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β-ARRESTIN DEPENDENT REGULATION OF CYTOSKELETON DYNAMICS AND SIGNALLING OF CHEMOKINE RECEPTOR ACKR2

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SUMMARY

Chemokines represent a family of secreted inflammatory mediators with a prominent role in leukocyte migration and angiogenesis, exerted through the activation of dedicated seven transmembrane domain receptors [1, 2]. Beyond conventional chemokine receptors, which directly induce cell migration through Gαi-mediated signalling events, a set of atypical chemokine receptors (ACKRs) have been described. ACKRs present high structural sequences homology with chemokine receptors but they do not activate Gαi-mediated signalling activity [3] due to alteration in domain involved in G-protein coupling. At present, ACKRs family include DARC (now called ACKR1) [4], D6 (now called ACKR2) [5], CXCR7 (now called ACKR3) [6], CCX-CKR (now called ACKR4) [7], CCRL2 (now called ACKR5) [8] and C5L2 [9]. Though being unable to directly support cell migration, ACKRs play non-redundant functions during innate and adaptive immune responses, controlling leukocyte mobilization, their recruitment in inflamed tissues, and traffic to draining lymph nodes [10]. ACKRs fulfil their biological functions by contributing to generate and maintain functional chemokine patterns by removal, transport, or concentration of chemokines [11].

ACKR2 is the best characterize member of the ACKR family [12]. ACKR2 is a scavenger receptor that binds with high affinity to 13 inflammatory CC chemokines [12]. It is expressed at high levels by endothelial cells of lymphatic vessels in several tissues [13], on invading trophoblast and syncytiotrophoblast cells in placenta [14], and at low levels in some leukocyte subsets [15, 16]. ACKR2 membrane expression is finely regulated by its intracellular traffic properties. Under homeostatic conditions, ACKR2 is mainly localized in intracellular stores associated with both early Rab4/5-positive and recycling Rab11-positive endosomes [17], with a minor pool of receptors undergoing constitutive internalization and recycling [18] and only 10% expressed on plasma membrane. At increasing levels of chemokines, ACKR2 increases plasma membrane abundance through an acceleration of Rab11-depedent recycling pathway, in order to optimize its chemokine degradatory activity [19]. By scavenging chemokines ACKR2 play an essential role in the resolution of inflammatory response and in the regulation of adaptive immunity [12, 20].

The actin and microtubules cytoskeleton has been reported to finely regulate the signalling, endocytosis and intracellular trafficking of chemokine receptors [21, 22]. Here, we reported the first evidence that both actin and microtubules dynamics have an essential role in regulating the intracellular distribution of ACKR2 in basal condition and its up-regulation after chemokine stimulation. ACKR2 trafficking properties are markedly affected by agonistic chemokines, which are bound and degraded with high efficiency, whereas other protease-inactivated chemokines bind the receptor with high affinity but

are not degraded and do not modify its cellular distribution, acting as neutral ligands [23]. As cofilin is one of the most important actin-depolimerizing factor, here we demonstrated that only active, but not neutral, ligands induce cofilin phosphorylation through a G-protein-independent β-arrestin-dependent Rac1-PAK1-LIMK1 signalling cascade downstream ACKR2, leading to actin cytoskeleton rearrangements [24]. The same Rac1-PAK1-LIMK1 signalling cascade is an important node not only in regulating actin polymerization through cofilin but also in inducing the microtubules network reorganization. This cytoskeletal regulation sustains ACKR2 up-regulation and its chemokine scavenging function, after chemokine stimulation.

Regulatory motor proteins are important in protein endocytosis and in regulating the intracellular trafficking of vesicles along cytoskeletal network [25]. We here reported that the membrane recycling system of ACKR2 is finely regulated at molecular level by myosin Vb, a motor protein for actin-based transport of recycling endosomes which associates multiple endocytic compartments and regulates vesicles exit from perinuclear compartments to the plasma membrane, along microtubule network [26-28].

β-arrestin has been reported to act as a scaffolding protein also for ACKRs, including ACKR3, ACKR4 and C5L2 [29-31]. The re-localization of β-arrestins after ACKR2 expression has been already demonstrated [17, 18], and here we analysed the β-arrestins association with ACKR2, showed that ACKR2 is constitutively associated to β-arrestin1 and β-arrestin2 both at plasma membrane that at intracellular compartment levels, but after active ligand stimulation, only β-arrestin1 is recruited to support the scavenging function of ACKR2.

In conclusion, the intracellular distribution of ACKR2 is maintained by cytoskeletal dynamics. After chemokine engagement, ACKR2 activate a G-protein-independent and β-arrestin-dependent Rac1-PAK1-LIMK1 signalling cascade to finely regulate the actin cytoskeletal and the microtubules network reorganization, to promote receptor up-regulation and scavenging function. ACKR2 is able to recruit and associates both β-arrestins in basal condition, at membrane and intracellular levels, but only β-arrestin1 is recruited after active ligand stimulation, in order to promote a β-arrestin1-dependent signalling pathway, required for supporting the myosin Vb-dependent ACKR2 up-regulation and scavenging properties. ACKR2 is not a “silent” receptor, as considered until now, but it presents some structural features that promote β-arrestin association and an unbalanced signalling pathway β-arrestin dependent.

INTRODUCTION

1. THE CHEMOKINES AND CHEMOKINE SYSTEM

1- A. THE CHEMOKINE SYSTEM

Cell migration is an important element involved in different processes of lymphoid tissues development, both in normal and pathological conditions, in the activation and induction of innate and adaptive immunity and in preventing infection in different body compartments by leukocytes. Leukocyte migration is enhanced by different classes of cytokines and growth factors, such as macrophage-colony stimulating factor (M-CSF) and vascular endothelial growth factor (VEGF), which are potent attractants for monocytes and macrophages. However, the main mediators of leukocyte trafficking are molecules such as the complement fragments C5a and C3a, bioactive lipids as sphingosine-1-phosphate, leukotrienes and platelet-activating factor, and chemokines, which are able to interact with G-protein-coupled receptors (GPCRs), belong to the rhodopsine-like family [11].

1- A.1 The repertoire of chemokines

Chemokines are a huge family of inflammatory mediators, belong to cytokines family. They play an important role in leukocytes recruitment, not only in driving directional migration to the site of inflammation through the creation of a chemokine gradient, but also by increasing leukocyte adhesion to vascular endothelium [11]. Over 40 related molecules have been discovered in humans and "chemo-kine" as a family of functionally related small secreted molecules, derived from leukocyte chemoattractant and cytokine-like activities including the regulation of angiogenesis and fibrosis [12], of proliferation of medulla precursor and apoptosis susceptibility [1], involvement in tumor development [32], in embryogenesis and neuronal functionality [33] and in HIV-1 infection [34]. Although most members share functional properties, only structural criteria governed the membership. The chemokine system is highly redundant: chemokines are active on different leukocytes populations that present receptors able to respond to different chemokines.

