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The atypical receptor CCRL2 as a modulator of aged neutrophil homing to clearance organs

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ABSTRACT

Tecniche avanzate di microscopia hanno permesso di dimostrare come i recettori delle chemochine possano essere organizzati sulla membrana cellulare in omodomeri e/o eterodimeri, implementando la complessità di modulazione delle risposte cellulari. Nel 2017 il nostro gruppo di ricerca ha dimostrato il ruolo funzionale dell'eterodimerizzazione fra il recettore convenzionale per chemochine CXCR2 e il recettore atipico CCRL2. CCRL2 è un recettore a sette domini transmembrana che condivide similarità strutturali con la famiglia dei recettori atipici per chemochine, anche se, tuttavia, non lega una chemochina ma una proteina chemotattica chiamata chemerina e non funge da mediatore di internalizzazione e riciclo del ligando. Quando è espresso insieme a CXCR2, CCRL2 può formare eterodimeri CCRL2-CXCR2, influenzando il signaling di CXCR2 che è cruciale per regolare il reclutamento dei neutrofili in risposta a CXCL8. Con l'obiettivo di individuare ulteriori possibili forme di interazione fra CCRL2 e altri recettori per chemochine, questa tesi dimostra come CCRL2 possa formare eterodimeri anche con altri recettori a 7 domini transmembrana, come CXCR4, un recettore per chemochine che, insieme con CXCR2, regola la migrazione dei neutrofili durante il loro ciclo vitale e la loro clearance. Infatti, l'asse CXCR4-CXCL12 gioca un ruolo fondamentale durante il rilascio e il ritorno dei neutrofili rispettivamente da e verso il midollo osseo, mentre CXCR2 è coinvolto nel reclutamento dei neutrofili nel sito infiammatorio.

Lo scopo principale di questo lavoro è quello di identificare la rilevanza biologica dell'eterodimerizzazione fra CCRL2 e CXCR4 durante la clearance di neutrofili senescenti. I dati sono stati prodotti tramite esperimenti di caratterizzazione fenotipica e funzionale di neutrofili murini *in vitro*, stimolazione pro-infiammatoria di neutrofili endogeni *in vivo* e esperimenti di adoptive transfer sfruttando inibitori selettivi o non selettivi di CXCR4 e CXCR2. I risultati ottenuti mostrano come CCRL2, in una condizione di possibile eterodimerizzazione con CXCR4, sia in grado, a seguito del legame con CXCL12, di modularne negativamente il signaling intracellulare sia *in vitro* che *in vivo*, diminuendo l'attivazione di RhoA, la fosforilazione di ERK1/2, la polimerizzazione dell'actina e, funzionalmente, anche il ritorno dei neutrofili senescenti al midollo osseo. Gli esperimenti di adoptive transfer hanno inoltre mostrato come CCRL2 possa influenzare il signaling di CXCR2 anche nel processo di clearance al fegato e non solo nel reclutamento al sito infiammatorio come già dimostrato. L'utilizzo di inibitori

selettivi (Repertaxin) e non selettivi (Pertussis Toxin) di CXCR4 e CXCR2, ha ulteriormente permesso di discriminare, quali, fra i processi osservati, fosse CXCR4- o CXCR2-dipendente. Invece, CCRL2 non sembra essere coinvolto nel processo di efferocitosi, ovvero di fagocitosi dei neutrofili senescenti da parte dei macrofagi tissutali.

Partendo dal presupposto che i neutrofili senescenti rappresentano un subset di rilievo in alcune condizioni patologiche di stampo cronico-infiammatorio, CCRL2, alla luce di questi dati, potrebbe quindi essere considerato come un fine modulatore della loro migrazione e, in un'ottica traslazionale, potrebbe rappresentare un futuro target terapeutico per modificarla. I nostri risultati possono quindi essere considerati come un punto di partenza per poter meglio definire il possibile coinvolgimento di CCRL2 nel reclutamento di neutrofili senescenti in diverse condizioni patologiche come, ad esempio, in patologie infiammatorie croniche del fegato o nei tumori.

Advance light microscopy techniques indicate that chemokine receptors can form both homo- and heterodimers with other receptors, adding layers of complexity in the modulation of cell responses. In 2017, our research group demonstrated the functional role of heterodimerization occurring between the conventional chemokine receptor CXCR2 and CCRL2. CCRL2 is a 7-transmembran domain receptor that shares structural similarities with atypical chemokine receptors family, even if it does not bind a chemokine but a chemotactic protein called chemerin and it does not act as an internalization and scavenger mediator. Through heterodimerization with CXCR2, CCRL2 can affect CXCR2 signaling, which plays a pivotal role in neutrophil CXCL8-dependent recruitment. With the purpose of finding further possible heterodimerization between CCRL2 and other chemokine receptors, this thesis demonstrates that CCRL2 can form heterodimers also with CXCR4, the receptor, together with CXCR2, that principally regulate neutrophil migration during their life-span and clearance. Briefly, CXCR4-CXCL12 axis plays a fundamental role in the release and homing of neutrophils from and to bone marrow, while CXCR2 is involved in neutrophil recruitment into the inflammatory sites.

The main aim of the study was to investigate the biological relevance of CCRL2 heterodimerization with CXCR4 during aged neutrophil clearance. The data were obtained by *in vitro* murine neutrophils phenotypic and functional characterization by flow cytometry, *in vivo* neutrophil stimulation and adoptive transfer experiments taking advantages from selective or not selective inhibitors of CXCR4 and CXCR2.

In particular, we demonstrated that CCRL2 can negatively affect CXCL12-dependent CXCR4 signaling both *in vitro* and *in vivo*, by diminishing RhoA activation, ERK1/2 phosphorylation, actin polymerization and, functionally speaking, also the homing of aged neutrophils to bone marrow. On the other hand, adoptive transfer experiments showed that CCRL2 can affect CXCR2 signaling not only, as already demonstrated, during neutrophil recruitment in inflammatory conditions, but also during aged neutrophil clearance to the liver. The use of selective or not selective inhibitors in adoptive transfer experiments, as Pertussis Toxin and Repertaxin, allowed to discriminate the different pathways CXCR4 or CXCR2 dependent.

However, CCRL2 doesn’t seem to play a role in the efferocytosis of aged neutrophils by tissue macrophages.

Assuming the pro-inflammatory relevance of senescent neutrophils in pathologic condition, CCRL2 can be considered a fine-tuning modulator of neutrophil migratory processes and, translationally, a possible target to affect them. Thus, our results can be considered as a starting point to further better define the role of aged neutrophils in different pathological conditions, such as inflammatory-dependent liver diseases and tumor processes.

INTRODUCTION

1. Chemokines and chemokine receptors

1.1 Chemokines: functions and classification

Cell migration is a key process in the development and distribution of immune cells in normal and pathological conditions, controlling activation and orientation of leukocytes of innate and adaptive immunity[1]. Different subfamilies of chemoattractants can induce leukocyte directional migration, for example, chemotactic lipids (leukotriene B4 or LTB4, lipoxins, phospholipids, platelet-activating factor, prostaglandin D2, and sterols), complement anaphylatoxins (C3a or C5a), formyl peptides (N-formyl-Met-Leu-Phe or fMLP) and small proteins of several classes (chemokines, defensins and non-chemokine attractant proteins)[2-4].

Chemokines (abbreviation for "***chemo****tactic cyto****kines"***) are a large family of small and secreted proteins that signal through G protein-coupled seven transmembrane chemokine receptors expressed on cell surface. Chemokines play a central role in the development and homeostasis of the immune system and are involved in immune and inflammatory responses. Chemokines can stimulate various types of directed and undirected migratory behaviour (chemotaxis haptotaxis, chemokinesis and haptokinesis) and, moreover, they can induce cell arrest and adhesion[1].

Chemokines are classified according to their structure that is characterized by a single polypeptide chains of 70-100 amino acids residues in length (8 to 12 kDa)[5,6] and a conserved couples of cysteines linked by disulphide bonds: a central three stranded β-sheet, a C-terminal α-helix and a short N terminus that plays a critical role in receptor activation, shaping together the characteristic "chemokine fold"[1,7]. Based on the cysteine residues number and location, four chemokine subfamilies have been identified. The largest group of chemokines has the first two of total four cysteines in the adjacent position (CC chemokines). Instead, the chemokines named CX3C have three amino acids between the two cysteines, CXC have only one amino acid and, if only one couple of cysteines is present, the chemokine is called XC. Moreover, another possible class of chemokines (CX) has been identified in zebrafish[8], which lacks one of the two N terminus cysteines but maintains the third and fourth ones; nevertheless, there is no prove that this latter type of chemokine is present also in mammals[9]. Although

chemokines were originally named according to specific functions, a systematic nomenclature was introduced in 2000 that includes a subfamily designation (CC, CXC, CX3C, or XC) followed by the letter “L” (denoting ligand) and a number according to when the gene was first isolated[10]. Chemokines bind to a subfamily of seven-transmembrane-G protein- coupled receptors, which are categorized as CCR, CXCR, CX3CR and XCR on the basis of the class of chemokines they bind (“R” denoting receptor).

In addition, chemokines can also be classified according to their production and role in homeostatic conditions (such as CXCL12, CXCL13, CCL14 and CCL19) and inflammatory conditions (such as CCL2, CCL5 and CXCL8)[6]. In the first case, chemokines represent a constitutive presence in organs like bone marrow (BM), lymph nodes (LNs) and thymus, suggesting a regulatory role in normal leukocyte production and distribution; in the second case, chemokines can be released by different cell types in condition of inflammation and infection, creating a complex pattern of chemokines expression that correlate with many inflammatory diseases both in human and in mice[6].

1.2 Chemokines in homeostasis and inflammation

Besides leukocyte migration, chemokines can also influence their survival and effector functions such as proliferation, differentiation, cytokines production and degranulation both in homeostatic and in pathological conditions[1].

Immune surveillance in homeostatic conditions requires constitutive expression of chemokines in order to guarantee the constant trafficking of leukocytes from bone marrow, lymph node and thymus into and out of peripherical tissues of the body. For example, CCL19, CCL21, and CXCL13 are constitutively expressed in secondary lymphoid organs and orchestrate the spatial interactions of dendritic cells and T cells within lymphoid follicles: so, the chemoattractant receptors CCR7 and CXCR5 are primarily responsible for lymphocytes recirculation and homing into lymphoid organs as well as for organization of functional lymphoid microenvironments during lymphoid organogenesis[1,11]. Another example of constitutively expressed chemokine is CXCL12 which has many indispensable functions, including its role in haematopoiesis where it is a key component of the niche that supports hematopoietic stem cells (HSCs) in the BM[1]. CXCL12 is the predominant signal that maintains $CXCR4^+$ HSCs within niches inside the bone

marrow. Thus, cells migration in and out of the BM follows opposite chemokines signals and different expression levels of their cognate chemokine receptors[11]. Consequently, CXCR5 knockout (KO) mice do not develop inguinal LNs or B cells areas in secondary lymphoid tissues[12] and CXCR4 KO mice have a severe reduction of myeloid progenitors in the bone marrow[13].

Chemokines have been implicated in a wide range of inflammatory diseases: when a damage or an infection occurs, the rapid recruitment of innate immune cells is essential to kill pathogens, avoid microbial dissemination, start inflammation and repair the tissue damage. Furthermore, many chemokines production can be strongly induced in response to a wide array of infectious and inflammatory stimuli[1]. For these reasons, high circulating expression of specific chemokines was found in patients with different inflammatory disorders, indicating that these molecules play important role in the development and progression of inflammatory diseases. Consequently, chemokines represent potential diagnostic markers or therapeutic targets, currently under investigation in clinical studies[1].

For example, increased levels of CC chemokines, especially CCL2, CCL3 and CCL5 in the joints of patients with rheumatoid arthritis, positively correlate with the accumulation of monocytes and T cells into synovial tissues[14]. The CC chemokine CCL3 is also found in pathologic specimens from patients with multiple sclerosis and its immunologic neutralization results to be strongly protective in the induction of the disease in the experimental mouse model[6]. Moreover, allergic inflammatory diseases, such as asthma, are characterized by the local production of CCL11, CCL7 and CCL5 and the increased expression of CCL11 receptor, CCR3, on eosinophils, basophils and Th2 cells, which accumulate at sites of allergic inflammation. Accordingly, the allergen-induced airway recruitment of eosinophils in CCL11 knockout mice is impaired[15]. Finally, consistent with the close association of CXCL8 with neutrophils mediated inflammatory disease, genetic deletion of the mouse CXCL8 receptor CXCR2, causes defective neutrophil recruitment with a reduced inflammatory reaction[16,17].

It is therefore established that chemokines can be overproduced by many tissues affected by different diseases including autoimmunity, allergy, Alzheimer's disease, chronic inflammatory disease, cardiovascular disease, and cancer[1,18-22]. However, the precise profile of chemokines produced in a given tissue will

depend on the nature of the inducing stimuli, the phase of the response and the genetics of the chemokines network in the affected individual[1].

Moreover, chemokines can act also on many non-leukocytic cell types, including epithelial cells, mesenchymal cells, endothelial cells and tumor cells, thereby contributing to different physiological and pathological conditions such as angiogenesis, haematopoiesis, organogenesis, tumor growth and metastasis[1,6].

1.3 Chemokine receptors: functions and classification

Chemokines exert their biological effects by binding to cell surface receptors. Chemokine receptors (CKRs) are seven transmembrane domain receptors (7TMRs) that mediate different functions, such as immune surveillance and embryonic development, by directly regulating cellular migration, adhesion, growth and survival. Different classes of leukocytes express different arrays of chemokine receptors making them able to respond to specific ligands. The ligand-receptor interaction is often promiscuous, since each chemokine can bind more than one receptor and vice versa, as shown in **Figure 1**, where chemokines and their cognate receptors are summarized. Such promiscuity most likely evolved to counteract external pathogens by creating robustness into leukocytes responses during infection[1]. Mammalian genomes encode approximately 20 chemokine receptors. Since the receptors were discovered after chemokines and most of them are selective for members of one chemokines subfamily, they are classified according to the subfamily of chemokines to which most of their ligands belong. Thus, receptors are named using the prefixes CCR, CXCR, CX3CR, and XCR followed by an identifying number[23].

Chemokine receptors are also involved in many pathological processes, and, for this reason, they have been considered as a target for drug development for many diseases such as atherosclerosis, human immunodeficiency virus (HIV) infection, tumor metastasis and autoimmune disorders. For examples, chemokines structures have been studied in order to model inhibitors and small molecules have been developed in order to antagonize specific chemokine receptors, for example Plerixafor for CXCR4 and Aplaviroc for CCR5[24].

Chemokine receptors are differentially expressed by leukocytes and non-hematopoietic cells and can be divided into two groups: conventional chemokine receptors (cCKRs) and atypical chemokine receptors (ACKRs). Chemokines that

bound cCKRs typically transduce signals through pertussis toxin-sensitive Gαi G-proteins, ultimately leading to cell migration, adhesion and/or a variety of other biological responses[1]. ACKRs, instead, play a role in shaping chemokine gradients and dampening inflammation by scavenging chemokines in a G protein independent manner[1].

Chemokine–CKR interactions are traditionally described by a two-step/two-site mechanism, in which the CKR N-terminus recognizes the chemokine globular core (site 1 interaction), followed by activation when the chemokine N-terminus is inserted into the receptor transmembrane bundle (site 2 interaction). This classical two-site model of chemokine/receptor interaction is probably oversimplistic, with recent studies suggesting that the two supposedly independent ligand-binding sites can be physically and allosterically linked, and that additional interactions between chemokine and receptor are likely to be involved in ensuring full receptor activation[24].

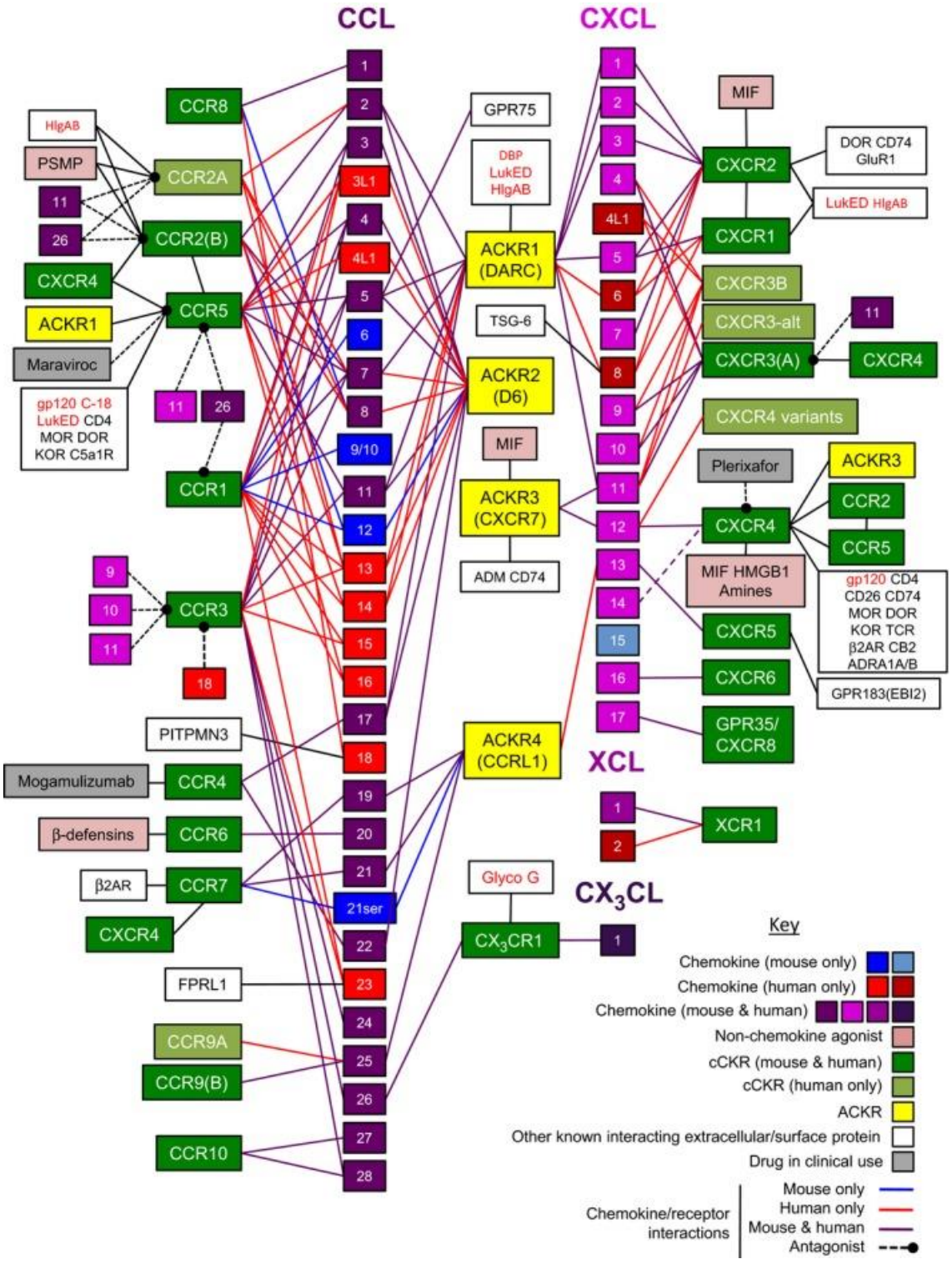

Figure 1: Chemokines and chemokine receptors interactions in human and mice.

From Huges & Nibbs, The FEBS Journal, 2018

1.4 Conventional chemokine receptors (cCKRs)

There are currently 18 cCKRs, including 10 CCRs, 6 CXCRs and a single CX3CR and XCR. Receptor specificity is complex: many chemokines bind to multiple cCKRs, and some cCKRs have many ligands. However, chemokines diversify their affinity for a particular cCKR and the precise pathways activated by a cCKR depend on which ligand it binds[1,25]. Moreover, some cCKRs can also be activated by non-chemokine ligands: for examples, β-defensins can activate CCR6[26] and the 'alarmin' high mobility group box 1 protein (HMGB1) is emerging as a key CXCR4 ligand[1,27].

cCKRs typically transduce signals through pertussis toxin-sensitive Gai G-proteins and β-arrestins, leading to cell migration, adhesion and other biological responses[1]. The Gi proteins belong to the family of heterotrimeric G proteins, which consist of three subunits: α (that owns GTPase activity), β, and γ. The ligand bound to the receptor promotes the exchange of GDP with GTP on the α subunit, and the GTP-bound Gα subunit is subsequently dissociated from the βγ subunits. The Gβγ subunits can regulate a number of effectors, including phospholipase (PLC) β2/3, phosphatidylinositide 3 (PI3K)γ, ion channels, G protein coupled receptor kinases, extracellular signal-regulated kinases (ERK1/2) and some adenylyl cyclases. Downstream, low molecular weight GTP-binding proteins (for example Rac, Rho, Cdc42) can be activated to mediate the final effects of receptor activation, such as actin polymerization, adhesion and membrane protrusion[28]. To start the active movement, cells have to polarize, assuming an elongated shape with a more blunted leading edge and narrower posterior, resulting from the formation of F actin-rich lamellipodia at the leading edge and actomyosin structure at the posterior to drive cell locomotion[28].

In this thesis work, focused on neutrophil clearance process, the neutrophil expression and functional properties of the two cCKRs CXCR4 and CXCR2 have been taken in consideration.

1.4.1 CXCR4: expression, ligand, signaling and role in health and disease

The 7-transmembrane G-protein coupled receptor CXCR4 is highly expressed by a variety of cell types, including lymphocytes, endothelial, epithelial and hematopoietic stem cells, stromal fibroblasts and cancer cells[29]. The basal expression of CXCR4 is mainly tuned by two transcription factors: the Nuclear

Respiratory Factor 1 (NRF-1), which positively modulates receptor transcription[30], and the negative regulator Ying Yang 1 (YY1)[29,31]. It can be also fine modulated by different signaling molecules in the environment: for example, second messengers (calcium, cyclic AMP), some cytokines (IL-2, IL-4, IL-7, IL-10, IL-15, TGF-β) and growth factors (bFGF, VEGF) positively increase CXCR4 transcription. On the contrary, CXCR4 transcription is down-regulated by the presence of other cytokines, such as TNF-α, IFN-γ, and IL-1β[29,32].

CXCR4 ligand is the chemokine CXCL12, a homeostatic chemokine ubiquitously expressed in both embryonic and adult tissues. A proteolytic cleavage is necessary for the formation of mature CXCL12 from the inactive primary form. Once secreted, CXCL12 binds to the negatively charged glycosaminoglycans (GAGs) on the cell surfaces leading to tight adhesion of leucocyte to the endothelium, inducing transmigration and enhancing cell migration[29].

The interaction between CXCL12 and CXCR4 takes place through a two-step mechanism[33]. The first CXCR4-CXCL12 contact happens at the extracellular domain (**Figure 2A**) inducing a conformational change of the receptor, which strengthens chemokine binding to a receptor pocket. Next, a second receptor conformational change activates the intracellular trimeric G protein by the dissociation of Gα subunit from the Gβ/Gγ dimer[29]. Once activated, Gαi inhibits adenyl cyclases and the consequent cAMP production stimulates the activation of the Ras/Raf/MEK/ERK pathway (**Figure 2B**). In fact, Pertussis toxin (PTX) that specifically inhibits GPCR/Gi coupling through the ADP-ribosylation of Gαi subunit, blocks several CXCR4-mediated effects[32]. In parallel, CXCR4-oriented migration is mediated by the phosphatidylinositide 3-kinases (PI3Ks) and furthermore, Gβ/Gγ dimer can trigger phospholipase C (PLC) activation (**Figure 2C**) which, in turn, catalyses the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). IP3 production results in Ca^{2+} mobilization from the intracellular stores, while DAG promotes the activation of protein kinase C (PKC) and mitogen associated protein kinase (MAPK)[29]. Gβγ subunits can also directly activate small GTPases Rho family (including Rho, Rac and Cdc42), whose activation is required for an efficient migration through actin polymerization and pseudopods formation[34] (**Figure 2D**).

Following CXCL12 binding, CXCR4 promotes the recruitment of G-protein coupled receptor kinases (GRKs) that induce site-specific phosphorylation at the C-terminus leading to association with β-arrestins[32] (**Figure 2E**). Arrestin recruitment results in the uncoupling of CXCR4 from G protein and induces receptor internalization in a process termed desensitization[35]. Following internalization, CXCR4 can be recycled back to the cell surface or degraded by lysosomes[29].

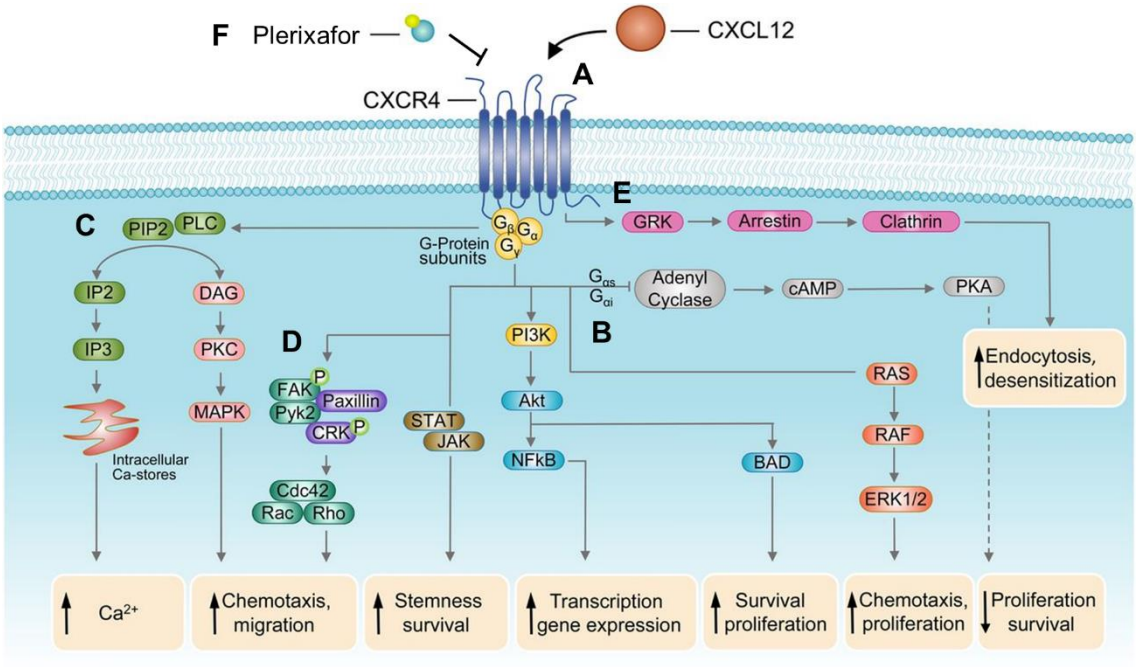

Figure 2: CXCR4 intracellular signaling after CXCL12 binding: (**A**) CXCL12 binds extracellular regions of CXCR4; (**B**) Gαi inhibits adenyl cyclases and the consequent cAMP production; (**C**) Gβ/Gγ dimer can trigger PLC activation cascade; (**D**) Activation of small GTPases Rho family; (**E**) GRKs induce site-specific phosphorylation at the C-terminus leading to association with β-arrestins; (**F**) Plerixafor is a selective CXCR4 antagonist.
Adapted from Walenkamp et al., The Journal of Nuclear Medicine, 2017

CXCR4 plays pleiotropic roles in both physiological and pathological conditions and for this reason it represents an interesting target in drug development. In addition to the well-defined functions in haematopoiesis and immune responses[29], CXCR4 plays a pivotal role in a range of physiological processes such as organogenesis, neurogenesis[36] and vascular formation[37] as attested by experiments performed with CXCL12 and CXCR4 KO mice[38,39].

