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Down-regulation of atypical chemokine receptor ACKR2/D6 expression by hematopoietic progenitors promotes myeloid cell mobilization and differentiation

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Abstract

Chemokines and chemokine receptors are key mediators of inflammation and important regulators of leukocyte migration in homeostatic conditions as well as during infection and cancer. The atypical receptor ACKR2 is a scavenger receptor for many inflammatory CC chemokines, it is expressed either by non-hematopoietic cells or by hematopoietic cells, and it has been shown to prevent the development of exacerbated inflammatory reactions.

In an effort to understand the contribution of this receptor in the regulation of myeloid cell mobilization and myeloid cell effector functions, we investigated the role of ACKR2 in a murine model of myeloid cell mobilization, and in a model of experimental metastasis.

The deficiency of ACKR2 was associated with increased mobilization of monocytes and neutrophils from the bone marrow (BM) and with increased number of monocytes confined to BM sinusoids compared to Wild-type (WT) mice. BM chimera experiments showed that the increased mobilization was due to the absence of ACKR2 in the hematopoietic compartment. The analysis of hematopoietic progenitor cells (HPCs) revealed that ACKR2 is expressed by $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ cells (LSK) to faint thereafter in more mature myeloid progenitor cells (MPCs) in contrast with the canonical chemokine receptor CCR2. Moreover, HPCs from *Ackr2*$^{-/-}$ mice expressed higher levels of CCR1, CCR2 and CCR5, but not of CXCR4 and they had higher differentiation rate compared to ACKR2 sufficient LSK. Although neutrophils express low levels of ACKR2 compared to LSK, we found that neutrophils from *Ackr2* deficient mice, as well as their HPCs, expressed higher level of CC chemokine receptors and exhibited a more activated phenotype compared to WT. Furthermore, neutrophil depletion and neutrophil adoptive transfer experiments demonstrated that only *Ackr2* deficient neutrophils were sufficient to control the metastatic seeding of B16 melanoma cells into the lung.

To enhance the metastatic protection observed in *Ackr2*$^{-/-}$ mice, we treated WT and *Ackr2*$^{-/-}$ tumor bearing mice with AMD3100, the competitive inhibitor of CXCR4, which is known to induce a rapid neutrophil mobilization from the BM. However, AMD3100 treatment did not further improve the metastatic protection in *Ackr2*$^{-/-}$ mice, whereas decreased the number of metastases in WT mice. Finally, by using the human promyelocytic cell line HL-60, we demonstrated that ACKR2 directly exerted a negative regulation of CC chemokine receptor expression and cell differentiation. Indeed, HL-60, when transfected with a vector overexpressing ACKR2, had decreased transcript levels of CCR2 and

CD11b. These data suggest the ACKR2 is involved in the regulation of chemokine availability and leukocyte recruitment. Moreover, ACKR2 directly controls HPC differentiation, myeloid cell mobilization and their effector function through the inhibition of CC chemokine receptor expression.

Introduction

1. The chemokine system

Cell migration is essential for the activation and orientation of innate and adaptive immunity during the processes of immune surveillance, inflammation, and development [1, 2]. Several cytokines and growth factors, such as macrophage-colony stimulating factors (M-CSF) and vascular endothelial factor (VEGF), regulate directional migration of leukocytes. The main mediators of leukocyte trafficking are chemokines, low molecular weight proteins (8-12 KDa) belonging to a family of small chemotactic cytokines secreted by cells. The name "chemo-kines" derived from their ability to induce chemotaxis in responsive cells, which express dedicated chemokine receptors [3].

Chemokine system is highly redundant and promiscuous: the same receptor can recognize more than one chemokine and multiple receptors can bind the same ligand [4]. To date, approximately 50 human chemokines and 20 receptors have been identified [5] (Figure 1).

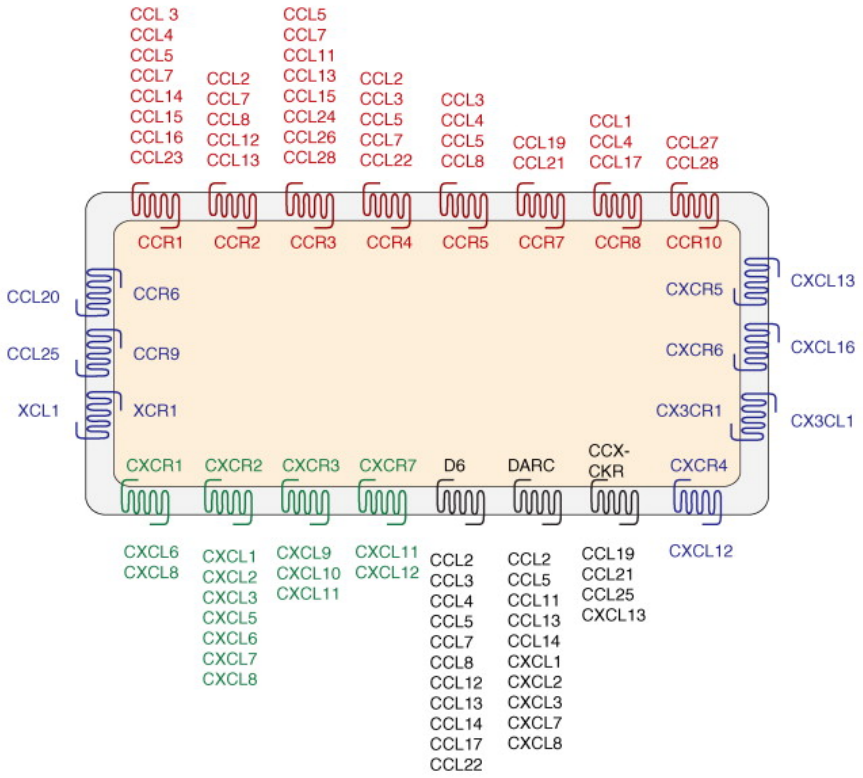


Figure 1: Chemokines and chemokine receptors. The same receptor can recognize more than one chemokine and multiple receptors can bind the same ligand. Only a minority of receptors has one ligand [6].

1.1 Chemokine function

Chemotactic cytokines were firstly identified for their role as potent chemo-attractant for leukocytes, such as monocytes and neutrophils, and were defined as important mediators of acute and chronic inflammation [2, 7].

During inflammatory process, chemokines (e.g. CCL2, CCL3, and CXCL8) play a key role in promoting leukocyte recruitment to the site of inflammation. Chemokines secreted in response to primary pro-inflammatory factors, such as interleukin-1 (IL-1) and tumor necrosis factor (TNF)-α, bind to glycosaminoglycans (GAGs) on endothelial cell surface. This binding is crucial for retaining chemokines and increasing chemokine concentration on blood vessels at the inflammatory site, providing cues for chemokine receptors and leukocyte migration. Next, the interaction between selectins expressed on the endothelium and leukocytes causes leukocyte adhesion and rolling along the cell surface; finally, the binding of integrin lymphocyte function-associated antigen 1 (LFA-1) on leukocyte surface to the adhesion molecule-1 (ICAM-1) expressed on endothelial cells, promotes leukocyte arrest and extravasation [8, 9]. Despite their predominant chemotactic activity, in the last decade several studies have shown that chemokines also regulate the development, the homeostasis and the pathogenesis of many human diseases. For instance, they are involved in ontogenesis of vascular and nervous system; they regulate angiogenesis and fibrosis, and control the differentiation and proliferation of HPCs [10-13].

1.2 Chemokine structure

Chemokines are single polypeptide chain composed of 70-100 amino acid residues in length, with a molecular weight between 8-12 KDa. Chemokines have low amino acid sequence homology, but they all have four well-conserved cysteine residues, which form two disulphide bonds between the first and the third cysteine and between the second and the fourth cysteine. These bounds confer to chemokines a conserved tertiary structure. This structure consists of a disordered amino-terminus, three-stranded antiparallel β-sheet and a carboxy-terminal α-helix [14].

1.3 Chemokine classification

Chemokines are classified on the basis of structural properties and according to their production. Indeed, depending on the position of cysteine residues in their N-terminus, chemokines can be classified into 4 subfamilies: CC, CXC, XC and CX_3C (Figure 2) [2, 15]. The CC chemokines are the largest family of chemokines and have the first two of the four cysteine residues in adjacent position. CC chemokines mainly attract mononuclear cells, such as monocytes, natural killer (NK) cells, T and B lymphocytes. This family include: CCL2, also called monocyte chemoattractant protein 1 (MCP-1) that is a potent agonist for monocytes, dendritic cells (DCs), memory T cells, and basophils; CCL3, also called macrophage inflammatory protein (MIP)-1α; CCL4 (MIP-1β) and CCL5 (RANTES) [16]. The second largest family of chemokines is represented by CXC chemokines, which have an amino acid between the first two cysteines; the CXC chemokines can be further classified in ERL^- and ERL^+ chemokines, based on the presence or absence of the ELR (Glu-Leu-Arg) motif [14]. ERL^- chemokine, except CXCL12, are angiostatic factors that inhibit the formation of blood vessels, whereas ERL^+ chemokine are angiogenic factors [12, 17]. Among CXC chemokines there are CXCL1 and CXCL2, which are important for polymorphonuclear leukocyte recruitment to the site of inflammation. CX_3C family is composed by only one member: CX_3CL1 (fractalkine), which has three amino acids separating the initial pair of cysteines in the N-terminus [18]. Finally, the XC subfamily is composed of chemokines with only two cysteines residues and only one N-terminal cysteine. XC chemokines include: XCL1 (lymphotactin-α) and XCL2 (lymphotactin- β) [19].

On the basis of their function, chemokines can be also distinguished in homeostatic and inflammatory chemokines [20]. Homeostatic chemokines (e.g. CXCL12, CXCL13, CCL14, and CCL19) are constitutively produced and regulate basal leukocyte trafficking, such as lymphocyte homing to secondary lymphoid organs. Inflammatory chemokines (e.g. CCL2, CCL5, and CXCL8) are inducible molecules secreted during inflammatory response, upon infection or tissue injury, and they drive leukocyte recruitment to the site of inflammation. However, this classification is not strict; indeed some chemokines exert both inflammatory and homeostatic functions, depending on the context, and are therefore called dual-function chemokines.

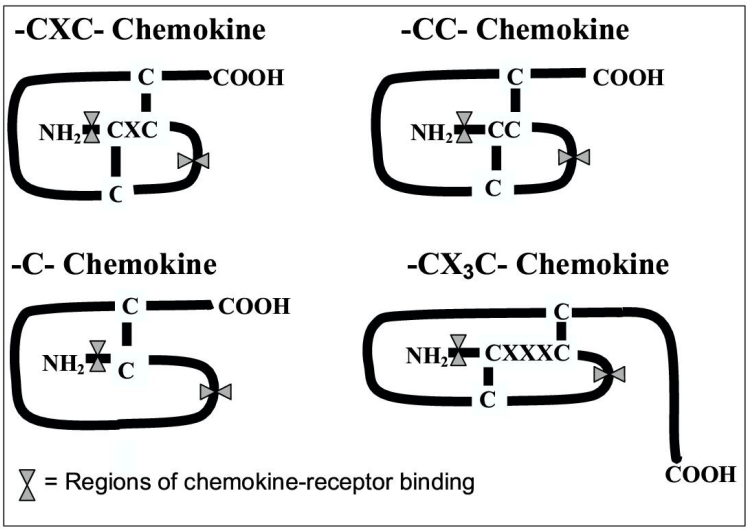


Figure 2: Chemokine subfamilies. Chemokines are classified into 4 subfamilies: CC, CXC, C and CX_3C depending on the position of cysteine residues in their N-terminus [21].

1.4 Chemokine receptors

Chemokines exert their specific function through the interaction with chemokine receptors expressed on their target cells [7]. Depending on the type of chemokine they bind, chemokine receptors can be classified in CCR that bind CC chemokines, CXCR that bind CXC chemokines, CXCR1 that binds CX3CL1, and XCR that bind the two XC chemokines [22]. Moreover, according to the type of chemokine they bind, these receptors can be either inflammatory or homeostatic chemokine receptors. Chemokine receptors are 7-transmembrane (7TM) receptors and they are coupled to hetero-trimeric GTP-binding proteins (G-protein) of the Gi type, sensitive to Bordetella pertussis toxin. These receptors are single polypeptide chain and have a high conserved structure characterized by an external N-terminus domain, seven transmembrane domains, three extracellular and three intracellular loops, and a serine/threonine-rich intracellular C-terminal domain. The N-terminal domain is important for the specificity of ligand binding, whereas the C-terminal domain, together with other motifs in the transmembrane domains and intracellular loops, mediates the interaction with signaling molecules and is important for receptor internalization [14]. The interaction between chemokines and their receptors involves two important sites of chemokine structure, the docking and the triggering domains. The docking domain is the rigid loop that follows the second cysteine and is recognized by the N-terminus of the receptor [23, 24]. Ligand binding to the receptor through the docking

domain reduces chemokine mobility and facilitates receptor interaction with triggering domain, which is in proximity of the N-terminal domain and promotes receptor activation. Upon ligand engagement, the G-protein α subunit dissociates from the βγ subunits of heterotrimeric complex and βγ subunits rapidly activate signal events [25]. Conserved motifs in the structure of chemokine receptors are important for signaling and they include: an aspartic acid residue, a Thr-X-Pro (TXP; where X denotes any amino acid) motif in the second transmembrane domain, and an Arg–Tyr–Leu–Ala–Ile–Val (DRYLAIV) motif between the third transmembrane domain and the second intracellular loop [14].

Besides their apparent redundancy, chemokine receptors differ in their capacity to induce a diversity of signal transduction pathways including: mitogen-activated protein (MAP) kinases, phospholipase Cβ2, phopshoinositide 3-kinase (PI3K) and RAS pathway (Figure 3) [25].

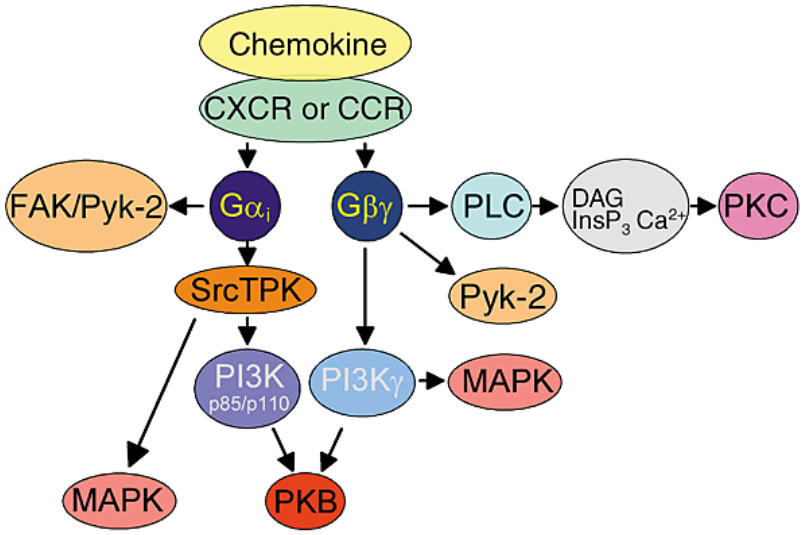


Figure 3: Chemokine receptor signaling pathways. Scheme of the most effectors involved in the chemokine signal transduction [25].

2. *Chemokines and chemokine receptors in cancer-related inflammation*

Chemokines and their receptors are important mediators of cancer-related inflammation, which is a critical component of tumor initiation, promotion and progression. Accordingly, recent discoveries about their role had facilitated the development of novel therapies to improve cancer treatment [26].

The link between cancer and inflammation can be schematically explained as consisting of an intrinsic or an extrinsic pathway [27]. The intrinsic pathway is represented by genetic alterations (e.g. oncogene) that cause tumor and induce the activation of inflammatory pathways. Different studies have demonstrated that chemokines and chemokine receptors are direct target of several oncogene activations. For example, oncogenic activation of RAS-RAF signaling pathway induces the activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and the production of inflammatory chemokines, such as CXCL8. Conversely, the extrinsic pathway is due to the establishment of inflammatory condition that increase the risk of cancer [27].

Inflammatory cells, including macrophages, neutrophils, subset of T cells and DCs, are commonly found infiltrating tumors, where they can produce cytokines, chemokines and growth factors that directly or indirectly support cancer growth [28].

On the other hand, effective anti-tumor immunity depends on inflammatory mediators that contribute to the activation of key immune cells such as DCs, for the induction of robust T cell response. In this view, CCL2 has been shown to promote the recruitment of pro-inflammatory monocytes in the tumor site and to be able to directly sustain tumor growth and spread. On the other hand, high levels of CCL4, CCL5, CXCL9, CXCL10, and CXCL11, which induce the recruitment of activated T cells, have been associated with good prognosis in many human cancers [29].

2.1 Sources of chemokines and chemokine receptors in tumors

The vast majority of transcription factors activated in tumor cells regulates the production of many chemokines, cytokines and prostaglandins [27]. These factors recruit various leukocytes and activate the same transcription factors in inflammatory cells, stromal cells and cancer cells, thus promoting the production of more inflammatory mediators and the establishment of a tumor promoting microenvironment. For instance, CXCL8, which is one of the most abundant chemokine produced by human tumors and stromal cells, is over-expressed in colon, lung, prostate, ovarian carcinoma and melanoma [30, 31], CXCL1 can be expressed by melanoma cells, CXCL5 by non-small cell lung cancer, CXCL6 by gastrointestinal tumors, CCL20 by pancreatic, renal and breast cancer, and papillary thyroid carcinoma, CCL5 can be produced by papillary thyroid carcinoma, CXCL12 by ovarian carcinoma and CCL19 by breast carcinoma [32-38]. In addition to produce chemokines, many cancer cells express a variety of chemokine receptors. CXCR4 is the receptor expressed by the majority of cancer cell types, including cancers of epithelial, mesenchymal and haematopoeitic origin [11]. For example, it is expressed by ovarian carcinomas, neuroblastoma, glioblastoma and tumor cells from breast, prostate, pancreatic and lung [39-45]; CXCR4 can be co-expressed with other CC or CXC chemokine receptors, indeed it has been found to be co-expressed with CCR7 in human breast cancer cells and melanoma cells [42, 46]. The receptor CCR7, as well as CXCR5 and CCR3 can be also expressed in leukaemia and lymphomas [47, 48]. Moreover, melanoma cells can express CCR9, CCR10, CXCR2 and CXCR3 [49-51]. The expression of chemokine receptors by tumor cells defines the metastatic tropism of cancer cells, depending on the chemokines produced at the metastatic site [6].

2.2 Chemokines and tumor infiltrating leukocytes

One of the main functions of chemokines produced within the tumor is to promote leukocyte migration and infiltration (Figure 4). Leukocyte infiltrate is present in most tumors and, depending on the tumor and chemokines that are produced it can be different and includes cells of both myeloid and lymphoid origin. All of these infiltrating cells may have both pro-tumoral and anti-tumoral role, thus impacting on tumor progression [52]. For instance, monocytes and macrophages are mainly recruited by CCL2 and CCL5 as demonstrated by murine model of breast cancer, melanoma, colon cancer, esophageal carcinoma, and prostate carcinoma [53-59]. In humans, the level of CC-inflammatory chemokines correlates with the number of tumor associated macrophages (TAMs), metastatization and poor prognosis [60, 61]. Neutrophil infiltration is mediated by the production of CXC chemokines, such as CXCL1, CXCL2, CXCL5, and CXCL6, which are expressed by several tumor types [62-64]. Potent attractant of DCs is CCL20. Indeed, tumors expressing high level of this chemokine, such as pancreatic, renal, breast and papillary thyroid carcinoma, show a strong DCs infiltrate [65, 66]. Other chemoattractants of DCs are CCL5 and CXCL12 [67, 68]. Finally, tumors can be characterized by tumor-infiltrating lymphocytes (TIL). TIL are mainly recruited by CXCL9 and CXCL10 by the interaction with CXCR3, and by CCL17 and CCL20 through the binding to CCR4. CXCR3 expressing cells recruited into the tumor are NK cells, $CD4^+$ Th1 cells, and $CD8^+$ cytotoxic T lymphocytes (CTL) which are important for an effective protective antitumor immune response [69-72]. Conversely, CCR4 positive cells are regulatory T cells (Treg) and polarized Th2 cells, which support tumor progression [73-75].

2.3 Chemokines and tumor growth

It is well established that chemokines and their receptors have an important role in tumor growth (Figure 4). Indeed, chemokines produced within the tumor can bind receptors expressed on cancer cells and activate signaling pathways, such as MAPK/ERK, PI3K/Akt, and p53 pathways that are involved in the proliferation of tumor cells [76]. One of the most well studied chemokine is CXCL12. This chemokine binds the receptor CXCR4 and is involved in the growth of many cancers including glioma, breast cancer, ovarian cancer, small cell lung cancer, acute lymphoblastic leukemia and chronic B cell leukemia [77-81]. Another chemokine, CXCL8, plays a role in the proliferation of esophageal cancer cells, human gastric carcinoma cells and melanoma [50, 82, 83]. In addition, melanoma growth and progression can be induced by CCL27 and by the chemokines CCL19 and CCL21, which bind to CCR10 and CCR7, respectively [84, 85].

2.4 Chemokines and angiogenesis

Angiogenesis is the process of formation of new blood vessels. It can be regulated by many chemokines and chemokine receptors, and plays a critical role in tumor progression (Figure 4). Chemokines can be either angiogenic or angiostatic, depending on their capability to promote or inhibit angiogenesis [12]. ELR^+ chemokine, such as CXCL1, CXCL3, CXCL5, CXCL6, CXCL8, CXCL12, and the chemokines CCL2, CCL11 and CCL16, are potent angiogenic factors [86]. These chemokines promote angiogenesis both directly by the interaction with receptors expressed on endothelial cells, or indirectly throught the recruitment of leukocytes that provide angiogenic factors. For example, CXCL12 has been shown to bind CXCR4 on endothelial cells promoting cell migration and proliferation [87]. By contrast, CXCL5, CXCL6 and CXCL8 contribute to angiogenesis by attracting neutrophils which express the dedicate receptors CXCR1 and CXCR2 [50, 88]. CCL2 is involved in the recruitment of TAMs expressing CCR2 [89]. Neutrophils and TAMs are cells able to produce angiogenic factors such as VEGF-β, platelet derived growth factor (PDGF), transforming growth factor (TGF)-β and matrix-metalloproteinases (MMP) such as MMP-2 and MMP-9 [90, 91]. Conversely, important angiostatic chemokines are CXCL4, CXCL9, CXCL10 and CXCL11, which inhibit neovascularization and endothelial cell proliferation [92, 93].

2.5 Chemokines and metastases

Metastatic spread is the leading cause of death in people with cancer. Metastatization is a very complex and organ-specific process, during which malignant tumor cells enter into the blood or lymphatic circulation and, from the original primary tumor, spread to other organs. During metastatic spread, tumor cell migration shares many similarities with leukocyte trafficking and, accordingly, it is regulated by chemokines and their receptors [94]. Indeed, tumor cells can take advantage of chemokine signalling pathways by expressing appropriate chemokine receptors, which confer them the capability to migrate to distant sites [94]. The most studied chemokine axis is CXCR4/CXCL12 that is involved in the metastatic dissemination of tumor cells, for example breast cancer cells, which express CXCR4 and migrate in response to CXCL12 produced in lymph nodes (LNs), lung, brain, liver and BM [42]. Another chemokine receptor CCR7, which is important in normal immune response to recruit naïve T cells and DCs to LNs, can be also expressed in breast cancer, leukemia and lymphomas. In this context, CCR7 mediates the migration of tumor cells to LNs where the ligands CCL19 and CCL21 are produced [95, 96]. Moreover, it has been demonstrated that combined CCR7 and CXCR4 expression by breast cancer cells correlates with LN metastases [42]. Additional studies showed also the involvement of CCR9/CCL25 axis and CCR10-CCL27/CCL28 axis in promoting melanoma metastases to the small intestine and to the skin, respectively [42, 94, 97]. In a mouse model of melanoma induced by injection of B16F10 melanoma cells, the expression of CXCR3 by cancer cells mediated metastases to LNs [51]. Finally, the expression of CXCR5, the receptor for CXCL13, has been associated to frequent metastases to LNs in certain lymphomas [47].

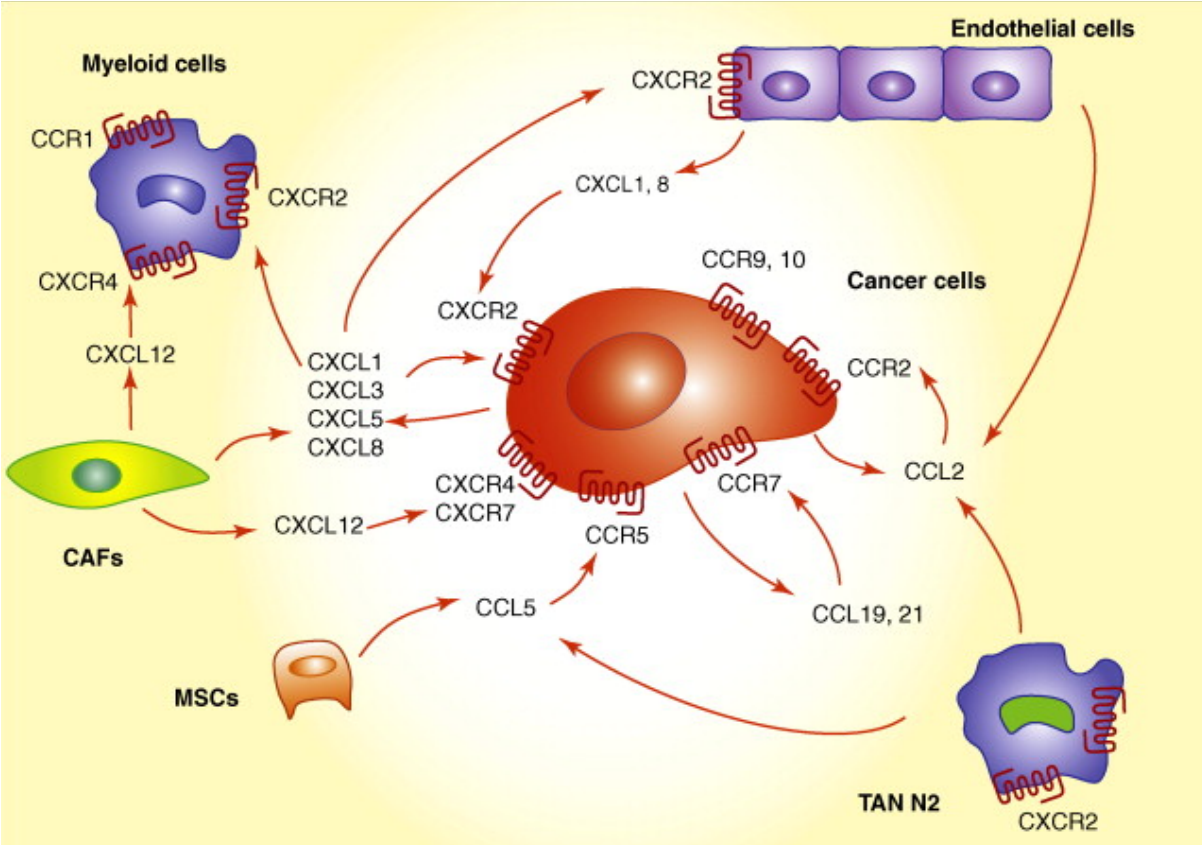


Figure 4: Chemokines and chemokine receptors expressed by cancer cells. Cancer cells express a vast repertoire of chemokines and chemokine receptors, which allow the interaction between cancer cell and tumor microenvironment [6].

