentamycin (0.8 mg/ml) in drinking water for 2 weeks starting 10 days before irradiation. WT and Ackr2$^{-/-}$ mice were lethally irradiated with a total dose of 900 cGy. After 2 h, mice were injected in the retro-orbital plexus with 4 x 10^6 nucleated BM cells obtained by flushing of the cavity of a freshly dissected femur from WT or Ackr2$^{-/-}$ donors. Experiment were performed 16 weeks after irradiation to allow complete myeloid repopulation.

3.1.9 In vitro cell killing assay

Neutrophils were isolated by magnetic separation as described above from blood of 14 days 4T1 tumor-bearing mice or from BM of untreated mice and seeded (1 x 105/well) in a 96 wells plate in which, 4 hours before, 5 x 10^3 4T1-luc cells were plated in Optimem (Thermo Fisher) + 0.5% FBS. Cells were incubated overnight in presence of Apocynin 100 μM (Sigma) or DMSO control. Firefly luciferase activity was detected with luciferase assay system (Promega) and Synergy H2 (Biotek). Cell killing was calculated as percentage of tumor lysis by the following formula: % cell killing = (1 - [luminescence of samples with neutrophils] / [luminescence of samples in medium]) x 100.

3.1.10 Transcript analysis by quantitative PCR (qPCR)

Total RNA was extracted from neutrophils using the TRIzol reagent (Thermo Fisher). Reverse transcription was done using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative PCR was performed with TaqMan Gene Expression Assays in a CFX Connect Real-Time PCR Detection System (BioRad) with probes listed in table 3.2. Relative mRNA expression was determined by using the 2^-ΔCt method, and normalized to the expression of the housekeeping gene Gapdh.

3.1.11 Statistical analysis

Data are represented as mean. In all figures sample variation is shown as SD. P value was generated using the unpaired t test (GraphPad Prism 5). * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, ns = not statistically different.

Table 3.1: List of murine antibodies.

Antigen	Fluorochrome	Clone	Supplier
CD45	PerCP	30-F11	Biolegend
CD45	V450	30-F11	BD Bioscience
CD45	BV605	30-F11	BD Bioscience
CD11b	Pacific Blue	M1/70	Biolegend
CD11b	PE	M1/70	BD Bioscience
CD11b	PerCP-Cy5.5	M1/70	BD Bioscience
Ly6G	PeCy7	1A8	BD Bioscience
Ly6G	FITC	1A8	BD Bioscience
Ly6C	FITC	AL-21	BD Bioscience
Ly6C	PE	AL-21	BD Bioscience
F4/80	Alexa 647	CI:A3-1	AbD Serotec
ICAM-1/CD54	APC	YN1/1.7.4	Biolegend
CD62L	PE	MEL14	eBioscience
Gr-1	APC	RB6-8C5	BD Bioscience

Table 3.2: List of murine Taqman probes for qPCR.

Gene name	Code
Ccr1	Mm01216147_m1
Ccr2	Mm_00438270_m1
Ccr5	Mm04207879_m1
Cxcr4	Mm_01292123_m1
Vegfa	Mm00437306_m1
Tnf-α	Mm00443258_m1
Alox5	Mm01182747_m1
Arg1	Mm_00475988_m1
Gapdh	Mm99999915_g1

3.2 Neutrophils in High grade glioma materials and methods

3.2.1 Patients

Al total of 6 patients with grade III glioma, 4 patients with IV grade glioma (glioblastoma) and 4 heathy controls were enrolled in the study. Patients were stratified based on histological classification. This study was approved by the clinical committee of Humanitas Clinical and Research Center. All patients signed an informed consent document.

3.2.2 Blood collection

Blood was collected the day before the surgery in vacutainer containing 2KD-EDTA spray coated tubes (BD Bioscience), washed in FACS buffer ($PBS^{-/-}$, 1% BSA, 0.05 % sodium azide), red blood cells were lysed, washed again and cells were stained as indicated.

3.2.3 Tissue collection and dissociation

Tumor core identified by PET analysis removed and washed in $PBS^{-/-}$. 0.2 g of tissue were put in C tube (Miltenyi Biotec) and mechanical disaggregated using GentleMACS (Miltenyi Biotec). Later, cells were filtered in 70 μm cell strainer and red blood cells were lysed, washed again and cells were stained as indicated.

3.2.4 Flow cytometry

Flow cytometry analysis were performed as previously described [222]. Fc receptors were blocked with incubation with 1% human serum (Sigma). Single cell suspension was stained with antibodies listed in table 3.3 and related isotype. All antibodies were purchased from BD Bioscience and BioLegend. Later, to exclude death cells from analysis, cells were stained with Violet dead cell stain kit (Biolegend). Flow cytometry

data were acquired using a FACS A5 Symphony (BD Bioscience) and data were analyzed with FlowJo Software (Tree Star).

3.2.5 Statistical analysis

Data are represented as mean. In all figures sample variation is shown as SD. P value was generated using the unpaired t test (GraphPad Prism 5). * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, ns = not statistically different.

Table 3.3: List of human antibodies.

Antigen	Fluorochrome	Clone	Supplier
L/D	BV510	Zombie	Biolegend
CD45	APC-Cy7	2D1	BD
CD14	FITC	M5E2	BD
CD15	BV710	W6D3	BD
CD16	PE 594	3G8	Biolegend
Syto16	FITC		Thermo Fisher
CD66b	AF700	G10F5	Biolegend
CD3	BV570	UCHT1	Biolegend
CD19	BV570	HIB19	Biolegend
CD56	BV570	5.1H11(NCAM)	Biolegend
CD11b	BV605	ICRF44	Biolegend
CD54	APC	HA58	Biolegend
CD62L	BV650	DREG56	Biolegend
CCR2	BV421	48607	BD
CXCR1	Percp-Cy5.5	8F1/CXCR1	Biolegend
CXCR2	PE-Cy7	E8/CXCR2	Biolegend

4 RESULTS

4.1 Study of the role of ACKR2 in the intrinsic pathway of cancer

To study the role of ACKR2 in the intrinsic pathway of cancer, we use NeuT mice as model of Her2+ breast cancer and we evaluated tumor growth and lung metastasis development as sign of disease severity.

4.1.1 Ackr2$^{-/-}$ mice are protected against tumor metastasis

In order to extend previous studies on ACKR2 in carcinogenesis, we crossed Balb/c WT and Ackr2$^{-/-}$ mice with Balb/c NeuT mice, which overexpress the rat HER2 (Neu) oncogene under the mouse mammary tumor virus (MMTV) promoter and spontaneously develop mammary carcinomas closely recapitulating human breast carcinogenesis [223]. We followed primary tumor development measuring time of appearance and volume, and we found that in NeuT/Ackr2$^{-/-}$ mice tumoral masses in mammary glands developed earlier and reached higher volumes as compared to NeuT/Ackr2$^{+/+}$ mice (Fig. 4.1A). This result is in accordance with previous reports showing that ACKR2 genetic deficiency results in increased growth of primary tumors [69, 224]. Unexpectedly, lung analysis revealed less metastatic lesions in NeuT/Ackr2$^{-/-}$ mice as compared to NeuT/Ackr2$^{+/+}$ mice (Fig. 4.1B and C).

In an effort to strengthen and extend these findings, we use also the mammary carcinoma tumor lines 4T1. 4T1 tumor cells were transplanted ortotopically in the mammary fat pad of WT and Ackr2$^{-/-}$ mice and no difference in primary tumor growth was detected (Fig. 4.1D), but again the number of spontaneous lung metastasis was significantly lower in Ackr2$^{-/-}$ mice (Fig. 4.1E and F). These findings reveal an opposite role of ACKR2 in tumor biology. ACKR2 deletion has a pro-tumoral role in primary tumor growth in NeuT model and, at the same time a protective role in lung metastasis development in NeuT and 4T1 models.

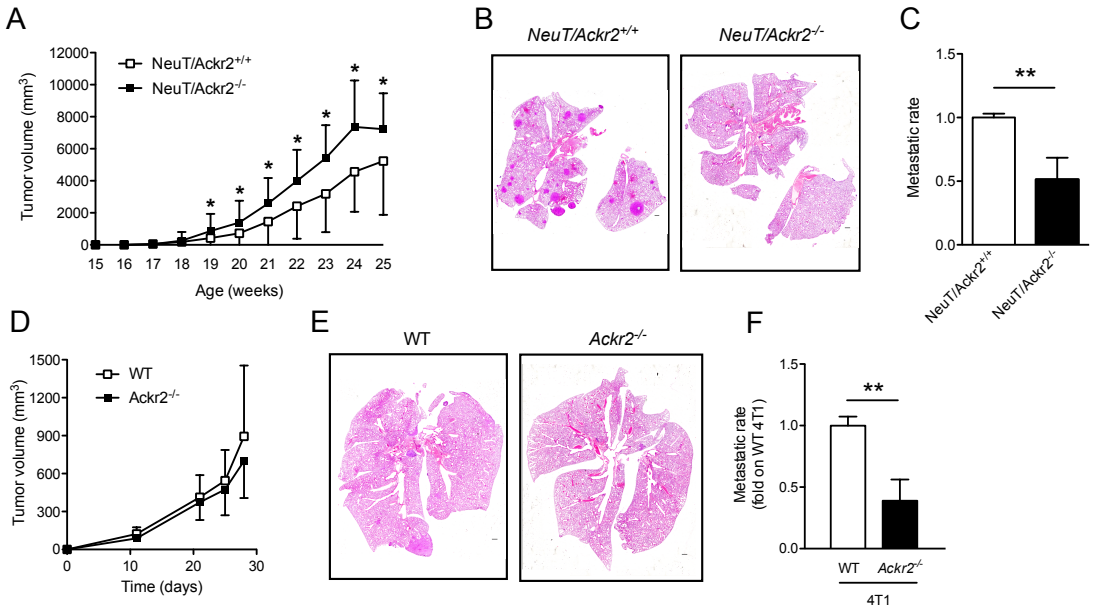


Figure 4.1: Ackr2$^{-/-}$ mice are protected from lung metastasis.