CXCR4-CXCL12 axis is fundamental for hematopoietic stem cells (HSCs) homeostasis inside the BM: in fact, the HSC niche is maintained by a number of cell types producing high amount of CXCL12, including stromal cells and CXCL12-abundant reticular (CAR) cells, osteoblasts and endothelial cells[29,40]. CXCR4-CXCL12 axis regulation is particularly important in order to preserve and retain inside the BM a stable HSC pool capable of providing the different blood cells for the entire lifetime of the organism[41].

Together with the regulation of BM homeostasis, CXCR4 plays a prominent role in orchestrating both innate and adaptive immune responses and leukocyte trafficking. For example, CXCR4 is highly expressed on activated cutaneous DCs and drives their homing to regional LNs initiating skin immune response[42]. Also, T cell homing to LNs and B cell and neutrophil homing and development involve CXCR4 activation[29,43].

The significant role of CXCR4 in several diseases such as cancer, autoimmunity, and immunodeficiencies has been widely documented. Since CXCL12/CXCR4 axis supports tumor cell survival, proliferation, migration and metastatic process[44], CXCR4 is designated as one promising candidate for novel therapeutic strategies against cancer[45] and its expression has been identified as a prognostic marker for several kinds of human cancers. Tumors in which CXCL12-CXCR4 axis plays a role include breast, ovarian and colorectal cancer[46], pancreatic adenocarcinoma[29,47] and multiple myeloma[48].

CXCR4 is also involved in disease pathogenesis acting as a co-receptor: CXCR4 is indeed the main co-receptor facilitating HIV entry in the host cell[49]. Following the interaction with the CD4 molecules, the envelope glycoprotein gp120 of the T-tropic HIV-1 strains binds to CXCR4, an event that triggers the entrance of the HIV virus into the target cells. The selective inhibition of CXCR4 through AMD3100, a bicyclam compound commercially available as Plerixafor[50], has been described as a strategy to block HIV infection at early stages in viral life cycle[50].

Furthermore, a heterozygous mutation of chemokine receptor CXCR4 causes a rare autosomal dominant primary immunodeficiency called WHIM syndrome (syndrome of Warts, Hypogammaglobulinemia, Infections and Myelokathexis). Most of the WHIM patients have a mutation that causes a truncation of the C-

terminal domain of CXCR4, crucial for ligand-induced internalization and receptor desensitization[51]: the mutated chemokine receptor causes an increased responsiveness and enhanced chemotaxis to CXCL12[52]. This mechanism explains the abnormal retention of neutrophils in the BM that characterizes WHIM patients, causing a strong immunodeficiency, clinically characterized by recurrent bacterial infections, HPV-induced warts of hands, feet and genitalia and potentially leading to cancer[53]. These symptoms in WHIM patients rise from a very low concentration of most blood leucocytes (leukopenia) and "myelokathexis", a term that describes a pathologic process in which mature neutrophils are retained in an hypercellular BM, bringing to neutropenia in the blood[54]. However, the few neutrophils released in blood circulation maintain a normal functionality[53]. WHIM syndrome was initially treated with elevated dosage of CXCL12 administration; however, this treatment only partially increased the number of circulating neutrophils and didn't affect the extent of neutrophil retention in the BM. A subsequent clinical indication for the WHIM syndrome is the administration of Plerixafor (**Figure 2F**), that is able to increase the frequency of circulating neutrophils by mobilizing these cells from the marginated pool in the lung and simultaneously decreasing the number of cells tracking back to the BM[55]. Plerixafor was approved by the Food and Drug Administration (FDA) in 2008, but in the subsequent years many other CXCR4 antagonists that bind the CXCR4 receptor transmembrane domain have been developed[56,57]. Moreover, new therapeutic indications for Plerixafor are under investigation, regarding pathological processes such as hepatopulmonary syndrome[58], liver fibrosis/steatosis[59] and tumor immunotherapy[60].

1.4.2 CXCR2: expression, ligand, signaling and role in health and disease

The first identified ligand of CXCR2 is the chemokine CXCL8[61,62]. CXCL8 is a powerful mediator able to induce morphologic changes, degranulation, chemotaxis and transmigration of neutrophils both *in vivo* and *in vitro*[63]. CXCL8 is secreted by different cell types including monocytes, alveolar macrophages, fibroblasts, endothelial cells and epithelial cells. CXCL8 expression is stimulated by various cytokines (IL-1, IL-6, CXCL12, and TNFα), reactive oxygen species (ROS), bacterial material and other environmental stresses, and it is mediated by transcription factors NF-κB and activator protein-1 (AP-1)[64]. In rodents, two main

ligands have the same role of human CXCL8, called CXCL1 (also known as keratinocyte-derived chemokine, KC) and CXCL2 (macrophage inflammatory protein, MIP-2). These chemokines have also been identified in humans as growth-regulated gene-alpha and -beta (GROα and GROβ), respectively[63]. During the last 30 years, other chemokines were found to bind CXCR2: CXCL3 (Gro-γ)[65], CXCL5 (ENA-78)[66], CXCL6 (GCP-2)[67] and CXCL7 (NAP-2)[68].

Human CXCL8, can bind two receptors: CXCR2 the principal receptor in chemotaxis processes, and CXCR1, which seems to mediate neutrophil activation and myeloperoxidase release[63]. CXCR1 homolog is lacking in mice, while the CXCR2, highly conserved among vertebrates, is 75% identical in humans and mice[69].

Following ligand binding, CXCR2 couples to PTX-sensitive G-protein starting phosphoinositide hydrolysis to generate diacylglycerol and inositol 1,4,5-trisphosphate, which then activate protein kinase C inducing the mobilization of calcium to start cellular responses, like chemotaxis and degranulation. CXCL8 also induces rapid and transient phosphorylation of extracellular signals related kinases (ERK1/2) and activation of members of Rho GTPase family[64]. This process allows the arrest and crawling of neutrophils on the surface of the endothelial cell monolayer and their extravasation. The G-protein signaling of CXCR1/2 is tightly regulated and quickly desensitized to prevent constitutive signaling: ligand–receptor complex is phosphorylated and endocytosed through clathrin-coated pits and, once internalized, CXCR2 may be either degraded or translocated back to the cell membrane after dephosphorylation[70].

CXCR2 is indeed abundantly expressed on neutrophils, but also on other leukocytes such as monocytes, macrophages, macrophage foam cells, basophils, T-lymphocytes and non-haematopoietic cells such as fibroblasts, circulating endothelial progenitor cells and oligodendrocytes[71,72].

CXCR2 KO mice showed neutrophilia and impairment in the recruitment of neutrophils during acute inflammatory conditions[73]. In fact, the primary physiological functions of CXCR2 in the innate immune system is to guard the body from infections and disease regulating neutrophil recruitment[74]. Moreover, the expression of CXCR2 on neutrophils surface plays a pivotal role in their physiological release from the BM[75].

Leukocytes are recruited into inflammatory sites by a series of different chemokines, including CXCL8[76]. Disruption in the CXCL8-CXCR2 axis often leads to impaired neutrophil recruitment and consequent decrease in bacterial clearance that can severely affect the host's immune mechanisms against infection[64]. However, the selective block or deletion of CXCR2, can also significantly reduce tissue damage and mortality in different models of inflammatory diseases. The reason behind this mechanism, is that leukocyte recruitment, during inflammation, has to be finely regulated since an excessive leukocyte extravasation may lead to the deterioration of the integrity of the organism and may worsen acute and chronic inflammation[64]. In fact, chemokine receptor CXCR2 and its ligands have been involved in a series of chronic inflammatory disorders, such as cystic fibrosis (CF), chronic obstructive pulmonary disorder (COPD), asthma, psoriasis, rheumatoid arthritis, inflammatory bowel diseases and liver fibrosis and steatosis[64,77], since protease secretion by neutrophils and other inflammatory cells in inflamed tissue leads to maintain and extend a chronic tissue damage[64,78]. In addition, CXCR2 has been involved in neuro-inflammatory and vascular diseases[64] and CXCR2/CXCL8 axis also recruits neutrophils in infarcted area after a myocardial or cerebral infarction event, leading to further injuries to the tissues. The use of CXCR2 antagonist or CXCR2 KO mice showed an improvement of the clinical outcome in both the pathologies[79,80].

Higher levels of CXCL8 have been reported also in serum of patients with chronic liver diseases such as fibrosis[77] and CXCR2 results to be strongly involved in neutrophil recruitment into the liver and accumulation in a series of other chronic liver diseases such as non-alcoholic steatohepatitis (NASH), alcoholic liver disease (ALD), viral hepatitis, drug induced liver injury (DILI) and hepatocellular carcinoma (HCC)[81-85]. Blocking CXCR2 through selective antagonists represents a novel therapeutic strategy for the management of chronic liver diseases[86]: for example, the non-competitive allosteric CXCR2 antagonist Repertaxin (RPTX) inhibits neutrophil recruitment and protects liver damage against reperfusion injury[17] and post ischemic hepatic syndromes[87].

Chronic inflammation is a major factor in tumor growth and progression[88]. Consequently, CXCR2 and its ligands have been implicated in tumor progression and growth, vessel formation and cancer cell proliferation, as well as in neutrophil

recruitment to the tumor microenvironment[64]. Once tumor-associated neutrophils (TANs) accumulate in tumor tissues, they show a dual functionality polarizing in two different states, N1 (tumor-suppressive phenotype) and N2 (pro-tumorigenic phenotype)[89]. CXCR2 inhibition seems able to reduce mouse lung tumorigenesis by reducing TANs accumulation[90]. Moreover, CXCL8 is secreted and expressed by various cancer cell types and it is able to stimulate cancer cell proliferation and migration in an autocrine way, actively contributing to pathogenesis of different tumor types (lung, colorectal, breast, prostate, ovarian, melanoma, pancreatic, liver and bladder cancer)[64]. The involvement of CXCR2 in tumor progression and angiogenesis is demonstrated by *in vivo* cancer models that showed that the depletion of the receptor significantly reduced lung tumor growth and microvessel density[91]. Similar results were obtained in prostate and pancreatic cancers, where CXCR2 KO mice have smaller tumors and reduced tumor vascularization[64,92].

Currently, several inhibitors and antibodies targeting CXCL8-CXCR2 pathway are under clinical development for inflammatory diseases and cancer treatment[64,93].

1.5 Atypical chemokine receptors (ACKRs)

Atypical chemokine receptors are a small subset of GPCRs characterized by high degree of homology with conventional chemokine receptors, able to bind chemokines[94]. However, they do not signal through G-protein according with the presence of a non-canonical DRYLAIV motif, that is not well conserved or totally absent in these receptors[94]. Originally classified as “silent” receptors for their inability to signal through a canonical pathway upon ligand engagement, some of them are now well characterized and their functions as “scavenger” or “decoy” have been widely demonstrated[95]. They are classified as “decoy” receptors for their capability to trap chemokines as a strategy to fine-tune and regulate innate and adaptive immunity[95]. The term chemokine “scavenging” defines the ability to internalize and then degrade chemokines through lysosomal compartment[96] or, in the case of polarized cells, to bring them to the opposite side of the cell monolayer[94]. This function has been better characterized for ACKR2 which, through endosomal vesicles recycling, is able to progressively regulate the gradient of chemokines[97]. Once detached from plasma membrane, these

vesicles deliver their cargo to various destinations: degradation or recycling back to cell surface. The main mechanism of internalization involves the clathrin recruitment and β-arrestin 1/2 as adaptor.

Recently the International Union of Pharmacology and Human Genome Organization Gene Nomenclature Committee formally approved the new nomenclature "ACKR#" for this group of atypical chemokine receptors[98]. The receptors classified in this family were six: ACKR1/DARC, ACKR2/D6, ACKR3/CXCR7, ACKR4/CCRL1, ACKR5/CCRL2 and ACKR6/PITPNM3[99]. However, in July 2021, Le Mercier and colleagues published a work in which they show that GPR182 is a new atypical chemokine receptor for CXCL10, CXCL12, and CXCL13. GPR182 seems to be involved in the regulation of hematopoietic stem cell homeostasis, acting like a scavenger in a ligand-independent manner[100]. ACKR1 and ACKR2 bind to a range of CC-and CXC chemokines, mostly inflammatory ones[101]. In particular, ACKR1 is the most promiscuous chemokine receptor, which binds over 20 chemokines and it is expressed by erythrocytes and endothelial cells[94]. ACKR2 is expressed by lymphatic endothelial cells but also by some leukocyte subtypes such as DCs and innate like B cells[102]. ACKR3 binds both CXCL12 (with higher affinity than the conventional receptor CXCR4) and CXCL11[46]. Homeostatic chemokines CCL19, CCL21 and CCL25 show high affinity for ACKR4[97]. CCL18 is the only ligand identified for ACKR6, that belongs to the Phosphatidyl-Inositol Transfer Protein (PITP) family. Regarding ACKR5/CCRL2, at date, the chemoattract protein chemerin, a small molecule belonging to the family of adipokines is the only ligand confirmed[103].

1.5.1 CCRL2: an atypical among the atypicals

(The content of this paragraph is part of a review article, which I coauthored[104])

Chemokine (C–C motif) receptor-like 2 (CCRL2, also called HCR or CRAM in humans and L-CCR in mice) is a seven transmembrane receptor closely structurally related to atypical chemokine receptors family.

In humans, two different CCRL2 splice variants are present, namely CCRL2A and CCRL2B[99,105]. By contrast, mouse CCRL2 consists only of one single variant corresponding to CCRL2B. The two isoforms may be differentially expressed and regulated: for example, CCRL2A expression is restricted to pre-B cells while other

B cell maturation stages express mainly CCRL2B[106]. Furthermore, CCRL2A can be specifically upregulated in certain pathological conditions, such as in breast cancer by IFN-γ[107]. These observations suggest that the two splice variants may possess so far unknown different biological roles and significance.

CCRL2 is expressed by cells in the hematopoietic and non-hematopoietic compartments. Among the hematopoietic cells, both CCRL2 mRNA and protein were detected in monocytes, macrophages, neutrophils, CD4 and CD8 positive T lymphocytes, B cells, monocyte-derived dendritic cells, and CD34 positive cells[99,106,108-112]. In agreement with the first description of CCRL2 as an early LPS-inducible gene in the mouse macrophage cell line RAW264[113], in most of the cases, CCRL2 expression is upregulated by proinflammatory stimuli. CCRL2 mRNA and protein were rapidly upregulated in mouse BM-derived DCs activated with LPS, Poly (I:C) or CD40L[114]. In human neutrophils, the expression of CCRL2 was increased by proinflammatory stimuli, such as LPS or TNF-α alone or in combination with IFN-γ or GMCSF[110] and in neutrophils isolated from inflamed joints of arthritis patients[111]. Similar CCRL2 expression kinetics was shown in mouse neutrophils[115]. Furthermore, in mouse mast cells, CCRL2 was found to be constitutively expressed and to be further upregulated *in vitro* in BM-derived cells[103]. Microglia and astrocytes were also shown to express CCRL2 both *in vitro* and *in vivo* under inflammatory conditions[116,117]. Within the nonhematopoietic compartment, CCRL2 mRNA was detected in inflamed bronchial epithelium[118], in hepatic stellate cells[119], in adipocytes[120], in skin[121] and in different cancer tissues including breast[107] and prostate cancers[122]. In primary human endothelial cells, CCRL2 was significantly upregulated by proinflammatory stimuli (e.g., the combination of LPS, IFN-γ, and TNF-α)[123]. In endothelial cells freshly isolated from mouse lung, CCRL2 was found constitutively expressed, while in mouse liver the expression was strongly increased by inflammatory stimuli[123]. CCRL2 regulation was detected also *in vitro* in lymphatic endothelial cells stimulated with retinoid acid[124]. Organ specific regulation may underscore specific functional properties of CCRL2 in different anatomical districts.

As the others atypical chemokine receptors, CCRL2 is unable to activate conventional G-protein dependent signaling and to induce cell directional migration, since it lacks the canonical high conserved DRYLAIV motif. In the past

few years, several chemokines were proposed as ligands for CCRL2, such as CCL2, CCL5, CCL7, CCL8[125], or CCL19[126], but these findings were not subsequently confirmed[99,103,127]. So far, the only commonly accepted CCRL2 ligand is not a chemokine but the non-chemokine chemotactic protein chemerin[103], a ligand shared with two other signaling receptors, namely Chemokine- Like Receptor 1 (CMKLR1) and G protein-coupled receptor 1 (GPR1)[127,128]. Chemerin binding to CCRL2 does not induce calcium mobilization, ligand scavenging or receptor internalization[103,127,129] and this atypical behaviour makes CCRL2 a unique member of the non-signaling GPCR chemotactic receptor family.

CCRL2/chemerin axis plays a role in leukocyte trafficking: when CCRL2 is expressed on the surface of barrier cells, such as endothelial and epithelial cells, can increase the local concentration of chemerin to form a membrane-bound chemotactic gradient for leukocytes expressing the functional chemerin receptor CMKLR1[103,123,124,128]. CCRL2 binds chemerin at the N-terminus leaving the C-terminal peptide sequence accessible for the interaction with CMKLR1[103]. By this mean, CCRL2 can promote *in vivo* the recruitment of CMKLR1- expressing cells, such monocytes/macrophages, dendritic cells, plasmacytoid dendritic cells, and NK cells[2,103,114,123,124,130-133] (**Figure 3**). The described role of CCRL2 has emerged by the use of CCRL2 KO mice tested in several experimental models of inflammatory diseases[114,115,124,134]. For example, in a model of ovalbumin (OVA) induced airways hypersensitivity, the genetic ablation of CCRL2 caused defective trafficking of antigen-loaded DCs from the lung to mediastinal LNs[114].

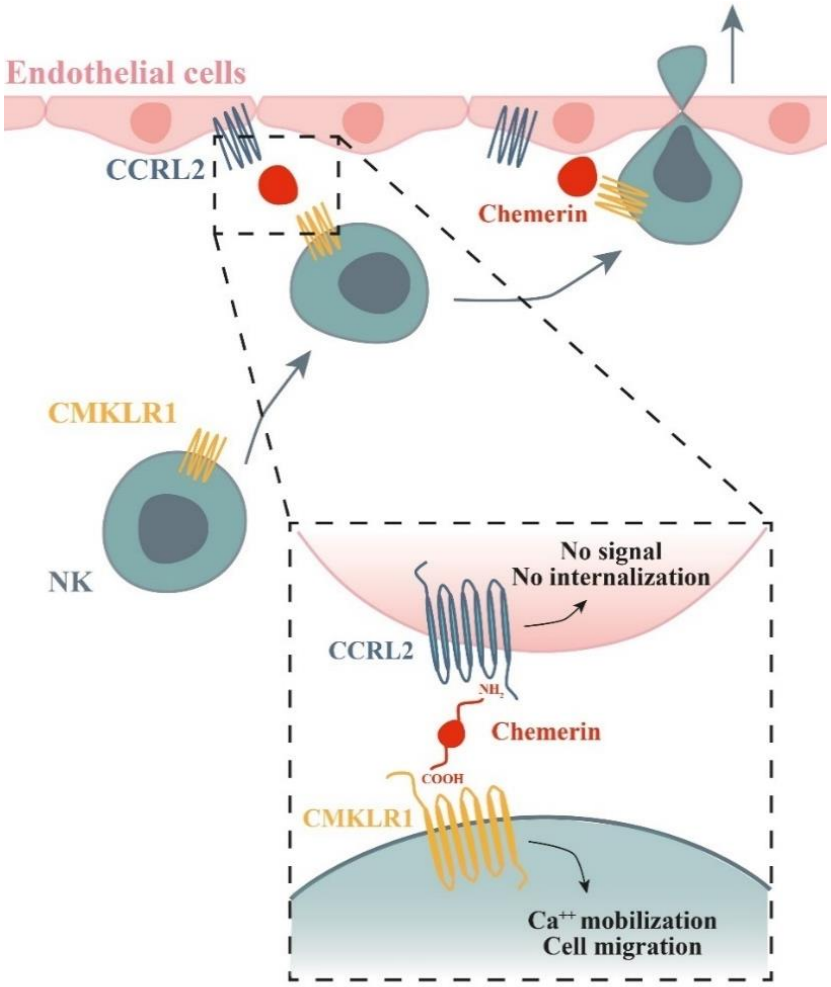

Figure 3: CCRL2 expressed on endothelial cells presents and concentrates chemerin creating a solid chemotactic gradient for cells of the immune system expressing CMKLR1 that consequently are recruited into the tissue.

Adapted from Schioppa et al., Frontiers in Cell and Development Biology, 2020

Moreover, a possible role for CCRL2 in the resolution phase of inflammation emerged in the chronic phase of MOG-induced experimental autoimmune encephalitis (EAE), a model that resembles the inflammatory process that characterizes multiple sclerosis[134] in the central nervous at the peak of clinical development of the disease. CCRL2 KO mice displayed increased mortality and severity of clinical score compared to control animals. In addition, the histopathological examination revealed enlarged demyelination areas and hyperactivation of microglia with unbalanced M1/M2 rate of polarization, especially during the recovery phase of the disease[134]. These findings highlight a potential involvement of the chemerin/CCRL2 axis in the dynamic process of

macrophage polarization, a fundamental step in the resolution of inflammation and tissue repair.

Also, the polymorphism CCRL2-167F was specifically associated with more rapid development of pneumocystis pneumonia (PCP) in HIV patients: considering the defined role of CCRL2 in lung immune cell trafficking, this atypical chemokine receptor may affect PCP through immune regulation and inducing inflammation[135].

CCRL2 expression was described in different cancer cells, including prostate and breast carcinoma, colorectal cancer liver metastasis and glioblastoma[122,136-138]. However, the functional role of CCRL2 in cancer is still unknown and needs further investigations. In non-small-cell lung cancer (NSCLC) patients, elevated expression of CCRL2 was found to have a beneficial effect on overall survival and correlated with better clinical outcome, particularly at the early phase of lung tumor progression[133,139]. In addition, CCRL2 deficiency was associated with increased tumor burden in urethane-induced lung carcinogenesis and in a genetic model of Kras/Tp53- driven (KrasG12D=C/p53LoxP) lung tumor. Similarly, CCRL2 KO mice were more permissive for tumor growth following orthotopic injection of a tumor cell line obtained from KrasG12D=C/p53LoxP mice. In all these experimental conditions, lung tumor microenvironment revealed the decrease of some myeloid cell subsets, such as monocytes, macrophages and neutrophils, and a consistent reduction of lung NK cell frequency. Since CCRL2 is not expressed by mouse NK cells, but was found expressed by CD31+, CCRL2 present on the surface of lung endothelial cells may act as a chemerin-presenting molecule regulating the recruitment of CMKLR1+ NK cells. By this mechanism, CCRL2 may shape the immune tumor microenvironment in lung cancer[133].

2. Chemokine receptors expression and functionality regulation

2.1 Chemokine receptors expression regulation

The presence of appropriate receptors in different cell populations and conditions defines the spectrum of action of different chemokines; indeed, chemokine receptors as their ligands are subjected to a tight control, so that several receptors are detected exclusively in specific cell states. For example, during maturation process, immune cells such as neutrophils and dendritic cells are characterized by a complete change in their membrane receptors pattern profile: dendritic cells (DCs) during maturation down-regulate inflammatory receptors (CCR2 and CCR5 among others) matched to selective upregulation of CCR7, which drives the mature DCs to draining LNs[2]. Moreover, neutrophils, in different states of their life-span, are recognised by a fine-tuning modulation of expression of the two chemokine receptors CXCR2 and CXCR4[140,141]. As a general rule, pro- and anti- inflammatory mediators display divergent effects on receptor production and expression, such as in the case of the CCL2/MCP-1-CCR2 axis on monocytes and dendritic cells[142].

It is important to underline that a variety of polymorphisms have been identified in either the coding or non-coding regions of chemokine receptors genes: these polymorphisms have the potential to alter expression levels, stability and interactions with chemokines or other receptors. Consequently, in some cases, these polymorphisms have been positively or negatively associated with disease incidence and progression[23]. For example, the CCR2 polymorphism 190 G/A, which gives rise to a conservative amino acid change from valine to isoleucine in the first transmembrane helix of the receptor, is associated with delayed progression of HIV, apparently because it indirectly reduces the cell surface expression of the HIV-co-receptor CCR5[143]; regarding CCR5, the CCR5-D32bp mutation, that brings to a truncated and not functional protein, provides strong protection against HIV-transmission and causes a delay in disease progression[144].

Finally, dimerization between chemokine receptors can influence their expression on cell membrane and functionality[145].

2.2 Homo- and heterodimerization of chemokine receptors

For many years GPCRs were thought to exist and exploit their function as monomers. A growing number of evidences has demonstrated that GPCRs can function as homodimers, heterodimers or even high order oligomers[146]. Fluorescence (FRET) and bioluminescence (BRET) resonance energy transfer techniques, performed in living cells, have been broadly used for detection of GPCR interactions. Both methods are based on the transfer of non-radiative energy from a donor molecule to an acceptor molecule that are in close vicinity and require transfection of the receptors coupled with acceptor and donor molecules. Moreover, evidences regarding the crystal structure of several chemokine receptors support the existence of dimeric entities in primary cells[147]. It has been demonstrated that dimerization, for some GPCRs, is required to stabilize the receptor and allow its correct exportation to the cell membrane. Dimerization can affect ligand binding, alter the endocytosis of the GPCR or activate distinct signaling pathways. Moreover, heterodimerization between chemokine receptors can have different biological significance such as fine-tuning of signaling, positive or negative cooperativity, lack of internalization, enhanced or diminished chemotaxis and decreased surface expression[145]. Two different models were proposed to describe GPCR dimerization. The first one, the most accepted, proposes that the two monomers take contact but remain structurally distinct. The second hypothesis, less widespread, states that two out of seven α-helices of one monomer replace two helices in the other monomer and vice versa[148].