3. Atypical chemokine receptors

Inflammation is a reaction of body to harmful stimuli consisting of an initiation stage, a maintenance stage, and a resolution stage [98]. Cytokines are important mediators of this process and their concentration needs to be tightly regulated and controlled for the correct development of the immune response and to avoid an exacerbated inflammation. As a consequence, vertebrates have adopted several mechanisms to control inflammation [99]. These mechanisms have been described to negatively regulate cytokine system acting both at transcriptional and post-transcriptional levels. One of the mechanisms by which the regulation at the post-transcriptional level is achieved, is represented by the activation of cytokine decoy receptors [14]. These receptors bind to their ligand with high affinity and specificity, but are structurally different from the canonical receptors and are unable to promote conventional signaling pathways. Among the group of decoy receptors, there is a subgroup of receptors, called atypical chemokine receptors (ACKRs) (Figure 5) [100, 101]. ACKRs share many similarities with canonical chemokine receptors but show structural modification in the DRYLAIV motif and, as result, are unable to couple to G protein and to promote cell migration [100]. By contrast, ACKRs activate a β-arrestin dependent pathway and modulate chemokine bioavailability by transporting their ligands to intracellular degradative compartment, thus acting as chemokine scavengers [102-107]. According to the new nomenclature, the family of ACKRs include: ACKR1, previously called Duffy antigen receptor for chemokines (DARC); ACKR2, also known as D6 or CCBP2; ACKR3, alias CXC chemokine receptor 7 (CXCR7) or RDC1; and ACKR4, previously called CC chemokine receptor-like 1 (CCRL1) and also known as CCX-CKR [108]. Other molecules, such as CCRL2 and PITPNM3 have been tentatively included in the ACKR family as ACKR5 and ACKR6, respectively, but are awaiting for functional confirmation [108]. Although low level of ACKRs expression has been detected on leukocytes, ACKRs are mainly expressed by non-leukocyte cell types, such as lymphatic or vascular endothelial cells and erythrocytes [100, 109]. ACKR1 is expressed by erythrocytes, vascular endothelial cells, and Purkinje cells; ACKR2 by lymphatic endothelial cells (LECs), leukocytes, keratinocytes, and trophoblast; ACKR3 by hematopoietic cells, LECs, mesenchymal cells, and neuronal cells; and ACKR4 by LECs and epithelial cells [110-114].

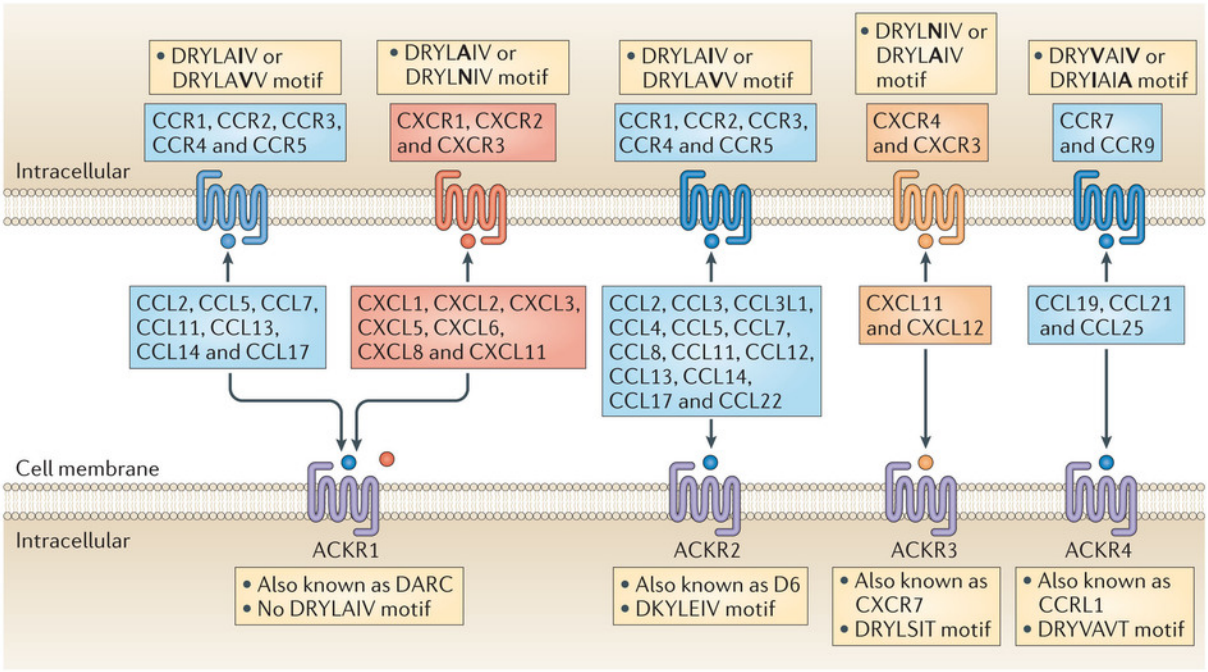


Figure 5: Chemokine binding profile of ACKRs. ACKRs share many ligands with canonical chemokine receptors. They are similar to canonical chemokine receptors but show structural modification in the DRYLAIV motif [100].

3.1 ACKRs in the resolution of inflammatory response

According with the crucial role of chemokines in inflammation, the biological activity of ACKRs has been demonstrated to be important in the resolution phase of the inflammatory response.

ACKR1 binds more than 20 inflammatory chemokines belonging to the CC and CXC families, such as CCL2, CCL5, CCL7, and CXCL8. ACKR1 is expressed by erythrocytes and by endothelial cells and regulates the bioavailability of circulating chemokines, whereas ACKR2 expression by endothelial cells reduces chemokine concentration in the inflamed tissues and creates a functional gradient allowing proper leukocyte migration [115-117]. As a consequence, ACKR1 deficient mice are protected in different pathological contexts, such as acute kidney damage induced by ischemia or LPS, atherogenesis, and bone fracture. Indeed, in these models the lack of ACKR1 increased the level of circulating chemokines and induces the desensitization of the cognate receptors thereby reducing neutrophil and monocyte recruitment, and tissue damage [115, 118-124].

ACKR3 binds the chemokines CXCL11 and CXCL12 [125]. This receptor plays an important role in regulating CXCL12 availability for CXCR4 [126]. Moreover, ACKR3 can modulate CXCR4 expression and function. Indeed, ACKR3 can form heterodimers with CXCR4 and can enhance or inhibit CXCL12- mediated signaling [126, 127]. ACKR3 is upregulated in inflammatory conditions, and its expression is associated with a pro-inflammatory phenotype, indeed ACKR3 enhances angiogenesis and promotes leukocyte extravasation [128, 129].

ACKR4 binds the homeostatic chemokines CCL19, CCL21, CCL25 and CXCL13. In the subcapsular sinus of LNs, ACKR4 creates functional gradients for CCL19 and CCL21, which are also ligand of CCR7, expressed by DCs [130]. By this way, ACKR4 promotes the migration of DCs and the induction of adaptive immune response [131].

3.2 ACKRs in cancer

In accordance with the crucial role of chemokines in cancer, preclinical and clinical observations provided evidence that the control of chemokine availability by ACKRs has an important role in cancer biology (Figure 6) [132]. For example ACKR1, in a mouse model of prostate cancer, has been demonstrated to have a protective role by limiting the level of the proangiogenic chemokines CXCL8 and CXCL2 [133]. ACKR3, in breast cancer, decreased CXCR4-mediated effects through the scavenging of CXCL12, thus limiting metastatic spread [134]. In tumors, expression of ACKRs was found in cancer cells themselves and can mediate different effects [132]. For example, expression of ACKR3 can promote tumor growth by the activation of signaling inducing the proliferation and by inhibiting apoptosis [135, 136]. In contrast, ACKR4 inhibits tumor growth and metastases by the degradation of its ligands [137-139].

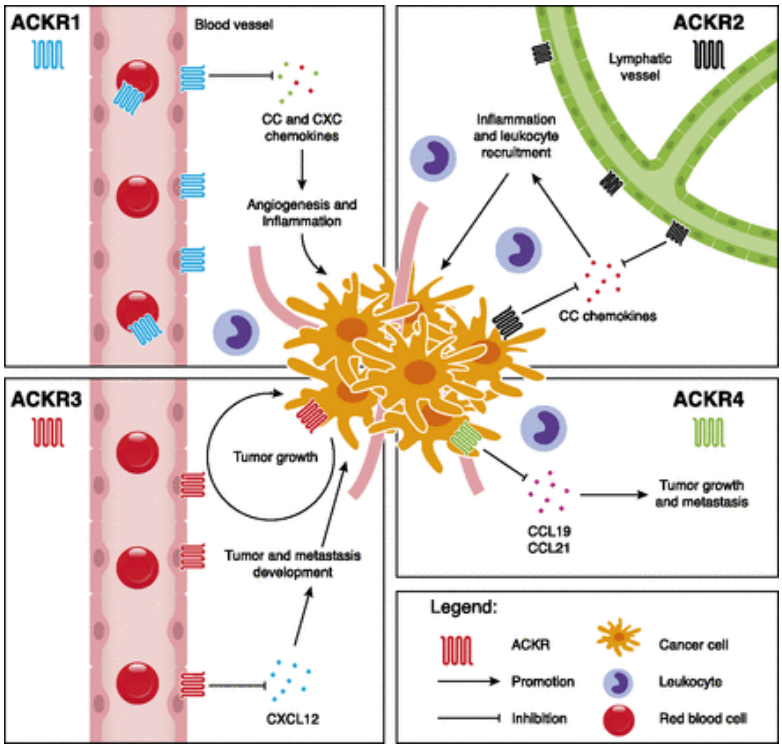


Figure 6: Role of ACKRs in cancer: ACKR1, expressed by erythrocytes and endothelial cells, reduces angiogenesis and tumor growth. ACKR2 scavenges inflammatory CC chemokine, thus inhibiting inflammation, leukocytes recruitment, and tumor growth. ACKR3 expressed by tumor cells accelerates tumor growth, whereas ACKR3 on vascular endothelial cells degrades CXCL12, thus inhibiting tumor growth and metastases. ACKR4 expressed by cancer cells constrains tumor growth [132].

4. Atypical chemokine receptor 2 (ACKR2)

In 1997 Steiner DF and Graham GJ have reported the cloning of a novel promiscuous receptor with high affinity binding for CC chemokines [140-142]. This receptor was named CCR9, CCR10 and D6, and the gene name ccbp2 was deposited in the GeneBank. Later, CCR9 and CCR10 nomenclatures were assigned to other receptors and D6 became the accepted name of this receptors. The name D6 referred to the coordinate on a multiwell plate, of the clone encoding this molecule [143]. However, according to the new nomenclature approved in 2014, D6 is now called ACKR2 and it is one of the best-characterized atypical chemokine receptor [108].

Although ACKR2 is highly promiscuous and binds a wide spectrum of chemokines, it selectively recognizes the agonists of the receptors CCR1, CCR2, CCR3, CCR4, and CCR5, which are CC-proinflammatory chemokines, such as CCL2, CCL3, CCL3L1, and CCL5 [141, 144]. Ligands of CCR6, CCR7, CCR8, CCR9, and CCR10 or other chemokine subfamilies are not recognized by ACKR2 [141].

Moreover, ACKR2 efficiently recognizes and degrades only the biologically active form of these chemokines. For this reason, ACKR2 ligands were subdivided into three classes: CC-inflammatory chemokines efficiently degraded, chemokines degraded with a less efficiency, and chemokines that are not degraded [145]. In particular, the chemokines degraded by ACKR2 are characterized by a proline in position 2 at the N-terminal, which can be cleaved by the protease CD26 [144]. For example, the chemokines CCL3 and CCL8, lacking this residue, are inactive, and thus they are not degraded by ACKR2 [145].

4.1 ACKR2 structure and properties

ACKR2 has a 7TM structure with a 30-35% range of similarity with canonical chemokine receptors, such as CCR1 and CCR5 [141]. However, ACKR2 shows some structural modifications in the transmembrane and C-terminal domains [146]. Indeed, it has a modified DRYLAIV motif mutated in DKYLEIV, which is conserved across the species and makes ACKR2 unable to couple to G-protein [147]. Moreover ACKR2 is characterized by the mutation of the aspartic acid into asparagine in the second transmembrane domain. Finally, the C-terminal domain of this receptor, starting from the amino acid 312 to the amino acid 384 is longer than the C-tail of conventional chemokine receptors (Figure 7). This domain is crucial for receptor internalization and recycling to the membrane after ligand stimulation [102]. In addition, it has a key role in preventing receptor degradation, indeed, its truncation decreases the stability of the receptor on cell membrane [148]. Despite all these modifications, the motif TxP, responsible for the activation of the receptor, is conserved [149].

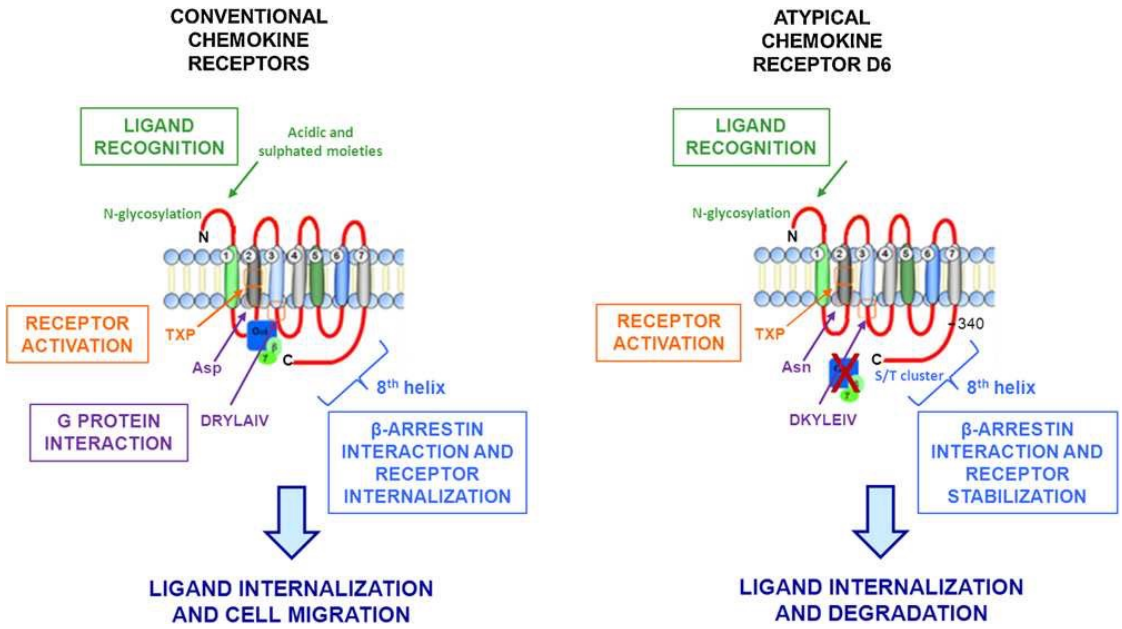


Figure 7: Structural differences between ACKR2 and canonical chemokine receptor: ACKR2 holds modifications in the transmembrane and C-terminal domains important for chemokine receptor signaling. In particular, ACKR2 has the DRYLAIV motif muted in DKYLEIV, the aspartic acid mutated into asparagine, and the C-terminus domain longer than C-tail of conventional chemokine receptors [150].

In basal conditions, ACKR2 is not highly expressed on the cell membrane and it is mainly stored in intracellular compartments. Upon chemokine engagement ACKR2 is mobilized from the intracellular compartments to the cell membrane through the $Rab11^+$ vesicles [151, 152].

In contrast to canonical receptors, which are internalized only after ligand engagement, ACKR2 is internalized constitutively in $Rab5^+$ vesicles and is recycled back to the plasma membrane through a $Rab4^+$ and a $Rab11^+$ pathway [103].

In particular, it has been demonstrated that ACKR2 traffic is induced by a β-arrestin1-dependent G- protein independent signaling pathway, involving the cascade of Rac1- p21-activated kinase-1 (PAK1)-LIMK1, and inducing the phosphorylation of cofilin and actin rearrangement (Figure 8) [102].

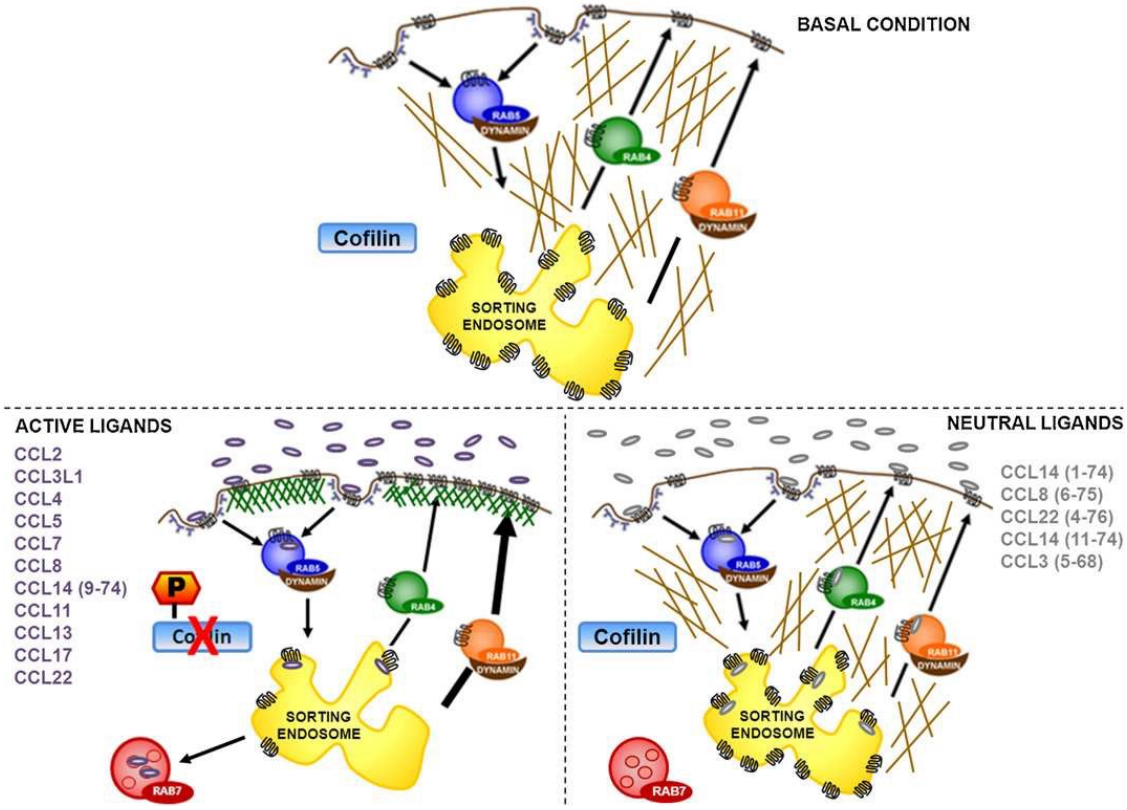


Figure 8: Actin dynamics sustain ACKR2 upregulation and degradatory activity. In basal condition ACKR2 is internalized in Rab5 vesicles and it is recycled to the plasma membrane through a Rab4/Rab11-pathway. Active cofilin maintains actin cytoskeleton organization and supports constitutive ACKR2 internalization and recycling. Stimulation with active ligands (purple) induces cofilin phosphorylation and its inactivation. This event induces modification in the actin cytoskeleton organization, thus leading to ACKR2 up-regulation and increasing chemokine degradation efficiency. Conversely, stimulation with neutral ligands (gray) does not change actin organization and cofilin activation, and does not affect ACKR2 distribution and scavenging efficiency [150].

4.2 Biological function of ACKR2

The first studies to assess ACKR2 function were performed by in vitro approaches and provided data regarding the ability of ACKR2 to degrade its ligands.

Next, the generation of monoclonal antibody against human ACKR2 provided the possibility to investigate the expression of the receptor. Nibbs and his colleagues demonstrated that ACKR2 is expressed by LECs of afferent lymphatic vessels (LVs) in barrier tissues such as skin, colon and lung [153]. Moreover, ACKR2 is strongly expressed in placenta by invading trophoblast and syncytiotrophoblast cells [111, 154]. Although at low level, the receptor has been found in some leukocyte populations, in particular in a subset of B cells and alveolar macrophages [111, 155, 156]. Finally, ACKR2 can be expressed by LVs in vascular tumors, in T cell malignances, including Kaposi's sarcoma, and can be upregulated by peritumoral lymphatic cells in breast cancer [153, 157].

Several studies in humans demonstrated that, at level of LVs, ACKR2 has increased expression in inflammatory conditions. For example, increased levels of ACKR2 expression by LVs have been found in the alveolar parenchyma of patients with chronic obstructive pulmonary disease (COPD) and in psoriatic skin compared with healthy controls [158, 159]. Moreover, McKimmie et al. demonstrated that ACKR2 expression can be induced by growth factors and cytokines, such as IL-6 and IFN-γ, further indicating that this receptor is over-expressed in inflammatory contexts [160].

Despite these observations, only the generation of gene modified mice deficient for ACKR2 allowed studies to assess the role of ACKR2 expression *in vivo* [161].

4.2.1 The role of ACKR2 in homeostatic conditions

The first evidences regarding the role of ACKR2 indicated that this receptor is mainly involved in the regulation of chemokine levels around afferent LVs, and ACKR2 is important to avoid an inappropriate accumulation of inflammatory leukocytes in the lymphatic system [162, 163]. McKimmie et al. demonstrated that ACKR2, by scavenging CC-proinflammatory chemokines, contributed to a selective presentation of CCR7 ligands, thus efficiently promoting the migration of DCs, crucial in maintaining immune surveillance [160]. Moreover, Savino et al. showed that, in resting condition, ACKR2

controls the traffic of CD11b^{+} Ly6C ^{high} monocytes with immunosoppressive phenotype and activity [164].

Finally, ACKR2, reciprocally with CCR2, is able to regulate LV density. In particular, Lee et al. described an increased LV density in the ears, diaphragms and popliteal LNs in *Ackr2*$^{-/-}$ mice [165]. Additional studies revealed that this phenotype is mediated by pro-lymphangiogenic macrophages present in proximity of developing LVs and recruited by CCL2; indeed, these macrophages are increased in *Ackr2*$^{-/-}$ mice and reduced in *Ccr2*$^{-/-}$ mice, which also have reduced LV density [165].

4.2.2 ACKR2 function during inflammation

The role of ACKR2 has been investigated in a range of inflammatory models, in which ACKR2 emerges as a key regulator of inflammation, by controlling chemokine distribution and bioavailability (Figure 9). For example, in models of cutaneous inflammation, such as phorbol ester skin painting and subcutaneous injection of complete Freund's adjuvant (CFA), *Ackr2*$^{-/-}$ mice developed an exacerbated inflammatory response [161, 166, 167]. Moreover, around the site of injection, *Ackr2*$^{-/-}$ mice showed increased areas of necrosis, increased angiogenesis and a higher number of infiltrating leukocytes compared to WT mice.

A role for ACKR2 has been described also in the control of inflammation, antimicrobial resistance and activation of immune response during infection. Indeed, Di Liberto et al. demonstrated that *Ackr2*$^{-/-}$ mice, infected with *Mycobacterium tuberculosis*, showed increased number of infiltrating leukocytes, aberrant production of pro-inflammatory cytokines (TNF-α, IL-1β, INF-γ) and CC chemokines (CCL2, CCL3, CCL4 and CCL5), increased tissue damage and reduced survival [168]. Furthermore, the blockage of inflammatory CC chemokines, in *Ackr2*$^{-/-}$ mice, reversed the inflammatory phenotype but led to increased susceptibility to *M. tuberculosis* infection [168].

In models of maternal systemic inflammation, the expression of ACKR2 has been demonstrated to be important in dampening inflammation at materno-fetal barrier. In fact, in this context, *Ackr2*$^{-/-}$ mice displayed increased level of inflammatory CC chemokines and leukocytes associated with enhanced susceptibility to miscarriage [111, 154].

Furthermore, the role of ACKR2 has been investigated in a model of colitis induced by the treatment of mice with dextran sulfate sodium (DSS). Vetrano et al. showed that *Ackr2*$^{-/-}$

mice had increased intestinal inflammation characterized by increased level of inflammatory chemokines and infiltrating leukocytes compared to WT mice [156]. On the contrary, Bordon et al. observed that *Ackr2*-deficient mice have reduced clinical symptoms and tissue pathology in response to DSS, and correlated this phenotype to an increased production of IL-17A by T cells [169].

In a murine model of graft versus host disease it has been found that the increased number of inflammatory monocytes with enhanced immunosuppressive activity protects *Ackr2* deficient mice from the development of the disease [164].

Finally, controversial results have been published regarding the role of ACKR2 in the context of autoimmune diseases [170, 171].

4.2.3 ACKR2 and cancer

Considering the relevance of ACKR2 in inflammatory conditions, the role of ACKR2 has been investigated in different models of cancer related inflammation (Figure 9). Nibbs et al. found that in a model of skin carcinogenesis *Ackr2*$^{-/-}$ mice showed increased susceptibility to skin cancer, and they also had increased level of inflammatory CC chemokines and tumor infiltrating leukocytes [172]. Similar phenotype was observed in a model of colon cancer induced by Azoxymethane (AOM) and DSS treatment [156].

Since ACKR2 is expressed by different tumors, the role of ACKR2 expressed by cancer cells was assessed in a model of breast cancer and Kaposi's Sarcoma.

Wu et al. demonstrated that the overexpression of ACKR2 in breast cancer cells, is associated with decreased chemokine levels (CCL2 and CCL5), LV density and TAM infiltration; Savino et. al showed that the overexpression of ACKR2 in KS-IMM cells regulates the level of inflammatory CC chemokine within the tumor and inhibits monocyte differentiation and VEGFA production, thus reducing angiogenesis and tumor growth [173].

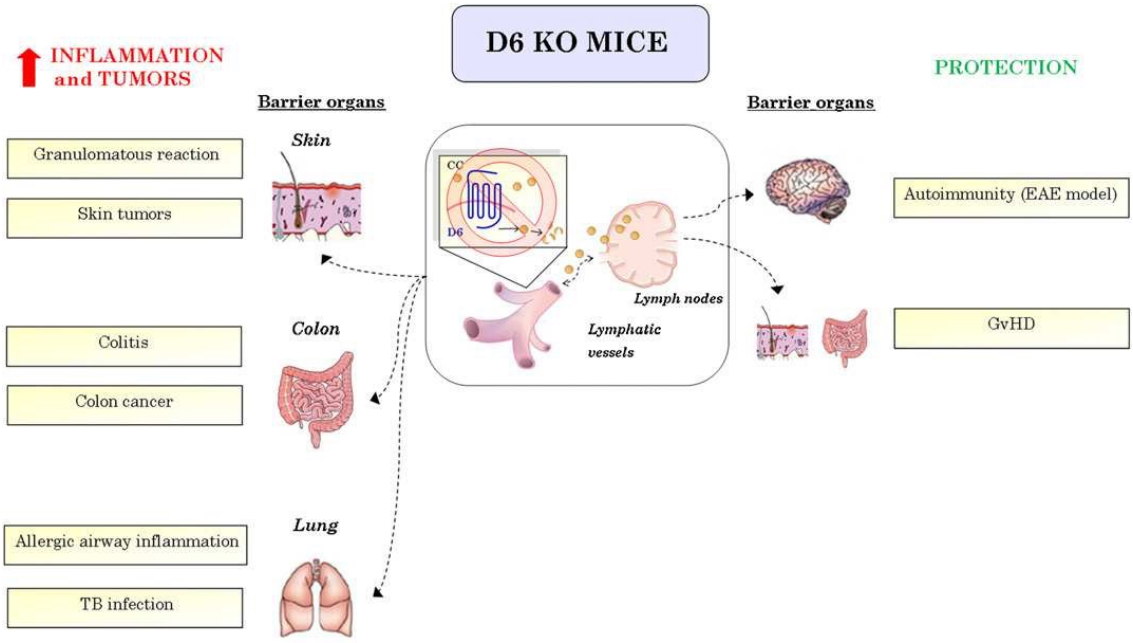


Figure 9: ACKR2 in vivo activity. ACKR2 has important regulatory functions on inflammation. Scavenging chemokines, ACKR2 controls local inflammatory response, and promotes the successful migration of dendritic cells (DCs) to draining lymph nodes (LNs). ACKR2 functions have been demonstrated in *Ackr2* deficient mice in different model of inflammation, including cancer related inflammation, allergy, infection, autoimmunity and graft versus host disease (GvHD) [150].

