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TESI DI DOTTORATO DI RICERCA

Role of CCL2 and its receptors CCR2 and D6 in the activation and polarization of tumor-associated macrophages

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Summary

Chemokines are well known to play a major role in tumor progression and metastasis. In particular CCL2 is over-expressed in several human cancers and their higher levels correlate with poor prognosis and shorter outcomes. Here we reported two different studies in which CCL2 receptors, the canonical CCR2 and the atypical D6 (or ACKR2) were examined for their involvement in tumor progression. In particular D6 was investigated for its expression and its ability to shape CCL2 gradient in Kaposi's sarcoma, whereas CCR2 has been analyzed as potential modeler of TAM polarization.

D6 is an atypical chemokine receptor acting as a decoy and scavenger for inflammatory CC chemokines expressed in lymphatic endothelial cells. Here, we report that D6 is also expressed by Kaposi's sarcoma (KS) which is a tumor ontogenetically related to lymphatic endothelium, yet its role in tumor progression was hitherto unknown. D6 expression was evaluated by immunohistochemistry in a cohort of KS patients and its role in cancer progression was investigated in an *in vivo* KS model. Both in human tumors and in the experimental model, D6 expression levels were inversely correlated with tumor aggressiveness, and directly correlated with increased chemokine-driven infiltration of macrophages and their acquisition of a pro-angiogenic phenotype. Inhibition of monocyte recruitment reduced growth of D6-incompetent tumors, while adoptive transfer of wt but not $CCR2^{-/-}$ macrophages increased the growth rate of D6-competent neoplasms. In the KS model, which presents the B-Raf V600E activating mutation, inhibition of B-Raf or

downstream ERK pathway induced D6 expression, and in progressing human KS tumors activation of the K-Ras/B-Raf/ERK pathway correlate with reduced levels of D6 expression. These results indicate that activation of the K-Ras/B-Raf/ERK pathway during KS progression down-regulates D6 expression, which unleashes chemokine-mediated macrophage recruitment and their acquisition of an M2-like phenotype supporting angiogenesis and tumor growth.

Thereafter, we wanted to deeper investigate how CCR2 support TAM M2 polarization firstly by using an *in vitro* system. Wt and $CCR2^{-/-}$ macrophages were polarized with M1 and M2 stimuli and analyzed for gene expression and cytokines production. While no difference was found in M2 polarized macrophages, $CCR2^{-/-}$ M1 or LPS activated macrophages showed higher expression of inflammatory genes and reduced production of the anti-inflammatory cytokine IL-10 and of the pro-angiogenic cytokine VEGF when compared to wt macrophages. The impaired IL-10 production was also confirmed by treating human monocytes with the CCR2 antagonist RS-504393. After LPS stimulation, $CCR2^{-/-}$ macrophages showed reduced activation of NF-kB and p38 MAPK when compared to wt macrophages indicating a cross talk between CCR2 and TLR4 signaling pathways. The contribution of CCR2 to cancer growth was evaluated with a transplantable lung cancer model that grew slower when co-injected with $CCR2^{-/-}$ macrophages, presenting a marked M1 phenotype of infiltrating TAM and a higher number of both CD4+ and CD8+ T cells, correlated with a decreased number of splenic T regulatory cells when compared to wt macrophages holding-tumors. Taken together these data indicate that CCR2 expression by macrophages not only induce their recruitment to tumor site but also affect their polarization and anti-tumor potential.

1. Introduction

1.1. The mononuclear phagocyte system

The monocyte-macrophage system has been first described by Metchnikoff in the late 19th century, depicting them as phagocytes, mesodermal cells able to internalize and digest bacteria and other cells [1]. In the 1970s Van Furth and Cohn established the mononuclear phagocyte system (MPS), a linear model proposing that committed bone marrow (BM) precursors differentiate into blood monocytes from which tissue macrophages derive, in the steady state and during inflammation [2]. In agreement with this model, circulating monocytes has been described to extravasate from the blood and give rise to inflammatory macrophages in response to inflammatory signals or other stimuli, like tumor progression and wound healing [3], [4], [5]. However, this model has always been disputed because of a large number of observations contrasting macrophages belonging to MPS. In fact from the literature it is known that during embryogenesis macrophages are already present in the yolk sac before primitive hematopoiesis [6, 7]. Moreover tissue-resident macrophages are not affected in monocytopenic mice [6, 8], do not exchange in parabiotic mice [9], [10] and are only partially replaced after irradiation and bone marrow replacement [11]. Concluding, now it has been established the existence of two models of myeloid cell differentiation that coexist during embryogenesis and adulthood.

1.1.1. Monocyte and macrophage ontogenesis

Adoptive transfer experiments and analysis of syngeneic BM transplantation have clearly shown that circulating monocytes, some macrophage subsets and the classical dendritic cell (DC) lineage [12] originate in vivo from hematopoietic stem cells (HSC). In murine hematopoiesis, the multipotent capability resides in a small fraction of BM cells called LSK, characterized as Lin- Sca-1+ c-kit+ (Figure 1.1). Within the LSK fraction it is possible to distinguish, by CD34 staining, the most primitive self-renewing HSC which have a long term reconstituting activity and are CD34-. The other components of LSK are CD34+ multipotent progenitors (MPP) only capable of a transient reconstitution [13]. Successive committed step in the BM include common lymphoid progenitor (CLP) and common myeloid progenitor (CMP) (Figure 1.1). The last one is characterized as IL-7Rα- c-Kit+ Lin- Sca-1- and can be further fractionated on the basis of the expression of FcγRII and FcγRIII (CD16/CD32) and CD34. CMP (FcγRII/III^{lo} CD34+) differentiate into granulocyte-macrophage precursors (GMPs: FcγRII/III^{hi} CD34+) and megakaryocyte-erythrocyte precursors (MEP: FcγRII/III^{lo} CD34-)[14], [15]. Subsequently CMP differentiate into a monocyte/macrophage and DC precursor (MDP), a subset of proliferating cells in the BM that give rise to monocytes and to the common DC precursor (CDP). Of note, the development of monocytes is dependent on the growth factor receptor Csf-1R (MCSF-R or CD115) [16], [17], indeed Csf1r deficiency causes a reduced number of circulating monocytes [18]. Recently, gene invalidation in mice allowed the identification of genes required for generation and maintenance of progenitor and hematopoietic stem cells. One of this genes is the transcription factor *Myb*, required for the development of HSPC and monocytes, macrophages and DC [19], [20].

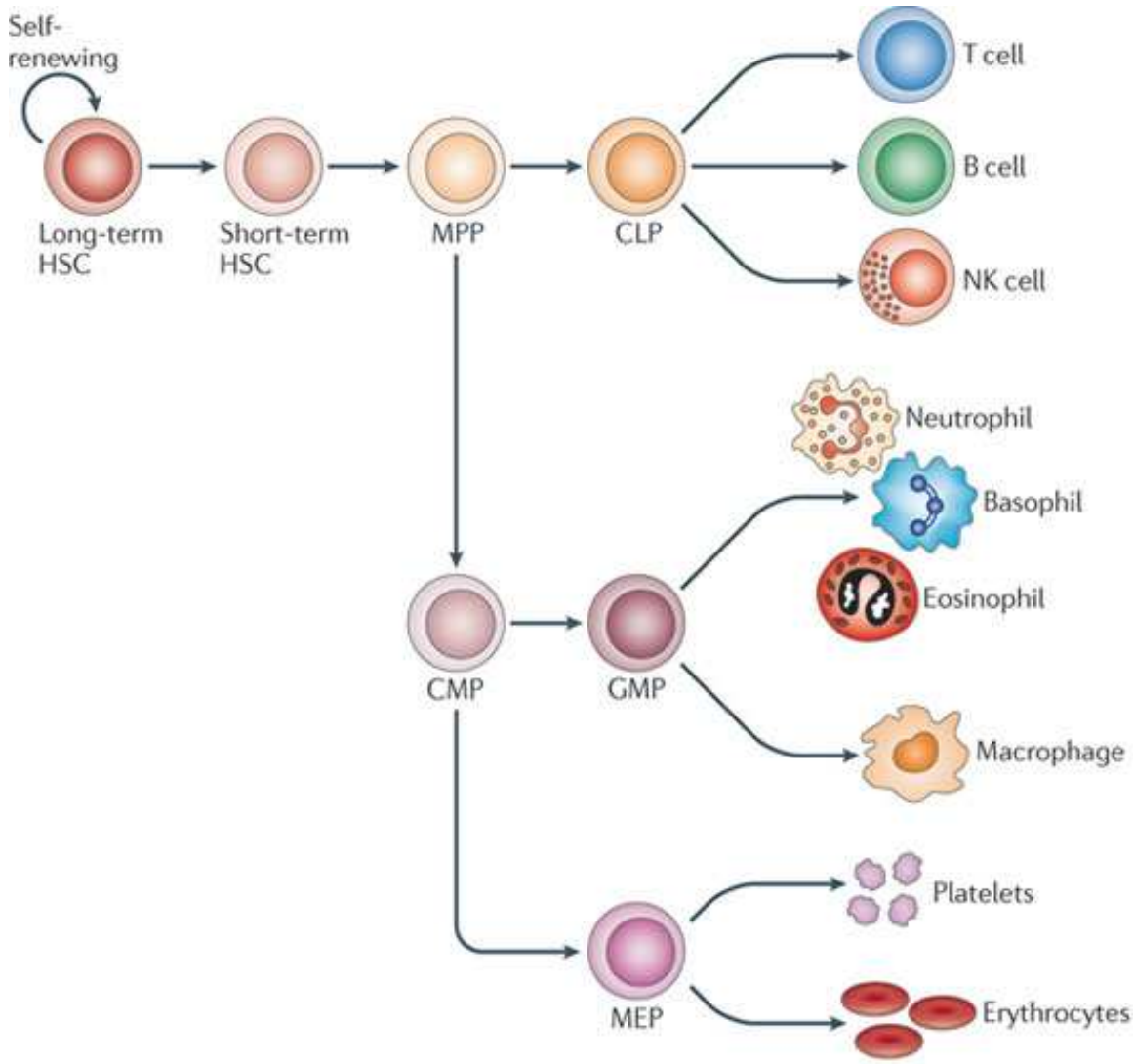

Figure 1.1 - The hematopoietic tree. Hematopoietic stem cells (HSCs) have the capacity to self-renew and to give rise to all the cell types of the bone marrow and peripheral blood. Other pluripotent progenitors, short-term HSCs and multipotent progenitors (MPPs) have less self-renewal capacity. Together, these three cell types constitute the hematopoietic stem and progenitor cell (HSPC) population. MPPs are thought to differentiate into the two main branches of hematopoietic development that arise from the common lymphoid progenitor (CLP) and the common myeloid progenitor (CMP). Mature peripheral blood cells, shown on the right, are derived from these progenitors. GMP, granulocyte and macrophage progenitor; MEP, megakaryocyte and erythrocyte progenitor; NK, natural killer [21].

In contrast to myeloid cells produced continually by the BM, tissue-resident macrophages develop in the embryo before the appearance of HSC and persist in adulthood [22]. In mice, macrophages start to develop at embryonic day 8 from the primitive ectoderm of the yolk sac and generate macrophages that do not have a monocytic progenitor and can be distinguished in several tissues as "F4/80 bright", for example liver Kupffer cells, epidermal Langerhans cells and microglia (Figure 1.2) [20]. Schulz et al [20]recently demonstrated, by fate-mapping studies, that this macrophages arise from precursor cells in the yolk sac independently from the transcription factor *Myb* whereas they are mainly regulated by CSF1R and its ligands, IL-34 and CSF1 [23]. Proliferative local expansion of tissue macrophages in neonatal period, followed by low-level of self-renewal during adulthood maintain tissue resident populations. Indeed two independent groups confirmed that tissue macrophages are able to self-renew independently from monocytes or BM progenitors [24], [25]. Nevertheless, experimental ablation of resident macrophages demonstrated the ability of BM-derived cells to replace self-renewing resident cells but it is not clear if these converted cells fully recapitulate the function of those replaced [26].

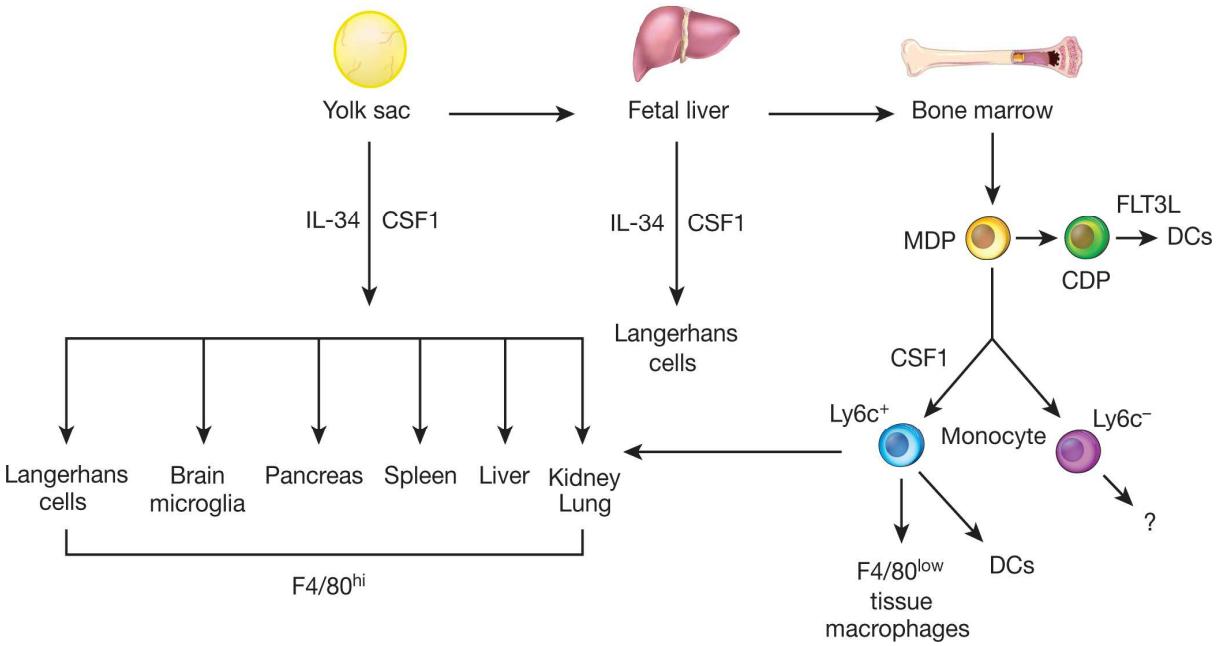

Figure 1.2 - A redefined model of macrophage lineages in mice. The mononuclear phagocytic system derives from at least two sources. The first is the yolk sac, which produces progenitors that populate

all tissues and that have progeny that persist throughout life as F4/80 bright resident macrophages. These lineages are mainly regulated by CSF1R and its ligands, IL-34 and CSF1. Moreover progenitors from the yolk sac can seed in the fetal liver and contribute to the production of adult Langerhans cells. The second lineage derives from the bone marrow (BM) to give circulating monocytes and their progeny F4/80^{low} macrophages, and dendritic cells (DCs). Other macrophages that are F4/80^{low} also emanate from Ly6C^{+} monocytes, and in some cases, such as in kidney and lung, they co-exist with those derived from the yolk sac to give chimeric organs. The exact role of the patrolling Ly6C^{-} macrophages, and the contribution of fetal liver to adult tissue macrophages, remain unclear. CDP, committed dendritic cell progenitor; MDP, monocyte dendritic cell progenitor [27].

1.1.2. Monocytes

Monocytes are a heterogeneous cell population consisting in several functional subsets that can be divided on the basis of chemokine receptor expression and the presence of specific surface molecules. In mice, they are classified in classical "inflammatory" and non-classical "resident" monocytes [5], [28], [4] (Figure 1.3). Classical monocytes, representing approximately 2-5% of circulating leukocytes, express high levels of Ly6C (Ly6C^{high}), the chemokine receptor CCR2, the adhesion molecule L-selectin (CD62L) and low levels of CX_3CR1. They owes the name to their pro-inflammatory phenotype since they secrete inflammatory cytokines, like TNF-α and IL-1 during infections or tissue damage and are selectively recruited to inflamed tissues and lymph nodes in vivo [28], [4]. Moreover upon inflammatory stimuli they translocate from the bone marrow into circulation. BM egress is CCR2-dependent but may also involve other pathways such as CXCR4/CXCL12 and when inflammatory signals hold off, Ly6C^{high} monocytes shuttle back to the bone marrow and down regulate CCR2 [3, 29]. During inflammatory events, Ly6C^{high} monocytes can differentiate into a variety of macrophages and DCs. Indeed during infection with *Listeria monocytogenes*, Ly6C^{high} monocytes have been reported to differentiate into DCs that produce inflammatory mediators such as TNF-α, nitric oxide and reactive oxygen species, known as TipDC [29], [30], whereas in several tumors, up to a half of myeloid derived suppressor cells (MDSC), cells that promote tumor growth by suppressing T cell response, are Ly6C^{high} [31].

The second subset of murine monocytes is characterized by high expression of CX_3CR1 and lack of expression of Ly6C ($Ly6C^{low}$), CCR2 and L-selectin [5, 28]. Firstly, they were called "resident" because of their long half-life in vivo and their localization in both resting and inflamed tissues after adoptive transfer [5]. Intravital microscopy revealed that they patrol blood vessels by crawling across the endothelium, suggesting that they are ideally located to survey endothelial cells and surrounding tissues for damage or infections [32]. Moreover, transcriptional profiling of extravaseted monocytes after *Listeria monocytogenes* infection show that $Ly6C^{low}$ monocytes initiate a macrophage differentiation program resembling the one describing for "alternative activated macrophages"[33]. Finally adoptive transfer experiments demonstrate that $Ly6C^{high}$ monocytes can shuttle between the blood and the bone marrow and lose Ly6C expression, suggesting that they give rise to $Ly6C^{low}$ monocytes [34, 35]. However, gene ablation and antibody-mediated depletion of $Ly6C^{high}$ monocytes did not affected the generation of $Ly6C^{low}$ monocytes [11, 36-38].

In humans, three subset of circulating monocytes are defined: classical monocytes (CD14++CD16-), intermediate (CD14+CD16+) and non-classical monocytes ($CD14^{low}$CD16+) (Figure 1.3) [39, 40]. Classical monocytes represent 80-90% of circulating monocytes and, expressing high levels of CCR2, have a phenotype resembling $Ly6C^{high}$ murine monocytes but, after LPS stimulation, they release the immune suppressing cytokine IL-10 [41, 42]. In turn, intermediate and non-classical monocytes express high levels of CX_3CR1 a low levels of CCR2. CD14+CD16+ also express Fc receptors CD64 and CD32 have high phagocytic activity and produce TNF-α and IL-1 in response to LPS, whereas non-conventional monocytes are poorly phagocytic but are more potent antigen presenting cells [43, 44].

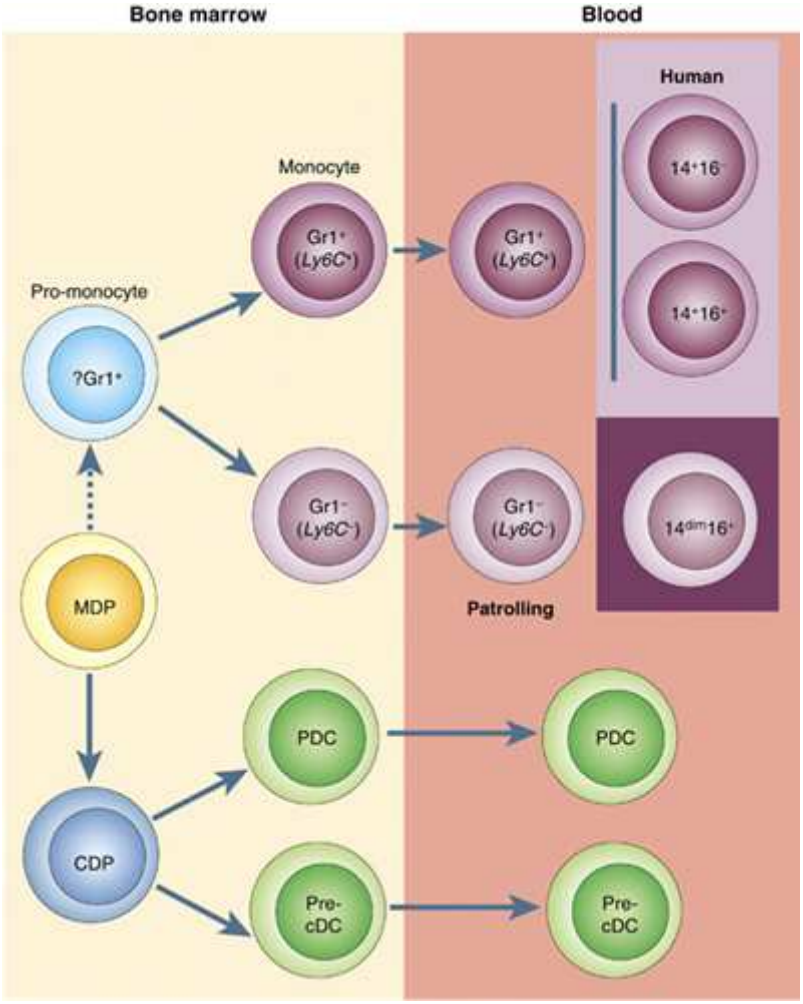

Figure 1.3 – Murine and human monocyte classification. Hematopoietic stem cells produce a monocyte, macrophage and dendritic cell precursor (MDP) in the bone marrow via a myeloid committed precursor (CMP). MDPs give rise to monocytes, possibly through a Gr1+ pro-monocyte (dashed line), and pre-classical dendritic cells (Pre-cDC) and plasmacytoid dendritic cells (PDC) via a common dendritic cell precursor (CDP). In the mouse, two monocyte subsets Ly6C^{high} and Ly6C^{low} leave the bone marrow to enter the circulation. The corresponding human monocyte subsets are shown in the light and dark purple boxes: inflammatory murine Ly6C^{high} and two human CD14+ subsets; and murine Ly6C^{low} and human CD14^{low} monocyte subsets. CD14+ monocytes respond to cells surface TLRs and are involved in inflammation and leukocyte recruitment, while CD14^{low} monocytes sense tissue damage and respond to viruses and nucleic acids via endosomal TLRs (TLR7,8) [45].

1.1.3. Macrophages

Mature macrophages are strategically located throughout the body and, reflecting the specialization of function that they adopted in different anatomical locations, they perform different roles in clearance, development, regulation of metabolism and an important immune surveillance function. For example, alveolar macrophages facilitate the removal of allergens from the lung [46-48], Kupffer cells participate in the clearance of pathogens and toxins from circulation and adipose tissue macrophage regulate insulin sensitivity and adaptive thermogenesis (Figure 1.4) [2]. Moreover, the gut is one of the richest sources of macrophages in the body, and isolation of macrophages from the lamina propria has highlighted a unique macrophage phenotype, characterized by high phagocytic and bactericidal activity but weak production of pro-inflammatory cytokines. This phenotype can be induced in peripheral-blood-derived macrophages by intestinal stromal-cell products, indicating that the tissue microenvironment can markedly influence the phenotype of tissue-resident macrophages [49].

1.1.3.1. Macrophage functions

Owing to their strategic location, closed to sites of injury, tissue resident macrophages play a central role in the innate recognition of a range of pathogens, by mounting proper and effective immune response. In fact, after pathogen recognition, tissue macrophages through inflammatory chemokines production, drive the influx of leukocytes, mainly neutrophils but also monocytes as precursors of inflammatory macrophages [50]. These monocyte-derived macrophages rapidly dominate many inflammatory lesions, being in greater number than resident macrophages. They show an inflammatory phenotype and secrete pro-inflammatory mediators as TNFα, NO and IL-1 but the excessive inflammation must be balance in order to protect tissue integrity [51]. Indeed, uptake of apoptotic neutrophils can stimulate macrophages to produce anti-inflammatory molecules, like IL-10, TGFβ, PGE_2 and VEGF which not only prevent the killing of tissue-resident cells but also contribute to repair after tissue injury. Moreover, it has been demonstrated that during

helminthic infection, IL-4 supports tissue-resident macrophages proliferation and consequently causes their accumulation resulting in the worm expulsion [52].

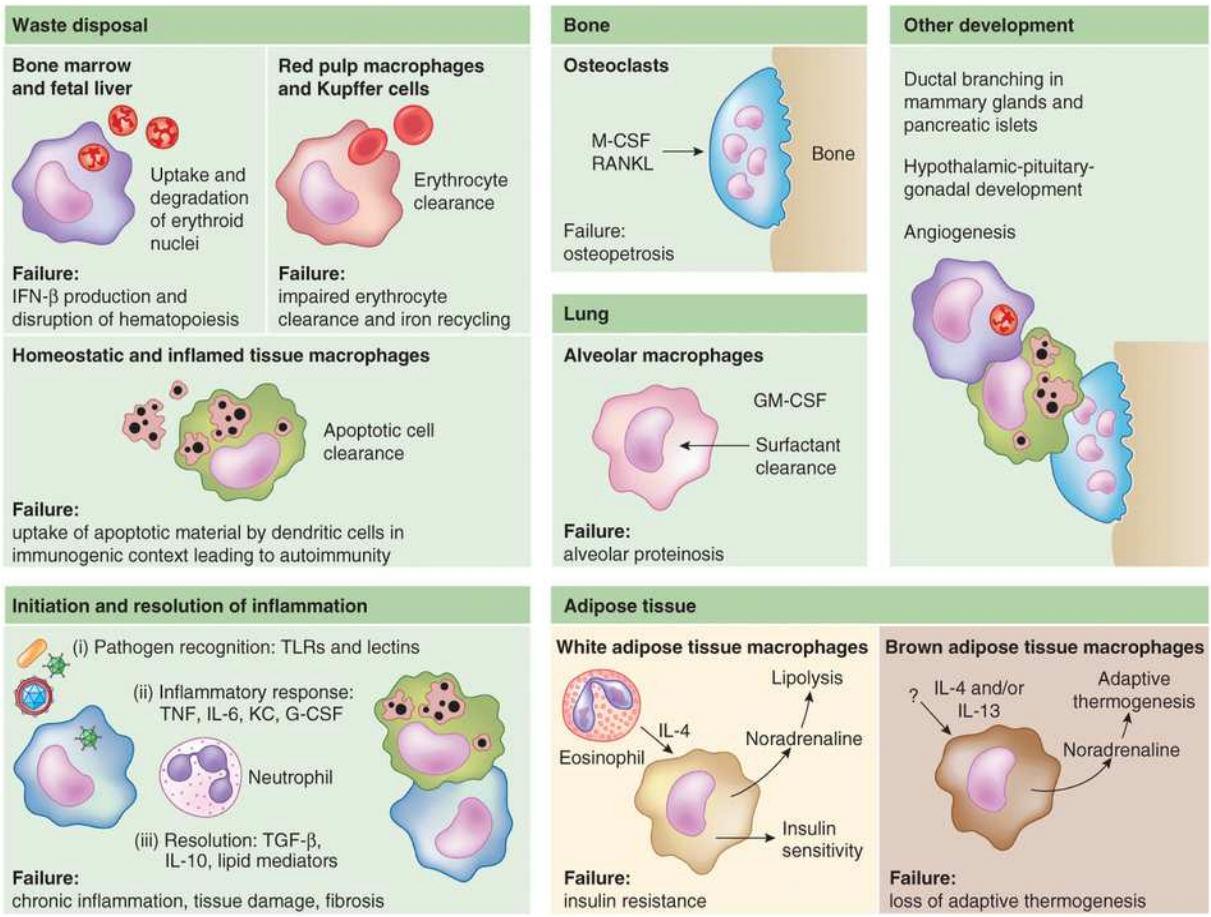

Figure 1.4 – **Different functions of tissue-resident macrophages**. Tissue-resident macrophages have broad roles in clearance (degradation of erythroid nuclei, senescent erythrocytes, apoptotic cells and pulmonary surfactant), development (bone degradation and angiogenesis) and the regulation of metabolism (regulation of insulin sensitivity and adaptive thermogenesis in adipose tissue). They also have a fundamental role as an immune sentinel, initiating inflammatory responses, clearing inflammatory debris and restoring homeostatic tissue environments [2].