Among chemokine receptors, the cCKRs CXCR1, CXCR2, CXCR3, CXCR4, CCR2, CCR3, CCR5, CCR7 and the ACKRs ACKR1 and ACKR3 were described to form constitutive homodimers, the majority of them stabilized or modulated by their ligands[145,147]. Conventional chemokine receptors can also form heterodimers with other cCKRs or with ACKRs. Well-established examples of heterodimerization between cCKRs are the ones between the pairs CCR2/CCR5 (which leads to cross-competition in ligand binding assays, negative cooperativity and activation of specific signaling pathways)[147,149] and CXCR1/CXCR2 (that brings to a decreased surface levels of both receptors and contributes to receptors assembly and trafficking to the cell membrane)[147,150]. Instead,

examples of heterodimerization between conventional and atypical chemokine receptors are ACKR1/CCR5 (inhibition of CCR5 activation and signaling)[147,151] and ACKR4/CXCR3 (inhibition of CXCR3 signaling)[147,152]. In particular, ACKR1 can form constitutive homodimers as well as constitutive heterodimers with CCR5: thus, in addition to act as chemokine "sink", ACKR1 plays also a role in immune response, weakening CCR5-driven signaling and chemotaxis[151].

Chemokine receptors can also heterodimerize with other types of GPCRs and even with non-GPCRs, among others opioid receptors and α1A/B-adrenergic receptors[153].

Chemokine receptors organization complexity at the cell membrane is represented in **Figure 4**.

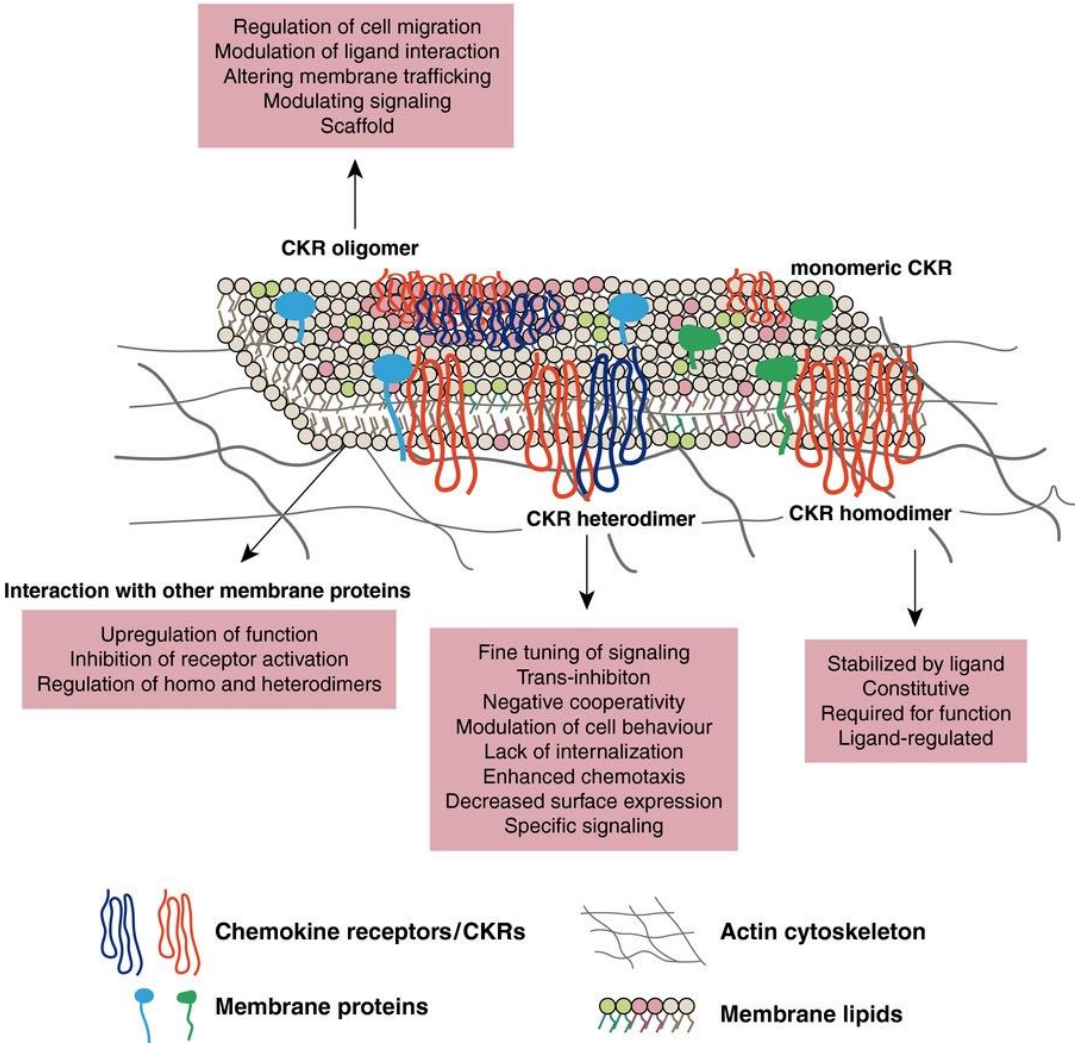

Figure 4: Schematic representation of the dynamic conformations of chemokine receptors at the level of cell membrane.

From Martìnez-Muños et al., The Journal of Leukocytes Biology, 2018

2.2.1 CXCR4 dimerization

Advanced light microscopy techniques showed that GPCRs can form both homo- and heterodimers and even higher-order oligomers, adding layers of complexity in the modulation of cell responses[147].

Five distinct and independent crystal structures of CXCR4 confirm the formation of constitutive CXCR4 homodimers through interactions that involve residues in the TM5 and TM6 helices[147,154]. However, FRET-based experiments have shown that treatment with a synthetic peptide of TM4 alters the homodimer formation, suggesting an involvement of TM4 helices in affecting the conformational orientation of the other transmembrane domains[155]. It has been proposed that CXCR4 has the ability to homodimerize in the absence of ligand soon after protein translation to the membrane[32,156]. However, it has been demonstrated that CXCR4 ligand CXCL12 can selectively enhance the receptor homodimerization[157]. Lastly, additional CXCR4 crystal structure characterizations suggest further interactions between CXCR4 homodimers generating higher order oligomers complexes[145].

Moreover, CXCR4 has been reported to dimerize with a series of other conventional and atypical chemokine receptors. Indeed, CXCR4 can dimerize with CCR2 and CCR5, and both complexes show negative binding cooperativity with their ligands, not only *in vitro* but also *in vivo*[154,158,159]. In particular, CXCR4-CCR2 heterodimers result to play a pivotal role in modulating T cell behaviour[160] and the specific ligand of one receptor is able to trans-inhibit the binding of chemokines to the other one[161]. Likewise, the heterodimerization between CXCR4 and CCR5 seems to work as a cross-inhibition decreasing ligand binding and cell migration[161] and is involved in the stabilization of the immunological synapse, sustaining T lymphocyte activation[29]. In addition, CXCR4 heterodimerizes also with CCR7, contributing to stabilize CCR7 expression and ligand binding on $CD4^+$ T cell surface[162], and with CXCR3, resulting in negative binding cooperativity[147,163].

Furthermore, CXCR4 can also heterodimerize with the atypical chemokine receptor CXCR7, also known as atypical chemokine receptor 3 (ACKR3). CXCR7 represents a second partner of CXCL12, showing an affinity for this ligand greater than CXCR4[29]. The binding of CXCL12 to CXCR4-CXCR7 heterodimers leads to the inhibition of G protein mediated signaling, while potentiates the β-arrestin-

dependent downstream signaling pathway that persists even after receptor internalization[29,164].

Finally, CXCR4 may form heterodimers with receptors different from chemokine receptors. In fact, CXCR4 can heterodimerize also with the chemotactic receptor Chemokine like receptor 1 (CMKLR1) also known as ChemR23[165], the G protein-coupled receptor for the chemoattractant adipokine chemerin. In particular, a negative binding cooperativity was detected between CXCR4 and ChemR23: in mouse BM-derived DCs, ChemR23-specific ligands cross-inhibit CXCL12 binding on CXCR4 in a ChemR23-dependent manner, while CXCR4-specific antagonist AMD3100 doesn't cross-inhibit chemerin binding in cells co-expressing ChemR23 and CXCR4[165]. Moreover, heterodimerization occurs between CXCR4 and TCR on T cells[166]. CXCR4-TCR heterodimer formation promotes the activation of different PLCs, such as PLC-γ1 and PLC-β3 which, respectively, modulates cell migration and leads to a sustained ERK activity and gene transcription[29].

2.2.2 CXCR2 dimerization

Both CXCR1 and CXCR2 have the capacity to form homo-/heterodimers and oligomers[150]. Following co-expression of these receptors in HEK293 cells, Wilson and colleagues demonstrated a constitutive interaction between CXCR1 and CXCR2 through co-immunoprecipitation and bioluminescence resonance energy transfer[150]. Interestingly, heterodimerization seems to occur not only on cell surface, but also during receptors synthesis and maturation at intracellular level prior to cell surface delivery[150]. Furthermore, Martinez-Muños et al.[167], using primary neutrophils and cell lines co-expressing the two receptors, found that first, CXCR1 expression interferes with CXCR2 homodimers and vice versa and, secondly, that CXCL8 alters CXCR1/CXCR2 heterodimers stabilizing both homodimeric forms triggering their internalization. Recently, CXCR2 heterodimerization complexes were described to occur with the not-signaling chemerin receptor CCRL2[115], structurally similar to the atypical chemokine receptors family. In this case, explained in detail in chapter 2.2.3 of the introduction, the heterodimerization results to play the role of fine-tuning modulator and to be necessary for an optimal CXCR2 signaling and, *in vivo*, to regulate neutrophil recruitment in the inflammatory sites[115].

Finally, CXCR2 can also form dimer interactions with surface receptors different from chemokine receptors: first CXCR2 heterodimers with AMPA-type glutamate receptor GluR1 result in an impairment of homodimers formation[147,168]; secondly, a CXCR2 antagonist can enhance the function of δ opioid receptors agonists as an allosteric regulator in a condition of heterodimerization[169].

2.2.3 CCRL2 dimerization

(The content of this paragraph is part of a review article, which I coauthored[104])

As in the case of conventional chemokine receptors, ACKRs have the ability to dimerize with other receptors.

In fact, a second function proposed for CCRL2 is unrelated to the interaction with its ligand chemerin and consists in the formation of heterodimers with chemokine receptors. In fact, CCRL2/CXCR2 heterodimers were shown to represent a mechanism of fine-tuning modulation of neutrophil migration in pathological contexts, such as inflammatory arthritis[115]. First, FRET analysis revealed that CCRL2/CXCR2 heterodimers were detectable both at the cell membrane and in the cytoplasm of transfected cells. Modulation of CXCR2 membrane expression by CCRL2 was shown both in transfected cells and in primary BM-derived neutrophils where CCRL2 deficiency was related with increased CXCR2 membrane expression[115], suggesting an involvement of CCRL2 in the intracellular retention of the CCRL2/CXCR2 heterocomplexes. As shown in **Figure 5**, CCRL2 expression was also associated with increased CXCR2 signaling through ERK1/2 and small GTPases phosphorylation, and activation of β2-integrin, as detected both *in vitro* and *in vivo* by underflow and intravital microscopy[115]. In fact, in CCRL2 KO mice, CXCL8-induced neutrophil recruitment to the peritoneal cavity was found to be impaired.

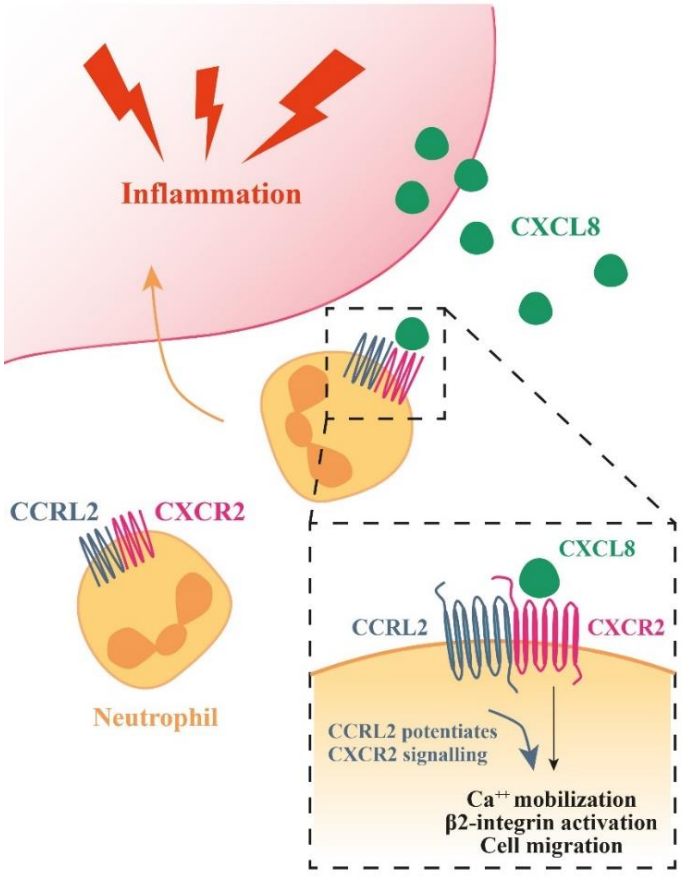

Figure 5: CCRL2 expressed on neutrophils surface can form heterodimers with CXCR2, increasing CXCR2 intracellular signaling in response to its ligand CXCL8 and thus neutrophil recruitment into the situ of inflammation.

Adapted from Schioppa et al., Frontiers in Cell and Development Biology, 2020

Accordingly, CCRL2 KO mice were also protected in experimental models of inflammatory arthritis, due to a defective neutrophil recruitment in the inflamed joints[115]. In fact, the process of tissue neutrophil infiltration is implicated in the pathophysiology of rheumatoid arthritis and is controlled by a well-defined temporally and spatially cascade of chemoattractants and their cognate receptors, being the CXCL8/CXCR2 axis a major player[170,171]. Neutrophil infiltration to inflamed joints was impaired in CCRL2 KO mice tested in collagen induced- and serum transfer induced-arthritis, two experimental models of inflammatory arthritis. In both experimental conditions, CCRL2 KO mice showed decreased severity of disease, lower incidence and delayed clinical onset, with reduced histopathological score[115]. Disease protection was reversed by the adoptive transfer of CCRL2 competent neutrophils. Intravital microscopy clearly

revealed that CCRL2 KO neutrophils displayed a strong reduction in their ability to adhere to the surface of endothelial cells in the vessels present in inflamed knee (**Figure 6**), with an increased number of rolling neutrophils on the endothelial surface. Similar results were obtained in experiments performed under flow conditions showing defective capacity of CCRL2 KO neutrophils to undergo rapid β2 integrin-mediated arrest in response to CXCL8[115].

Collectively, these findings suggest the pivotal role of CCRL2 in the regulation of optimal CXCR2 functionality.

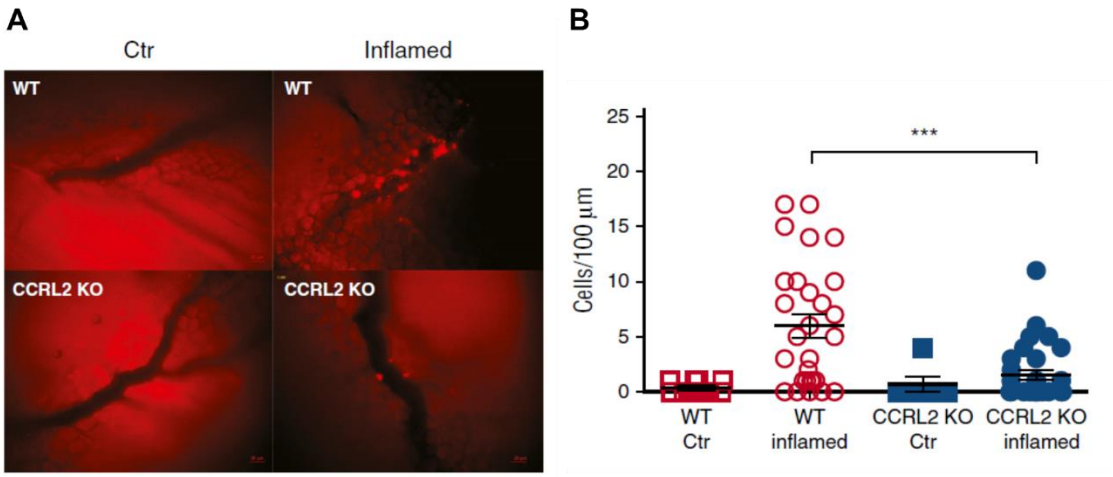

Figure 6: (**A**) Intravital microscopy of the interaction between leukocytes and endothelial cells in the synovial microvasculature in WT and CCRL2-deficient (KO) mice, after antigen (left) and saline (right) injection into the knees. (**B**) Corresponding quantitative analysis of cells adherent to the synovial endothelium.

Adapted from Del Prete et al., Blood, 2017

Finally, in 2021, Yin and colleagues[172] published a paper in which they report a positive association between tumoral CCRL2 expression and the intensity of anti-tumor T-cell immunity in human cancer. The authors showed that CCRL2 can interact with the membrane-expressed TLR4 and retain its expression in cell surface of classically activated macrophages, thereby amplifying membrane TLR4-mediated inflammatory signaling to enhance their immunostimulatory phenotype[172].

The involvement of CCRL2 in the regulation of other chemotactic receptors through heterodimerization needs to be further explored.

3. Neutrophils

Innate immune system is the first responder to invaders, facing daily exposure to pathogens. In particular, neutrophils are one of the major arms of the innate immune system, during acute inflammation and invasion of pathogens including bacteria, fungi, and protozoa. Recruitment of neutrophils into sites of infection is so critical that a decrease of neutrophils in blood leads to serious immunodeficient conditions in humans. In fact, neutrophil production in the bone marrow, during period of infection, can be boosted 10-fold up to 10^{12} cells per day[173].

Neutrophils are a type of myeloid leukocytes of ~7–10 μm in diameter, that in humans represent the 50–70% of all circulating leukocytes, while in mice, represent the 10–25% of circulating leukocytes[173]. Neutrophils are also known as polymorphonuclear (PMN) granulocytes, respectively for the segmented shape of their nucleus and the presence of granules and secretory vesicles inside the cytoplasm.

Despite the traditional thought that neutrophils are present only in acute phases of inflammation to eliminate pathogens, recent findings have demonstrated a more versatile role of neutrophils in both health and disease[173-175].

3.1 Functionality: activation, degranulation and pathogens killing

Neutrophils are the first leukocytes recruited during acute inflammation where they can employ several mechanisms to eliminate pathogens. Circulating neutrophils are quiescent and in physiological conditions most of them terminate their life without meeting any activating signal. In the case of infection or sterile injury, however, they are the first immune cells to respond through two steps of activation: a first signal that “prime” the cells and then a second one that makes the cells fully activated[176]. Priming is an indispensable step for an effective immune response, because unprimed neutrophils respond very little even to the strongest activating signals. Also, primed cells can spontaneously return to the quiescent state, a regulatory mechanism that reflects the need to control exaggerated immune response that can produce tissue damage[176]. Priming agents include inflammatory signals such as chemokines, cytokines, alarmins, integrins, pathogen-derived molecules and mechanical forces[174]. Examples of priming agents are: N-formyl peptides, whose production is restricted to bacteria

and mitochondria, recognized by formyl peptide receptor 1 (FPR1); leukotriene B4, which is endogenously produced in response to inflammation; C5a, an anaphylatoxin released from cleavage of complement component C5[174]. Moreover, in addition to FPR1, neutrophils express a vast repertoire of pattern recognition receptors (PRR), including the majority of the members of the Toll-like receptors (TLR) family, the C-type lectin receptors dectin 1 (also known as CLEC7A), CLEC2 (also known as CLEC1B), and cytoplasmic sensors of ribonucleic acids (RIG-I and MDA5)[174]. The sensing of pathogens or tissue damage through these PRRs activates the effector functions of neutrophils.

Once primed and activated, neutrophils can eliminate pathogens by three distinct mechanisms: degranulation, phagocytosis and neutrophil extracellular trap (NET) formation[177].

Degranulation is a process that consists in the release of molecules from cytoplasmic secretory vesicles, called granules. Three types of neutrophil granules are formed sequentially during differentiation, since different proteins are expressed with distinct timing and they are associated with characteristic pro-inflammatory proteins[177]. Primary (azurophil) granules contain the main microbicidal substances such as myeloperoxidase, cathepsin G, elastase and proteinase 3. Secondary (specific) granules carry lactoferrin, transcobalamin II and lysozyme, while tertiary (gelatinase) granules, that are rich of gelatinase, matrix metalloproteinase 9 (MMP9) and antibacterial proteins, are involved in the processes of extravasation and migration[177]. Vesicles can rapidly transport their content to the cell surface, where the granule proteins are incorporated into surface membrane. Upon neutrophil activation, secretory vesicles first transport molecules that are required for cell adhesion (for example, β2 integrins); then, gelatinase granules deliver proteases that can digest basement membrane and/or extracellular matrix, thus allowing neutrophil transmigration[177].

Neutrophils can eliminate pathogens also by phagocytosis, a process that consists in the engulfment of large (≥ 0.5 μm) particles, including microorganisms, in phagosomes which are vacuoles derived from the plasma membrane. The newly formed phagosome must undergo maturation into phagolysosome to acquire its microbicidal features, by fusion with intracellular granules. Inclusion of anti-microbic enzymes from granules, reduction of pH, production of ROS and nitric oxide inside the mature phagolysosome, induce impairment of bacterial

metabolism and replication. In particular, neutrophils kill the pathogens using NADPH oxygenase-dependent mechanisms (ROS) or antibacterial proteins (cathepsins, defensins, lactoferrin and lysozyme) which are released from granules not only into the extracellular milieu but also into phagosomes[177].

Highly activated neutrophils can eliminate pathogens by releasing NETs. NETs are composed of a core of DNA to which histones, proteins and enzymes released from neutrophil granules are attached. NETs can immobilize pathogens, preventing them from spreading but also facilitating their subsequent engulfment. Through the release of antimicrobial histones and proteases, NETs are also thought to directly kill pathogens[178]. Example of molecule released and trapped into DNA core are lactoferrin, cathepsins, MPO and elastase[177].

It is well established that neutrophils express and produce a variety of pro- and anti-inflammatory cytokines, including chemokines, TNF family members, immunoregulatory cytokines, and colony-stimulating, angiogenic, fibrogenic and growth factors[174]. Cytokines production by neutrophils is positively modulated by IFNγ[177] and negatively by IL-10[179]. After synthesis, some cytokines can be stored in intracellular compartments and then released upon opportune stimulation, for example B-cell activating factor (BAFF), TNF-related apoptosis-inducing ligand (TRAIL), CXCL8, CCL20 and IL-1 receptor antagonist (IL-1RA)[174]. Neutrophil-derived cytokines can have different roles involved in pathogen recognition and elimination, B cell survival and maturation, tumor growth and progression.

3.2 Neutrophil life-span: focus on the role of chemokines

3.2.1 Granulopoiesis

In steady-state conditions, two thirds of the BM volume are involved in neutrophil and monocytes production[180].

The first step of differentiation from the haematopoietic stem cell is the lymphoid-primed multipotent progenitor (LMPP) that further matures into granulocyte-monocyte myeloid progenitor (GMP), as shown in **Figure 7A**. Stimulation by granulocyte-colony stimulating factor (G-CSF) or granulocyte–macrophage-colony stimulating factor (GM-CSF) leads to the maturation of GMPs into neutrophil population, following precise steps: myeloblast, promyelocyte, myelocyte, metamyelocyte, band neutrophil and segmented neutrophil[180]. As the

neutrophil differentiation occurs, the morphology of the nucleus changes from round to ring-like to segmented; also, the characteristic intracellular granules progressively appear during differentiation, starting from primary azurophil (at the promyelocytic stage), followed by secondary granules (at the myelocytic to metamyelocytic stage) and finally tertiary granules (starting from the band cell stage)[181].

Undifferentiated hematopoietic stem cells create the stem cells pool, while granulocytic progenitor cells that are proliferating and differentiating make up the mitotic pool. Completely mature and differentiated neutrophils form the postmitotic pool, from where they are ready to be released[173] (**Figure 7B**). BM niche, in which neutrophil differentiation occurs, is rich of the chemokine CXCL12: thus, immature neutrophils are retained into BM through CXCL12 cognate receptor CXCR4 that results to be highly expressed on their surface[182]. Deletion of CXCR4 causes a shift in the release of mature neutrophils from BM to circulation, while mutations of CXCR4 that result in increased signaling, were described in the clinical syndrome WHIM, characterized by deficiency of circulating neutrophils and by increased accumulation of mature neutrophils in the BM[183].

Neutrophil production and turnover can be accelerated or delayed during pathological and inflammatory conditions.

3.2.2 Neutrophil release

During the process of neutrophil differentiation and maturation inside the BM, their pattern of membrane proteins expression strongly changes. Proteins KIT, VLA4 (also known as integrin β1) and CXCR4 are downregulated, while CXCR2 and Toll-like receptor 4 are upregulated[180].

In homeostatic conditions, KIT ligand, vascular cell adhesion molecule 1 (VCAM1) and CXCL12 are constitutively produced by osteoblasts and other BM stroma cells and retain neutrophils inside the BM binding their respective ligands KIT, VLA4 and CXCR4. Outside the BM niche, different type of cells such as endothelial cells and megakaryocytes produce CXCL1, CXCL2, CXCL5 and CXCL8, creating a chemotactic gradient that stimulates neutrophil mobilization via CXCR2 (**Figure 7C**). Deletion of CXCR2 causes a myelokathexis phenotype

with retention of mature neutrophils in the BM; a double deletion of both CXCR2 and CXCR4 gives a phenotype similar to the CXCR4 deletion[75,183].

Thus, mature neutrophils are retained or release in/from the BM by an antagonistic interplay between CXCR4 and CXCR2 expressed on neutrophil surface in different stages of their life cycle[180]. Under homeostatic conditions, the equilibrium is towards the retention, and circulating mature neutrophils account for only 1–2% of all neutrophils throughout the body[180].

G-CSF is not only a positive influencer of granulopoiesis, but in addition, it is also a well-known disruptor of neutrophil retention: a positive modulation of G-CSF production reduces CXCL12 expression from BM stromal cells, downregulates CXCR4 on neutrophil surface and upregulates CXCR2 ligands production from megakaryocytes. Consequently, neutrophils are released from BM in response to G-CSF. As feedback, macrophages and DCs that phagocytose apoptotic neutrophils decrease IL-23 secretion, thus lowering IL-17 production by T cells, which controls G-CSF production. Reduction of G-CSF inhibits the release of neutrophils from the BM[177,180,184]. In fact, the lack of the G-CSF receptor causes a strong neutropenia both in mice and humans[173].