5. The hematopoietic system

The hematopoietic system is a hierarchy of hematopoietic cell populations that became progressively mature and in which hematopoietic stem cells (HSCs) reside at the top (Figure 10) [174]. HSCs represent a rare population of cells in the BM and are characterized by an extensive self-renewal capability and pluripotency. Based on their temporal ability to generate the whole spectrum of mature blood cells, HSCs can be classified in long-term, intermediated-term and short-term hematopoietic stem cells (LT-HSC, IT-HSC, ST-HSC) [174].

HSCs division can result in the production of additional HSCs or HPCs [175]. HPCs have a limited self-renewal capability and consist of: multipotent progenitors (MPPs), with no longer self-renewal ability and endowed with full lineage differentiation potential; and lymphoid-primed multipotent progenitors (LMPPs), common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs), in which multipotency is restricted to a specific lineage [174, 176, 177].

CMPs give rise to granulocytes-macrophages progenitors (GMPs) and megakaryocyte-erythrocyte progenitors (MEPs). The differentiation of CLPs, GMPs and MEPs give rise to the largest pool of hematopoietic cells which is made up of mature blood cells, including B cells, T cells, NK cells, NKT cells, monocytes, macrophages, granulocytes, platelets, erythrocytes, and DCs. Blood mature cells have no self-renewal capability, with the exception of certain B cell, and T cell subsets, tissue-resident macrophages and DCs [176, 177].

In mice, the fraction of multipotent cells is called LSK and is characterized as $Lin^- Sca\text{-}1^+$ $c\text{-}kit^+$. Within the LSK fraction it is possible to distinguish, depending on the level of CD34 expression, the HSCs that are $CD34^-$ and MPPs that are $CD34^+$. The CMPs are characterized as $IL\text{-}7R\alpha^-$ $c\text{-}Kit^+$ Lin^- $Sca\text{-}1^-$ and can be further fractionated on the basis of the expression of FcγRII and FcγRIII (CD16/CD32) and CD34. Finally, GMPs and MEPs that originate from CMP ($Fc\gamma RII/III^{lo}$ $CD34^+$) are characterized as $Fc\gamma RII/III^{hi}$ $CD34^+$ and $Fc\gamma RII/III^{lo}$ $CD34^+$, respectively [176].

The process by which HSCs proliferate and differentiate into mature blood cellular components is called hematopoiesis [178]. In adult life haematopoiesis takes place into the BM [178]. However, in some cases, the liver, the thymus, and the spleen may assume an hematopoietic function; this is called extramedullary hematopoiesis and it is typical of fetal development, since bone and thus BM develop later [178]. Interestingly, macrophages that

have been traditionally known to originate from monocyte differentiation in the BM, may also derive from different precursors cells and do not have a monocytic progenitor [179]. Indeed, in embryos, tissue-resident macrophages in liver (Kupffer cells), brain (microglia), epidermis (Langerhans cells) and lung (alveolar macrophages), have been demonstrated to originate from precursors cells in the yolk sac and to be able to self-renew [180].

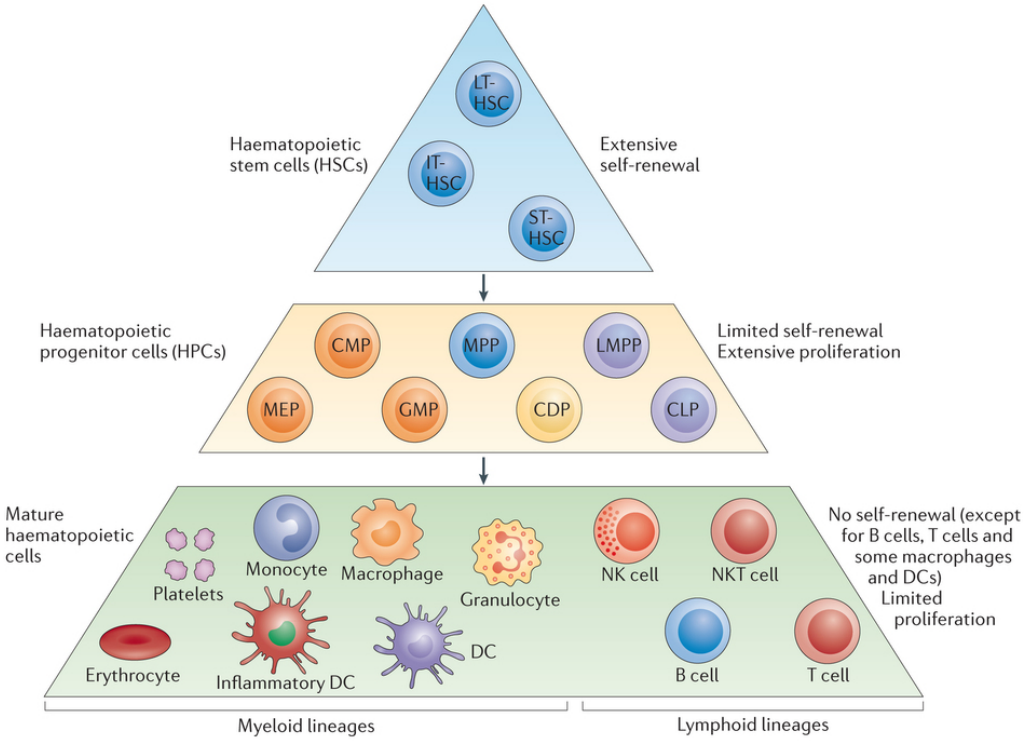


Figure 10: The hematopoietic hierarchy: The hematopoietic system is a hierarchy of hematopoietic cell populations that became progressively mature and in which hematopoietic stem cells (HSCs) reside at the top. Depending on their temporal ability to differentiate, HSCs can be classified in long-term, intermediated-term and short-term hematopoietic stem cells (LT-HSC, IT-HSC, ST-HSC). HSCs division can result in the production of additional HSCs or into hematopoietic progenitor cells (HPCs) that give rise to the whole spectrum of mature blood cells [181].

5.1 HSC bone marrow niche

The niche is the tissue microenvironment that maintains and regulates stem cells [182, 183]. During adulthood HSCs reside mainly within the BM, which is a complex organ, consisting of both hematopoietic and non-hematopoietic cell types, surrounded by vascularized and innervated bone. The interface between bone and BM is called endosteum. Near the endosteum, there is a rich supply of arterioles and sinusoids, which are small specialized and fenestrated vessels, allowing the cells to pass in and out the circulation, and providing a sort of barrier between the hematopoietic compartment and the peripheral circulation [178]. Within the BM, HSCs can be found in the endosteal zone closed to osteoblastic cells, or adjacent to sinusoids. Osteoblastic cells, together with osteoclastic and mesenchymal-derived stromal cells, form the osteblastic niche, where long term quiescent HSCs can be found [184]. Conversely, BM sinusoids represent the vascular niche that contains cells more prone to proliferate and differentiate (Figure 11). The function of quiescent HSCs is to replace active recycling HSCs in the vascular niche, whereas active HSCs replace lost quiescent cells in the osteblastic niche and also create a negative feedback to prevent the activation of quiescent HSCs [183, 185]. Interestingly, because of their localization and their capability to actively proliferate and differentiate, cells in the BM vascular niche are those immediately released to support the daily production of blood cells [186]. In BM, HSCs interact with osteoblast and endothelial cells. This interaction is mainly mediated by the CXCR4/CXCL12 axis. Indeed CXCL12, also known as stromal derived factor-1 (SDF-1), produced by stromal cells in the niche, binds to the receptor CXCR4 expressed by HSCs and induces their localization in both osteoblatic and vascular niches [187]. Another important factor is represented by stem cell growth factor (SCF) that binds to cKit on HSCs inducing their self-renewal [188]. In addition, other molecules such as P-selectin, E-selectin, and vascular cell adhesion molecules (VCAM-1), are expressed by niche stromal cells and control HSC maintenance and function by the interaction with their specific receptors on HSCs [186].

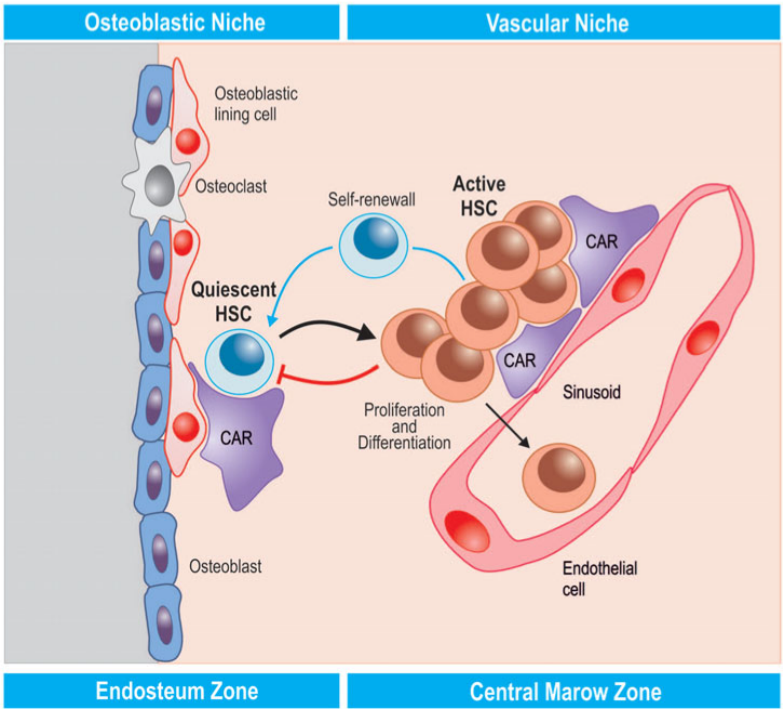


Figure 11: Proposed model of bone marrow (BM) osteoblastic and vascular niches of hematopoietic stem cells (HSCs). Long term quiescent HSCs can be found in the osteblastic niche, consisting of osteoblastic cells, together with osteoclastic and mesenchymal-derived stromal cells. Proliferating and differentiating cells are located in the BM sinusoids, which represent the vascular niche. Quiescent HSCs replace active recycling HSCs in the vascular niche, whereas active HSCs replace lost quiescent cells in the osteblastic niche and also create a negative feedback to prevent the activation of quiescent HSCs [186].

5.2 Regulation of HSC differentiation and mobilization

The relationship between HSCs and their niches are highly dynamic and depend on the requirements of circulating hematopoietic cells. Indeed, in order to maintain physiological homeostasis and to achieve the balance between HSC renewal and differentiation, the niche elaborates also chemokines, cytokines and growth factors, which orchestrate hematopoietic development [183].

These factors, such as hormone erythropoietin (EPO), SCF, IL-3, IL-6 and colony-stimulating factors (CSFs), are important for the initial rounds of cell division and differentiation of HSCs in HPCs, and promote the formation of colonies that will give rise to more mature cellular component [189-193].

CSFs are the major orchestrators of myelopoiesis, they were defined by their abilities to generate *in vitro* colonies of mature myeloid cells from BM precursor cells, and they

include: macrophage CSF (M-CSF; also known as CSF1), which supports macrophage differentiation from medullar precursors and differentiation of tissue macrophages; granulocyte-macrophage colony stimulating factor (GM-CSF; also known as CSF2) that stimulate the proliferation and differentiation into monocytes and DCs; and granulocyte colony stimulating factor (G-CSF; also known as CSF3), which is essential for the differentiation of neutrophil progenitors and precursors [193].

In steady state, myelopoiesis is regulated by the activation of several transcription factors. For example, G-CSF-induced granulopoiesis is mediated by CCAAT-enhancer-binding protein-α (C/EBPα), signal transducer and activator of transcription-3 (STAT3), and extra-cellular regulated kinase 1 (ERK1), whereas M-CSF supports monocyte differentiation by activating transcription factors PU.1 and Interferon regulatory factor-8 (IRF8) [194-197].

Interestingly, it has been found that G-CSF, by increasing the number of neutrophils, also promotes the mobilization of HSCs [186]. These cells, released from BM, act as sentinel and rapid source of leukocytes in peripheral circulation [181, 186]. The mechanisms inducing their mobilization are aimed to inhibit cell-cell contacts between HSCs and BM niche cells, and include the cleavage of adhesion molecules and the desensitization of chemokine signaling, mainly the SDF1/CXCR4 axis [186, 198]. Proteolytic enzymes, such as Elastase and Cathepsin-G, play a major role in disrupting this axis by cleaving the N-terminal signal sequence of SDF-1 and the receptor CXCR4 [198].

HSC mobilization in response to G-CSF is due to the release of neutrophil proteases, including MMP-9, resulting in enhanced cleavage of ckit, SDF-1, CXCR4, VCAM and its receptor Very Late Antigen-4 (VLA-4) [186].

HSC recruitment in peripheral circulation is mediated by CXCL12 production at the periphery, and although HSCs express chemokine receptors, such as CCR3 and CCR9, they do not migrate in response to the ligands of these receptors [199].

5.3 The role of the chemokine system in myelopoiesis

The role of chemokines and their receptors in myelopoiesis encompasses the regulation of proliferation as well as the survival of HSCs and HPCs [200, 201]. Based on several findings, at least 24 chemokines belonging to CC, CXC and C families, act as hematopoietic suppressor molecules, and they include: CCL3, CCL6, CCL19, CXCL5, CCL11, CCL20, CXCL6, CXCL2, CCL15, CCL16, CCL1, CXCL8, CXCL10, XCL1,

CCL2, CCL3, CCL23, CCL9/10, CXCL4, CCL21, and CCL25 [200, 202]. In addition, these chemokines had myelosuppressive activity in vivo. Indeed, they induced a dose dependent inhibition in the absolute number and cycling of HSCs [202, 203].

Among these chemokines, one of the best studied is CCL3/MIP-α, which was first identified as a suppressor molecule for spleen colony formation units (CFU-s), and then for multi-growth factor responding MPCs (e.g. CMP) [204, 205]. Conversely, CCL3 was shown to enhance the proliferation of more mature MPCs that respond to a single growth factor and are more committed progenitors [206]. To identify through which chemokine receptor CCL3 exerted its myelosuppressive activity, strategies for the cloning of CCL3 receptor were developed. Interestingly, by this way ACKR2 was discovered [141]. However, given the redundancy of chemokine system, the role of CCL3 receptor in mediating chemokine myelosuppressive activity has been assessed by generating gene knock out mice deleted for specific chemokine receptor. K. Ottersbach and colleagues examinated the response of CCR1, CCR3, CCR5 and ACKR2 null BM cells to CCL3 and saw that BM cells had a full inhibitory response, indicating that none of CCL3 identified receptors was mediating the stem cell inhibitory effects of CCL3 [207]. Thus, yet unidentified receptor may be involved.

Even if *Ccr1*$^{-/-}$ mice did not show reduced myelosuppression in response to CCL3, they elucidated the role of CCR1 in mediating myelopoietic effect of CCL3 on more mature MPCs (e.g. GMP) [206]. Indeed, *Ccr1*$^{-/-}$ MPCs stimulated to proliferate either by GM-CSF or M-CSF, in presence of CCL3, formed less colonies containing only granulocytes and macrophages compared to WT [206].

Again by using gene target mice, also the role of CCR2 and CXCR2 in the negative regulation of HPC proliferation was assessed. It was found that CCR2 controls the proliferation and programmed cell death (apoptosis) of HPCs [208]. Indeed, mice lacking CCR2 showed increased cycling status of MPCs in BM, and no difference in the absolute number of HPCs in BM and spleen compared to WT. This effect was demonstrated to be due to a balance between enhanced proliferation and enhanced apoptosis of *Ccr2*$^{-/-}$ HPCs [208]. The suppressive effect of CCR2 may be mediated by CCL2, CCL13 as well as CCL12, since these CCR2 ligands, but not CCL8 or CCL7, are myelosuppressive [200]. In *Cxcr2*$^{-/-}$ mice a selective insensitivity of MPCs to inhibition by CXCL8 and CXCL2, and a large expansion of MPCs have been described. Thus CXCR2 has been identified as a negative regulator of MCP proliferation by mediating the myelosuppressive activity of CXCL8 and CXCL2 [209].

5.4 The role of chemokine system in myeloid cell mobilization

The chemokine system, in addition to control the proliferation of HSCs and MPCs, has a crucial role in the mobilization of mature myeloid cells, such as monocytes and neutrophils, from BM to the peripheral blood [2]. Depending on their activation states, monocytes and neutrophils can be distinguished in different cell subpopulations with a distinct pattern of chemokine receptor expression, which confers different migration and homing capabilities to the cells [210].

The release of monocytes from BM is mainly controlled by CCR2. In mice, this receptor is highly expressed on a subpopulation of blood monocytes, also distinguished for differential expression of the antigen Ly6C and the chemokine receptor CX3CR1 [210]. $Ly6C^{high}$ $CCR2^{high}$/ $CX3CR1^{low}$ monocytes are the classical or proinflammatory monocytes that are recruited to inflammatory sites where they produce high levels of inflammatory cytokines and differentiate into tissue macrophages [210]. The expression of CCR2 by this cells starts during hematopoiesis, indeed as HSCs differentiate along the myeloid lineage, CCR2 expression increases and mediates also the trafficking of HSCs and MPCs to the site of inflammation [211]. The relevance of CCR2 in controlling inflammatory monocyte recruitment has been demonstrated by the fact that the number of these cells is markedly decreased in the blood of *Ccr2*$^{-/-}$ mice [212]. Moreover, analysis of mice lacking CCL2, CCL7, CCL12, or CCL8 plus CCL12 revealed that CCL2 and CCL7 are the CCR2 agonists most critical for the maintenance of normal blood monocyte counts [213].

Conversely, $Ly6C^{high}$ $CCR2^{low}$/ $CX3CR1^{high}$ monocytes are the non-classical, patrolling, or alternative monocytes [210, 214]. Their recruitment is mediated by CX3CR1 and they are considered to be important in tissue repair through the production of IL-10 and their differentiation in pro-resolving macrophages [214]. Whether the different subsets of monocytes originate as distinct populations from a common precursor or by transition from $Ly6C^{high}$ to $Ly6C^{low}$ is a still debated issue.

Monocytes also express CCR1 and CCR5, which are receptors for a variety of chemokines, including CCL3 and CCL5 [2, 215, 216]. *In vitro* transmigration assays have shown that CCR1 supports the arrest of monocytes in the presence of shear flow, CCR5 contributes to monocyte 'spreading', and both CCR1 and CCR5 mediate trans- endothelial chemotaxis towards CCL5 [217].

Although less studied, also CCR6, CCR7, CCR8 and CXCR2 have been suggested to be involved in monocyte recruitment [2].

Regarding neutrophils, their trafficking from BM is regulated by the antagonistic activity of the chemokine receptors CXCR2 and CXCR4 [218]. Indeed, a constitutive expression of CXCL12 in the BM provides a retention signal for neutrophil precursors in the BM through the activation of their receptor CXCR4. CXCR4 down-regulation, induced by type I cytokines such as interferon (INF)-γ, IFN-α, GM-CSF and G-CSF, during medullary maturation, promotes the release of neutrophils from the BM [219]. This release has been demonstrated to be facilitated by the up-regulation of CXCR2 expression, in fact by using mouse mixed BM chimeras reconstituted with *Cxcr2*$^{-/-}$ and WT neutrophils, it was shown that *Cxcr2*$^{-/-}$ neutrophils are preferentially retained in the BM [218]. However CXCR4 inhibition plays a dominant role in neutrophils mobilization, indeed neutrophils lacking both CXCR2 and CXCR4 displayed constitutive mobilization [218]. Interestingly, CXCR4 that is down-regulated in circulating neutrophils may be up-regulated in senescent neutrophils to promote their homing back to the BM [220, 221].

Moreover, in various myeloid cell lines, CCR2 gene has been identified as one of the several targets of G-CSF during neutrophilic differentiation, and recently, CCR2 expression, which was previously supposed to be restricted to monocytes, has been described in a subset of murine BM neutrophils [222, 223]. This expression has been demonstrated to influence neutrophil egress from the BM. Indeed, analysis performed in CCR2-RFP$^{+/-}$ mice (with intact CCR2 expression) and CCR2-RFP$^{+/+}$ mice (CCR2 deficient) had shown that CCR2-RFP$^{+/+}$ mice have a small but statistically significant decrease in the number of neutrophils in the blood, LNs and spleen [223].

5.5 Emergency granulopoiesis

Myeloid cells are the first line of defence against invading organisms, and in steady state they are continuously generated to ensure their immediate availability for the containing of pathogens. However, during immunologic stress, such as systemic infection and cancer, the first-line of defense is overwhelmed and the demand of myeloid cells markedly increases. Most importantly, in this condition, neutrophils are consumed in large quantities [181]. To provide a supply to the increased demand of neutrophil, the hematopoietic system modifies the hematopoietic output through a process called emergency granulopoiesis, characterized by increased MPC proliferation and enhanced granulocytic differentiation within the BM [181].

One of the most important cytokine regulating granulopoiesis is G-CSF as demonstrated by the strong reduction of circulating neutrophils in G-CSF and G-CSF receptor (G-CSFR) deficient mice [224] [225]. G-CSF production is regulated by IL-17A, which can be synthetized by γδ and NK cells, and by IL-23 that is produced by tissue-resident macrophages and DCs [226-229]. Since IL-17A is upstream of G-CSF, low levels of IL-17A are associated with reduced expression of G-CSF and reduced release of neutrophils from the BM [229].

In steady state, G-CSF, upon binding to G-CSFR that is expressed on MPCs, stimulates multiple intracellular signaling pathways via the activation of the Janus family kinases (JAK) and the STAT family proteins, such as STAT3 and STAT5 [194]. Moreover, G-CSF induces the activation of the major transcriptional regulator of steady-state granulopoieisis C/EBPα [230]. C/EBPα promotes the expression of many genes encoding proteins that are important for the proliferation of MPCs and granulocytic differentiation, for example the G-CSFR [231] (Figure 12).

However, at the same time, both C/EBPα and STAT3-mediated G-CSF-induced signaling activate mechanisms to limit excessive cell proliferation. Indeed, C/EBPα also inhibits the expression of genes encoding for proteins, which are crucial for cell cycle progression, such as MYC, cyclin-dependent kinase (CDK)-2 and CDK4, whereas STAT-3 induces the expression of suppressor of cytokine signaling 3 (SOCS3) [232, 233]. SOCS3 is recruited to tyrosine residues on G-CSFR, and inhibits signal transduction through JAK–STAT3 [233]. Furthermore, G-CSF stimulation induces the expression of the inhibitor of nuclear factor-κB (IκB) family member B cell lymphoma 3 protein (BCL-3) in a STAT3-dependent manner [234]. BCL-3, originally identified as a proto-oncogene, also acts as an anti-inflammatory regulator by limiting the transcription of nuclear factor-κB (NF-κB)-dependent genes [235, 236]. Thereby BCL-3 is required to limit MPC proliferation and differentiation in NF-κB dependent-manner [235, 236].

Conversely, emergency granulopoiesis is characterized by the activation of transcription factors different from those activated in steady-state granulopoiesis, and the mechanisms preventing excessive cell proliferation are lost [181]. Indeed, G-CSFR signaling through JAK leads to nuclear translocation of phosphorylated STAT3 (pSTAT3), which directly stimulates the expression of C/EBPβ that is the major transcriptional regulator of emergency granulopoiesis [237, 238]. C/EBPβ directly stimulates *Myc* transcription and also replaces C/EBPα at the *Myc* promoter through competition for binding, thereby leading to the inhibition of the transcriptional repression that C/EBPα exerts on *Myc*

expression [238]. As a result, the proliferative effects of C/EBPβ outweigh the anti-proliferative effects of C/EBPα, resulting in enhanced MPC proliferation and neutrophil generation [181, 238] (Figure 12).

The switch from steady state to emergency granulopoiesis can be induced by the pathogen sensing through pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), and might be direct or indirect. Indeed, HSCs are capable to sense infection and inflammation, directly through the expression of PRRs or indirectly via inflammatory cytokines produced by other cells (both mature hematopoietic and non-hematopoietic cells) [181]. However, the signaling pathways that control the shift from C/EBPα-dependent steady-state granulopoiesis to C/EBPβ-dependent emergency granulopoiesis are poorly understood.

As well as all inflammatory conditions, also in tumor, cytokine production is prominently amplified, and as consequence, emergency granulopoiesis is activated [239]. In particular, tumor may either directly or indirectly produces G-CSF, indeed cancer cells themselves are a potent source of G-CSF, and also produce cytokines, which in turn induce the recruitment of tumor-associated leukocytes that are involved in G-CSF production. For example, TAMs are a well-known source of IL-1β, which is a potent inducer of IL-17/G-CSF axis [240]. Recently, another factor named retinoic-acid-related orphan receptor (RORC1) has been identified as regulator of tumor-emergency granulopoiesis by suppressing Socs3 and Bcl3 and inducing C/EBPβ [241].

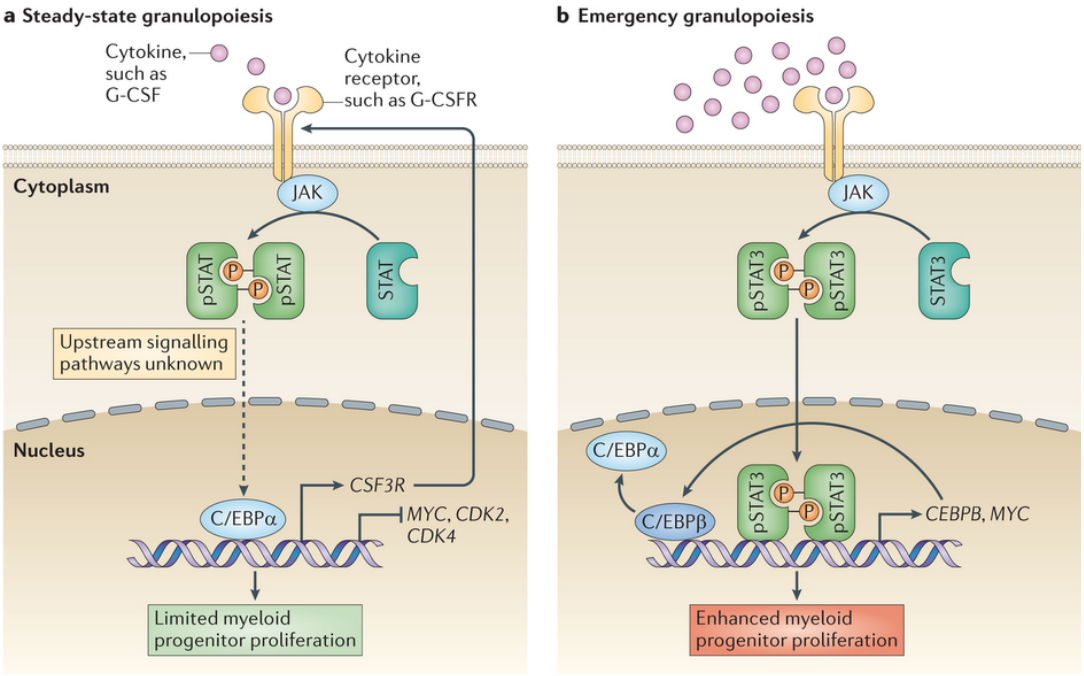


Figure 12: Signal transduction and transcriptional activation in steady-state and emergency granulopoiesis. In steady-state G-CSFR signaling through the Janus family kinase (JAK) leads to the activation of C/EBPα that promotes the expression of many genes encoding proteins that are important for the proliferation of myeloid progenitor cells (MPCs) and granulocytic differentiation. C/EBPα simultaneously limits excessive proliferation by inhibiting the expression of *Myc*, cyclin-dependent kinase 2 (*Cdk2*) and *Cdk4* that are crucial for cell cycle progression. Conversely, during emergency granulopoiesis G-CSFR signaling through JAK leads to nuclear translocation of phosphorylated STAT3 (pSTAT3), which directly stimulates the expression of C/EBPβ that is the major transcriptional regulator of emergency granulopoiesis. Directly binding to the proximal *Myc* promoter, C/EBPβ also inhibits the C/EBPα binding to the promoter, thereby suppressing the transcriptional repression exerted by C/EBPα on *Myc* expression [181].