Chemokines, as cytokines, are mainly secreted by leukocytes in an autocrine or paracrine way, both in constitutive or in an inducing way, and they act through the interaction with seven transmembrane receptors on different leukocytes targets (**Figure 1**) [11]. Chemokines are also classified according to their production, as reported in **Figure**

1. Homeostatic chemokines are produced constitutively, as CXCL12, CXCL13, CCL14 and CCL19 (reported in green in **Figure 1**), while inflammatory chemokine, as CCL2, CCL5 and CXCL8, are produced in response to inflammatory or immunological stimuli (reported in red in **Figure 1**).

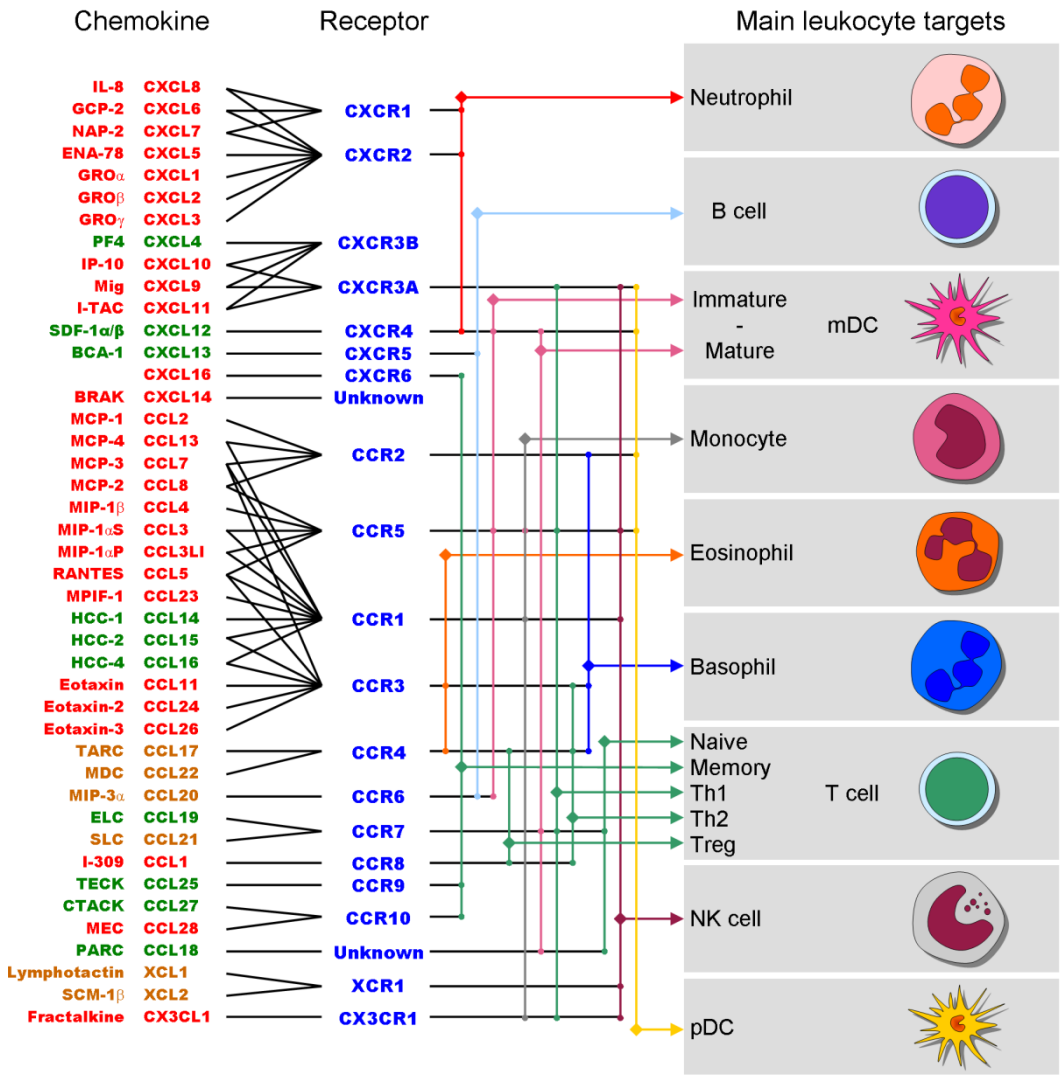

Figure 1: The human chemokine system. In the left column are listed chemokines, identified with the old acronym and the new systematic nomenclature. In red are reported pro-inflammatory chemokines, in green homeostatic one and in yellow those with mixed function. Abbreviations: DC, dendritic cell; NK, natural killer; TH, T helper; Treg cell, regulatory T cell (Figure derived from [11]).

Chemokines are single polypeptide chains of 70-100 amino acid residues in length, with a molecular weight between 8-10 kDa, having 20-95% sequence identity to each other. They present the same structural motif including 4 conserved cysteine residues that create two disulphide bonds between the first and the third cysteine and between the

second and the fourth cysteine [35]. Based on the cysteine spacing and numbering, chemokines are divided into four subfamilies [11, 36] (**Figure 2**).

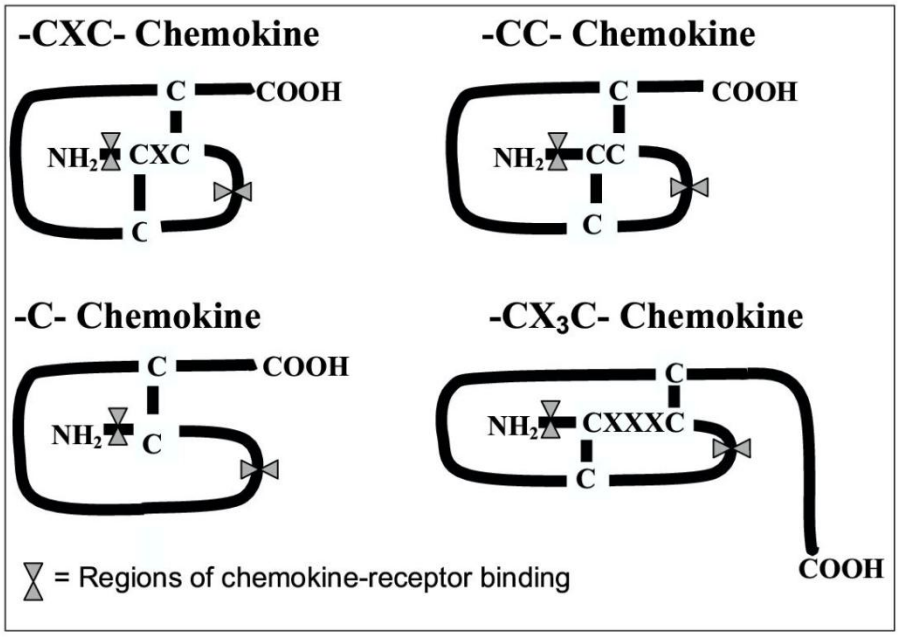

Figure 2: Chemokines subfamily schematic representation. The position of the conserved cysteine residue near the N-terminus is the basis of the classification in four subfamilies. The -CXC- or α chemokines have one amino acid (X) separating two of four conserved cysteine residues. The -CC- or β chemokines have two adjacent cysteine residues. The -C- chemokines have only one conserved cysteine residue. The -CX3C- chemokine, in which the only member is fractalkine, has three amino acids separating two of four cysteines. In figure are also represented the regions of chemokine/receptor interaction (Figure from [37]).