3.2.3 Neutrophil recruitment cascade

Once in the circulation, neutrophils can interact with the endothelium. Endothelial cells (EC) are able to sense inflammatory mediators (including histamine, cysteinyl-leukotrienes and cytokines) that are released by sentinel leukocytes in the surrounding tissues in case of damage or presence of pathogens (**Figure 7D**); otherwise, EC can also be activated directly by PRR-mediated detection of pathogens. In both cases, EC increase expression of adhesion molecules such as P-selectin, that is stored in Weibel–Palade bodies and E-selectin, which is synthesized *de novo*[177]. These two selectins have partially overlapped functions and maximize neutrophil recruitment. Recruitment to the site of inflammation follows ordered steps: tethering, rolling, adhesion, crawling and transmigration[177]. Free-flowing neutrophils expressing P-selectin glycoprotein ligand 1 (PSGL1) are *tethered* (captured) to endothelial surface through P- and E-selectin; subsequently, they are able to *roll* along the vessel in the direction of blood flow. Circulating neutrophils also express L-selectin that facilitates their secondary tethering during rolling phase[177]. Neutrophils roll on endothelium at shear stress

of 1 to 10 dynes per cm^2: moving in these conditions requires a balance between formation and disruption of adhesive bonds, that include not only the one between P-sel and PSGL1, but also between lymphocyte function-associated antigen 1 (LFA1) on neutrophil surface and intercellular adhesion molecule 1 or 2 (ICAM1-ICAM2) on endothelium[177]. Moreover, the rolling of neutrophils facilitates their contact with chemokines that are transported to the luminal side of EC and immobilized on endothelium by a binding to heparan sulphates. The binding between the chemokine and the cognate chemokine receptor on neutrophils, initiates a conformational change of cell surface-expressed integrins that is essential for firm *adhesion*[177]. The ligation of integrins with their ligands activates signaling pathways inside the neutrophil, thus stabilizing adhesion and initiating cell motility[177].

In fact, adherent cells are able to scan the surroundings with pseudopods and to actively start *crawling* looking for endothelial cell-cell junctions; crawling may be directional if a chemotactic gradient is present[185] and it depends on the interaction of endothelial cell-expressed ICAM1 with neutrophil-expressed MAC1[177]. Crawling under shear stress is a phenomenon that has a mechanotactic component, since neutrophils are able to sense shear; it also depends on a wide range of intracellular signals that include VAV1 (a guanine exchange factor for the Rho-family GTPase Rac) and Cdc42[177].

To finally leave the blood flow, neutrophils can pass across (*transmigrate*) the endothelium following either the transcellular route (through an endothelial cell) or the paracellular route (between endothelial cells), starting where the proteins of extracellular matrix are less dense[177]. The paracellular process, that is preferentially chosen by neutrophils, requires the disruption of junctional intercellular protein bonds, such as those formed by vascular endothelial (VE)-cadherin. However, neutrophils also use the transcellular pathway, even if it is time consuming and less efficient[177].

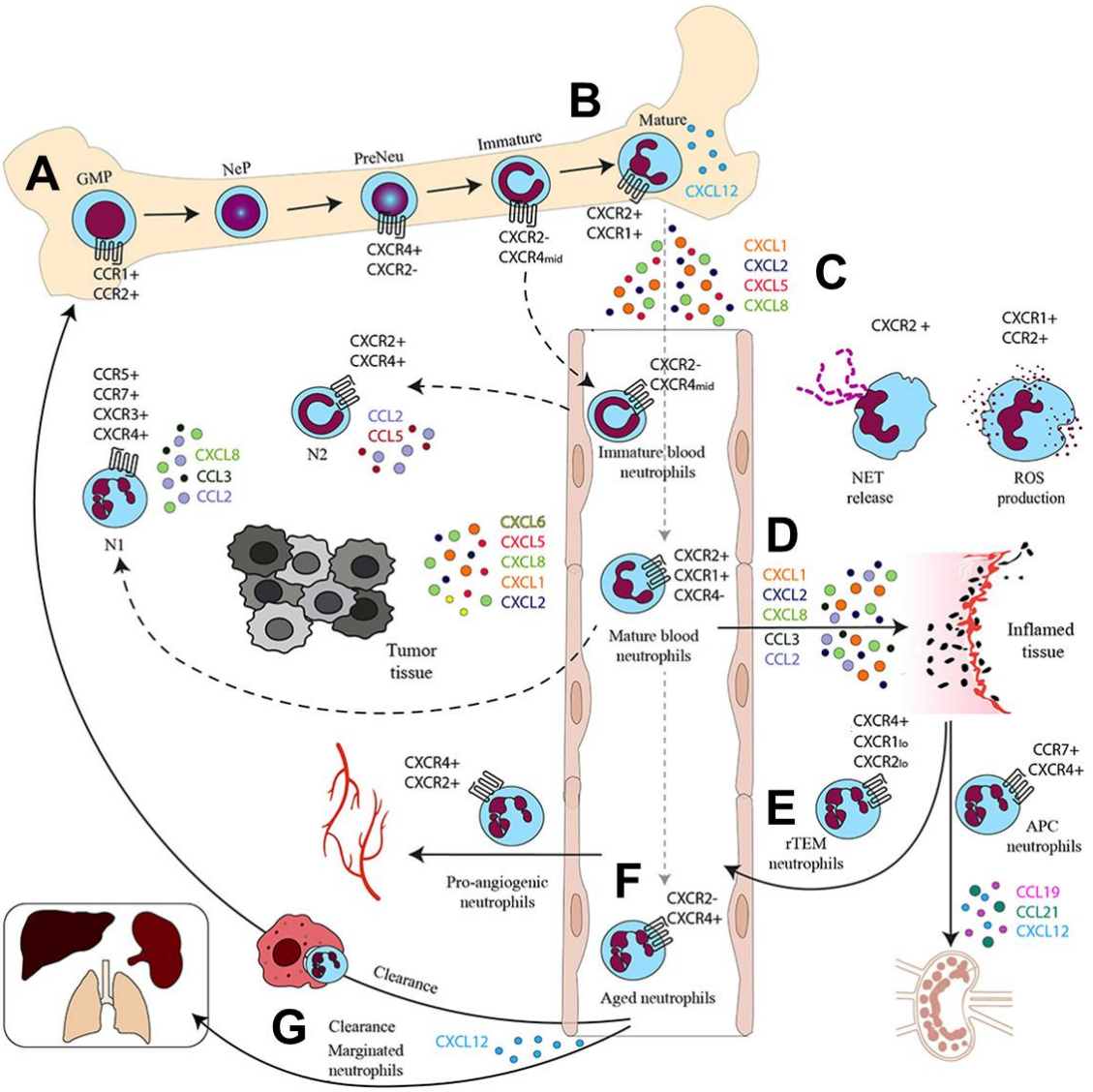

Figure 7: Representative summary of neutrophil life-span and subsets, focusing on the role of chemokines and their cognate chemokine receptors: (**A**) GMPs maturation; (**B**) Mature and differentiated neutrophils form the postmitotic pool; (**C**) Chemotactic gradient that stimulate mature neutrophil mobilization via CXCR2; (**D**) Neutrophils recruitment into the inflammatory site; (**E**) rTEM neutrophils come back to blood circulation; (**F**) Aged neutrophils are present in the circulation ready to be cleared; (**G**) Aged neutrophil clearance and efferocytosis.

Adapted from Capucetti et al., Frontiers in Immunology, 2020

3.2.4 Reverse trans-endothelial migration (rTEM)

Neutrophil recruitment at the site of inflammation is crucial for protection against injuries; however, their persistence in the inflamed tissue can lead to tissue damage and chronic inflammation. Traditionally, neutrophil-mediated damage was thought to be resolved by neutrophil apoptosis and their clearance by macrophages[186,187]. However, recent findings show a different mechanism: experiments in zebrafish model have shown the ability of neutrophils to migrate away from the wound back to the vasculature, a reverse migration that was proposed as a mechanism of inflammation resolution[188]. Afterwards, also human neutrophils *in vitro* showed a bidirectional movement that was called reverse trans endothelial migration rTEM[189]. In 2017 Wang et al. demonstrated that neutrophil rTEM also occurs *in vivo* in mammalian model, performing intravital imaging experiments in mice model of thermal hepatic injury: they also showed that neutrophils are required during the initial phase of inflammation but, within 24 hours, the 90% of them migrate from the injured tissue back to blood circulation in order to reach lung first and subsequently BM, where they undergo apoptosis[190].

The concentration of chemoattractants is one of the major determinant for accelerating rTEM, according with the dynamic regulation of chemokine receptors expressed by neutrophils (**Figure 7E**): neutrophils that perform rTEM result to have diminished expression of the chemokine receptors CXCR1 and CXCR2 but increased expression of CXCR4, CD11b and apoptosis markers, showing a phenotype that overlaps with the one of aged neutrophils; moreover, blocking CXCR4, their homing to BM was shown to be impaired[187,189,191,192].

Finally, the compound Tanshinone IIA (a natural lipophilic compound extracted from *Salvia miltiorrhiza*) was identified as a promoter of neutrophil reverse migration, opening the possibility of a therapeutic approach for the resolution of chronic inflammation[193].

3.2.5 Neutrophil clearance and death

Neutrophil homeostasis is maintained through an accurate balance between neutrophil development, storage, release into blood circulation, recruitment into peripheral tissues, aging and finally death[194].

Neutrophil life-span is a topic still debated: its range, based on published works, goes from 6 hours to 4-5 days in human and 18 hours in mice[182]. However, recent data obtained *in vitro* and *in vivo* support the hypothesis of prolonged neutrophil survival under conditions of inflammation and argue against a short half-life[140,195]. According to this, aged neutrophils are still recognizable in mouse tissue after 48 hours from BrdU injection[196] and neutrophil life-span range calculated in different organs by mathematical models goes from 30 to 60 hours[197]. Moreover, the discovery of neutrophil rTEM supports the hypothesis of a life-span longer than what was described in the past.

Aged neutrophils (**Figure 7F**) are not exhausted cells that just have to be replaced: they are a subset of circulating and "experienced" neutrophils[196] characterized by a high reactivity and cell toxicity resulted from a robust disposition of degranulation. Higher production of ROS and NETosis have been identified in aged neutrophils that, for these reasons, are closely connected with chronic inflammation and pro-tumorigenic environment[198]. Hence, it's important that senescent neutrophils are promptly cleared from blood circulation, preventing the release of their toxic content in case of neutrophil death in the circulation or in peripheral tissues. Bone marrow, spleen and liver result to be responsible of around 30% each of neutrophil clearance[199] (**Figure 7G**). In particular, liver seems to be the preferential organ of clearance in case of neutrophils activated by pro-inflammatory signals[196,200]. In clearance organs or in peripheral tissue in case of pathological condition, aged neutrophils are mainly in an apoptotic state and are then phagocytized by local macrophages in a process called efferocytosis[201,202]. Apoptosis is a non-inflammatory process of cell death activated in senescent neutrophils by some cytokines and growth factors or after phagocytosis and ROS production in inflammatory conditions[203]. Moreover, neutrophils can undergo cell death also through necroptosis, necrosis, pyroptosis, NETosis and autophagy[203]. It is important to underline that some of the neutrophil death pathway such as NETosis and necroptosis, could induce a pro-inflammatory environment by releasing, their toxic content[194]. The process of phagocytosis of apoptotic neutrophils usually initiates with exposure of the phospholipid phosphatidylserine (PS) on neutrophil surface, that represents an "eat me" signal for the resident macrophages. Then, an expanding repertoire of "eat me" and "find me" (ATP, UTP and others) signals are released or exposed

by dying or activated neutrophils[140]. Finally, PS exposure can promote their phagocytosis, which is facilitated by the PS binding protein MFG-E8 (Milk fat globule-EGF factor 8 protein) and Annexin 1[203].

3.2.6 Fresh vs aged neutrophils

In physiological conditions, neutrophils undergo, with the passing of the time, to the aging process which is associated with phenotypical changes compared to fresh neutrophils released from BM (**Figure 8**). Among these changes, the increase of CXCR4 membrane expression, both in human and mice, is the most relevant factor for aged neutrophil migratory processes[204]: CXCL12 levels in the BM follow a circadian rhythm and neutrophils are cleared by this organ from the blood when CXCL12 levels reach the peak[205].

In a pathological condition, it has been shown as neutrophils with characteristic features of aged neutrophils, can reversely migrate to selectively reach the BM in a CXCR4-dependent way[190]. Therefore, it appears that the increased CXCR4 expression on aged neutrophils is the master regulator of their trafficking both in homeostatic and in pathological conditions.

Together with CXCR4 increased expression, aged neutrophils show a downregulation of CXCR2, the receptor that promote the release of neutrophils into the circulation and their migration into inflammatory sites[141]. These observations might suggest that aged neutrophils respond less efficiently to inflammatory signals; however, it has been shown that aged neutrophils are competent or even superior mediators of inflammation compared to fresh neutrophils[206].

Aged neutrophils also present reduced levels of CD62L (also known as L-selectin); aged neutrophils, identified as $CD62L^{low}$ $CXCR4^{high}$, displayed circadian oscillations over time and they almost completely disappeared from the circulation in the evening, coinciding with the beginning of the active phase of the animal[141]. Aged neutrophils also express high levels of the integrin subunits CD11b and CD49d, suggesting a more efficient adhering capability to the inflamed endothelium[141]. Further, elevated expression of TLR4, ICAM-1, CD11c, CD24, and CD45 were reported, together with a reduction of surface LY6C/G expression[206].

At the same time of increased expression of “eat me” and “find me” signals, the exposure of the “don’t eat me” molecule CD47 is slightly reduced in aged neutrophils, enhancing the possibility of recognition and phagocytosis by macrophages[140,141].

Together with surface receptors expression modifications, also morphological and functional alterations occur in aged neutrophils; they are smaller, less granular and characterized by the presence of a multilobullar nucleus[205], suggesting a spontaneous propension of degranulation over the time. In addition, aging predisposes neutrophils to overactivation also increasing NETs formation[206]. It seems that all these changes in aged neutrophils precede apoptosis, although they preserve cellular integrity longer than other cell types[141]. In fact, it has been observed increased apoptosis markers on neutrophil surface such as PS and Annexin V[141,184]. A hypothesis behind this observation is that delayed death would give neutrophils time to reach areas of phagocytosis, thus minimizing the potential release of their toxic content[141].

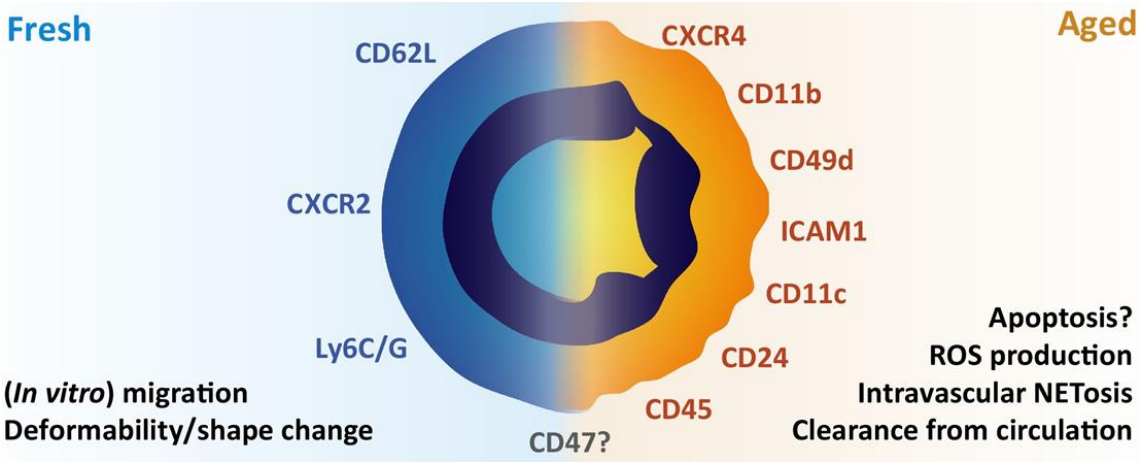

Figure 8: Some of the phenotypical and functional changes that occur during neutrophils aging
Adapted from Adrover et al., Trends in Immunology, 2016

3.3 Role of neutrophils in pathology

3.3.1 Cancer, Inflammation and Autoimmunity

Neutrophils are important players in innate immune surveillance and resolution of infections; however, they can be also involved in supporting diseases progression, such as in cancer, inflammation and autoimmunity conditions.

Tumor cells and tumour-infiltrating leukocytes can present an altered production and release of chemokines and other molecules that regulate neutrophil trafficking and functions, inducing often a massive recruitment of neutrophils into tumor microenvironment. The presence of TAN in the malignant mass may have opposing effects depending on the signals they receive from the environment: TGFβ and G-CSF make TANs pro-tumoral, while IFNβ makes them tumor-inhibiting[180]. In particular, pro-tumoral activity is attributed to a particular population of pathologically activated neutrophils called PMN myeloid-derived suppressor cells (PMN-MDSCs): their activity consists in suppressing the functions of T lymphocytes, B lymphocytes and natural killer (NK) promoting tumor escape and progression[207]. For this reason, their presence in cancer patients is associated with poor prognosis and outcome[208]. PMN-MDSCs are characterized by the expression of activation markers (CD54/ICAM-1, CD63, CD274/PD-L1), chemokine receptors (CXCR2, CXCR4) and functional markers such as ARG1 and Lox-1[209]. Recently, this population has been better characterized by single-cell RNA-seq analysis showing the existence of a subset of activated PMN-MDSCs expressing, among other markers, CCRL2[207]. Finally, it has been also demonstrated that neutrophils influence metastasis formation promoting cancer cell migration by TNF and entrapment of circulating cancer cells by NET release[180].

Likewise, neutrophils are emerging as important determinants in chronic inflammation. One example is COPD, a disease characterized by pathological limitation of airflow in the airway, mostly caused by tobacco smoking[174]. In COPD, the number of neutrophils is increased in the bronchoalveolar lavage, sputum, bronchial glands and airway smooth muscle. Neutrophils can release oxidants and proteinases that favour a progressive destruction of the pulmonary parenchyma and stimulate the secretion of mucus which determines airflow limitation[210]. Furthermore, external agents such as tobacco smoking stimulate the secretion of IL-8 and IFN-α, which, in turn, contribute to neutrophilic inflammation, immunosuppression and recurrent exacerbations of the disease[210].

Neutrophil recruitment in the liver is involved also in the pathological progression of chronic inflammatory-dependent disease as NAFLD, ALD, Viral Hepatitis, hepatocellular carcinoma (HCC), Liver Ischemia/Reperfusion (I/R) injury and

others[81-84]. In particular, neutrophil accumulation into the liver is positively correlated with bad outcome in HCC patients [84].

In some cases, neutrophils are involved also in the pathogenesis of autoimmune diseases, since antibodies against neutrophil-related antigens are found in the circulation of patients. For instance, in patients affected by systemic lupus erythematosus, a multiorgan autoimmune disease, anti-nuclear antibodies and NET-specific antibodies are often found. Also, the presence of anti-neutrophil cytoplasmic antibodies is evident in small-vessel vasculitis patients[174].

Therefore, it is not surprising that the blood neutrophil to lymphocyte ratio (NLR) has been recently proposed as a predictor of the onset, progression, and prognosis of several chronic inflammatory diseases, autoimmune diseases and cancer[210,211].

3.3.2 Aged neutrophils in pathology

As reported in paragraph 3.2.6 of the introduction, aged neutrophils in homeostasis represent a highly activated neutrophils subset that increases in some chronic inflammatory models, including sickle cell disease[198]. Since part of their functionality is to enhance the defence against invading pathogens, aged neutrophils result increased in gut microbiome-associated inflammatory states[196,206].

Moreover, during acute ischemic stroke (IS), neutrophils present an aged and hyperactivated phenotype including lower L-selectin expression and higher CD11b expression at the cell surface, increased ROS and elastase production. IS patients also have higher percentages of senescent neutrophil subset (identified as $CXCR4^{high}/CD62L^{low}$) and neutrophils with a rTEM phenotype. Importantly, these neutrophil populations are associated with the clinical severity of the stroke, highlighting a new potential therapeutic approach by rebalancing the ratio of senescent neutrophils[212].

Aged neutrophils express several tumor-promoting factors, including NETs, Mac-1, ROS, vascular endothelial growth factors, and MMP-9, all previously shown to enhance successful metastatic seeding, for example in the liver[198,213]. For instance, NETs promote cancer metastasis, through microenvironment proteolytic remodelling that favours tumor cells adhesion, proliferation, migration, and invasion. NETs have also been shown to physically protect tumor cells from

T cell or NK cell-mediated toxicity, favouring tumor cells escape from immunesurveillance[214]. Thus, the CXCR4^{hi}CD62L^{lo} aged neutrophils that accumulate in metastatic tumor, result to contribute to the pro-tumorigenic TAN population and are the dominant mature neutrophil subset that drive inflammation and promote metastasis[198].

Recently, Peng et al. show that, in experimental metastatic melanoma and breast cancer models, aged neutrophils preferentially accumulate in the environment and more robustly promote tumor migration and metastasis compared to naïve neutrophils[198]. In fact, it is possible to enhance metastatic processes to the liver[198] with adoptive transfer of aged neutrophils in tumor-bearing mice.

Thus, in order to reduce cancer metastasis, targeting aged neutrophils and better controlling their migration can represent new potential therapeutic approaches that need to be further and deeply investigated.

AIM of the THESIS

CCRL2 is a 7-transmembrane domain receptor that shows structural similarities and sequence homology to the members of the atypical chemokine receptors (ACKRs) family. Like ACKRs, CCRL2 does not induce any chemotactic response or G protein-mediated intracellular signaling[104]. However, CCRL2 behaviour diverges from ACKRs because CCRL2 cannot perform scavenging activity, internalization or beta-arrestin coupling[129]. In addition, CCRL2 doesn't bind chemokines. The only ligand confirmed until now is the chemotactic protein, chemerin, a ligand shared with the G protein-coupled, chemotactic receptor CMKLR1[104].

ACKRs have been described to be able to modulate chemokine receptors functions also by heterodimerization[1]. A paper recently published by our group shows that also CCRL2 can undergo heterodimerization with the main neutrophil chemotactic receptor CXCR2[115]. The CCRL2/CXCR2 heterocomplex showed functional relevance, since CCRL2 was demonstrated to be required for CXCR2-dependent neutrophil recruitment and tissue damage[115].

Unpublished data, obtained by our research group in collaboration with Prof. Mario Mellado from Centro Nacional de Biotecnología of Madrid, have shown by Förster Resonance Energy Transfer (FRET) analysis, the possible heterodimeric interactions of CCRL2 as a more general mechanism of regulation of conventional chemokine receptors. Indeed, CCRL2 is able to form heterocomplexes also with the chemokine receptors CCR7 and CXCR4. In particular this thesis project is focused on the identification of a possible biological role of the heterodimerization between CCRL2 and CXCR4. CXCR4 is a receptor that plays a key role in the retention of immature neutrophils in the BM and during aged neutrophil clearance[215].

The first aim of this project was to identify the optimal *in vitro* experimental condition in which CCRL2 can be co-expressed with CXCR4 on cell surface of murine neutrophils, recapitulating a biological state compatible with a possible heterodimerization of the two receptors. Thus, the hypothesis that CCRL2 could affect CXCR4 intracellular signaling and chemotactic behaviour was investigated by *in vitro* experiment using murine neutrophils expressing both the receptors.

The second purpose of this work was to understand the functional role of CCRL2/CXCR4 heterodimerization *in vivo,* recapitulating the neutrophil clearance process through adoptive transfer experiments using selective or not selective inhibitory molecules to better define the precise role of the chemokine receptors of interest. In particular, the attention was focused on neutrophil clearance by BM and liver. Finally, the potential involvement of CCRL2 in the modulation of efferocytosis of aged neutrophils by tissue macrophages was investigated.

MATERIAL and METHODS

1. Animals

Wild type (WT) and CCRL2 knockout (KO) C57BL/6J mice were used for experiments. Mice were aged (8-12 weeks) and sex matched. Procedures involving animals handling were conformed to institutional guidelines in compliance with national (D.L. N.26, 4-3-2014) and international (Directive 2010/63/EU revising Directive 86/609/EEC, September 22, 2010) law and policies.

2. Murine neutrophil characterization *in vitro*

2.1 Neutrophil isolation from murine bone marrow (BM)

WT and CCRL2 KO mice were sacrificed by cervical dislocation and both femur and tibia were harvested and kept in sterile PBS. In sterile condition, BM was flushed using for each bone 1 mL of RPMI added with glutamine (g), Penicillin/Streptomycin solution (P/S), 10% of fetal bovine serum (FBS) and 2 mM EDTA (ethylenediamine tetraacetic acid). Red blood cells lysis was performed trough hypotonic solutions: cells were resuspended with 3 mL of 0,2% NaCl solution in order to create a hypotonic condition leading to red blood cells lysis; after 30 seconds 7 mL of 1,2% NaCl solution were added to stop lysing process restoring a homeostatic condition. After centrifugation, cells were counted and processed for negative selection of neutrophils, according to manufacturer's protocol (Neutrophil Isolation Kit, mouse; 130-097-658; Miltenyi Biotec). The purity of neutrophil population so isolated, identified as $CD11b^+$ and $Ly6G^+$, was routinely between 85% and 95% as shown in **Figure 9**.

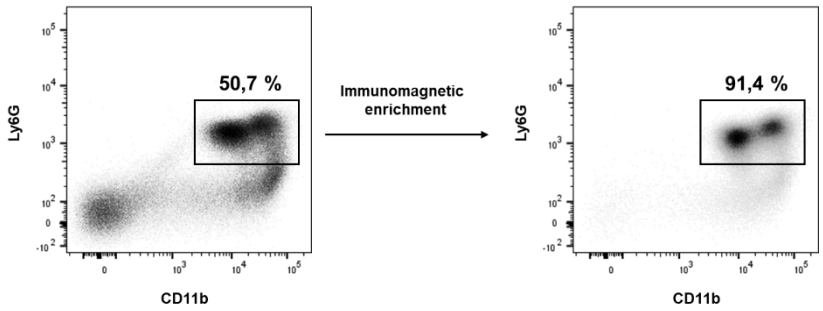

Figure 9: Cytofluorimetric plot represented an example of percentages of neutrophils, identified as $Ly6G^+$ and $CD11b^+$, before and after the immunomagnetic enrichment.

2.2 Neutrophil culture and stimulation

Isolated neutrophils were resuspended in RPMI + g + P/S + 10% FBS and put in culture at the concentration of 1 mln/mL with different combinations of stimuli (or with equal volume of PBS, recapitulating a resting condition): LPS (100 ng/mL), TNF-α (20 ng/mL) and mIFNγ (50 ng/mL)[115]. Cells were harvested with cold PBS after different time points, from 6 to 30 hours.

2.3 Antibodies staining for flow cytometry

Primary murine cells were CD16/32 (2.4G2) blocked for 15 minutes at 4°C and stained with antibodies showed in **Table 1** for other 15 minutes at 4°C.

Antigen	Fluorophore conjugation	Dilution	Brand	CAT
CD11b	PE-Vio770	1:500	Miltenyi Biotec	130-113-246
CD11b	VioBright	1:50	Miltenyi Biotec	130-113-243
Ly6G	FITC	1:40	Miltenyi Biotec	130-120-820
CXCR2	PE-Vio770	1:50	Miltenyi Biotec	130-115-636
CD62L	VioBlue	1:100	Miltenyi Biotec	130-112-841
CXCR4	APC	1:50	ThermoFisher Scientific	17-9991-80
CCRL2	PE	1:40	BD	564946

Table 1: Directly conjugated antibodies used for *in vitro* murine neutrophil phenotype characterization.