A) NeuT/Ackr2$^{+/+}$ (white symbols) and NeuT/Ackr2$^{-/-}$ (black symbols) mice were evaluated for tumor growth calculated as described in the Materials and Methods section (n = 42 and 23 for NeuT/Ackr2$^{+/+}$ and NeuT/Ackr2$^{-/-}$ mice, respectively). B) Representative images of hematoxylin and eosin staining of NeuT/Ackr2$^{+/+}$ and NeuT/Ackr2$^{-/-}$ lungs at 25 weeks of age. Magnification: 10X. Scale bar: 500 μm. C) Lung metastatic rate of NeuT/Ackr2$^{+/+}$ (white column) and NeuT/Ackr2$^{-/-}$ (black column) mice, calculated as described in the Materials and Methods section (n = 26 and 16 for NeuT/Ackr2$^{+/+}$ and NeuT/Ackr2$^{-/-}$ mice, respectively). D) Tumor volume in WT (white symbols) and Ackr2$^{-/-}$ (black symbols) mice injected orthotopically with 4T1 cells. (n = 14 and 13 for WT and Ackr2$^{-/-}$ mice, respectively). E) Representative images of hematoxylin and eosin staining of WT and Ackr2$^{-/-}$ lungs at day 28 after 4T1 cell injection. Magnification: 10X. Scale bar: 500 μm. F) Lung metastatic rate of WT (white columns) and Ackr2$^{-/-}$ (black columns) mice at day 28 after orthotopic injection of 4T1 cells (n = 14 WT and 13 Ackr2$^{-/-}$ mice).

4.1.2 ACKR2 in the hematopoietic compartment is responsible of the protection

In order to determine the contribution of Ackr2 expressed by hematopoietic cells in the metastatic phenotype, we performed BM chimera experiments. We found that WT mice reconstitute with Ackr2$^{-/-}$ BM recapitulate the lung metastasis levels found in Ackr2$^{-/-}$ control (Fig. 4.2A) indicating that the protective phenotype is due to lack of ACKR2 by hematopoietic cells.

The two breast cancer models used in our experiments are known to induce expansion and mobilization of myeloid cells, which then promote tumor growth [169, 225]. Interestingly, when animals were challenged with the 4T1 sibling cell line 66cl4, which on the opposite is unable to induce myeloid cell expansion because lack in the production of cytokine that influence myeloid cell recruitment such as G-CSF and GM-CSF [226, 227], we did not find any difference in the number of metastatic lesions between WT and Ackr2$^{-/-}$ mice (Fig. 4.2B).

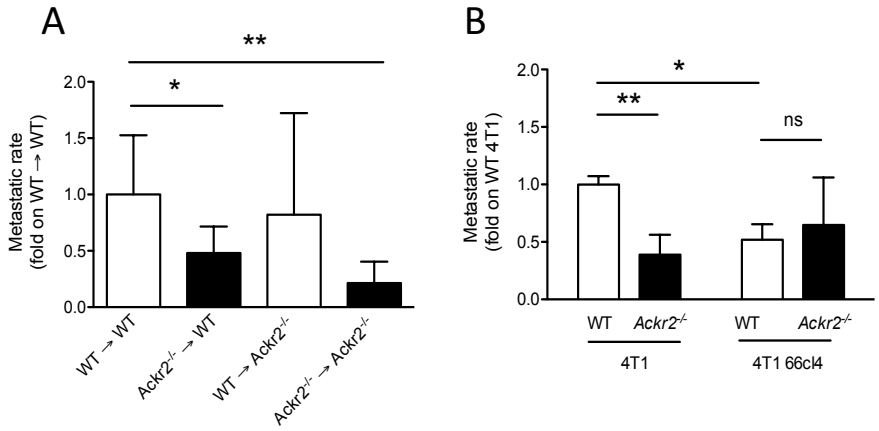


Figure 4.2: Hematopoietic expression of ACKR2 cause lung metastasis protection.

A) Lung metastatic rate using 4T1 model in bone marrow chimeric mice (Donor → Recipient) calculated as described in the Materials and Methods section (n = 6 for WT and 4 for Ackr2$^{-/-}$ recipient mice, respectively). B) Lung metastatic rate of WT (white columns) and Ackr2$^{-/-}$ (black columns) mice at day 28 after orthotopic injection of 4T1 or 4T1 66cl4 cells (n = 14 WT and 13 Ackr2$^{-/-}$ mice for 4T1, 4 WT and 4 Ackr2$^{-/-}$ mice for 4T1 66cl4).

4.1.3 Neutrophil and monocytes are increased in blood and lung of Ackr2$^{-/-}$ mice

We therefore focused on effects of Ackr2 genetic inactivation on the myeloid compartment of tumor-bearing mice as a potential mechanism of protection from metastasis. As previously reported, in resting conditions Ackr2$^{-/-}$ mice present increased number of inflammatory monocytes compared to WT mice while there is no difference in the number of other circulating leukocytes [56]. When we analyzed circulating and lung infiltrating leukocyte subsets in tumor-bearing mice (Gating strategy fig. 4.3A and B), we found, as expected, an increased circulating Ly6G^{+} neutrophils and Ly6C^{high} inflammatory monocytes in NeuT/Ackr2$^{+/+}$ mice (Fig. 4.4A and B, respectively) compared with resting WT and Ackr2$^{-/-}$ mice.

When we crossed Ackr2$^{-/-}$ mice with NeuT mice we found that the absolute number of neutrophils and monocytes was further increased compared to NeuT/Ackr2$^{+/+}$ mice (Fig. 4.4A and B, respectively). Increased number of inflammatory neutrophils and monocytes, but not alveolar or interstitial macrophages, was detected also in the lung of NeuT/Ackr2$^{-/-}$ as compared to NeuT/Ackr2$^{+/+}$ mice (Fig. 4.4C). Lung immunohistochemistry also showed a higher number of Ly6G^{+} neutrophils in the parenchyma of NeuT/Ackr2$^{-/-}$ lungs compared with NeuT/Ackr2$^{+/+}$ mice, confirming flow cytometry data (Fig. 4.4D and E). In 4T1 model, analysis of myeloid cells in the BM at day 14 after tumor injection showed a reduced number of monocytes and neutrophils in the BM of Ackr2$^{-/-}$ animals compared to WT mice (Fig. 4.4F) while no differences were found in vehicle-injected mice. These results indicate that, under tumor challenge, Ackr2$^{-/-}$ mice show enhanced release from BM of myeloid cells, which then accumulate in the blood stream and seed peripheral organs.

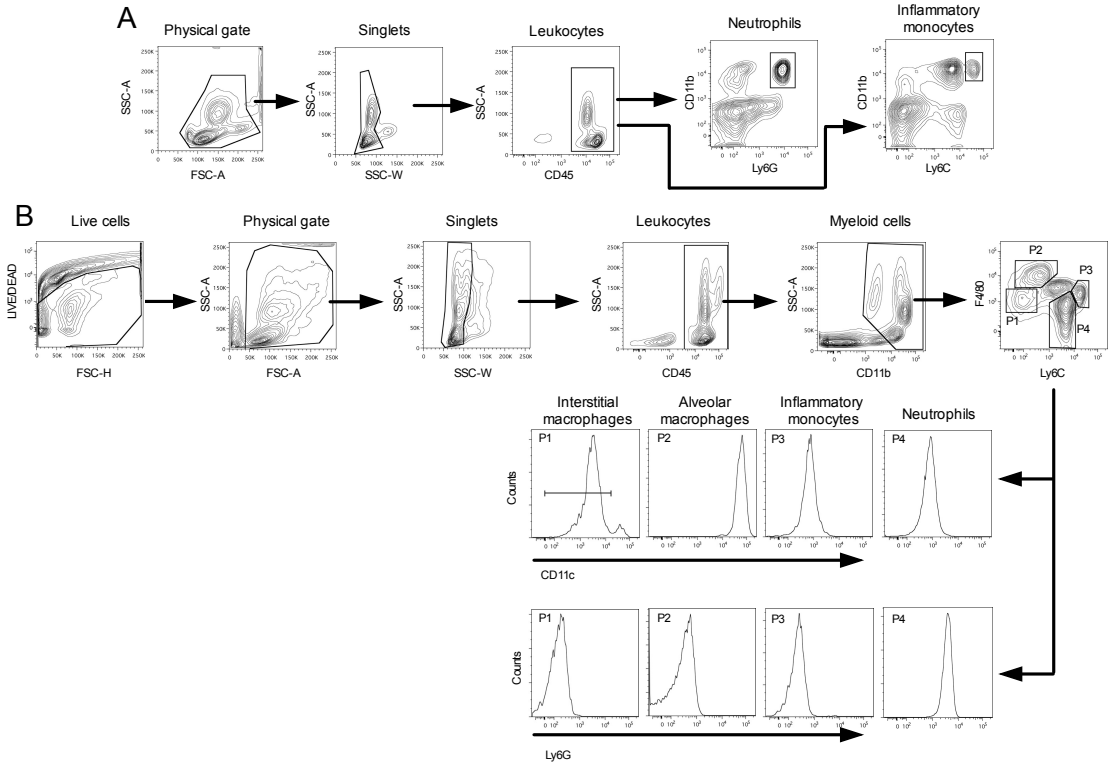


Figure 4.3: Flow cytometry gating strategy for the analysis of circulating and lung infiltrating neutrophil and inflammatory monocytes.