1.1.3.2. Macrophage polarization

Macrophages have been functionally grouped in two classes: M1 or “classically activated” and M2 or “alternatively activated” (Figure 1.5). Classically activated macrophages develop in response to NK and Th1 product IFN-γ, in concomitant exposure to microbes or microbial products such as LPS [53]. The hallmark of classically activated macrophages is the increased secretion of pro-inflammatory cytokines and enhanced ability to kill intracellular pathogens [54]. Their inflammatory repertoire is characterized by the secretion of pro-inflammatory mediators, like TNFα, IL-6, IL-12, IL-1β, inflammatory chemokines as CCL2, CCL5 and CXCL8, and the release of reactive oxygen and nitrogen intermediates (ROI and RNI) [55, 56]. Other genes associated to M1 phenotype encode the enzyme indoleamine-pyrrole 2,3 dioxygenase and NO synthase 2 (iNOS2) [57] which are involved in microbicidal activity and co-stimulatory molecules such as CD80 and CD86 [58]. On the contrary alternatively activated macrophages have anti-inflammatory function and regulate wound healing. They have recently been divided into three groups: M2a, induced by IL-4 and IL-13; M2b induced by immune complexes and agonists of TLRs or IL-1 receptors; and M2c, induced by IL-10 and glucocorticoid hormones [59]. M2 macrophages produce anti-inflammatory cytokines like IL-10, IL-19 and IL-20 and are characterized by the up-regulation of Yim1/2 and mannose receptor whereas down-regulate inflammatory cytokines [60]. Moreover they express high levels of the enzyme arginase-1 (ARG1) and down-regulate iNOS2, thus skewing the metabolic pathway of NO to the production of proline. Consequently these cells fail to produce NO and are significantly compromised in their microbicidal ability for intracellular pathogens, compared to M1 activated macrophages (although they may acquire microbicidal activity to other pathogens) [61]. Alternatively activated macrophages also produce growth factors that stimulate epithelial cells and fibroblasts, including transforming growth factor-β1 (TGFβ1) and PDGF which contribute to tissue regeneration and wound repair [62].

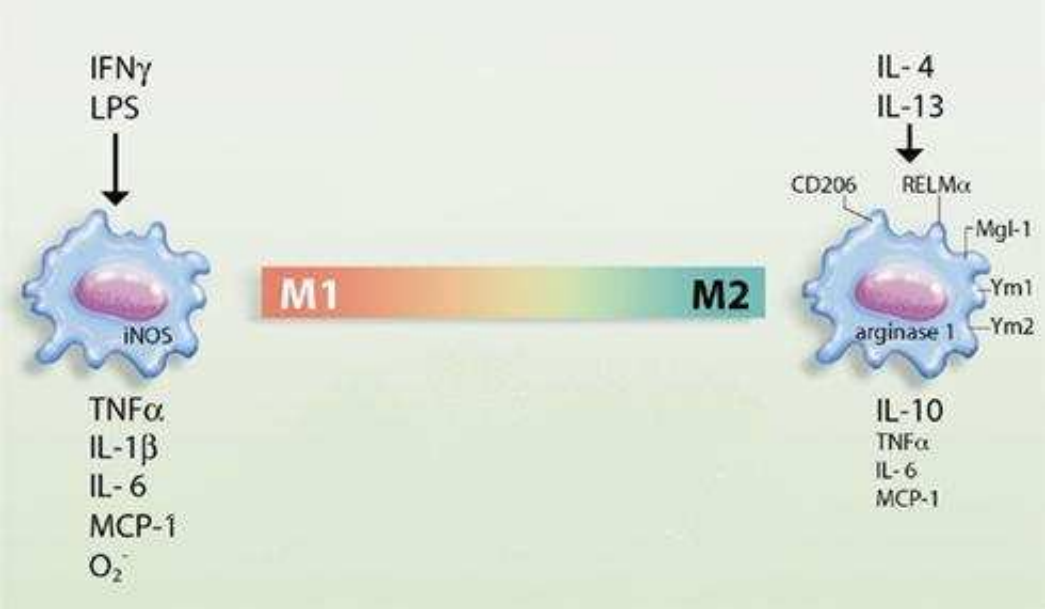

Figure 1.5 – Schematic representation of macrophages polarization. M1 or classically activated macrophages are highly inflammatory and release pro-inflammatory cytokines/chemokines as TNF-α, IL-1β, IL-6, MCP-1 and superoxide anion. These macrophages also express iNOS and are activated by IFN γ and LPS. Conversely, M2 or alternative activated macrophages release high levels of IL-10 in parallel with reduced levels of TNFα, IL-6, and MCP-1. M2 macrophages are alternatively activated macrophages, originally identified after IL-4 and IL-13 stimulation, that up-regulate scavenger, mannose (CD206), RELM-α, and show chitinases Ym1 and Ym2 expression and arginase 1 activity [63].

1.1.3.3. Tumor associated macrophages

Tumor microenvironment is inflammatory and that activation of the immune system plays a role in progression of cancer [64-66]. Indeed, plasticity of macrophage function is well described, and evidences suggest that tumor-associated macrophages (TAM) are involved in complex crosstalk with tumor cells and other cells of tumor microenvironment [67-69]. Moreover there is an extensive literature showing that macrophages are not only important for tumor progression but also in promoting tumor cell survival, angiogenesis and metastatization, in fact they have been demonstrated fundamental for malignant cell migration and invasion in different tumor models [70]. Tumor microenvironment, by the presence of tumor cell products, including extracellular matrix components, IL-10, CSF-1, and chemokines (CCL2, CCL18, CCL17, and CXCL4), is thought to “educate” TAM towards a

tumor-promoting M2 phenotype mediating a switch from M1 to M2 phenotype during tumor progression [71]. Indeed, classically activated M1-polarized macrophages exhibit antitumor activity and elicit tumor tissue disruption [72], whereas TAM mainly display an M2-like phenotype, expressing high levels of IL-10, Mannose receptor and low levels of IL-12. TAM infiltration is generally associated with poor prognosis, as shown in Hodgkin disease, glioma, cholangiocarcinoma, and breast carcinoma [73, 74]. However, TAMs with various functional states can coexist in the same tumor [66, 75].

1.2. Chemokines

Chemotactic cytokines, or *chemo-kines*, are a large subfamily of cytokines that direct the recruitment of circulating leukocytes to sites of inflammation. To date, approximately 50 human chemokines and 20 receptors have been discovered. They are a single polypeptide chain proteins composed of 70-100 aminoacid residues in length, with a molecular weight of 8-12 KDa and 20-95% sequence identity to each other including conserved cysteine residues that have also been used for subfamily definition and nomenclature. A characteristic of the chemokine system is the binding promiscuity of chemokines and chemokine receptors: most chemokine receptors interact with more than one chemokine ligand and most ligands recognize multiple receptors. This applies in particular for the inflammatory chemokines, whereas homeostatic chemokines display a more selective relationship with their receptors [76, 77]. Moreover, chemokines display also high degree redundancy in leukocyte migration, despite the cell-specific expression of defined chemokine receptors. This property is the result of recurrent gene duplications during evolution [78] [79]. Most chemokine genes are clustered in defined chromosomal locations. These clusters originate from repeated duplications of genomic regions and contain genes encoding many ligands which interact with only a selective set of receptors and may have similar effector functions.

1.2.1. Chemokine classification

Chemokines can be classified into subgroups on the basis of their structure or function [78, 80-82]. Looking at the function, they can be divided in inflammatory and homeostatic chemokines. Inflammatory chemokines are inducible and secreted during inflammatory responses after infection or tissue injury and they are responsible for recruitment of effector leukocytes to the site of inflammation. In contrast, homeostatic chemokines are constitutively produced by tissue cells and control basal leukocyte trafficking, such as lymphocyte homing to secondary lymphoid organs and lymphocyte recirculation through peripheral tissues. Some chemokines exert both inflammatory and homeostatic functions, depending on the biological context or pathological state, and are therefore called dual-function chemokines. On the other hand, chemokines and their receptors have been traditionally structurally classified into four subfamilies based on the location of the two NH2-terminal cysteine residues: CC, CXC, C, and CX3C (Figure 1.6). In 2000, a system of nomenclature has been introduced identifying each ligand and receptor by its subfamily and given an identifying number [83, 84]. The bigger family consists of CC chemokines, which have the first two of the four cysteine residues in adjacent positions. CC chemokines mainly attract mononuclear cells, such as monocytes, T and B lymphocytes, to sites of inflammation. The most deeply characterized CC chemokine is CCL2, also called monocyte chemoattractant protein 1 (MCP-1) that is a potent agonist for monocytes, dendritic cells, memory T cells, and basophils. Other CC chemokines include CCL3 (macrophage inflammatory protein (MIP)-1α), CCL4 (MIP-1β) and CCL5 (RANTES) [85]. The second bigger family of chemokines consists of CXC chemokines, which have a single amino acid residue interposed between the first two canonical cysteines. Most CXC chemokines attract polymorphonuclear leukocytes to sites of inflammation. The third family is the CX3C family composed of only one member: fractalkine (CX3CL1) [86] [87]. It is expressed as transmembrane molecules, having the chemokine domain fused to a mucin-like stalk. It can be cleft by tumor necrosis factor (TNF)-α–converting enzyme and so being able to function as a soluble chemoattractant. The last family is also composed of one member, called lymphotactin (XCL1), which has a single cysteine residue [88].

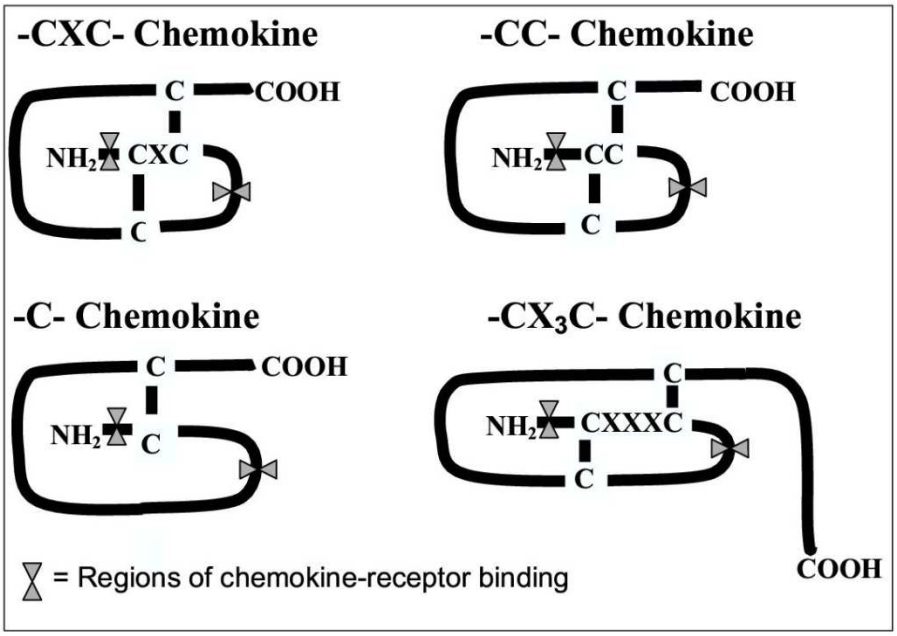

Figure 1.6: – **Chemokine structure and classification**. Chemokines are subdivided into 4 families on the basis of location of the two NH2-terminal cysteine residues: CC, CXC, C, and CX3C [89].

1.2.2. Chemokine structure

In general, chemokines contain four well conserved cysteine residues, which form two cysteine bridges that are responsible for their specific tertiary structure. Indeed, despite the low overall amino acid sequence homology among chemokines, they all share remarkably conserved tertiary structure. This consist of a disordered N-terminus of 6–10 amino acids, which functions as a key signaling domain in all chemokines characterized to amino acids, which functions as a key signaling domain in all chemokines characterized to date. This region is followed by the N-loop, a long loop containing important binding determinants that ends in a α-helix, a three-stranded β-sheet, and a C-terminal helix. Disulfide bonds stabilize the overall topology. Moreover, many chemokines form dimers and higher order oligomers alone in solution or upon binding to glycosaminoglycans (GAGs) [90] [91], whereas only few chemokines are known to form tetrameric structures.

1.2.3. Chemokine functions

The principal and more studied function of chemokines is to direct cell migration. This process involve may sequential interactions involving adhesion molecules, GAGs, but obviously also chemoactractant ligands and their receptors [92] [93]. First, selectins expressed on the endothelium interact with mucin receptors on leukocytes through labile interactions, causing a rolling behavior of the leukocyte along the cell surface [94]. Chemokines, secreted in response to proinflammatory cytokines, bind GAGs on the endothelial cell surface creating a cell surface gradient [85]. The role of inflammatory chemokines is to induce the migration of leukocytes to the injured or infected site, but, their roles are more complicated and depend on the physiological context. In the last few years chemokines have been recognized as important mediators in the pathogenesis of many human diseases and have assumed growing relevance in clinical pathology as markers of disease onset, progression, and remission. Indeed, chemokines play also non chemotactic functions. For example, they modulate angiogenesis and fibrosis [95], regulate differentiation processes and proliferation of hematopoietic progenitors [80], are involved in ontogenesis of vascular and nervous system [96], control cell survival and co-stimulate T cell [97].

1.3. Chemokine receptors

Chemokines exert their biological functions through the interaction with their membrane receptors, which belong to seven transmembrane domain G protein coupled receptors (GPCR) and in particular to the rhodopsin-like family [98] [99]. The structure of chemokine receptors (CKR) consists of a single polypeptide chain crossing seven times the cell membrane, consequently generating three extracellular and three intracellular loops. The acidic amino-terminal extracellular domain is fundamental for ligand binding and contributes to the specificity of ligand recognition. The conserved transmembrane sequences, the cytoplasmic loops and the C-terminal domain, rich in phosphorilable serine/threonine are involved in receptor internalization and signal transduction. Chemokine

receptor's structure hold structural motifs, important for signaling: an aspartic acidic residue, a Thr-X-Pro (TXP; where X denotes any amino acid) motif in the second transmembrane domain and the DRYLAIV motif in the second intracellular loop which determine the receptor coupling with G-proteins [100].

The interaction between chemokines and their receptor is a two-step process, involving two important sites present in chemokine structure and kept in close proximity by a disulphide bonds: *docking* and *triggering domain*. The first one is represented by the conformationally rigid loop that follows the second cysteine and is firstly recognized by the N-terminus of the receptor. This interaction restricts the mobility of the chemokine and presumably facilitates the proper orientation of the *triggering domain* that corresponds to the chemokine N-terminal domain and is thought to lead the receptor activation [98, 101-103].

Chemokine receptors have been demonstrated to be coupled to hetero-trimeric GTP-binding proteins (G-protein) of the Gi type, sensitive to *Bordetella pertussis* toxin. Upon receptor engagement, G-proteins are activated by exchanging GDP with GTP and inducing the dissociation of α subunit from Gβγ complex. Whereas α subunit inhibits adenylate cyclase, the βγ subunit induces the reorganization of the actin cytoskeleton, cell migration, increase in intracellular calcium and activation of specific gene transcription [104]. All these processes are supported by the βγ subunit activation of phospholipase Cβ2 (PLC-β2) which causes the formation of inositol-1,4,5- trisphosphate (InsP3) and diacylglycerol (DAG) and a transient increase of intracellular free calcium concentration. DAG production leads to protein kinase C (PKC) activation which contributes to receptor phosphorylation and consequently desensitization and inhibition of functional responses, through the β-arrestin recruitment. Other pathways activated after chemokine binding comprehend phosphatidylinositol 3 kinase γ (PI3K γ) which activate MAPKs [105] (Figure 1.7).

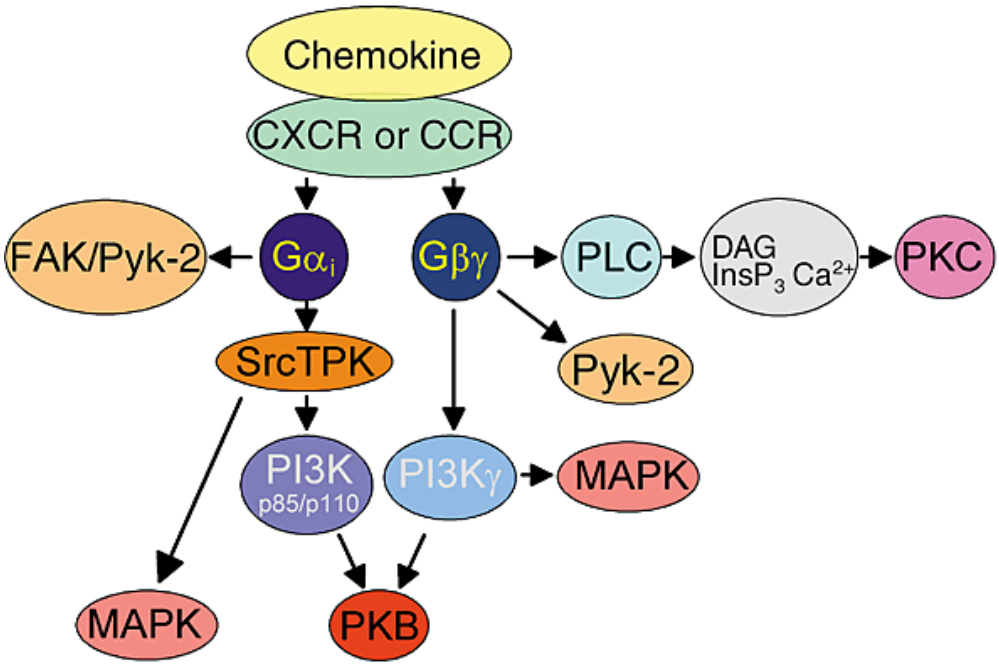

Figure 1.7: – **Chemokine receptor signaling pathway**. Scheme of the most effectors of the signal transduction present in chemokine signal transduction [104].

1.3.1. CCR2

CCR2 is a chemokine receptor that belongs to the subclass of CC receptors and binds inflammatory chemokines such as CCL2 (MCP-1), CCL8 (MCP-2), CCL7 (MCP-3), CCL13 (MCP-4), CCL11 (Eotaxin-1), CCL24 (Eotaxin-2), CCL26 (Eotaxin-3), with different affinity. It is expressed by hematopoietic cells, in particular by monocytes, but also by non-hematopoietic cells, such as endothelial cells [106], fibroblasts [107], mesenchymal stem cells [108] and several tumor cell lines including prostate, lung, breast cancer, and myeloma cell lines. The most studied CCR2 ligand is CCL2 also known as monocyte chemoattractant protein-1 (MCP-1). This chemokine is a potent *in vitro* monocyte activator and is one of the main responsible for monocyte traffic. CCL2 is a 76 amino acid polypeptide whose gene is localized in a cluster of genes encoding for cytokines on the q-arm of chromosome 17 [109]. It was purified in 1989 and since then it has been found to chemoactract not only monocytes, but also memory T cells, natural killer (NK) cells, and perhaps dendritic cells.

1.3.1.1. CCR2 and monocyte traffic

CCR2 and its two ligands CCL2 and CCL7 play a crucial role in monocyte egression from bone marrow and their recruitment at site of inflammation. The important role of CCR2 in mobilization of hematopoietic cells from BM was firstly individuated by Charo et al in 2007 [110]. In this study they found $CCR2^{-/-}$ mice to show in homeostatic conditions a marked decrease in circulating $Ly6C^{high}$ inflammatory monocytes, whereas they showed a normal or increased number of monocytes in the bone marrow. These data suggested that $CCR2^{-/-}$ mice present a defect in monocyte mobilization rather than monocyte differentiation and CCL2 and CCL7 have been demonstrated to be the CCR2 ligands responsible for that defect. In fact only mice deficient for these two chemokines showed the same defect present in $CCR2^{-/-}$ animals, whereas MCP-$5^{-/-}$, $MCP2^{-/-}$ mice exhibit a normal blood monocyte number.

By other studies CCR2 resulted to be crucial not only for homeostatic monocyte mobilization, but also in response to inflammatory stimuli. In fact, Serbina et al examined the importance of CCR2 in different stages of monocyte migration in a setting of *L. monocytogenes* infection [3] and found that $Ly6C^{high}$ monocyte egression into circulation is enhanced by inflammation or infections. Moreover, further studies demonstrated that during infections low concentrations of TLR ligands in the bloodstream induce CCR2-dependent emigration of monocytes from bone marrow. This is caused by rapid production of CCL2 by bone marrow mesenchymal stem cells and CXCL12 abundant reticular (CAR) cells in response to TLR ligands or bacterial infections [111] (Figure 1.8).

However CCR2 importance in monocyte egression from BM is confirmed, its role in monocyte recruitment at inflammation site in still controversial. Indeed, in setting of *L. monocytogenes* infection monocyte recruitment in spleen was demonstrated to be CCR2-independent. However, others studies reported that in West Nile Virus (WNV) and protozoal infections the axis CCL2-CCR2 is crucial not only for the monocyte traffic from the bone marrow to the circulation but also for their recruitment to the inflammation sites [112].

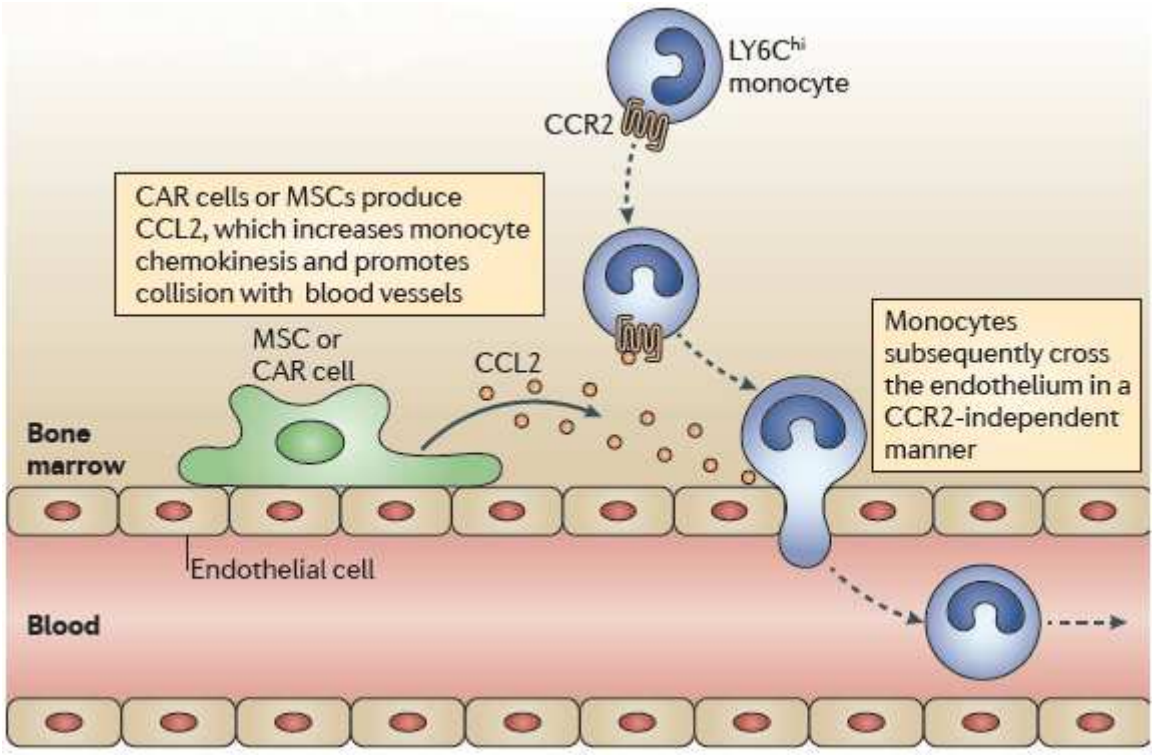

Figure 1.8: – CCR2-dependent LY6C^{high} monocytes egression from the bone marrow. CCL2 is produced in homeostatic conditions and increased during infection, by BM MSC or CAR cells and induce monocyte egression from BM into the bloodstream [113].

1.3.1.2. CCR2 and cancer

CCL2/CCR2 axis has a crucial role in several chronic inflammatory conditions such as atherosclerosis [114, 115], multiple sclerosis [116], rheumatoid arthritis [117] and cancer. Indeed, CCL2 is expressed in different tumors, like prostate, breast, lung, melanoma, ovary, gastric, renal, colorectal, esophageal and multiple myeloma. In prostate cancer CCR2 expression levels has been correlated with tumor aggressiveness and higher expression have been found in the more aggressive cell lines [118]. Moreover CCL2 expression in tumor cells significantly correlates with tumor-associated-macrophage (TAM) infiltration, and in breast cancer CCL2 expression has been positively correlated with VEGF production, TAM infiltration, angiogenesis, and poor survival [119]. Tumor supporting effects of CCL2 are different and comprehend the induction of cancer cell proliferation through the activation of (PI3K)/AKT pathway, promotion of cancer cell extravasation and metastatization and induction of angiogenesis (Figure 1.9) [118, 120]. In colonic adenoma CCL2 has been

demonstrated to stimulate the production of the pro-angiogenic cytokine VEGF through pathway of COX-2/PGE2 in both autocrine and paracrine manner [121]. All these evidences suggested that CCL2/CCR2 axis could be a target for cancer treatment. In vivo experiments indicate that administration of neutralizing antibody against CCL2 significantly reduce tumor blood vessel density and decrease tumor burden [120, 122]. Moreover, CCR2 antagonist in prostate cancer patients has been shown to decrease cancer cell proliferation and invasion in vitro [123]. Growing evidences in several cancer types, like prostate, breast cancer and myeloma, suggest that CCL2 can directly promote cancer epithelial cell migration and invasion, enhancing metastatic potential [124, 125].

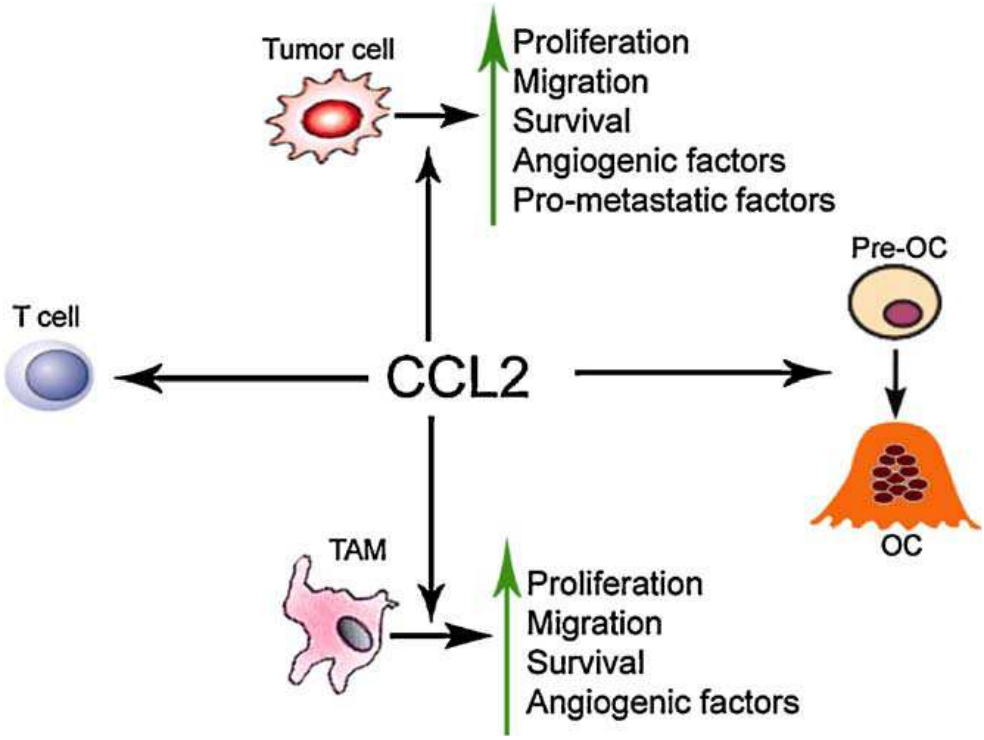

Figure 1.9: – **Tumorigenic activity of CCL2**. CCL2 is tumorigenic through its direct effects on tumor cells and its influence on normal host cells (OC: osteoclasts) [89, 126].