After washing, cells were incubated with LIVE/DEAD Fixable Dead Cell Stain Violet or near IR (Thermo Fisher Scientific) for 10 minutes at room temperature in the dark. Cells were washed, fixed with PFA 1% for 10 minutes at room temperature, acquired with MACSQuant 10 or 16 (Miltenyi Biotec) and analysed by FlowJo software.

2.4 Neutrophil apoptosis staining

After isolation, culture and stimulation for different time points, neutrophils were incubated with annexin V and SYTOX AADvanced Dead Cell Stain for 30 minutes at room temperature as indicated by datasheet (Pacific Blue Annexin V/SYTOX

AADvanced Apoptosis Kit; Invitrogen). Samples were acquired with MACSQuant 10 or 16 and analysed by FlowJo software. Apoptotic cells were identified as $AnnV^+$ and AAD^-.

3. Study of neutrophil migration and intracellular signaling

3.1 Boyden chamber chemotaxis assay

Neutrophil migration was evaluated using a 48-well chemotaxis chamber (Neuroprobe) and polycarbonate filters (5-µm pore size; Neuroprobe). Neutrophils (250.000) were charged and incubated for 50 minutes. Results are expressed as number of migrated cells counted in an average of 5 high-power fields (100X) in response of different concentration of CXCL12 (3-30-300 ng/mL) or in basal condition (without the addition of the chemotactic stimuli).

3.2 ERK1/2 and small GTPases intracellular staining

Neutrophils were incubated for different time points (1-2-5 minutes) with different concentration of CXCL12 (100-300-1000 ng/mL), fixed with PFA 4% and permeabilized. Then, cells were incubated with the antibodies shown in **Table 2**. Since antibodies anti-active RhoA-GTP and Rac1-GTP are not conjugated with a fluorophore, they needed, before staining, a direct conjugation through Zenon Alexa Fluor 647 labelling kit (Invitrogen, Z25208) following manufacturer's instructions. After washing, cells were incubated with LIVE/DEAD Fixable Dead Cell Stain Violet (Thermo Fisher Scientific) for 10 minutes at room temperature. Cells were acquired with MACSQuant 10 or 16 and analysed by FlowJo software. ERK1/2 phosphorylation and Rho/Rac1 activation were evaluated in $CD11b^+/Ly6G^+$ gated population, as the increase of fluorescence mean compared to cells not incubated with CXCL12.

Antigen	Fluorophore conjugation	Dilution	Brand	CAT
CD11b	PE-Vio770	1:500	Miltenyi Biotec	130-113-246
Ly6G	FITC	1:40	Miltenyi Biotec	130-120-820
ERK1/2	Alexa Fluor 647	1:20	BD	612593
Active RhoA -GTP	Unconjugated	1:20	NewEast Biosciences	26904
Active Rac1-GTP	Unconjugated	1:20	NewEast Biosciences	26903

Table 2: Directly conjugated or unconjugated antibodies used for ERK1/2 phosphorylation and RhoA/Rac1 activation intracellular stainings.

3.3 Actin polymerization assay

Neutrophils were incubated for different time points (5-10-20 seconds) with 100 ng/mL of CXCL12, fixed with PFA 4% and permeabilized. Then, cells were incubated for 15 minutes at 4°C with CD11b and Ly6G antibodies (Miltenyi Biotec) and Alexa Fluor 488 conjugated Phalloidin (A12379, Invitrogen). After washing, cells were incubated with LIVE/DEAD Fixable Dead Cell Stain Violet (Thermo Fisher Scientific) for 10 minutes at room temperature. Cells were acquired with MACSQuant 10 or 16 and analysed by FlowJo software. Actin polymerization was evaluated in $CD11b^+/Ly6G^+$ gated population, as increased of FITC fluorescent mean compared to cells not incubated with CXCL12.

4. *In vivo* adoptive transfer experiment to study neutrophil homing to clearance organs

WT and CCRL2 KO neutrophils were isolated from BM and cultured as described in points 2.1-2.2. After 30 hours, cells were collected and differentially stained with 1 μM of CellTrace CFSE/Violet Cell Proliferation Kit (Invitrogen) and incubated for 20 minutes at room temperature, following manufacturer's protocol. Cells were washed twice and then equally mixed (1mln:1mln in 200 μL). 200 μL of the cells mix (corresponding to 2 million of cells) was retro-orbitally injected in WT recipient mice. A sample of cells mix was acquired before and after the injection at flow cytometer to take note of the proportion of the two different

genotypes, in order to normalize the results based on the ratio between the cells injected.

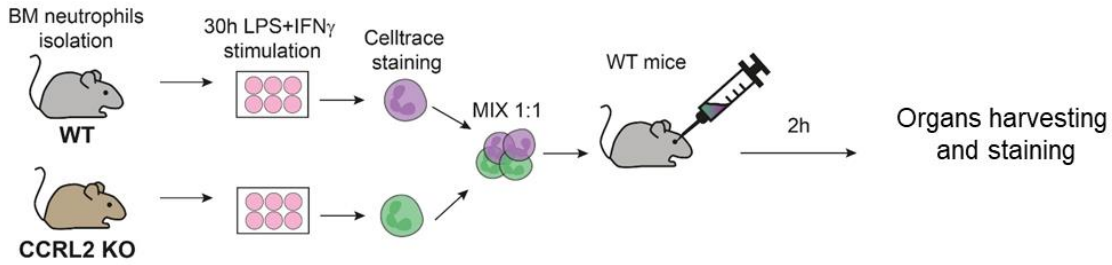

Figure 10: Scheme of competitive adoptive transfer experiments.

After 2 hours from the injection, recipient mice were sacrificed by cervical dislocation and organs (BM, liver, spleen, blood and lung) were harvested, processed and stained as following described. The schematic representation of adoptive transfer experiments is shown in **Figure 10**.
Samples were acquired with MACSQuant 10 or 16 and analysed by FlowJo software. Neutrophils injected and migrated to each organ was identified as CellTrace Violet or CFSE positive in the gate of $CD11b^+$ and $Ly6G^+$ cells.

4.1 Cells collection from BM

BM total cells were collected from WT recipient mice as described in point 2.1. In this case, the staining was performed on counted total BM cells as described in point 4.6.

4.2 Cells collection from liver

Livers from WT recipient mice were perfused from portal vein first with HBSS w/o Ca/Mg (20 mL) and then with HBSS+Ca+Mg with 0,04% of Collagenase IV (20 mL). Liver was harvested and kept in cold PBS. In sterile condition, it was gently teased in a Petri dish with HBSS+Ca+Mg with 0,02% of Collagenase IV for 20 minutes at room temperature. Digested liver was collected, smashed on a 70 μm filter and then centrifugated at 35g for 3 minutes at maximum brake in order to separate parenchymal from non-parenchymal cells. Pellet was discarded and supernatant pelleted. Pellet was resuspended in 5 mL of ACK for red blood cells

lysis for 30 sec on ice. Lysis was stopped with HBSS added with 2 mM of FBS. After filtration on a 40 μm filter, cells suspension was centrifugated, resuspended in PBS and counted before staining as following described in point 4.6.

4.3 Cells collection from spleen

Spleens from WT recipient mice were smashed over a 70 μm filter into a falcon using 5 mL of RPMI + g + P/S. Cells suspension was centrifuged at 1200 rpm for 7 minutes at 4°C and then red blood cells were lysed with 800 mL of ACK for 5 minutes on ice. Lysis was stopped with 5 mL of RPMI + g + P/S + 10%FBS, and cells were spin at 1200 g for 7 minutes, resuspended in 1 mL of PBS, counted and stained as following described in point 4.6.

4.4 Cells collection from lung

Lungs from WT recipient mice were intracardially perfused with 7 mL of physiological solution and then harvested. Lung tissue was gently teased in a Petri dish with digestion medium (1mg/ml di Collagenase IV + 0.02mg/ml Dnasi I in RPMI 1% FBS) for 30 minutes at 37°C and then smashed on a 70 μm filter. Cells suspension was centrifuged and the supernatant discarded. Pellet was resuspended, for red blood cell lysis, in 1 mL of ACK on ice; lysis was stopped with 1mL di RPMI + 5% FBS + 2 mL di FACS-EDTA buffer (PBS + 5% EDTA-TREATED FBS). Pellet was resuspended in 1mL di FACS-EDTA buffer and passed through a 70 μm filter, counted and stained as described in point 4.6.

4.5 Cells collection from blood

Blood was collected from the heart of isoflurane anesthetized recipient mice and transferred a in a tube with EDTA to avoid coagulation. Red blood cells lysis was performed with ACK, cells was resuspended in PBS and stained as following described in point 4.6.

4.6 Antibodies staining for flow cytometry

Cells suspensions from each organ were CD16/32 blocked for 15 minutes at 4°C and stained with a mix of antibodies showed in **Table 3** for other 15 minutes at 4°C.

Antigen	Fluorophore conjugation	Dilution	Brand	CAT
CD11b	PE-Vio770	1:500	Miltenyi Biotec	130-113-246
Ly6G	APC	1:50	Miltenyi Biotec	130-120-734
CD45	VioGreen	1:100	Miltenyi Biotec	130-110-665

Table 3: Directly conjugated antibodies used for the gating strategy in *in vivo* adoptive transfer experiments.

Cells were incubated with LIVE/DEAD Fixable Dead Cell Stain near IR (Thermo Fisher Scientific) for 10 minutes at room temperature, washed, fixed with PFA 1%, acquired with MACSQuant 10 or 16 and analysed by FlowJo software.

4.7 Pertussis toxin (PTX) adoptive transfer experiment (Figure 11)

After 30 hours of culture and stimulation, WT and CCRL2 KO BM-derived neutrophils were collected. One half of each neutrophil genotype was incubated for 1 hour at 37°C with PTX 1 µg/mL[199] (Sigma), while the second part was incubated as control. WT and CCRL2 KO neutrophils that were incubated with PTX were then differently stained with CellTrace and equally mixed. Same procedure was carried out for the control neutrophils. The two different cell mixes were injected in two different groups of WT recipient mice; after 2 hours, recipient mice were sacrificed and BM and liver harvested, processed and stained as shown in point 4.1, 4.2 and 4.6.

4.8 Repertaxin (RPTX) adoptive transfer experiment (Figure 11)

After 30 hours of culture and stimulation, WT and CCRL2 KO BM-derived neutrophils were collected, differently stained with CellTrace and equally mixed. Neutrophil mix was divided in two equal parts, in one of which were added 30 µg/mL[216] of RPTX (Reparixin, Sigma). Moreover, RPTX was injected i.p. (30 mg/Kg[217,218]) in one group of recipient mice 30 minutes before adoptive transfer of the cells. The control group was injected i.p. with PBS. RPTX-incubated cells were then injected i.v. in the animal group that was treated with RPTX; after 2 hours, recipient mice were sacrificed and BM and liver harvested, processed and stained as shown in point 4.1, 4.2 and 4.6.

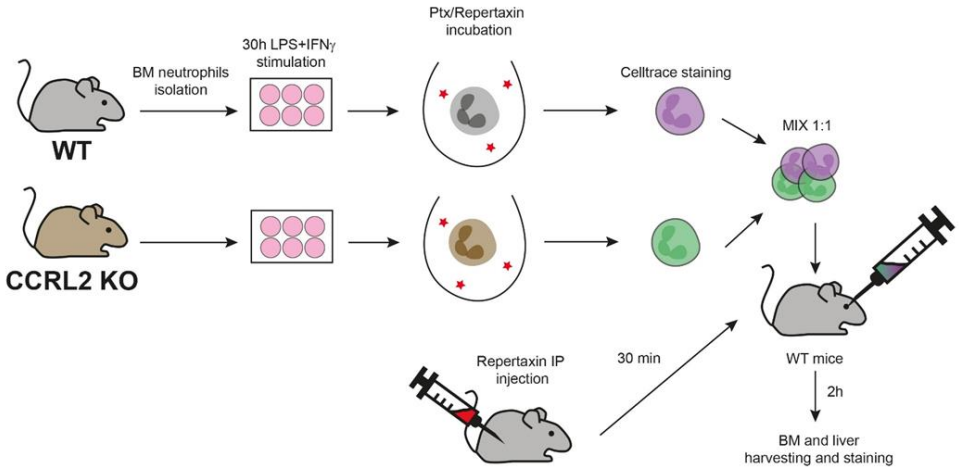

Figure 11: Schematic representation of adoptive transfer experiments performed inhibiting G coupled receptors and CXCR2 respectively with PTX and RPTX.

5. *In vivo* inflammatory stimulation

WT and CCRL2 KO mice were injected i.p. with 1 µg of LPS and 150 ng of IFNγ[219] (or equal volume of PBS, control). Mice were sacrificed after 3 hours from the stimulation, BM and liver were harvested, processed (see points 4.1 and 4.2) and stained for neutrophil characterization with the same antibodies anti-CXCR4, CCRL2, CD11b and Ly6G shown in **Table 1**.

5.1 *In vivo* BrdU labelling of endogenous neutrophils (Figure 12)

Mice were treated with a single injection i.p. of 5-bromo-29-deoxyuridine (BrdU, 1,5 mg per mouse; FITC BrdU Flow kit, BD). Three hours before sacrifice (45 hours after BrdU injection), i.p. administration of 1 µg of LPS and 150 ng of mIFNγ (or with equal volume of PBS, for control mice) was performed.

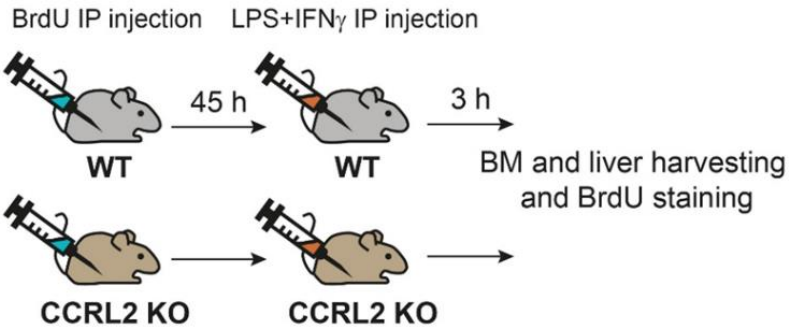

Figure 12: Schematic representation of BrdU *in vivo* experiments.

BM and liver were harvested, processed (see point 4.1 and 4.2) and stained with surface antibodies shown in **Table 4** as described in point 4.6. After surface markers staining, cells were fixed and permeabilized according to BrdU Flow kit instructions. In order to expose incorporated BrdU, cells were then incubated with 300 μg/mL of DNAse for 1 hour at 37°C. Cells were washed and finally stained with 50 μL of 1:50 diluted anti-BrdU antibody for 20 minutes at room temperature. Following washing, samples were acquired with MACSQuant 10 or 16 and analysed by FlowJo software.

Antigen	Fluorophore conjugation	Dilution	Brand	CAT
CD11b	PE-Vio770	1:500	Miltenyi Biotec	130-113-246
Ly6G	APC	1:50	Miltenyi Biotec	130-120-734
CD45	VioGreen	1:100	Miltenyi Biotec	130-110-665
BrdU	FITC	1:50	BD	559619 (CAT of the kit containing the antibody)

Table 4: Directly conjugated antibodies used for evaluate the quote of aged and not aged neutrophils in the organs of interest.

6. Study of neutrophil efferocytosis by macrophages

6.1 *In vitro* neutrophil efferocytosis by peritoneal macrophages

WT murine neutrophils were isolated, cultured and stimulated as described in point 2.1. At the end of neutrophil stimulation, peritoneal exudate cells (PEC) were collected from WT mice by peritoneal lavage with 7 mL of PBS. A quote of PEC was stained with antibodies presented in **Table 5** in order to evaluate the percentage of peritoneal macrophages in the sample. Neutrophils were stained with CellTrace 1 μM before being mixed in a proportion 1:1 with peritoneal macrophages and incubated at 37°C. After 2 hours, cells mixed were stained with antibodies shown in **Table 5** and then acquired by flow cytometer.

Antigen	Fluorophore conjugation	Dilution	Brand	CAT
CD11b	PE-Vio770	1:500	Miltenyi Biotec	130-113-246
Ly6G	APC	1:50	Miltenyi Biotec	130-120-734
CD45	VioGreen	1:100	Miltenyi Biotec	130-110-665
F4/80	PerCP-Vio700	1:50	Miltenyi Biotec	130-118-466

Table 5: Directly conjugated antibodies used for evaluate neutrophil efferocytosis by macrophages *in vitro* and *in vivo*.

The level of neutrophil efferocytosis was calculated based on the fluorescence mean of the CellTrace (used to stained neutrophils) in the gate of $CD11b^+$ and $F4/80^+$ macrophages. In order to investigate a possible role of chemerin in neutrophil engulfment by macrophages, a concentration of 200 nM of chemerin was added where indicated. Samples were acquired with MACSQuant 10 or 16 and analysed by FlowJo software.

6.2 *In vivo* adoptive transfer experiments to study neutrophil efferocytosis by tissue macrophages

Adoptive transfer experiments were carried out as described at point 4 of Methods. After 2 hours, BM and liver were harvested, processed (see point 4.1 and 4.2) and stained with antibodies of **Table 5**. Cells were washed and incubated with LIVE/DEAD Fixable Dead Cell Stain Near IR (Thermo Fisher Scientific) for 10 minutes at room temperature. Cells were washed and fixed with PFA 1% for 10 minutes at room temperature.

After washing, cells were acquired with MACSQuant 10 or 16 and analysed by FlowJo software. Neutrophils engulphed by resident macrophages were identified as CellTrace positive events in the gate $Ly6G^-$, $CD11b^+$ and $F4/80^+$ macrophages.

6.3 Gadolinium chloride ($GdCl_3$) adoptive transfer experiment

In order to selectively deplete liver Kupffer cells from recipient mice, they were injected i.p. with $GdCl_3$ (20 mg/Kg)[220] 24 hours before neutrophils adoptive transfer, as schematically described by **Figure 13**.

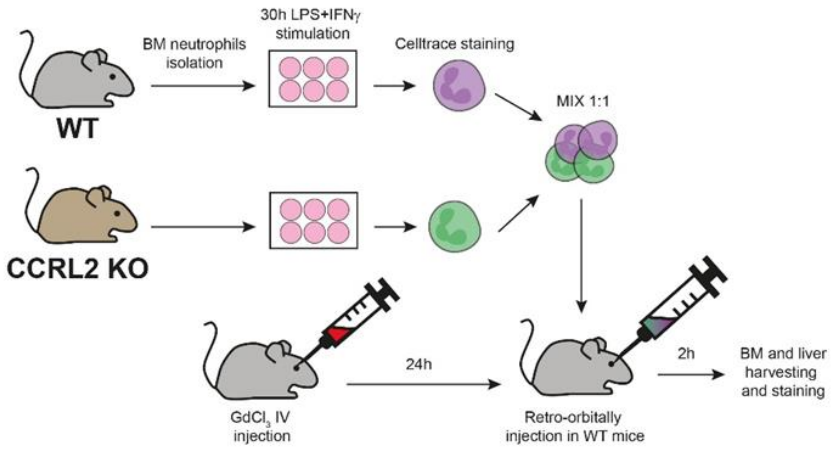

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Figure 13: Schematic representation of adoptive transfer experiments performed with $GdCl_3$ administration in order to deplete Kupffer cells in recipient mice.

After 2 hours, BM and liver were harvested, processed (see point 4.1 and 4.2) and stained with antibodies of **Table 2.** Samples were acquired with MACSQuant 10 or 16 (Miltenyi Biotec) and analysed by FlowJo software.

7. Statistical analysis

Statistical analyses were performed by using paired or unpaired Student t test and ordinary one-way ANOVA, as appropriate. Results were analysed by GraphPad PRISM 7.0 software.

RESULTS

1. CCRL2 and CXCR4 can heterodimerize both in mice and in human

My thesis project started from data obtained by a collaboration of our research group with the laboratory of Prof. Mario Mellado from Centro Nacional de Biotecnología of Madrid, as they have a strong experience in Förster Resonance Energy Transfer (FRET) technique. FRET technique is based on the transfer of non-radiative energy from a donor molecule to an acceptor molecule that are in close vicinity (<100 Å) and it is performed in living cells, requiring transfection of the receptors coupled to acceptor and donor molecules[147]. Through FRET experiments, it is possible to assess whether two receptors can interact on cell membrane and form heterocomplexes.

Since our research group recently demonstrated that CCRL2 can form heterodimers with CXCR2 affecting its functionality on neutrophil surface[115], the aim of this collaboration was to investigate the possibility of a heterodimerization between CCRL2 and other chemokine receptors. HEK293 cells were co-transfected with murine CCRL2-CFP (donor) and murine CXCR4- or CCR7-YFP (acceptor). FRET efficiency was positively detected by photobleaching method both between CCRL2-CXCR4 and CCRL2-CCR7: as shown in **Figure 14A** and **14B**, FRET efficiency for CXCR4 and CCR7 was respectively almost 4% and 6%, while was undetectable for histamine 3 receptor (H3R) that was used as a negative control.

Finally, FRET saturation curves were performed using HEK293 cells co-transfected with human CCRL2, CXCR4 and CCR7. Positive FRET was observed even between hCCRL2-hCXCR4 and hCCRL2-hCCR7 (**Figure 14C**).

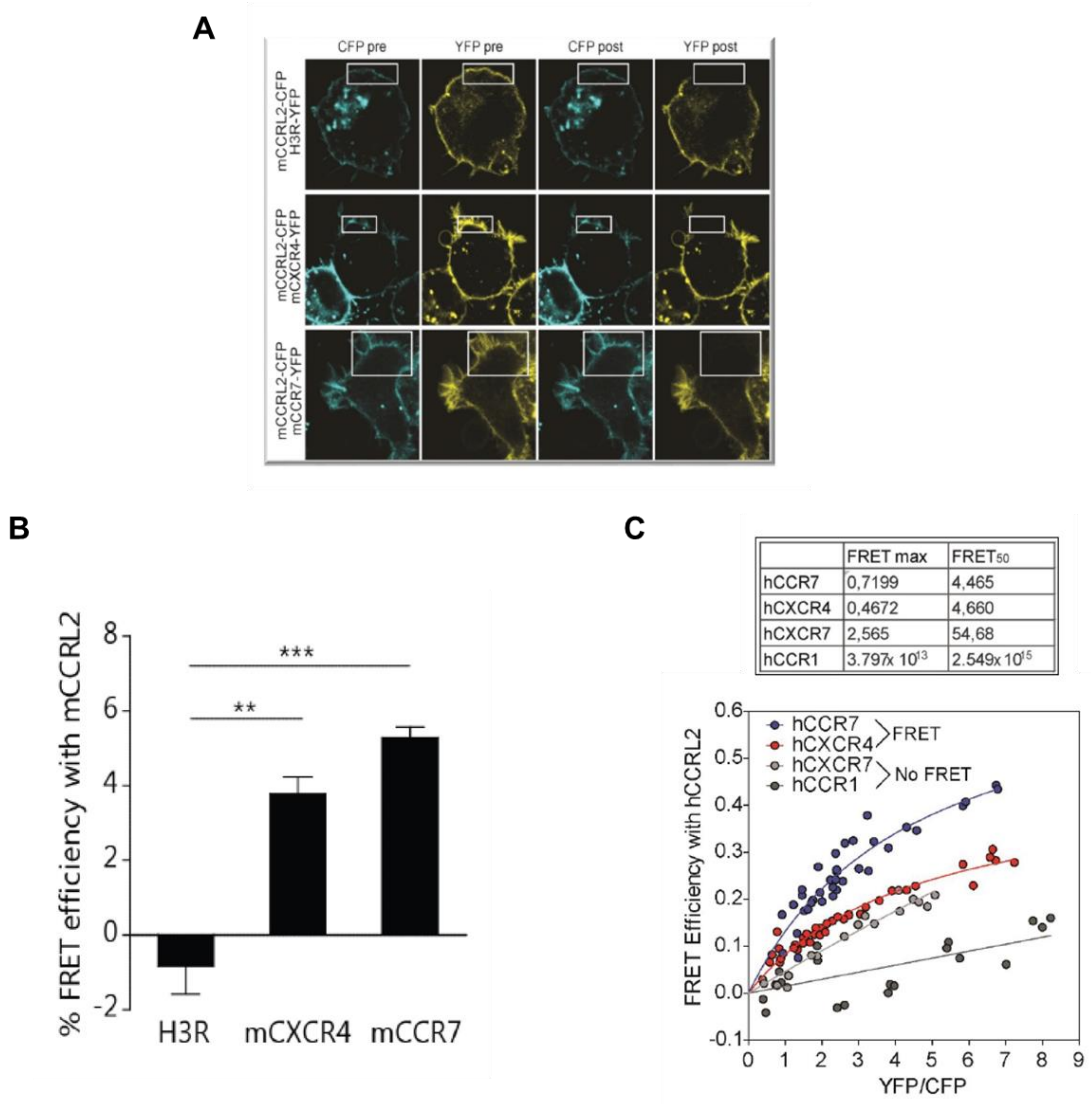

Figure 14: **CCRL2-CXCR4 form heterodimers on human and mouse neutrophils.**

(**A**) FRET analysis by acceptor photobleaching of mCCRL2 co-transfected with hH3R, mCXCR4 or mCCR7 in HEK293T cells. Shown are representative images of CFP and YFP fluorescence before photobleaching (CFP Pre, YFP Pre) and after photobleaching (CFP Post, YFP Post) and the photobleached areas is indicated by a white outline. (**B**) By using ImageJ 1.43 software, FRET efficiency was determined on a pixel-by-pixel basis and calculated in percent as [(ICFPpost – ICFPpre)/ICFPpost] x 100, where ICFPpre and ICFPpost are the background-corrected CFP fluorescence intensities before and after YFP photobleaching, respectively. Median FRET efficiency was calculated from about 20 images from each experiment. Data are expressed as the median ± interquartile range of 2-6 independent experiments. *p < 0.05; **p < 0.01; ns, not significative by Student's t-test. (**C**) FRET saturation curves for hCCRL2 co-transfected with hCCR7, hCXCR4, hCCR1, or hCXCR7 in HEK293T cells. Curves were obtained by using cells transiently co-transfected with either the vector encoding CCRL2-cyan fluorescent protein (CFP) and increasing amounts of the second yellow fluorescent protein (YFP)-conjugated plasmid. FRET50 and FRETmax value were calculated by using a nonlinear regression equation for a single binding site model (GraphPad Prism).