The largest group of chemokines has the first two of total four cysteines in adjacent position (CC or β chemokines) [38]. The major cellular target for this cluster of molecules is represented by mononucleate cells, including T and B lymphocyte, natural killer (NK) cells, monocytes and dendritic cells (DCs). Of the same family, there are also some chemokines as CCL11, that act on neutrophils or eosinophils and basophils cells [39].

The other large group of chemokines has the two cysteines at the N-terminal separated by an amino acid (CXC or α chemokines). The major cellular target is represented by neutrophils, T and B lymphocytes [11].

Both CC and CXC subfamilies include many members, resembling each other more than they resemble members of other subfamilies. More recently, a small number of chemokines that does not fit these structural profiles has been described. A third subfamily includes molecules with only two cysteine residues (C or γ chemokines). This subfamily

includes only two members: lymphotactine or XCL1, with an anti-tumoral activity if associated to interleukine-2 (IL-2) [40], and SCM-1β or XCL2, both of them active on T lymphocytes and NK cells. Only one molecule with three intervening amino acid in-between the first two cysteine residues have been described so far. This molecule is the first representative of a putative fourth chemokine subfamily indicated as CX3C or δ chemokines. The only member of this new family, fraktalkine, may also present a quite new structural organization, since this chemokine has a transmembrane domain which allows it to be tethered to the cell surface. Fraktalkine, acts on monocytes, NK cells and T-helper 1 (Th1) lymphocytes [41].

Chemokine nomenclature is based both on structural features related to cysteine residues position and receptor usage [42]. CC chemokines, being CCR ligands, are indicated as CCL followed by a number provided by the corresponding SCYb coding gene. Similarly, CXC chemokines are named CXCL, C chemokines XCL and CX3C chemokines as CX3CL. Basically, chemokines are now identified by a name providing information on the respective structural subfamily and the type of receptor they engage, followed by a consecutive number provided by and referring to the respective coding gene.

1- A.2 Sources and targets of chemokines

The chemokine sources, targets and regulation vary widely to provide specificity and flexibility in leukocytes trafficking [39]. Some chemokines are produced by different cells type as well as by one. The chemokine production could be constitutive or inducible, and depending on which cell type they are expressed on, they could be both induced or constitutively produced. The inducing agent specificity determines which inducible chemokine is produced. Many chemokines are up-regulated by pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and IL-1; others are up-regulated specifically by interferon-gamma (IFN-γ) and most are down-regulated by the anti-inflammatory cytokine IL-10 [43]. An exception is represented by CCL16, which is up-regulated by IL-10 and could switch off signals during the inflammatory response [44]. Most inducible chemokines are regulated at the transcriptional level, but some are stored for immediate release, as in the case of CXCL4 in platelet alpha granules [45].

Chemokines could act on different cells subtype with different spectra of action. In the CXC subfamily are mainly found chemokine acting on neutrophils, while the CC subfamily attracted mainly monocyte/macrophages, basophils and eosinophils. The target specificity

is also determined by the presence of the tripeptide Glu-Leu-Arg motif (ELR in short) near the N terminus, presents on CXC chemokines that preferentially act on neutrophils. This chemokine subfamily tends to be more restricted, while CC chemokines presents a multiple broad-spectrum. Both CC and CXC subfamilies also contain members specific for T lymphocyte and Th-1 lymphocytes are characterized by specific CC receptors (CXCR3, CCR5) as well as Th2 subsets, expressing CCR3 and CCR4 [46]. While some chemokines act only on a specific receptor, such as CXCL12 acting on CXCR4 in many cell types excluding mature B cells and CXCL13 acting on CXCR5 on mature B cells, other chemokines promiscuously signal on different receptors, as CCL5 acting on CCR1, CCR3, and CCR5, which are differentially expressed on basophils, eosinophils, monocytes, and T cells [39]. Different receptors for the same chemokine can be co-expressed at similar levels on the same cell type, even on the same cell. One proposing model is that different receptors could balance desensitizing and activating signals, promoting the formation of a successive chemotactic gradient.

1- A.3 Chemokines and the multistep model of leukocyte trafficking

The lymphocytes migration to the site of inflammation is driven by selectins, a family of cell surface adhesion molecules produced during the inflammatory responses by the endothelial cells of the vessel walls of the circulatory system. In particular, E and P-selectin direct lymphocytes migration to specific areas of infection, contributing to inflammation resolving and utilized by both the innate and adaptive immune system [47]. Their expression is induced by TNF-α and IL-1, enhanced leukocyte rolling on the endothelial surface, through their interaction with Sialyl Lewis x (sLex)-like glycans, expressed in relatively high numbers by circulating leukocytes. This leukocyte rolling allows other molecules to interact with leukocyte, promoting their extravasation. The binding with E and P-selectin is reversible and tight adhesions to the rolling leukocytes are enhanced by other molecules, as reported in **Figure 3**. Intercellular adhesion molecule-1 (ICAM-1) expressed on endothelial cells binds to the integrin LFA-1 on leukocyte surface, promoting leukocyte arrests and extravasation.

At inflamed sites, leukocytes interact with chemokines immobilized on cells surface by proteoglycans [48], preventing washout by blood flow and promoting the formation of a chemokine gradient, which is followed by leukocytes to the site of inflammation. The full activation of leukocytes is achieved through the simultaneous action of chemokines and

integrins, together with primary cytokines. This process enhances phagocytosis, superoxide production, granule release, and bactericidal activity in vitro.