6. Neutrophils

Neutrophils are key component of innate immunity and essential effectors of the inflammatory response [242]. In humans, they are the most abundant leukocyte population in circulation. The current paradigm is that circulating neutrophils have a short half-life in the bloodstream (6-12 h for mice and humans), followed by a rapid clearance from blood [243], and they are continuously produced and released from BM, especially in case of severe inflammation [181]. However, in vivo experiments have demonstrated that non-circulating neutrophils can survive in tissue for several days [244, 245]. Moreover, tumor microenvironment can support their survival. Indeed, Mantovani and his colleagues showed that many cytokines produced by tumor cells prolong neutrophil survival in culture [246]. Neutrophils are characterized by a segmented nucleus and a cytoplasm enriched with granules and secretory vesicles. Three types of neutrophil granules can be distinguished; these are azurophilic (primary) granules, which are characterized by a high content of myeloperoxidase (MPO); specific (secondary) granules, which are characterized by a high content of lactoferrin; and gelatinase (tertiary) granules, which are characterized by a high content of MMP9 [242].

During inflammation, neutrophils are the first cells to be recruited at the site of injury. They migrate by following a chemotactic gradient, and express two proteins, which are crucial for the recognition of endothelial inflammatory signals. Indeed, they express the P-selectin glycoprotein ligand-1 (PSGL-1) and L-selectin, also known as CD62L; these molecules engage the P and E-selectins expressed by inflamed endothelial cells, resulting in neutrophil capture to endothelial wall. Then, the interaction of ICAM-1 on endothelial cells and LFA-1 on neutrophils, promotes neutrophil arrest and endothelial transmigration [243].

Upon arriving at the infection site, neutrophils can eliminate invading pathogen through different mechanisms. Indeed, they can release the antibacterial proteins (e.g. cathepsins, defensins, lactoferrin and lysozyme) contained in neutrophil granules either into phagosomes, upon pathogen phagocytosis or into the extracellular environment, thus killing pathogens by an intra- or extracellular mechanism, respectively. Moreover, neutrophils can fight pathogens by releasing reactive oxygen species (ROS, by NADPH oxidase activity) and reactive nitrogen species (RNS, by nitric oxide synthase activity), and by releasing neutrophil extracellular traps (NETs). NETs are fibers composed of processed chromatin and proteins, and they are an important strategy to immobilize invading

microorganisms, thus preventing their spread but also facilitating phagocytosis of trapped microorganisms [242].

Since neutrophils are the first cells to arrive to the site of infection, in addition to kill invading pathogens, they also play a crucial role in establishing the proper environmental conditions to promote the activation of immune response. Indeed, they produce several cytokines and chemokines, which induce the recruitment of new immune cells, such as monocytes, macrophages, DCs, NK cells, and T cells [242].

6.1 Neutrophil heterogeneity

Recent findings have uncovered new interesting aspects regarding the biology of neutrophils. Indeed, studies analysing neutrophils in several inflammatory conditions have shown that these cells can display different degrees of maturation and activation, and they can have different functional properties being either immunosuppressive or pro-inflammatory neutrophils [247]. These heterogeneous populations with distinct characteristics have been identified with a variety of name, including polymorphonuclear myeloid-derived suppressor cells PMN-MDSCs, low-density neutrophils (LDNs), and high-density neutrophils (HDNs) [248, 249]. PMN-MDSCs are morphologically and phenotypically similar to neutrophils, and they have been described as immature neutrophils with immunosuppressive activity [248]. LDNs are so called because after density gradient centrifugation of blood, they sediment within the low-density mononuclear cell layer rather than the expected HDN fraction [247, 249]. LDNs include both immature and mature cells with immunosuppressive or pro-inflammatory functions. Finally, HDNs have been described as mature and pro-inflammatory neutrophils [247, 249]. However these ways to identify neutrophils are still matter of debate.

6.2 Neutrophil subpopulations

As well as for monocytes, in the last few years, the existence of different subsets was also described for neutrophils [250]. Indeed, neutrophils, after leaving the BM, enter into the circulation and acquire several phenotypic features depending on homeostatic or inflammatory conditions [251].

In mice, the majority of circulating neutrophils are Ly6G^{high} CD62L^{high}. When an inflammatory situation occurs, a fraction of circulating neutrophils down-regulates CD62L /L-selectin and CXCR2, and up-regulates ICAM-1/CD54, CCR1, and CCR2 [250]. These circulating neutrophils are activated and ROS, which confer them increased killing ability [91, 252].

Heterogeneity of circulating neutrophils has also been reported in the human setting. Indeed, in healthy donors is present a single population of neutrophils identified as CD66b^{bright}/CD16^{high}/CD62L^{high}/CXCR2^{high}, whereas in inflammatory conditions, two populations can be found: CD66b^{bright}/CD16^{dim}/CD62L^{bright} that are immature forms of neutrophils and CD66b^{bright}/CD16^{bright}/CD62L^{dim} neutrophils that have an enhanced anti-microbial function and ROS generation ability [253, 254].

Neutrophils after extravasation or after stimulation with proinflammatory cytokines, acquire a different pattern of chemokine receptor expression. They up-regulate the chemokine receptors CCR1, CCR2, CCR3, and CCR5 that promote respiratory burst activity and bacterial killing [255].

Both in humans and mice, neutrophils recruited to the inflammatory site, can then transmigrate back into the circulation. These neutrophils are called reverse transmigrated neutrophils (rTEM), they express high levels of ICAM-1, and show enhanced ROS production. In addition they express low levels of the chemokine receptor CXCR1 [256]. Moreover, circulating neutrophils can spontaneously up-regulate the expression of CXCR4, which has been propose to mediate the migration of “senescent” neutrophils back to BM where the level of CXCL12 are constantly high [220].

6.3 Neutrophil polarization in cancer

Neutrophils represent a high percentage of infiltrating leukocytes in many human cancers, and even though they have been mostly considered for their anti-microbial functions, recently they are emerging for their involvement in cancer biology [257, 258].

In mice, it was demonstrated that tumor associated neutrophils (TAN) can exist in two different functional states: proinflammatory, antitumoral neutrophils (N1) and anti-inflammatory tumor-promoting neutrophils (N2) [259].

N1 neutrophils are hypersegmented, produce high levels of proinflammatory cytokines (e.g. TNF-α, CCL3) and radical oxygen species (H_2O_2, and NO) and are able to stimulate

T cell response. N1 polarization is determined by IFN-β and inhibited by TGF-β, which lead TANs to assume a proinflammatory and cytotoxic phenotype. Furthermore, neutrophils can be polarized to N1 by the proto-oncogene MET, which is induced on neutrophils by proinflammatory stimuli [260]. MET stimulation by its ligand HGF leads to neutrophil endothelial transmigration and NO production that promotes cancer cell killing and reduces tumor growth and metastases [260].

Conversely, N2 polarization is mediated by TGF-β and by IFN-β inhibition. Indeed, *Ifn-β* deficient mice showed enhanced growth of B16F10 melanoma and MCA205 fibrosarcoma tumors. This effect was due to increased infiltration of neutrophils expressing high levels of the transcription factors c-Myc and STAT3, known to regulate the expression of the proangiogenic factors VEGF and MMP9, and the homing receptor CXCR4 [261].

Some studies have proposed that TAN infiltrating early tumors are N1 and they become N2 with tumor progression. In fact, in murine models of Lewis lung carcinoma (LLC) and mesothelioma (AB12), at early stages of tumor development TANs are cytotoxic toward tumor cells and produce high levels of TNF-α and NO, whereas in established tumors, these functions are reduced and TANs acquire a more pro-tumorigenic phenotype [262]. The same picture was found in an experimental model of breast cancer liver metastases, where infiltrating neutrophils showed an N2 phenotype during tumor progression and sustained tumor growth [263]. Similarly, in humans, TANs isolated from early stages of lung cancer as $CD11b^{+}CD15^{hi}CD66b^{+}$ cells, resemble N1 neutrophils. Indeed, they have an activated phenotype ($CD62L^{lo}$ ICAM/$CD54^{+}$), express a distinct repertoire of chemokine receptors (CCR5, CCR7, CXCR3, and CXCR4), produce high level of proinflammatory chemokines and cytokines (CCL2, CXCL8, CCL3, and IL-6), and express increased levels of costimulatory molecules that stimulate T cell proliferation [264].

6.4 Neutrophil protumoral functions

A recent meta-analysis of published papers showed that the presence of neutrophils in tumor tissues represents a poor prognostic indicator in various cancers [265]. Indeed, as shown in several studies, TANs are able to sustain cancer progression through several mechanisms.

TANs release enzymes, contained in their granules, such as neutrophil elastase, MMP8 also known as neutrophil collagenase, and MMP9 also known as neutrophils gelatinase B,

that promote tumor cell invasion by remodeling extracellular matrix (ECM) or directly acting on tumor cells [266]. Neutrophils also produce cytokines and growth factors (EGF, TGF-β, PDGF, HGF, VEGF) with pro-tumoral activity. Indeed, hepatocyte growth factor (HGF) has been shown to promote the invasion of human pulmonary adenocarcinoma cells [267]. Moreover, neutrophils can promote the angiogenesis through the release of MMP9 that activate VEGF, and through the production of oncostatin M, a member of IL-6 family, known to induce the production of VEGF [268, 269]. Additionally, neutrophils enhance tumorigenesis through the release of ROS and RNS that contribute to DNA damage and genetic instability [270].

Furthermore, neutrophils can produce molecules, which are able to directly promote tumor cell proliferation. For example, neutrophil elastase, prostaglandin E2 (PGE2) and leukotrienes activate intracellular signaling cascades, which lead to tumor cell proliferation [271, 272]. In particular, rachidonate 5-lipoxygenase (ALOX-5) mediated leukotriene synthesis supports the proliferation of metastasis initiating cells in different mouse model of breast cancer [273].

Neutrophils can promote tumor metastasis by facilitating the adhesion of tumor cells to endothelial cells at the extravasation site. Indeed tumor cells can be trapped within the NETs and increase their adhesion to hepatic and pulmonary microvasculature. NETs can also promote cancer cell proliferation by inhibiting apoptosis [274].

Finally, neutrophils exert their protumoral activity by inhibiting the antitumoral immune responses. In fact, upon CXCL8 stimulation, neutrophils produce arginase 1 (Arg1), an inhibitor of T cell function [275]. It was also described that neutrophils express programmed death-ligand 1 (PD-L1), which suppresses T-cell proliferation [276]. Moreover, neutrophils were shown to produce TGF-β, a cytokine with immunosuppressive effect on other immune cells [277].

6.5 Neutrophil antitumoral functions

Despite the predominant outline of neutrophils protumoral functions, other studies revealed the capabilities of these cells to exert anti tumoral effects [278].

For example, although neutrophils, by producing ROS, promote cell death and tissue damage in the tumor microenvironment, they can be cytotoxic against tumor cells. Accordingly, it has been described that neutrophils can inhibit the metastatic seeding of

breast carcinoma cells in the lungs by producing high levels of hydrogen peroxide and killing tumor cells [252].

Another killing mechanism is reported for IFN-activated neutrophils that release biologically active TNF-related apoptosis-inducing ligand (TRAIL/APO2 ligand), a molecule that exerts a selective apoptotic activity toward tumor cells [279, 280]. Moreover, neutrophils also mediate Fas-mediated apoptosis of cancer cells [281].

Finally, neutrophils are able to promote the adaptive immune response against tumors. They can either directly stimulate T cells through the expression of OX-40L and 4-1BBL, which are co-stimulatory molecules that enhance the proliferation of $CD4^+$ and $CD8^+$ T cells, or indirectly via cytokine and chemokine-mediated leukocyte recruitment [264, 282].

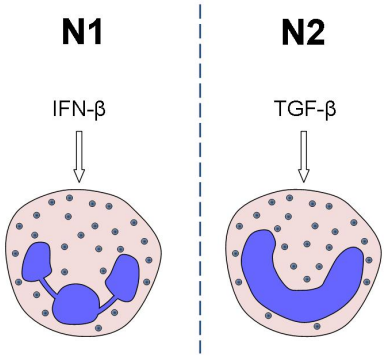
6.6 Insights into the role of chemokine system in neutrophil recruitment and effector functions in tumors

Recently, it is emerging that the differential expression of chemokine receptors on neutrophil subpopulations, can affect not only their recruitment but also their effector functions [258]. For example, it has been shown that CCR2 is not only important for neutrophil mobilization and tumor infiltration but also for neutrophil antitumoral activity. Indeed $CCR2^+$ neutrophils exert an anti-metastatic activity by producing ROS in a CCR2-depent manner [252]. Similarly, CXCR2 that also have an important role in neutrophil mobilization promotes tumor infiltration of neutrophils that produce pro-angiogenic factors, which support tumor growth. Accordingly, the expression of CXCR2 ligands by tumors plays a role in promoting the development and metastases of several cancer types [63, 283]. CXCL5 was found to recruit neutrophils that support tumor growth in a mouse model of hepatocellular carcinoma (HCC) [64]. CXCL6 induces neutrophil infiltration and increases tumor angiogenesis and melanoma growth, whereas CXCL1 and CXCL2 recruit neutrophils expressing S100SA8/9 that promote cancer cell survival and resistance to chemotherapy [62, 63]. As a consequence, the blockage of CXCR2 signaling results in inhibited infiltration of neutrophils into the tumor, retarded tumor growth and reduced angiogenesis, in both B16F10 and MCA205 tumor models [284], whereas in breast carcinoma model CXCR2 inhibition reduces the recruitment of neutrophils into the tumor and increases the efficacy of chemotherapy [62].

Although targeting of CXCR2 has a beneficial effect on tumor growth, opposing results were published on metastases. In renal cell carcinoma (RCC) CXCL5 and CXCL8 produced by tumor cells have been described to recruit antimetastatic neutrophils that inhibit the seeding of tumor cells [285].

Thus, it is still unclear if CXCR2 expression can be considered an N1 or N2 marker. CXCR2 expression in murine neutrophils is not modified by the treatment with the N1 polarizing cytokine IFN-β, whereas in human lung patients CXCR2 is down-regulated in TANs with N1 phenotype [264, 284].

Finally, it has been found that CXCR4 is down-regulated by IFN-β stimulation, and is overexpressed in a subpopulation of circulating neutrophils promoting angiogenesis and tumor progression, thus suggesting that CXCR4 is up-regulated in N2 neutrophils [261].



	N1	N2
Adhesion molecules and receptors	ICAM-1 ^{pos} CD62L ^{low} MET	ICAM-1 ^{neg} CD62L ?
Inflammatory mediators	TNF-α IL-12 ROS	VEGF-A MMP9 Arg-1
Chemokines	CCL3 CXCL8 CCL9	CCL2 CCL5
Chemokine receptors	CCR2 CCR5 CXCR2 ?	CXCR4 CXCR2

Figure 13: Neutrophil plasticity in cancer: N1 polarization is induced by IFN-β and inhibited by TGF-β, whereas TGF-β stimulation and IFN-β inhibition promote N2 polarization. N1 and N2 phenotype can be distinguished by the expression of distinct molecules and chemokine receptors, and by the production of different chemokines and inflammatory mediators [258].

Aim of the thesis

ACKR2 plays an essential role in the regulation of inflammatory chemokine concentration, and its activity has been demonstrated to be important to promote the appropriate leukocyte recruitment into inflamed tissue and to sustain the resolution of inflammatory response in several inflammatory conditions. The general objective of this PhD thesis was to gain insight into the role of ACKR2 in BM mobilization and effector functions of myeloid cells.

Thus, taking advantage from *Ackr2*-gene targeted mice, FACS analysis, RT-PCR, primary cell cultures and cell lines, the purpose of this investigation was to address the role ACKR2 in the regulation of neutrophil and monocyte mobilization, the contribution of ACKR2 in the regulation of HPC differentiation, the impact of ACKR2 expression on CC chemokine receptor expression and finally the relevance of the data obtained in a model of experimental metastasis in order to address the role of myeloid cells in this context.

Material and methods

1. Animals

Ackr2$^{-/-}$ mice have been generated as described and were maintained on a C57BL/6J genetic background [161]. WT mice were obtained from Charles River Laboratories, Calco, Italy. All colonies were housed and bred in the Specific Pathogen Free (SPF) animal facility of Humanitas Clinical and Research Center in individually ventilated cages. Mice used for experiment were from 8 to 12 weeks old. Procedures involving animal handling and care were conformed to protocols approved by the Humanitas Clinical and Research Center (Rozzano, Milan, Italy) in compliance with national (D.L. N.26, 4-3-2014) 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.

2. Chemokine administration

For mobilization experiments, mice were intraperitoneally injected with 3 μg of CCL3L1 (R&D) per mouse and one hour later, blood was collected for flow cytometry analysis.

To evaluate the frequency of monocytes in BM sinusoids, mice were injected with 3 μg of CCL3L1 (R&D), and 2 minutes before the end of experiment they were intravenously injected with 1 μg Ly6C-PE (clone AL21, BD Bioscience) antibody.

3. AMD3100 administration

AMD3100, the competitive inhibitor of CXCR4, was obtained from Sigma-Aldrich, Inc. (St. Louis, MO). Each mouse received a daily subcutaneous injection of AMD3100 (4 mg/kg) or PBS for the entire duration of the experiment.

4. Cell lines

HL-60 cells were purchased from ATCC and growth in Iscove's Modified Dulbecco's Medium (IMEM) (Lonza), 20% FBS (Sigma), 1% Penicillin/Streptomycin (Lonza), 1% L-glutamine (Lonza), 1% Sodium Pyruvate (Lonza). B16F10 cells expressing Melan A were kindly gift by Massimiliano Mazzone from VIB Vesalius Research Center (Leuven), and were growth in DMEM (Lonza), 10% FBS (Sigma), 1% Penicillin/Streptomycin (Lonza), 1% L-glutamine (Lonza), 1% HEPES (Lonza).

5. HL-60 transfection

HL-60 cells were transfected by using the Nucleofector Kits for HL60 (LONZA) according to the manufacturer's instructions.

6. Tissue collection

Blood was collected from the retro-orbital plexus and by cardiac puncture as previously described [286]. Briefly, blood was collected and transferred in 2KD-EDTA spray coated tube (BD Bioscience). Erythrocytes were lysed with ACK buffer. After washing in FACS buffer ($PBS^{-/-}$, 1% BSA, 0,05 % sodium azide), the cells were stained for flow cytometry analysis. BM cells were flashed out from the bone cavity of femurs and tibias of C57BL/6J WT and *Ackr2*$^{-/-}$ mice. Total BM cells were filtered through a 70 μm cell strainer. Erythrocytes were lysed with ACK buffer. After washing in FACS buffer, the cells were stained for flow cytometry analysis.

7. Generation of bone marrow chimeras

WT and *Ackr2*$^{-/-}$ mice were lethally irradiated with a total dose of 900 cGy. Then, 2 hours later, mice were injected in the retro-orbital plexus with $4x10^6$ nucleated BM cells obtained by flushing of the cavity of a freshly dissected femur from WT or *Ackr2*$^{-/-}$ donors. Recipient mice received gentamycin (0.8 mg/ml in drinking water) starting 10 days before irradiation and maintained during 2 weeks. Blood was analysed 15 weeks after BM transfer.

8. FACS analysis

The phenotypic analysis of cells was performed using the combination of following antibodies: anti-CD45-PerCP or –V450 (Clone 30-F11), anti-CD11b-PB or PE (clone M1/70), anti-Ly6G-PeCy7 or -FITC (clone 1A8), anti-Ly6C-FITC (clone AL-21), anti-F4/80-APC (clone CI:A3-1), anti-ICAM-1-APC (clone YN1/1.7.4), anti-CD62L-PE (clone MEL14), anti-Gr-1-APC (clone RB6-8C5), anti-CCR2 (gently gift by Matthias Mack) , anti-CXCR4-PE (clone 2B11), anti-Lin cocktail-eFluor 450 (clone 17A2, RA3-6B2, M1/70, TER-119, RB6-8C5), anti-Sca-1-PeCy7 (clone D7), anti-c-Kit-APCeFluor780 (clone 2B8), anti-CD34-FITC (clone RAM34), anti-FcγR-PerCP-Cy5.5 (clone 93), and isotype-matched control antibodies. Antibody anti-CCR2 was not fluorochrome-labelled, thus for CCR2 staining, after incubation with the rat anti-mouse CCR2, the cells were incubated with a monoclonal biotinylated mouse anti-rat IgG2b and finally with Steptavidin-PE or PB. All antibodies were purchased from BD Bioscience, BioLegend, eBioscience or AbD Serotec. Cell viability was determined by Aqua LIVE/Dead-405nm staining (Invitrogen), negative cells were considered viable. The absolute number was determined by using TruCount beads (BD Biosciences) according to the manufacturer's instructions. Flow cytometry was performed by using FACSCanto II (BD Bioscience) and LSR Fortessa (BD Bioscience). Data were analyzed with FACSDiva software (BD Bioscience) and FloJo Software (Tree Star).

9. Hematopoietic progenitor isolation and culture

Lineage negative cells (Lin^-) were isolated from BM WT and *Ackr2*$^{-/-}$ using Lineage Cell Depletion Kit, mouse (Miltenyi Biotec), and the LS columns from Miltenyi Biotec according to manufacturer's instructions. Negative fraction of cell preparation was stained with anti-Streptavidin-PB, anti-Sca-1, anti-c-kit, anti-CD34 and anti-FcγR antibodies and sorted with FACSAria III (BD Bioscience). Antibody anti-Streptavidin was used to further discriminate Lin^+ cells that were not accurately depleted by the Lineage Cell Depletion Kit. After sorting, LSK, identified as Lin^-, $Sca\text{-}1^+$, $cKit^+$, were seeded in round bottom 96 wells plate ($1x10^4$ cells/well) in IMDM (Lonza) supplemented with 10% FCS (Sigma), 1% Glutamine (Lonza), SCF 10ng/ml (Peprotech), IL-6 10 ng/ml (Peprotech), IL-3 10 ng/ml (Peprotech).

10. Evaluation of lung metastases

Lung metastases were evaluated 10 days after intravenous injection of $2x10^5$ B16-F10 cells, and they were macroscopically counted as dark nodules on the lung surface.

11. Monocyte and neutrophil depletion

To inhibit monocyte differentiation, mice were treated the day before the injection of B16F10 with 100 μg of αCD115 antibody (clone AFS98, Bioxcell) and then, every two days with 100 μg for the entire duration of the experiment. To deplete neutrophil, mice were treated the day before the injection of B16F10 with 200 μg of αLy6G antibody (clone 1A8, Bioxcell) and then, every three days with 100 μg for the entire duration of the experiment.

12. Neutrophil isolation

Neutrophils were isolated from BM WT and *Ackr2*$^{-/-}$ using the Neutrophil Isolation Kit, mouse (Miltenyi Biotec) and the autoMACS Pro separator (Miltenyi Biotec), according to manufacturer's instructions. The purity of neutrophil cell preparation was evaluated by flow cytometry using antibodies anti-CD45, anti-CD11b and anti-Ly6G. Neutrophils were used only when the frequency of contaminating leukocytes in neutrophil preparations was <6% for neutrophil adoptive transfer, and <3% for RT-PCR analysis.

13. Neutrophil adoptive transfer

Neutrophils were isolated from BM WT and *Ackr2*$^{-/-}$. WT mice were intravenously injected with $5x10^6$ neutrophils of WT or *Ackr2*$^{-/-}$ mice every 3 days for the entire duration of the experiment.

14. Quantitative PCR

Total RNA was extracted from HPCs with miRNA easy Mini kit (Qiagen), and from HL-60 cells, neutrophils and BM total cells by using Trizol reagent (Invitrogen) following the manufacturer's recommendations. Reverse transcription was done using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative real-time PCR (RT-

PCR) was performed with TaqMan Gene Expression Assays using TaqMan fast advanced Master Mix, or with SYBR Green method using SybrGreen PCR Master Mix (Applied Biosystems), and was conducted in a CFX Connect Real-Time PCR Detection System (BioRad). For TaqMan Gene Expression Assays (Thermo Fisher) specific probes for murine ACKR2 (Mm_00445551_m1), murine CCR2 (Mm_00438270_m1), murine CCR1 (Mm01216147_m1), murine CCR5 (Mm04207879_m1), murine CXCR4 (Mm_01292123_m1), murine β-Actin (Mm_00607939_s1), murine GAPDH (Mm_99999915_g1), murine VEGFA (Mm00437306_m1), murine TNFα (Mm00443258_m1), murine Alox5 (Mm01182747_m1), murine Arg1 (Mm_00475988_m1), human ACKR2 (Hs_00174299_m1), human CCR2 (Hs00704702_s1), human CXCR4 (Hs_00607978_s1), human CD11b (Hs00355885_m1), and human GAPDH (Hs_99999905_m1) were used. For SYBR Green Assay, primers for STAT3, CEBPβ, PU.1, IRF8, Bcl3, Socs3, and RORC1 mRNA were gently given by Antonio Sica and are available upon request [241]. Relative mRNA expression was determined by using the Δ^2Ct method (Applied Biosystems, Real-Time PCR Applications Guide). Data were normalized based on the GAPDH or β-Actin expression determined in the same sample.

15. Statistics

All values were expressed as mean ± SEM. Unpaired Student's t test, was used as specified. $P \leq 0.05$ was considered significant. Statistics were calculated with GraphPad Prism version 6, GraphPad Software.

Results

1. Ackr2$^{-/-}$ mice display increased mobilization of myeloid cells

Given the relevance of the chemokine system in controlling leukocyte trafficking and BM mobilization, we addressed the effect of ACKR2 chemokine scavenging in a mouse model of BM mobilization. To this purpose, we used CCL3L1, which is an inflammatory chemokine, binding ACKR2 and other CC chemokine receptors, and known to induce a rapid mobilization of both neutrophils and monocytes [287]. We intraperitoneally injected CCL3L1 into WT and *Ackr2*$^{-/-}$ mice and, one hour after chemokine injection, blood and BM were collected. As expected, CCL3L1 injection led to an increasing of neutrophils (Figure A) and monocytes (Figure B) into the blood of WT and *Ackr2*$^{-/-}$ mice. However, the absolute number of these cells was higher in *Ackr2*$^{-/-}$ mice compared to WT. Moreover, when we evaluated the frequency of neutrophils and monocytes into the BM of WT and *Ackr2*$^{-/-}$ animals, we observed that CCL3L1 injection caused a decrease in the frequency of these cells in the BM, and this reduction was more prominent in *Ackr2*$^{-/-}$ mice (Figures 1C and 1D). These data indicate that *Ackr2*$^{-/-}$ mice show increased CCL3L1-induced mobilization of myeloid cells from the BM.