A) Gating strategy to identification of circulating neutrophils ($CD45^+/CD11b^+/Ly6G^+$) and inflammatory monocytes ($CD45^+/CD11b^+/Ly6C^{hi}$). B) Gating strategy for the identification of interstitial macrophages (P1; $CD11b^+/F4/80^{int}/Ly6C^-/CD11c^-/Ly6G^-$) alveolar macrophages (P2: $CD11b^{low}/F4/80^{hi}/Ly6C^{int}/CD11c^+/Ly6G^-$), inflammatory monocytes (P3; $CD11b^+/F4/80^{int}/Ly6C^{hi}/CD11c^-/Ly6G^-$), and neutrophils (P4; $CD11b^+/F4/80^-/Ly6C^{int}/CD11c^-/Ly6G^+$) in the lungs of tumor-bearing mice.

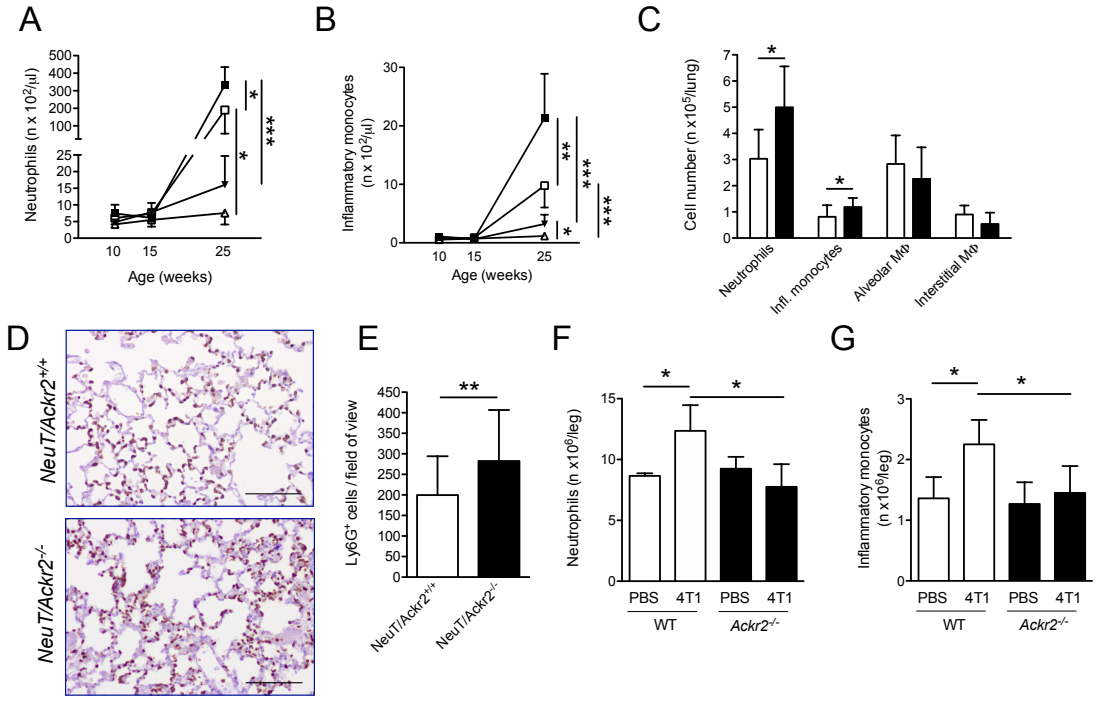


Figure 4.4: Protection from metastasis in Ackr2$^{-/-}$ mice is associated with increased numbers of monocytes and neutrophils in blood and lungs.

A) Absolute number of circulating neutrophils (CD45^{+}/CD11b^{+}/Ly6G^{+}) and B) inflammatory monocytes (CD45^{+}/CD11b^{+}/Ly6C^{hi}) in NeuT/Ackr2$^{+/+}$ (white squares), NeuT/Ackr2$^{-/-}$ (black squares), WT (white triangles) and Ackr2$^{-/-}$ (black triangles) mice (n = 6 – 9 for NeuT/Ackr2$^{+/+}$ mice, 5 – 7 for NeuT/Ackr2$^{-/-}$ mice, 3 - 5 for WT and Ackr2$^{-/-}$ mice). C) Absolute number of neutrophils, inflammatory monocytes, alveolar (CD11b^{low}/F4/80^{+}/Ly6C^{int}/CD11c^{+}/Ly6G^{-}) and interstitial macrophages (CD11b^{+}/F4/80^{int}/Ly6C^{-}/CD11c^{-}/Ly6G^{-}) in the lungs of NeuT/Ackr2$^{+/+}$ (white columns) and NeuT/Ackr2$^{-/-}$ (black columns) mice at 15 weeks of age (n = 12 and 6 for NeuT/Ackr2$^{+/+}$ and NeuT/Ackr2$^{-/-}$ mice, respectively). D) Representative immunohistochemical images of Ly6G staining in NeuT/Ackr2$^{+/+}$ and NeuT/Ackr2$^{-/-}$ lungs at 25 weeks of age. Magnification: 20X. Scale bar: 100 µm. E) Quantification of Ly6G immunohistochemical images as DAB positive cells on field of view (n = 9 and 8 for NeuT/Ackr2$^{+/+}$ and NeuT/Ackr2$^{-/-}$ mice, respectively). F) Absolute number of neutrophils and inflammatory monocytes in the BM of WT (white columns) and Ackr2$^{-/-}$ (black columns) mice on day 14 after orthotopic injection of PBS or 4T1 cells (n = 4 for both WT and Ackr2$^{-/-}$ mice).

4.1.4 Neutrophils are required for metastasis protection in Ackr2$^{-/-}$ mice

To investigate the relevance of the increased myeloid cell mobilization found in Ackr2$^{-/-}$ mice in the metastatic process, the B16F10 melanoma cell line was injected i.v. in a classic "artificial" hematogenous metastasis model.

Also in this experimental setting, Ackr2$^{-/-}$ mice showed a significant reduction in the number of metastatic foci in the lungs as compared to WT animals (Fig. 4.5A). In order to understand which myeloid cells are responsible for metastasis protection, we performed depletion experiments using monoclonal antibodies. Macrophages depletion by treatment with an α-CD115 monoclonal antibody significantly decreased the number of metastasis in WT mice, but did not reverse the protection observed in Ackr2$^{-/-}$ mice (Fig. 4.5B). B cell depletion (performed with α-CD20 administration) did not modify the metastatic rate in WT and Ackr2$^{-/-}$ (Fig. 4.5C). Interestingly, neutrophil depletion with α-Ly6G monoclonal antibody caused a reduction in metastasis in WT mice while, on the contrary, an increase in metastasis in Ackr2$^{-/-}$ mice, which in the absence of neutrophils lost their protection and developed a number of metastasis comparable to WT animals (Fig. 4.5D).

The protective role of neutrophils in Ackr2$^{-/-}$ mice was also demonstrated performing depletion experiments with the ortotopically transplanted tumor lines 4T1. Also in this tumor, the number of metastasis in WT mice are reduced by neutrophil depletion while it is increased in Ackr2$^{-/-}$ mice (Fig. 4.5E).

The role of Ackr2$^{-/-}$ neutrophils in metastasis protection was further supported by adoptive transfer experiments. Transfer of Ackr2$^{-/-}$ but not WT neutrophils into WT tumor-bearing mice significantly reduced the number of metastasis (Fig. 4.5F) to numbers comparable to those observed in Ackr2$^{-/-}$ tumor-bearing mice (Fig. 4.5A). These results indicate Ackr2$^{-/-}$ neutrophils as leukocytes population with antimetastatic activity.