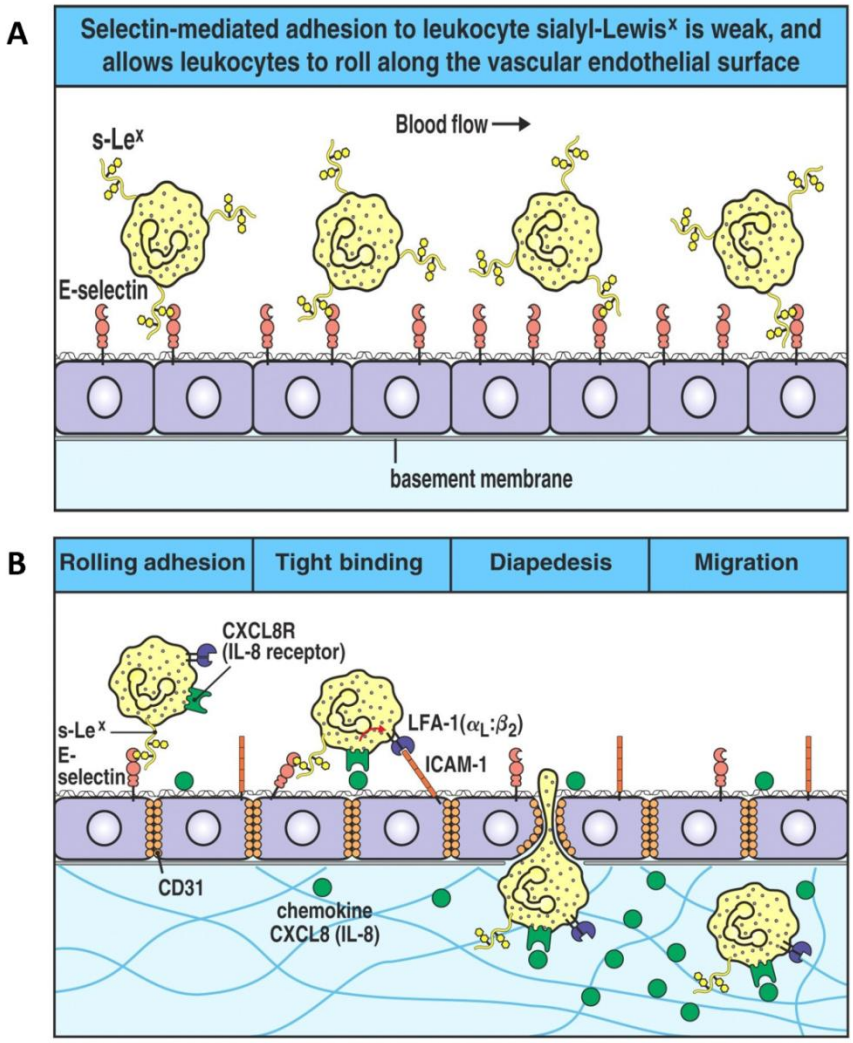

Figure 3: Leukocyte rolling and extravasation. E-selectin expression induced by IL-1 and TNF-α promote leukocyte rolling through the interaction with Sialyl Lewis x (sLex)-like glycans, as shown in panel **A**. A tight binding is achieved after LFA-1 interaction with ICAM-1 expressed on endothelial cells, in order to promote leukocyte extravasation (panel **B**). The leukocyte migration is driven to the site of inflammation by the formation of a chemokine gradient. Chemokines are immobilized on cells surface by proteoglycans and leukocyte interacts with chemokine with seven transmembrane receptors, coupled to G proteins (Figure derived from [47]).

Chemokines drive leukocytes migration to the site of inflammation through the interaction with seven-transmembrane receptor (7TMR) coupled to heterotrimeric G-protein (GPCR), called chemokine receptors.

1- B. CHEMOKINE RECEPTORS

Chemokines drive leukocytes migration through the interaction with chemokine receptors, belong to the rhodopsin-like family, coupled to G proteins.

1- B.1 Chemokine receptors classification and structure

Actually, 19 chemokine receptors, able to bind multiple chemokines in a subclass-restricted manner, are known (**Figure 4**). Thus, their names include the chemokine subclass specificity followed by a number (CCR1-10, CXCR1-6, XCR1, CX3CR1). As for ligands, also for receptors is observed 20-85% amino acid identity to each other, focused in particular in transmembrane and intracellular domains. The significant identity score and some structural aspects indicate that both ligand and receptor families arose from common ancestors by repetitive gene duplication.

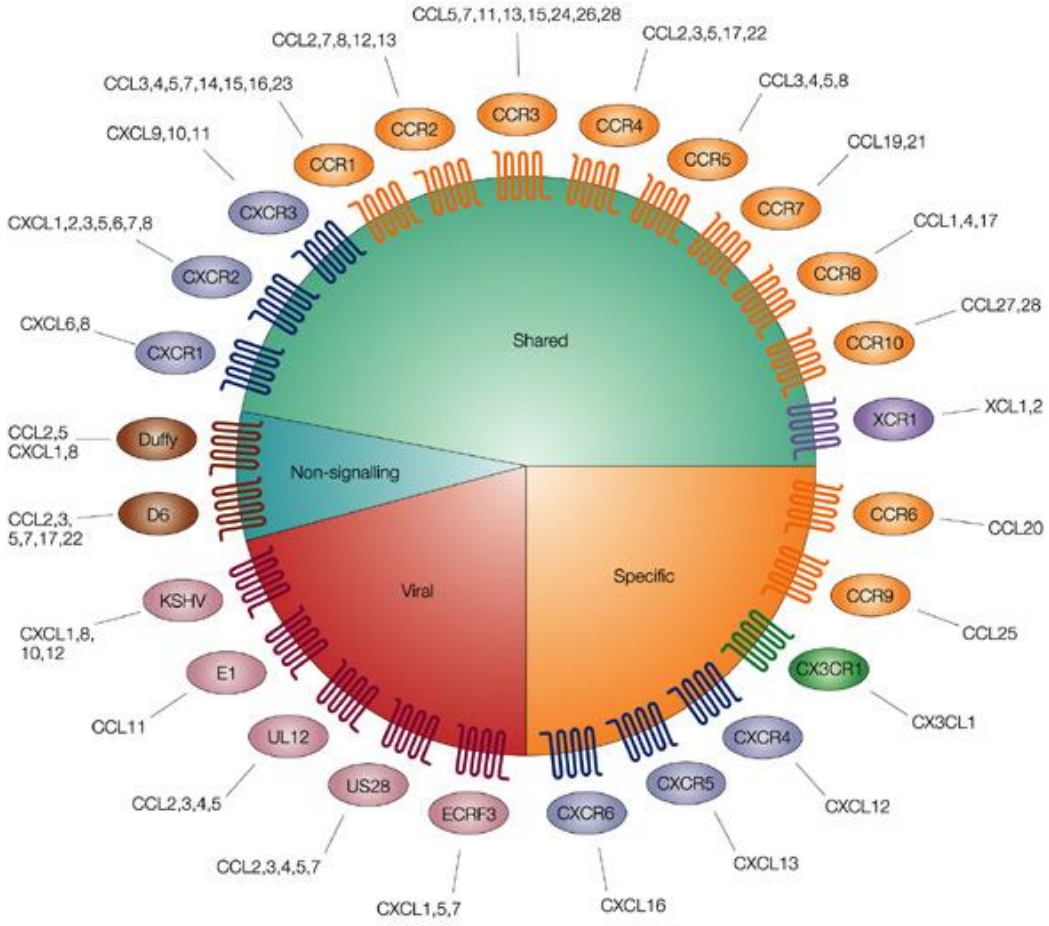

Figure 4: Chemokine receptor classification. Receptors CXCR1-CXCR3, CCR1-CCR5, CCR7, CCR8, CCR10 and XCR1 bind several chemokine. On the other hand, CCR6, CCR9, CX3CR1 and CXCR4-CXCR6 bind only one ligand each. Actually, we distinguish 10 receptors for CC chemokines, identified as CCR1-10, and 6

receptors for CXC chemokines, as CXCR1-6. DARC and D6 (now called ACKR1 and ACKR2, respectively) are considered decoy receptors, able to bind ligands but not to signal, acting as a negative feedback for chemokine (Figure derived from [32]).