1.4. Atypical chemokine receptors

Chemokines activity is thinly regulated at transcriptional, translational and post-translational levels. One of the mechanisms regulating chemokine bioavaibility is represented by a sub-class of chemokine receptors, previously called decoys, interceptors, scavengers, or chemokine-binding proteins. Recently it has been formalized the new name of atypical chemokine receptors in the new Nomenclature Committee of the International Union of Pharmacology and Human Genome Organization Gene Nomenclature Committee [127]. This family of receptors is characterized by their promiscuous chemokine binding, with the exception of CXCR7, and their inability to couple G proteins and induce conventional G protein-coupled signaling. This inability is the consequence of the presence of a modified or missing canonical DRYLAIV motif in the second intracellular loop of the chemokine receptor, which is indispensable for G proteins coupling. However, recent evidences suggest that these receptors do signal through G protein-independent signaling pathways [128-130].

The family of atypical chemokine receptors is very heterogeneous in structure and function. Some of them have clearly been shown to scavenge chemokines and therefore to manipulate chemokine responses in vivo, whereas others appear to be important for the local regulation of chemokine presentation and function. The members of the atypical chemokine receptor family are: ACKR1, previously known as Duffy Antigen Receptor for Chemokines (DARC); ACKR2, formerly D6 or CCBP2; ACKR3, alias CXCR7; ACKR4, formerly CCRL1 or CCX CKR. Two more receptors could be part of this family, but pending confirmation: CCRL2 or ACKR5, and PITPNM3, also known as the CCL18/PARC receptor that would be ACKR6 [127] (Figure 1.10).

ACKR1 or DARC is able to bind, with different affinity, more than 20 different inflammatory CC and CXC chemokines [131-133]. It show different functions depending on cell type in which it is expressed. Its expression on erythrocytes historically sticks at DARC the function of “sink” for chemokines, being able to regulate blood levels of inflammatory chemokines. On the other hand, it can be also expressed by polarized cells, in particular in vascular endothelial cells, like capillaries and venules in several tissues and organs. Here, DARC acts as transporter; in fact it binds chemokines from the baso-lateral layer and brings

them to the apical surface where chemokines remain associated with DARC, leading to chemokine presentation on the luminal microvilli [134].

ACKR3, alias CXCR7, has two ligands: CXCL12, which is the unique ligand of CXCR4 and it is bound by CXCR7 with very high affinity. The second ligand is CXCL11, whose conventional receptor is CXCR3, but it is bound with much lower affinity than CXCL12 [135, 136]. ACKR3 is expressed by different cell types, including hematopoietic, mesenchymal, and neuronal cells [137]. In particular, its expression has been identified in different leukocyte population, for example in human B cells and in B cells located in murine splenic marginal zone [138, 139], but also in barrier organs, like placenta or brain microvessel endothelium. Functionally, ACKR3 has been shown to play a fundamental role in regulating CXCL12 availability for CXCR4. Indeed, CXCR7 knock-out mice display a lethal phenotype, corresponding to le phenotype of CXCR4 or CXCL12 KO mice. Animals die at birth or perinatal because of defect in heart valves[140-142]. Moreover, this phenotype is also shown in mice keeping a conditional deletion of the gene in endothelial cells, suggesting a crucial role of ACKR3 expressed in this cell type [140]. Finally, CXCR7 has been reported to be expressed in different type of tumors, both of hematopoietic and mesenchymal origin [143-145], such as lymphomas, sarcomas, prostate and breast cancer [146, 147]. Its expression in tumors is often matched with CXCR4 expression, but the role of ACKR3 in cancer still remains unclear.

ACKR4, formerly CCRL1 or CCX CKR, binds homeostatic CC chemokines, in particular CCL19, CCL21, CCL25 but also, with lower affinity, CXCL13 [148]. After receptor engagement, it is internalized and drives chemokines to the degradation in lysosomal compartment [149]. ACKR4 expression is still under debate, in fact it has been reported to be expressed in heart, lung, gut, skin keratinocytes, several lymphoid organs and thymic epithelial cells but, recently, CCX CKR reporter mice did not confirm the expression in heart tissues [148, 150]. ACKR4-/- mice, which are viable and healthy, confirmed the chemokine scavenging ability of this receptor, in fact higher levels of its ligands have been detected in serum and lymph nodes [151]. Few studies on ACKR4 in pathologies have been performed, and preliminary results describe it having a role in basal traffic of dendritic cells to lymph nodes and of embryonic thymic precursors to the thymus [151].

There are two other atypical chemokine receptors, but their belonging to this family is still not confirmed. The first one, ACKR5, is CCRL2 and its putative murine orthologous is L-CCR. It shares 40% of similarity with the amino acid sequence of CCR1, CCR2, CCR3 and CCR5 and it has been recently demonstrated to bind CCL19 and the chemoactractant protein chemerin but without a consequently induction of cell migration and calcium fluxes [152, 153]. CCRL2 expression has been detected in several leukocytes, such as monocytes, macrophages, neutrophils, DCs, T and B lymphocytes, NK cells, mast cells and also in hematopoietic progenitor cells [154, 155]. Conversely, its murine orthologous expression is restricted to DCs and macrophages [156]. The in vivo role of CCRL2 is thought to be the regulation of chemerin levels, increasing them locally in the inflammatory sites, in order to facilitate chemerin binding to its functional receptor ChemR23 [152]. The last potential member of the ACKR family is PITPNM3, whose new name would be ACKR6. It is member of the phosphatidyl-inositol transfer protein (PIPT) family, which comprehends six transmembrane-domain proteins, and it has been recently demonstrated to bind CCL18 and to be involved in breast cancer metastasis [74].

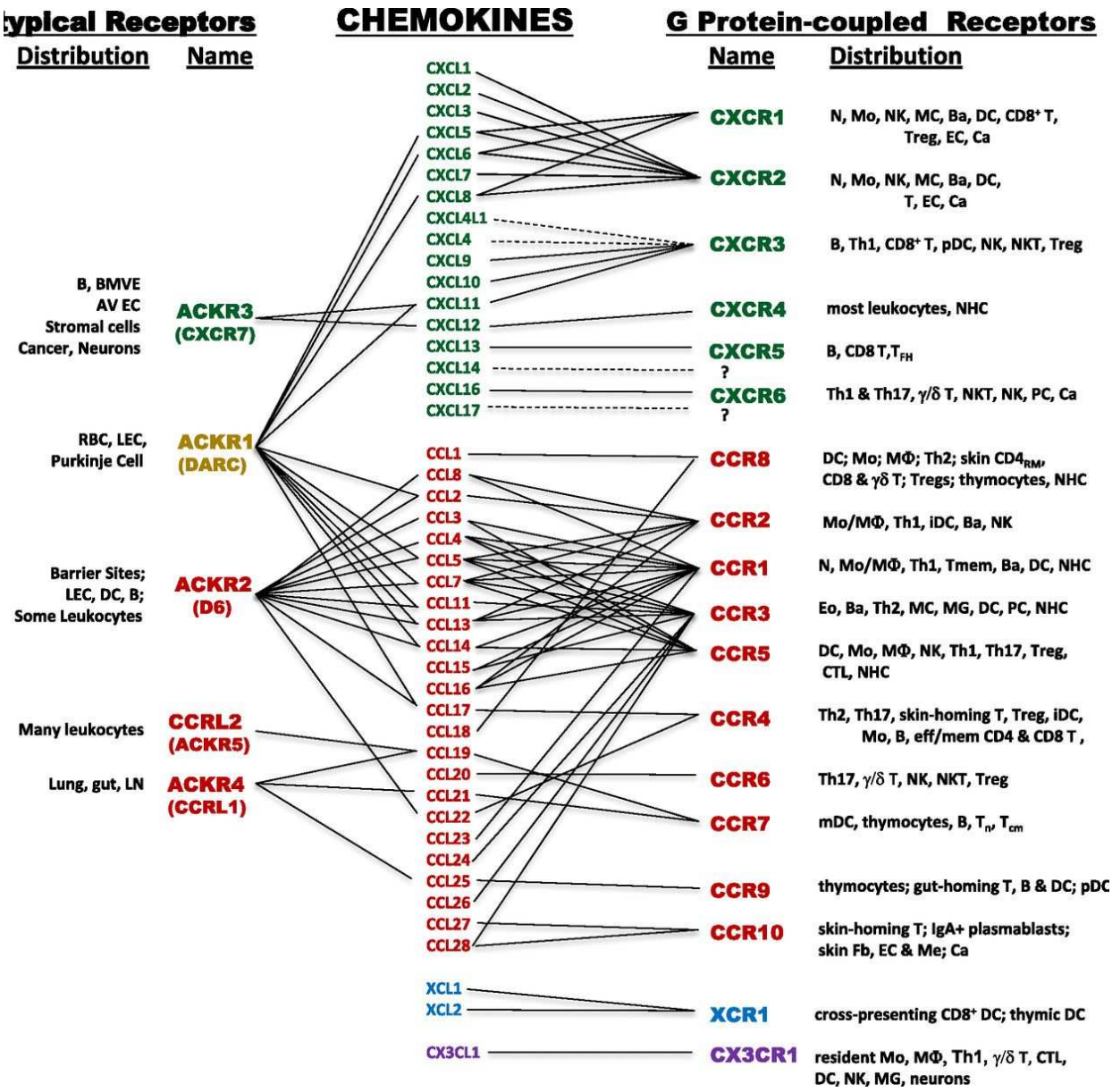

Figure 1.10: – Canonical and atypical chemokine receptors. Schematic representation of canonical and atypical chemokine receptor specificity for ligands and leukocytes [127].