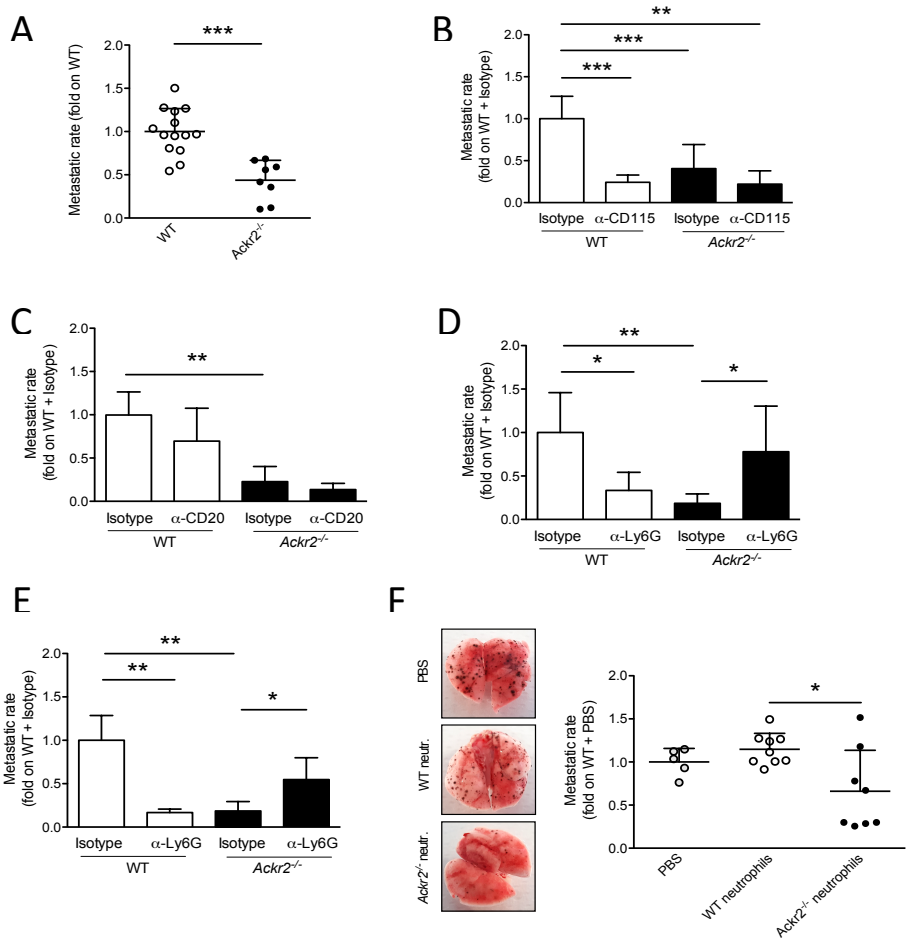


Figure 4.5: Ackr2$^{-/-}$ neutrophils are responsible of metastasis protection.

A) Lung metastatic rate in WT and Ackr2$^{-/-}$ mice 10 days after i.v. injection of B16-F10 cells. Number of metastasis was normalized on WT mice. B-D) Metastatic rate in WT and Ackr2$^{-/-}$ mice depleted for macrophages with α-CD115 (B), B cells with α-CD20 (C) and neutrophils with α-Ly6G (D) 10 days after i.v. injection of B16-F10 cells. Number of metastasis was normalized on WT mice treated with an irrelevant antibody (IgG) (n = 5 – 10 for WT mice, 5 – 8 for Ackr2$^{-/-}$ mice). E) Metastatic rate in WT and Ackr2$^{-/-}$ mice depleted for neutrophils with α-Ly6G 28 days after orthotopic injection of 4T1 cells (n = 3 for WT mice, 3 – 5 for Ackr2$^{-/-}$ mice). Number of metastasis was normalized on WT mice treated with an irrelevant antibody (IgG). F) Metastatic rate in WT mice 10 days after i.v. injection of B16-F10 cells and adoptive transfer of WT (white dots) or Ackr2$^{-/-}$ neutrophils (black dots) or PBS (grey dots). Number of metastasis was normalized on PBS injected mice. Representative images of excised lungs are shown on the left.

4.2 Analysis of neutrophil phenotype in Ackr2$^{-/-}$ mice

We demonstrated that ACKR2 expression in hematopoietic compartment is responsible of the protection and neutrophils are the leukocytes involved in the protection. Our objective was to analyze neutrophils phenotype in Ackr2$^{-/-}$ mice to describe ACKR2 role on neutrophils biology.

4.2.1 Hematopoietic Ackr2 expression impairs in vivo neutrophil mobilization

To investigate the role of ACKR2 in neutrophil infiltration in lungs, we performed experiments of leukocyte mobilization in BM chimeric mice. After one hour from i.p. injection of CCL3L1, a ACKR2, CCR1, CCR3 and CCR5 ligand known to induce rapid neutrophil mobilization [228], we analyzed blood and lung infiltrating neutrophils in bone marrow chimeric mice to assess if hematopoietic ACKR2 expression altered neutrophils mobilization. I.p. injection causes a systemic spread of CCL3L1 and cause an increase of neutrophil extravasation in tissues including the lung.

Results showed an increase in the number of circulating (Fig. 4.6A) and lung infiltrating neutrophils (Fig. 4.6B) in either WT or Ackr2$^{-/-}$ host animals when transplanted with Ackr2$^{-/-}$ but not whit WT hematopoietic cells, demonstrating that the increased neutrophil mobilization induced by CCL3L1 injection was caused by the absence of ACKR2 on the hematopoietic compartment. To exclude the role of other hematopoietic cells, we performed adoptive transfer experiment. We transfer WT and Ackr2$^{-/-}$ CD45.2 neutrophil in WT CD45.1 recipient challenged with CCL3L1. After one hour from the injection, we registered an increased infiltration in the lung of Ackr2$^{-/-}$ CD45.2 neutrophils in WT CD45.1 mice (Fig. 4.6C) with a concomitant decrease in the blood (Fig. 4.6D) indicating and increase activity to extravasate from blood to lung.

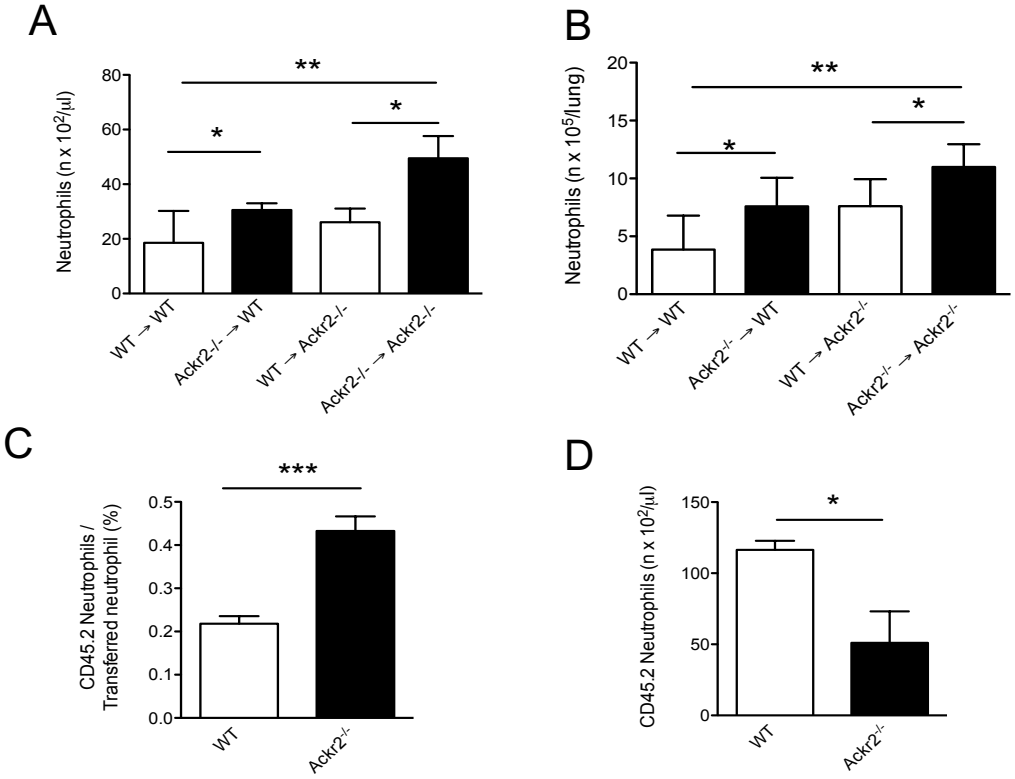


Figure 4.6: Hematopoietic ACKR2 expression increased neutrophils mobilization and lung infiltration in vivo.

A and B) Absolute number of circulating (A) and lung infiltrating (B) neutrophils in bone marrow chimeric mice (Donor → Recipient) after one hour from i.p. injection of CCL3L1 (n = 6 for WT and 4 for Ackr2$^{-/-}$ recipient mice, respectively). C) WT and Ackr2$^{-/-}$ CD45.2 neutrophils percentage on transferred neutrophils after one hour from i.p. injection of CCL3L1 (n = 3 recipient for each group). D) Absolute number of circulating WT and Ackr2$^{-/-}$ CD45.2 neutrophils after one hour from i.p. injection of CCL3L1 (n = 3 recipient for each group).

4.2.2 ACKR2 impairs CC chemokines expression in neutrophils

To elucidate the increased neutrophil mobilization in Ackr2$^{-/-}$ mice, we investigated chemokine receptor expression. Being CCL3L1 a ligand of CCR1, CCR3 and CCR5 we investigated the role of these and other chemokine receptor in neutrophils. We exclude from the analysis CCR3 because it is not expressed of neutrophils [229]. We added analysis of CCR2 as a chemokine receptor involved in neutrophils migration and functional activity [149] and CXCR4 known to have an important role in neutrophil egression from the bone marrow [230]. RT-PCR analysis from WT and Ackr2$^{-/-}$ resting bone marrow neutrophils revealed an increase of the expression of CCR1, CCR2 and CCR5 (Fig. 4.7A) but not CXCR4. This result indicates that the increased capacity of neutrophil to migrate in vivo are associated with a selectively increased expression of chemokine receptors.