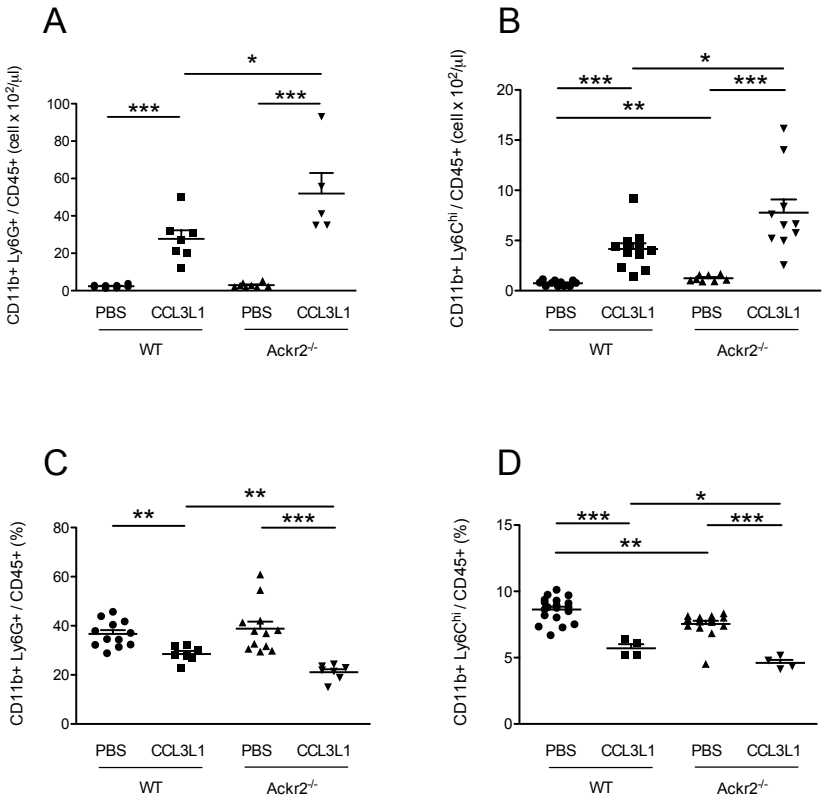


Figure 1: Increased monocyte and neutrophil chemokine-induced mobilization in *Ackr2*$^{-/-}$ mice. A, B: Absolute number of circulating neutrophils (A) and inflammatory monocytes (B) in the bloodstream of WT and *Ackr2*$^{-/-}$ mice. C, D: Frequency of BM neutrophils (C) and inflammatory monocyte (D) in basal conditions and 1 hour after i.p. injection of CCL3L1. Neutrophils were identified as CD45^{+}CD11b^{+}Ly6G^{+}, whereas inflammatory monocytes as CD45^{+}CD11b^{+}Ly6C^{high}. Data were represented as (mean ± SEM). *: p<0.05, **: p<0.01, ***: p<0.001.

2. Ackr2$^{-/-}$ mice have increased number of monocytes in BM sinusoids

Then we evaluated whether ACKR2 was affecting the number of cells in the BM sinusoids, which represent the BM vascular niche. Differently from quiescent HSCs, which are located in the BM osteblastic niche, actively proliferating and differentiating HPCs, and mature cells immediately release into the blood are located in the BM sinusoids. To evaluate the number of cells in this compartment, we intraperitoneally injected CCL3L1 into WT and *Ackr2*$^{-/-}$ mice, and 2 minutes before the end of experiment, we intravenously injected an anti-Ly6C labelled antibody. Being anti-Ly6C injected intravenously, when we harvested BM, this technic allowed us to identified only Ly6C^{+} cells located in BM vascular compartment [288]. By analyzing the frequency of Ly6C^{+} cells in the BM, we saw that it was higher in *Ackr2*$^{-/-}$ mice compared to WT, thus indicating that *Ackr2*$^{-/-}$ mice had more cells located in the BM sinusoids (Figures 2A and 2B). These results suggest that the increased mobilization observed in *Ackr2*$^{-/-}$ mice could be due to a bigger pool of cells ready to be release in the BM vascular niche.

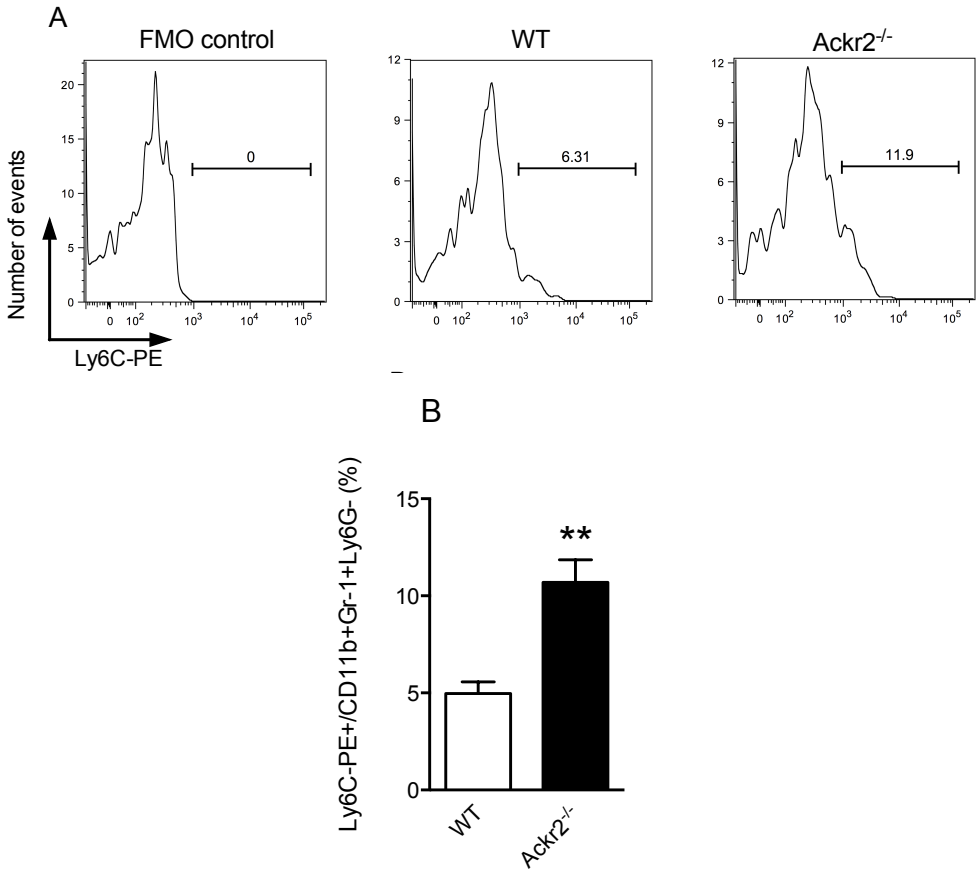


Figure 2: Increased number of monocytes in the BM vascular niche of *Ackr2*$^{-/-}$ mice upon CCL3L1 injection. A, B: Representative histogram of FACS analysis (A) and frequency (B) of Ly6C labelled monocytes of WT and *Ackr2*$^{-/-}$ mice 1 hour after i.p. injection of CCL3L1. Ly6C antibody was injected i.v. 2 minutes before the end of the experiment. Monocytes were identified as $CD45^{+}CD11b^{+}Gr1^{+}Ly6G^{-}$. Data were represented as (mean ± SEM). **: $p<0.01$, *Ackr2*$^{-/-}$ versus WT.

3. ACKR2 expression in the hematopoietic compartment affects myeloid cell mobilization from BM

To understand whether the increased mobilization observed in *Ackr2*$^{-/-}$ mice was due to the lack of ACKR2 in hematopoietic cells or BM stromal cells, we performed BM chimera experiments by transplanting WT or *Ackr2*$^{-/-}$ BM cells into WT and *Ackr2*$^{-/-}$ hosts. To ensure the appropriate BM reconstitution, we performed the BM mobilization experiment 15 weeks after BM transfer, and we saw that both WT and *Ackr2*$^{-/-}$ animals, transplanted with *Ackr2*$^{-/-}$, but not with WT hematopoietic cells, had an increased mobilization of myeloid cells in response to CCL3L1, as indicated by the increased number of circulating neutrophils (Figure 3A) and monocytes (Figure 3B) into the blood of those mice. These results indicate that the increased chemokine-induced mobilization of *Ackr2*$^{-/-}$ mice is due to the absence of ACKR2 in the hematopoietic compartment.

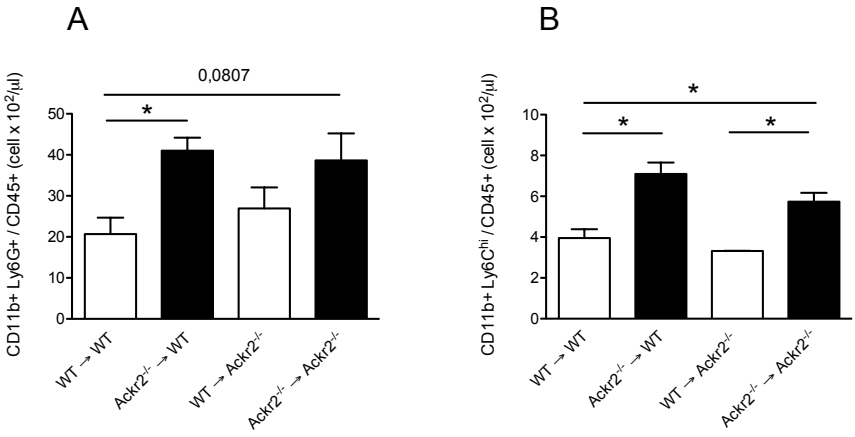


Figure 3: Increased myeloid cell mobilization in mice transplanted with BM *Ackr2*$^{-/-}$ cells. A, B: Absolut number of neutrophils (A) and inflammatory monocytes (B) in the bloodstream of WT and *Ackr2*$^{-/-}$ mice reconstituted either with WT or *Ackr2*$^{-/-}$ BM after i.p. injection of CCL3L1. Neutrophils were identified as $CD45^+CD11b^+Ly6G^+$, whereas inflammatory monocytes as $CD45^+CD11b^+Ly6C^{high}$. Data were represented as (mean ± SEM). *: $p<0.05$.

4. ACKR2 is expressed by hematopoietic progenitors

Since BM chimera experiments demonstrated that the absence of ACKR2 in cells of the hematopoietic compartment was responsible for the increased myeloid mobilization observed in *Ackr2*$^{-/-}$ mice, we evaluated ACKR2 expression in hematopoietic cells in the BM by RT-PCR analysis. In particular, we analyzed the expression of ACKR2 in sorted hematopoietic precursors of the myeloid lineage identified as shown in Figure 4A. We found that ACKR2 was expressed by Lin^{-}, Sca-1^{+}, cKit^{+} (LSK) cells and its expression was down-regulated in the more mature CMP (Lin^{-}, Sca-1^{-}, cKit^{+}, CD34^{+}, FcγR^{+}) and GMP (Lin^{-}, Sca-1^{-}, cKit^{+}, CD34^{+}, FcγR^{high}) (Figure 4B). We also analyzed the expression of CCR2 in these populations and we confirmed data already reported in literature, indeed CCR2 was weakly expressed by LSK and its expression increased during maturation through myeloid lineage [211] (Figure 4C). Thus, ACKR2 is expressed in most immature progenitors to faint thereafter in more mature MPCs in contrast with the canonical chemokine receptor CCR2, whose expression is acquired during maturation.

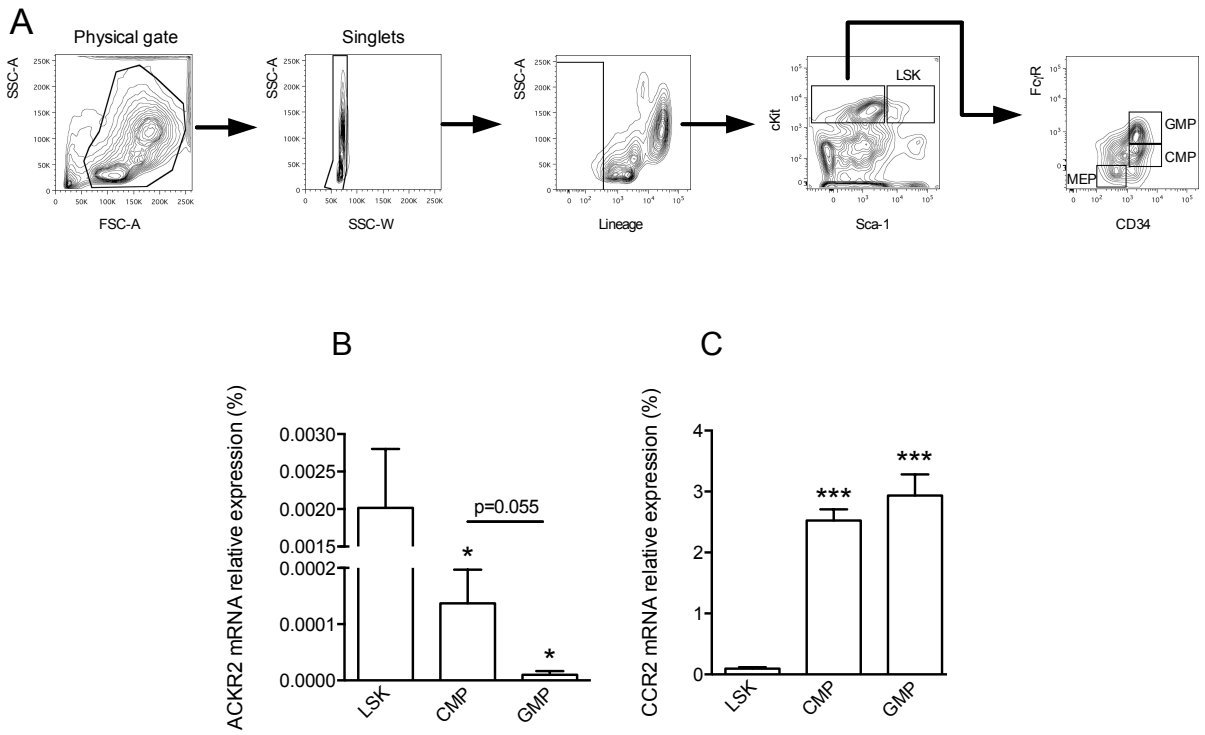


Figure 4: Expression of ACKR2 and CCR2 in hematopoietic progenitors. A: Gating strategy used to identify LSK, CMP, and GMP. B, C: Analysis of the expression of ACKR2 (B) and CCR2 (C) by RT- PCR in LSK, CMP and GMP cells sorted from BM of WT mice. Data were relative to β-actin expression and represented as mean ± SEM. *: $p<0.05$, ***: $p<0.001$, unpaired Student's t test, CMP and GMP versus LSK.

5. *CC-Chemokine receptor expression is up-regulated in Ackr2$^{-/-}$ hematopoietic progenitors*

The analysis of HPCs revealed that there was an inverse correlation between the expression of ACKR2 and CCR2 on those cells. Thus, we investigated the expression of CCR2 in *Ackr2*$^{-/-}$ HPCs. RT-PCR analysis showed that LSK, CMP and GMP isolated from *Ackr2*$^{-/-}$ mice expressed higher levels of CCR2 compared to the same cells isolated from WT mice (Figure 5A). FACS analysis confirmed an increased expression of CCR2 in *Ackr2*$^{-/-}$ LSK, CMP and GMP cells and revealed that this up-regulation was restricted to monocyte and neutrophil progenitors, indeed there was no difference in the expression of CCR2 between WT and *Ackr2*$^{-/-}$ erythrocyte progenitors (Lin^{neg}, Sca-1^{-}, cKit^{+}, CD34^{-}, FcγR^{-}, MEP) (Figure 5B). Furthermore, we evaluated the expression of CCR1 and CCR5 in HPCs by RT-PCR analysis and, in line with the data observed for CCR2, LSK, CMP and GMP isolated from *Ackr2*$^{-/-}$ mice expressed higher levels of CCR1 and CCR5 compared to WT (Figures 5C and 5D). These results indicate that ACKR2 in HPCs controls the expression of inflammatory chemokine receptors known to be involved in myeloid cell release from BM.

Moreover, given the relevance of CXCR4 in controlling cell mobilization from BM, we evaluated CXCR4 expression, and we did not find differences in mRNA and protein levels of CXCR4 between WT and *Ackr2*$^{-/-}$ HPCs (Figures 5E and 5F). Thus, suggesting that the increased mobilization seen in *Ackr2*$^{-/-}$ mice is not due to CXCR4 down-regulation in HPCs.

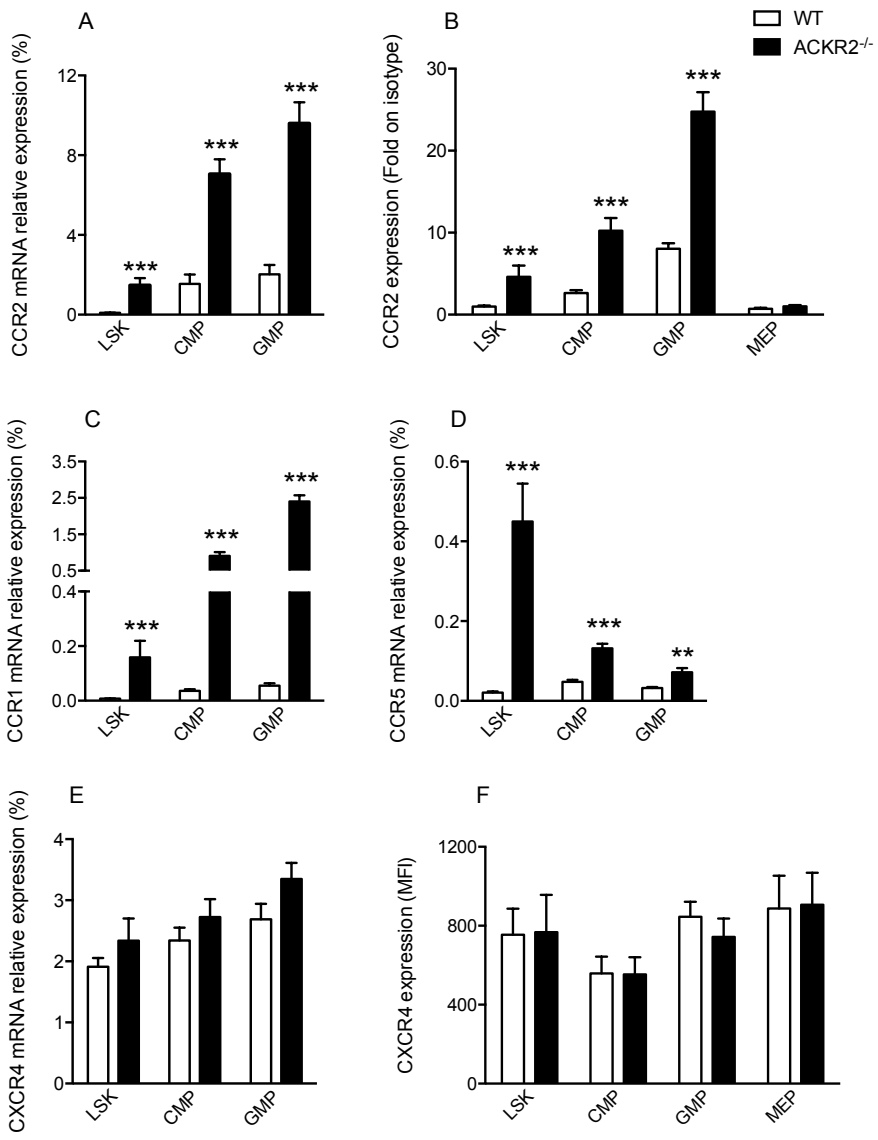


Figure 5: Increased expression of CC chemokine receptors in hematopoietic progenitors of *Ackr2*$^{-/-}$ mice. A: Analysis of CCR2 expression by RT-PCR in LSK, CMP, and GMP cells sorted from BM of WT and *Ackr2*$^{-/-}$ mice. B: Analysis of CCR2 expression by FACS on LSK, CMP, GMP, and MEP of WT and *Ackr2*$^{-/-}$ mice. C, D, E: Analysis of CCR1 (C), CCR5 (D), and CXCR4 (E) expression by RT-PCR in LSK, CMP, and GMP cells sorted from BM of WT and *Ackr2*$^{-/-}$ mice. F: Analysis of CXCR4 expression by FACS on LSK, CMP, GMP, and MEP (F) of WT and *Ackr2*$^{-/-}$ mice. Data were relative to β-actin expression (A, C, D, E), expressed as mean fluorescence intensity (MFI) ratio on isotype (B), or as MFI (F). Results were represented as mean ± SEM. **: $p<0.01$, ***: $p<0.001$, unpaired Student's t test, *Ackr2*$^{-/-}$ versus WT.

6. *Ackr2*$^{-/-}$ *LSK cells display increased differentiation rate*

Since the expression of chemokine receptors is regulated during maturation, we investigated whether the increased expression of CC chemokine receptors in *Ackr2*$^{-/-}$ HPCs was associated with altered maturation of LSK. To this aim we sorted LSK cells from BM WT and *Ackr2*$^{-/-}$, we cultured them and we analyzed the expression of differentiation markers after 3 and 6 days of culture. We found that *Ackr2*$^{-/-}$ LSK cells cultured in vitro had increased expression of the myeloid differentiation markers CD11b and Ly6C after 3 days of culture (Figures 6A and 6C), and increased expression of CD11b, Ly6G, and Ly6C (Figures 6A, 6B and 6C) after 6 days of culture, as compared to WT. These data confirm that the receptors CCR1, CCR2 and CCR5 do not mediate any myelosuppressive effect, whereas the absence of ACKR2 results in increased differentiation of LSK cells.

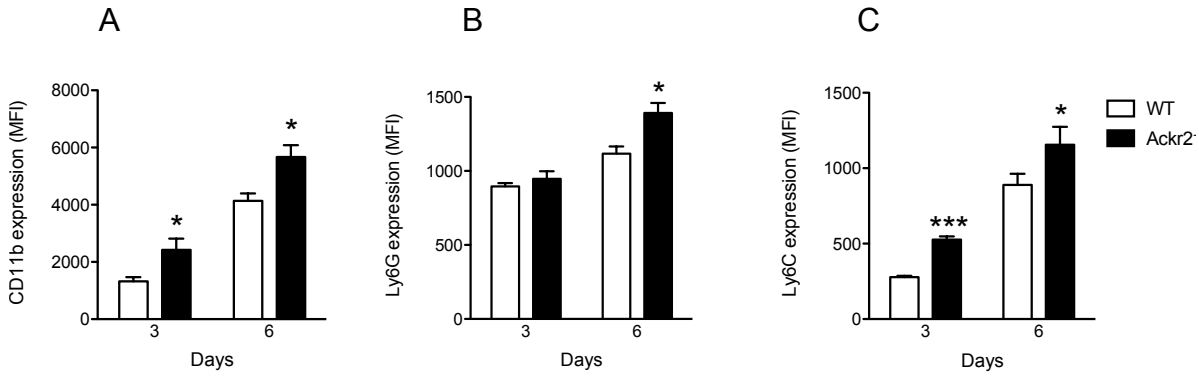


Figure 6: Increased differentiation rate of *Ackr2*$^{-/-}$ LSK cells. A, B, C: FACS analysis of CD11b (A), Ly6G (B), and Ly6C (C) expression on LSK sorted from BM of WT and *Ackr2*$^{-/-}$ mice after 3 and 6 days of culture. Data were expressed as mean fluorescence intensity (MFI), and represented as mean ± SEM. *: $p<0.05$, ***: $p<0.001$, unpaired Student's t test, *Ackr2*$^{-/-}$ versus WT.

7. ACKR2 overexpression in human promyelocytic leukemia cell line (HL-60) reduces CCR2 and CD11b expression

In a second set of experiment, we took advantage of an in vitro system to assess the contribution of ACKR2 in regulating the maturation of HL-60. The latter is a promyelocytic cell; it expresses CCR2 and to lesser extent also ACKR2 (Figure 7A). Thus, in order to study ACKR2, we transfected HL-60, with either mock vector (Mock) or ACKR2 expressing vector (hACKR2). PCR analysis at 24 hours after cell transfection confirmed the expression of ACKR2 in transfected cells (Figure 7B). RT-PCR analysis revealed a significantly reduction of CCR2 and CD11b expression levels in HL-60 overexpressing ACKR2 (Figure 7C). The expression level of CXCR4 was not affected (Figure 7C). Together, these results suggest that ACKR2 controls the expression of CCR2 and the maturation of HPCs.

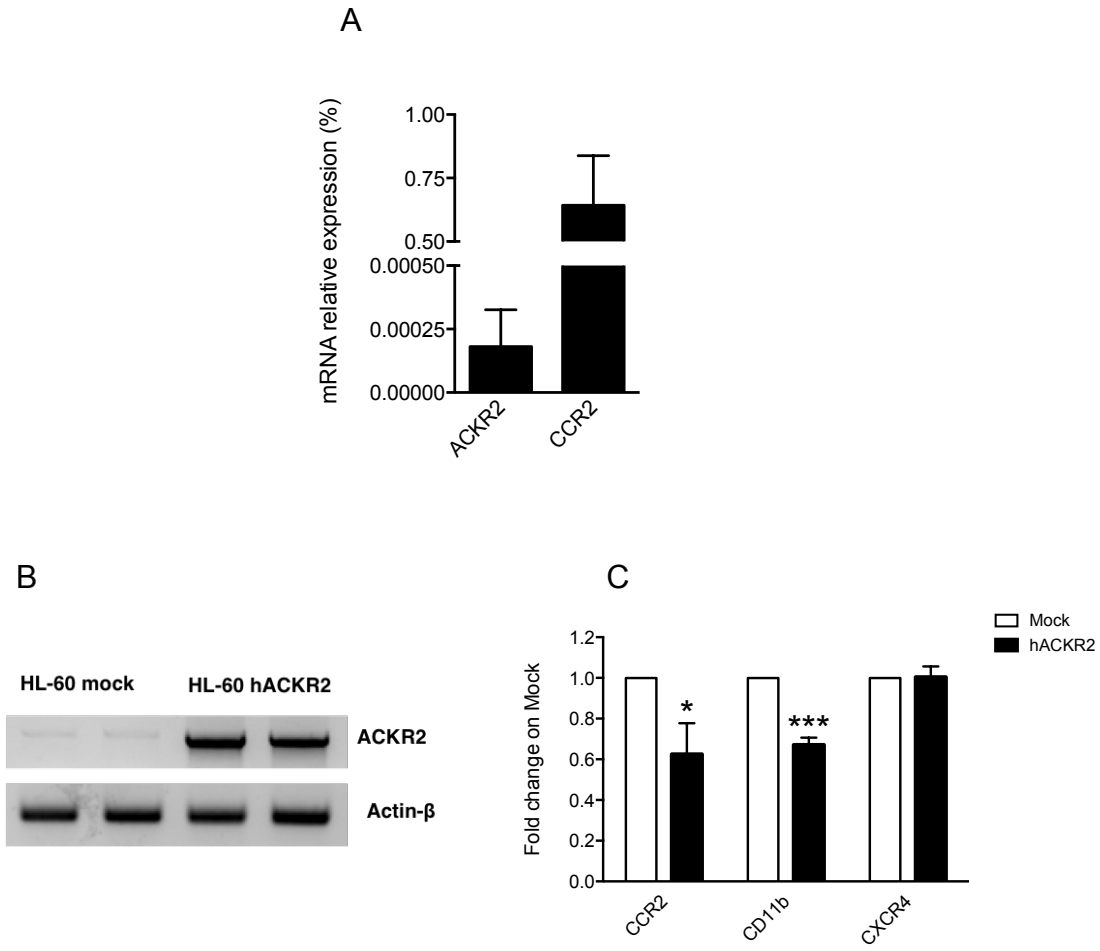


Figure 7: ACKR2 over-expression results in down-regulation of CCR2 and CD11b in HL-60 cells. A: Analysis of ACKR2 and CCR2 expression by RT-PCR in HL-60 cells. B: Analysis of ACKR2 expression in HL-60 transfected cells by PCR. C: Analysis of CCR2, CD11b, and CXCR4 expression by RT-PCR in HL-60 cells transfected either with mock or ACKR2 expressing vector. Data were relative to GAPDH expression, and expressed as percentage (A) or fold on mock (C). Results were represented as mean ± SEM. *: $p<0.05$, ***: $p<0.001$, unpaired Student's t test, hACKR2 vs Mock.