1.4.1. D6 (ACKR2)

D6, now called ACKR2, is encoded in the gene cluster of CC chemokine receptors, wherewith it shares 30-35% of the overall sequence identity [157]. It was cloned from placenta and hematopoietic stem cells in the middle of 1990s [157, 158]. It is known to bind promiscuously inflammatory CC chemokines, but nor homeostatic ones neither chemokines belonging to other classes (Figure 1.11) [159, 160]. Different evidences support the importance of a proline residue in position 2 (P2) in the sequence of D6 ligands [160, 161]. In fact, D6 ligands can be subdivided into three classes: CC inflammatory chemokines efficiently degraded by D6 (CCL2, CCL3L1, CCL4, CCL22, CCL14(9-74)), chemokines degraded with a less efficiency (CCL14 (11-74), CCL3, CCL8 (6-75)) and chemokines that are not degraded (CCL14 (1-74) e CCL3 (5-70)). Moreover, chemokines degraded by D6 have been demonstrated to modify its intracellular traffic and sequence analysis revealed that all members of these classes of D6 ligands, with the only exception of CCL17, have a proline residue in position 2. These evidences suggest a relevant role for this amino acid in the induction of D6 adaptive redistribution on the cell membrane resulting in efficient chemokine degradation (Figure 1.9) [160]. Furthermore, D6 ligands can be modified by CD26 protease, which remove the two last amino acids at the N-terminal domain of chemokines holding P2 residue [160].

```
CCL2            -----QPDAINAPVTCCYNFTNRKISVQRLASYRRITSSKCPKEAVIFKTIVAKEICADPKQKWVQDSMDHLDKQTQTPKT
CCL3            -----ASLAADTPTACCFSYTSRQIPQNFIADY-FETSSQCSKPGVIFLTKRSRQVCADPSEEWVQKYVSDLELSA-----
CCL3(5-68)      ---------ADTPTACCFSYTSRQIPQNFIADY-FETSSQCSKPGVIFLTKRSRQVCADPSEEWVQKYVSDLELSA-----
CCL3L1          -----APLAADTPTACCFSYTSRQIPQNFIADY-FETSSQCSKPSVIFLTKRGRQVCADPSEEWVQKYVSDLELSA-----
CCL4            -----APMGSDPPTACCFSYTARKLPRNFVVDY-YETSSLCSQPAVVFQTKRSKQVCADPSESWVQEYVYDLELN-------
CCL8            -----QPDSVSIPITCCFNVINRKIPIQRLESYTRITNIQCPKEAVIFKTKRGKEVCADPKERWVRDSMKHLDQIFQNLKP
CCL8(6-75)      ----------SIPITCCFNVINRKIPIQRLESYTRITNIQCPKEAVIFKTKRGKEVCADPKERWVRDSMKHLDQIFQNLKP
CCL14           TKTESSSRGPYHPSECCFTYTTYKIPRQRIMDY-YETNSQCSKPGIVFITKRGHSVCTNPSDKWVQDYIKDMKEN-------
CCL14(9-74)     ---------GPYHPSECCFTYTTYKIPRQRIMDY-YETNSQCSKPGIVFITKRGHSVCTNPSDKWVQDYIKDMKEN-------
CCL14(11-74)    ----------YHPSECCFTYTTYKIPRQRIMDY-YETNSQCSKPGIVFITKRGHSVCTNPSDKWVQDYIKDMKEN-------
CCL17           -----ARGTNVGR-ECCLEYFKGAIPLRKLKTW-YQTSEDCSRDAIVFVTVQGRAICSDPNNKRVKNAVKYLQSLERS----
CCL22           -----GPYGANMEDSVCCRDYVRYRLPLRVVKHF-YWTSDSCPRPGVVLLTFRDKEICADPRVPWVKMILNKLSQ--------
                              **       :.   :  :   *.  *.. .::: *   : :*::*    *:  :  :.
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Figure 1.11: – **Amino acid sequence of D6 ligands:** alignment of CC chemokines recognized by D6 using the program clustalW2 (http://www.ebi.ac.uk). Amino acid residues conserved in all aligned sequences are indicated by "*"; conserved substitutions are indicated by ":"; semi-conserved substitutions are indicated by ".". Grey boxed chemokines are the ones efficiently scavenged by D6.

1.4.1.1. D6 structure and signaling properties

D6 structure is similar to that of conventional chemokine receptors, but it contains alterations in conserved domains that are essential for G protein coupling and signal transduction (Figure 1.12). These modifications could be the cause of D6 unconventional signaling. The receptor N-terminal domain contains charged aminoacid important for ligand recognition that is also present in conventional CKR structure. In the second transmembrane domain of D6 is also localized the TxP (Thr-Xaa-Pro) motif present in conventional CKR structure and responsible for their activation [162]. Conversely, D6 does not hold the aspartic acid in position 92, being mutated into an arginine. This amino acidic residue is thought to be required for receptor activation but its role in D6 activity has not yet been identified. Another important determinant in CKR structure is the DRYLAIV motif present at the boundary of the second intracellular loop with the third transmembrane domain. This motif in D6 structure is converted in DKYLEIV and it has been demonstrated to give a weak ligand-induced signaling activity [163]. D6 also shows a C-terminal tail longer than canonical CRK and containing a serine cluster with a putative 8th helix. The C-terminal domain is not require for receptor internalization but is fundamental to prevent receptor degradation in late endosomes and to allow receptor recycling after chemokine degradation [164, 165].

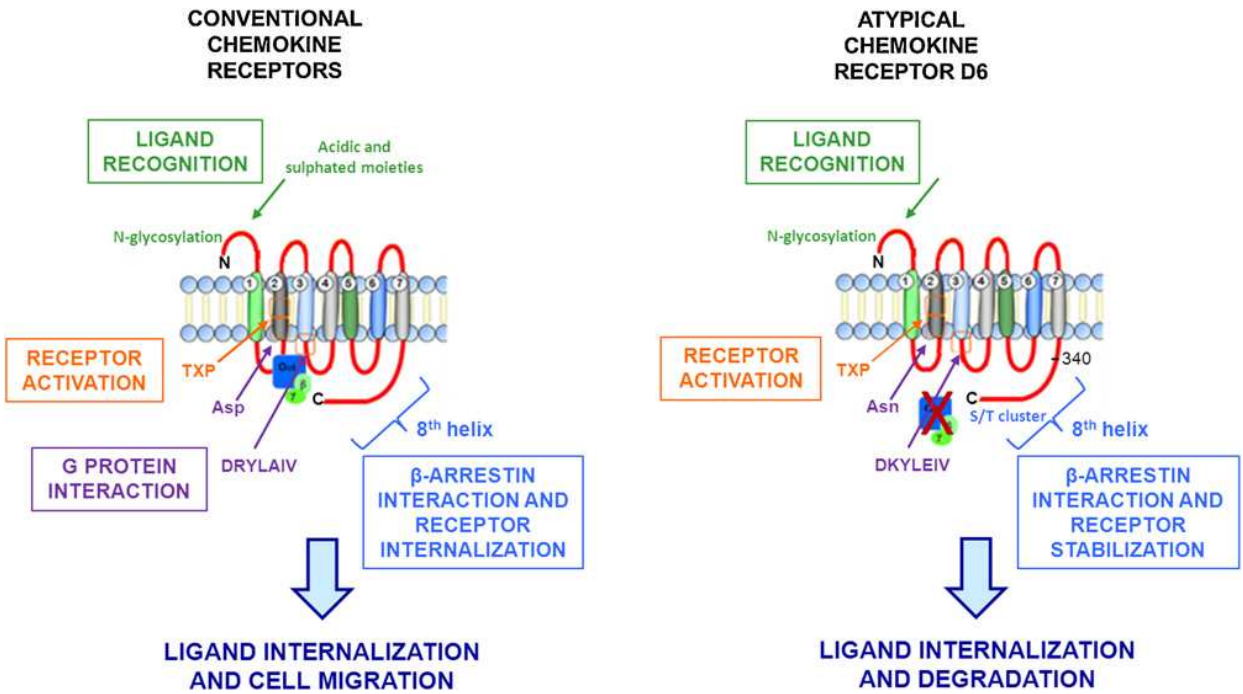

Figure 1.12: – Structural motif in canonical CKR and ACKR2: ACKR2 (D6) hold alterations in several structural motif mainly important for CKR signaling, in particular in DRYLAIV motif and in the Asp residue [166].

In homeostatic conditions, D6 is mainly stored in intracellular peri-nuclear compartments and only few molecules reside on the cell membrane [167, 168]. D6 is able to internalize not only after chemokine engagement, as conventional CKR, but also constitutively in Rab5+ vesicles that bring the receptor to early endosomes, which is then recycled back to the plasma membrane through a rapid Rab4+ and a slow Rab11+ pathway (Figure 1.13) [169]. After chemokine engagement D6 is mobilized from intracellular pool and up-regulated on the plasma membrane through the Rab11+ vesicles with the effect of optimizing its degradative activity [169]. Finally, a recent work demonstrated that D6 traffic after chemokine engagement is β-arrestin1-dependent and that it induces a non-conventional chemokine receptor signaling. Indeed, D6, through a β-arrestin1-dependent G-protein independent signaling pathway, involving the cascade of Rac1- PAK1- LIMK1 cascade, finally induces cofilin phosphorylation and actin rearrangement [130].

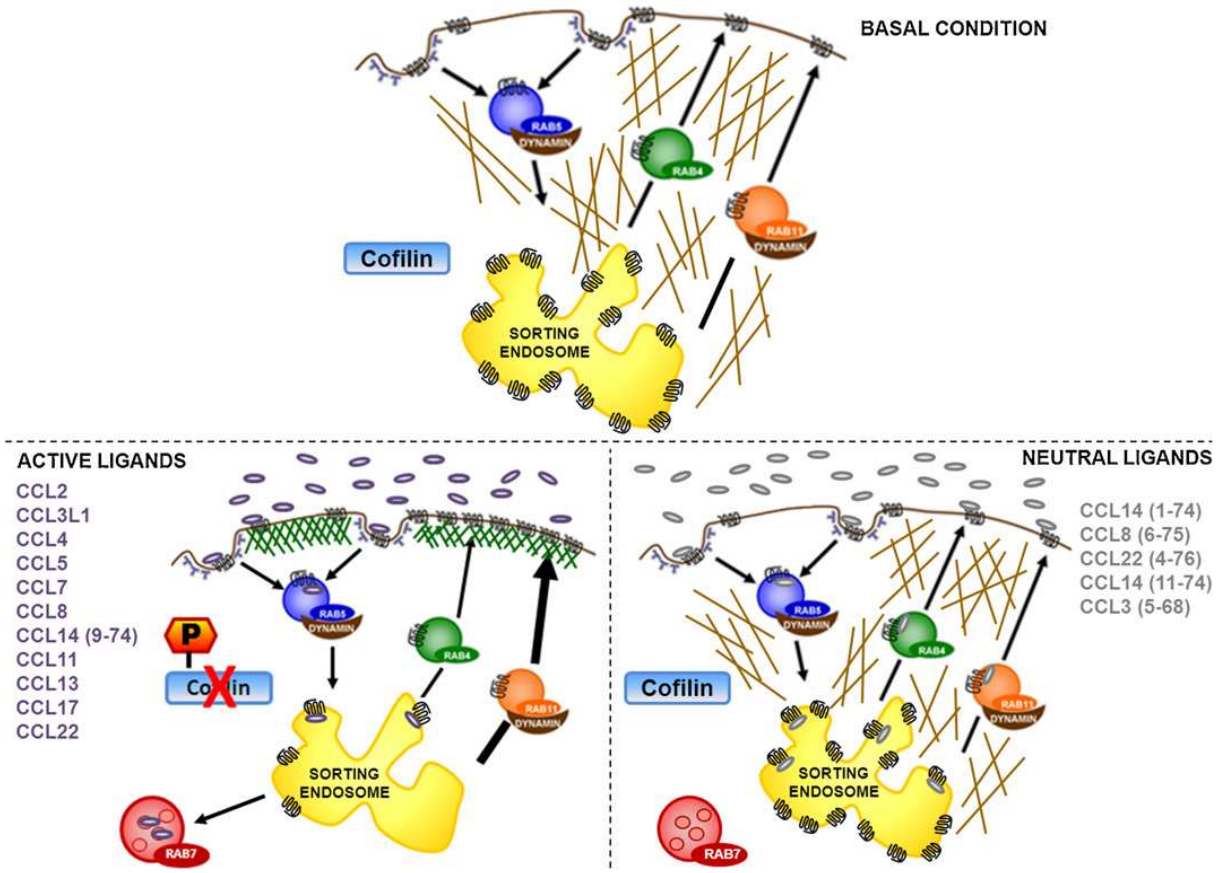

Figure 1.13: – Actin dynamics sustain D6 up-regulation and degradatory activity: In basal condition D6 is internalized in a Rab5-dependent way and it recycles to the plasma membrane through a Rab4/Rab11-dependent mechanism. Active cofilin maintains actin cytoskeleton organization in stress fibers (brown), supporting constitutive internalization and recycling of D6 to the plasma membrane. Stimulation with active ligands induces cofilin phosphorylation and its inactivation. In this way actin polymerize at plasma membrane in cortical actin (green), allowing D6 up-regulation and increasing its scavenging efficiency and chemokine degradation. On the other hand, D6 stimulation with neutral ligands (gray) does not change actin organization and cofilin activation state without affecting its distribution and scavenging efficiency [166].

1.4.1.2. Biological functions of D6

D6 is constitutively expressed by lymphatic endothelial cells (Figure 1.14) [170], in particular in the skin, gut and lung and by placenta trophoblasts [171, 172]. Moreover it has been reported to be expressed by several leukocytes, for example innate-like B cells (IBC) such as MZ B cells, B1a and B1b [173], but also some tissue macrophages [172, 174, 175], alveolar macrophages from chronic obstructive pulmonary disease (COPD) patients [176] and in a subset of vascular tumors [170].

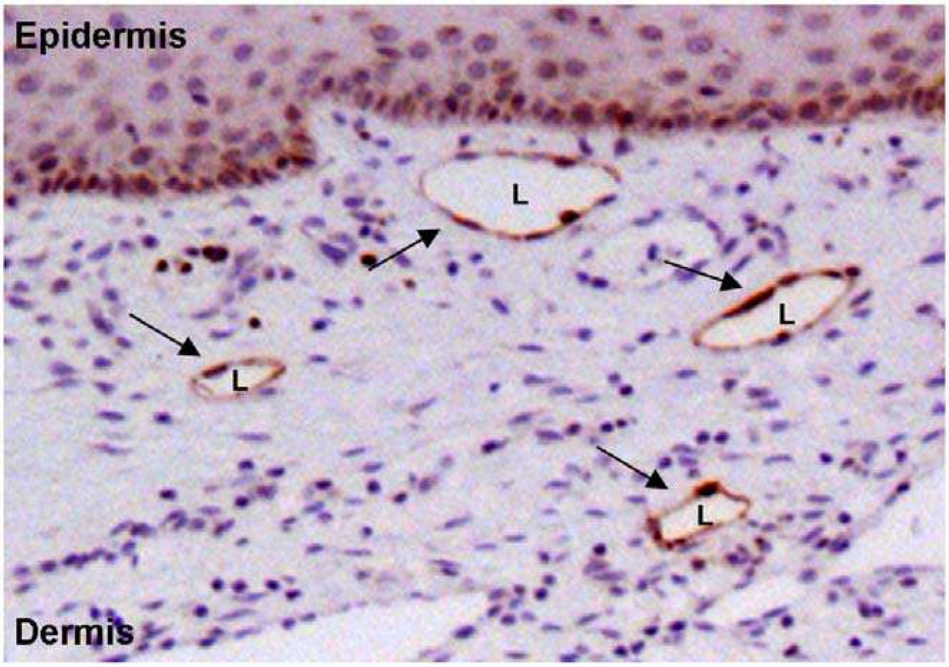

Figure 1.14: D6 expression on lymphatic endothelial cells in human skin. Human skin stained by anti-D6 antibody. "L"marks subcutaneous lymphatic vessels that show D6 immunoreactivity.

1.4.1.2.1. Homeostatic functions

D6 has been studied for several years, but only recently it has been shown to play an important role in homeostatic ones and not only in the inflammatory response. In fact, Savino et al reported that D6-deficient mice, in resting conditions, showed a selective increase of Ly6C^{high} monocytes both in the bloodstream and in the spleen, whereas they were decreased within the bone marrow. They also resulted to exhibit a more immature phenotype, expressing lower levels of CD11b, CD115 and F4/80 than wt cells. By chimera experiments, this defect was demonstrated to be dependent of D6 absence on the non-hematopoietic compartment [177]. Furthermore, lack of D6 in homeostatic conditions also provokes an enhanced B1-cell response to CXCL13, an impairment of peritoneal B1 cell number and anti PC antibodies present in the serum [173], as well as increased serum concentration of CCL11 [172] and CCL2 [177].

Finally , a recent work demonstrated that D6 contributes to a selective presentation of CCR7 ligands by eliminating inflammatory chemokine binding to lymphatic endothelial cell (LEC) surface and regulating their capacity to discriminate mature and immature DCs. The role of D6 in correctly direct DCs migration toward lymph nodes was also reported by Lee et al. [178]. Moreover, McKimmi et al demonstrated that D6 expression can be regulating by growth factors, cytokines and tumor microenvironment, in particular by IL-6 and IFNγ, indicating that this receptor is over-expressed in inflammatory contexts [179].

1.4.1.2.2. Protective role of D6 during inflammatory conditions

D6 has been shown to play a fundamental role in controlling inflammatory chemokine gradients and so inflammatory responses in different *in vivo* models (Figure 1.15). Indeed, in two models of local inflammation D6 has been investigated. Firstly, in phorbol ester skin painting model, D6-null mice developed an exacerbated inflammatory response dependent on TNFα and then sustained by inflammatory CC chemokines, not efficiently cleared from inflamed tissues. Consequently, $D6^{-/-}$ mice arose psoriasis-like lesions, characterized by an aberrant recruitment of T cells, mast cells and a vast keratinocyte proliferation and neovascularization [180]. In the second model of subcutaneous injection of CFA, Martinez de la Torre et al demonstrated that D6-deficient mice showed a worst disease characterized by premature lesions, increased necrosis and neovascularization. At shorter time points (e.g. day 7) inflammation evolved in macroscopic granuloma-like lesions in a significant percentage of $D6^{-/-}$ animals, and only in a minority of wild-type littermates, whereas no differences were evident at later time points [181]. Interestingly, in both models increased levels of inflammatory CC chemokines were detected and pre-treatment with chemokine receptors blocking antibodies prevented lesion development, confirming that the increased inflammatory response is caused by inefficient control of the chemokine system in absence of D6.

Maternal-fetal interface inflammation is also controlled by D6, which is expressed by invading trophoblast and syncytiotrophoblast cells, which are located between maternal bloodstream and fetus. The role of D6 in this context was studied by using two different *in*

vivo model of fetal loss[172]: injection in pregnant mice of LPS or anti-phospholipid autoantibodies purified from patients affected by antiphospholipid syndrome (APS). In both cases lack of D6 resulted in increased fetal loss rate due to uncontrolled levels of CC inflammatory chemokines and consequently increased number of infiltrating leukocytes. Moreover, also in humans D6 has been indirectly shown to be fundamental during pregnancy, in fact D6-ligand chemokines are decreased in plasma of pregnant women, even in the ones affected by pre-eclampsia [171].

It seems that D6 expression in organ barriers, such as skin and placenta is directed to emphasize its activity of inflammation regulator, controlling chemokine distribution and bioavaibility into lymphatic vessels and decidua.

Polymorphisms in D6 gene sequence were described to be correlated with grading of hepatic inflammation of chronic hepatitis C infected patients [182], whereas studying D6 in a model of liver damage induced by CCl4, an extended liver damage was obtained in mice lacking D6, associated to high levels of intrahepatic inflammatory chemokines and increased infiltration of T and NK cells [183].

In the literature there are controversial results concerning the role of D6 in colon inflammation. Vetrano et al described $D6^{-/-}$ mice to fail the resolution in DSS-induced colitis, because of an exacerbated inflammatory response at the expense of D6 absence on stromal and lymphatic compartment, as understood through chimera experiments [175]. Opposite results were obtained by Bordon et al, who described D6-deficient mice to have a reduction susceptibility to colitis and correlating it with the increased production of IL-17A by T cells. The two papers showed opposite results, nevertheless both reported D6 to be expressed in normal mucosa and up-regulated in colon samples from inflammatory bowel disease patients [184].

D6-defincient mice were also subjected to a model of antigen induced disease, showing D6 as controller of chemokines and inflammation also in the lung [185]. In a model of intranasal injection of low doses of Mycobacterium tuberculosis $D6^{-/-}$ mice resulted in dramatic local and systemic inflammation which exerts in mice death [186].

Finally, a recent work demonstrated D6 to be up-regulated in F4/80^{high} peritoneal macrophages during resolution of peritonitis[174] and in alveolar macrophages of COPD patients, indicating a possible role of D6 in promoting immune response [176].

1.4.1.2.3. D6 and cancer

Inflammatory chemokines have been demonstrated to affect different steps of tumor development and progression [187] and D6, due to its important role in controlling the inflammation, has been subjected to different cancer models and in particular in inflammation-driven tumorigenesis (Figure 1.15). Indeed, D6$^{-/-}$ mice in phorbol ester-induced skin tumorigenesis model and in an azoxymethane/sodium dodecylsulphate model of colon cancers resulted to be more susceptible than wt mice, caused by excessive inflammation and leukocyte infiltration. Moreover, Nibbs et al reported that in a mouse strain susceptible for papilloma formation D6 transgenic expression was protective, suggesting that D6 scavenger activity exerts in tumor suppression [188].

D6 expression has been reported in different tumors, including large granular lymphocytes leukemia cells[189], malignant vascular tumors [170] and breast cancers [190]. In particular, in human breast carcinoma D6 expression is regulated by different cytokines, like IL-1 and TNFα and inversely correlated with lymph node metastasis and clinical disease stage, whereas positively correlates with disease-free survival rate. Interestingly, in vivo and in vitro experiments confirmed that D6 plays an inhibitory role in tumor biology, in fact D6-expressing breast cancer cell lines, injected in vivo, are associated to decreased inflammatory chemokine levels, decreased vessel density and TAM infiltration at tumor site [190].

1.4.1.2.4. D6 in immune response

D6, in two different model of disease, has been reported to play a role in the development of specific immune response (Figure 1.15). Firstly, in experimental autoimmune encephalomyelitis (EAE) induced by myelin oligodendroglial glycoprotein (MOG) peptide 35–55 administration in CFA, the absence of D6 causes aberrant inflammatory response associated to CD11c+ DCs accumulation in inflamed skin. The retention of DCs at the skin consequently produced a poor priming of encephalitogenic T cells and their decreased proliferation and IFNγ production. The poor T cell response resulted in protection of $D6^{-/-}$ mice from encephalomyelitis [191]. Secondly, D6-deficient mice were investigated in a model of graft versus host disease (GvHD) where $D6^{-/-}$ mice display a partial protection. Here the protection was caused by the accumulation of myeloid derived suppressor cells (MDSC), in particular of $Ly6C^{high}$ monocytes in secondary lymphoid organs, which exhibit an enhanced immunosuppressive activity and so inhibit the development of adaptive immune responses, partially protecting mice from the development of GvHD [177].

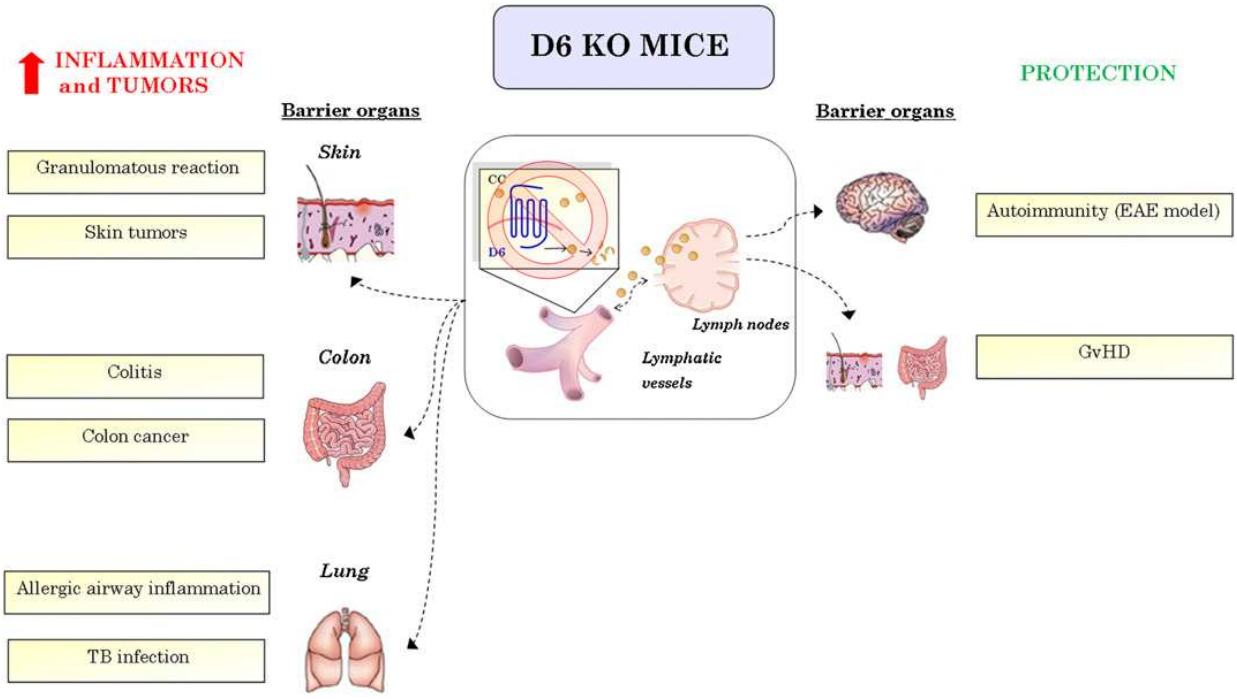

Figure 1.15: D6 in vivo activity. D6 is an important regulator for orchestration of the immune response by containing the local inflammatory response and controlling the migration to secondary lymphoid organs of DC and a subset of myeloid population with immunosuppressive properties. These activities have been demonstrated by the use of D6-deficient mice in different disease models, here subdivided in two classes: inflammatory and tumor models (on the left) and models involving specific immune response (on the right) [166].

1.5. Chemokines and cancer

Chemokines and chemokine receptors are key players in several stages of tumor progression, in particular in leukocyte recruitment at tumor site, but also in promoting angiogenesis and metastatization [192]. Different animal models confirmed their important function in crosstalk between tumor cells, leukocytes and tumor surrounding stromal cells (Figure 1.16).

1.5.1. Chemokines and tumor infiltrating leukocytes

A common feature of cancer is the infiltration of inflammatory leukocytes at tumor site, which is largely mediated by chemokines produced by neoplastic cells, stromal cells or even leukocytes themselves. Tumor infiltrating leukocytes, depending on tumor model, can be different and they would be recruited by corresponding chemokines. For example monocytes and macrophages have been demonstrated to be mainly recruited by CCL2 and CCL5 [59, 193, 194] in several murine model of tumors, such as breast cancer [195, 196], melanoma [197] esophageal carcinoma [198], colon cancer [199], prostate carcinoma [120] and pancreas tumor[120, 126, 147, 200, 201]. Moreover, in human tumors these two CC inflammatory chemokines have been correlated with TAM number, lymph node metastatization and poor prognosis [195, 196, 202]. Furthermore, high numbers of neutrophils infiltrating the tumor have been associated to overexpression of ELR+ CXC chemokines, in particular of CXCL8, and to negative outcomes [203]. Others CXC chemokines are correlated to neutrophil recruitment at tumor site: CXCL1 has been detected in gastric and colon carcinoma [204] and malignant melanoma [205]; CXCL5 is expressed by non-small cell lung cancer [206]; CXCL6 by gastrointestinal tumors [207]. Cancer cell lines over-expressing CXCL8 display an increased PMN infiltration and a reduced growth when injected in mice [208], suggesting that PMN may have a protective role, but, on the contrary, in human tumor biopsies the presence of PMN has been correlated with poor prognosis [209-211]. The major chemoattractant of dendritic cells is CCL20, which has been reported to be expressed by several tumors strongly infiltrated by DCs, including pancreatic, renal, breast