Chemokine receptors are single polypeptide chains spanning 7 times the membrane (transmembrane domain, TM), with three extracellular domains and three intracellular domains. The acidic N-terminal domain is mainly involved in chemokine binding, while the C-terminal is rich of serine/threonine residues, important for signalling transduction and receptor internalization.

The acidic N-terminal extracellular domain includes several charged residues most likely involved in ligand binding and recognition, including acidic amino acids and sulphated moieties, presumably tyrosine-linked [49, 50]. In the TM2 domain, the TxP motif is a highly conserved structural determinant, playing an important role in receptor activation, but not in ligand binding. In the same domain, an aspartic acid is present in most GPCRs and is required for receptor activation and signalling, together with the well conserved DRYLAIV motif at the boundary of the second intracellular loop and the TM3 domain. This motif is important in signal transduction, through the coupling with G-protein [11] (**Figure 5**). The C-tail, also referred to 8th helix, contains a serine/threonine-rich domain, involved in receptor phosphorylation and in β-arrestins interaction, which mediate receptor internalization and desensitization [51, 52] (**Figure 5**). Two disulphide bonds between the N-terminal domain and the second extracellular loop and the first and third extracellular loops are normally required for the definition of the molecular structure.

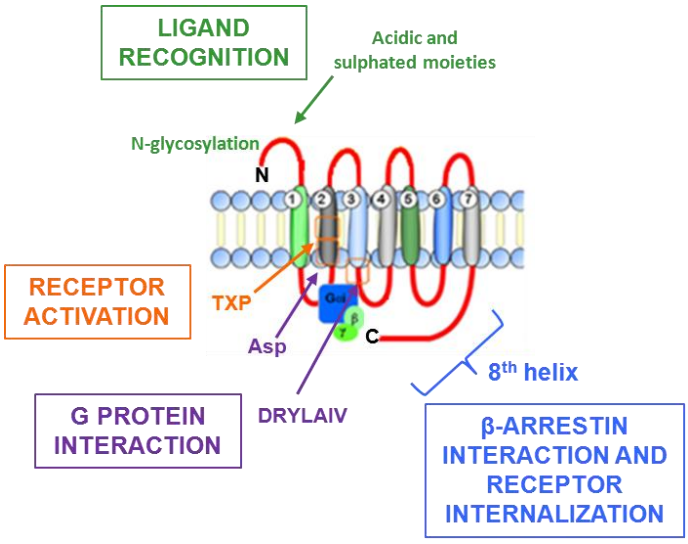

Figure 5: Structure-function relation of chemokine receptors. N-terminal domain shows acidic amino acids, sulphated moieties and N-linked glycosylation sites, which are important for ligand recognition.

The TM2 includes a highly conserved TxP motif, an aspartic acid and the DRYLAIV motif, relevant for receptor and G-protein activation. The C-terminal domain is involved in β-arrestin interaction and receptor internalization (Figure derived from [53]).

1- B.2 Chemokine–chemokine receptors interaction

Structural and mutational studies have been performed in order to identify domains mainly involved in chemokine binding and in receptor activation. In particular, two domains have been identified to be important for chemokine interaction. These two domains are closed by a disulphide bond. The first one is at the N-terminus, and the second one is a static domain between the second and the third cysteine [35, 54].

The receptor activation is mediated by the chemokine N-terminus: for some chemokines, this domain is represented by a single amino acid, as for CXCL12 that after cleavage of the lysin at the N-terminus completely lost its agonist activity and gain an antagonist function on CXCR4 [55]. Otherwise, this motif is represented by the ELR motif, highly conserved on chemokine acting on neutrophils. In general, this motif is represented by the preceding sequence before the first cysteine.

The residues between the N-terminus and the first TM are considered important in conferring specificity to the binding with ligands [56].

The schematic binding is easily represented by the model of interaction between CXCR4-CXCL12 [55]. Following this model, the interaction between ligand and receptor occurs in two steps. Initially, the basic domain including the first two cysteine and the β-sheet of chemokines recognize the N-terminus of the receptor (*docking* domain, reported in **Figure 6** in blue). This first interaction reduces chemokine mobility and enhances the right chemokine orientation. Subsequently, the chemokine N-terminus interacts with the second and third receptor extracellular domain, in order to stabilize the interaction and induce receptor activation (*triggering* domain, reported in **Figure 6** in orange) [55].

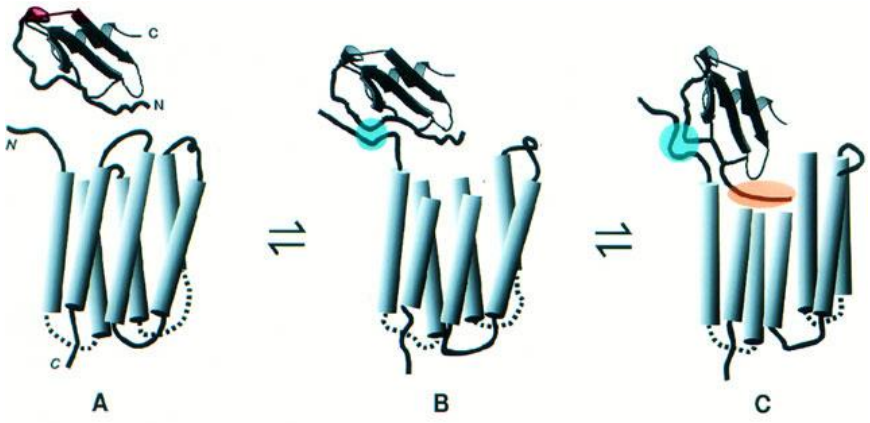

Figure 6: A schematic model for interaction between CXCL12 and CXCR4. (**A**) The receptor and the ligand are indicated separately prior to any interaction. (**B**) The N-terminal domain of CXCL12 interacts with the N-terminal segment of the receptor (reported in blue). (**C**) The N-terminal region of CXCL12 bound with the extracellular loop of CXCR4 (reported in orange). Binding of the N-terminal region results in the activation of the receptor, which is depicted in (**C**) by the change in conformation of the receptor helices compared with (**B**) (Figure derived from [55]).

This model should be considered also for the other chemokine receptors. The docking domain corresponds to the highly affinity domain, while the triggering domain has less affinity. The triggering domain with low affinity is used by the immune system to block the receptor and the immune response. For CCR5, the docking and the triggering domain are the same, in the second extracellular loop [57].