8. *Ackr2$^{-/-}$ and WT BM cells do not differ in the expression of transcriptional regulators of myelopoiesis*

Next, we assessed whether the change in chemokine receptor expression and the maturation seen in *Ackr2$^{-/-}$* HPCs was due to the alteration of transcription factors regulating myelopoiesis. To answer this question we analyzed the transcript levels of STAT3 that induces granulopoiesis, CEBPβ and RORC1, which regulate emergency granulopoiesis, PU.1 and IRF8, which support monocyte differentiation, and finally Bcl3 and Socs3 that negatively regulate myelopoieisis. As shown in Figure 8A, neither factor which positively or negatively regulates myelopoiesis was differentially expressed between WT and *Ackr2$^{-/-}$* BM total cells, thus suggesting that none of those factors was responsible of enhanced LSK maturation in *Ackr2$^{-/-}$* mice.

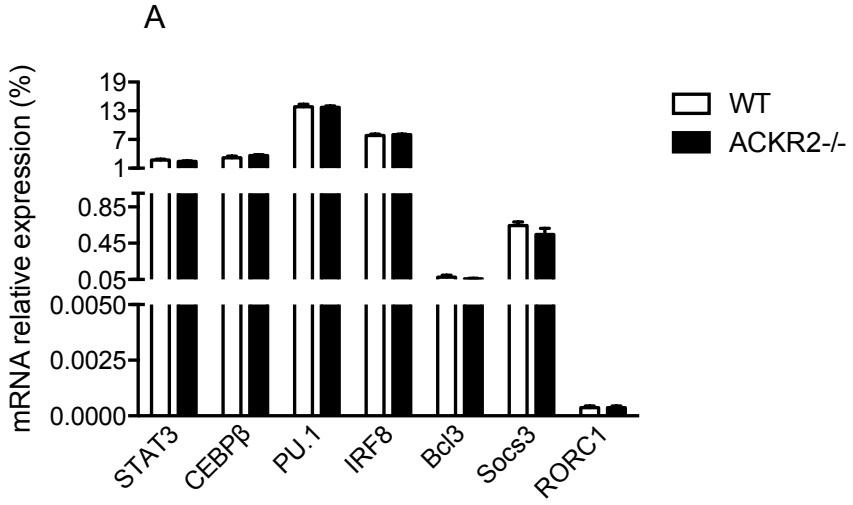


Figure 8: Expression of transcriptional regulators of myelopoiesis. A: Analysis of STAT3, CEBPβ, PU.1, IRF8, Bcl3, Socs3, and RORC1 expression by RT-PCR. Data were relative to GAPDH expression, and represented as mean ± SEM.

9. Neutrophil mobilization is required for metastases protection in Ackr2$^{-/-}$ mice

Given the importance of myeloid cell development in cancer, we wanted to delineate the effect of increased CC chemokine receptor expression and maturation observed in *Ackr2*$^{-/-}$ LSK cells, in tumor challenged mice. To address this point we used an experimental model of lung metastasis by injecting B16F10 melanoma cells intravenously through the tail vein. As shown in Figure 9A, *Ackr2*$^{-/-}$ mice displayed a significant reduction in the number of metastatic foci, compared to WT.

Next, we sought to understand whether myeloid cells were involved in *Ackr2*$^{-/-}$ mice protection from metastases. To this purpose, we evaluated the effect of monocyte or neutrophil depletion in tumor bearing mice. Thus, we injected B16F10 melanoma cells intravenously into the tail vein of WT and *Ackr2*$^{-/-}$ mice, and then we treated animals with an anti-CD115 monoclonal antibody to inhibit monocytes differentiation into macrophage or with an anti-Ly6G monoclonal antibody to deplete neutrophils. Although the inhibition of monocyte differentiation caused a significant reduction in the number of metastases in WT mice, it did not reverse the protection found in *Ackr2*$^{-/-}$ mice, whereas neutrophil depletion caused a reduction of metastases in WT mice and an increase in *Ackr2*$^{-/-}$ mice, which developed a number of lesions similar to untreated WT animals (Figure 9B).

To further confirm that neutrophils were responsible for metastatic protection observed in *Ackr2*$^{-/-}$ mice, we performed an adoptive transfer experiments. We isolated neutrophils from WT and *Ackr2*$^{-/-}$ mice and 4 hours after intravenously injection of B16F10 melanoma cells into WT mice, we transferred WT or *Ackr2*$^{-/-}$ neutrophils. We found that *Ackr2*$^{-/-}$ neutrophils significantly reduced the number of lung metastases as compared to those animals injected with WT neutrophils (Figures 9C and 9D).

Taken together, these results suggest that *Ackr2* deficient neutrophils play a role in inhibiting the seeding of cancer cells into the lung, thus protecting *Ackr2*$^{-/-}$ mice from metastases.

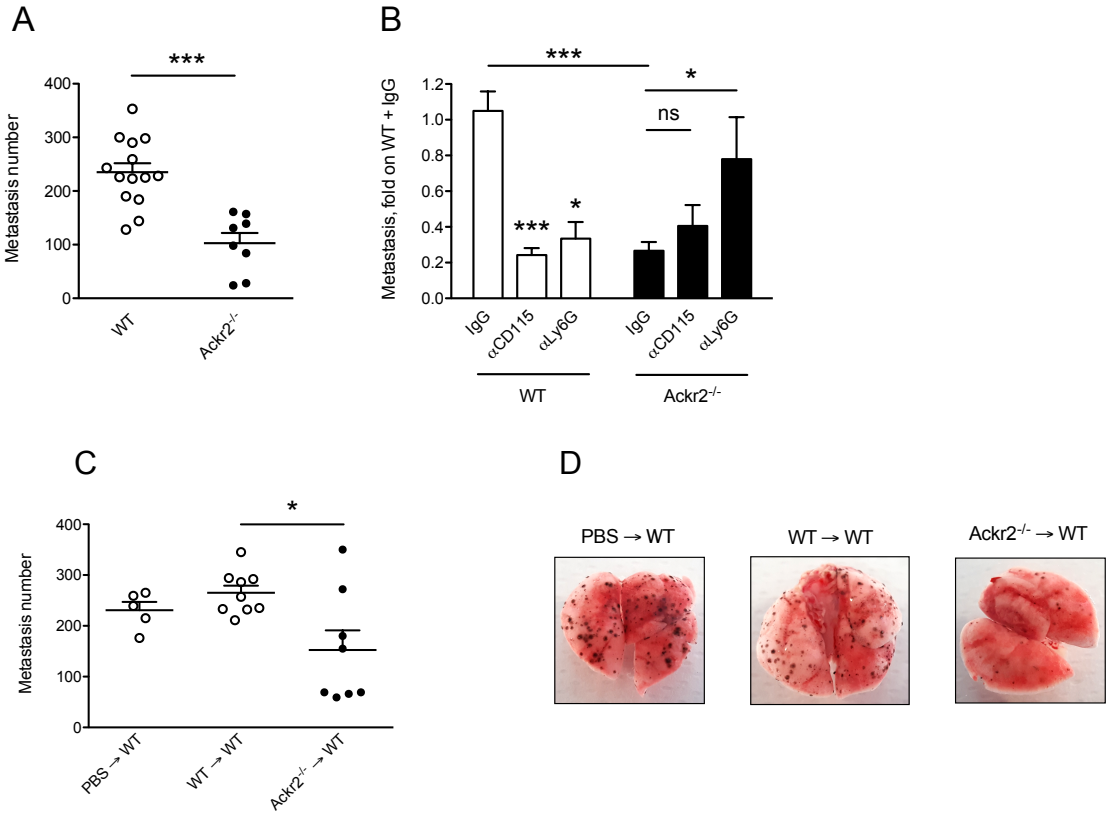


Figure 9: *Ackr2*$^{-/-}$ neutrophils are required for metastases protection. A: Number of lung metastases in WT and *Ackr2*$^{-/-}$ mice 10 days after i.v. injection of 2X10^5 B16-F10 cells. B: Number of lung metastases in WT and *Ackr2*$^{-/-}$ mice depleted for monocytes (αCD115) and neutrophils (αLy6G) 10 days after i.v. injection of 2X10^5 B16-F10 cells. Number of metastases was expressed as the ratio between the number of metastases of WT and *Ackr2*$^{-/-}$ mice treated with αCD115 or anti Ly6G and the number of metastases of WT and *Ackr2*$^{-/-}$ mice treated with IgG antibody. C: Number of lung metastases in WT mice 10 days after i.v. injection of 2X10^5 B16-F10 cells and adoptive transfer of neutrophil isolated from BM WT or *Ackr2*$^{-/-}$. D: Representative image of lungs 10 days after i.v. injection of 2X10^5 B16-F10 cells. Data were represented as (mean ± SEM). *: $p<0.05$.

10. Ackr2$^{-/-}$ neutrophils have an activated phenotype

The decrease in the number of metastases in WT mice upon anti-CD115 and anti-Ly6G treatment was in agreement with the pro-tumoral role that these cells may have in tumor [289]. Hence, considering that neutrophils in cancer can be differently polarized, we next sought to determinate whether the opposite effect induced by the anti-Ly6G treatment in WT and *Ackr2*$^{-/-}$ animals, was due to a different phenotype of *Ackr2*$^{-/-}$ neutrophils. To this aim, we evaluated the activation state of neutrophils into the blood of WT and *Ackr2*$^{-/-}$ tumor-bearing mice. As shown in Figures 10A and 10B, neutrophils from *Ackr2*$^{-/-}$ animals expressed lower levels of CD62L (Figure 10A) and higher levels of ICAM-1 (Figure 10B), coherent with a more activated and mature phenotype. RT-PCR analysis revealed that genes, which are known to be differentially regulated in activated neutrophils, including TNFα, Alox5, VEGF-A, and Arg1, were equally expressed in BM neutrophils isolated from WT and *Ackr2*$^{-/-}$ mice (Figure 10C). Conversely, when we analyzed chemokine receptors expression, we found that CC chemokine receptors CCR1, CCR2 and CCR5 were higher expressed in *Ackr2*$^{-/-}$ neutrophils, compared to WT, whereas no difference in the expression level of CXCR4 was observed (Figure 10D). To understand if ACKR2 was also controlling the expression of CC chemokine receptors in mature neutrophils, we sought whether WT neutrophils expressed ACKR2. We found that ACKR2 was very weakly expressed on WT neutrophils, and its level was much lower compared to LSK (Figure 10E). Taken together, these results suggest that ACKR2 deficiency in hematopoietic precursors, in addition to accelerate the maturation of myeloid cells, results in an increased expression of inflammatory chemokine receptors in mature neutrophils, which are more efficiently released from the BM and also exert an anti-metastatic function.

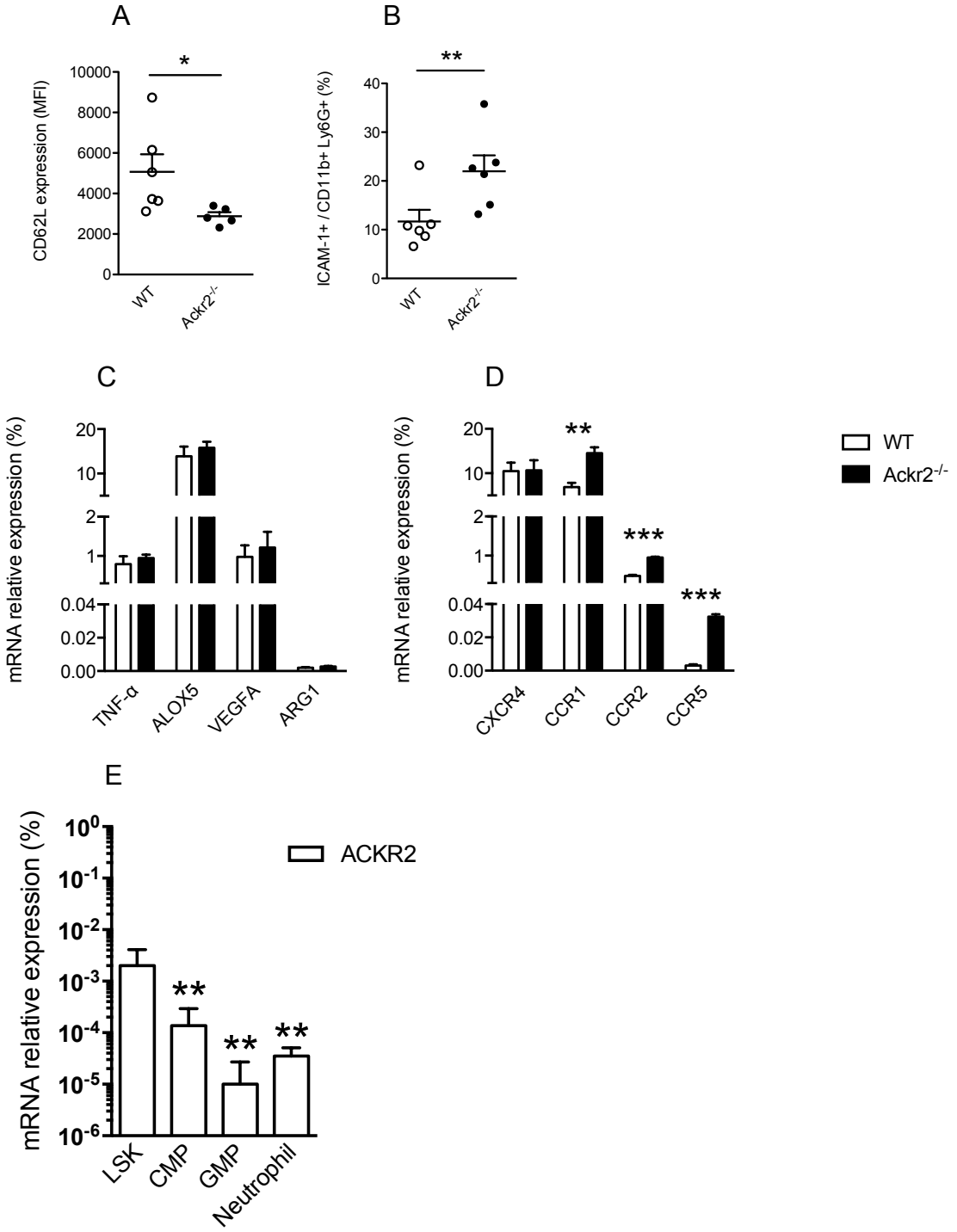


Figure 10: *Ackr2*$^{-/-}$ neutrophils display activated phenotype and increased expression of CC chemokine receptors. A, B: FACS analysis of CD62L expression on WT and *Ackr2*$^{-/-}$ neutrophils identified as CD45^{+} CD11b^{+} Ly6G^{+} (A), and frequency of ICAM^{+} neutrophils (B). C, D: Analysis of TNFα, Alox5, VEGF-A, and Arg1 (C), and CXCR4, CCR1, CCR2 and CCR5 (D) by RT-PCR. E: Analysis of ACKR2 expression on LSK, CMP, GMP and neutrophils isolated from BM WT. Data were relative to GAPDH (C, D), or β-actin (E) expression. Results were represented as mean ± SEM. *: $p<0.05$, ***: $p<0.001$, unpaired Student's t test, Ackr2$^{-/-}$ versus WT (A, B, C, D) and CMP, GMP and neutrophils versus LSK (E).

11. AMD3100-treatment reduces the number of metastases in WT mice but does not affect metastases in Ackr2$^{-/-}$ mice

Finally, we sought to determine whether increasing neutrophil mobilization would have resulted in improved metastatic protection in *Ackr2*$^{-/-}$ tumor bearing mice. To this aim we used AMD3100, the competitive inhibitor of CXCR4. AMD3100 treatment has been demonstrated to rapidly revert neutropenia in human patients; it acts by inhibiting the chemokine receptor CXCR4 thus disrupting the CXCL12/CXCR4 axis and promoting BM mobilization. We injected B16F10 melanoma cells intravenously through the tail vein of WT and *Ackr2*$^{-/-}$ mice and we treated animals with AMD3100. The treatment induced a significant reduction in the number of metastatic foci in WT animals compared to untreated WT mice, however *Ackr2*$^{-/-}$ mice developed a number of metastases similar to those observed in WT animals treated with AMD3100 (Figures 11A and 11B). Being CXCR4 highly expressed by tumor cells it is difficult to delineate its role in TAN biology. Moreover, CXCR4 expression by neutrophils has a pro-tumoral effect being CXCR4 positive neutrophils pro-angiogenetic, as consequence CXCR4 inhibition may results in a reduction of neutrophil pro-tumoral activity both in WT and *Ackr2*$^{-/-}$ mice. However, these preliminary results suggest that CXCR4 inhibition can reduce metastatic seeding in WT mice, but is not able to enhance the metastatic protection in *Ackr2*$^{-/-}$ mice.

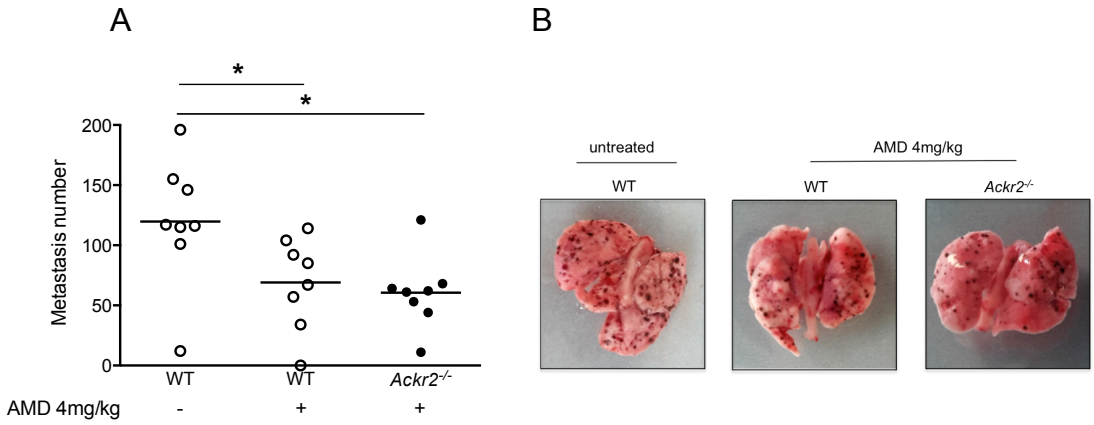


Figure 11: CXCR4 inhibition reduces the number of metastases in WT mice. A, B: Number of lung metastases (A) in WT mice either treated or untreated with AMD3100, and *Ackr2*$^{-/-}$ mice treated with AMD3100 10 days after i.v. injection of 2X10^5 B16-F10 cells. Representative image of lungs (B) of WT mice treated or untreated with AMD3100, and *Ackr2*$^{-/-}$ treated with AMD3100 10 days after from i.v. injection of 2X10^5 B16-F10 cells. Data were represented as (mean ± SEM). *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$.

Discussion

Chemokines and chemokine receptors are known to play a major role in the regulation of leukocyte recruitment in both homeostatic and inflammatory conditions, including cancer. Dampening the signal of almost all inflammatory CC chemokines and limiting their bioavailability in tissues, the atypical chemokine receptor ACKR2 represents an important mechanism of regulation of the chemokine system.

Experimental approaches have unequivocally proven the regulatory function of ACKR2 in different model of inflammation, including infection, allergy, and cancer. The exacerbated inflammatory reactions observed in *Ackr2*-deficient mice have been attributed to the ability of ACKR2 to restrain the local inflammatory response, thus promoting the appropriate leukocyte recruitment and the successful migration of DCs to draining LNs. Moreover, by inhibiting the recruitment of inflammatory cells, ACKR2 limits Kaposi's sarcoma growth and aggressiveness, controls TPA/DMBA and AOM/DSS-induced carcinogenesis, and in particular the inflammatory processes associated with tumor development.

The general aim of this PhD thesis was to understand the role of ACKR2 in the context of myeloid cell BM mobilization and HPC differentiation, and to address the role of ACKR2 in controlling myeloid cell effector functions.

Most of CC chemokines, including CCL3, the ligand of ACKR2 have been identified as myelosuppressive and myeloprotective chemokines. Though none of the chemokine receptors has been already demonstrated to mediate this myelosuppressive effect, we found that *Ackr2* deficiency in the hematopoietic compartment led to increased differentiation of HPCs, and enhanced monocyte and neutrophil mobilization in response to CCL3L1 injection. Accordingly to the inhibitory activity of ACKR2 on HPC differentiation, ACKR2 was expressed in LSK cells and its expression was down-regulated in more differentiated CMP and GMP cells, in opposite way to the canonical chemokine receptor CCR2. Interestingly, the increased differentiation of *Ackr2* deficient HPCs was associated to a higher expression of CC chemokine receptors CCR1, CCR2, and CCR5, which are important to sustain myeloid cell mobilization from BM, in those cells.

The role of myeloid cells in cancer is still debated and controversial since they have been shown to play both pro-tumoral and anti-tumoral activity [258, 259, 290, 291]. However, taking advantage from a model of experimental metastasis we found that *Ackr2*$^{-/-}$ mice were protected from the development of lung metastases, and that the protection was

mediated by neutrophils. Indeed, depletion of neutrophils but not of monocytes reversed the protective phenotype observed in *Ackr2*$^{-/-}$ mice. Furthermore, the adoptive transfer of *Ackr2*-deficient but not WT neutrophils clearly reduced metastases in WT mice.

Neutrophils are the most abundant leukocyte population, and essential effectors of innate immune response. Emerging evidences have indicated that neutrophils exert important functions in the tumor context, indeed they can have different phenotypic and functional properties, which depend either on their differentiation or activation state, and are able to alter tumor behaviour. N1 polarization is induced by IFN-β and inhibited by TGF-β, which lead neutrophils to assume a proinflammatory and cytotoxic phenotype towards tumor cells, whereas TGF-β stimulation and IFN-β inhibition promote N2 polarization that is related to an immunosuppressive and tumor-supporting growth phenotype [259]. N1 and N2 phenotypes can be distinguished by the expression of distinct molecules and chemokine receptors, hence in an effort to understand how neutrophils were mediating the metastatic protection in *Ackr2* deficient mice, we analysed the phenotype of *Ackr2*$^{-/-}$ circulating neutrophils.

The critical role for *Ackr2* deficient neutrophils was further supported by the activated phenotype that these cells displayed. Indeed, we found that *Ackr2*$^{-/-}$ neutrophils expressed lower levels of CD62L/L-selectin and increased levels of ICAM-1, compared to WT. Moreover, neutrophils lacking ACKR2, as well as HPCs, showed increased expression of the chemokine receptors CCR1, CCR2 and CCR5 compared to WT mice. CD62L down-regulation and ICAM-1 up-regulation are associated to an activated phenotype of neutrophils. Moreover, many studies have demonstrated that the up-regulation of CC chemokine receptors in neutrophils is functionally relevant since the stimulation with the cognate chemokines activates neutrophil effector functions such as respiratory burst activity and bacterial killing [252]. For example, despite the role of CCR2 in mediating the recruitment of monocytes that support tumor growth, evidences have demonstrate that CCR2 expression is crucial to induce BM mobilization of neutrophils with anti-metastatic activity, by producing ROS able to kill tumor cells in a CCR2-dependent manner [252]. In the future, we will, thus, deeper investigate this aspect by evaluating the possible differential production of ROS in response to CCR2 ligands between neutrophils WT and *Ackr2*$^{-/-}$, and eventually the differential cytotoxic activity of WT and *Ackr2*$^{-/-}$ neutrophils towards tumor cells.

The analysis of other genes differentially regulated in N1 and N2 neutrophils, such as TNFα, Alox5, VEGF-A, Arg1, and CXCR4 did not reveal any difference between WT and

Ackr2$^{-/-}$ neutrophils.

In an effort to improve the metastatic protection observed in *Ackr2*$^{-/-}$ mice, we treated WT and *Ackr2*$^{-/-}$ tumor bearing mice with AMD3100, the competitive inhibitor of CXCR4, which is known to induce a rapid mobilization of neutrophils from the BM. However, AMD3100 treatment did not induce difference in the development of metastases in WT and *Ackr2*$^{-/-}$ mice. Since CXCR4 is also express by B16F10 melanoma cells and by neutrophils with proangiogenic and pro-tumoral role, it is possible that *Ackr2* deficiency was less prominent then CXCR4 inhibition, which was sufficient to reduced metastases both in WT and *Ackr2*$^{-/-}$ mice [261, 292].

The increased expression of CC chemokine receptors in HPCs and neutrophils of *Ackr2*$^{-/-}$ deficient mice was clarified by using the promyelocytic cell line HL-60, and demonstrating that ACKR2 exerted a negative regulation on CC chemokine receptors expression and cell differentiation. Indeed, the overexpression of ACKR2 in HL-60 resulted in decreased transcript levels of CCR2 and CD11b. Since ACKR2 is weakly expressed in neutrophils, it is unlikely that ACKR2 is inhibiting the expression of CC chemokine receptors in those cells in WT mice. However, it is possible that the differential ontogenesis of WT and *Ackr2*$^{-/-}$ neutrophils influenced their chemokine receptor expression pattern and also their effector function.

Even though ACKR2 overexpression in HL-60 proved the regulatory function exerted by ACKR2, the signaling pathway potentially involved is still unknown. In our laboratory it was demonstrated that the scavenger activity of ACKR2 is associated to a β-arrestin1-dependent G protein-independent pathway which is functionally relevant for ACKR2 internalization and recycling to the cell membrane [102]. Thus, we hypothesize that ACKR2 by activating the β-arrestin dependent signaling, also controls the expression of CC chemokine receptors by negatively regulating pathways involved in HPC differentiation and chemokine receptor expression. For example, the G-CSF pathway is important for HPC differentiation into neutrophils, and, in various myeloid cell lines, G-CSF has been demonstrated to be able to induce CCR2 expression [193, 222]. Thus, it would be interesting to test whether the β-arrestin dependent signaling of ACKR2 leads to the inhibition of the G-CSF signaling, and negatively controls HPC proliferation and CCR2 expression, which is relevant for neutrophil mobilization and antimetastatic activity. In our set of experiment, we cultured LSK cells in presence of IL-6, IL-3, and SCF, which are important for LSK differentiation [190-192]. Whether the β-arrestin dependent signaling of ACKR2 is also inhibiting the signaling pathway of those molecules, and if

Ackr2$^{-/-}$ mice express different level of the receptors for IL-6, IL-3 and SDF-1 also remains to be investigated.

Preliminary data regarding the analysis of STAT3, CEBPβ, PU.1, IRF8, Bcl3, Socs3, and RORC1 in BM total cells did not reveal any difference in the expression of these transcription factors between WT and *Ackr2*$^{-/-}$ mice. Therefore, in future, we will enrich LSK population in effort to find out whether analysing only the population where ACKR2 is more expressed, we can appreciate differences in the expression of those transcription factors, involved in myelopoiesis, between WT and *Ackr2*$^{-/-}$.

In summary, our data indicate that ACKR2, through a pathway still undetermined, negatively regulates the expression of CC chemokine receptors in HPCs, inhibits their differentiation and, as consequence, the mobilization of myeloid cells. We thus provide another mechanism by which ACKR2 controls inflammation; however, we also found that ACKR2 by negatively regulating myeloid cell mobilization from the BM restrains the potential protective role that neutrophils may play in tumor context.

By proving that besides its well-known chemokine scavenging activity and its regulatory functions on inflammation, ACKR2 restrict neutrophils anti-metastatic potential, we thus infer that ACKR2 inhibition might be a new strategy to target tumor microenvironment and to induce neutrophil polarization through a N1 anti-tumoral phenotype.