and papillary thyroid carcinoma [212, 213]. Other major attractants of immature DC include CCL5 in papillary thyroid carcinoma [214], CXCL12 in ovarian carcinoma [215] and CCL19 in breast carcinoma [216]. Tumors can contain variable numbers of tumor-infiltrating lymphocytes (TIL). In some tumors, in particular in colon carcinoma, the presence of TIL is a strong predictor of a positive outcome [217-219]. On the contrary in other tumors, like melanoma, the protective role of TIL is compromised by the high proportion of T regulatory cells (Treg) that down-regulate the activation and proliferation of tumor-reactive lymphocytes [220]. TILs are manly chemoattracted at tumor site by CXCL9 and CXCL10 through their interaction with CXCR3, and evidences in human gastric and colon-rectal carcinoma defined that their expression correlates with a good prognosis [221, 222]. On the other hand, high levels of CCL17 and CCL20 have been associated with poor prognosis in breast [223, 224], ovarian [225], prostate [226] and gastric carcinoma [227] because they have been found to recruit CCR4+ Treg and polarized Th2 cells, which support tumor progression.

1.5.2. Chemokines and angiogenesis

CXC chemokines on the basis of the tri-peptide Glu-Leu-Arg (ELR) at the N-terminal domain can be divided in two main categories: ELR+ and ELR- CXC chemokines. The first ones are angiogenic whereas the other ones are angiostatic as assessed by their effects on endothelial cell proliferation and chemotaxis *in vitro* and in animal models, such as rat cornea and tumor regression in vivo [95, 228]. Angiogenic CXC chemokines, like CXCL5 and CXCL8, promote the migration and proliferation of endothelial cells, acting on CXCR1 and CXCR2, which are expressed by endothelial cells. They stimulate endothelial cell proliferation and inhibition of apoptosis. Moreover, engagement of CXCR2 induced up-regulation of metallo-proteases such as MMP-2 and MMP-9 that are involved in extracellular matrix degradation and in releasing of other angiogenic factors such as VEGF and FGF2 [229]. Another important angiogenic chemokine is CXCL12, whose receptor CXCR4 is expressed by endothelial cells and its activation supports endothelial cell migration and proliferation, and recruitment at tumor site of endothelial precursors [230]. Conversely, CXCL4,CXCL9, CXCL10

and CXCL11, via CXCR3 activation, are potent angiostatic factors, inhibiting neovascularization and endothelial cell proliferation [231].

1.5.3. Chemokines and metastasis

Chemokines and chemokine receptors also play a fundamental role in metastasis. In this context the most studied chemokine receptor is CXCR4 together with its ligand CXCL12, which has been found to be up-regulated in several tumors and to be expressed also on cancer stem cells (CSC). In fact, CXCR4+ CSC have been isolated from glioblastoma [232] and pancreas carcinoma [233]. CXCR4 is also associated with lymph node metastasis in oral squamous cell carcinoma, human nasopharyngeal carcinoma, pancreatic cancer, non-small lung cancer, human colorectal cancer, and human breast cancer. Other chemokine receptors have been correlated with tumor metastasis, for example CXCR1, CXCR5 and CCR7. In particular, CCR7 is up-regulated in several tumor models, leading cell dissemination through lymphatic vessels into lymph nodes. Indeed, it is now considered a potential marker for metastasis prediction in breast and colon-rectal cancer [234].

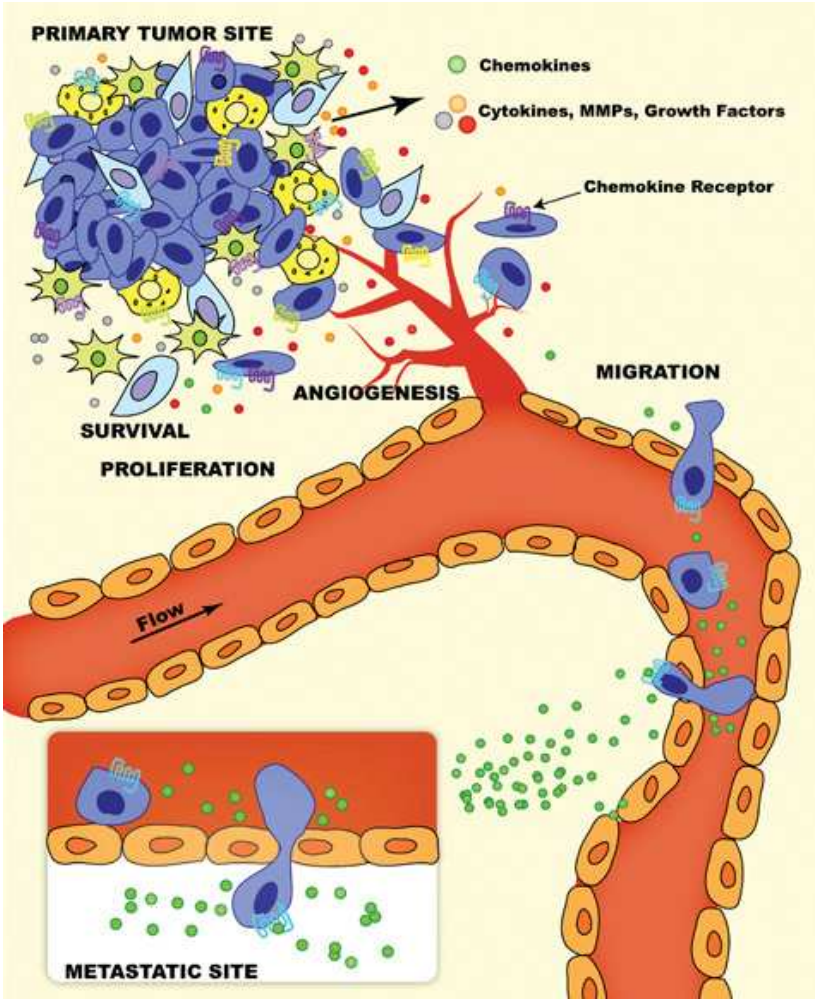

Figure 1.16: – **Chemokine and chemokine receptor in cancer**. Chemokines and chemokine receptors affect several step of tumor progression, including survival and proliferation of tumor cells, recruitment of leukocytes at tumor site, angiogenesis, migration and metastatization of tumor cells [192].

1.6. Kaposi's sarcoma

Kaposi's Sarcoma (KS) is the most common malignancy associated with HIV infection [235] and the most common tumor in African men [236]. It is a highly vascularized tumor holding endothelial lymphatic origin [237, 238] and characterized by extravasation of erythrocytes, infiltration of inflammatory cells and neoangiogenesis [239]. Tumor cells have a characteristic spindle shape, are poorly differentiated and highly proliferative [240]. In 1994, a novel viral DNA sequence in KS biopsies was discovered and named KS-associated herpes virus (KSHV) but it was later classified as human herpes virus 8 (HHV-8) [241]. Successive studies found that Kaposi's sarcoma (KS) is directly caused by infection of LEC by the human herpes virus 8 (HHV8). KS tumor cells are considered to be derived from the LEC lineage and expresses several LEC markers, including VEGFR3, LYVE-1, and PROX1 [242]. Interestingly, it has been shown that KS can also derive from HHV8-infected blood vascular cells undergoing a lymphatic reprogramming which creates a more favorable microenvironment for the tumor growth [243].

1.6.1. Epidemiology

Despite the fact that HHV8 infection is widespread, KS occurs only sporadically in immunocompetent individuals whereas it is relatively common in immunocompromized patients, in particular in HIV patients or transplanted patients treated with immunosuppressive drugs. KS can be classified in four classes: the indolent form affecting older men was referred to as "classical" or "sporadic"; more aggressive cases from subequatorial Africa are named "endemic"; KS associated to immunosuppression treatment of transplanted patients are defined "iatrogenic" and finally KS derived from HIV infection is called "epidemic" or "AIDS-related" KS.

Classical KS is usually characterized by slow-progressing skin lesions and limited dissemination ability, on the contrary in immunocompromized patients it displays a more aggressive phenotype, with widespread diffusion on the skin, frequent involvement of visceral organs, and life-threatening complications [244]. Moreover, HIV-infected individuals

show a risk to develop KS a thousand times higher than general population [245-247] but luckily KS has been shown to regress during HAART treatment [248-251]. This is epidemiologically highlighted by strong decreases of KS incidence in AIDS patients since the use of HAART became widespread [252, 253]. It is also reported that incidence of KS in patients treated with immunosuppressors because of organ transplantation is 500-1000 times greater than in the general population [254], indeed 0.5-5% of organ transplant recipients develop Kaposi's Sarcoma, depending on their geographical origin and the type of organ received (Figure 1.17) [255]. Little information is available on the risk of KS among immunocompetent HHV-8-positive individuals. HHV-8 is known to be transmitted by blood or blood products [256] but also virus transmission among family members, associated with close contact and crowding, has been reported [257-260]. There is little evidence of the vertical transmission of HHV-8 during pregnancy. Among homosexual men, there is consensus that virus transmission is sexual, whereas among heterosexuals correlates with sexual transmission have not been consistently established. As current therapeutic options are only palliative, KS is also a leading cause of morbidity and mortality in AIDS patients [261, 262].

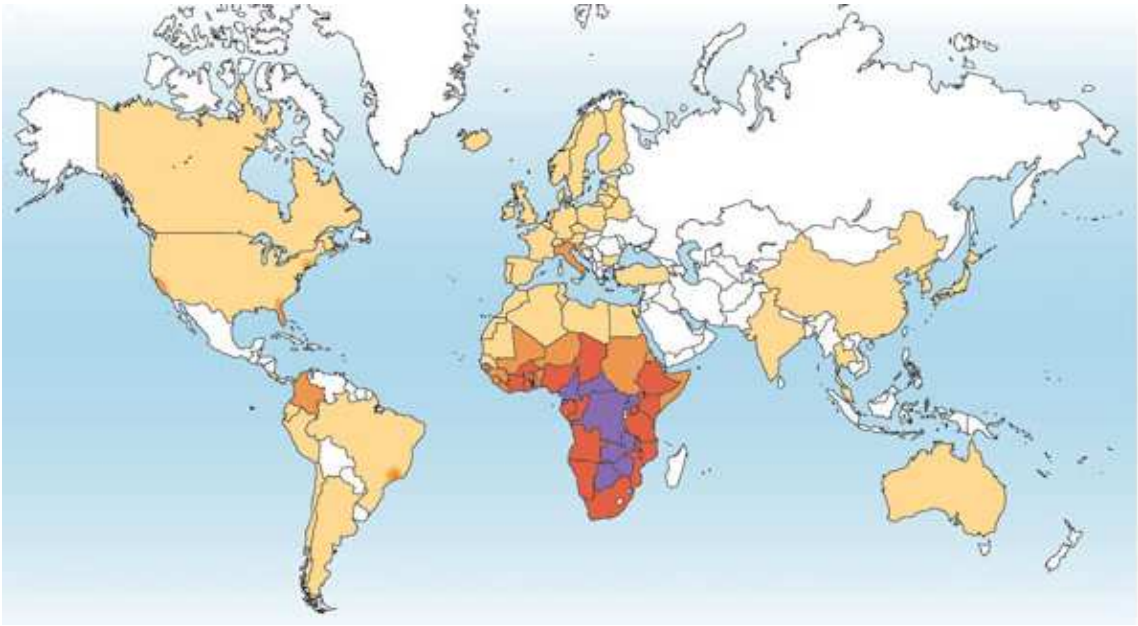

Figure 1.17: –. **Geographical prevalence of KS**. Standardized incidence of Kaposi's sarcoma (KS) in males obtained from the International Agency for Research on Cancer Incidence in Five Continents publication [261].

1.6.2. Clinical features

KS lesions appear as multiple foci highly heterogeneous in their composition depending on the disease stage. The first lesions detectable in the dermis, called patch lesions, are composed by few HHV8-infected spindle cells, an abundant inflammatory infiltrate mainly composed by T and B lymphocytes and monocytes, and a prominent angiogenic process. Then lesions progress to the plaque stage, characterized by solid, edematous, and violaceous in color lesions, and finally to maculo-nodular lesions, with abundant abnormal leaky vessels with edema and red blood cells extravasation and a predominance of spindle cells [263]. At the clinical level, KS lesions are classified in four stages, such as stage I: maculo-nodular lesions; stage II: infiltrative; stage III: florid; stage IV: disseminated. As for still unknown reasons lesions at the same stage show highly variable progression rate, they are further subdivided retrospectively according to the speed of disease evolution in A: slow progressive lesions; B: rapid progressive lesions [264].

Usually, the disease appear as violaceous skin lesions and it can then precede with oral, visceral or nodal involvement but lesions are typically multifocal and do not have a predictable order of progression. They can be solitary modules or can form plaques or even occur in clusters.

In many cases, edema is associated with KS either locally or at distal site. It is thought to be caused by mechanical obstruction of draining lymphatic vessels and also supported by production of permeability factors in KS lesions, as VEGF-A. Moreover, new vessels formed inside KS lesions are leaky and cause extravasation of cells and plasma proteins into surroundings tissues, improving edema formation.

Other organs can be involved in the disease, for example one of the most affected tissues is the gastro-intestinal tract that can be so damaged to result in massive bleeding. Also pulmonary involvement is frequent and based on localization can turn out in bronchial irritation, pleural effusions, hemoptysis or in the more severe cases the parenchyma can be involved with a consequent life-threatening respiratory problems and high mortality.

1.6.3. Pathogenesis

1.6.3.1. HHV8 as oncogene

Kaposi's sarcoma is a tumor caused by infection of lymphatic endothelial cells by KS-associated herpes virus (KSHV), now re-named human herpes virus 8 (HHV-8). Kaposi tumor spindle cells express markers typical of lymphatic vessels, as VEGFR-3, podoplanin and PROX-1 [242]. Moreover, it has been demonstrated that HHV-8 infection of endothelial vascular cells induces their reprogramming towards lymphatic phenotype, up-regulating lymphatic specific genes like PROX-1 and LYVE-1 and down-regulating vascular endothelial genes [242].

HHV-8 is thought to be necessary to develop KS in all its form, but it also causes primary effusion lymphoma (PEL) and multicentric Castelman's disease. It belongs to the family of γ-herpes virus and its genome is composed by a double-stranded DNA whose size can range from 140 to 170 kb [265, 266]. HHV-8 genome contains several open reading frames (ORF), named from K1 to K5 according to their location into the viral genome, and several viral genes, mostly copied from host genome (Figure 1.18) [267].

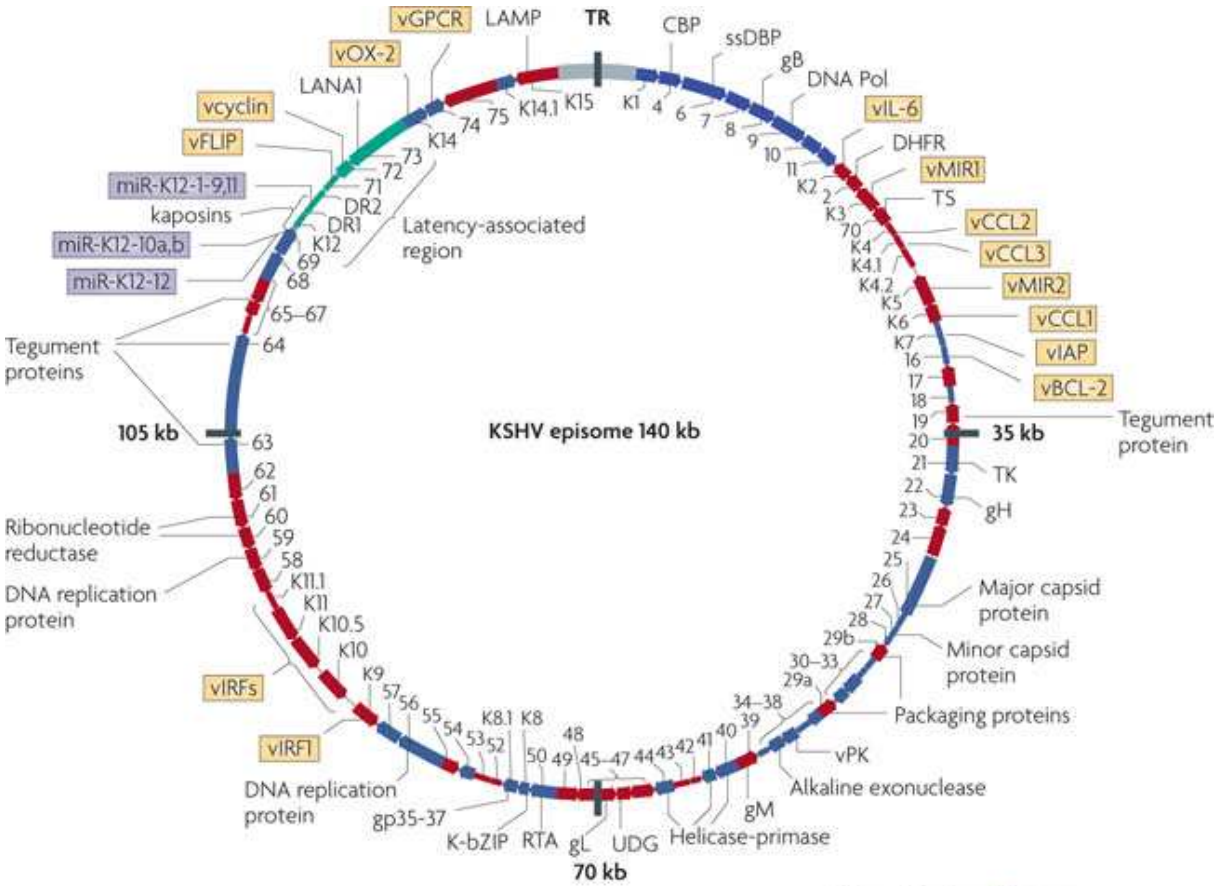

Figure 1.18: – **KSHV genome**. KSHV or HHV-8 encodes 87 open reading frames (ORF), many of these encode cellular orthologous, in particular cytokines, chemokines and a viral GPCR (ORF74). Putative latent transcripts are indicated in green, and cellular orthologous in yellow [261].

HHV-8 lifecycle is composed by two phases: latent and lytic phase. During the first one, viral genome is circularized and not integrated in host chromosomes and no viral genes are transcribed [268]. On the contrary, during the lytic phase the viral genome is linearized and viral genes are transduced in proteins. It is thought that some viral proteins show oncogenic properties whereas others are growth or angiogenic factors, essential for tumor development. One of these proteins is latency-associated nuclear antigen (LANA) which is able to perturb host cellular pathway. In particular, LANA binds p53 inhibiting its ability to induce gene transcription and apoptosis [269] and can inactivate the tumor suppressor retinoblastoma (Rb) protein (Figure 1.19).

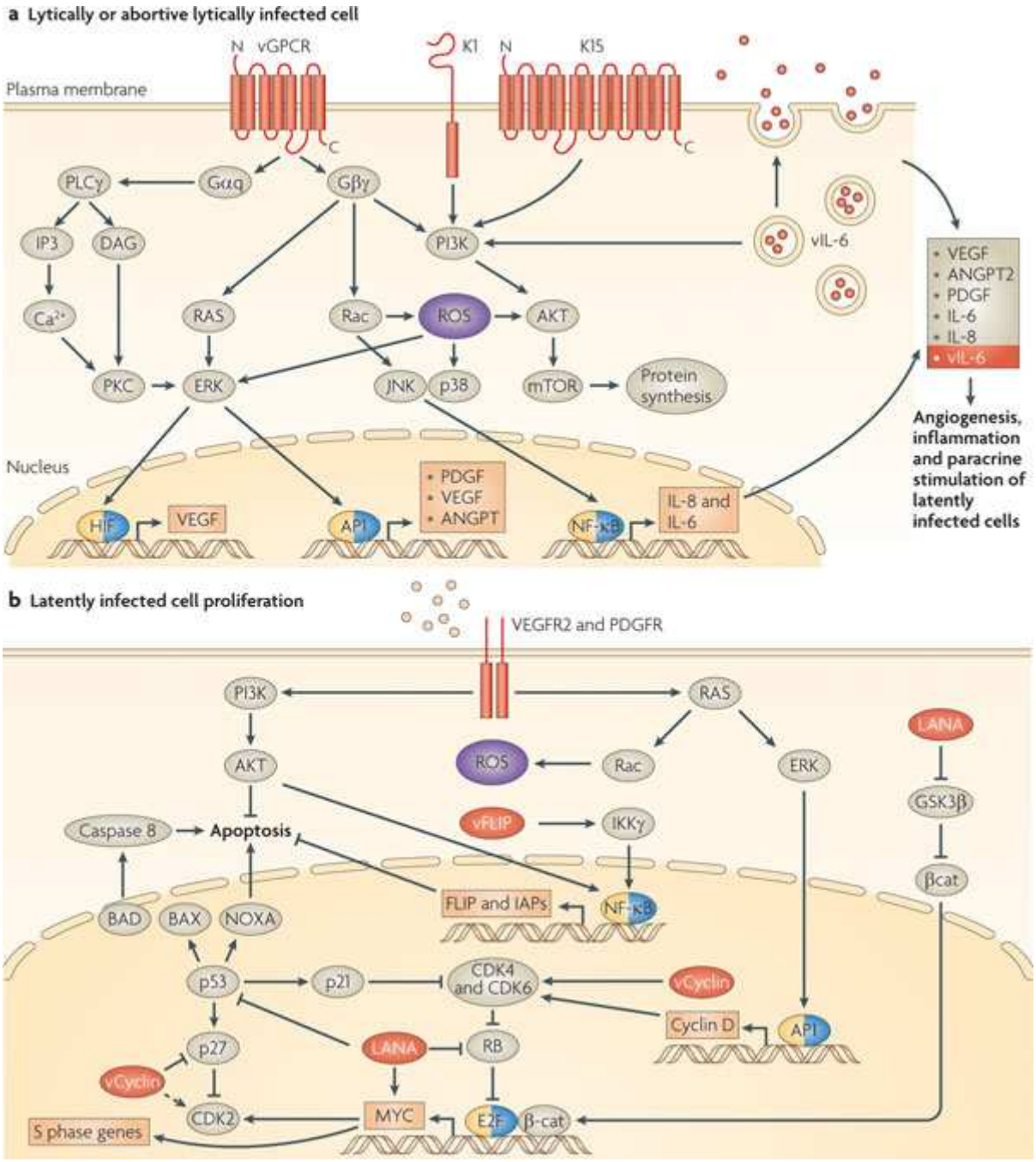

Figure 1.19: – Proposed mechanism of HHV-8-induced sarcoma. In lytic cells HHV-8 expresses early lytic genes like vGPCR and vIL-6 subverting host signaling pathway, activating oncogenes, for example K-RAS and consequently MAPKs [261].

1.6.3.2. HHV-8: tumor growth and subversion of the immune responses

KS growth has been demonstrated to be associated with lytic HHV-8 markers that lead to abnormal production of inflammatory cytokines and chemokines, leading to immune-dysregulation [270]. Spindle cells do not display some characteristics of transformed cells, being euploid and not clonal, and with a growth potential *in vitro* and *in vivo* largely dependent by the autocrine/paracrine activity of angiogenic and inflammatory cytokines, including VEGF-A, bFGF2, IL-6, and IL-1β [244, 271]. It has been proposed that spindle cells produce cytokines that recruit leukocytes and vessels, which in turn produce growth factors required for their proliferation [263, 272, 273]. Consistent with this, KS lesions often occur at inflammatory sites or in scaring tissues, a condition known as Koebner phenomenon, and in patients with the Immune Reconstitution Inflammatory Syndrome [274].

Among inflammatory mediators, chemokines have been extensively investigated in KS pathogenesis because HHV8 has hijacked the chemokine system in several ways [275]. HHV8 through ORF74 encodes a constitutively active GPCR (vGPCR) which shares similarities with CXCR1 and CXCR2 recognizing both angiostatic ELR- and angiogenic ELR+ CXC chemokines and acts as a transforming receptor in transgenic mice [276]. vGPCR displays a constitutive signaling activity, leading to phosphorylation of PI3-kinase and MAPK ERK1/2 and p38 [277]. HHV-8 is also able to produce three CC chemokines (vMIP-I, -II, and -III) which interact with CCR3, CCR8 and CCR4, receptors expressed at high levels in Th2 and Treg cells [278, 279]. These viral inflammatory CC chemokines represent a strategy to subvert and divert effective antiviral and antitumor immunity. In addition, HHV8-infected endothelial cells display increased production of several chemokines, including CCL2, CCL5, CXCL8, and CXCL16 [280, 281].

2. Aim

This thesis is focused on the role in tumor progression of the inflammatory chemokine CCL2 and its two receptors: the canonical CCR2 and the atypical chemokine receptor D6.

In the first part the role of D6 in Kaposi's sarcoma biology has been investigated by using a Kaposi's sarcoma cell line overexpressing D6 injected in nude mice. Moreover D6 expression was also analyzed in human KS lesion confirming *in vivo* evidences.

In the second part, we investigated the possible role of CCR2 in tumor associated macrophage (TAM) polarization and its abilities in shaping tumor microenvironment and supporting tumor progression.

3. Materials and methods

3.1. Part 1

Immunohistochemistry

Formalin-fixed paraffin-embedded cutaneous nodular KS lesions from HIV-seropositive and -seronegative cases were obtained from Luigi Sacco Hospital and Ospedale Maggiore Policlinico, Milan, Italy, respectively. Cutaneous nodular KS lesions from HIV-seronegative patients were classified according to a staging system [264] comprising 4 clinical stages (stage I: maculo-nodular lesions; stage II: infiltrative; stage III: florid; stage IV: disseminated), each further divided according to the speed of disease evolution (A: slow and B: rapid). Ethics approval for D6 expression analysis was obtained from the local Institutional Review Committee and a signed informed consent was obtained from all participants. Sections were incubated with rat anti human D6 monoclonal antibody (clone 196124, 1:100 dilution; R&D Systems) and mouse anti human KSHV Orf73 monoclonal antibody (1:100 dilution; Dako). For murine tumors, frozen sections were incubated with rat anti mouse CD31 monoclonal antibody (1:100 dilution; obtained as described in ref. [282].

Cell culture and transfection

The KS-IMM cell line, originally isolated from a KS occurring in a kidney transplanted immunosuppressed patient [283], was grown in DMEM (Lonza) with 10% FCS. KS-IMM cells were transfected with the hD6/pEFGP-N1 or empty pEFGP-N1 expression plasmids by using Lipofectamine 2000 (Invitrogen) and selected using 400 μg/ml G418 (Invitrogen). For growth curve, KS-IMM cells were seeded in 6-well plates (5 x 10^4 cells/well) and grown under normal conditions for 1-4 days. Every day, cells were harvested with trypsin from three wells for group, diluted in Trypan blue to assess viability and counted. For preparation of cell culture supernatant, KS-IMM cells (1 x 10^6 cells) were cultured in 75 cm^2 flasks with complete medium. After 48 h, culture medium was discarded and fresh medium without G418 resistance was added to the flask for 24 h. Supernatant was collected and centrifuged.

Immunofluorescence microscopic analysis

KS-IMM cells (10^5) were seeded in 24-well plates and grown at 37°C for 18 h. Cells were fixed with 4% paraformaldehyde, then incubated with DAPI. High-resolution images (1024 x 1024 pixels) were acquired sequentially with a 60x/1.4 NA Plan-Apochromat oil immersion objective was used with a FV1000 laser scanning confocal microscope (Olympus). Differential interference contrast (Nomarski technique) was also used. Images were assembled and cropped using the Photoshop software (Adobe Systems).

Reverse-transcriptase polymerase chain reaction

Total RNA was extracted from cell pellets using the TRIZOL reagent (Invitrogen). The reverse transcriptase polymerase chain reaction (RT-PCR) was performed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. Primers for human D6 (forward: 5'-GGGTTTCTCCTTCCACTCCT-3', reverse: 5'-TATTCCCCACATCCTCCTTG-3') and human β-actin (forward: 5'-GCTCGTCGTCGACAACGGCT-3', reverse 5'-CAAACATGATCTGGGTCATCTTCTCT-3'). TaqMan real-time RT-PCR (probes of Applied Biosystems) was used to detect D6 RNA in KS-IMM

seeded in DMEM 1%FCS overnight and stimulated for 8 or 24 h with UO126 (10 μM; Calbiochem) and PLX4032 (1μM; Selleckchem)

Chemokine scavenging assay

KS-IMM transfectants were plated the day before the experiment in 96-well (3 x 10^4 cells/well), then incubated with 10 ng/ml of CCL3L1 (R&D Systems) for increasing times. The supernatant was collected and chemokine concentration was evaluated by sandwich ELISA (R&D Systems).

Animals and treatment