2. CCRL2 and CXCR4 are co-expressed by murine neutrophils in a condition of inflammatory aging

Based on our previous report showing the CCRL2-CXCR2 heterodimerization occurring during inflammatory recruitment[115] and considering that both CXCR4 and CCRL2 can be expressed by neutrophils[115,204], we decided to investigate the biological role of CXCR4-CCRL2 heterodimerization on murine primary neutrophils isolated from BM. CXCR4 is known to be upregulated on aged neutrophil surface, where it plays a key role in their clearance by BM[215]. In order to identify the experimental condition where both the receptors CXCR4 and CCRL2 can be co-expressed on primary neutrophil surface, WT BM-derived neutrophils were isolated and expression of the two receptors of interest was studied during a process of *in vitro* aging with or without pro-inflammatory stimuli (see point 2 of Material and Methods). At different time points, cells were collected and stained for the two receptors of interest. Neutrophils that got aged in resting condition**,** progressively increased CXCR4 expression, reaching almost 80% of positivity after 30 hours and were indicated as T30 REST neutrophils. However, just 3-4% of T30 REST neutrophils expressed CCRL2 (**Figure 15A**, left). CCRL2 is a receptor that is reported to be upregulated under pro-inflammatory stimulation; in particular, the synergistic activities of LPS, IFNγ and TNFα (called MIX), have been shown to strongly upregulate CCRL2 expression on endothelial cells and neutrophils[115,123]. Based on this observation, we cultured BM-derived neutrophils with MIX and studied CCRL2 expression on their membrane at different time points as shown in **Figure 15A** (middle panel). We found that CCRL2 was upregulated in a time-dependent way, reaching 80% of positivity after 30 hours. However, since this proinflammatory condition did not allow a simultaneous upregulation of CXCR4 (just the 20% of neutrophils were CXCR4+ after 30 hours), suggesting a not full completed aging process (**Figure 15A**, middle), we incubate neutrophils with a different combination of pro-inflammatory stimuli. When stimulated with LPS and IFNγ combination, neutrophils upregulated the expression of both receptors and, after 30 hours from the beginning of stimulation, around 50-60% of them co-expressed CXCR4 and CCRL2 (**Figure 15A**, right). Neutrophils cultured and stimulated for 30 hours with LPS and IFNγ, were indicated as T30 L+γ neutrophils and represented the model that was used

in this thesis to study the possible role of CCRL2 in the modulation of CXCR4 signaling.

In order to evaluate the biological significance of CCRL2 and CXCR4 co-expression, BM-derived freshly isolated neutrophils (T0 neutrophils) were collected also from CCRL2 KO mice and cultured as T30 REST and T30 L+γ neutrophils. At basal condition, CXCR4 expression was significantly different between WT and CCRL2 KO T0 neutrophils, however, this difference was not maintained neither in T30 REST nor in T30 L+γ neutrophils model (**Figure 15B**). The ability of CCRL2 to modulate expression of other receptors has been already reported in literature, as CXCR2 on fresh neutrophils[115] and TLR4 on macrophages[172].

Martin et al[204]. demonstrated that different CXCR4 expression levels mirror a different BM clearance behaviour *in vivo*. To gain insight into this mechanism, WT T30 REST and T30 L+γ neutrophils were stained with fluorescent dyes, adoptively transferred in different groups of WT recipient mice and their migration to BM was assessed. **Figure 15C** shows that T30 REST neutrophils were cleared up from circulation by BM in a faster and more abundant way; otherwise, the process of clearance of T30 L+γ neutrophils was slower and their persistence in blood circulation was prolonged, coherently with the CXCR4 lower expression on these cells. In fact, even after 2 hours from the injections, T30 L+γ neutrophils are still 70% less cleared by BM compared to T30 REST neutrophils.

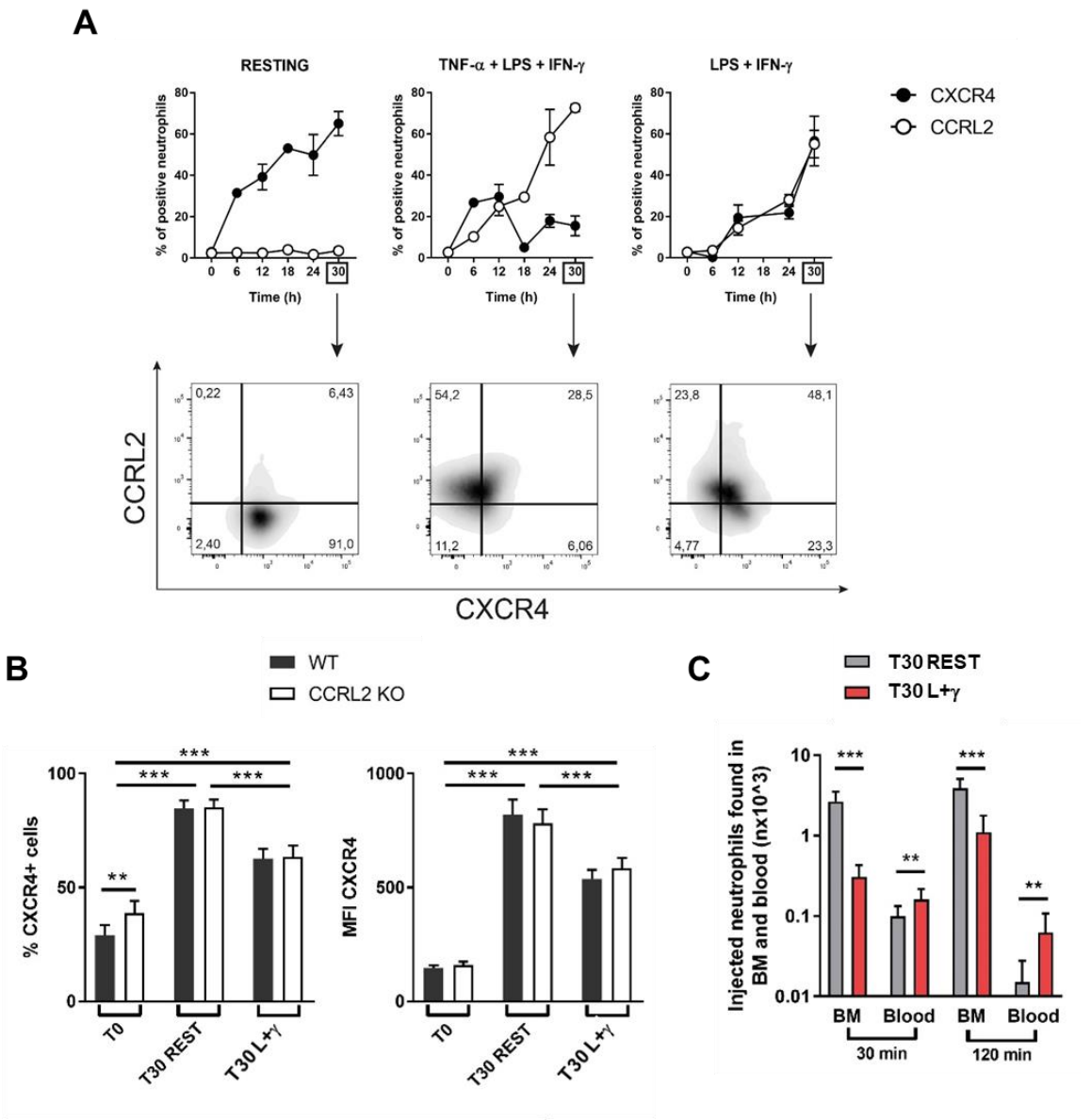

Figure 15: **CXCR4 and CCRL2 are co-expressed on murine primary neutrophils in a model of stimulated senescence.** (**A**) Cytofluorimetric profiles of CXCR4 (**black** circles) and CCRL2 (white circles) expression on BM-derived neutrophils purified from WT mice and stimulated with or without IFNγ (50 ng/mL), LPS (100 ng/mL) and TNFα (20 ng/mL) in different combinations and for different time points. (**B**) Variations in CXCR4 expression (left: % of $CXCR4^+$ neutrophils; right: CXCR4 MFI) on T0 neutrophils, T30 REST neutrophils and T30 L+γ neutrophils from both WT (**black** bars) and CCRL2 KO (white bars) mice. ** $P < 0.01$; *** $P < 0.001$ by unpaired Student t test. (**C**) Number of WT T30 REST (**grey** bars) and T30 L+γ (**red** bars) neutrophils that homes to BM or that remains in blood circulation, after being injected in WT recipient mice. ** $P < 0.01$; *** $P < 0.001$ by unpaired Student t test.

Based on these observations, we decided to better define whether T30 L+γ neutrophils really expressed an “aged” phenotype, deeply investigating their phenotypic characteristics. First, as illustrated in **Figure 16A** and **16B**, these neutrophils showed, by flow cytometry, a statistically significant decrease of dimensional and granular parameters compared to T0 neutrophils. As described[141], these changes are due to a major propension to degranulation of aged neutrophils. Secondly, aged neutrophils cell membrane is enriched in apoptotic markers such as Annexin V[141,184] (AnnV), whose expression is upregulated over time also in our model of T30 L+γ neutrophils, starting from 0% of T0 neutrophils to 40% of T30 L+γ neutrophils (**Figure 16C**). Regarding apoptotic assay, it is important to stress that the percentage of alive and apoptotic neutrophils during aging and stimulating process, were not different between WT and CCRL2 KO (**Figure 16C**), thus suggesting that our results were not influenced by the apoptotic process.

Aged neutrophils are often identified as $CXCR4^{hi}$ and $CD62L^{lo}$[196,206]: likewise, we observed a decrease in the expression of CD62L on T30 L+γ neutrophils compared to T0 neutrophils (**Figure 16F**) and, at the same time, an enrichment of events in $CXCR4^{hi}CD62L^{lo}$ gate (**Figure 16D**). Finally, also CXCR2 membrane expression is described to be reduced on aged neutrophils[141]. This is confirmed also in our model of T30 L+γ neutrophils: we found a slight reduction in CXCR2 expression as Mean Fluorescent Intensity (MFI) compared to T0 neutrophils (**Figure 16E-F**).

Based on these observations, our model of T30 L+γ neutrophils seems to recapitulate the phenotype of aged neutrophils.

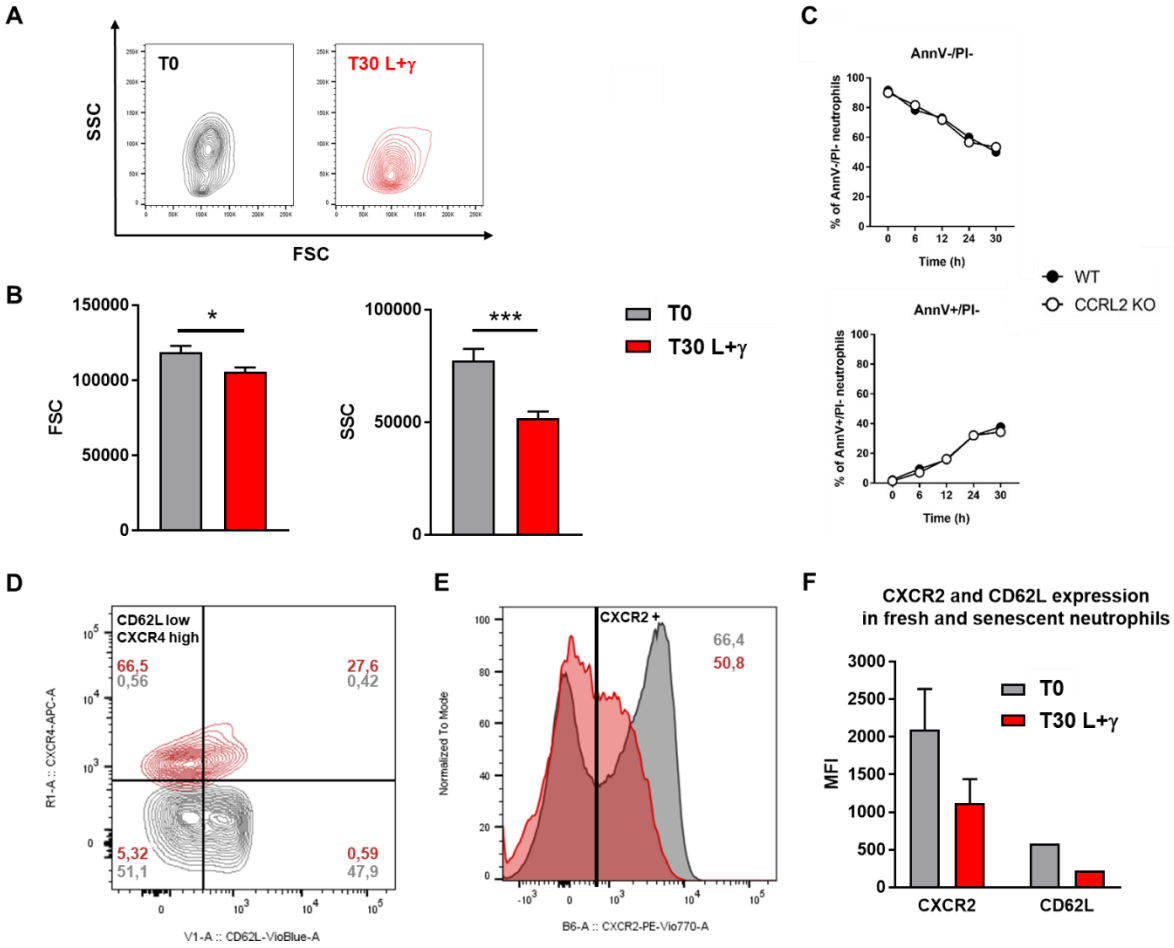

Figure 16: **T30 L+γ neutrophils present an aged phenotype.** (**A**) Plot showing a decreased of size and granularity parameters of our model of T30 L+γ neutrophils compared to T0 neutrophils. (**B**) Quantification of dimensional (FSC) and granular (SSC) parameters, expressed in linear scale, of T0 and T30 L+γ neutrophils. * $P < 0.05$; *** $P < 0.001$ by unpaired Student t test. (**C**) Vitality staining with Annexin V and PI of WT (**black** circles) and CCRL2 KO (white circles) neutrophils in different time points during aging and LPS+mIFNγ stimulating process. (**D**) Representative cytofluorimetric profiles of $CXCR4^{hi}CD62L^{lo}$ gate of T0 neutrophils (**grey**) and T30 L+γ neutrophils (**red**). (**E**) Representative variation in CXCR2 expression between T0 (**grey**) and T30 L+γ neutrophils (**red**) expressed as % of positive cells. (**F**) Average variations in CD62L and CXCR2 expression between T0 (**grey**) and T30 L+γ neutrophils (**red**), expressed as MFI.

3. CCRL2 inhibits CXCR4 intracellular signaling and migration in response to CXCL12 in a dose-dependent way

WT and CCRL2 KO T30 L+γ neutrophils, were used to perform *in vitro* functional assays in order to assess whether the presence or absence of CCRL2 could affect CXCR4 signaling in response to the ligand CXCL12. For this reason, T30 L+γ WT and CCRL2 KO neutrophils were incubated at different time points with 100 ng/mL of CXCL12 and then various mediators of CXCR4 intracellular signaling were evaluated by flow cytometry (see points 3.2 and 3.3 of Material and Methods). After 1 minute of incubation with CXCL12, RhoA activation resulted to be 20% higher in CCRL2 KO T30 L+γ neutrophils compared to WT (**Figure 17A**, left); otherwise, no differences were observed in Rac1 activation between WT and CCRL2 KO T30 L+γ neutrophils (**Figure 17A**, right). In addition, actin polymerization in response to CXCL12 was investigated through Phalloidin AF488 conjugated staining: CCRL2 KO T30 L+γ neutrophils presented a 5% increased and faster actin polymerization compared to WT (**Figure 17B**).

Then, dose dependent ERK1/2 phosphorylation was performed in CCRL2 KO T30 L+γ neutrophils compared to WT, after incubation with different doses of CXCL12. ERK1/2 phosphorylation resulted 50% higher in CCRL2 KO neutrophils compared to WT at the lowest dose tested, but the differences decrease as CXCL12 concentration increase (**Figure 17C**): at the highest CXCL12 dose tested the differences between WT and CCRL2 KO were annulled. Likewise, neutrophil chemotaxis evaluated by Boyden chambers, showed a trend to an increased chemotactic response to CXCL12 by T30 L+γ CCRL2 KO neutrophils compared to WT. However, the difference reached statistical significance only at the lowest CXCL12 dose tested. No differences were found in the chemotactic response of T30 REST neutrophils (**Figure 17D**).

The idea of a more relevant role for CCRL2 at low concentration of CXCL12, is coherent with our preliminary FRET data showing that CXCL12 was able to negatively influence the efficiency of heterodimerization between CCRL2 and CXCR4 (**Figure 17E**). We can speculate that, the heterocomplex between CCRL2 and CXCR4 are not stable in presence of high concentration of CXCL12 when CXCR4 homodimers formation seems to be preferential.

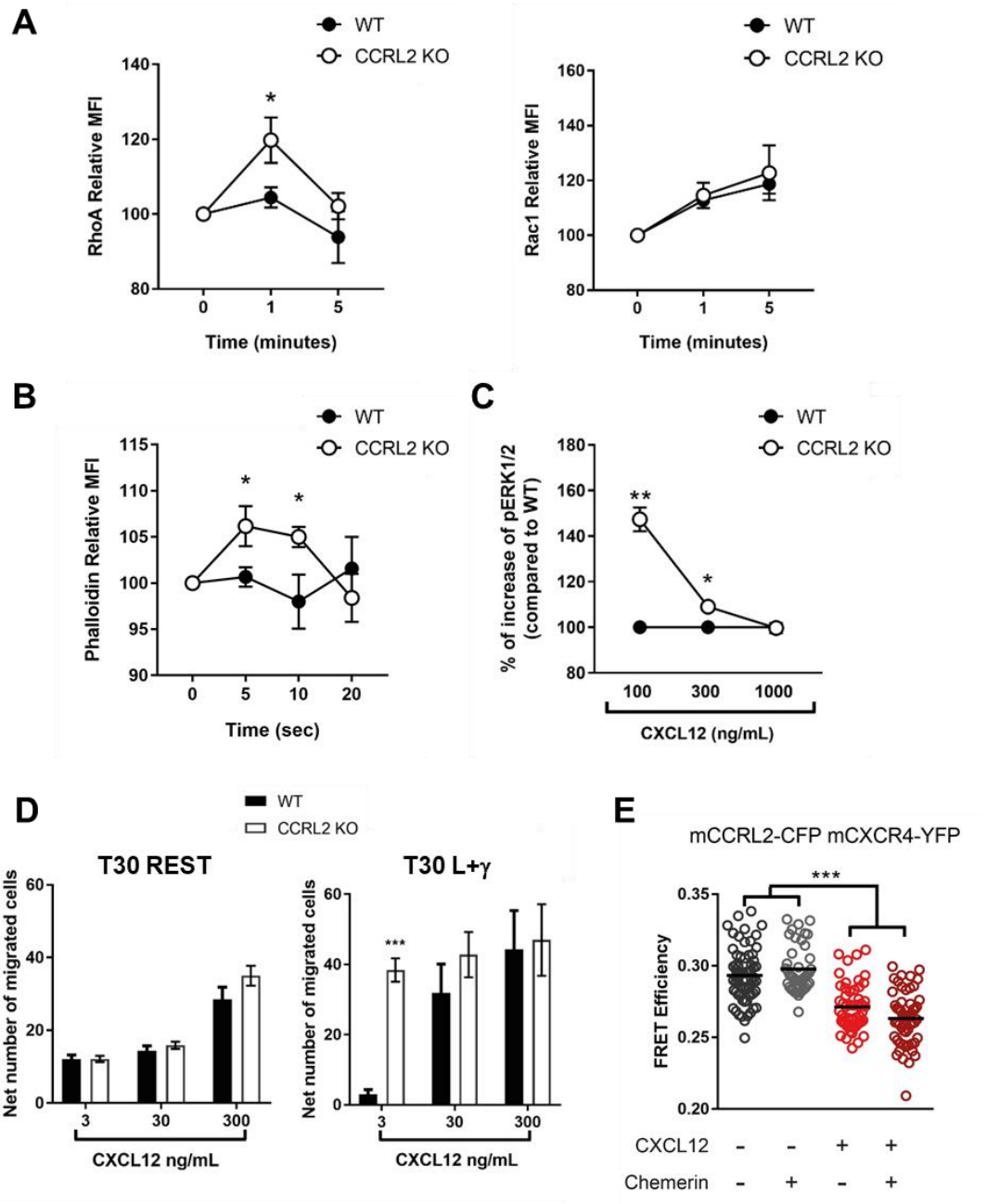

Figure 17: **CCRL2 affects CXCL12-dependent signaling *in vitro*.** (**A**) Rho/Rac1 activation was evaluated in CD11b$^+$/Ly6G$^+$ gate of T30 L+γ neutrophils, after the incubation with CXCL12 (100 ng/mL) at the indicated time points. Results are expressed as percent of increase of MFI of CXCL12 stimulated cells over unstimulated cells. Shown is the mean of three independent experiments performed in triplicate. * P < 0.5 by unpaired Student t test. (**B**) Levels of actin polymerization identified as increase of Phalloidin AF488 conjugated MFI, evaluated in CD11b$^+$/Ly6G$^+$ gate of T30 L+γ neutrophils, after incubation with CXCL12 (100 ng/mL) at the indicated time points. Results are expressed as percent of increase of MFI of CXCL12 stimulated cells over unstimulated cells. Shown is the mean of three independent experiments performed in triplicate. * P < 0.5 by unpaired Student t test. (**C**) % of increase of ERK1/2 phosphorylation of

CCRL2 KO T30 L+γ neutrophils compared to WT (=100%); * P < 0.5; ** P < 0.01 by unpaired Student t test. (**D**) Migration of T30 REST and T30 L+γ neutrophils isolated from WT and CCRL2 KO mice in response to CXCL12 3-30-300 ng/mL by Boyden chambers. Migrated cells were counted in high-power fields (100X). Results are expressed as the net number of cells migrated compared to control ± SEM of four independent experiments performed in triplicate. *** P < 0.001 by unpaired Student t test. (**E**) mCCRL2/mCXCR4 FRET efficiency in presence of their respective ligands chemerin (100 nM) and CXCL12 (100 nM). *** P < 0.001 by ordinary one-way ANOVA.

4. CCRL2 affects neutrophil homing to clearance organs

In order to evaluate whether CCRL2 is able to affect CXCR4 signaling and aged neutrophil migration also in an *in vivo* experimental model, adoptive transfer experiments and appropriate gating strategy were set up: **Figure 18** shows the gating strategy used to identified injected neutrophils in BM (**Figure 18A**) and liver (**Figure 18B**) of recipient mice. Briefly, our model of T30 REST or T30 L+γ BM-derived neutrophils from WT and CCRL2 KO mice were differently stained with CFSE/Violet CellTrace, mixed in equal proportion and injected i.v. in WT recipient mice; BM, lung, spleen and liver of recipient mice were harvested and processed (see point 4 of Material and Methods) to evaluate the ratio of the two genotypes of neutrophils migrated to clearance organs.

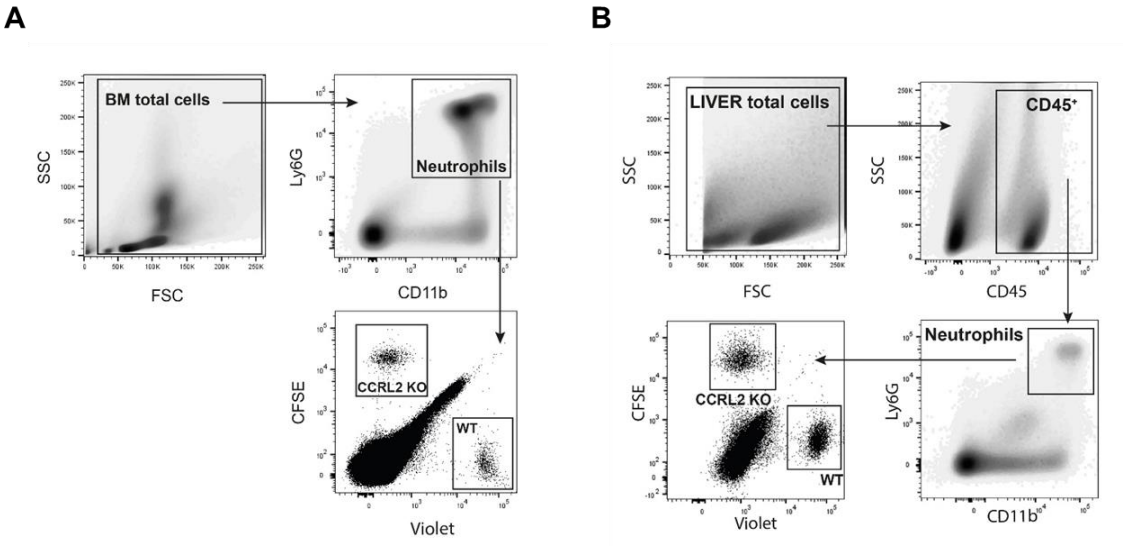

Figure 18: Gating strategy used to identify WT and CCRL2 KO neutrophils in BM (**A**) and in liver (**B**) of recipient mice. WT and CCRL2 KO injected neutrophils are identified as Ly6G$^+$, CD11b$^+$ and CellTrace$^+$ (CFSE or Violet) inside the gate of CD45$^+$ events.

4.1 CCRL2 inhibits CXCR4-dependent neutrophil clearance to BM

After being adoptively transferred in recipient mice, WT and CCRL2 KO T30 REST neutrophils were equally cleared by BM and they were significantly more recruited into BM compared to T30 L+γ (**Figure 19A**). When T30 L+γ neutrophils were injected, we observed a different migratory behaviour to BM between WT and CCRL2 KO: CCRL2 KO neutrophils were recruited by BM on average 53% more compared to WT neutrophils (respectively on average 1926 vs 1303 neutrophils recruited in one tibia/femur pair), suggesting that a negative modulation of CXCR4 signaling by CCRL2 could occur also in *in vivo* settings (**Figure 19A**).

Moreover, with the purpose of assess whether the results obtained were dye-dependent, we repeated the experiments switching the CellTrace dyes for the two genotypes: we observed that still significantly more CCRL2 KO T30 L+γ neutrophils homed to BM compared to WT. We could conclude that the evidence observed was completely dye-independent (**Figure 19B**).