1- B.3 Chemokine receptors signalling

The chemokine receptors have been best characterized to signal through heterotrimeric G-proteins, primarily involving Gi, a signalling pathway *Bordetella Pertussis* Toxin (PTX) sensitive [58]. After chemokine binding, the receptor is activated and Gi protein interacts with the intracellular loop, in particular the DRYLAIV motif [59]. The G-proteins have three subunits: α, β and γ. The Gα subunit interacts directly with the GPCR C-terminal domain, the intracellular loop 2 and 3, and with the G-protein β subunit, which forms a tight complex with the γ subunit. In the inactive state, the Gα subunit binds GDP. Upon ligand binding and receptor activation, GDP dissociates from Gα and it is replaced by GTP, inducing the Gα subunit dissociation from the receptor and from Gβγ complex. Although α subunit of G-proteins has traditionally been regarded to as the major signalling subunit, the βγ subunits are crucial for the activation of many chemokine-induced

pathways that ultimately lead to the physiological response. Two of the major pathways activated by Gβγ are phosphoinositide 3-kinase-γ (PI3Kγ) and phospholipase C (PLC), whereas Gαi proteins mainly inhibit adenylate cyclase and transduce signals through tyrosine kinases such as Src. Taken together, these signalling pathways induce actin reorganization, calcium fluxes and the transcriptional activation of some genes, in order to enhance directional cell migration and cell responses [60] (**Figure 7**).

Cells have developed a mechanism of negative feedback, in order to switch off signals and migration. Receptors are internalized in a mechanism that is not G-protein dependent. After receptor activation, the conformational changes of the receptor induce C-tail exposition and its phosphorylation by G-protein receptor kinases (GRKs), in serine/threonine residues [61, 62]. The phosphorylation promotes binding of arrestins, which sterically block further G-protein interaction and mediate receptor internalization through clathrin-coated pits, inducing signalling desensitization [63] (**Figure 7**). Receptor endocytosis can lead to either receptor lysosomal degradation or its recycling back to the plasma membrane for re-sensitization. Receptor degradation reduce the total number of receptor available on cell surface and consequently cell responses to ligands [64].

Recently, GPCRs have been demonstrated to elicit signals through interaction with the scaffolding proteins β-arrestins, independently of heterotrimeric G-protein coupling. β-arrestin functions as receptor-activated scaffold able to coordinate upstream and downstream component of particular signal transduction pathways, including PI3K/Akt, mitogen-activated protein kinases (MAPKs), the small GTPase RhoA and the actin filament severing protein cofilin, as well as the inhibition of other molecules such as NF-kB and LIMK, leading to a variety of cellular responses [65, 66] (**Figure 7**).

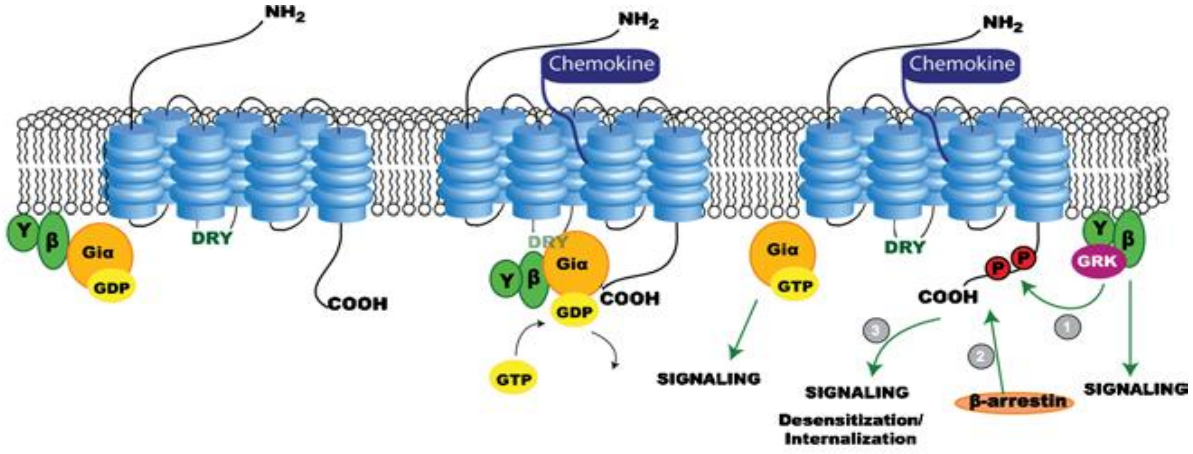

Figure 7: Receptor activation and signalling pathway. After chemokine binding to the extracellular side of the receptor, G-proteins bind intracellular loop and are activated, through GDP-exchange into GTP. α

subunit dissociates from the receptor and from βγ subunits and both of these complexes subsequently activate a variety of downstream effectors that lead to the physiological responses. The C-terminal tail of the receptor is phosphorylated by GRKs (G-protein receptor kinases), promoting binding of β-arrestins and receptor internalization. The receptor could recycled back to the cell surface (in a re-sensitization process) or is degraded (in a desensitization process). In addition, β-arrestins can function as signal transducers by activating different signalling pathways which lead to a variety of cellular responses (Figure derived from [60]).

In general, receptors are capable of activation of G protein-dependent pathways and subsequent regulation and signalling through GRKs and β-arrestin. It is now evident that certain ligands can preferentially activate β-arrestins while blocking or minimally activating G proteins or vice versa [67, 68]. Kohout TA et al. reported a differential CCR7 activation after stimulation with CCL19 and CCL21. Taken together, these data show that, despite CCL19 and CCL21 are equally potent at activating CCR7, only CCL19 is capable of inducing receptor desensitization and phosphorylation, leading to a different β-arrestin recruitment. In addition, only CCL19 induces MAPK activation in a β-arrestin-dependent pathway, suggesting that CCL19 induces a distinct receptor conformational changes, which lead to a similar G-protein coupling properties but different ability to induce C-tail phosphorylation, β-arrestin recruitment and MAPK signalling pathway [69]. For this reason, ligands can be classified also in β-arrestin-biased ligands, as CCL19, for their ability to induce a preferential β-arrestin signalling pathway. On the opposite, Steen A. et al. demonstrated that CCR5 single mutation at the interface between TM6 and TM7 shift the equilibrium in a more active state and in a preferentially G-protein-coupled signalling instead of β-arrestin binding [70]. TM6 and TM7 include key residues to promoting active state of chemokine receptor, and compounds targeting this area very likely could have biased properties. This could be important for the development of biased ligands, β-arrestin but not G protein or the opposite promoting ligands.

Recently, CCR1 has been reported to be a constitutive active receptor that can activate G protein and stimulate cell migration in an agonist-independent manner. It is constitutively phosphorylated and internalized in a β-arrestin-dependent manner, showing complexes formation with β-arrestin and G protein. The β-arrestin complex formation has been suggested to be important for scavenging activity of CCR1, through a G protein independent internalization of chemokines [71].