WT and CCR2$^{-/-}$ C57/Bl6 mice and nude CD-1 mice were purchased from Jackson Laboratories and Charles River Italia (Calco), respectively. Animals were housed in pathogen-free conditions, and used at 8-12 weeks of age. Mice were injected s.c. in the flank with 5 x 10^6 KS-IMM cells mixed with liquid matrigel (BD Biosciences). The two major tumor diameters were measured every 2-3 days with a caliper and tumor volume was estimate applying the formula $(d_1 x d_2^2)/2$. To deplete neutrophils *in vivo*, the purified anti-Ly6G rat mAb (clone 1A8; BioXCell) was administered to mice (i.p.; 0.2 mg) 1 day prior to KS-IMM inoculation and every 3 days thereafter. Control animals received purified whole rat IgG2a (BioXCell). Pharmacological inhibition of CCR2 was achieved treating mice daily with 2 mg/kg RS-504393 (Santa Cruz Biotechnology) by oral gavage from the day of KS-IMM injection until the day animals were sacrificed. In some experiments, BMDM (10 ng/ml mM-CSF) generated from WT and CCR2$^{-/-}$ C57/Bl6 mice were mixed in liquid matrigel at 1:2 ratio with KS-IMM cells and s.c. injected. Animal housing and procedures were in accordance with national (D.L. N.116, reviewed and approved Gazzetta Ufficiale della Repubblica Italiana, supplement 40, 18-2-1992) and international law and policies (European Economic Community Council, 1987, Directive 86/609, Official Journal of European Community L 358.1; and Institute of Laboratory Animal Resources, Committee on Life Sciences, National Research Council, 1996, Guide for the Care and Use of Laboratory Animals). Animal procedures were also reviewed

and approved by the Institutional Ethical Committee at Humanitas Clinical and Research Center.

Bone marrow-derived macrophages (BMDM)

BM cells were obtained from femurs of 8 weeks old male WT and CCR2$^{-/-}$ C57/Bl6 mice and plated with DMEM with 5% FCS for 4 h at 37°C. Non-adherent cells were recovered and plated at 3 x 10^5 cells/well in 24 well ultra-low attachment plates (Corning Costar) and cultured for 7 days in the presence of 25% conditioned media from D6$^-$KS-IMM or D6$^+$KS-IMM cultures or with 20 ng/ml mM-CSF (Miltenyi) added every two days. When indicated indomethacin (Sigma) was added to cultures at 10 μM from day 0. After 7 days, cells were detached with cold Accutase (Millipore).

FACS analysis and sorting

Flow cytometry was performed using FACSCanto II flow cytometer and FACSDiva 6.1.1 software (BD Biosciences) or Flowjo (Treestar). KS-IMM cells were sorted for EGFP expression by using a FACS Aria flow cytometer (BD Biosciences). For tumor infiltrate analysis, tumor explants were harvested at the indicated time points, minced and incubated in DMEM with 1% FCS plus collagenase IV (Sigma-Aldrich Chemicals) for 30 min at 37ºC, then passed through a 70 μm nylon mesh filter (BD Falcon). Erythrocytes were lysed with ACK buffer. Cells were stained with anti-mouse: CD45-PercP, CD11b-APC, Ly6G-PE, Ly6C-FITC, I-A/I-E-Biotin, anti-CD206-Biotin (BD Biosciences), and F4/80 (Serotec). Dead cells were excluded by LIVE/DEAD Fixable Cell Stain Kit (Invitrogen). The absolute number was determined using TruCount beads (BD Biosciences) according to the manufacturer's instructions.

Cytokine levels

KS-IMM cells were seeded into 24-well microplates (9 x 10^5 cells/well) and grown under normal conditions for 24 h. The medium was replaced by DMEM with 1% FCS, and in a

particular experiment 10 ng/ml hIL-1β (R&D Systems) were added. Supernatants were collected after 24 h and hCCL2, hIL-6 and hVEGF-A were measured using sandwich ELISA (R&D Systems). mVEGF-A was measured in tumor lysates and BMDM supernatants using ELISA test (R&D Systems) and murine chemokines using bio-plex protein assay (Bio-Rad).

Western blot

0.7 x 10^6 cells were treated as indicated and lysed with a buffer containing 50 mM Tris-HCl (pH 8), 150 mM NaCl, 5 mM EDTA, 1.5 mM $MgCl_2$, 10% glycerol, 1% triton X-100, and protease/phosphatase inhibitors. The lysates were electrophoresed and immunoblotted with the antibody rabbit anti-human ph-ERK1/2 (Cell Signaling) using standards conditions. Chemiluminescence was acquired by ChemiDoc XRS Imaging System, densitometric analysis was performed by Image Lab software (Bio-Rad) and protein band intensity was calculated by normalization over α-tubulin band intensity.

Statistical analysis

Data were compared using unpaired Student's t test. Immunohistochemical data were analyzed by the two-tailed unpaired t test with Welch's correction (GraphPad Prism4 software). *, $P<0.05$; **, $P<0.005$; ***, $P<0.0005$. Linear regression analysis was obtained using Prism4 software.

3.2. Part 2

Bone marrow-derived macrophages (BMDM)

BM cells were obtained from femurs of 8 weeks old male WT and CCR2$^{-/-}$ C57/Bl6 mice and plated in IMDM with 10% FCS overnight at 37°C. Non-adherent cells were resuspended at 5 x 10^6 cells/dish in complete bone marrow macrophage medium (IMDM, 10% FCS, 150 µM MTG, 1%P/S, 1%Glut, 10 ng/mL M-CSF) in low attachment culture dish (Corning Costar) and cultured for 7 days replacing medium at day 3. To prepare BMDM to further stimulations, cells were detached with 2mL of Accutase and replate at the concentration of 0.5 x10^6 cells/mL in complete bone marrow macrophage medium without M-CSF in multiwell 6 or 24 wells low attachment (500uL/well). After overnight culture BMDM were stimulated with 100 ng/mL LPS (Sigma) plus mIFNγ (Peprotech) 20ng/mL or 100 ng/mL LPS alone or 20 ng/mL mIL-4 (Peprotech) for indicated time points.

Human monocytes from buffy coat

Human monocytes were obtained from healthy donor buffy coats by 2-step gradient centrifugation using Ficoll (Biochrom) and Percoll (Amersham). 2 x 10^6 cells were plated in 6 wells in RPMI 1640 medium (Lonza) without FCS for 20 min. Non-adherent cells were discarded, and cells were incubated in RPMI 1640 medium supplemented with 10% FCS overnight. Monocyte polarization was obtained by removing the culture medium and culturing cells in RPMI 1640 medium supplemented with 1% FCS and 100 ng/mL of LPS (Sigma) for indicated time points.

FACS analysis

Flow cytometry was performed using FACSCanto II flow cytometer, LRSFortessa cell analyzer and FACSDiva 6.1.1 software (BD Biosciences). BMDM were stained on membrane surface with anti-mouse: CD11b-APC, Ly6C-FITC, I-A/I-E-Biotin, CD206-Biotin, Streptavidin-PercP (BD Biosciences), and F4/80 PE (Serotec). Intracellular staining of Relmα (Peprotech)

was perform with BD Cytofix/Cytoperm™ Kit (BD Biosciences). Annexin V/7AAD staining was performed on human monocytes according to the manufacturer's instructions (Annexin V: PE Apoptosis Detection Kit I - BD Biosciences). For tumor infiltrate analysis, tumor explants were harvested at the indicated time points, minced and incubated in DMEM with 1% FCS plus collagenase IV (Sigma-Aldrich Chemicals) for 30 min at 37ºC, then passed through a 70 µm nylon mesh filter (BD Falcon). Erythrocytes were lysed with ACK buffer. Dead cells were excluded by LIVE/DEAD Fixable Cell Stain Kit (Invitrogen). Tumor macrophages and PMN were stained with anti-mouse: CD45-PercP, CD11b-PE-CF594, Ly6G-PE, Ly6C-BV605, CD206-FITC (BD Biosciences), I-A/I-E-AlexaFluor700 (eBioscience), F4/80- PacificBlue (Serotec); tumor lymphocytes with CD19-PacificBlue, CD3-FITC, CD4-PeCy7(BD Biosciences), CD25-PE, Foxp3-APC (eBioscence). The absolute number was determined using TruCount beads (BD Biosciences) according to the manufacturer's instructions.

Cytokine levels

Supernatants were collected from BMDM culture at indicated time points and mIL-10 and mVEGF-A were measured using sandwich ELISA (R&D Systems).

Reverse transcription (RT) and qPCR

Total RNA was extracted from cell pellets using miRNeasy Mini Kit (Qiagen). The reverse transcriptase polymerase chain reaction (RT-PCR) was performed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). To measure iNOS2, Arg-1 and IL-12p40 expression in BMDM, SYBR Green real-time PCR was used. Primers for murine iNOS2 (forward: 5'-gccaccaacaatggcaaca-3', reverse: 5'-cgtaccggatgagctgtgaatt-3'), murine Arg-1 (forward: 5'-cagaagaatggaagagtcag-3', reverse 5'-cagatatgcagggagtcacc-3') and murine IL-12p40 (forward: 5'-ggaagcacggcagcagaata-3', reverse 5'-aacttgagggagaagtaggaatgg-3').

Western blot

1 x 10^6 of WT and $CCR2^{-/-}$ BMDM cells or human monocytes were treated as indicated and lysed with a buffer containing 50 mM Tris-HCl (pH 8), 150 mM NaCl, 5 mM EDTA, 1.5

mM $MgCl_2$, 10% glycerol, 1% triton X-100, and protease/phosphatase inhibitors. The lysates were electrophoresed and immunoblotted with the antibodies rabbit anti-human phospho-NF-Kb p65, phospho-p38 MAPK and phospho-p44/42 MAPK using standards conditions. All antibodies were purchased from Cell Signaling. Chemiluminescence was acquired by ChemiDoc XRS Imaging System, densitometric analysis was performed by Image Lab software (Bio-Rad) and protein band intensity was calculated by normalization over α-tubulin band intensity or total p38 MAPK.

Animals and treatment

WT and $CCR2^{-/-}$ C57/Bl6 mice were purchased from Jackson Laboratories. Animals were housed in pathogen-free conditions, and used at 8-12 weeks of age. Animal housing and procedures were in accordance with national (D.L. N.116, reviewed and approved Gazzetta Ufficiale della Repubblica Italiana, supplement 40, 18-2-1992) and international law and policies (European Economic Community Council, 1987, Directive 86/609, Official Journal of European Community L 358.1; and Institute of Laboratory Animal Resources, Committee on Life Sciences, National Research Council, 1996, Guide for the Care and Use of Laboratory Animals). Animal procedures were also reviewed and approved by the Institutional Ethical Committee at Humanitas Clinical and Research Center.

WT mice were s.c. injected with BMDM generated from WT and $CCR2^{-/-}$ C57/Bl6 mice mixed in liquid matrigel (BD Biosciences) at 1:1 ratio with 3LL cells (Lewis lung carcinoma cell line). The two major tumor diameters were measured every 2-3 days with a caliper and tumor volume was estimate applying the formula $(d_1 x d_2^2)/2$.

Statistical analysis

Data were compared using unpaired Student's t test of Prism4 software. *, $P<0.05$; **, $P<0.005$; ***, $P<0.0005$.

4. Results

4.1. D6 inhibits CCR2+ macrophage-dependent angiogenic switch in Kaposi's sarcoma

4.1.1. D6 is expressed in KS lesions

The atypical chemokine receptor D6 is known to be expressed by the afferent lymphatic endothelium and in particular by the lymphatic endothelial cells (LEC) [170]. Due to the fact that this cells are considered the precursors of the KS spindle cells [242], D6 expression in lesions of KS patients was investigated by immunohistochemistry. A broad expression of the receptor was detected in Kaposi's lesions and in particular in tumor spindle cells that were also positive for the HHV8-encoded nuclear antigen LANA-1 (Figure 3.1A). Moreover D6 expression was evident on membrane surface and in intracellular compartments, as previous reported in transfectant cells [168]. Analyzing lesions from HIV-seronegative or seropositive patients, a significant increased expression of D6 was observed in indolent cutaneous nodular lesions of HIV- patients as compared to aggressive lesions of HIV+ ones (Figure 3.1B). Furthermore lesions from seronegative patients at the maculo-nodular stage (stage I) were analyzed and classified, based on the speed of the disease progression, in slow (A) and rapid (B) progressive [264]. Upon this classification, D6

expression resulted to be significantly higher in patients who showed a slow disease progression rate (stage IA) as compared to those characterized by a rapid progression rate (stage IB) (Figure 3.1C).

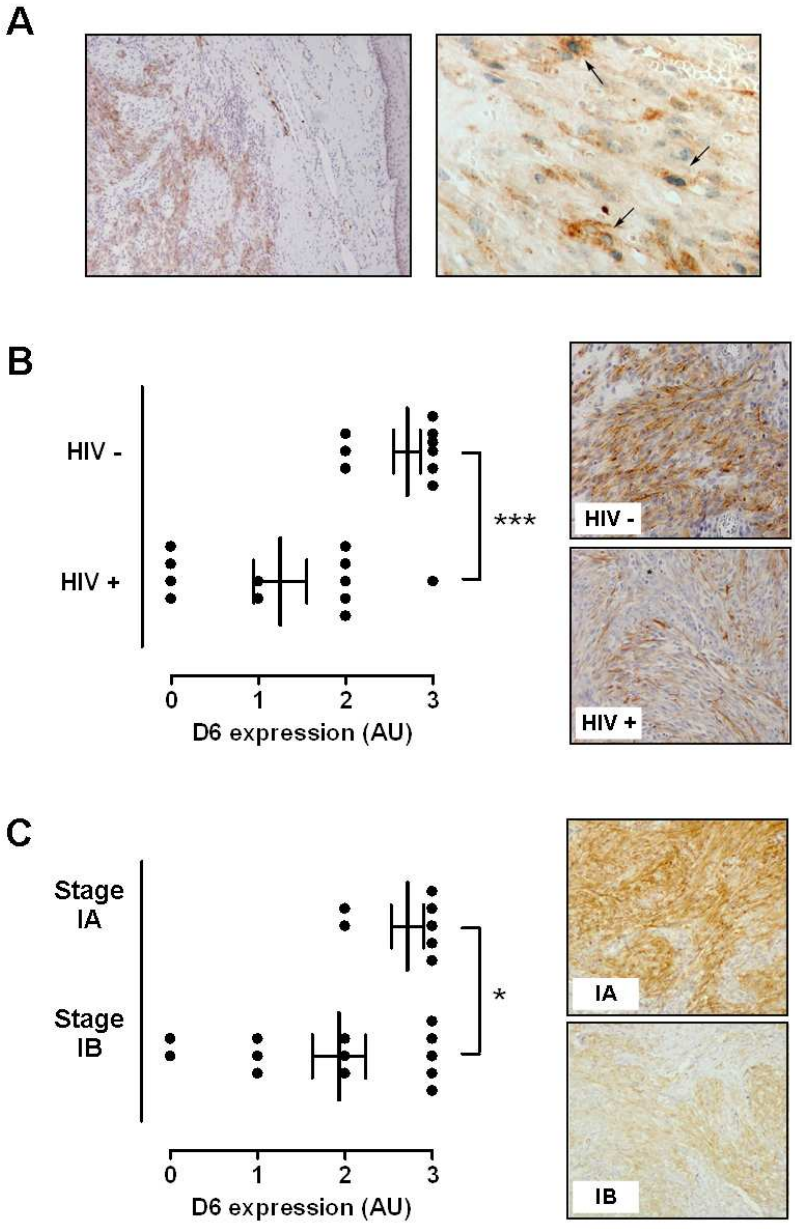

Figure 3.1 - D6 expression in human KS lesions and KS-associated HHV8+ spindle cells. (A) Representative immunohistochemical analysis of a cutaneous KS lesion from a HIV-seronegative patient stained for human D6 (brown) and viral antigen LANA-1 (blue). KS spindle cells are indicated by arrows. Magnification 10x (left panel) and 40x (right panel). (B) D6 immunohistochemical analysis

in cutaneous maculo-nodular KS lesions from HIV-seropositive and seronegative patients (n=22) and from HIV-seronegative patients affected by disease with slow (stage IA) and rapid (stage IB) progression rate (panel C, n=21). Representative panels of D6 staining are shown on the right (magnification 20x). Scatter plots report mean values ± SEM from five random fields evaluated for number of positive cells using a semiquantitative scale (0: no positive cells; 1: 0÷25% positive cells; 2: 25÷75% positive cells; 3: >75% positive cells). *p<0.05, ***p<0.0005.

4.1.2. Generation and characterization of D6+ and D6- KS-IMM transfectants

To gain insight the role of D6 in KS, we used the human cell line KS-IMM, originally derived from a biopsy of an iatrogenic KS. It is a not-subclonal population which have a Kaposi's Sarcoma characteristic spindle shape and is able to grow in nude mice as highly vascularized tumors closely resembling human KS lesions [283].

KS-IMM were analyzed by RT-PCR for the expression of D6 and other chemokine receptors. They resulted to be negative for D6 but also for CCR2 CCR4 and CCR5, receptors that share some ligand with D6. The lack of D6 may be explained by the fact that often lymphatic endothelial cells can lose expression of specific markers when cultured in vitro, as already demonstrated for the lymphatic endothelial cell line MELC [284]. Therefore, in order to obtain a cell population expressing D6, cells were transfected with the empty pEGFP-N1 vector or with D6-pEGFP-N1. Three independent populations with each vector were achieved and were called D6-KS or D6+KS respectively. The over-expression of the receptor was confirmed by RT-PCR (Figure 3.2 A), FACS (Figure 3.2 B) and confocal analysis (Figure 3.2 C). All the transfectants showed expression of GFP and human adhesion molecule ICAM-1, whereas only D6+KS cells expressed D6. However, by flow cytometry only 25% of D6+KS resulted to be positive for D6 staining, suggesting an intracellular compartmentalization of the receptor. In fact fluorescence microscopy displayed the receptor to be mainly stored in intracellular perinuclear compartments consistent with the distribution of D6 observed in KS spindle cells (Figure 3.2 C) and other D6 transfectants [169]. In order to confirm the functional activity of the receptor, cells were incubated with exogenusly provided CCL3L1, one of the active ligand of D6 and, at different time points, supernatants were collected and measured by ELISA test. Only D6+KS were found to be able to degrade chemokines, showing

high scavenging activity at 6 hours (85 ± 5%) and 24 hours (97 ± 3%), establishing that the receptor is capable of its typical scavenger activity. Furthermore CCL2 autocrine production in basal conditions or after IL-1β stimulation [283] was reduced in the supernatant of D6 expressing cells when compared to D6-KS (Figure 3.2 D) while no difference in IL-6 or VEGF-A amounts was found, consistent with D6 specificity for chemokines. Finally, in order to understand if D6 overexpression modified KS-IMM growth, cells were plated at the same concentration and counted for the following four days. The same growth rate of D6-KS and D6+KS cells was detected with a calculated duplication time of 23,5 ± 2,3 hours and 25,7 ± 2,8, respectively (Figure 3.2 F). In conclusion, KS-IMM cells after transfection express a fully functional D6 scavenger receptor, which has an impact on the chemokine milieu but not on the production of other cytokines neither on cell growth in vitro.

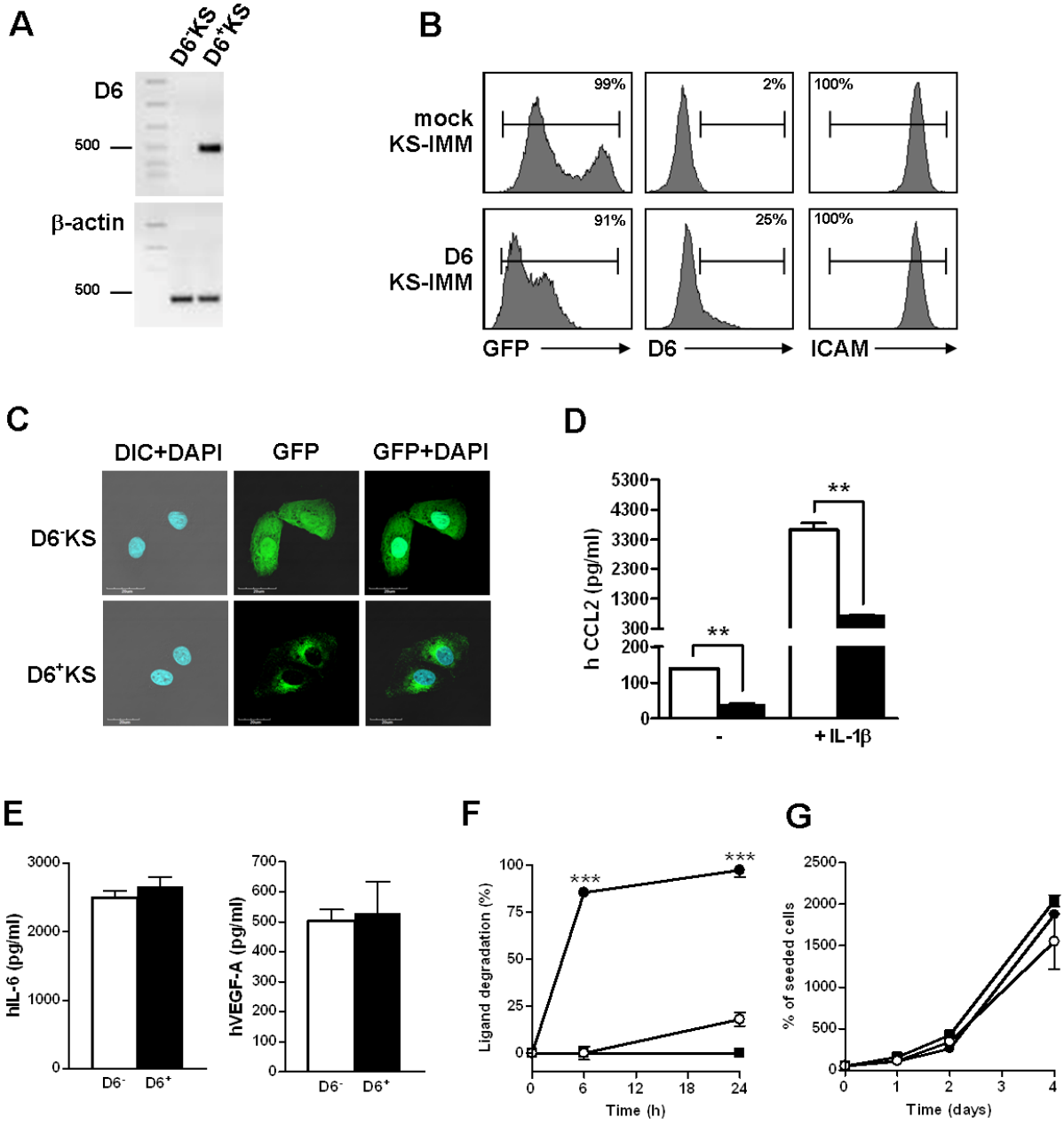

Figure 3.2 - Characterization of D6+KS. (A) RT-PCR of D6 expression in a representative KS-IMM cell line transfected with D6-pEGFPN1 expression vector (D6+KS) and the corresponding control vector pEGFP-N1 (D6-KS). (B) FACS analysis of GFP, D6 and ICAM-I expression in a representative D6-KS (upper panel) and D6+KS transfectants (lower panel). (C)Confocal microscopy of a representative D6+ (lower panel) and D6- (upper panel) KS cells. (D) Hccl2 production by D6+ (black bar) and D6- (white bar) KS cells in homeostatic conditions or after IL-1β stimulation. (E) Human IL-6 and VEGF-A production by D6+ (black bar) and D6- (white bar) KS cells cultured in a medium containing low FCS (1%) for 24 h. (F) Kinetics of CCL3L1 scavenging in representative KS cell lines (KS-IMM wild-type ■, D6+KS ●,D6-KS ○). (G) Growth curve of KS cell lines (KS-IMM wild-type ■, D6+KS ●,D6-KS ○). Data are expressed as the percentage of seeded cells. In all panels data are the mean ± SEM of at least three independent experiments. **p<0.005,***p<0.0005 D6-KS versus D6+KS.

4.1.3. D6 expression restrains KS growth in vivo

In order to investigate the in vivo role of D6 in KS biology, three independent populations of D6+KS and D6-KS were implanted in the flank region of CD-1 nude mice and tumor growth was measured with the caliper twice a week. The two groups of animals showed a comparable tumor uptake, growing in a similar way until day 19 after injection. However, from that time on D6-KS were significantly larger than D6+KS and, at the conclusion of the experiment, they were on average 2.77±1.28 times bigger than D6+KS. These results suggest that the expression of D6 did not influence tumor uptake but strongly reduce tumor growth (Figure 3.3).

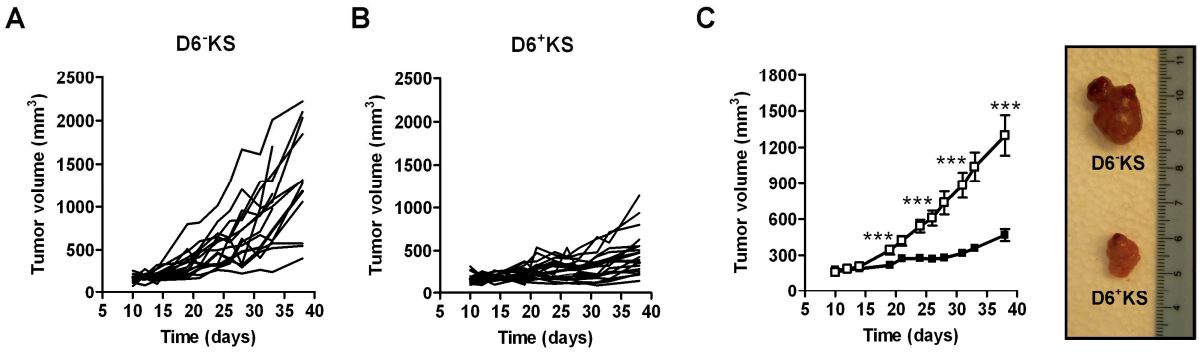

Figure 3.3 - D6 expression restrains KS tumor growth in vivo. Panels A and B report the growth rate of tumors generated in the flank of each CD-1 nude mouse injected with 5 x 106 D6-KS (n=23) or D6+KS (n=17) cells, respectively. Panel C shows average volumes ± SEM of tumors derived from D6+KS (■) and D6-KS (□). Representative tumor explants derived from D6-KS (upper) and D6+KS (lower) are shown on the side. ***p<0.0005.

4.1.4. Reduced levels of inflammatory chemokines in D6+KS tumors

In order to identify the mechanism by which D6 overexpression at tumor site inhibited KS-IMM growth, tumor lysates from D6-KS and D6+KS were analyzed by ELISA for cytokine content. Inflammatory cytokines and chemokines are key elements in the pathogenesis of Kaposi's Sarcoma, being an highly vascularized tumor characterized by an abundant inflammatory infiltrate, mainly composed by T and B lymphocytes and monocytes, and a prominent angiogenic process. Leukocytes and tumor cells produce many inflammatory cytokines, including IL-6, TNF-α, TNF-β, INF-γ, IL-1α and IL-1β, and angiogenic growth factors, for example VEGF, that acts in autocrine and paracrine manner promoting tumor progression.