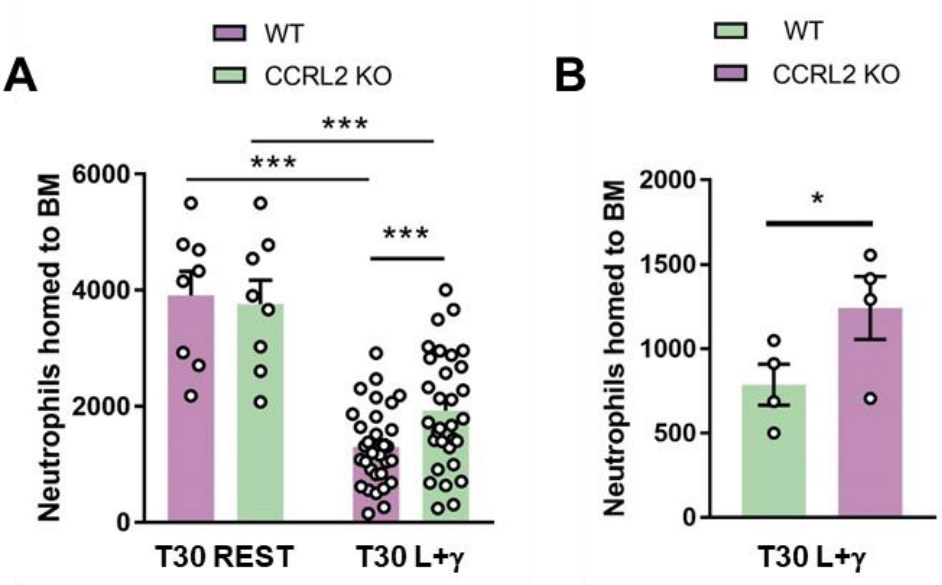

Figure 19: **CCRL2 inhibits neutrophil CXCR4-dependent homing to BM regardless of the dye used.** (**A**) Number of T30 REST or T30 L+γ WT (violet bars) and CCRL2 KO (green bars) neutrophils that homed to BM after being adoptively transferred into WT recipient mice. The number of homed neutrophils were normalized based on the proportion of the neutrophils mix injected into recipient mice. *** P < 0.001 by paired Student t test (unpaired Student t test for comparison between T30 REST and T30 L+γ condition). (**B**) Number of T30 L+γ WT (green bars) and CCRL2 KO (violet bars) neutrophils homed to BM using inverted colours of CellTrace staining. * P < 0.05 by paired Student t test.

4.2 CCRL2 affects migration of aged neutrophils to spleen, lung and liver

During adoptive transfer experiments, also migration of T30 L+γ WT and CCRL2 KO neutrophils to others clearance organs was analysed. CCRL2 KO T30 L+γ neutrophils injected in recipient mice were significantly more recruited not only in the BM, but also in the lung (+46%) (**Figure 20A**) and the spleen (+43%) (**Figure 20B**) compared to WT. Lungs are a site of marginated neutrophils pool and also a step for neutrophils that underwent reverse trans-endothelial migration (rTEM) before homing to BM[55,190]. As reported in literature, aged neutrophils are found to migrate also to lung, where pulmonary vasculature express CXCL12, in order to supply the pool or to respond to inflammatory injuries[55,221]. These observations together with our results, allow us to speculate that CCRL2 is able to negatively modulate neutrophil migration in the lungs mirroring the same condition of the BM.

Spleen is a site of neutrophil clearance, which is mediated by a Pertussis Toxin (PTX)-dependent mechanisms[199], including CXCR4-CXCL12 axis[221]. Our results show that CCRL2 could inhibit aged neutrophil recruitment also in this organ, even if further investigations are needed to identify the specific mechanism involved.

Finally, also the number of T30 L+γ neutrophils homed to liver was evaluated (**Figure 20C**), since it is the main neutrophil clearance organ involved during infections or inflammatory condition[196], a context in which CCRL2 is generally upregulated[115,123]. In contrast to what observed in the other clearance organs analysed, in the liver a significant higher number of WT T30 L+γ neutrophils compared to the CCRL2 KO ones (respectively on average 2327 vs 1948 neutrophils, corresponding to +17%) occurred, suggesting that the presence of CCRL2 on neutrophil surface could favour their migration to liver. Based on these data, we decided to focus our attention on neutrophil migration to BM and liver, since the first is the principal clearance organ involved through CXCR4-CXCL12 axis and the second is the main site of neutrophil clearance during inflammation, of which CCRL2 represents a marker.

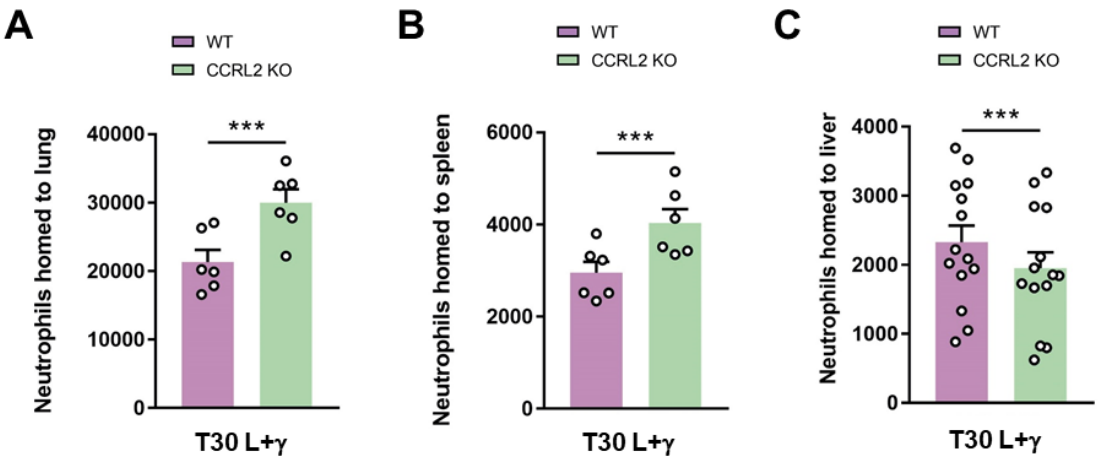

Figure 20: **CCRL2 differently affects neutrophil migration to other clearance organs.** Number of T30 L+γ WT (**violet** bars) and CCRL2 KO (**green** bars) neutrophils that homed to lung (**A**), spleen (**B**) and liver (**C**), after being adoptively transferred into WT recipient mice. The number of homed neutrophils were normalized based on the proportion of the neutrophils mix injected into recipient mice. *** P < 0.001 by paired Student t test.

5. CCRL2 differentially affects CXCR4 and CXCR2 during aged neutrophil homing to BM and liver

It has been demonstrated that neutrophil migration and fate are antagonistically controlled by CXCR4 and CXCR2 expression[75]. WT T30 L+γ neutrophils in our model express both CXCR4 and CXCR2 and, in around 50% of them, the two receptors are co-expressed, together with CCRL2 (**Figure 21A**).

To evaluate directly how CXCR4 and CXCR2 are involved in the different homing observed between WT and CCRL2 KO T30 L+γ neutrophils, we performed adoptive transfer experiments using selective or not selective inhibitors of chemokine receptors: Pertussis Toxin (PTX, able to inhibit both CXCR4 and CXCR2 signalling, since it prevents the activation of G protein-coupled receptors intracellular communication) and Repertaxin (RPTX, a non-competitive allosteric inhibitor of CXCR2) were used (see points 4.7-4.8 of Material and Methods).

In agreement with previous observations of Furze&Rankin[199], our results show that PTX is able to inhibit senescent neutrophil recruitment in the BM through the blocking of CXCR4: PTX reduced of 80% BM homing of T30 L+γ neutrophils, and, moreover, no differences between the number of WT and CCRL2 KO neutrophils

were observed (**Figure 21B**, left). These data confirm that CXCR4 is involved in the difference observed between T30 L+γ WT and CCRL2 KO neutrophils during their migration to BM. Also, the number of T30 L+γ neutrophils recruited into the liver after PTX incubation decreased, without reaching statistical significance, as confirmed by previous report[199]. However, interestingly, the significant increased migration of WT T30 L+γ neutrophils compared to CCRL2 KO in the liver wasn't observed anymore after PTX incubation. This data suggests that a G protein-coupled receptor could be responsible for the different recruitment of T30 L+γ WT and CCRL2 KO neutrophils into the liver (**Figure 21B**, right).

CXCL8, the cognate ligand of CXCR2, was described to be one of the main chemotactic factors involved in neutrophil recruitment into the liver especially during inflammation and pathological conditions[81,82,84,222,223]. In adoptive transfer experiments performed in the presence of RPTX (selectively inhibiting CXCR2 intracellular signals), the total number of T30 L+γ neutrophils recruited by liver didn't significantly change compared to control, but, again, no differences between the two genotypes were found after incubation and administration of RPTX (**Figure 21C**, right). These results suggest that CCRL2 seems to be necessary for an optimal CXCR2 signaling also during neutrophil clearance, as already observed during neutrophil recruitment in the inflammatory sites[115]. Finally, it is important to stress that RPTX didn't affect neither the number of T30 L+γ neutrophils recruited into the BM nor the difference between WT and CCRL2 KO neutrophils in this clearance organ, as expected (**Figure 21C**, left).

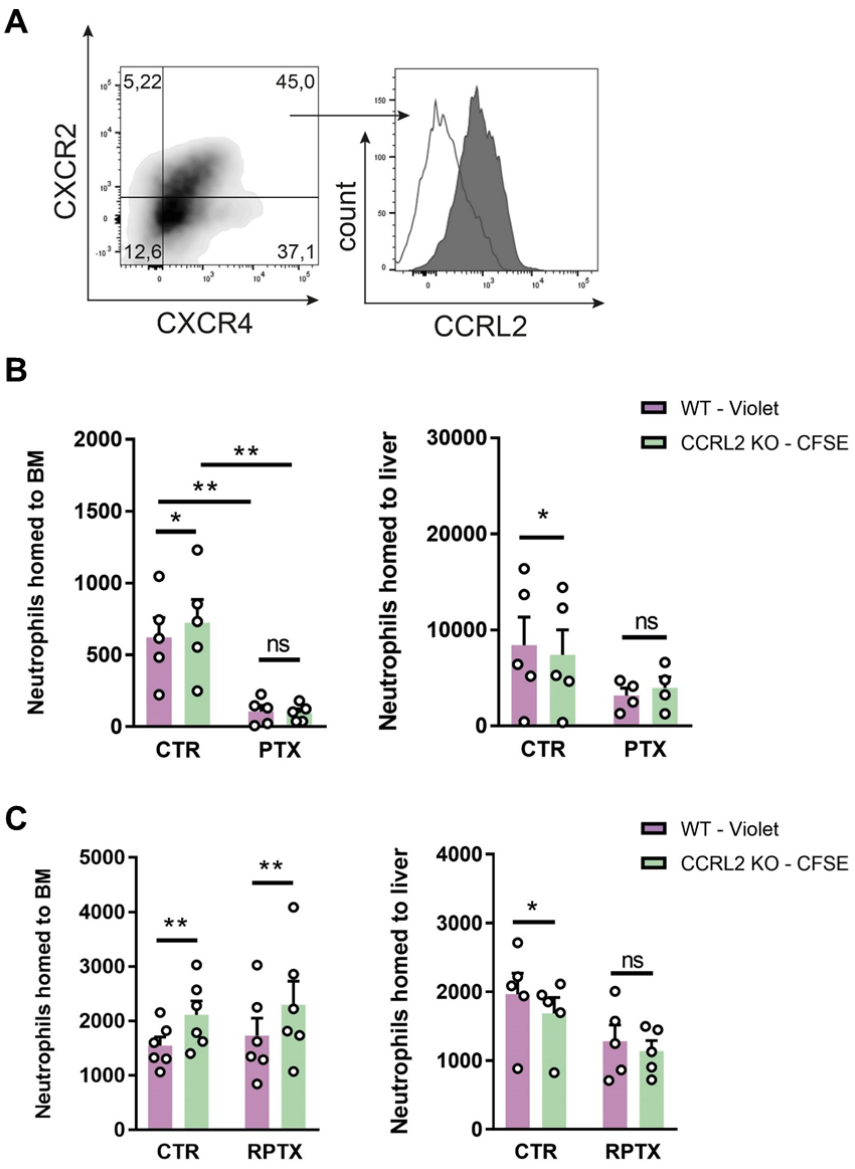

Figure 21: **CCRL2 affects T30 L+γ neutrophil migration to BM and liver in a G protein coupled - receptor dependent manner.** (**A**) Flow cytometry plots about CXCR4-CXCR2 and histogram of CCRL2 surface expression of WT T30 L+γ neutrophils used in adoptive transfer experiment. (**B**) Number of T30 L+γ WT (**violet** bars) and CCRL2 KO (**green** bars) neutrophils migrated to BM and liver, after being incubated with 1 µg/mL PTX (or control, CTR) and adoptively transferred in WT recipient mice. * P < 0.05; ** P < 0.01 by paired/unpaired Student T test (unpaired Student T test was used to compared CTR vs PTX conditions). (**C**) Number of T30 L+γ WT (**violet** bars) and CCRL2 KO (**green** bars) neutrophils migrated to BM and liver, after being incubated with 30 µg/mL RPTX (or control, CTR) and adoptively transferred in WT recipient mice that were injected 30 min before with RPTX (30 mg/Kg) or control (CTR). * P < 0.05; ** P < 0.01 by paired/unpaired Student T test (unpaired Student T test was used to compared CTRT vs RPTX conditions).

6. Endogenous neutrophils in condition of *in vivo* inflammatory stimulation express both CXCR4 and CCRL2

To recapitulate *in vivo* the phenotype obtained *in vitro* with the T30 L+γ neutrophils model, we stimulated WT and CCRL2 KO mice with i.p. injection of LPS and mIFNγ[219] (or PBS in control mice); after 3 hours mice were sacrificed and BM and liver processed and cells stained to identify endogenous neutrophils expressing both CCRL2 and CXCR4 receptors (see point 5 of Material and Methods). First, according to literature, we noticed that both in WT and CCRL2 KO, the percentage of neutrophils in BM of control mice (on average 60% of $CD45^+$) was significantly higher compared to the percentage of neutrophils found in stimulated mice (on average 52% of $CD45^+$) (**Figure 22A**, left). This observation could be due to a major release of neutrophils from the hematopoietic compartment combined with a minor homing of senescent neutrophils back to bone marrow in a condition of acute inflammation[196,224]. No differences were found between the percentage of total neutrophils in BM of WT and CCRL2 KO mice after stimulation (**Figure 22A**, left). At the same time, according to literature[196,225], the percentage of neutrophils identified in the liver compartment was significantly higher in stimulated condition compared to control one (on average 2,5% of $CD45^+$) (**Figure 22A**, right). Interestingly, after stimulation, the percentage of total neutrophils recruited in the liver of CCRL2 KO mice was significantly lower (on average 15,7% of $CD45^+$) compared to the number of total neutrophils recruited into liver of WT mice (on average 23,7% of $CD45^+$) (**Figure 22A**, right). To better characterize tissue neutrophils in term of chemokine receptors expression after systemic inflammation, we noticed that just WT liver neutrophils express CCRL2 (on average the 40% in $LY6G^+/CD11b^+$ gate), while no detectable CCRL2 was identified on WT BM neutrophils (**Figure 22B**). Moreover, around 30% of total liver neutrophils co-expressed CXCR4 and CCRL2 (**Figure 22B**). These results supported the importance of CCRL2 expression on neutrophils during their migration into the liver in inflammatory conditions. These data also strengthen the evidence obtained by adoptive transfer experiments in which a role of CCRL2 emerged in the reduction of neutrophil clearance to BM, in favour of neutrophil migration to the liver.

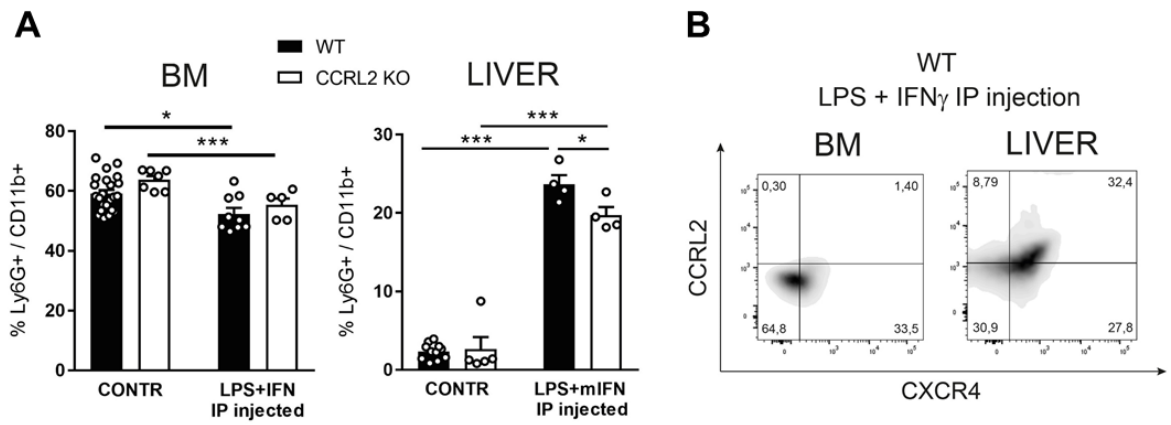

Figure 22: **CCRL2 and CXCR4 are co-expressed by endogenous neutrophils in the liver.** (**A**) Percentage of neutrophils ($Ly6G^+$ and $CD11b^+$) in $CD45^+$ BM and liver population of WT (**black** bars) and CCRL2 KO (white bars) mice after *in vivo* stimulation (i.p. injection of 1 ug LPS and 150 ng mIFNγ) compared to control. Mice were sacrificed 3 hours after the stimulation. *P < 0.05; *** P < 0.001 by unpaired Student t test. (**B**) Flow cytometry plots of CXCR4 and CCRL2 surface expression of WT BM and liver neutrophils after *in vivo* stimulation.

7. Aged neutrophil recruitment in CCRL2 KO mice is defective in liver but enhanced in BM compared to WT neutrophils

In order to evaluate the effective number of endogenous senescent neutrophils recruited into BM and liver in condition of inflammation both in WT and CCRL2 KO mice, we used BrdU *in vivo* labelling model, to distinguish between aged and not aged neutrophils (see point 5.1 of Material and Methods). Based on the method set up by Uhl et al.[196], we injected a BrdU solution in WT and CCRL2 KO mice. BrdU, an analogue of thymidine, can be incorporated by the cells that are in mitotic phase at the moment of the injection (immature), while cells already mature are not able to incorporate it. Thus, after 48 hours, BrdU negative cells were identified as senescent cells (**Figure 23A**). Three hours before mice sacrifice, LPS and mIFNγ i.p. injection was performed[196]. Interestingly, the decrease of senescent neutrophil homing to BM compared to control was significantly higher in WT mice compared to CCRL2 KO mice as shown in **Figure 23B** (left panel). In WT mice, the 20,8% of aged neutrophils were found to be cleared compared to control (=100%), while in CCRL2 KO mice the rate of aged neutrophils recruited was around 37,4% compared to control (=100%). Moreover, the opposite trend was observed in the liver, where, in both WT and CCRL2 KO

mice, an increase of neutrophil recruitment was observed: in particular, aged neutrophils in WT mice were 4 times more recruited in the liver compared to CCRL2 KO mice (**Figure 23B**, right). These data further corroborated the role of CCRL2 in affecting aged neutrophil homing to BM and liver by inhibiting CXCR4 and simultaneously enhancing CXCR2 signalings[115].

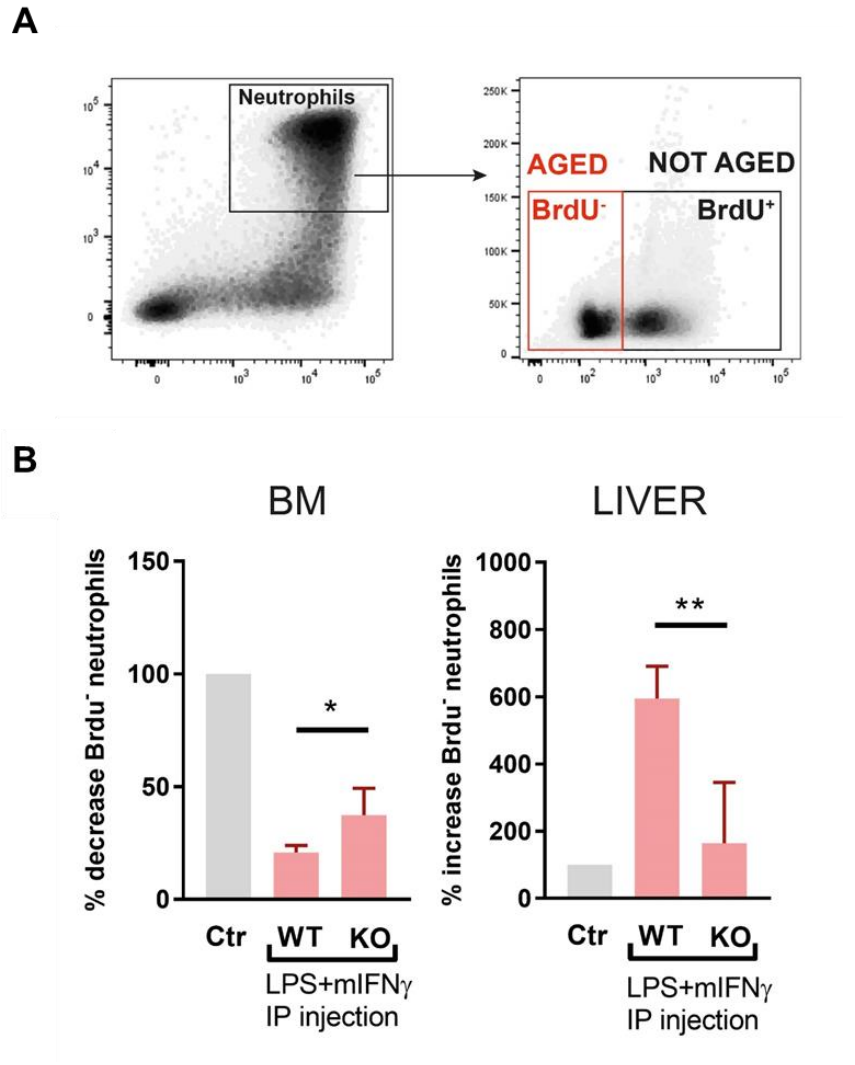

Figure 23: **BrdU$^-$ aged neutrophils in CCRL2 KO mice are differently cleared by BM and liver compared to WT.** (**A**) Gating strategy for discriminating between aged and not aged neutrophils after BrdU staining. (**B**) Percentage of decrease (BM, left) and increase (liver, right) of BrdU$^-$ senescent neutrophils in *in vivo* stimulated WT and CCRL2 KO mice, compared to control (=100%). * $P < 0.05$; ** $P < 0.01$ by unpaired Student t test.

8. CCRL2/chemerin axis is not involved in neutrophil efferocytosis *in vitro* and *in vivo*

The final step of senescent neutrophil life-span is efferocytosis: aged neutrophils recruited into clearance organs are then phagocytized by tissue macrophages[201,205]. We perform *in vitro* and *in vivo* experiments to investigate whether CCRL2 can be considered an “eat-me” signal and CCRL2/chemerin axis can influence neutrophil engulfment by macrophages.

8.1 Chemerin doesn’t affect neutrophil efferocytosis *in vitro*

To investigate a possible modulation of neutrophil efferocytosis by CCRL2/chemerin axis, an *in vitro* phagocytosis experiment was set up (see point 6.1 of Material and Methods). T30 L+γ WT neutrophils were CellTrace Violet stained, equally mixed with peritoneal macrophages and incubated with or without chemerin (200 nM). After different time points, macrophages that had engulfed neutrophils were identified as CellTrace$^+$. After 1 hour of incubation, around 15% of macrophages (F480$^+$ and CD11b$^+$) resulted to be CellTrace+. As shown in **Figure 24A**, the percentage of this population decreased in a time dependent way, possibly caused by a dye degradation in the intracellular macrophage environment. These results suggest that CCRL2/chemerin axis is not probably involved in neutrophil engulfment by macrophages.

8.2 WT and CCRL2 KO neutrophils are equally engulfed by tissue macrophages *in vivo*

It has been proved that neutrophils adoptively transferred in recipient mice are able to be engulfed by tissue macrophages. We adapted a gating strategy already described by Casanova-Acebes et al.[205] in order to identify, both in BM and liver of recipient mice, tissue macrophages that had phagocytised previously labelled and injected T30 L+γ WT or CCRL2 KO neutrophils (Ly6G$^-$, F480$^+$, CD11b$^+$ and CellTrace CFSE/Violet) (**Figure 24B**). As shown in **Figure 24C**, WT and CCRL2 KO T30 L+γ neutrophils were equally phagocytised by tissue macrophages, both in BM and in liver. We can conclude from these data that the presence of CCRL2 is relevant for neutrophil migration but not for their engulfment by macrophages.

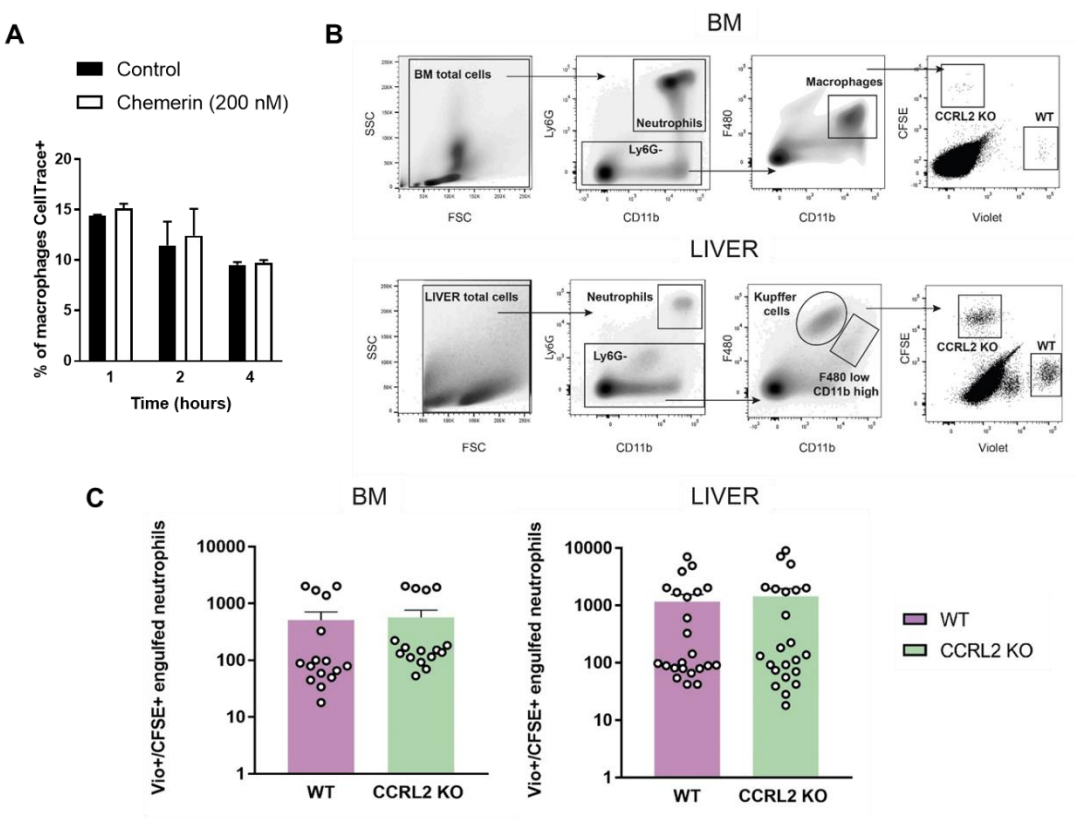

Figure 24: **CCRL2 doesn't affect neutrophil clearance by tissue macrophages in BM and liver.** (**A**) Percentage of peritoneal macrophages Violet CellTrace^{+} incubated with CellTrace stained T30 L+γ neutrophils with or without chemerin. The data were analysed by unpaired Student t test. (**B**) Gating strategy used for the analysis of adoptive transfer experiments in BM and in liver to evaluate the quote of engulfed neutrophils by BM resident macrophages or by Kupffer cells in the liver. (**C**) Number of T30 L+γ WT and CCRL2 KO neutrophils engulfed by BM or liver macrophages after being adoptively transferred into WT recipient mice. The number of phagocytized neutrophils were normalized based on the proportion of the neutrophils MIX injected in recipient mice. The data were analysed by paired Student t test.