1- C. CHEMOKINE RECEPTOR TRAFFIC

An important aspect of chemokine receptor function is its intracellular trafficking. Chemokine receptors constitutively internalize and could be degraded or recycled back to the plasma membrane, in a ligand-independent manner. After ligand binding, the chemokine receptor internalization is enhanced, promoting receptor trafficking and dynamics of re-sensitization instead of desensitization, as is promoted the receptor recycling versus degradation (**Figure 8**).

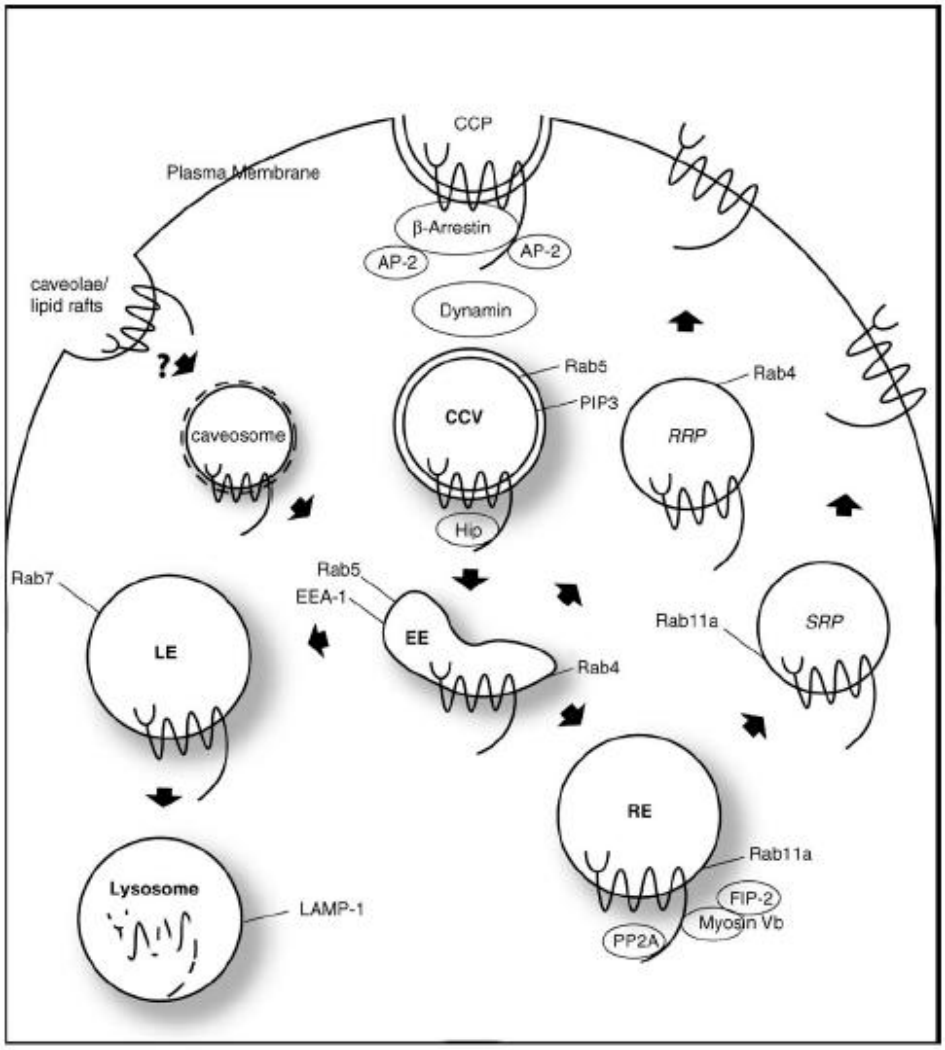

Figure 8: Schematic representation of endocytosis processes. Abbreviation reported are: CCP: clathrin-coated pit, CCV: clathrin-coated vesicle, EE: early endosome, LE: late endosome, RE: recycling endosome, RRP: rapid recycling pathway, SRP: slow recycling pathway, EEA-1: early endosomal antigen-1, FIP-2: Rab11-family interacting protein-2, LAMP-1: lysosomal-associated membrane protein-1 (Figure modified from [72]).

As previously mentioned, the intracellular trafficking properties of chemokine receptors may vary depending on the presence or absence of ligands. Receptors could be internalized in two different ways: clathrin-mediated endocytosis or lipid raft/caveolae-dependent internalization. Some receptors, as CCR5, could be internalized using both of these pathways, while others follow preferentially one pathway. The cell type in which the receptor is expressed may in part determine the utilization of one pathway as compared to another. After ligand stimulation, the receptor fate once internalized may depend on the length, strength, or type of intracellular signals generated. In addition, the post-translational modifications of the receptor can also have major effects on ligand-mediated signalling.

Till now, the intracellular chemokine receptor trafficking is not completely understood. For example, β-arrestin was originally considered to be important for internalization and desensitization. However, recent studies on GPCRs and some chemokine receptors support roles for β-arrestin as an intracellular signalling scaffold and as a mediator of recycling.

1- C.1 The clathrin-mediated endocytic pathway

Clathrin-mediated endocytosis is the preferred mechanism by which chemokine receptors undergo ligand-induced endocytosis (**Figure 8**) [17, 73, 74]. After receptor phosphorylation, adaptor molecules are recruited at C-tail, promoting receptor links to a lattice of clathrin and facilitating receptor internalization. Adaptin 2 (AP-2) and β-arrestin are the two major adaptor molecules demonstrated to have an important role in chemokine receptor internalization. β-arrestin binds with high affinity the phosphorylated receptor, to the β2-adaptin subunit of the AP-2 heterotrimeric protein complex [75, 76] through AP-2 binding directly to some chemokine receptors, in particular at highly conserved di-leucine motifs at the C-terminus [77], in order to mediate endocytosis. The association of receptors with these adaptor molecules mediate the endocytosis, resulting in recruitment of clathrin and formation of clathrin-coated pits, with the creation of clathrin-coated vesicles through the action of dynamin and Rab5 [17, 73, 78]. These vesicles are then uncoated and the receptor-ligand complexes enter the early endosomal (EE) compartment. As suggested by recent findings, β-arrestin is not involved only in desensitization and internalization processes, but it has been reported to control the intracellular trafficking of chemokine receptors. As shown by Orsini et al., β-arrestin

accompanies CXCR4 to EE compartments after CXCL12-induced internalization, as demonstrated by immunofluorescence staining and confocal microscopy analysis [79]. However, it is not completely clear if this endosomal colocalization points to an active contribution of β-arrestin to the endosomal trafficking of CXCR4 or whether it is just a consequence of the β-arrestin binding to both clathrin and CXCR4 during internalization.