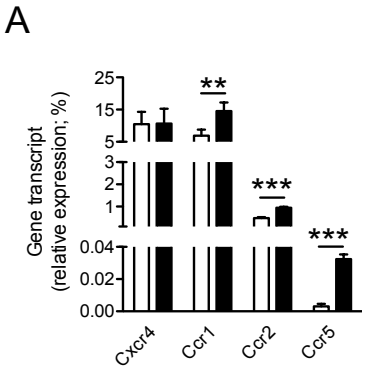


Figure 4.7: ACKR2 impairs CC but not CXC chemokine receptor expression.
A) qPCR analysis of chemokine receptors in FACS sorted WT (white columns) and Ackr2$^{-/-}$ (black columns) neutrophils (n = 4 for both WT and Ackr2$^{-/-}$ mice, two independent experiments). Data are relative to GAPDH expression.

4.2.3 ACKR2-deficient neutrophils have an activated phenotype

Neutrophil, as previously discussed, can be polarized in N1 (anti-tumoral) and N2 (pro-tumoral) phenotype. To asses if Ackr2$^{-/-}$ neutrophil show features belonging to polarized phenotype, we performed flow cytometry analysis of circulating neutrophils. We analyzed surface markers know to be involved in neutrophil activation: CD62L/L-selectin that is shed prior to neutrophil extravasation [119] and ICAM-1 that after binding with ICAM-1 on the endothelium allow neutrophils diapedesis [82].

In B16F10 model, we observed decrease of CD62L expression in Ackr2$^{-/-}$ mice compared to WT control (Fig. 4.8A and B) indicating an increased potential of neutrophil extravasation. At the same time, we found an increased expression of ICAM-1 in Ackr2$^{-/-}$ neutrophil (Fig. 4.8C and D) indicating the increased capacity to extravasate confirming an activated phenotype of Ackr2$^{-/-}$ neutrophils. No differences again were found in basal control for CD62L and ICAM-1 expression (Fig. 4.8B and D). We analyzed also by q-PCR a panel of gene involved in neutrophil biology described to be regulated in neutrophil polarization [10] and we found no difference in the expression of Tnf-α, Alox5, Vegfa and Arg1 (Fig. 4.8E). In 4T1 model, we observed a decreased level of CD62L in basal conditions between WT and Ackr2$^{-/-}$ neutrophil but not in tumor bearing mice (Fig. 4.8F). Similar data was obtained in 4T1 model: ICAM-1 is increased in Ackr2$^{-/-}$ tumor bearing mice and no difference were found in basal control (Fig. 4.8G). These results indicate an activated phenotype of Ackr2$^{-/-}$ neutrophils that associated with antimestatic activity in B16F10 model previously described (Fig. 4.5F) indicate a N1 phenotype both in term of expression of cell surface molecules and in vivo functional activity.

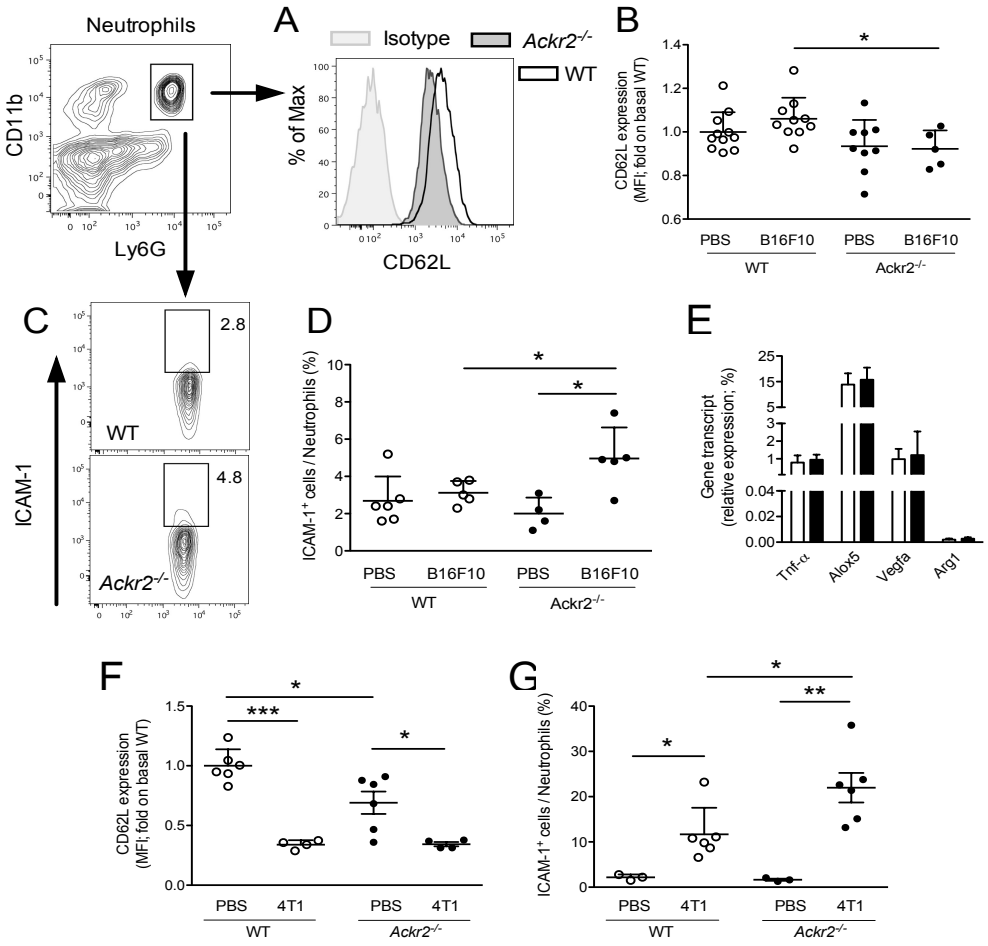


Figure 4.8: Ackr2$^{-/-}$ neutrophils have activated phenotype and increased expression of inflammatory CC chemokine receptors.

A) Representative histograms and B) quantifications of CD62L expression. CD62L mean fluorescence intensity (MFI) was normalized on WT neutrophils injected with PBS. C) Representative plot and D) quantification of percentage of ICAM-1 positive circulating neutrophils (CD45^{+}/CD11b^{+}/Ly6G^{+}) in WT and Ackr2$^{-/-}$ mice 10 days after i.v. injection with B16-F10 cells. Negative gate was set on isotype control. E) qPCR analysis of activation markers in FACS sorted WT (white columns) and Ackr2$^{-/-}$ (black columns) neutrophils (n = 4 for both WT and Ackr2$^{-/-}$ mice, two independent experiments). Data are relative to GAPDH expression. F) Quantifications of CD62L expression. CD62L mean fluorescence intensity (MFI) was normalized on WT neutrophils injected with PBS. G) Quantification of percentage of ICAM-1 positive circulating neutrophils (CD45^{+}/CD11b^{+}/Ly6G^{+}) in WT and Ackr2$^{-/-}$ mice 7 days after orthotopic injection with 4T1 cells. Negative gate was set on isotype control.

4.2.4 ACKR2-deficient neutrophils increased ROS production

To investigate functional molecule involved in neutrophil polarization, we analyzed reactive oxygen species (ROS) production of circulating neutrophil from tumor bearing mice by flow cytometry. We found an increase of ROS production in Ackr2$^{-/-}$ neutrophil in both B16F10 (Fig. 4.9A) and 4T1 model (Fig. 4.9B). Chemokine receptor are known to induce neutrophil ROS production [109] and our objective was to investigate if the increased transcript expression of CCR2 in Ackr2$^{-/-}$ have a functional role in our system. We noticed that Ackr2$^{-/-}$ neutrophil have an increased ROS activity even in basal condition (Fig. 4.9C) and after CCR2 stimulation through the ligand CCL2, Ackr2$^{-/-}$ neutrophil display an increased ROS production indicating the receptor have a function role in Ackr2$^{-/-}$ neutrophil biology (Fig. 4.9C).

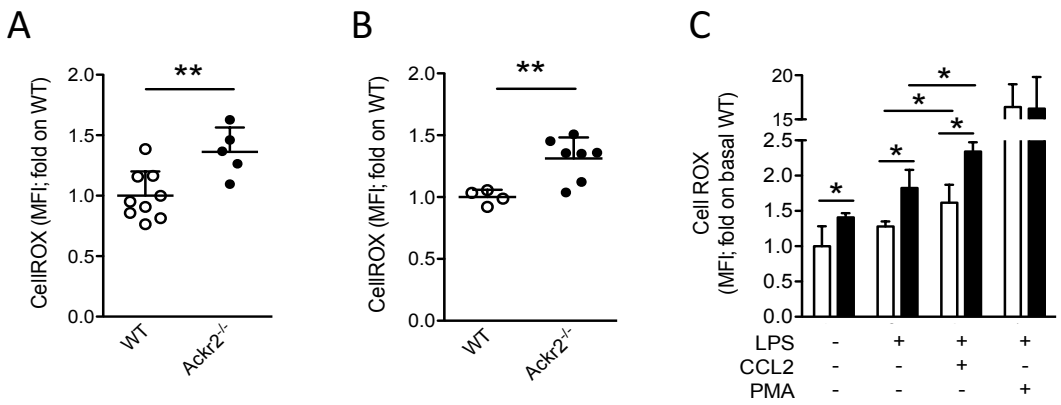


Figure 4.9: ACKR2 impair neutrophil ROS production in vivo and in vitro.
A) MFI of cellROX in WT and Ackr2$^{-/-}$ neutrophils taken from mice 10 days after orthotopic injection of B16F10 cells. CellROX MFI was normalized on WT neutrophils. B) MFI of cellROX in WT and Ackr2$^{-/-}$ neutrophils taken from mice 7 days after orthotopic injection of 4T1 cells. CellROX MFI was normalized on WT neutrophils. C) CellROX MFI in WT (white columns) and Ackr2$^{-/-}$ (black columns) neutrophils preincubated with PBS or LPS (100 ng/ml, 20 min) and stimulated with CCL2 (500 ng/ml, 30 min) or PMA (50 ng/ml, 30 min). CellROX MFI was normalized on basal WT group (n = 4, two independent experiments for both WT and Ackr2$^{-/-}$ mice).