References

1. Griffith, J.W., C.L. Sokol, and A.D. Luster, *Chemokines and chemokine receptors: positioning cells for host defense and immunity.* Annu Rev Immunol, 2014. **32**: p. 659-702.
2. Charo, I.F. and R.M. Ransohoff, *The many roles of chemokines and chemokine receptors in inflammation.* N Engl J Med, 2006. **354**(6): p. 610-21.
3. Luster, A.D., *Chemokines--chemotactic cytokines that mediate inflammation.* N Engl J Med, 1998. **338**(7): p. 436-45.
4. Mantovani, A., *The chemokine system: redundancy for robust outputs.* Immunol Today, 1999. **20**(6): p. 254-7.
5. Bonecchi, R., et al., *Chemokines and chemokine receptors: an overview.* Front Biosci (Landmark Ed), 2009. **14**: p. 540-51.
6. Lazennec, G. and A. Richmond, *Chemokines and chemokine receptors: new insights into cancer-related inflammation.* Trends Mol Med, 2010. **16**(3): p. 133-44.
7. Baggiolini, M., *Chemokines and leukocyte traffic.* Nature, 1998. **392**(6676): p. 565-8.
8. Weber, C., *Novel mechanistic concepts for the control of leukocyte transmigration: specialization of integrins, chemokines, and junctional molecules.* J Mol Med (Berl), 2003. **81**(1): p. 4-19.
9. Springer, T.A., *Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm.* Cell, 1994. **76**(2): p. 301-14.
10. Butcher, E.C. and L.J. Picker, *Lymphocyte homing and homeostasis.* Science, 1996. **272**(5258): p. 60-6.
11. Balkwill, F., *Cancer and the chemokine network.* Nat Rev Cancer, 2004. **4**(7): p. 540-50.
12. Strieter, R.M., et al., *CXC chemokines in angiogenesis.* Cytokine Growth Factor Rev, 2005. **16**(6): p. 593-609.
13. Broxmeyer, H.E., *Chemokines in hematopoiesis.* Curr Opin Hematol, 2008. **15**(1): p. 49-58.
14. Mantovani, A., R. Bonecchi, and M. Locati, *Tuning inflammation and immunity by chemokine sequestration: decoys and more.* Nat Rev Immunol, 2006. **6**(12): p. 907-18.
15. Rollins, B.J., *Chemokines.* Blood, 1997. **90**(3): p. 909-28.
16. Proudfoot, A.E., et al., *Glycosaminoglycan binding and oligomerization are essential for the in vivo activity of certain chemokines.* Proc Natl Acad Sci U S A, 2003. **100**(4): p. 1885-90.
17. Strieter, R.M., et al., *CXC chemokines: angiogenesis, immunoangiostasis, and metastases in lung cancer.* Ann N Y Acad Sci, 2004. **1028**: p. 351-60.
18. Bazan, J.F., et al., *A new class of membrane-bound chemokine with a CX3C motif.* Nature, 1997. **385**(6617): p. 640-4.
19. Kelner, G.S., et al., *Lymphotactin: a cytokine that represents a new class of chemokine.* Science, 1994. **266**(5189): p. 1395-9.
20. Le, Y., et al., *Chemokines and chemokine receptors: their manifold roles in homeostasis and disease.* Cell Mol Immunol, 2004. **1**(2): p. 95-104.
21. Townson, D.H. and A.R. Liptak, *Chemokines in the corpus luteum: implications of leukocyte chemotaxis.* Reprod Biol Endocrinol, 2003. **1**: p. 94.

22. Murphy, P.M., *The molecular biology of leukocyte chemoattractant receptors.* Annu Rev Immunol, 1994. **12**: p. 593-633.
23. Crump, M.P., et al., *Solution structure and basis for functional activity of stromal cell-derived factor-1; dissociation of CXCR4 activation from binding and inhibition of HIV-1.* EMBO J, 1997. **16**(23): p. 6996-7007.
24. Fernandez, E.J. and E. Lolis, *Structure, function, and inhibition of chemokines.* Annu Rev Pharmacol Toxicol, 2002. **42**: p. 469-99.
25. Thelen, M., *Dancing to the tune of chemokines.* Nat Immunol, 2001. **2**(2): p. 129-34.
26. Zlotnik, A., *Chemokines and cancer.* Int J Cancer, 2006. **119**(9): p. 2026-9.
27. Mantovani, A., et al., *Cancer-related inflammation.* Nature, 2008. **454**(7203): p. 436-44.
28. Balkwill, F., K.A. Charles, and A. Mantovani, *Smoldering and polarized inflammation in the initiation and promotion of malignant disease.* Cancer Cell, 2005. **7**(3): p. 211-7.
29. Galon, J., et al., *The continuum of cancer immunosurveillance: prognostic, predictive, and mechanistic signatures.* Immunity, 2013. **39**(1): p. 11-26.
30. Liu, Q., et al., *The CXCL8-CXCR1/2 pathways in cancer.* Cytokine Growth Factor Rev, 2016. **31**: p. 61-71.
31. Lee, L.F., et al., *IL-8 reduced tumorigenicity of human ovarian cancer in vivo due to neutrophil infiltration.* J Immunol, 2000. **164**(5): p. 2769-75.
32. Richmond, A., J. Yang, and Y. Su, *The good and the bad of chemokines/chemokine receptors in melanoma.* Pigment Cell Melanoma Res, 2009. **22**(2): p. 175-86.
33. Dhawan, P. and A. Richmond, *Role of CXCL1 in tumorigenesis of melanoma.* J Leukoc Biol, 2002. **72**(1): p. 9-18.
34. Kowalczuk, O., et al., *CXCL5 as a potential novel prognostic factor in early stage non-small cell lung cancer: results of a study of expression levels of 23 genes.* Tumour Biol, 2014. **35**(5): p. 4619-28.
35. Gijsbers, K., et al., *GCP-2/CXCL6 synergizes with other endothelial cell-derived chemokines in neutrophil mobilization and is associated with angiogenesis in gastrointestinal tumors.* Exp Cell Res, 2005. **303**(2): p. 331-42.
36. Rubie, C., et al., *CCL20/CCR6 expression profile in pancreatic cancer.* J Transl Med, 2010. **8**: p. 45.
37. Popple, A., et al., *The chemokine, CXCL12, is an independent predictor of poor survival in ovarian cancer.* Br J Cancer, 2012. **106**(7): p. 1306-13.
38. Cassier, P.A., et al., *Prognostic value of the expression of C-Chemokine Receptor 6 and 7 and their ligands in non-metastatic breast cancer.* BMC Cancer, 2011. **11**: p. 213.
39. Scotton, C.J., et al., *Epithelial cancer cell migration: a role for chemokine receptors?* Cancer Res, 2001. **61**(13): p. 4961-5.
40. Geminder, H., et al., *A possible role for CXCR4 and its ligand, the CXC chemokine stromal cell-derived factor-1, in the development of bone marrow metastases in neuroblastoma.* J Immunol, 2001. **167**(8): p. 4747-57.
41. Rempel, S.A., et al., *Identification and localization of the cytokine SDF1 and its receptor, CXC chemokine receptor 4, to regions of necrosis and angiogenesis in human glioblastoma.* Clin Cancer Res, 2000. **6**(1): p. 102-11.
42. Muller, A., et al., *Involvement of chemokine receptors in breast cancer metastases.* Nature, 2001. **410**(6824): p. 50-6.

43. Taichman, R.S., et al., *Use of the stromal cell-derived factor-1/CXCR4 pathway in prostate cancer metastases to bone.* Cancer Res, 2002. **62**(6): p. 1832-7.
44. Koshiba, T., et al., *Expression of stromal cell-derived factor 1 and CXCR4 ligand receptor system in pancreatic cancer: a possible role for tumor progression.* Clin Cancer Res, 2000. **6**(9): p. 3530-5.
45. Burger, M., et al., *Functional expression of CXCR4 (CD184) on small-cell lung cancer cells mediates migration, integrin activation, and adhesion to stromal cells.* Oncogene, 2003. **22**(50): p. 8093-101.
46. Pinto, S., et al., *Intracellular coexpression of CXC- and CC- chemokine receptors and their ligands in human melanoma cell lines and dynamic variations after xenotransplantation.* BMC Cancer, 2014. **14**: p. 118.
47. Lopez-Giral, S., et al., *Chemokine receptors that mediate B cell homing to secondary lymphoid tissues are highly expressed in B cell chronic lymphocytic leukemia and non-Hodgkin lymphomas with widespread nodular dissemination.* J Leukoc Biol, 2004. **76**(2): p. 462-71.
48. Kleinhans, M., et al., *Functional expression of the eotaxin receptor CCR3 in CD30+ cutaneous T-cell lymphoma.* Blood, 2003. **101**(4): p. 1487-93.
49. Kuhnelt-Leddihn, L., et al., *Overexpression of the chemokine receptors CXCR4, CCR7, CCR9, and CCR10 in human primary cutaneous melanoma: a potential prognostic value for CCR7 and CCR10?* Arch Dermatol Res, 2012. **304**(3): p. 185-93.
50. Singh, S., et al., *CXCR1 and CXCR2 enhances human melanoma tumourigenesis, growth and invasion.* Br J Cancer, 2009. **100**(10): p. 1638-46.
51. Kawada, K., et al., *Pivotal role of CXCR3 in melanoma cell metastases to LNs.* Cancer Res, 2004. **64**(11): p. 4010-7.
52. Chow, M.T. and A.D. Luster, *Chemokines in cancer.* Cancer Immunol Res, 2014. **2**(12): p. 1125-31.
53. Luboshits, G., et al., *Elevated expression of the CC chemokine regulated on activation, normal T cell expressed and secreted (RANTES) in advanced breast carcinoma.* Cancer Res, 1999. **59**(18): p. 4681-7.
54. Ueno, T., et al., *Significance of macrophage chemoattractant protein-1 in macrophage recruitment, angiogenesis, and survival in human breast cancer.* Clin Cancer Res, 2000. **6**(8): p. 3282-9.
55. Conti, I. and B.J. Rollins, *CCL2 (monocyte chemoattractant protein-1) and cancer.* Semin Cancer Biol, 2004. **14**(3): p. 149-54.
56. Harlin, H., et al., *Chemokine expression in melanoma metastases associated with CD8+ T-cell recruitment.* Cancer Res, 2009. **69**(7): p. 3077-85.
57. Hu, H., et al., *Tumor cell-microenvironment interaction models coupled with clinical validation reveal CCL2 and SNCG as two predictors of colorectal cancer hepatic metastases.* Clin Cancer Res, 2009. **15**(17): p. 5485-93.
58. Ohta, M., et al., *Monocyte chemoattractant protein-1 expression correlates with macrophage infiltration and tumor vascularity in human esophageal squamous cell carcinomas.* Int J Cancer, 2002. **102**(3): p. 220-4.
59. Loberg, R.D., et al., *CCL2 as an important mediator of prostate cancer growth in vivo through the regulation of macrophage infiltration.* Neoplasia, 2007. **9**(7): p. 556-62.
60. Valkovic, T., et al., *Correlation between vascular endothelial growth factor, angiogenesis, and tumor-associated macrophages in invasive ductal breast carcinoma.* Virchows Arch, 2002. **440**(6): p. 583-8.

61. Itatani, Y., et al., *The Role of Chemokines in Promoting Colorectal Cancer Invasion/Metastases.* Int J Mol Sci, 2016. **17**(5).
62. Acharyya, S., et al., *A CXCL1 paracrine network links cancer chemoresistance and metastases.* Cell, 2012. **150**(1): p. 165-78.
63. Van Coillie, E., et al., *Tumor angiogenesis induced by granulocyte chemotactic protein-2 as a countercurrent principle.* Am J Pathol, 2001. **159**(4): p. 1405-14.
64. Zhou, S.L., et al., *Overexpression of CXCL5 mediates neutrophil infiltration and indicates poor prognosis for hepatocellular carcinoma.* Hepatology, 2012. **56**(6): p. 2242-54.
65. Kleeff, J., et al., *Detection and localization of Mip-3alpha/LARC/Exodus, a macrophage proinflammatory chemokine, and its CCR6 receptor in human pancreatic cancer.* Int J Cancer, 1999. **81**(4): p. 650-7.
66. Bell, D., et al., *In breast carcinoma tissue, immature dendritic cells reside within the tumor, whereas mature dendritic cells are located in peritumoral areas.* J Exp Med, 1999. **190**(10): p. 1417-26.
67. Scarpino, S., et al., *Papillary carcinoma of the thyroid: hepatocyte growth factor (HGF) stimulates tumor cells to release chemokines active in recruiting dendritic cells.* Am J Pathol, 2000. **156**(3): p. 831-7.
68. Zou, W., et al., *Stromal-derived factor-1 in human tumors recruits and alters the function of plasmacytoid precursor dendritic cells.* Nat Med, 2001. **7**(12): p. 1339-46.
69. Wendel, M., et al., *Natural killer cell accumulation in tumors is dependent on IFN-gamma and CXCR3 ligands.* Cancer Res, 2008. **68**(20): p. 8437-45.
70. Groom, J.R. and A.D. Luster, *CXCR3 in T cell function.* Exp Cell Res, 2011. **317**(5): p. 620-31.
71. Hensbergen, P.J., et al., *The CXCR3 targeting chemokine CXCL11 has potent antitumor activity in vivo involving attraction of CD8+ T lymphocytes but not inhibition of angiogenesis.* J Immunother, 2005. **28**(4): p. 343-51.
72. Ohtani, H., et al., *Abundant expression of CXCL9 (MIG) by stromal cells that include dendritic cells and accumulation of CXCR3+ T cells in lymphocyte-rich gastric carcinoma.* J Pathol, 2009. **217**(1): p. 21-31.
73. Gobert, M., et al., *Regulatory T cells recruited through CCL22/CCR4 are selectively activated in lymphoid infiltrates surrounding primary breast tumors and lead to an adverse clinical outcome.* Cancer Res, 2009. **69**(5): p. 2000-9.
74. Olkhanud, P.B., et al., *Breast cancer lung metastases requires expression of chemokine receptor CCR4 and regulatory T cells.* Cancer Res, 2009. **69**(14): p. 5996-6004.
75. Curiel, T.J., et al., *Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival.* Nat Med, 2004. **10**(9): p. 942-9.
76. Steelman, L.S., et al., *Roles of the Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR pathways in controlling growth and sensitivity to therapy-implications for cancer and aging.* Aging (Albany NY), 2011. **3**(3): p. 192-222.
77. Zhou, Y., et al., *CXCR4 is a major chemokine receptor on glioma cells and mediates their survival.* J Biol Chem, 2002. **277**(51): p. 49481-7.
78. Hall, J.M. and K.S. Korach, *Stromal cell-derived factor 1, a novel target of estrogen receptor action, mediates the mitogenic effects of estradiol in ovarian and breast cancer cells.* Mol Endocrinol, 2003. **17**(5): p. 792-803.

79. Kijima, T., et al., *Regulation of cellular proliferation, cytoskeletal function, and signal transduction through CXCR4 and c-Kit in small cell lung cancer cells.* Cancer Res, 2002. **62**(21): p. 6304-11.
80. Juarez, J., et al., *Effects of inhibitors of the chemokine receptor CXCR4 on acute lymphoblastic leukemia cells in vitro.* Leukemia, 2003. **17**(7): p. 1294-300.
81. Burger, J.A., et al., *Blood-derived nurse-like cells protect chronic lymphocytic leukemia B cells from spontaneous apoptosis through stromal cell-derived factor-1.* Blood, 2000. **96**(8): p. 2655-63.
82. Wang, B., et al., *A growth-related oncogene/CXC chemokine receptor 2 autocrine loop contributes to cellular proliferation in esophageal cancer.* Cancer Res, 2006. **66**(6): p. 3071-7.
83. Kitadai, Y., et al., *Regulation of disease-progression genes in human gastric carcinoma cells by interleukin 8.* Clin Cancer Res, 2000. **6**(7): p. 2735-40.
84. Murakami, T., et al., *Immune evasion by murine melanoma mediated through CC chemokine receptor-10.* J Exp Med, 2003. **198**(9): p. 1337-47.
85. Wang, J., et al., *Autocrine and paracrine chemokine receptor 7 activation in head and neck cancer: implications for therapy.* J Natl Cancer Inst, 2008. **100**(7): p. 502-12.
86. Keeley, E.C., B. Mehrad, and R.M. Strieter, *CXC chemokines in cancer angiogenesis and metastases.* Adv Cancer Res, 2010. **106**: p. 91-111.
87. Kryczek, I., et al., *CXCL12 and vascular endothelial growth factor synergistically induce neoangiogenesis in human ovarian cancers.* Cancer Res, 2005. **65**(2): p. 465-72.
88. Singh, S., et al., *CXCR1 and CXCR2 silencing modulates CXCL8-dependent endothelial cell proliferation, migration and capillary-like structure formation.* Microvasc Res, 2011. **82**(3): p. 318-25.
89. Rehman, J., et al., *Peripheral blood "endothelial progenitor cells" are derived from monocyte/macrophages and secrete angiogenic growth factors.* Circulation, 2003. **107**(8): p. 1164-9.
90. Mantovani, A., et al., *Role of tumor-associated macrophages in tumor progression and invasion.* Cancer Metastases Rev, 2006. **25**(3): p. 315-22.
91. Sionov, R.V., Z.G. Fridlender, and Z. Granot, *The Multifaceted Roles Neutrophils Play in the Tumor Microenvironment.* Cancer Microenviron, 2015. **8**(3): p. 125-58.
92. Lasagni, L., et al., *An alternatively spliced variant of CXCR3 mediates the inhibition of endothelial cell growth induced by IP-10, Mig, and I-TAC, and acts as functional receptor for platelet factor 4.* J Exp Med, 2003. **197**(11): p. 1537-49.
93. Ehlert, J.E., et al., *Identification and partial characterization of a variant of human CXCR3 generated by posttranscriptional exon skipping.* J Immunol, 2004. **173**(10): p. 6234-40.
94. Sarvaiya, P.J., et al., *Chemokines in tumor progression and metastases.* Oncotarget, 2013. **4**(12): p. 2171-85.
95. Ding, Y., et al., *Association of CC chemokine receptor 7 with LN metastases of esophageal squamous cell carcinoma.* Clin Cancer Res, 2003. **9**(9): p. 3406-12.
96. Takanami, I., *Overexpression of CCR7 mRNA in nonsmall cell lung cancer: correlation with LN metastases.* Int J Cancer, 2003. **105**(2): p. 186-9.
97. Amersi, F.F., et al., *Activation of CCR9/CCL25 in cutaneous melanoma mediates preferential metastases to the small intestine.* Clin Cancer Res, 2008. **14**(3): p. 638-45.

98. Soehnlein, O. and L. Lindbom, *Phagocyte partnership during the onset and resolution of inflammation.* Nat Rev Immunol, 2010. **10**(6): p. 427-39.
99. Bonecchi, R., et al., *Cytokine decoy and scavenger receptors as key regulators of immunity and inflammation.* Cytokine, 2016. **87**: p. 37-45.
100. Nibbs, R.J. and G.J. Graham, *Immune regulation by atypical chemokine receptors.* Nat Rev Immunol, 2013. **13**(11): p. 815-29.
101. Bonecchi, R. and G.J. Graham, *Atypical Chemokine Receptors and Their Roles in the Resolution of the Inflammatory Response.* Front Immunol, 2016. **7**: p. 224.
102. Borroni, E.M., et al., *beta-arrestin-dependent activation of the cofilin pathway is required for the scavenging activity of the atypical chemokine receptor D6.* Sci Signal, 2013. **6**(273): p. ra30 1-11, S1-3.
103. Bonecchi, R., et al., *Regulation of D6 chemokine scavenging activity by ligand- and Rab11-dependent surface up-regulation.* Blood, 2008. **112**(3): p. 493-503.
104. Watts, A.O., et al., *beta-Arrestin recruitment and G protein signaling by the atypical human chemokine decoy receptor CCX-CKR.* J Biol Chem, 2013. **288**(10): p. 7169-81.
105. Heinzel, K., C. Benz, and C.C. Bleul, *A silent chemokine receptor regulates steady-state leukocyte homing in vivo.* Proc Natl Acad Sci U S A, 2007. **104**(20): p. 8421-6.
106. Rajagopal, S., et al., *Beta-arrestin- but not G protein-mediated signaling by the "decoy" receptor CXCR7.* Proc Natl Acad Sci U S A, 2010. **107**(2): p. 628-32.
107. Pruenster, M., et al., *The Duffy antigen receptor for chemokines transports chemokines and supports their promigratory activity.* Nat Immunol, 2009. **10**(1): p. 101-8.
108. Bachelerie, F., et al., *New nomenclature for atypical chemokine receptors.* Nat Immunol, 2014. **15**(3): p. 207-8.
109. Graham, G.J., et al., *The biochemistry and biology of the atypical chemokine receptors.* Immunol Lett, 2012. **145**(1-2): p. 30-8.
110. Chaudhuri, A., et al., *Expression of the Duffy antigen in K562 cells. Evidence that it is the human erythrocyte chemokine receptor.* J Biol Chem, 1994. **269**(11): p. 7835-8.
111. Martinez de la Torre, Y., et al., *Protection against inflammation- and autoantibody-caused fetal loss by the chemokine decoy receptor D6.* Proc Natl Acad Sci U S A, 2007. **104**(7): p. 2319-24.
112. McKimmie, C.S., et al., *Hemopoietic cell expression of the chemokine decoy receptor D6 is dynamic and regulated by GATA1.* J Immunol, 2008. **181**(11): p. 8171-81.
113. Gosling, J., et al., *Cutting edge: identification of a novel chemokine receptor that binds dendritic cell- and T cell-active chemokines including ELC, SLC, and TECK.* J Immunol, 2000. **164**(6): p. 2851-6.
114. Townson, J.R. and R.J. Nibbs, *Characterization of mouse CCX-CKR, a receptor for the lymphocyte-attracting chemokines TECK/mCCL25, SLC/mCCL21 and MIP-3beta/mCCL19: comparison to human CCX-CKR.* Eur J Immunol, 2002. **32**(5): p. 1230-41.
115. Novitzky-Basso, I. and A. Rot, *Duffy antigen receptor for chemokines and its involvement in patterning and control of inflammatory chemokines.* Front Immunol, 2012. **3**: p. 266.
116. Rot, A., *Contribution of Duffy antigen to chemokine function.* Cytokine Growth Factor Rev, 2005. **16**(6): p. 687-94.

117. Graham, G.J. and M. Locati, *Regulation of the immune and inflammatory responses by the 'atypical' chemokine receptor D6.* J Pathol, 2013. **229**(2): p. 168-75.
118. Lee, J.S., et al., *Duffy antigen facilitates movement of chemokine across the endothelium in vitro and promotes neutrophil transmigration in vitro and in vivo.* J Immunol, 2003. **170**(10): p. 5244-51.
119. Lee, J.S., et al., *The Duffy antigen modifies systemic and local tissue chemokine responses following lipopolysaccharide stimulation.* J Immunol, 2006. **177**(11): p. 8086-94.
120. Zarbock, A., et al., *Chemokine homeostasis vs. chemokine presentation during severe acute lung injury: the other side of the Duffy antigen receptor for chemokines.* Am J Physiol Lung Cell Mol Physiol, 2010. **298**(3): p. L462-71.
121. Zarbock, A., et al., *The Duffy antigen receptor for chemokines in acute renal failure: A facilitator of renal chemokine presentation.* Crit Care Med, 2007. **35**(9): p. 2156-63.
122. Rundle, C.H., S. Mohan, and B. Edderkaoui, *Duffy antigen receptor for chemokines regulates post-fracture inflammation.* PLoS One, 2013. **8**(10): p. e77362.
123. Wan, W., et al., *Atypical chemokine receptor 1 deficiency reduces atherogenesis in ApoE-knockout mice.* Cardiovasc Res, 2015. **106**(3): p. 478-87.
124. Zimmerman, P.A., et al., *Red blood cell polymorphism and susceptibility to Plasmodium vivax.* Adv Parasitol, 2013. **81**: p. 27-76.
125. Burns, J.M., et al., *A novel chemokine receptor for SDF-1 and I-TAC involved in cell survival, cell adhesion, and tumor development.* J Exp Med, 2006. **203**(9): p. 2201-13.
126. Levoye, A., et al., *CXCR7 heterodimerizes with CXCR4 and regulates CXCL12-mediated G protein signaling.* Blood, 2009. **113**(24): p. 6085-93.
127. Thelen, M. and S. Thelen, *CXCR7, CXCR4 and CXCL12: an eccentric trio?* J Neuroimmunol, 2008. **198**(1-2): p. 9-13.
128. Werner, L., et al., *Reciprocal regulation of CXCR4 and CXCR7 in intestinal mucosal homeostasis and inflammatory bowel disease.* J Leukoc Biol, 2011. **90**(3): p. 583-90.
129. Chatterjee, M., et al., *Platelet-derived CXCL12 regulates monocyte function, survival, differentiation into macrophages and foam cells through differential involvement of CXCR4-CXCR7.* Cell Death Dis, 2015. **6**: p. e1989.
130. Ulvmar, M.H., et al., *The atypical chemokine receptor CCRL1 shapes functional CCL21 gradients in LNs.* Nat Immunol, 2014. **15**(7): p. 623-30.
131. Comerford, I., et al., *The atypical chemokine receptor CCX-CKR scavenges homeostatic chemokines in circulation and tissues and suppresses Th17 responses.* Blood, 2010. **116**(20): p. 4130-40.
132. Massara, M., et al., *Atypical chemokine receptors in cancer: friends or foes?* J Leukoc Biol, 2016. **99**(6): p. 927-33.
133. Shen, H., et al., *The Duffy antigen/receptor for chemokines (DARC) regulates prostate tumor growth.* FASEB J, 2006. **20**(1): p. 59-64.
134. Hernandez, L., et al., *Opposing roles of CXCR4 and CXCR7 in breast cancer metastases.* Breast Cancer Res, 2011. **13**(6): p. R128.
135. Sun, X., et al., *CXCL12 / CXCR4 / CXCR7 chemokine axis and cancer progression.* Cancer Metastases Rev, 2010. **29**(4): p. 709-22.
136. Gao, W., et al., *ShRNA-mediated knock-down of CXCR7 increases TRAIL-sensitivity in MCF-7 breast cancer cells.* Tumour Biol, 2015. **36**(9): p. 7243-50.

137. Feng, L.Y., et al., *Involvement of a novel chemokine decoy receptor CCX-CKR in breast cancer growth, metastases and patient survival.* Clin Cancer Res, 2009. **15**(9): p. 2962-70.
138. Shi, J.Y., et al., *CC chemokine receptor-like 1 functions as a tumour suppressor by impairing CCR7-related chemotaxis in hepatocellular carcinoma.* J Pathol, 2015. **235**(4): p. 546-58.
139. Zhu, Y., et al., *CCX-CKR expression in colorectal cancer and patient survival.* Int J Biol Markers, 2014. **29**(1): p. e40-8.
140. Nibbs, R.J., et al., *Cloning and characterization of a novel murine beta chemokine receptor, D6. Comparison to three other related macrophage inflammatory protein-1alpha receptors, CCR-1, CCR-3, and CCR-5.* J Biol Chem, 1997. **272**(19): p. 12495-504.
141. Nibbs, R.J., et al., *Cloning and characterization of a novel promiscuous human beta-chemokine receptor D6.* J Biol Chem, 1997. **272**(51): p. 32078-83.
142. Bonini, J.A., et al., *Cloning, expression, and chromosomal mapping of a novel human CC-chemokine receptor (CCR10) that displays high-affinity binding for MCP-1 and MCP-3.* DNA Cell Biol, 1997. **16**(10): p. 1249-56.
143. Graham, G.J., *D6/Ackr2.* Front Immunol, 2015. **6**: p. 280.
144. Bonecchi, R., et al., *Differential recognition and scavenging of native and truncated macrophage-derived chemokine (macrophage-derived chemokine/CC chemokine ligand 22) by the D6 decoy receptor.* J Immunol, 2004. **172**(8): p. 4972-6.
145. Savino, B., et al., *Recognition versus adaptive up-regulation and degradation of CC chemokines by the chemokine decoy receptor D6 are determined by their N-terminal sequence.* J Biol Chem, 2009. **284**(38): p. 26207-15.
146. Nibbs, R.J., et al., *Structure-function dissection of D6, an atypical scavenger receptor.* Methods Enzymol, 2009. **460**: p. 245-61.
147. Hansell, C.A., C.E. Hurson, and R.J. Nibbs, *DARC and D6: silent partners in chemokine regulation?* Immunol Cell Biol, 2011. **89**(2): p. 197-206.
148. McCulloch, C.V., et al., *Multiple roles for the C-terminal tail of the chemokine scavenger D6.* J Biol Chem, 2008. **283**(12): p. 7972-82.
149. Govaerts, C., et al., *The TXP motif in the second transmembrane helix of CCR5. A structural determinant of chemokine-induced activation.* J Biol Chem, 2001. **276**(16): p. 13217-25.
150. Cancellieri, C., et al., *Review: Structure-function and biological properties of the atypical chemokine receptor D6.* Mol Immunol, 2013. **55**(1): p. 87-93.
151. Weber, M., et al., *The chemokine receptor D6 constitutively traffics to and from the cell surface to internalize and degrade chemokines.* Mol Biol Cell, 2004. **15**(5): p. 2492-508.
152. Bonecchi, R., et al., *Chemokine decoy receptors: structure-function and biological properties.* Curr Top Microbiol Immunol, 2010. **341**: p. 15-36.
153. Nibbs, R.J., et al., *The beta-chemokine receptor D6 is expressed by lymphatic endothelium and a subset of vascular tumors.* Am J Pathol, 2001. **158**(3): p. 867-77.
154. Madigan, J., et al., *Chemokine scavenger D6 is expressed by trophoblasts and aids the survival of mouse embryos transferred into allogeneic recipients.* J Immunol, 2010. **184**(6): p. 3202-12.
155. Hansell, C.A. and R.J. Nibbs, *The odd couple: innate-like B cells and the chemokine scavenger D6.* Cell Cycle, 2011. **10**(21): p. 3619-20.