8.3 CCRL2 is not involved in neutrophils-Kupffer cells crosstalk in liver

Finally, a possible involvement of CCRL2 in the cross-talk between neutrophils and Kupffer cells was investigated. Kupffer cells are a subset of liver resident macrophages closely correlated with intense phagocytosis, antigen presentation, and cytokines secretion, actively participating to pro- and anti-inflammatory immune response[226]. A role for Kupffer cells in recruitment and in efferocytosis of neutrophils was reported in inflammatory conditions both in humans[227] and in animal models[228,229]. Moreover, Kupffer cells result to be a source of CXCL8, that

play a key role in neutrophil recruitment from liver sinusoids[230,231]. An experimental model of selective depletion of Kupffer cells with Gadolinium Chloride ($GdCl_3$) was set up (see point 6.3 of Material and Methods). Following $GdCl_3$ administration, the percentage of resident Kupffer cells, identified as $F480^{high}$ and $CD11b^{low}$ in non-parenchymal liver cells of recipient mice[232,233], significantly decreased (**Figure 25A**). However, we didn't observe a significant reduction of neutrophils recruitment into the liver, and, moreover, WT neutrophils were still significantly more recruited in the liver compared to CCRL2 KO (**Figure 25B**). For these reasons, we can assume that CCRL2 is not involved in the process of efferocytosis of senescent neutrophils or in the interaction with Kupffer cells.

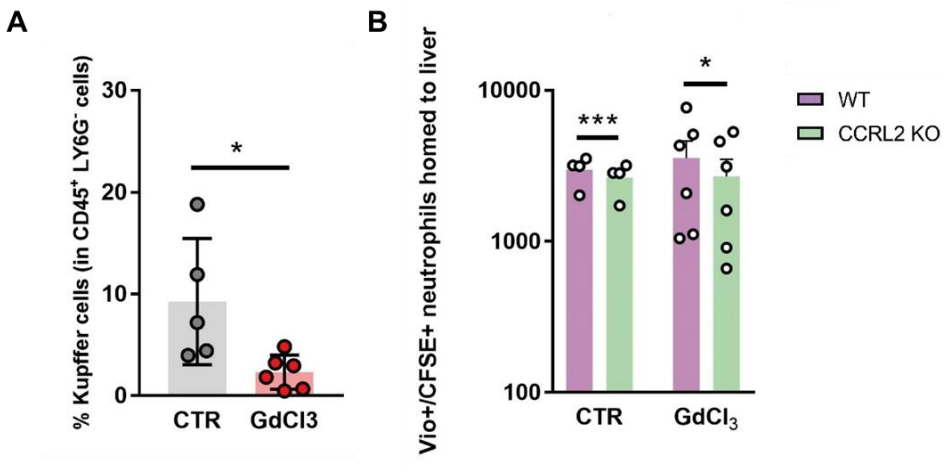

Figure 25: **CCRL2 doesn't affect neutrophils-Kupffer cells crosstalk in the liver.** (**A**) Percentage of Kupffer cells identified in $CD45^+/Ly6G^-/F480^{high}/CD11b^{low}$ gate of control (CTR) and $GdCl_3$ treated recipient mice. * $P < 0.05$ by unpaired Student t test. (**B**) Number of T30 L+γ WT and CCRL2 KO neutrophils migrated to liver, adoptively transferred in WT recipient mice control (CTR) or treated with $GdCl_3$. * $P < 0.05$; *** $P < 0.001$ by paired Student t test.

DISCUSSION

CCRL2 is a 7-transmembran domain receptor that shares structural similarities with atypical chemokine receptors family[104]. However, it does not bind a chemokine but a chemotactic protein called chemerin and it does not show the two functional hallmarks of this class of receptors (internalization and scavenging of ligands)[129]. A promising role of CCRL2 emerged in the last years, not only as a creator of a solid gradient of chemerin[104], but also as a fine-tuning modulator of expression and functionality of other transmembrane receptors[115,172]. For example, we recently demonstrated that CCRL2 is able to modulate CXCR2 signaling through the formation of heterodimers on neutrophil surface, as confirmed by the defective neutrophil recruitment via CXCL8 in CCRL2 KO mice under inflammatory conditions[115].

Both neutrophil recruitment and homing to clearance organs are regulated by chemokine receptors and their cognate chemokines[221]. CXCR4 expression on immature neutrophils prevents their early release and its absence on competent neutrophils, together with the upregulated expression of the chemokine receptor CXCR2, led them to exit into the blood circulation and to be recruited into the site of inflammation. Once neutrophils in the circulation become senescent, they undergo to a switch of chemokine receptors phenotype, downregulating CXCR2 and upregulating again CXCR4 that plays a key role in driving senescent neutrophil migration into the bone marrow. For these reasons, it is possible to identify an antagonistic balance between CXCR2 and CXCR4, whose expression and signalling on neutrophil surface, can define their “age” and their chemotactic behaviour[75,204,215,234].

Based on the assumption that CCRL2 can heterodimerize with CXCR2 modulating its signaling[115], the main aim of our work was to evaluate whether CCRL2 can also form heterodimers with CXCR4 affecting its functionality during neutrophil clearance. Data obtained in collaboration with Prof. Mario Mellado from National Center of Biotechnology in Madrid, proved the ability of CCRL2 to form heterodimers with other chemokine receptors, including CXCR4.

In this thesis work, we characterized, by *in vitro* and *in vivo* experimental settings, the functional role of CCRL2 in the regulation of CXCR4 and CXCR2 signaling during aged murine neutrophil clearance.

Martin et al.[204] demonstrated as neutrophils, when cultured *in vitro*, upregulate in a time-dependent way their membrane expression of CXCR4; CXCR4 high expressing neutrophils were also shown to home to BM, where they are cleared by macrophages. This clearance process is CXCR4-dependent, since neutrophils expressing a lower rate of CXCR4 homed back to BM in a less efficient way[204]. In our models of aged neutrophils, we reproduced the CXCR4-dependent BM clearance process. Also, T30 L+γ neutrophils, presented other classical characteristics of an aged phenotype, according to recent literature[141,184,196,206]. Thus, we can assert that T30 L+γ neutrophils, used to perform *in vitro* and *in vivo* experiments, represented a correct model of aged neutrophils, as shown by morphologic and phenotypic markers. In addition, we can speculate that T30 L+γ neutrophils also showed the phenotypic characteristic of neutrophils that performed reverse trans-endothelial migration (rTEM) after inflammation: in fact, Wang et al.[190] and others[187,189,191], characterized rTEM neutrophils as high expressing CXCR4 and Annexin V. It is possible to speculate that T30 L+γ neutrophils model could represent a condition of aged neutrophils that perform rTEM after inflammation, since they were cultured under pro-inflammatory stimuli in order to upregulate CCRL2.

Our research group already demonstrated that freshly isolated neutrophils obtained from CCRL2-deficient mice were characterized by altered membrane CXCR2 expression[115], suggesting a role for CCRL2 in the regulation of chemokine receptors expression. This role for CCRL2 was also confirmed by the significant difference observed in CXCR4 expression on freshly isolated neutrophils compared to WT neutrophils. Accordingly, CCRL2 was shown also to modulate expression of other receptors, such as TLR4 on macrophages[172].

We also proved that CCRL2 negatively affects CXCR4 signalling both *in vitro* and *in vivo*, with a consequent decrease of aged neutrophil homing to BM. Furze and Rankin[199] proved that aged neutrophil CXCR4-dependent homing to BM can be cancelled by Pertussis Toxin (PTX) administration. We also observed a dramatic decreased of aged neutrophil recruitment to BM when incubated with PTX; moreover, in this condition, we didn't observe anymore a CXCR4 modulation by CCRL2, demonstrating that CCRL2 directly modulate the G protein-dependent CXCR4 signaling. In fact, CCRL2 KO neutrophils showed increased levels of

RhoA activation, ERK1/2 phosphorylation and actin polymerization in response to CXCL12, compared to WT neutrophils.

Uhl and colleagues[196] studied in deep aged neutrophil clearance during inflammation, showing that inflamed aged neutrophils are preferentially cleared by liver instead of BM. Through the same BrdU *in vivo* assay, we could confirm that, in inflammatory conditions, aged neutrophils ($BrdU^-$) are more recruited into the liver compared to physiological condition.

CXCR2-CXCL8 axis is known to play a key role in neutrophil recruitment into the liver during inflammation[81-85], in fact, the blocking of CXCR2 signaling through the antagonist molecule Repertaxin (RPTX) protects liver damage against inflammatory diseases such as reperfusion injury[17] and post ischemic hepatic syndromes[87]. Despite this, our results, accordingly to data already obtained by Furze and Rankin[199], proved that aged neutrophil recruitment into the liver is not totally G protein-coupled receptor dependent. However, our experiments showed that CCRL2 is a positive modulator of CXCR2 signaling in a quote of aged neutrophils recruited by the liver. This result corroborates the already published data regarding the optimal CXCR2 signaling and migration by CCRL2 heterodimers formation[115].

Taken together these data strongly suggest a role for CCRL2 as a fine-tuning modulator of both CXCR4 and CXCR2 on aged neutrophil surface during their clearance. However, CCRL2 doesn't seem to act as a "eat me" or "don't eat me" signal, which regulates aged neutrophil efferocytosis by macrophages.

Our hypothetical model showing the regulatory role of CCRL2 on CXCR4 and CXCR2 signaling is schematically represented in **Figure 26**. Immature neutrophils with high membrane expression of CXCR4 are retained in BM niche through CXCL12 gradient (**26A**). When neutrophils get mature, they are released into the blood circulation after the simultaneous CXCR4 downregulation and CXCR2 upregulation (**26B**). CCRL2 is expressed on neutrophil surface following pro-inflammatory stimuli (coming from tissue injuries or infections) and it can heterodimerize with CXCR2 affecting its signaling and consequently neutrophil recruitment in the inflammatory sites (**26C**). In a condition of rTEM, in which neutrophils maintain inflammatory marker and upregulate senescence characteristics as CXCR4 and Annexin V expression[189,190,235] (**26D**), it is possible

to speculate that CCRL2 can modulate through heterodimerization both CXCR4 and CXCR2 signaling: in particular, CCRL2 could favour neutrophil clearance into liver by modulating CXCR2 signaling (**26E**), while it could inhibit neutrophil clearance to BM by affecting CXCR4 functionality (**26F**).

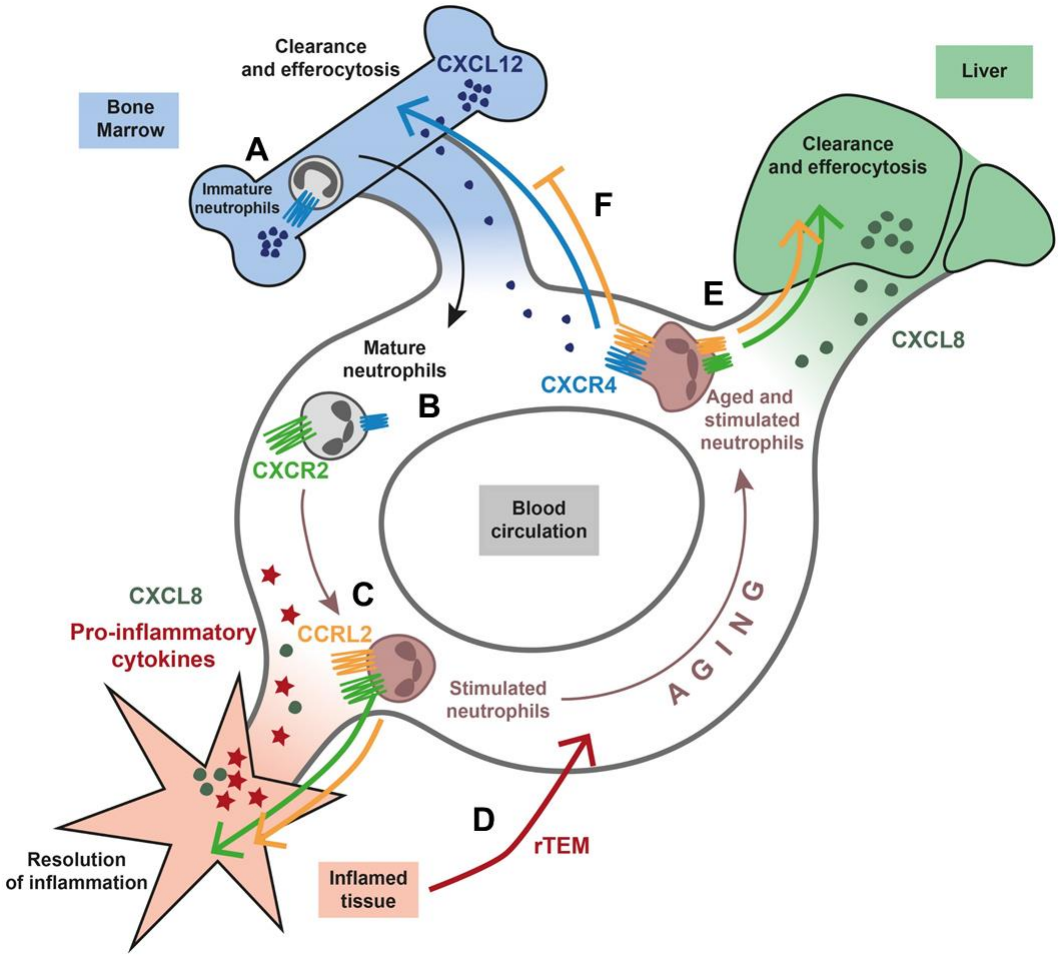

Figure 26: **Representative scheme of the role of CCRL2 in neutrophil life-span.** CCRL2 is upregulated in inflammatory conditions and it is able to potentiate CXCR2 signaling in response to CXCL8 through heterodimerization on mature neutrophils. On aged and stimulated neutrophil surface, CCRL2 could heterodimerize both with CXCR2 and CXCR4: in this condition, CCRL2 affects CXCR2 signaling promoting clearance of aged neutrophils into the liver while it decreases CXCR4 signaling, reducing the number of neutrophils cleared by BM.

Assuming the pro-inflammatory relevance of senescent neutrophils in pathologic conditions[196,198], CCRL2 can be considered a fine-tuning modulator of neutrophil migratory processes and, translationally, a potential pharmacological target.

Neutrophil recruitment in the liver is involved in the pathological progression of inflammatory-dependent diseases such as Non-Alcoholic Fatty Liver Disease

(NAFLD), Alcholic Liver Disease (ALD), Viral Hepatitis, HepatoCellular Carcinoma (HCC), Liver Ischemia/Riperfusion (I/R) injury and others[81-84]. In particular, neutrophil accumulation into the liver is positively correlated with negative outcome of diseases, as in HCC[84]. In this condition, inflamed and aged neutrophils could be crucial in the formation of a chronic pro-inflammatory environment and the potential reduction of their migration into the liver could lead to a partial resolution of the inflammatory context.

Another pathological model in which CXCR4 and CXCR2 can be modulated by CCRL2 on neutrophil surface is represented by polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs), a subset of pathologically activated neutrophils with immunosuppressive activity in tumor microenviroment[236]. PMN-MDSCs express high levels not only of CXCR4 and CXCR2, but also of CCRL2[237] when activated; in facts, CCRL2 has been recently indicated as a selective marker for the activated PMN-MDSCs[207]. These observations suggest that CCRL2 could probably modulate the migration of this neutrophil subset into the tumor area, and, eventually, it can be targeted to affect its migratory potential.

In conclusion, our results identify a novel role of CCRL2 in the regulation of CXCR4 signaling trough heterodimerization resulting in a negative modulation of intracellular CXCL12-dependent downstream. We observed these results both *in vitro* assay and *in vivo* adoptive transfer experiments. In our model, CCRL2 affects CXCR4-dependent homing of aged neutrophils to BM, potentiating the neutrophil clearance into the liver in a CXCR2-dependent way. From a translationally point of view, these results can be considered as a starting point to better define both the role of aged neutrophils recruited during inflammatory conditions, such as liver diseases and the possible involvement of CCRL2 in modulating CXCR4 and CXCR2 signalings during the recruitment of PMN-MDSCs in tumoral tissues.

SIDE RESEARCH ACTIVITIES and PUBLICATIONS

February 2021 – October 2021

Attendance period at Laboratory of Immunology and Immunopathology of Sapienza Università di Roma, in collaboration with Prof. Silvano Sozzani

Epithelial tissues act as barriers between two compartments and the epithelial barrier function is provided by the epithelial cells and the tight junctions (TJs) that connect them. Altered expression or functions of TJs can lead to a remodelling of barrier permeability; these variations play a key role in some pathological condition, as colon cancer cancerogenesis, in which an increase of tissue permeability can lead to an higher risk of developing the disease[238].

As already described, CCRL2 can be expressed also by non-hematopoietic cells and, for example, it can be expressed by colon epithelial cells. As part of a study regarding the role of CCRL2 in colon cancer carried out by our research group, the possible involvement of CCRL2 in modulating the expression of cell junction (occludin, claudin 7 and ZO-1) was investigated to check if CCRL2 can influence the colon tissue permeability.

At the laboratory of Immunology and Immunopathology of Sapienza Università di Roma, we produced and kept in culture murine colon organoids in order to perform immunofluorescence staining and functional assays. The three-dimensional organ-like structures of organoids is composed of functional, live cells that can self-renew and spatially organize[239]. Organoids overcome many of the limitations of standard cell lines: they reproduce the three-dimensional architecture of mice/human tissue and can be derived from almost any normal intestinal or cancer sample for long-term propagation[239]. Organoid culturing has also been applied to study gastrointestinal diseases, intestinal-microbe interactions, and colorectal cancer[239].

We isolated colon crypts from WT and CCRL2 KO mice. Isolated colon crypts can be grown long-term as organoids suspended in a 3D Matrigel covered by a medium containing soluble factors as Noggin, R-spondin 1 and Wnt3a that favour the maintaining of a staminal state[239]. At the microscope, an organoid at the staminal state appears as a spheroid (**figure 27A**), while, eliminating the pro-staminal factors from the medium, the organoid starts differentiating assuming a more complex 3D structured (**figure 27B**).

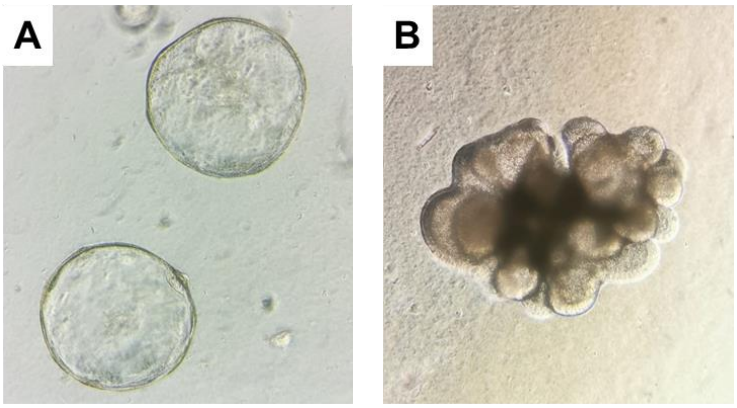

Figure 27: **Murine colon organoids in different states of differentiation.** (**A**) Murine colon organoids in a staminal spheroid state; (**B**) Murine colon organoids in an advanced differentiated state.

WT an CCRL2 KO organoids suspended in a 3D matrigel were fixed and permeabilized in order to be stained for different cell junctions as the tight junctions ZO-1 and claudin 7. In **figure 28A** and **28B** are shown respectively a staining for ZO-1 and claudin7 (red), performed using a rabbit anti-mouse primary antibody and a goat anti-rabbit AlexaFluor 647 secondary antibody.

These developed techniques represent a starter point to better characterized tight junction expression and functionalities in CCRL2 KO colon organoids compared to WT. Moreover, colon crypts from mice model of colitis-associated colon cancer will be isolated, to generate organoids and investigate the permeability condition of the two genotypes in a pathological context.

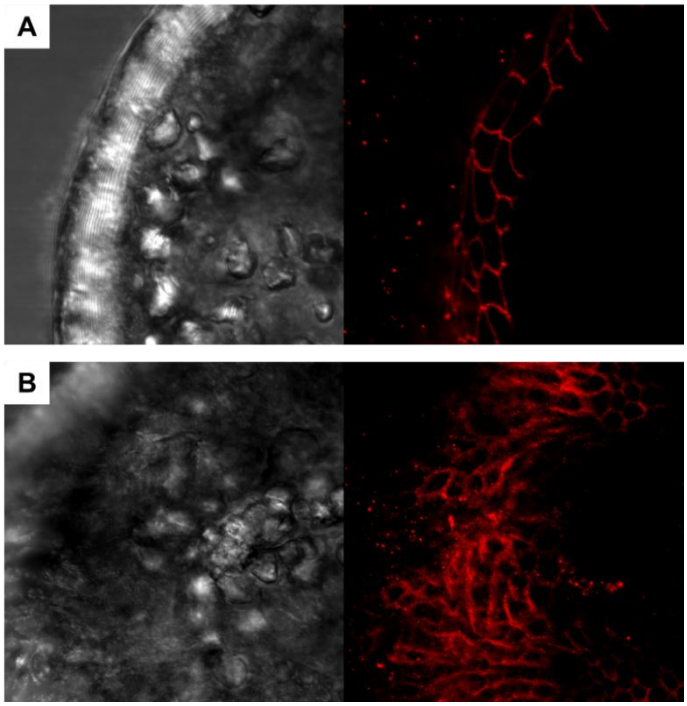

Figure 28: **Murine colon organoids stained for epithelial cells junctions.** (**A**) Confocal microscope acquisition of WT colon organoid stained for Zonula occludens-1 (ZO-1, **red**) with a rabbit anti-mouse primary antibody and a goat anti-rabbit AF647 secondary antibody; (**B**) Confocal microscope acquisition of WT colon organoid stained for Claudin-7 (Cldn7, **red**) with a rabbit anti-mouse primary antibody and a goat anti-rabbit AF647 secondary antibody.

Publications

- **Reviews**

Functional Role of Dendritic Cell Subsets in Cancer Progression and Clinical Implications

International Journal of Molecular Sciences, May 2020

Annalisa Del Prete, Francesca Sozio, **Ilaria Barbazza**, Valentina Salvi, Laura Tiberio, Mattia Laffranchi, Angela Gismondi, Daniela Bosisio, Tiziana Schioppa and Silvano Sozzani

https://www.mdpi.com/1422-0067/21/11/3930

During the PhD program I've contributed in writing a review regarding the broad range of dendritic cells (DCs) functionalities involved in tumor progression and anti-tumor immune response. DCs population is composed by different subsets that share common functions but that also embody many divergent aspects. All dendritic cell subsets undergo a complex trafficking program related to their stage of maturation and they are all able to prime T cell response; however, DCs are implicated in a wide spectrum of functionalities that comprehend both protective and detrimental immune responses. Although cDC1s are the most potent subset in tumor antigen cross-presentation, they need close interaction and cooperativity with the other dendritic cell subsets (cDC2s and pDCs) in order to induce full-strength anti-tumor cytotoxic T cell response. Understanding the specificity of dendritic cell subsets will allow to gain insights on role of these cells in pathological conditions and to design new selective promising therapeutic approaches.

This review takes into consideration different aspects of DCs biological relevance, including the functional role of dendritic cell subsets in both promoting and suppressing tumor growth, the mechanisms underlying their recruitment into the tumor microenvironment, as well as the prognostic value and the potentiality of DCs as therapeutic target.

Molecular Basis for CCRL2 Regulation of Leukocyte Migration

Frontiers in Cell and Developmental Biology, December 2020

Tiziana Schioppa, Francesca Sozio, **Ilaria Barbazza**, Sara Scutera, Daniela Bosisio, Silvano Sozzani, Annalisa Del Prete

https://www.frontiersin.org/articles/10.3389/fcell.2020.615031/full

In 2020, I've also contributed in writing a review with the purpose of summarize updated knowledge regarding CCRL2 expression, regulation and functionalities in different cell types and its involvement in different pathological models. Some chapters of this review have been inserted in point 1.5.1 and 2.2.3 of the introduction of this thesis, dedicated to CCRL2 description and functionalities.

CCRL2 is a 7-transmembran domain receptor that shares structural similarities with atypical chemokine receptors family, even if it does not bind a chemokine but a chemotactic protein called chemerin and it does not act as an internalization and scavenger mediator. CCRL2 is expressed both by leukocytes and non-hematopoietic cells. The genetic ablation of CCRL2 in animal model has been used to elucidate the role of this receptor as a modulator of inflammation. CCRL2 modulates leukocyte migration by two main mechanisms. First, when CCRL2 is expressed by barrier cells, such endothelial cells, it acts as a presenting molecule, contributing to the formation of a solid chemerin chemotactic gradient for leukocytes expressing CMKLR1, the functional chemerin receptor. This mechanism was shown to be crucial in NK cells recruitment in lung tumor microenvironment, actively contributing to immune surveillance during cancer progression and metastasis. Second, by forming heterocomplexes with other chemokine receptors: CCRL2/CXCR2 heterodimers were shown to regulate the activation of β2-integrins in mouse neutrophils.

- **Original papers**

SARS-CoV-2-associated ssRNAs activate inflammation and immunity via TLR7/8

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The inflammatory and Interferon (IFN) pathways of innate immunity play a key role in the resistance and pathogenesis of coronavirus disease 2019 (COVID-19). Innate sensors and SARS-CoV-2–associated molecular patterns (SAMPs) remain to be completely defined. In this work, we have identified single-stranded RNA (ssRNA) fragments from the SARS-CoV-2 genome as direct activators of endosomal TLR7/8 and MyD88 pathway. The same sequences induced human DC activation in terms of phenotype and function, such as IFN and cytokine production and Th1 polarization. A bioinformatic scan of the viral genome identified several hundreds of fragments potentially activating TLR7/8, suggesting that products of virus endosomal processing potently activate the IFN and inflammatory responses downstream of these receptors.

In particular, I've been involved in this work during the phase of *in vivo* experiments: the identified ssRNA administrated in mice, induced MyD88-dependent lung inflammation characterized by accumulation of proinflammatory and cytotoxic mediators and immune cell infiltration.

Taken together, these results identified TLR7/8 as a crucial cellular sensor of ssRNAs encoded by SARS-CoV-2 involved in host resistance and the disease pathogenesis of COVID-19.

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