4.2.5 ACKR2-deficient neutrophils have increased cytotoxic activity

The increased ROS production in Ackr2$^{-/-}$ mice could have a role in direct cell killing of cancer cell by neutrophil. We assessed in vitro cell killing in order to evaluate anti-metastatic potential of neutrophils and we performed ROS inhibition to dissect ROS contribution in cell killing. WT and Ackr2$^{-/-}$ neutrophils separated from 4T1 tumor bearing mice were co-cultured with 4T1 luciferase expressing cells to determinate cell killing assay. We registered an increased cell killing by Ackr2$^{-/-}$ neutrophil compared to WT control (Fig. 4.10A). ROS inhibition performed with apocynin, an inhibitor of NADPH oxidase complex [109], inhibits cell killing in WT mice and in Ackr2$^{-/-}$ but not abrogates difference between WT and Ackr2$^{-/-}$ neutrophils (Fig. 4.10A).
Similar results were obtained using neutrophils from bone marrow of resting mice. Ackr2$^{-/-}$ neutrophils display an increased tumoral killing activity (Fig. 4.10B), ROS inhibitor apocynin decreases cells killing in WT and Ackr2$^{-/-}$ neutrophils but not abrogates the different cell killing ability between WT and Ackr2$^{-/-}$ neutrophils (Fig. 4.10B).

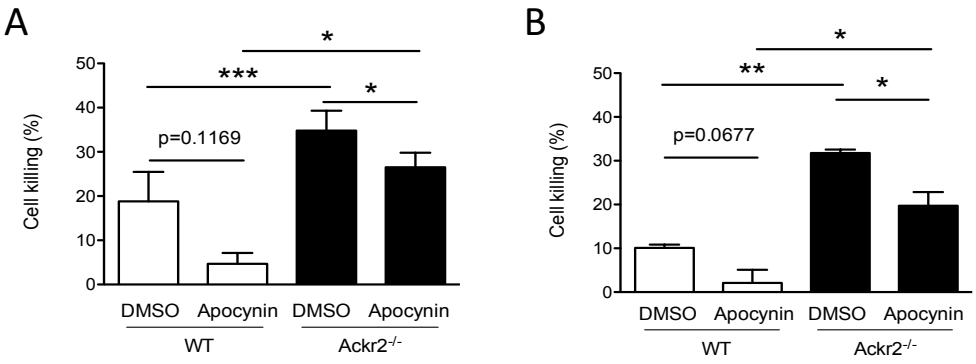


Figure 4.10: Ackr2$^{-/-}$ neutrophil show a ROS-dependent cell killing activity in vitro.

A) In vitro cell killing activity against 4T1-luc cells of circulating neutrophils magnetically separated from WT and Ackr2$^{-/-}$ mice after 21 days from orthotopic 4T1 injection. DMSO or Apocynin (100 μM) was added to inhibit ROS activity (n = 3, two independent experiments for both WT and *Ackr2*$^{-/-}$ mice). B) In vitro cell killing activity against 4T1-luc cells of resting bone marrow neutrophils magnetically separated from WT and Ackr2$^{-/-}$ mice. DMSO or Apocynin (100 μM) was added to inhibit ROS activity (n = 3, two independent experiments for both WT and *Ackr2*$^{-/-}$ mice).

4.3 Analysis of human neutrophils in glioma patients

In the previous paragraph, we assessed an anti-tumoral role of neutrophil in ACKR2-deficient mice. Our aim was to investigate the neutrophil phenotype in a human cancer context. We select high grade glioma (grade III and GBM) because are tumor types in which a strong systemic immunosuppressive environment is described [188]. We select also this type of cancer because we have the practical access to fresh blood and tissue samples, fundamental for neutrophils flow cytometry analysis. We analyzed relative neutrophil abundance on total leukocytes, their phenotype and subsets the day before the surgery and we correlated with TANs abundance.

4.3.1 Detection of circulating neutrophils and monocytes in high grade glioma patients

In order to investigate human circulating neutrophil rate and phenotype we perform multiparametric flow cytometry analysis on total blood leukocytes. We identified neutrophils as $CD45^{+}/CD3^{-}/CD19^{-}/CD56^{-}/CD15^{+}/CD16^{+}$ (Fig. 4.11A). We analyzed by flow cytometry neutrophil rate to total leucocytes (CD45 positive cells) in healthy patients, glioma grade III and GBM patients. While in healthy control and grade three glioma patients we observed about 65% of circulating neutrophils, in GBM patients we found an increased neutrophil rate of 82% (SD = 6.74) (Fig. 4.11B). This result confirms previous data [202] that indicate neutrophilia as a feature of GBM patients due to increase granulocytosis and neutrophil mobilization for increased CXCL8 [231], G-CSF [203, 204]. With the same staining, we are able to identify monocytes as $CD45^{+}/CD3^{-}/CD19^{-}/CD56^{-}/CD15^{-}/SSC^{int}$ (Fig. 4.11A). We found no difference are found in term of relative monocytosis, all the groups have the same rate of circulating monocytes (Fig. 4.11C). Based on this evidences, we foucus our attention on neutrophils evaluating their subsets and activatory status.

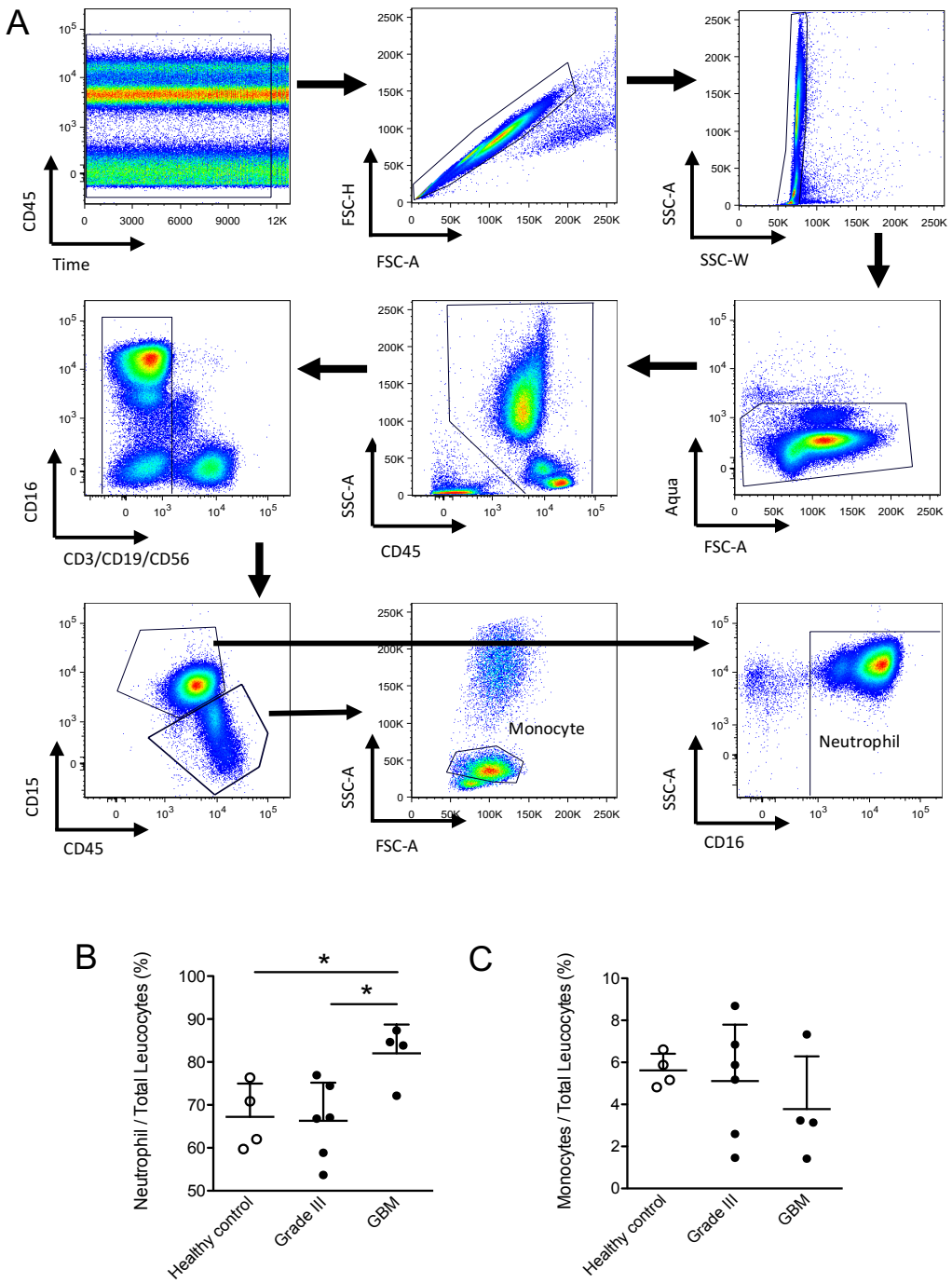


Figure 4.11: GBM patients have relative neutrophilia but not monocytosys.