As expected, when compared to D6-KS lysates, D6+KS lysates contained lower amount of host-derived inflammatory chemokines mCCL2, mCCL5 and mCCL3, which are efficiently degraded by D6 (Figure 3.4 A, 3.4 B and 3.4 C, respectively). Very low concentrations of hCCL2 were detected (data not shown), indicating that the main source of chemokines in the system were infiltrating host cells rather than tumor cells, as reported for some human tumors [187].

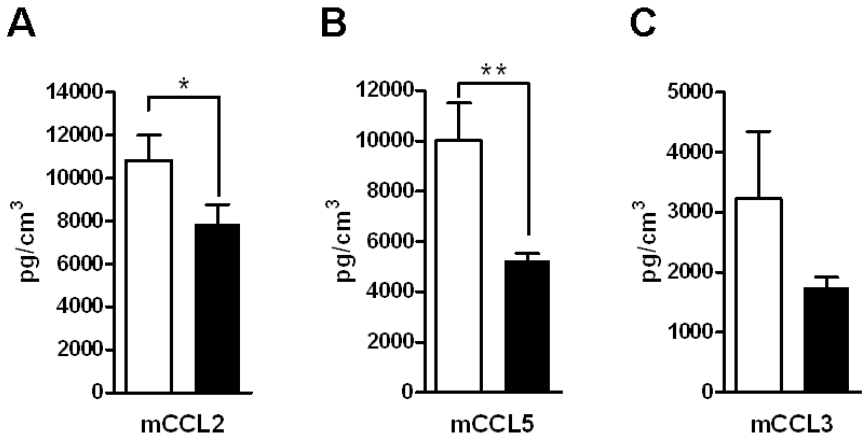

Figure 3.4 - Increased inflammatory chemokine content in D6-negative tumors. mCCL2 (A), mCCL5 (B), and mCCL3 (C), and were measured in homogenized tumors at day 14 after inoculation. Data are expressed as the mean concentration ± SEM of the chemokine levels normalized for the tumor volume (n= 4/each group). In all figures histograms represent the mean ± SEM of at least three independent experiments (n = 5 tumors/group) of D6+KS (black bar) or D6-KS (white bar). *p<0.05, **p<0.005.

4.1.5. D6 expression inhibits tumor growth by modulating leukocyte infiltrate

As D6 expression influences the intratumoral chemokine milieu, tumors at different time points after inoculation were explanted and characterized for their cellular composition by flow cytometry analysis. Tumor masses were composed by about 50% of tumor cells, identified as FSC^{high}, SSC^{high}, EGFP+, CD45- cells while the remaining 50% of the tumor masses was represented by infiltrating leukocytes (EGFP-/CD45+) (Figure 3.5 A). D6+KS tumors displayed a significantly reduced number of both tumor cells and infiltrating leukocytes when compared to D6-KS tumors (Figure 3.5 B-C). Furthermore the expression of D6 not only had significant impact on the number of tumor cells and infiltrating leukocytes, but also qualitative effects on infiltrating leukocytes. In fact, in the absence of D6, KS tumors showed a significant increase in infiltrating neutrophils, identified as CD11b+, $Ly6C^{int}$, F4/80-, Ly6G+ (Figure 3.5D). Moreover TAM were recognized as CD11b+, Ly6C-, F4/80+ (R3, Figure 3.5E), while two populations of monocytes were classified as CD11b+, $Ly6C^{high}$, F4/80- (R1) and CD11b+, $Ly6C^{high}$, F4/80+ (R2). On the basis of this gating strategy D6-KS tumors were found to have increased TAM but a reduced number of monocytes. Moreover we checked for the polarization skewing of tumor associated macrophages, analyzing the expression of the M1 marker MHC-II and the M2 marker mannose receptor, also known as CD206. Compared to D6+KS, TAM infiltrating D6-KS tumors had decreased levels of MHC-II and a tendency to have more CD206 (Figures 3.5F and 3.5G, respectively).

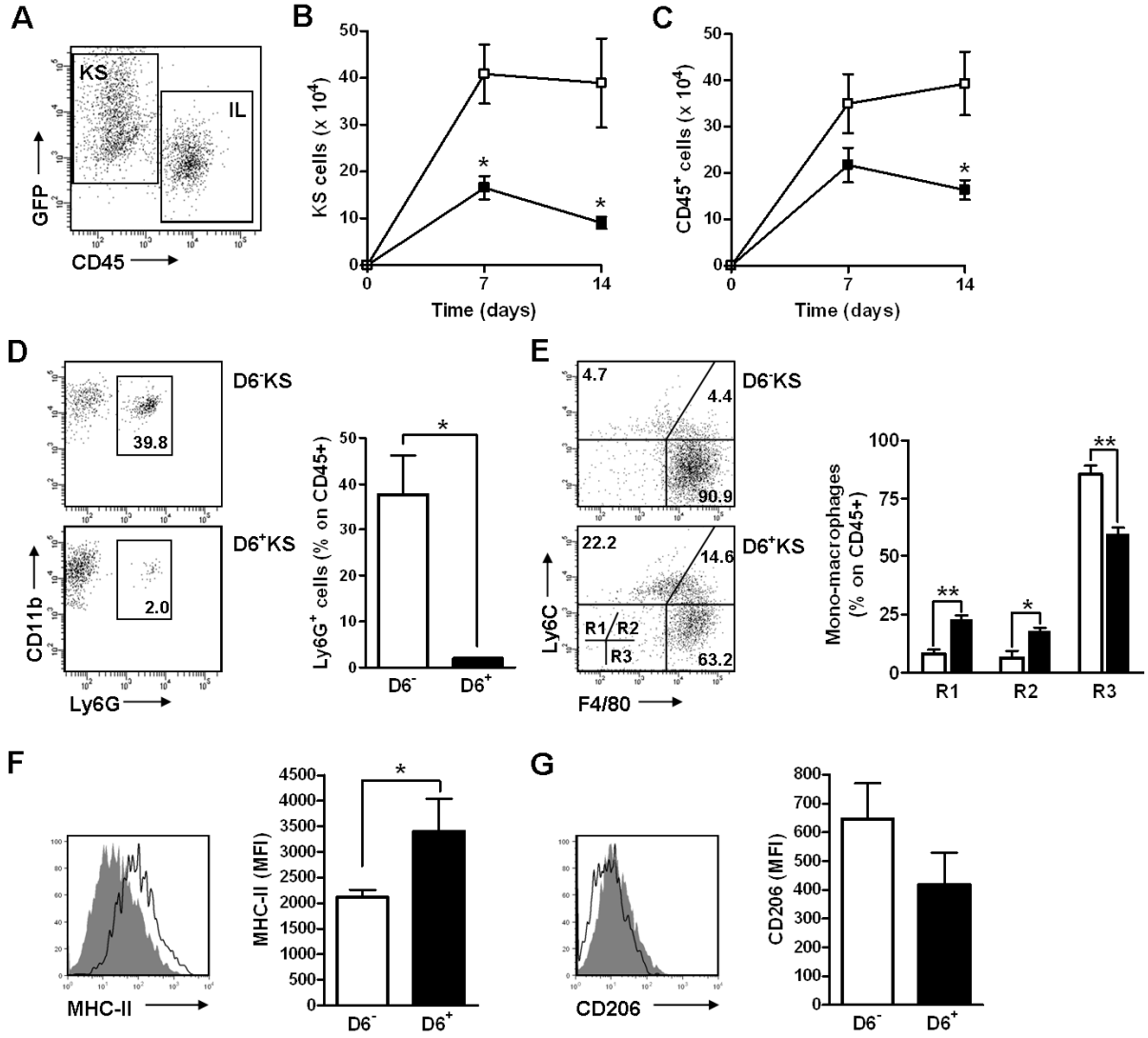

Figure 3.5 - Increased leukocyte infiltrate in D6-negative tumors. (A) Representative dot plot analysis of an enzymatically digested tumor stained and analyzed by flow cytometry showing gating strategy to detect tumor cells (KS) and infiltrating leukocytes (IL) analyzing live cells and singlets in single cell suspension for the expression of GFP and CD45. Mean ± SEM of the total number of GFP+ KS cells (B), and CD45+ infiltrating leukocytes (C) at day 7 and 14 after inoculation of D6+KS (■) and D6-KS (□) are shown. (D) Representative dot plot and mean of the percentage of CD11b+ Ly6G+ neutrophils in the CD45+ gate and (E) immature monocytes (R1: Ly6Chigh, F4/80-), monocytes (R2: Ly6Chigh, F4/80+) and macrophages (R3: Ly6C-, F4/80+) in the CD11b+ Ly6G- gate at day 7. Representative histogram plot and mean fluorescence intensity (MFI) of MHC-II (F) and CD206 (G) expression of R3 gated cells in D6+ (black line) and D6-KS (grey area) tumors. All gates are based on isotype controls. In all figures histograms represent the mean ± SEM of at least three independent experiments (n = 5 tumors/group) of D6+KS (black bar) or D6-KS (white bar). *p<0.05, **p<0.005.

Similar results were also obtained differentiating in vitro bone marrow derived macrophages (BMDM) with tumor conditioned medium (TCM) derived from D6+ or D6- KS cells. In fact BMDM after 7 days of culture with D6-TCM showed higher percentage of macrophages as compared to BMDM cultured with D6+KS TCM (Figure 3.6A). Moreover increased levels of the M2 marker CD206 (Figure 3.6 B) and reduced levels of the M1 marker MHC-II (Figure 3.6 C) were induced by TCM from D6-KS as compared to D6+KS cells. All together *in vitro* and *in vivo* data indicate that reduced levels of D6 are associated with increased TAM recruitment at tumor site and their local maturation towards M2-like polarization.

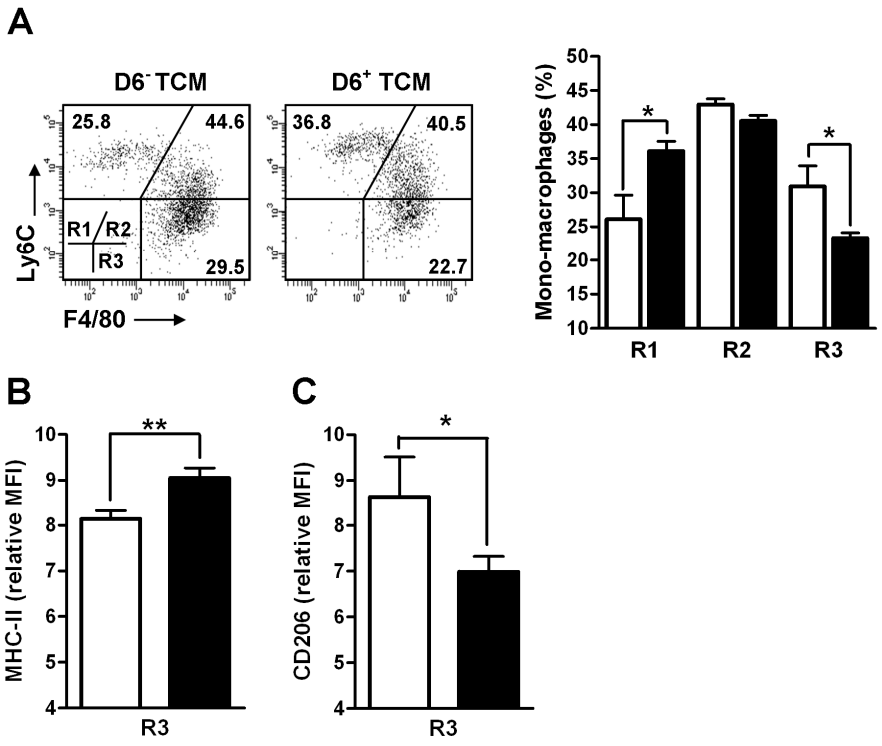

Figure 3.6 - Increased M2-like macrophage differentiation induced by D6-KS supernatant. (A) Representative dot plot analysis and percentage of immature monocytes (R1), monocytes (R2) and macrophages (R3) of WT BMDM cultured with D6-KS (white bar) or D6+KS (black bar) TCM labeled and gated as described in Figure 3H. Relative MFI of MHC-II (B) and CD206 (C) on R3 gated macrophages cultured with D6-KS (white bar) or D6+KS (black bar) TCM. Mean ± SEM of at least three independent experiments is shown. *p<0.05, **p<0.005.

4.1.6. CCR2-dependent TAM recruitment is required for KS growth

Analyzing tumor infiltrated leukocytes we found that D6 expression has a significant impact on the number of neutrophils, monocytes and tumor associated macrophages but it also has qualitative effects on infiltrating macrophages, skewing them toward a more M1 phenotype. To directly assess the role of different populations of tumor-infiltrating leukocytes on KS growth, depletion experiments were performed.

4.1.6.1. Neutrophils depletion did not affect D6-KS growth

Firstly neutrophils, which have been observed to be significantly increased in tumors lacking D6, were depleted. The depletion was achieved by injecting mice 1 day prior to KS-IMM inoculation and every 3 days thereafter with anti-Ly6G 1A8 MoAb whereas control animals received purified whole rat IgG2a. Anti-Ly6G administration completely depleted neutrophils present in the blood (Figure 3.7 A) and in the KS tumors (Figure 3.7 B) but no reductions or modifications in the progression of D6- and D6+KS tumors were seen (Figure 3.7 C). This data indicate that neutrophils are not involved in supporting D6-KS tumor growth.

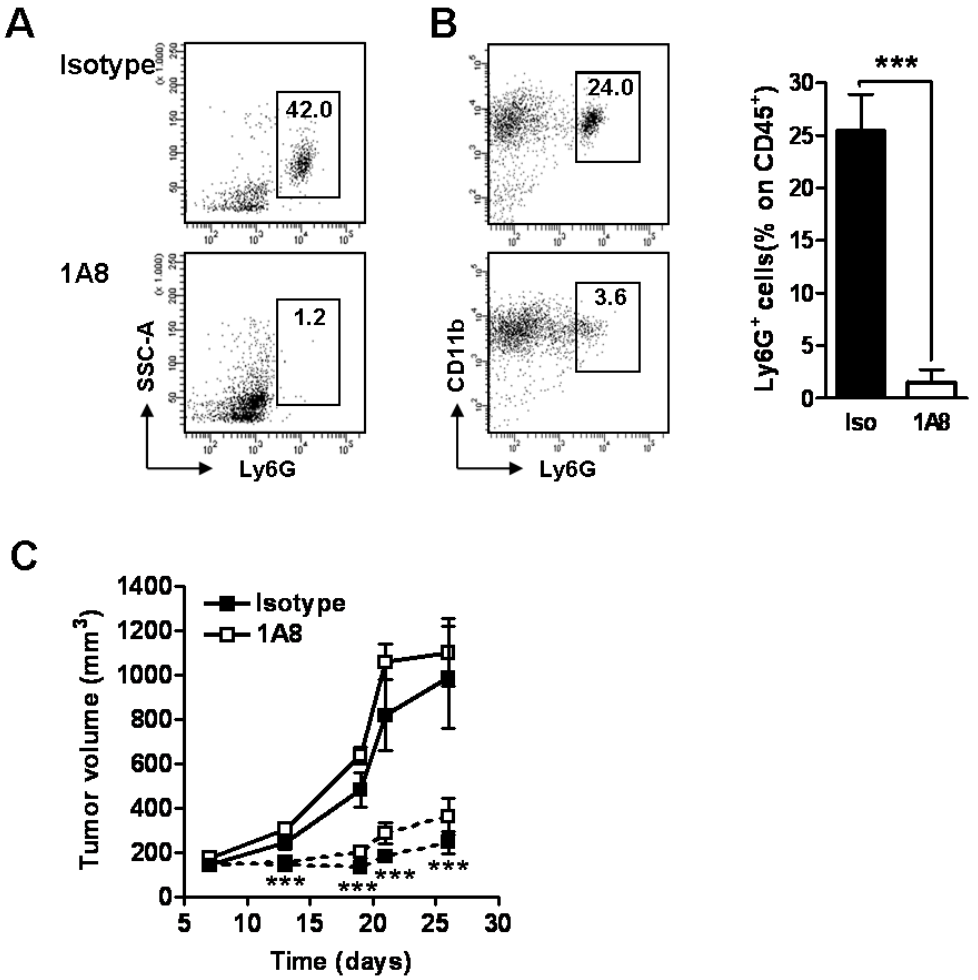

Figure 3.7 – Neutrophil depletion did not affect KS tumor growth. Mice inoculated with D6-KS or D6+KS cells were treated with a blocking antibody against Ly6G (1A8). Representative dot plot analysis at day 25 of circulating (A) and tumor-associated (B) PMN (CD11b+, Ly6G+) of D6-KS bearing mice before and after 1A8 treatment. The graph reports the percentage ± SEM of PMN in D6-KS tumors. (C) D6- (solid line) and D6+KS (dashed line) tumor volume after s.c. inoculation in PBS or 1A8-treated mice. Results are expressed as mean ± SEM. ***p<0.0005, D6+ versus D6-KS.

4.1.6.2. Inhibition of CCR2-dependent macrophage recruitment restrains D6-KS growth

Once neutrophils were excluded as population mainly involved in supporting tumor growth, monocyte and macrophage recruitment at tumor site was inhibited by antagonizing CCR2, chemokine receptor fundamental for directional monocyte traffic. Mice were treated with the antagonist of CCR2 called RS-504393 by oral gavage from the day of KS-IMM injection until the day animals were sacrificed. The treatment and the associated reduction of circulating monocytes (Figure 3.8 A) and infiltrating TAM (Figure 3.8 B) had no effect on D6+KS growth, but significantly inhibited D6-KS growth rate at early time points (Figure 3.8 C). The fact that a CCR2 antagonist only partially inhibited the growth of D6-KS is consistent with the fact that these lesions contain increased levels of other monocyte attracting chemokines (Figure 3.4 B and 3.4 C).

Finally in order to evaluate the importance of CCR2-expressing macrophages in endorsing tumor, WT or $CCR2^{-/-}$ BMDM were co-injected with D6+KS in nude mice. When co-injecting with WT but not $CCR2^{-/-}$ BMDM D6+KS growth improved and became similar to D6-KS one (Figure 3.8D). In conclusion data indicate the relevance of CCR2 expression by TAM for their pro-tumoral activity beyond their local recruitment

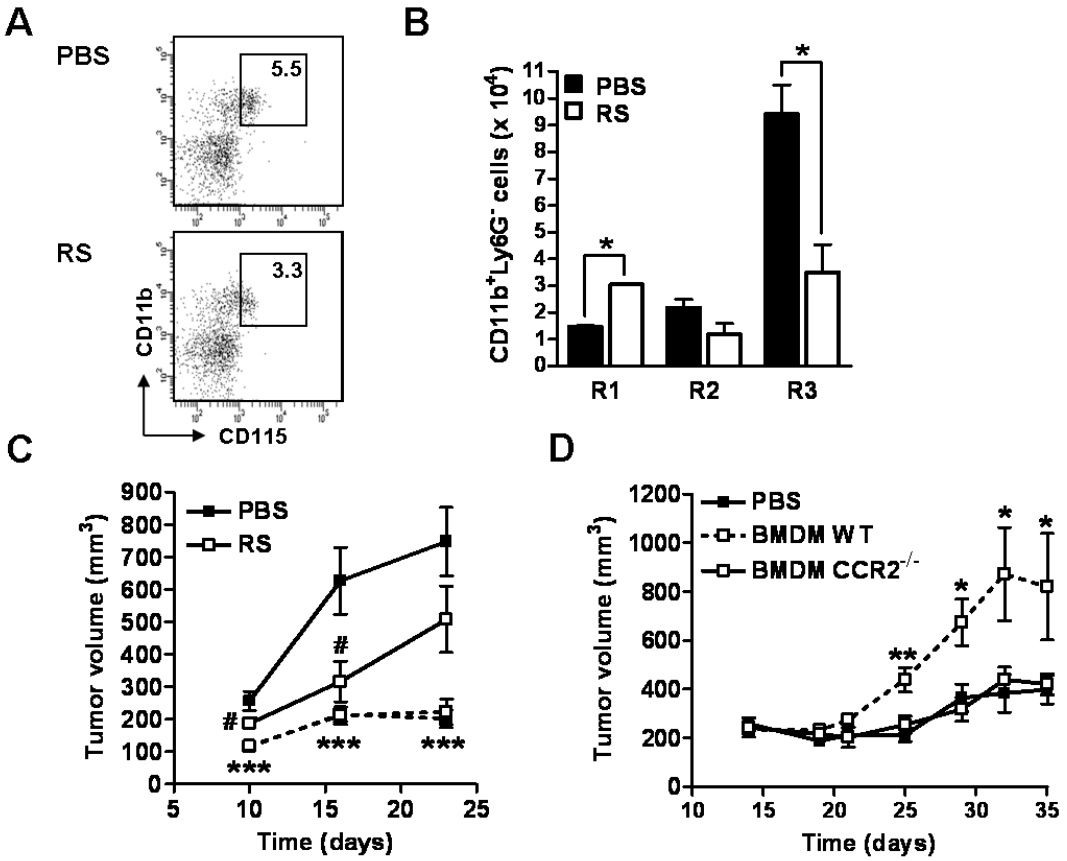

Figure 3.8 – KS growth is sustained by TAM recruitment. Mice inoculated with D6-KS or D6+KS cells were treated with the specific CCR2 antagonist RS-509343 (RS). (A) Representative flow cytometry gating scheme at day 25 of circulating monocytes (CD11b+, CD115+). (B) CD11b+Ly6G- tumor infiltrate in PBS-treated versus RS-treated D6-KS tumors. The monocyte-macrophage population was gated as described in figure 5E and number ± SEM is reported in the graph. (C) D6-KS (solid line) and D6+KS (dashed line) tumor volumes after treatment with PBS or RS. Panel D shows tumor growth of D6+KS, injected alone (■) or with WT (dotted line) or $CCR2^{-/-}$ BMDM (□). In all panels data are representative of two independent experiments (n = 8 mice/group). Results are expressed as mean ± SEM. *p<0.05, ***p<0.0005, D6+ versus D6-KS; #p<0.05 treatment versus control.

4.1.7. Increased production of VEGF-A and angiogenesis in D6-KS

From *in vivo* experiments we established the importance of CCR2 expressed by macrophages at tumor site, underlining a relevant effect of the receptor beyond cell recruitment. In order to identify the mechanism by which CCR2+ TAM promote KS growth, the content of the angiogenic factor VEGF-A and the expression of the endothelial cell marker CD31 were investigated. In fact by the literature there are evidences indicating that CCR2 engagement can induce VEGF-A production through an autocrine pathway requiring COX2 activity [285], [286]. While no difference was found for human VEGF-A content (data not shown), D6-incompetent tumors showed higher levels of murine VEGF-A as compared to D6-competent tumors (Figure 3.9 B). Moreover immunohistochemistry analysis with anti-CD31 performed on sections of KS tumors showed that in D6 expressing tumors few vascular structures were present, whereas D6-KS tumors were highly vascularized (Figure 3.9 A). No differences were seen between D6- and D6+KS tumors in terms of necrosis, as assessed by hematoxylin-eosin staining of histological sections (data not shown). Finally to define D6-KS derived factor influencing VEGF-A production in vitro experiments were performed. BMDM were cultured with MCSF and 25% of D6+ or D6-KS TCM and levels of VEGF-A were measured. The conditioned medium of D6-KS but not D6+KS cells increased the production of mVEGF-A by BMDM (Figures 3.9 C). Interestingly, VEGF-A production induced by D6-KS TCM was reverted when $CCR2^{-/-}$ BMDM were used (Figures 3.9 C), indicating a key role of tumor-derived CCR2 agonists in the acquisition of a pro-angiogenic phenotype. Furthermore to check the mechanism involved in CCR2-induced VEGF production, the COX2 inhibitor indomethacin was used to treat BMDM during their culture. The treatment with indomethacin inhibited VEGF-A production by BMDM stimulated with D6-KS TCM (Figures 3.9 C). In conclusion CCR2 agonists, which are efficiently degraded in D6+KS tumors, induce a pro-angiogenic phenotype of TAM that sustain D6-KS growth.

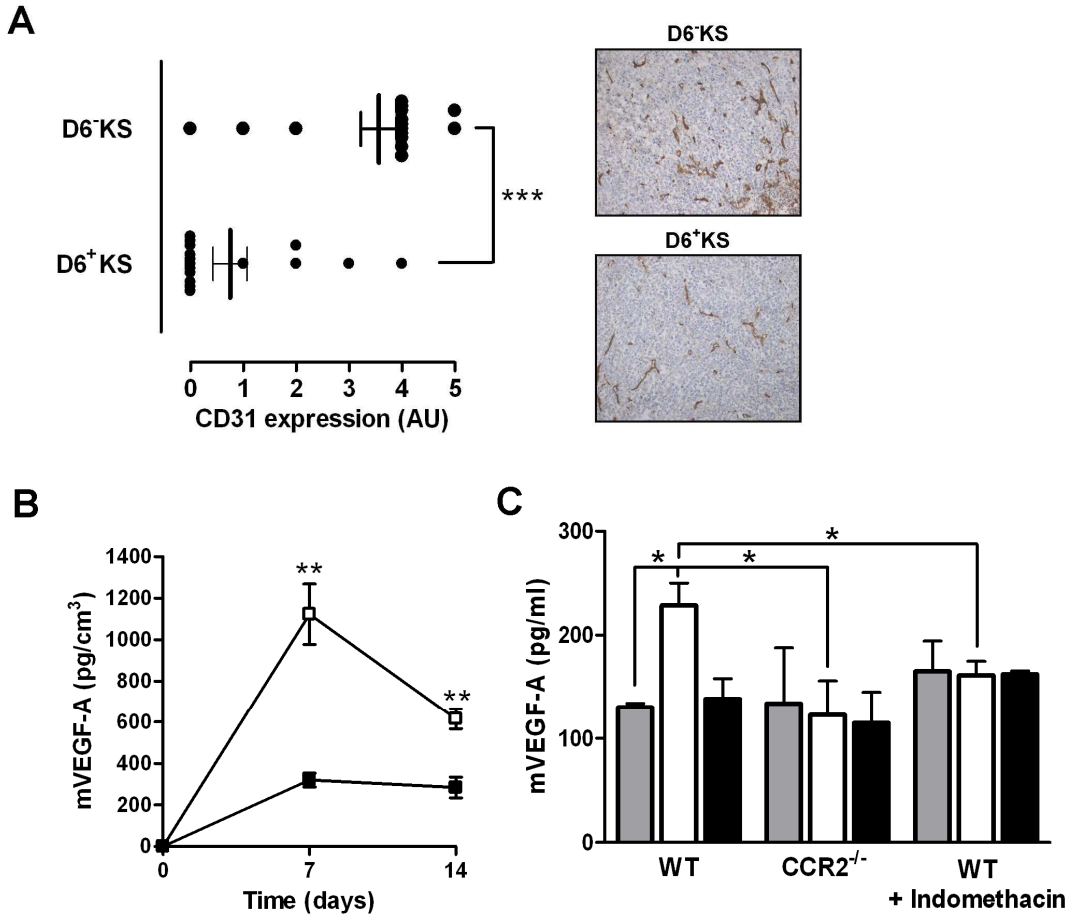

Figure 3.9 – CCR2^{+} TAM sustain angiogenesis in D6-KS tumors. Panel A shows mean ± SEM of the number of CD31+ cells (semiquantitative score: 0: no positive cells; 1: 0÷20% positive cells; 2: 20÷40% positive cells; 3: 40÷60% positive cells; 4: 60÷80% positive cells; 5: > 80% positive cells). Score was given as the mean from five random fields for each sample. Representative images of mCD31 immunohistochemical analysis in D6+KS and D6- KS tumors at day 7 after implantation are shown (magnification 20x). Panel B shows mVEGF-A concentration in homogenized D6+KS (■) and D6-KS (□) tumors at indicated time points. Data are expressed as the mean ± SEM (n = 4/each group) normalized for the tumor volume. Panel C shows mVEGF-A production by WT and CCR2$^{-/-}$ BMDM stimulated for 6 days with D6+KS (■) or D6-KS (□) TCM or with normal medium (■) and where indicated with indomethacin. Mean ± SEM of at least three independent experiments is shown. Results are expressed as mean ± SEM. *p<0.05, **p<0.005, ***p<0.001.

4.1.8. Role of the ERK pathway in the downregulation of D6 expression

From *in vivo* experiments D6 resulted to act as oncosupressor molecule, inhibiting KS growth by shaping tumor microenvironment. At the same time, analysis of KS sections from patients demonstrated an inverse correlation between D6 expression and tumor aggressiveness, supporting *in vivo* results. Therefore, to understand the mechanism by which tumor cells are able to down-regulate D6 expression, *in vitro* experiments using KS-IMM cell line were performed. Firstly K-Ras/B-Raf/MEK pathway was investigated, being it involved in HHV8 reactivation process in KS [287]. Furthermore K-Ras activating mutations or amplifications are frequently found in KS lesions, particularly in advanced stages [288], [289]. Indeed KS-IMM cell line was analyzed to examine the presence of mutations on this pathway. While no mutations in the K-Ras gene were detected, the B-Raf V600E oncogenic mutation was present (Figure 3.10 A). Consistent with the known ability of activated B-Raf to phosphorylate MEK, that respectively activates ERK1/2 [290], KS-IMM, in basal conditions, showed high levels of ERK1/2 phosphorylation (Figure 3.10 B), which were inhibited by B-Raf inhibitor PLX4032 and MEK inhibitor U0126. The suppression of the constitutive activation of the ERK pathway by these inhibitors resulted in a concomitant up-regulation of D6 expression in KS-IMM, indicating that, in KS-IMM, D6 expression is down-regulated as a consequence of the constitutive activation of B-Raf/MEK/ERK pathway (Figure 3.10 C).

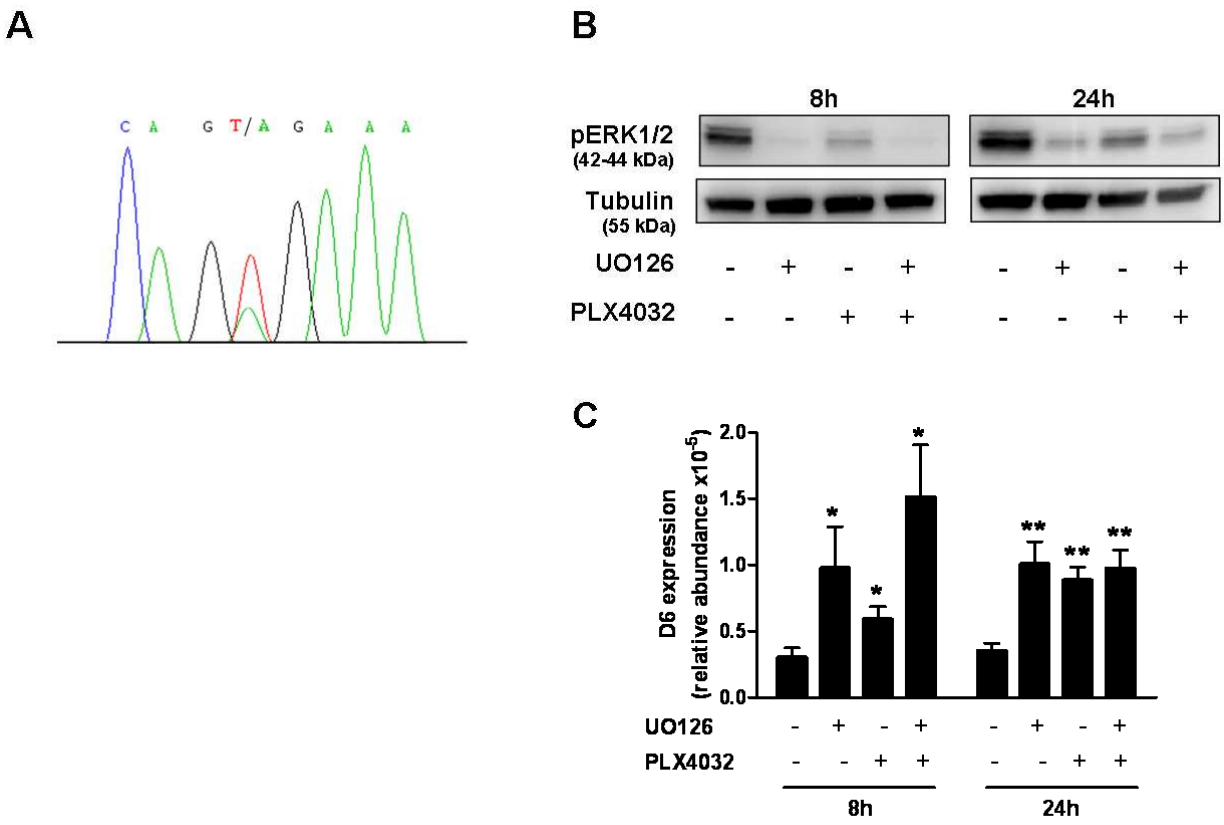

Figure 3.10 – Downregulation of D6 expression by ERK activation. (A) Sequence chromatogram of BRAF exon 15 of DNA from KS-IMM cells. The thymine (T) to adenine (A) transition introduces a substitution of amino acid valine to glutamic acid at codon 600 (V600E). (B) Western blot analysis of ERK1/2 phosphorylation and (C) qRT-PCR of D6 expression in KS-IMM cells treated with UO126, PLX4023 or their combination. *p<0.05, **p<0.005.

4.1.9. ERK activation, macrophage infiltration and D6 expression in human KS

Once identify the mechanism involved in the down-regulation of D6 in KS-IMM, we wanted to assess its actual clinical relevance in patients. For that reason sections from KS patients at stage IB were stained by immunohistochemistry for ERK phosphorylation and, in agreement with *in vitro* data, an inverse correlation between ERK1/2 activation (anti-pERK staining) and D6 expression (n=10) (Figure 3.11 A) was observed. Moreover we wanted to validate the hypothesis obtained with the in vivo model concerning the ability of D6 to shape KS microenvironment toward an anti-tumoral phenotype. Indeed immunohistochemical analysis for identification of TAM and their polarization were performed, respectively with anti-CD68 and anti-CD163 staining. The results showed increased infiltration of CD68+ and CD163+ (Figure 3.11B) macrophages in KS lesions characterized by a rapid progression rate (stage IB) as compared with slow progressing ones (stage IA) and they also highlight a significant inverse correlation between D6 expression levels and the number of CD68+ ($p=0.039$, $n=18$) and CD163+ cells ($p=0.003$ $n= 18$) (Figure 3.11C). Taken together, these results are consistent with data obtained in the KS-IMM-based experimental model, supporting the notion that in KS progression activation of the B-Raf/MEK/ERK pathway mediates down-regulation of D6, resulting in increased CCR2-dependent TAM infiltration and their local activation towards a tumor-promoting M2-like phenotype.

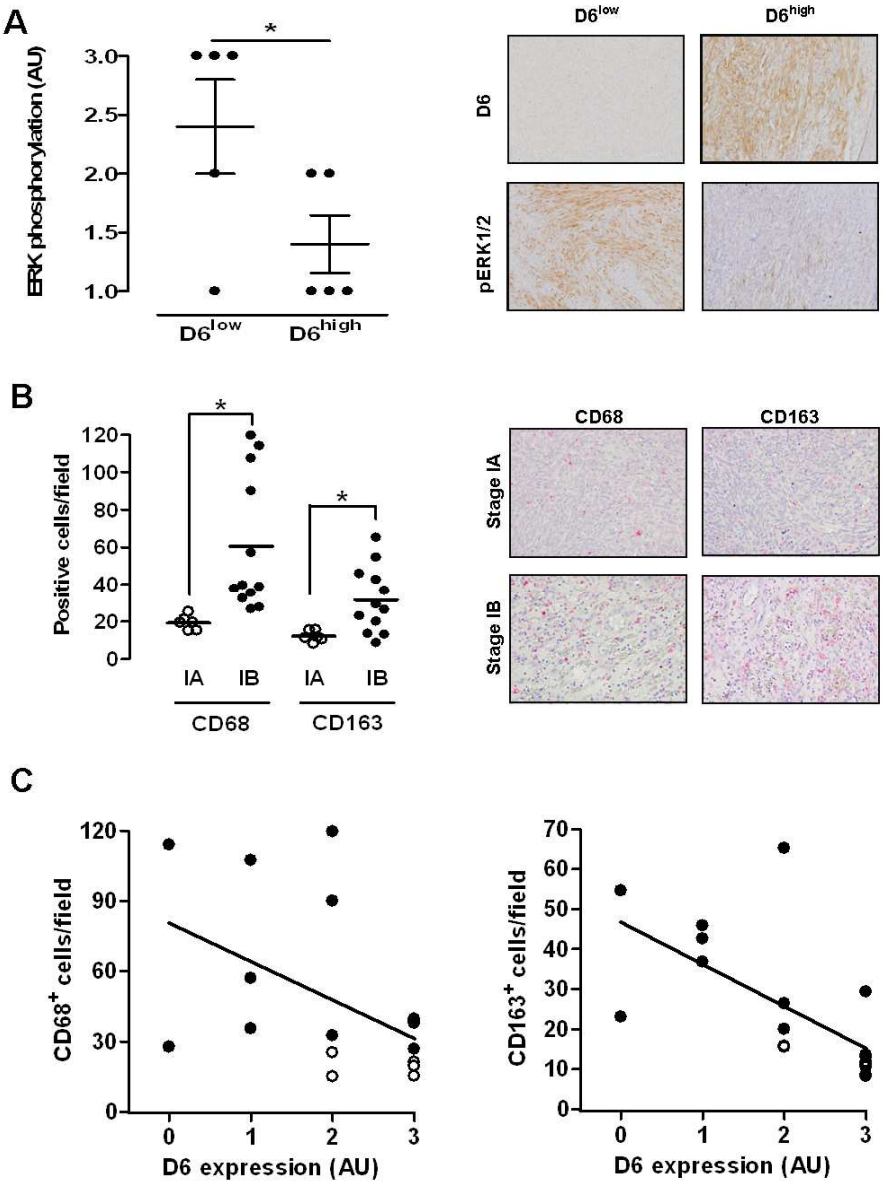

Figure 3.11 – inverse correlation between D6 expression and ERK activation, macrophage infiltration and M2 macrophage phenotype. (A) D6 and pERK1/2 immunohistochemical analysis in cutaneous maculo-nodular KS lesions from stage IB 28 patients. Scatter plot reports mean values ± SEM from five random fields evaluated for number of pERK1/2 positive cells using a semiquantitative scale (1: 0÷25% positive cells; 2: 25÷50% positive cells; 3: 50÷75% positive cells; 4: 75÷100% positive cells) in $D6^{low}$ (score 1-2) and $D6^{high}$ (score 3- 4) lesions. In the right panel representative immunohistochemical analysis of KS lesions from two patients stained for human D6 (score 1 on the left and 3 on the right) and pERK1/2 (score 3 on the left and 1 on the right). Magnification 20x. (B) Mean and SEM of CD68 and CD163 positive cells and (right panel) representative immunohistochemical staining (magnification 10X; positive cells are pink) in maculo-nodular lesions from HIV-negative patients with slow (stage IA) and rapid (stage IB) progression rate. Five random fields for each sample were counted. (C) Linear regression analysis of D6 expression levels and CD68 (left panel) and CD163 (right panel) number of positive cells/fields in cutaneous maculo-nodular KS lesions from stage IA (○) and IB patients (●). *p<0.05, **p<0.005.

4.2. CCR2 expression by macrophages promotes tumor progression through induction of IL-10 and VEGF production

Chemokines and chemokine receptors have been intensively studied in tumor biology, in fact they are key players in several stages of tumor progression, in particular in leukocyte recruitment at tumor site, but also in promoting angiogenesis and metastatization [192]. One of the best characterized chemokine in supporting tumor development is CCL2, indeed, this chemokine, together with its receptor CCR2, displays different roles in tumor biology. Firstly CCR2 has been demonstrated to be crucial in monocyte mobilization from bone marrow into the circulation [110, 291, 292] and in monocytes-macrophage recruitment at tumor site [126, 293] but in the literature there are also data highlighting its potential role in macrophage polarization [201].

4.2.1. Lack of CCR2 skew macrophages toward M1 phenotype