156. Vetrano, S., et al., *The lymphatic system controls intestinal inflammation and inflammation-associated Colon Cancer through the chemokine decoy receptor D6.* Gut, 2010. **59**(2): p. 197-206.
157. Wu, F.Y., et al., *Chemokine decoy receptor d6 plays a negative role in human breast cancer.* Mol Cancer Res, 2008. **6**(8): p. 1276-88.
158. Bazzan, E., et al., *Expression of the atypical chemokine receptor D6 in human alveolar macrophages in COPD.* Chest, 2013. **143**(1): p. 98-106.
159. Singh, M.D., et al., *Elevated expression of the chemokine-scavenging receptor D6 is associated with impaired lesion development in psoriasis.* Am J Pathol, 2012. **181**(4): p. 1158-64.
160. McKimmie, C.S., et al., *An analysis of the function and expression of D6 on lymphatic endothelial cells.* Blood, 2013. **121**(18): p. 3768-77.
161. Jamieson, T., et al., *The chemokine receptor D6 limits the inflammatory response in vivo.* Nat Immunol, 2005. **6**(4): p. 403-11.
162. Locati, M., et al., *Silent chemoattractant receptors: D6 as a decoy and scavenger receptor for inflammatory CC chemokines.* Cytokine Growth Factor Rev, 2005. **16**(6): p. 679-86.
163. Nibbs, R., G. Graham, and A. Rot, *Chemokines on the move: control by the chemokine "interceptors" Duffy blood group antigen and D6.* Semin Immunol, 2003. **15**(5): p. 287-94.
164. Savino, B., et al., *Control of murine Ly6C(high) monocyte traffic and immunosuppressive activities by atypical chemokine receptor D6.* Blood, 2012. **119**(22): p. 5250-60.
165. Lee, K.M., et al., *The chemokine receptors ACKR2 and CCR2 reciprocally regulate lymphatic vessel density.* EMBO J, 2014. **33**(21): p. 2564-80.
166. Martinez de la Torre, Y., et al., *Increased inflammation in mice deficient for the chemokine decoy receptor D6.* Eur J Immunol, 2005. **35**(5): p. 1342-6.
167. Graham, G.J. and C.S. McKimmie, *Chemokine scavenging by D6: a movable feast?* Trends Immunol, 2006. **27**(8): p. 381-6.
168. Di Liberto, D., et al., *Role of the chemokine decoy receptor D6 in balancing inflammation, immune activation, and antimicrobial resistance in Mycobacterium tuberculosis infection.* J Exp Med, 2008. **205**(9): p. 2075-84.
169. Bordon, Y., et al., *The atypical chemokine receptor D6 contributes to the development of experimental colitis.* J Immunol, 2009. **182**(8): p. 5032-40.
170. Liu, L., et al., *Cutting edge: the silent chemokine receptor D6 is required for generating T cell responses that mediate experimental autoimmune encephalomyelitis.* J Immunol, 2006. **177**(1): p. 17-21.
171. Hansell, C.A., et al., *The atypical chemokine receptor ACKR2 suppresses Th17 responses to protein autoantigens.* Immunol Cell Biol, 2015. **93**(2): p. 167-76.
172. Nibbs, R.J., et al., *The atypical chemokine receptor D6 suppresses the development of chemically induced skin tumors.* J Clin Invest, 2007. **117**(7): p. 1884-92.
173. Savino, B., et al., *ERK-dependent downregulation of the atypical chemokine receptor D6 drives tumor aggressiveness in Kaposi sarcoma.* Cancer Immunol Res, 2014. **2**(7): p. 679-89.
174. Kondo, M., et al., *Biology of hematopoietic stem cells and progenitors: implications for clinical application.* Annu Rev Immunol, 2003. **21**: p. 759-806.
175. Arai, F., *Self-renewal and differentiation of hematopoietic stem cells.* Rinsho Ketsueki, 2016. **57**(10): p. 1845-1851.

176. Akashi, K., et al., *A clonogenic common myeloid progenitor that gives rise to all myeloid lineages.* Nature, 2000. **404**(6774): p. 193-7.
177. Kondo, M., I.L. Weissman, and K. Akashi, *Identification of clonogenic common lymphoid progenitors in mouse bone marrow.* Cell, 1997. **91**(5): p. 661-72.
178. Birbrair, A. and P.S. Frenette, *Niche heterogeneity in the bone marrow.* Ann N Y Acad Sci, 2016. **1370**(1): p. 82-96.
179. Yona, S., et al., *Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis.* Immunity, 2013. **38**(1): p. 79-91.
180. Lichanska, A.M. and D.A. Hume, *Origins and functions of phagocytes in the embryo.* Exp Hematol, 2000. **28**(6): p. 601-11.
181. Manz, M.G. and S. Boettcher, *Emergency granulopoiesis.* Nat Rev Immunol, 2014. **14**(5): p. 302-14.
182. Li, L. and T. Xie, *Stem cell niche: structure and function.* Annu Rev Cell Dev Biol, 2005. **21**: p. 605-31.
183. Wilson, A. and A. Trumpp, *Bone-marrow haematopoietic-stem-cell niches.* Nat Rev Immunol, 2006. **6**(2): p. 93-106.
184. Nilsson, S.K., H.M. Johnston, and J.A. Coverdale, *Spatial localization of transplanted hemopoietic stem cells: inferences for the localization of stem cell niches.* Blood, 2001. **97**(8): p. 2293-9.
185. Wilson, A., et al., *Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair.* Cell, 2008. **135**(6): p. 1118-29.
186. Suarez-Alvarez, B., A. Lopez-Vazquez, and C. Lopez-Larrea, *Mobilization and homing of hematopoietic stem cells.* Adv Exp Med Biol, 2012. **741**: p. 152-70.
187. Sugiyama, T., et al., *Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches.* Immunity, 2006. **25**(6): p. 977-88.
188. Thoren, L.A., et al., *Kit regulates maintenance of quiescent hematopoietic stem cells.* J Immunol, 2008. **180**(4): p. 2045-53.
189. Fang, J., et al., *EPO modulation of cell-cycle regulatory genes, and cell division, in primary bone marrow erythroblasts.* Blood, 2007. **110**(7): p. 2361-70.
190. Ogawa, M., et al., *Expression and function of c-kit in hemopoietic progenitor cells.* J Exp Med, 1991. **174**(1): p. 63-71.
191. Bernad, A., et al., *Interleukin-6 is required in vivo for the regulation of stem cells and committed progenitors of the hematopoietic system.* Immunity, 1994. **1**(9): p. 725-31.
192. Heike, T. and T. Nakahata, *Ex vivo expansion of hematopoietic stem cells by cytokines.* Biochim Biophys Acta, 2002. **1592**(3): p. 313-21.
193. Metcalf, D., *Hematopoietic cytokines.* Blood, 2008. **111**(2): p. 485-91.
194. Rosenbauer, F. and D.G. Tenen, *Transcription factors in myeloid development: balancing differentiation with transformation.* Nat Rev Immunol, 2007. **7**(2): p. 105-17.
195. Akagi, T., et al., *Impaired response to GM-CSF and G-CSF, and enhanced apoptosis in C/EBPbeta-deficient hematopoietic cells.* Blood, 2008. **111**(6): p. 2999-3004.
196. Kamezaki, K., et al., *Roles of Stat3 and ERK in G-CSF signaling.* Stem Cells, 2005. **23**(2): p. 252-63.
197. Friedman, A.D., *Transcriptional control of granulocyte and monocyte development.* Oncogene, 2007. **26**(47): p. 6816-28.
198. Semerad, C.L., et al., *G-CSF potently inhibits osteoblast activity and CXCL12 mRNA expression in the bone marrow.* Blood, 2005. **106**(9): p. 3020-7.

199. Wright, D.E., et al., *Hematopoietic stem cells are uniquely selective in their migratory response to chemokines.* J Exp Med, 2002. **195**(9): p. 1145-54.
200. Youn, B.S., C. Mantel, and H.E. Broxmeyer, *Chemokines, chemokine receptors and hematopoiesis.* Immunol Rev, 2000. **177**: p. 150-74.
201. Broxmeyer, H.E., *Regulation of hematopoiesis by chemokine family members.* Int J Hematol, 2001. **74**(1): p. 9-17.
202. Broxmeyer, H.E. and C.H. Kim, *Regulation of hematopoiesis in a sea of chemokine family members with a plethora of redundant activities.* Exp Hematol, 1999. **27**(7): p. 1113-23.
203. Broxmeyer, H.E., et al., *Effects of CC, CXC, C, and CX3C chemokines on proliferation of myeloid progenitor cells, and insights into SDF-1-induced chemotaxis of progenitors.* Ann N Y Acad Sci, 1999. **872**: p. 142-62; discussion 163.
204. Broxmeyer, H.E., et al., *Enhancing and suppressing effects of recombinant murine macrophage inflammatory proteins on colony formation in vitro by bone marrow myeloid progenitor cells.* Blood, 1990. **76**(6): p. 1110-6.
205. Graham, G.J., et al., *Identification and characterization of an inhibitor of haemopoietic stem cell proliferation.* Nature, 1990. **344**(6265): p. 442-4.
206. Broxmeyer, H.E., et al., *Dominant myelopoietic effector functions mediated by chemokine receptor CCR1.* J Exp Med, 1999. **189**(12): p. 1987-92.
207. Ottersbach, K., et al., *Macrophage inflammatory protein-1alpha uses a novel receptor for primitive hemopoietic cell inhibition.* Blood, 2001. **98**(12): p. 3476-8.
208. Boring, L., et al., *Impaired monocyte migration and reduced type 1 (Th1) cytokine responses in C-C chemokine receptor 2 knockout mice.* J Clin Invest, 1997. **100**(10): p. 2552-61.
209. Broxmeyer, H.E., et al., *Involvement of Interleukin (IL) 8 receptor in negative regulation of myeloid progenitor cells in vivo: evidence from mice lacking the murine IL-8 receptor homologue.* J Exp Med, 1996. **184**(5): p. 1825-32.
210. Geissmann, F., S. Jung, and D.R. Littman, *Blood monocytes consist of two principal subsets with distinct migratory properties.* Immunity, 2003. **19**(1): p. 71-82.
211. Si, Y., et al., *CCR2 mediates hematopoietic stem and progenitor cell trafficking to sites of inflammation in mice.* J Clin Invest, 2010. **120**(4): p. 1192-203.
212. Lu, B., et al., *Abnormalities in monocyte recruitment and cytokine expression in monocyte chemoattractant protein 1-deficient mice.* J Exp Med, 1998. **187**(4): p. 601-8.
213. Tsou, C.L., et al., *Critical roles for CCR2 and MCP-3 in monocyte mobilization from bone marrow and recruitment to inflammatory sites.* J Clin Invest, 2007. **117**(4): p. 902-9.
214. Auffray, C., et al., *Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior.* Science, 2007. **317**(5838): p. 666-70.
215. Mack, M., et al., *Expression and characterization of the chemokine receptors CCR2 and CCR5 in mice.* J Immunol, 2001. **166**(7): p. 4697-704.
216. Kaufmann, A., et al., *Increase of CCR1 and CCR5 expression and enhanced functional response to MIP-1 alpha during differentiation of human monocytes to macrophages.* J Leukoc Biol, 2001. **69**(2): p. 248-52.
217. Weber, C., et al., *Specialized roles of the chemokine receptors CCR1 and CCR5 in the recruitment of monocytes and T(H)1-like/CD45RO(+) T cells.* Blood, 2001. **97**(4): p. 1144-6.

218. Eash, K.J., et al., *CXCR2 and CXCR4 antagonistically regulate neutrophil trafficking from murine bone marrow.* J Clin Invest, 2010. **120**(7): p. 2423-31.
219. Nagase, H., et al., *Cytokine-mediated regulation of CXCR4 expression in human neutrophils.* J Leukoc Biol, 2002. **71**(4): p. 711-7.
220. Adrover, J.M., J.A. Nicolas-Avila, and A. Hidalgo, *Aging: A Temporal Dimension for Neutrophils.* Trends Immunol, 2016. **37**(5): p. 334-45.
221. Casanova-Acebes, M., et al., *Rhythmic modulation of the hematopoietic niche through neutrophil clearance.* Cell, 2013. **153**(5): p. 1025-35.
222. Iida, S., et al., *Identification of CCR2, flotillin, and gp49B genes as new G-CSF targets during neutrophilic differentiation.* J Leukoc Biol, 2005. **78**(2): p. 481-90.
223. Fujimura, N., et al., *CCR2 inhibition sequesters multiple subsets of leukocytes in the bone marrow.* Sci Rep, 2015. **5**: p. 11664.
224. Lieschke, G.J., et al., *Mice lacking granulocyte colony-stimulating factor have chronic neutropenia, granulocyte and macrophage progenitor cell deficiency, and impaired neutrophil mobilization.* Blood, 1994. **84**(6): p. 1737-46.
225. Liu, F., et al., *Impaired production and increased apoptosis of neutrophils in granulocyte colony-stimulating factor receptor-deficient mice.* Immunity, 1996. **5**(5): p. 491-501.
226. Stark, M.A., et al., *Phagocytosis of apoptotic neutrophils regulates granulopoiesis via IL-23 and IL-17.* Immunity, 2005. **22**(3): p. 285-94.
227. Cua, D.J. and C.M. Tato, *Innate IL-17-producing cells: the sentinels of the immune system.* Nat Rev Immunol, 2010. **10**(7): p. 479-89.
228. Buonocore, S., et al., *Innate lymphoid cells drive interleukin-23-dependent innate intestinal pathology.* Nature, 2010. **464**(7293): p. 1371-5.
229. Yu, J.J. and S.L. Gaffen, *Interleukin-17: a novel inflammatory cytokine that bridges innate and adaptive immunity.* Front Biosci, 2008. **13**: p. 170-7.
230. Iida, S., et al., *Essential role of C/EBPalpha in G-CSF-induced transcriptional activation and chromatin modification of myeloid-specific genes.* Genes Cells, 2008. **13**(4): p. 313-27.
231. Zhang, P., et al., *Upregulation of interleukin 6 and granulocyte colony-stimulating factor receptors by transcription factor CCAAT enhancer binding protein alpha (C/EBP alpha) is critical for granulopoiesis.* J Exp Med, 1998. **188**(6): p. 1173-84.
232. Johansen, L.M., et al., *c-Myc is a critical target for c/EBPalpha in granulopoiesis.* Mol Cell Biol, 2001. **21**(11): p. 3789-806.
233. Croker, B.A., et al., *SOCS3 is a critical physiological negative regulator of G-CSF signaling and emergency granulopoiesis.* Immunity, 2004. **20**(2): p. 153-65.
234. Kreisel, D., et al., *Bcl3 prevents acute inflammatory lung injury in mice by restraining emergency granulopoiesis.* J Clin Invest, 2011. **121**(1): p. 265-76.
235. Wulczyn, F.G., M. Naumann, and C. Scheidereit, *Candidate proto-oncogene bcl-3 encodes a subunit-specific inhibitor of transcription factor NF-kappa B.* Nature, 1992. **358**(6387): p. 597-9.
236. Dechend, R., et al., *The Bcl-3 oncoprotein acts as a bridging factor between NF-kappaB/Rel and nuclear co-regulators.* Oncogene, 1999. **18**(22): p. 3316-23.
237. Zhang, H., et al., *STAT3 controls myeloid progenitor growth during emergency granulopoiesis.* Blood, 2010. **116**(14): p. 2462-71.
238. Hirai, H., et al., *C/EBPbeta is required for 'emergency' granulopoiesis.* Nat Immunol, 2006. **7**(7): p. 732-9.

239. Dranoff, G., *Cytokines in cancer pathogenesis and cancer therapy.* Nat Rev Cancer, 2004. **4**(1): p. 11-22.
240. Ueda, Y., et al., *IL-1R type I-dependent hemopoietic stem cell proliferation is necessary for inflammatory granulopoiesis and reactive neutrophilia.* J Immunol, 2009. **182**(10): p. 6477-84.
241. Strauss, L., et al., *RORC1 Regulates Tumor-Promoting "Emergency" Granulo-Monocytopoiesis.* Cancer Cell, 2015. **28**(2): p. 253-69.
242. Amulic, B., et al., *Neutrophil function: from mechanisms to disease.* Annu Rev Immunol, 2012. **30**: p. 459-89.
243. Kolaczkowska, E. and P. Kubes, *Neutrophil recruitment and function in health and inflammation.* Nat Rev Immunol, 2013. **13**(3): p. 159-75.
244. Vincent, P.C., et al., *The intravascular survival of neutrophils labeled in vivo.* Blood, 1974. **43**(3): p. 371-7.
245. Cheretakis, C., et al., *Timing of neutrophil tissue repopulation predicts restoration of innate immune protection in a murine bone marrow transplantation model.* Blood, 2006. **108**(8): p. 2821-6.
246. Colotta, F., et al., *Modulation of granulocyte survival and programmed cell death by cytokines and bacterial products.* Blood, 1992. **80**(8): p. 2012-20.
247. Scapini, P., et al., *Human neutrophils in the saga of cellular heterogeneity: insights and open questions.* Immunol Rev, 2016. **273**(1): p. 48-60.
248. Marvel, D. and D.I. Gabrilovich, *Myeloid-derived suppressor cells in the tumor microenvironment: expect the unexpected.* J Clin Invest, 2015. **125**(9): p. 3356-64.
249. Sagiv, J.Y., et al., *Phenotypic diversity and plasticity in circulating neutrophil subpopulations in cancer.* Cell Rep, 2015. **10**(4): p. 562-73.
250. Beyrau, M., J.V. Bodkin, and S. Nourshargh, *Neutrophil heterogeneity in health and disease: a revitalized avenue in inflammation and immunity.* Open Biol, 2012. **2**(11): p. 120134.
251. Tsuda, Y., et al., *Three different neutrophil subsets exhibited in mice with different susceptibilities to infection by methicillin-resistant Staphylococcus aureus.* Immunity, 2004. **21**(2): p. 215-26.
252. Granot, Z., et al., *Tumor entrained neutrophils inhibit seeding in the premetastatic lung.* Cancer Cell, 2011. **20**(3): p. 300-14.
253. Pillay, J., et al., *A subset of neutrophils in human systemic inflammation inhibits T cell responses through Mac-1.* J Clin Invest, 2012. **122**(1): p. 327-36.
254. Hao, S., M. Andersen, and H. Yu, *Detection of immune suppressive neutrophils in peripheral blood samples of cancer patients.* Am J Blood Res, 2013. **3**(3): p. 239-45.
255. Hartl, D., et al., *Infiltrated neutrophils acquire novel chemokine receptor expression and chemokine responsiveness in chronic inflammatory lung diseases.* J Immunol, 2008. **181**(11): p. 8053-67.
256. Buckley, C.D., et al., *Identification of a phenotypically and functionally distinct population of long-lived neutrophils in a model of reverse endothelial migration.* J Leukoc Biol, 2006. **79**(2): p. 303-11.
257. Mantovani, A., et al., *Neutrophils in the activation and regulation of innate and adaptive immunity.* Nat Rev Immunol, 2011. **11**(8): p. 519-31.
258. Bonavita, O., M. Massara, and R. Bonecchi, *Chemokine regulation of neutrophil function in tumors.* Cytokine Growth Factor Rev, 2016. **30**: p. 81-6.
259. Fridlender, Z.G., et al., *Polarization of tumor-associated neutrophil phenotype by TGF-beta: "N1" versus "N2" TAN.* Cancer Cell, 2009. **16**(3): p. 183-94.

260. Finisguerra, V., et al., *MET is required for the recruitment of anti-tumoural neutrophils.* Nature, 2015. **522**(7556): p. 349-53.
261. Jablonska, J., et al., *Neutrophils responsive to endogenous IFN-beta regulate tumor angiogenesis and growth in a mouse tumor model.* J Clin Invest, 2010. **120**(4): p. 1151-64.
262. Mishalian, I., et al., *Tumor-associated neutrophils (TAN) develop pro-tumorigenic properties during tumor progression.* Cancer Immunol Immunother, 2013. **62**(11): p. 1745-56.
263. Tabaries, S., et al., *Granulocytic immune infiltrates are essential for the efficient formation of breast cancer liver metastases.* Breast Cancer Res, 2015. **17**: p. 45.
264. Eruslanov, E.B., et al., *Tumor-associated neutrophils stimulate T cell responses in early-stage human lung cancer.* J Clin Invest, 2014. **124**(12): p. 5466-80.
265. Shen, M., et al., *Tumor-associated neutrophils as a new prognostic factor in cancer: a systematic review and meta-analysis.* PLoS One, 2014. **9**(6): p. e98259.
266. Dumitru, C.A., S. Lang, and S. Brandau, *Modulation of neutrophil granulocytes in the tumor microenvironment: mechanisms and consequences for tumor progression.* Semin Cancer Biol, 2013. **23**(3): p. 141-8.
267. Wislez, M., et al., *Hepatocyte growth factor production by neutrophils infiltrating bronchioloalveolar subtype pulmonary adenocarcinoma: role in tumor progression and death.* Cancer Res, 2003. **63**(6): p. 1405-12.
268. Nozawa, H., C. Chiu, and D. Hanahan, *Infiltrating neutrophils mediate the initial angiogenic switch in a mouse model of multistage carcinogenesis.* Proc Natl Acad Sci U S A, 2006. **103**(33): p. 12493-8.
269. Queen, M.M., et al., *Breast cancer cells stimulate neutrophils to produce oncostatin M: potential implications for tumor progression.* Cancer Res, 2005. **65**(19): p. 8896-904.
270. Tazawa, H., et al., *Infiltration of neutrophils is required for acquisition of metastatic phenotype of benign murine fibrosarcoma cells: implication of inflammation-associated carcinogenesis and tumor progression.* Am J Pathol, 2003. **163**(6): p. 2221-32.
271. Ruan, D. and S.P. So, *Prostaglandin E2 produced by inducible COX-2 and mPGES-1 promoting cancer cell proliferation in vitro and in vivo.* Life Sci, 2014. **116**(1): p. 43-50.
272. Houghton, A.M., et al., *Neutrophil elastase-mediated degradation of IRS-1 accelerates lung tumor growth.* Nat Med, 2010. **16**(2): p. 219-23.
273. Wculek, S.K. and I. Malanchi, *Neutrophils support lung colonization of metastases-initiating breast cancer cells.* Nature, 2015. **528**(7582): p. 413-7.
274. Cools-Lartigue, J., et al., *Neutrophil extracellular traps sequester circulating tumor cells and promote metastases.* J Clin Invest, 2013.
275. Rotondo, R., et al., *IL-8 induces exocytosis of arginase 1 by neutrophil polymorphonuclears in nonsmall cell lung cancer.* Int J Cancer, 2009. **125**(4): p. 887-93.
276. He, G., et al., *Peritumoural neutrophils negatively regulate adaptive immunity via the PD-L1/PD-1 signalling pathway in hepatocellular carcinoma.* J Exp Clin Cancer Res, 2015. **34**: p. 141.
277. Aoyagi, Y., et al., *Overexpression of TGF-beta by infiltrated granulocytes correlates with the expression of collagen mRNA in pancreatic cancer.* Br J Cancer, 2004. **91**(7): p. 1316-26.

278. Yan, J., et al., *Human polymorphonuclear neutrophils specifically recognize and kill cancerous cells.* Oncoimmunology, 2014. **3**(7): p. e950163.
279. Cassatella, M.A., et al., *Interferon-activated neutrophils store a TNF-related apoptosis-inducing ligand (TRAIL/Apo-2 ligand) intracellular pool that is readily mobilizable following exposure to proinflammatory mediators.* J Leukoc Biol, 2006. **79**(1): p. 123-32.
280. Kemp, T.J., et al., *Neutrophil stimulation with Mycobacterium bovis bacillus Calmette-Guerin (BCG) results in the release of functional soluble TRAIL/Apo-2L.* Blood, 2005. **106**(10): p. 3474-82.
281. Chen, Y.L., et al., *Fas ligand on tumor cells mediates inactivation of neutrophils.* J Immunol, 2003. **171**(3): p. 1183-91.
282. Suttmann, H., et al., *Neutrophil granulocytes are required for effective Bacillus Calmette-Guerin immunotherapy of bladder cancer and orchestrate local immune responses.* Cancer Res, 2006. **66**(16): p. 8250-7.
283. Verbeke, H., et al., *Isotypic neutralizing antibodies against mouse GCP-2/CXCL6 inhibit melanoma growth and metastases.* Cancer Lett, 2011. **302**(1): p. 54-62.
284. Jablonska, J., et al., *CXCR2-mediated tumor-associated neutrophil recruitment is regulated by IFN-beta.* Int J Cancer, 2014. **134**(6): p. 1346-58.
285. Lopez-Lago, M.A., et al., *Neutrophil chemokines secreted by tumor cells mount a lung antimetastatic response during renal cell carcinoma progression.* Oncogene, 2013. **32**(14): p. 1752-60.
286. Bonavita, O., et al., *Flow Cytometry Detection of Chemokine Receptors for the Identification of Murine Monocyte and Neutrophil Subsets.* Methods Enzymol, 2016. **570**: p. 441-56.
287. Baba, T. and N. Mukaida, *Role of macrophage inflammatory protein (MIP)-1alpha/CCL3 in leukemogenesis.* Mol Cell Oncol, 2014. **1**(1): p. e29899.
288. Allende, M.L., et al., *S1P1 receptor directs the release of immature B cells from bone marrow into blood.* J Exp Med, 2010. **207**(5): p. 1113-24.
289. Engblom, C., C. Pfirschke, and M.J. Pittet, *The role of myeloid cells in cancer therapies.* Nat Rev Cancer, 2016. **16**(7): p. 447-62.
290. Zhang, M., et al., *A high M1/M2 ratio of tumor-associated macrophages is associated with extended survival in ovarian cancer patients.* J Ovarian Res, 2014. **7**: p. 19.
291. Almatroodi, S.A., et al., *Characterization of M1/M2 Tumour-Associated Macrophages (TAMs) and Th1/Th2 Cytokine Profiles in Patients with NSCLC.* Cancer Microenviron, 2016. **9**(1): p. 1-11.
292. D'Alterio, C., et al., *Inhibition of stromal CXCR4 impairs development of lung metastases.* Cancer Immunol Immunother, 2012. **61**(10): p. 1713-20.

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