A) Gating strategy to identification of circulating neutrophils ($CD45^+/CD3^-/CD19^-/CD56^-/CD15^+/CD16^+$) and monocytes neutrophils ($CD45^+/CD3^-/CD19^-/CD56^-/CD15^-/SSC^{int}$) B) Relative neutrophil rate on total leukocytes ($CD45^+$ cells). C) Relative monocytes rate on total leukocytes ($CD45^+$ cells).

4.3.2 Circulating neutrophil subsets in GBM patients

As previously described, granulopoiesis is altered in GBM patients. We set a flow cytometry approach to gate circulating and infiltrating neutrophils and their subsets. It is reported that neutrophils are a heterogeneous population and neutrophils subset can be identified in pathological situation such as sepsis and cancer evaluating expression of CD62L and CD16 [122]. We identified those populations also in our patients cohort (Fig. 4.12A) and we found in GBM patients a strong increase of immature $CD16^{low}/CD62L^{+}$ neutrophils that are not present in healthy controls (Fig. 4.12B). We observed also a concomitant reduction of "classical" neutrophils $CD62L^{+}/CD16^{+}$ rate in GBM compared to healthy controls (Fig. 4.12C). Finally, only a trend in the increase of activated neutrophils $CD62L^{neg}/CD16^{+}$ in GBM patients was detected (Fig. 4.12D). These results indicate a differential presence of neutrophils subsets in GBM patients indicating an altered neutrophil release from bone marrow and a different maturation status.

To assess neutrophils activation status in HGG patients compared to heatlhy subjects, we analyzed a panel of functional molecules involved in neutrophil biology. We noticed any difference in the expression on total neutrophil from healthy subjects and patients of CD11b (Fig. 4.12E), ICAM-1 (Fig. 4.12F), CXCR1 (Fig. 4.12G), CXCR2 (Fig. 4.12H) and CCR2 (Fig. 4.12I).

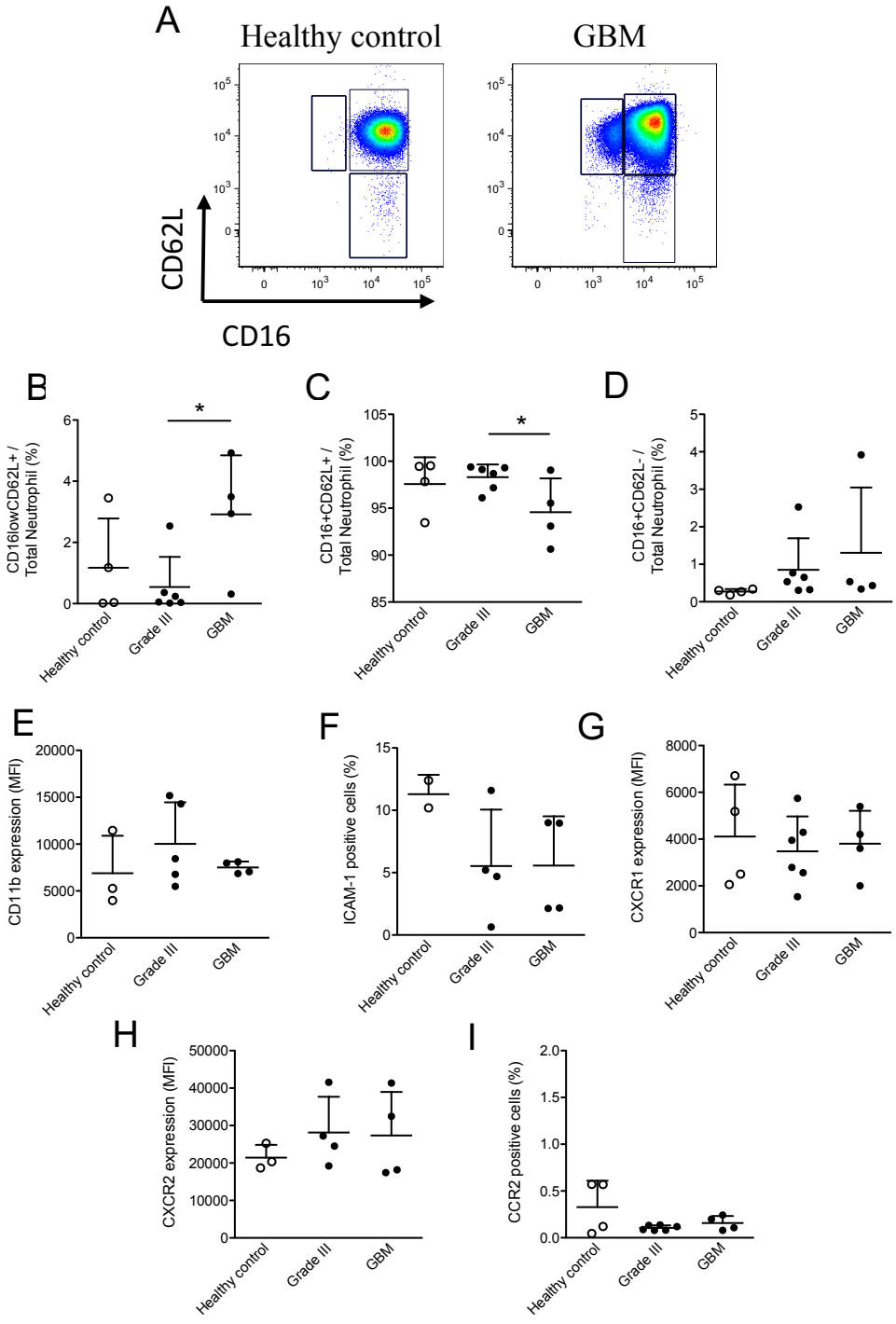


Figure 4.12: GBM patients display altered neutrophils subsets ration.

A) Representative dot plot of healthy control or GBM patient showing an altered neutrophil subsets ratio. B – D) Relative abundance on total neutrophils of (B) $CD16^{low}/CD62L^{+}$ cells, (C) $CD62L^{+}/CD16^{+}$, (D) $CD62L^{neg}/CD16^{+}$. E – I) MFI or percentage of expression on total neutrophil of (E) CD11b, (F) ICAM-1 (G) CXCR1, (H) CXCR2, (I) CCR2.

4.3.3 Increased neutrophilia is associated with increased neutrophil infiltration

Neutrophils infiltrate tumor lesion in the brain [200] and to assess if increased neutrophilia in blood is associated with increased neutrophils infiltration rate we performed flow cytometry analysis on immune infiltrate in glioma lesion. We set protocol to dissociate and stain infiltrating neutrophils in brain lesion (Fig. 4.13A) and we found a positive correlation between circulating neutrophils rate and infiltrating neutrophils rate on total CD45+ cells (Fig. 4.13B). These results indicate that increased circulating neutrophils rate is reflected on infiltrating neutrophil abundance.

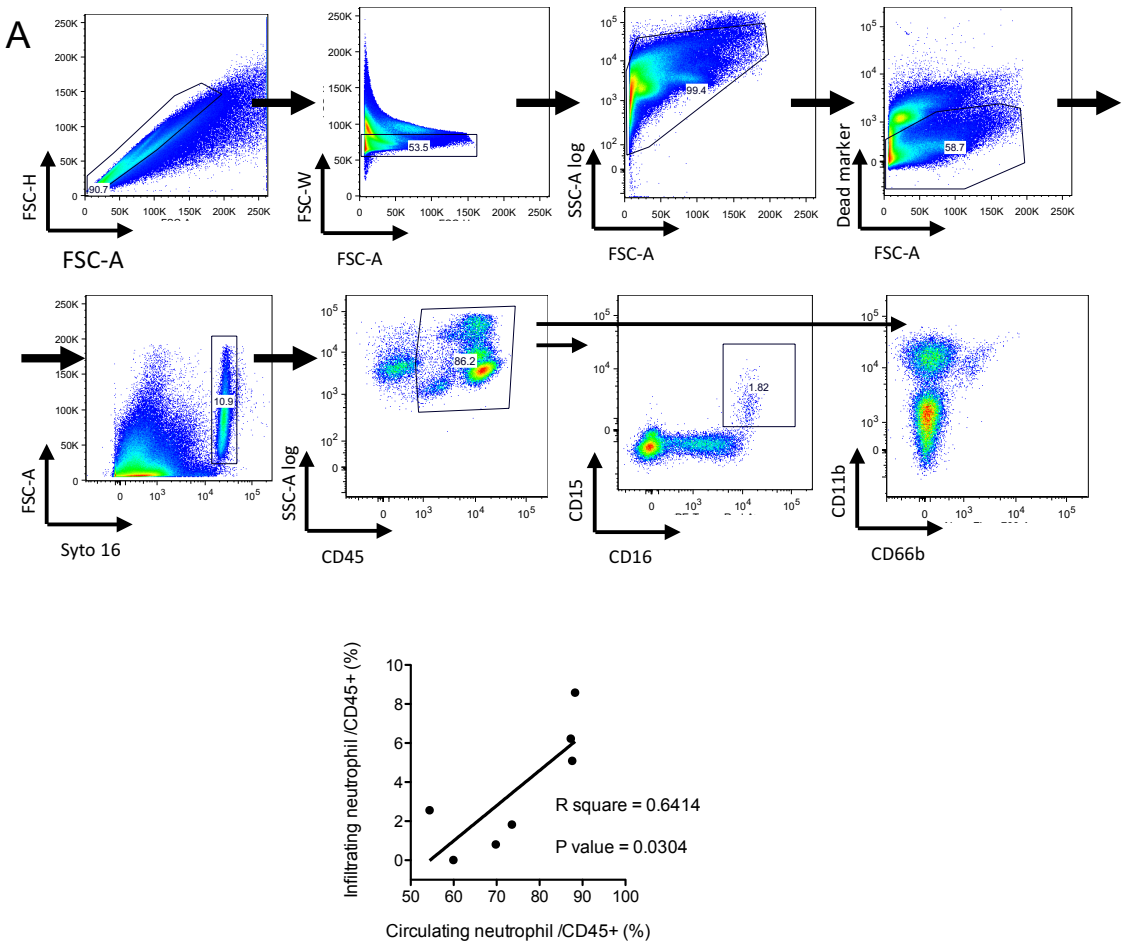


Figure 4.13: Circulating neutrophils rate is positively associated with infiltrating neutrophils rate.