In order to better understand the possible implication of CCR2 in macrophage polarization wt and $CCR2^{-/-}$ bone marrow derived macrophages (BMDM), cultured for 7 days with M-CSF, were polarized toward M1 or M2 phenotype, by using LPS+IFNγ or IL-4, respectively. After 24 hours of stimulation, wt and $CCR2^{-/-}$ BMDM were analyzed for M1 and M2 marker expression by flow cytometry and quantitative PCR. No differences were found by FACS analysis, nor in expression of the M1 marker MHCII do neither in the M2 marker Relmα (Figure 3.2.1 A, B). Furthermore, quantitative PCR of M2 genes, such as Relmα and Arginase-1 (Figure 3.2.1 E, F), did not show differences between wt and $CCR2^{-/-}$ M2-polarized BMDM. Nevertheless, when M1-polarized BMDM were analyzed for iNOS and IL-12p40 (Figure 3.2.1 C,D) expression, $CCR2^{-/-}$ macrophages showed an enhanced expression of these typical M1 genes. Moreover, macrophages lacking the chemokine receptor also displayed a decreased production of the anti-inflammatory cytokine IL-10 (Figure 3.2.1 G).

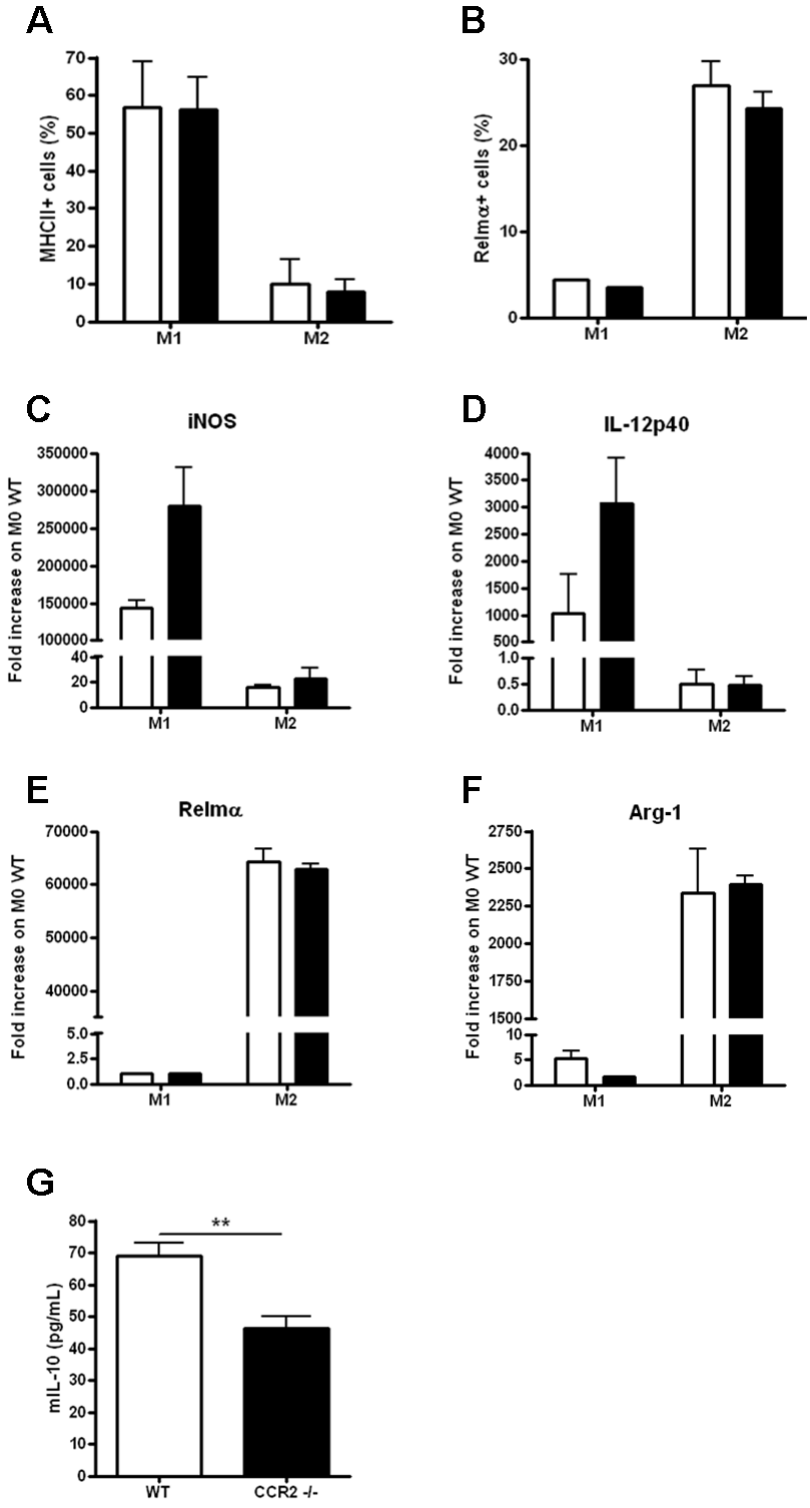

Figure 3.2.1 – Increased expression of inflammatory gene in CCR2$^{-/-}$ M1-polarized BMDM. M1 and M2-polarized wt (white bar) and CCR2$^{-/-}$ (black bar) BMDMs were analyzed by flow cytometry, for expression of MHCII (A) and Relmα (B), or by qPCR for measuring expression of iNOS (C), IL-12p40 (D), Relmα (E) and Arg-1 (F). Production of IL-10 was measured by ELISA in M1-supernatants. Data are expressed as the mean concentration ± SEM of at least three independent experiments. **p<0.005

4.2.2. The improvement of inflammatory gene expression in CCR2-/- BMDM correlates with an impairment of IL-10 and VEGF production

M1 polarization is modelled *in vitro* by interferon-γ (IFNγ) and lipopolysaccharide (LPS) stimulation, dictating a transcriptional response that shapes the phenotype and function of macrophages. In order to dissect the signaling pathway interacting with the one of CCR2, wt and CCR2$^{-/-}$ BMDMs were stimulated for 24h with LPS alone. Once again CCR2$^{-/-}$ macrophages showed an increased expression of inflammatory genes, such as iNOS and IL-12p40 (Figure 3.2.2 A, B), suggesting that CCR2 signaling could interact with TLR4 pathway. Moreover, we also found that macrophages lacking CCR2 produced lower levels of the M2-related cytokines IL-10 and VEGF (Figure 3.2.2 C, D).

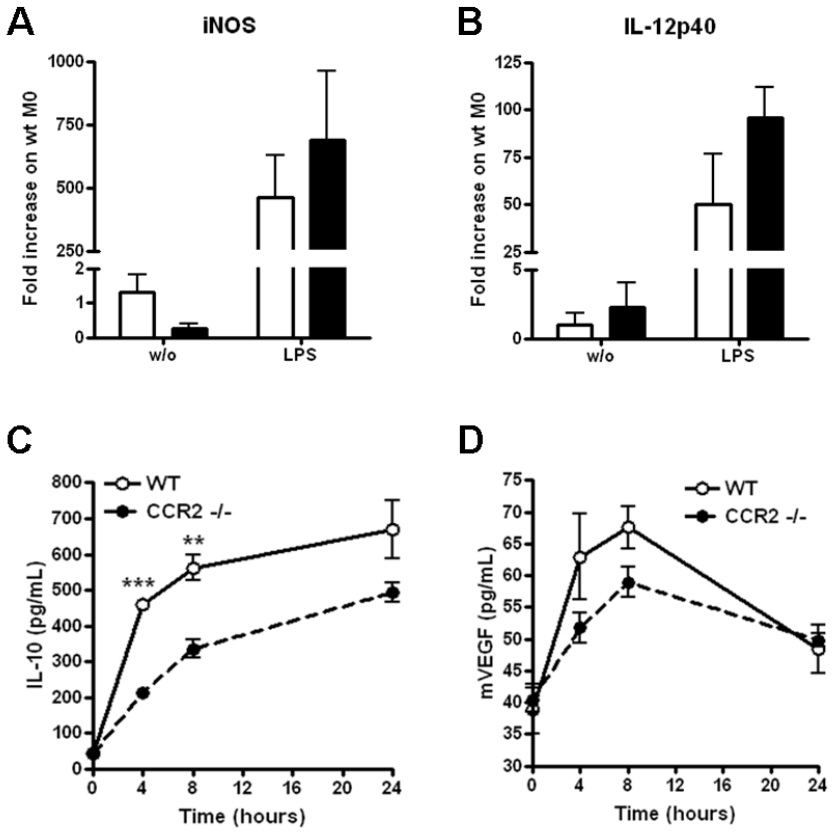

Figure 3.2.2 - Increased inflammatory gene expression in CCR2$^{-/-}$ LPS-stimulated BMDM. Wt (white bar) and CCR2$^{-/-}$ (black bar) BMDMs were analyzed after 24h of LPS stimulation by qPCR for expression of iNOS (A) and IL-12p40 (B). Production of IL-10 and VEGF was measured by ELISA in BMDM supernatants after 4, 8 and 24h of LPS stimulation. Data are expressed as the mean concentration ± SEM of at least two independent experiments. **p<0.005

4.2.3. CCR2 inhibition on monocytes affects IL-10 production

Because previous experiments highlighted a possible crosstalk between TLR4 and CCR2 pathways, we wanted to confirm also in the human setting that inhibition of CCR2 signaling would turn into a decreased production of IL-10. Indeed, human monocytes were treated with the CCR2 antagonist RS-504393 that inhibited ERK1/2 phosphorylation after CCL2 stimulation (Figure 3.2.3 A) and it has been demonstrated to be not toxic with Annexin V/7AAD staining (data not shown). LPS stimulated monocytes treated with RS displayed reduced IL-10 mRNA and protein (Figure 3.2.3 B, C, D). RS has a dose-response effect and at 5μM inhibits nearly 30% of IL-10 present in supernatants.

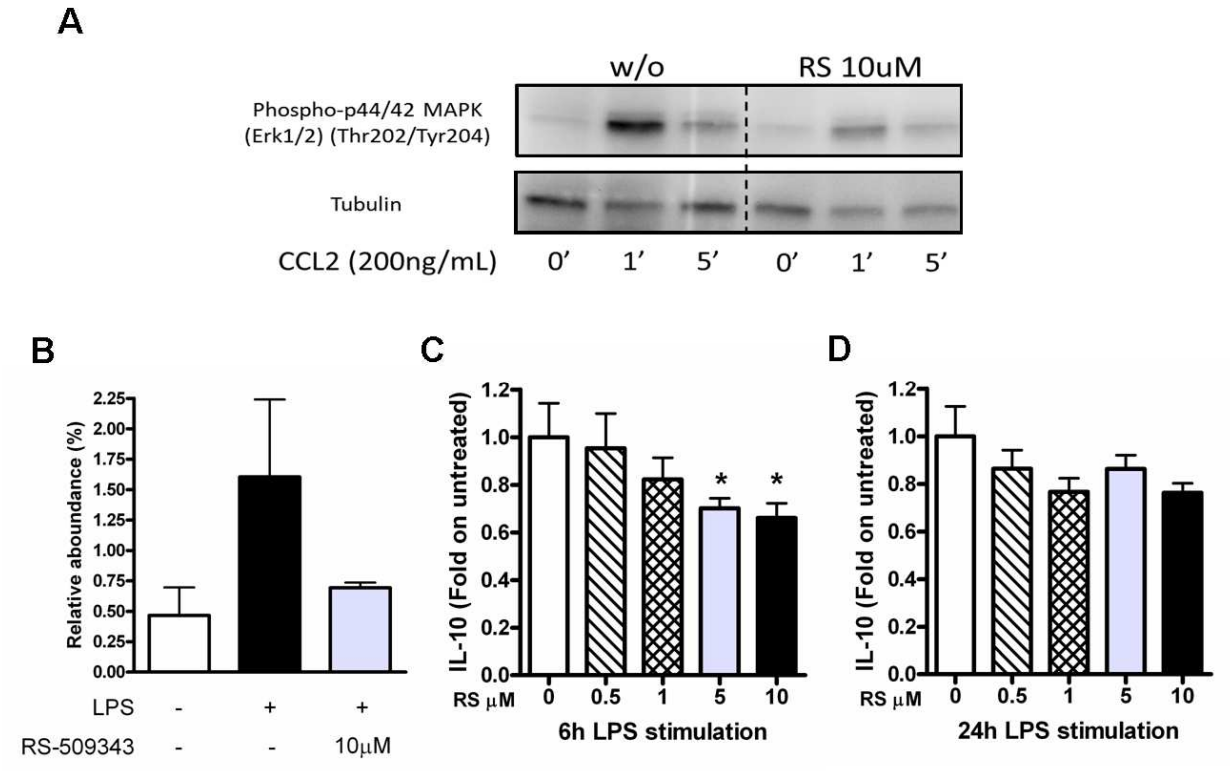

Figure 3.2.3 – Decreased IL-10 production in RS-treated human monocytes. ERK1/2 phosphorylation after CCL2 stimulation (A) and viability analysis by annexin V/7AAD (B) of RS-treated human monocytes. (C) IL-10 mRNA production was measured by qPCR after 16 hours of LPS stimulation. Detection of IL-10 protein by ELISA assays in supernatants was performed after 6 (D) and 24h (E) of LPS stimulation. Data are expressed as the mean concentration ± SEM of at least two independent experiments. *p<0.05

4.2.4. CCR2 signaling interacts with NF-κB and p38 pathways

The inhibition of IL-10 and the increase expression of inflammatory genes suits with a defect in TLR4 pathway activation. In fact, TLR4 engagement entails the phosphorylation of NF-kB and p38/MAPK. It consequently leads to expression of inflammatory genes and then to the production of IL-10, which in turn, switch off the inflammation.

The activation of NF-kB and p38/MAPK pathways was analyzed by western blot in wt and CCR2$^{-/-}$ BMDMs after 10, 30 and 240 minutes after LPS stimulation. NF-kB p65-subunit is early phosphorylated, with the maximum level of activation at 10 minutes (Figure 3.2.4 A, B), whereas p38/MAPK (Figure 3.2.4 A, C) started to be phosphorylated 30 minutes after LPS stimulation. Both pathways have been detected to be restrained in CCR2$^{-/-}$ macrophages (Figure 3.2.4 B, C).

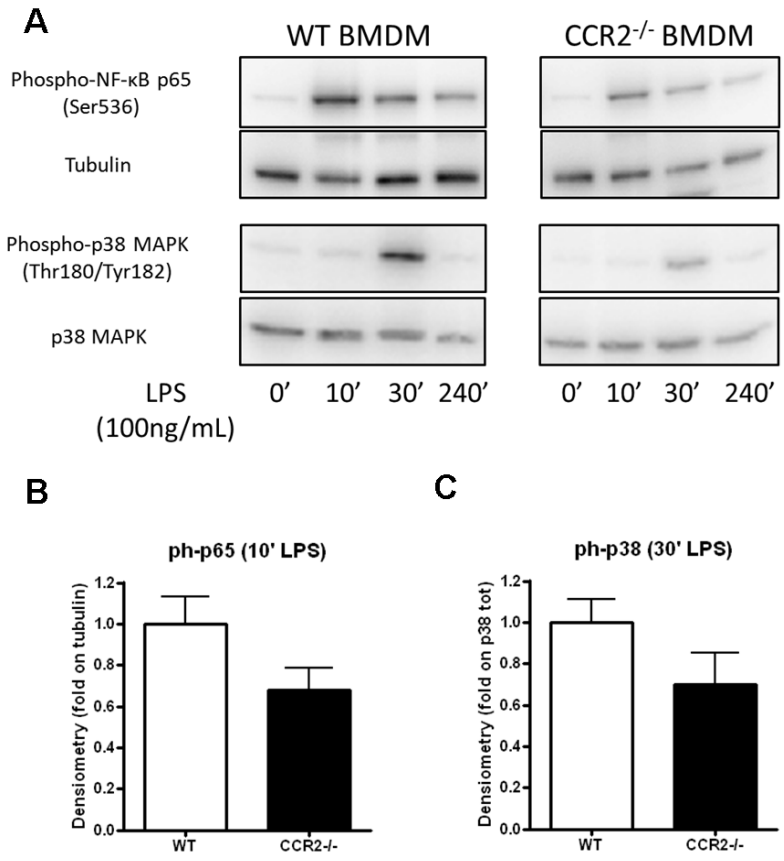

Figure 3.2.4 – Restraint NF-kB and p38 activation in CCR2$^{-/-}$ macrophages. NF-Kb (A, B) and p38 (A, C) phosphorylation after LPS stimulation was analyzed by western blot and bands were quantified by densitometry. Data are expressed as the mean concentration ± SEM of at least two independent experiments.

4.2.5. CCR2 expressed by TAM improves tumor growth

Our data indicate that macrophages lacking CCR2 hold a more inflammatory phenotype and express lower levels of M2-like cytokines, such as the anti-inflammatory cytokine IL-10 and the pro-angiogenic VEGF. Because of these evidences we decided to investigate the biological role of CCR2 expressed by TAM in tumor biology. The Lewis lung carcinoma cell line, LLC or 3LL, was co-injected together with wt or $CCR2^{-/-}$ macrophages, with a ratio of 1:1, in the flank of C57BL/6 mice. The results showed that co-injection of 3LL with $CCR2^{-/-}$ macrophages provoke an impairment of tumor growth (Figure 3.2.5 A), an increase of mice survival (Figure 3.2.5 B) associated to a decreased production of VEGF-A (Figure 3.2.5 C). Furthermore tumor infiltrated leukocytes were analyzed. No differences were in monocytes and macrophages percentages (Figure 3.2.5 E), whereas there is a tendency to have more neutrophils (Figure 3.2.5 D) in tumors containing $CCR2^{-/-}$ macrophages than wt counterpart. Nevertheless, analyzing TAM phenotype we observed that the one derived from tumors co-injected with $CCR2^{-/-}$ BMDMs showed a more M1 phenotype, expressing higher levels of MHCII and lower levels of CD206 (Figure 3.2.5 F-G). Looking then at lymphocytes, tumors holding $CCR2^{-/-}$ macrophages resulted to have an higher number of T cells (Figure 3.2.5 H) and analyzing their phenotype, both CD4+ T-helper and CD8+ T-cytotoxic cells were increased (Figure 3.2.5 I-L), correlating with the impaired tumor growth. Expansion of T lymphocytes can be modulated by the activity of FOXP3+ regulatory T cells, which are primary located in lymphoid organs, such as lymph nodes and spleen, acting by suppressing tumor-specific immune response. FOXP3+ regulatory T cells in spleens of mice holding tumors co-injected with $CCR2^{-/-}$ macrophages were detected to be decreased (Figure 3.2.5 M).

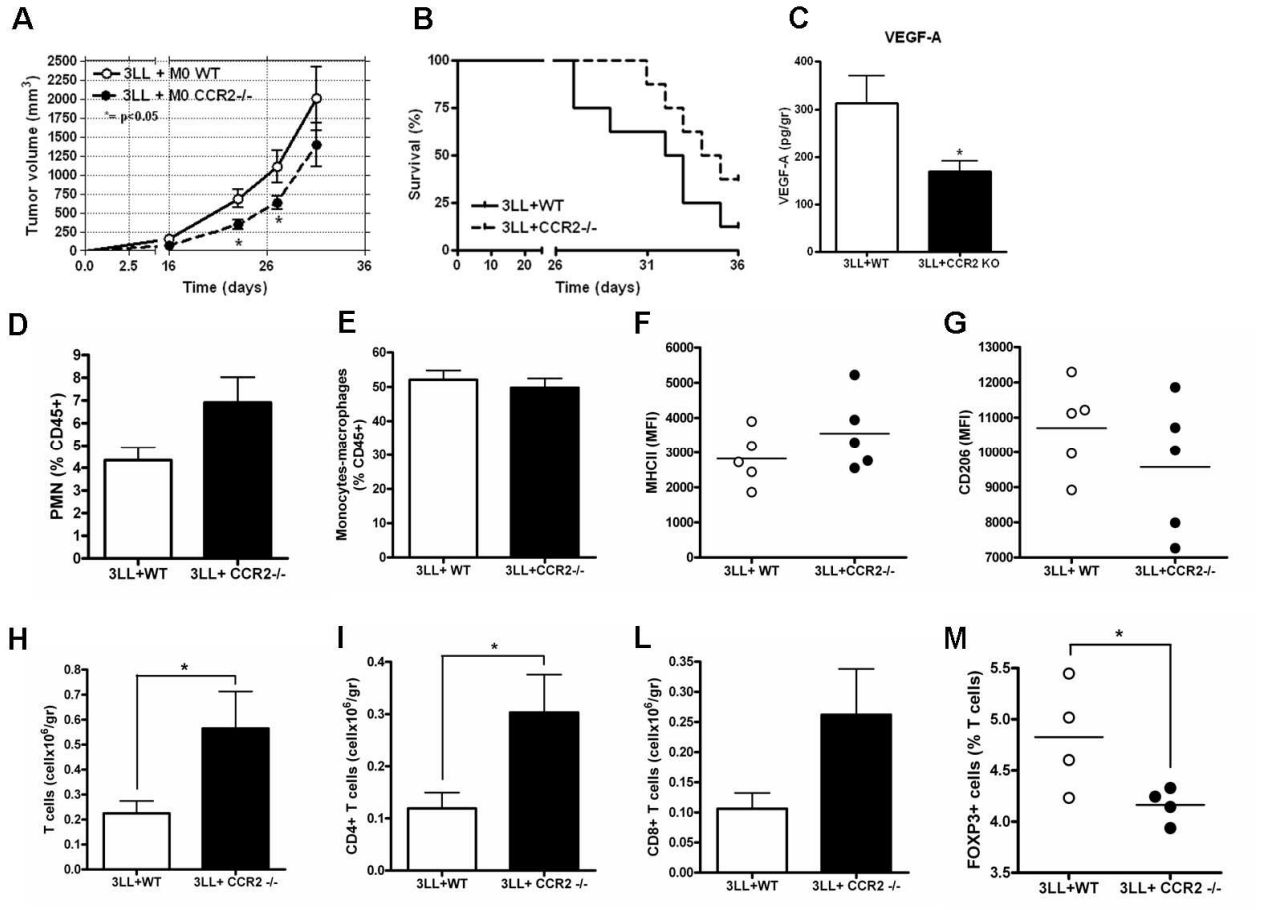

Figure 3.5 – Impaired tumor growth and increased mice survival of tumors co-injected with CCR2$^{-/-}$ macrophages. Panels A and B report the growth rate and percentage of survival of tumors co-injected with wt (black line) or CCR2$^{-/-}$ (dashed line) macrophages. After 14 days from tumor injection, tumors were explanted and analyzed for VEGF-A (C) production and for tumor infiltrating leukocytes: neutrophils (D), monocyte-macrophage cells (E), total number of T lymphocytes (H), CD4+ (I) and CD8+ (L) T lymphocytes. Mean fluorescence intensity (MFI) of MHC-II (F) and CD206 (G) expression of TAM. (M) Percentage of FOXP3+ Treg cells in spleens of mice injected with tumor cells and wt or CCR2$^{-/-}$ macrophages . (n = 5 tumors/group). *p<0.05.

5. Discussion

Chemokines are important components of cancer-related inflammation affecting tumor progression in multiple pathways, including tumor cell proliferation and survival, invasion and metastasis, leukocyte recruitment, and angiogenesis [294-296]. By targeting to degradation most inflammatory CC chemokines and limiting their bioavailability in tissues, the atypical chemokine receptor D6 represents an emerging mechanism of regulation of the chemokine system [166]. D6 has a well-established non-redundant role in the control of the inflammatory response, regulating the traffic and the activity of cells of the mononuclear system, in particular inflammatory monocytes [177], and also has a negative role in inflammation-driven tumor development in experimental models [175, 188]. In the human setting, D6 has been reported to be expressed by choriocarcinoma cell lines [172], in breast [190] and vascular tumors [170, 179], but its actual relevance in human cancer has not yet been established.

KS is a malignancy caused by the interplay of LEC infection with HHV8, oncogenic events, and a tumor-promoting chronic inflammatory milieu [273]. Considering the major role of inflammatory chemokines in KS pathogenesis and the fact that LEC express the atypical chemokine receptor D6 [243], analysis of its expression was performed on biopsies of KS lesions. D6 was found expressed by HHV8-infected spindle-shape KS cells, and its expression was inversely correlated with tumor aggressiveness, both when comparing maculonodular KS lesions retrospectively classified according to their progression rate

(group A versus group B stage I lesions) and when comparing maculo-nodular lesions of HIV-seronegative and -seropositive patients, which typically show a slow and rapid progression rate, respectively.

To put into a test the hypothesis emerging from these observations that reduction of D6 expression might be part of the natural KS progression process, we set up an experimental model based on the tumor cell line KS-IMM, which has been derived from a human KS lesion and induces KS-like sarcomas retaining most features of the parental tumor when injected subcutaneously in nude mice [283]. When compared to D6-expressing KS, D6-incompetent KS display increased tumor growth *in vivo* but not *in vitro*, suggesting that D6 expression may influence KS growth by interfering with the tumor microenvironment. Consistent with this, D6-negative tumors were found to be characterized by increased levels of inflammatory chemokines and profound changes in the intratumoral leukocytic infiltrate composition. D6-negative tumors showed an increased infiltration of TAM and tumor associate neutrophils. Despite tumor associate neutrophils have been reported to be important for tumor growth by activating angiogenic factors [210], the selective depletion of Ly6G+ cells did not affect KS growth. Conversely, inhibition of monocyte recruitment and TAM infiltration by the use of a CCR2 antagonist inhibited the growth rate of D6-KS tumors. Direct assessment of the requirement of TAM for KS growth was demonstrated by adoptive transfer experiments showing that coinjection of BMDM promoted D6+KS growth. D6 expression downregulation (in patients KS lesions) or absence (in the KS-IMM model) was also correlated with an increased M2-like polarization of TAM. Furthermore, the same M2-skewed phenotype was found in BMDM cultured with TCM taken from D6-negative tumors. These results collectively indicate that downregulation of this chemokine scavenger receptor at the tumor site not only allowed inflammatory CC chemokines to recruit more monocytes, but also directly promoted their pro-tumoral M2-like polarization [201].

Several studies have indicated that TAM have a pivotal role in the regulation of tumor angiogenesis, especially at early stages of tumor progression [297]. Here we report that D6-incompetent tumors, in addition to having more TAM, had increased amounts of VEGF-A and intratumoral angiogenesis. Using KS-conditioned media it was found that increased VEGF-A

production by BMDM required CCR2 expression and an autocrine loop triggered by CCR2 agonists involving induction of COX2 and PGE2 production [285, 286]. Furthermore, requirement of CCR2 expression on macrophages for KS growth was directly demonstrated by adoptive transfer experiments, in which wt but not $CCR2^{-/-}$ BMDM were able to sustain KS tumor growth. Several studies have correlated CCR2+ TAM infiltration with increased tumor angiogenesis and VEGF-A production [293], but the direct role of this chemokine receptor in the production of VEGF-A has not been assessed. By providing direct evidence that besides its well-known chemotactic function CCL2 can also directly affect the angiogenic potential of TAM, these results may be relevant to tumors other than KS characterized by elevated CCL2 levels, including breast and prostate tumors [187].

In an effort to elucidate the molecular basis responsible for D6 downregulation associated with tumor progression, we focused on the K-Ras/B-Raf/MEK/ERK1/2 pathway, as oncogenic mutations or amplifications interfering with the activity of this pathway are frequently observed in late-nodular KS lesions and angiosarcomas [288, 298], where its activity has been shown to support enhanced expression of growth factors and cytokines, including VEGF-A [299]. Consistently with their aggressiveness *in vivo*, KS-IMM cell line, originally established from an advanced KS lesion, harbors an activating oncogenic mutation in the B-Raf gene and shows high levels of constitutive ERK1/2 activity. In this cell system, D6 expression was found to be under control of this signaling pathway, as its inhibition either by B-Raf or ERK inhibitors resulted in upregulation of D6. This functional link was then confirmed in KS lesions, where D6 expression levels were found to be inversely correlated with tumor aggressiveness, ERK1/2 activation, and number of infiltrating TAM, whose number and M2-like phenotype was on the other hand directly correlated with tumor aggressiveness. The results presented here suggest that during KS progression oncogenic events activate the ERK1/2 pathway which induces D6 downregulation in tumor cells. This allows tumor-derived inflammatory chemokines, and in particular CCR2 ligands, to recruit monocytes and sustain their local polarization toward M2-like TAM, which support a VEGF-A-dependent angiogenetic switch promoting tumor growth. Targeting these components of

the KS tumor microenvironment may thus represent alternative or complementary therapeutic strategies.

From KS study CCL2 resulted to be an important player in tumor promotion, considering that it affects angiogenic potential of TAM, by inducing VEGF production and it also seems to directly promote their pro-tumoral M2-like polarization. Moreover, in the literature there are increasing evidences implicating CCL2 in cancer progression and metastatization. Indeed, CCL2 has been demonstrated to be expressed in several tumors, such as sarcomas, gliomas, and cervical, ovarian, bladder, breast, and lung tumors [68, 300] and more recently it has been described in prostate cancer bone metastasis [301]. In certain carcinomas, CCL2 also strongly correlated to TAM infiltration and it has been suggested to be involved in Th2 polarization [302], whereas *in vitro* data demonstrated CCL2 to support M2-like phagocytic functions promoting tumor growth in human monocytic cells, by induction of CD206 expression and macrophage autophagy [201]. Taken together all these evidences indicate that chemokines and in particular CCL2 can be fundamental in supporting tumor promotion through TAM polarization, but the mechanism whereby it happens is still unknown. Therefore, we decided to investigate the polarization process in macrophages deficient for CCL2 receptor, CCR2. Whereas, unexpectedly, no differences were found in M2 polarization, an over-expression of inflammatory genes was shown in $CCR2^{-/-}$ macrophages both polarized toward M1 phenotype or stimulated with LPS alone. The increased inflammation is associated with a defect in the production of the anti-inflammatory cytokine IL-10, which plays a fundamental role in turning off inflammatory gene expression in order to avoid an excessive inflammatory response [303-305]. The decreased IL-10 production was confirmed in human monocytes, by treating them with the CCR2 antagonist RS-504393, supporting the evidence that, during LPS stimulation, CCR2 signaling interacts with TLR4 pathway straightening the production of IL-10. The complex response to LPS is mediated by different proteins and comprehends the activation of NF-kB and p38/MAPK [306, 307]. We investigated the phosphorylation state of this two proteins and we found that $CCR2^{-/-}$ macrophages, after LPS stimulation, showed a decreased activation of both p65 NF-kB-

subunit and p38/MAPK than wt, confirming that CCR2 signaling acts in synergism with TLR4 pathway for controlling the inflammatory response through the production of IL-10.

Consequently, in order to assess the *in vivo* role of CCR2 expressed by TAM in skewing their polarization toward an M2 phenotype. When $CCR2^{-/-}$ macrophages were co-injected in mice with tumor cells (3LL), an impaired tumor growth and an increased mice survival were obtained. These tumors were also found to produce less VEGF-A than tumors co-injected with wt macrophages, confirming the autocrine loop sustained by CCR2 in macrophage production of the pro-angiogenic cytokine VEGF-A [285, 286, 293]. Looking then to TAM, a skewing toward a more M1 phenotype was showed by TAM of $CCR2^{-/-}$ macrophage-holding tumors, than wt counterpart, highlighting the importance of CCR2 in modeling tumor microenvironment and shaping macrophage functions. Furthermore, tumors co-injected with $CCR2^{-/-}$ BMDMs also showed an increased number of tumor infiltrating T lymphocytes, both CD4+ and CD8+ T cells, which play an important contribution in tumor suppression. The number of T cells and their proliferation ability can be repressed by T regulatory cells which produce high levels of anti-inflammatory cytokines and in particular of IL-10 and TGFβ. IL-10 is also known to play a direct role in T-reg genesis [309]. Indeed, in our tumor model, the number of T-reg cells was found to be decreased in the spleen of animals bearing tumor co-injected with $CCR2^{-/-}$ macrophages, which produce lower amount of IL-10, in comparison with wt counterpart. In conclusion, the concurrence of CCR2 and TLR4 signaling and their synergic control of the inflammatory response, resulted in macrophage skewing toward M2 resolving-phenotype but also in shaping tumor microenvironment, inducing angiogenesis and T-reg proliferation, finally promoting tumor growth.

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