A) Gating strategy for the identification of infiltrating neutrophils ($CD45^+/CD15^+/CD16^+/CD11b^+/CD66b^+$) B) Linear correlation between circulating and infiltrating neutrophils in brain lesion.

5 DISCUSSION

Chemokines are small cytokine with chemotactic function and play a pivotal role in the inflammation process and in cancer [31]. Negative control of inflammation is an essential aspect of resolution of inflammation and its target represents a concrete therapeutic to limit tumor growth and metastatic spread [25]. Atypical chemokine receptors are a distinct family of receptors that control chemokine bioavailability. They scavenge, transport chemokines and represent one important mechanisms of control of the chemokine system [37]. ACKR2 (also known as D6) is a negative regulator of inflammation because it leads to degradation many inflammatory CC chemokines [40]. Previously studies using ACKR2 gene-targeted mice describe an important role for this receptor in the control of inflammation because it dampens excessive inflammatory responses and accumulation of leukocytes with pro-inflammatory phenotype [232]. Also in cancer model, $Ackr2^{-/-}$ mice treated with inflammatory and mutagen compounds that recapitulate the extrinsic pathway of tumor development, shown an increased tumor growth [38]. These data indicate an important role for ACKR2 in the control of the inflammatory process that can promote carcinogenesis.

The aim of this thesis was to study the role of ACKR2 in the intrinsic pathway of cancer. We used NeuT mice, that express the rat oncogene HER2 under the mouse mammary tumor virus (MMTV) promoter, and develop breast cancer. $Ackr2^{-/-}$ mice crossed with NeuT mice showed an increased tumor growth compared to NeuT/$Ackr2^{+/+}$ mice recapitulating the role of ACKR2 found in skin and colorectal cancer models [61, 69]. Surprisingly, despite enhanced tumor growth, NeuT/$Ackr2^{-/-}$ mice were partially protected from lung metastasis, indicating a double role of ACKR2 in this tumor model. Lung metastasis protection in $Ackr2^{-/-}$ mice was also demonstrated in mice injected orthotopically with the breast cancer cell line 4T1 and in mice injected i.v. in a classic “artificial” hematogenous metastasis model with the melanoma cell line B16F10.

In order to understand if ACKR2 plays a role in the hematopoietic or stromal compartment, we performed bone marrow chimera experiment in the 4T1 model and we recapitulate the protection of $Ackr2^{-/-}$ mice transferring $Ackr2^{-/-}$ bone marrow in WT host. These results indicate that ACKR2 expression in the hematopoietic

compartment is responsible of the protection. We associated also metastasis protection to increased number of circulating neutrophils and monocytes in NeuT/Ackr2$^{-/-}$ mice. To assess which leukocyte population was involved in the metastasis protection, we performed cell depletion using monoclonal antibody. Only when neutrophils were depleted, but not monocytes or B cells, Ackr2$^{-/-}$ mice increased metastatic rate compared to WT levels in B16F10 and 4T1 model. We prove also that neutrophil-mediated protection in Ackr2$^{-/-}$ mice was due to neutrophils performing adoptive transfer experiments. Only the transfer of Ackr2$^{-/-}$ neutrophils in B16F10 WT bearing mice recapitulated the phenotype of Ackr2$^{-/-}$ mice. Following these results, we focused our attention to Ackr2$^{-/-}$ neutrophils in order to understand which was the mechanism of protection. We analyzed circulating neutrophil phenotype in tumor-bearing mice with flow cytometry techniques. Ackr2$^{-/-}$ neutrophils, compared to WT neutrophils, had increased ICAM-1 expression and lower levels of CD62L, indicating N1 polarization or an activate state of neutrophils. Moreover, we demonstrated that Ackr2$^{-/-}$ neutrophils, in a model of in vivo mobilization with the administration with CCL3L1 chemokine, have a cell intrinsic increased mobilization from bone marrow to blood and are more able to infiltrate lungs. Increased cell-autonomous mobilization of neutrophils in Ackr2$^{-/-}$ mice was previously observed also in skin in a psoriasis model [65]. To further demonstrate the cell intrinsic increased mobilization, we performed the same experiment transferring CD45.2 WT and Ackr2$^{-/-}$ neutrophils into CD45.1 WT mice. We found, again, an increase rate of Ackr2$^{-/-}$ neutrophils in the lung of WT mice compared to controls and a concomitant decrease of Ackr2$^{-/-}$ neutrophils in the blood due to the increased extravasation activity. These results were correlated to increased transcript level of chemokine receptor CCR1, CCR2 and CCR5 in Ackr2$^{-/-}$ neutrophils in resting condition that indicate a direct role of ACKR2 in the regulation not only of chemokines bioavailability, but also of the receptors that share the ligands with ACKR2. Ackr2$^{-/-}$ neutrophils have also an increased ROS production compared to WT controls in both B16F10 and 4T1 model. We demonstrated also that ROS production is CCR2-dependent and Ackr2$^{-/-}$ neutrophils have increased CCR2-dependent ROS production compared to WT. Finally, we prove that Ackr2$^{-/-}$ neutrophils have increased direct cancer cell activity performing co-culture of WT and Ackr2$^{-/-}$ neutrophils with 4T1 luciferase-expressing cells and we found an increased cell killing rate of Ackr2$^{-/-}$ neutrophils compared to WT controls. ROS inhibition

abrogates cell killing by WT neutrophils and cause a cell killing decrease in Ackr2$^{-/-}$ neutrophils but not abrogates the difference between WT and Ackr2$^{-/-}$ neutrophils. These results indicated a not complete ROS-dependent mechanism of cell killing by Ackr2$^{-/-}$ neutrophils. Extensive experiments are needed to elucidate the cell killing mechanism in Ackr2$^{-/-}$ neutrophils. All these achievements indicate ACKR2 as a negative regulator of the mobilization of neutrophils with anti-metastatic activity [233].

Neutrophils were considered for many years as neutral players in cancer, but in the last decade neutrophils were emerged as a leukocyte population with different roles in carcinogenesis, primary tumor growth and metastatic spread [84]. Cytokines released in the tumor microenvironment and at systemic level modulate neutrophil maturation, mobilization from bone marrow and polarization [10]. These observations indicate that neutrophils are potential target of antitumor therapy for their direct activity against cancer cells [109] and for their indirect role such as regulator of angiogenesis [212]. Our findings reveal that ACKR2 genetic ablation in neutrophils resulted in an activated phenotype that inhibit tumor metastasis [233].

In order to understand if human neutrophils are present in different activation states in the blood of tumor patients, we performed flow cytometry analysis. We started to phenotype neutrophils in high grade glioma patients. High grade gliomas are brain tumors that include glioblastoma. They have a mean survival time of fifteen months after diagnosis. In high grade glioma patients, a severe immunosuppression is described with a concomitant neutrophilia in blood and presence of TANs in the brain lesion [188]. Increased neutrophil to lymphocytes ratio is associated with a worse pathology outcome while few data describe neutrophils phenotype [188, 205, 207-209]. All these features make high grade gliomas an interesting context for the study of neutrophils phenotype. Our preliminary data confirm a strong neutrophilia before the surgery in GBM patients compared to grade III patients and heathy controls [202]. On the contrary, no differences were observed for monocytes. In healthy condition, neutrophils are described as a homogeneous population but during pathology, such as sepsis and cancer, neutrophil subsets with differential activation and maturation were described [122]. We demonstrated that also in GBM patients three distinct neutrophil subsets were detected based on the differential expression of CD16 and CD62L. this indicated that in glioma patients neutrophil maturation and activation is affected, but

at the moment we have not detected differences in the expression of a panel of markers involved in neutrophil biology. Nevertheless, we found a positive correlation between circulating and tumor infiltrating neutrophil abundance on total leukocytes indicating that increased neutrophilia is associated to enhance neutrophils activation. Further analyses will be necessary to better describe neutrophil phenotype and to associate abundance of circulating and infiltrating neutrophils and their phenotype with survival rate and therapy effectiveness.

Collectively taken, our findings reveal that canonical and atypical chemokine receptor have an active role in the maturation and the activation of neutrophils with anti-metastatic activity. Neutrophils and their chemokine receptors are so indicated as functional target during cancer pathologies in order to block pro-tumoral role and unleash their anti-tumoral and anti-metastatic potential.

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