1- C.2 Internalization via lipid rafts and caveolae

The intracellular trafficking and endocytosis processes of some chemokine receptors has been reported not be regulated by clathrin-dependent mechanisms. These pathways are mediated by lipid rafts or by caveolae, cholesterol-rich structures [80]. In fact, several GPCRs have been shown to signal in lipid rafts/caveolae [81, 82]. At present, at least two chemokine receptors, CCR5 and CXCR4, have been identified to constitutively associate with some raft proteins, as CD4, supporting their raft localization [83, 84]. In addition, the CCR5 membrane levels are highly affected by inhibition of glycosphingolipid synthesis, suggesting association of CCR5 with rafts for the correct receptor transport. Palmitoylation and other post-translational modifications of chemokine receptors are probably crucial for receptor association with lipid domains present in the membrane.

Lipid rafts contribute to the structure and function of caveolae. Caveolin-1, -2 and -3 determine the shape and the structural organization of caveolae, through their organization and self-assembly in high mass oligomers in order to form a cytoplasmic coat on the membrane invaginations. The endocyted vesicles enter in compartment known as the caveosome and then fuse with EE [85], through a mechanism that remains to be elucidated. As recycling compartments are highly enriched in cholesterol and other raft components, caveolae/lipid raft-dependent trafficking pathways appear to have a role in recycling [86].

The chemokine receptor can then either enter the perinuclear recycling compartment, recycling endosomes (RE) by which receptors can be recycled back to the plasma membrane by a rapid recycling pathway (RRP), usually Rab4-dependent, or by a slow recycling pathway (SRP), Rab11-dependent (**Figure 8**), or it can enter the late endosomes (LE) compartment where it will be sorted to the lysosomal compartment, Rab7-positive, for degradation.

1- C.3 Regulation of trafficking by Rab GTPases

Rabs are small GTPases that regulate cellular trafficking events and exist in an active state, GTP-bound, or in an inactive state, GDP-bound. The exchange of GDP into GTP, GTP hydrolysis, and GDP displacement are regulated by guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and GDP dissociation inhibitors (GDIs), respectively. Interestingly, individual Rab family members are associated with particular endocytic compartments. For example, Rab4/Rab5 associate with early endocytic compartment and Rab11 associates with perinuclear recycling compartment (**Figure 8**).

Rab5 mediates the EE response and vesicle endocytosis. Rab5 binds PI3K and promotes production of phosphatidylinositol 3-phosphate (PI_3P) [87] and together promote vesicle fusion with EE through the recruitment of early endosomal antigen-1 (EEA-1) and other proteins. Rab5 is not important only in chemokine receptor endocytosis, but also in receptor intracellular trafficking, as reported for CXCR2. At early time point after ligand stimulation, the internalized receptor colocalized with Rab5 in EE. The CXCR2 internalization requires Rab5 GTP hydrolysis, as demonstrated by the use of a Rab5 dominant negative mutant, which significantly impairs CXCR2 internalization. [88]. In addition, also CXCR4 and CCR5 internalization is inhibited by Rab5 dominant-negative mutant expression [89].

Vesicles recycle back to the plasma membrane with two main endosomal recycling pathways, a slow and a rapid one, Rab11- and Rab4-depedent, respectively. Rab11 localizes mainly at perinuclear recycling compartment and it is involved in receptors slow recycling processes [90], regulating the intracellular trafficking and the cell responses mediated by chemokine receptors. After chemokine engagement, CXCR2 localizes in Rab11-positive compartment. The receptor recycling is significantly inhibited by the dominant negative mutant of Rab11 (Rab11-S25N) [88]. The recycling properties of chemokine receptors are controlled by two main proteins that interact with Rab11: myosin Vb [28] and Rab11-Family Interacting Protein 2 (FIP2) (**Figure 8**). Expression of the myosin Vb tail (a mutant form of the actin-associated motor protein that is missing the motor domain) and Rab11-FIP2 (129–512) truncated mutant inhibits recycling of CXCR2 and impairs its re-sensitization and it is reported to impair CXCR2- and CXCR4-mediated chemotaxis [88]. These studies demonstrate the potential importance of recycling in chemokine receptor function. The rapid recycling pathway controlled by Rab4 bypasses the Rab11-positive perinuclear endosomes, but the mechanisms by which it occurs remain unclear [91].

Finally, Rab7 mediates vesicles degradation promoting their movement from late endosomes to lysosomes through the interaction with microtubule motor proteins [92]. The desensitization of chemokine receptor is achieved through lysosomal receptor degradation, mediated by Rab7, after prolonged exposure of chemokine receptors to ligands. Expression of a dominant negative mutant of Rab7 (Rab7-T22N) reduced localization of CXCR2 to lysosomal compartment after prolonged ligand treatment and increased its colocalization with early and recycling compartment, Rab5- and Rab11-positive, respectively. These data clearly suggest a Rab7 role in regulating CXCR2 transfer to lysosome after prolonged chemokine exposure [88].

1- C.4 Regulation of receptor recycling

The mechanisms regulating chemokine receptor recycling and factors mediating the fate of the internalized chemokine receptor are not completely clear. The differential recycling of chemokine receptor is regulating by different factors, including the duration and concentration of ligand stimulation as well as sorting motifs located in the intracellular domains of the receptor. In fact, the duration of ligand stimulation seems to determine the fate of chemokine receptor, the recycling/degradation sorting decision. For example, at early time points after chemokine engagement, CCR5 is reported to localize at plasma membrane and at recycling endosome and at late time points, it localizes at late endosomal compartments[93].

Due to the redundancy of chemokine system, in which receptors bind multiple ligands with high affinity, the receptor recycling dynamics could be regulated by the various ligands differentially bound. For example, studies on the CCR4 receptor show different internalization and recycling patterns for the two ligands, CCL22 and CCL17. CCL22 causes a more rapid internalization and recycling of the receptor when compared to CCL17 [94].

The recycling mechanisms are reported to be regulated by actin dynamics, as demonstrated for CXCR1 and CXCR2, in which the receptor recycling occurs through actin filaments and is regulated by actin-related kinases. Cytochalasin D treatment, an actin depolymerizing agent, of CXCR1 and CXCR2 positive cells results in receptor decreased recycling. Further investigation into actin-dependent recycling of CXCR1 and CXCR2 reveals that the recycling of these receptors is regulated by actin-related kinases, which requires phosphorylated residues in the C-tail of receptors. The C-tail phosphorylation has been associated to alterations in actin rearrangements, required for the movement of

endosomes [95]. Also CCR5 internalization and recycling have been reported to be regulated by actin polymerization and activation of small G proteins in a Rho-dependent manner [21]. CCR5 recycling is finely regulated by actin cytoskeletal reorganization, as suggested by cells treatment with cytochalasin D [96]. Taken together, these results suggest the importance of the chemokine receptor C-terminus in the regulation of intracellular recycling pathways. Recent findings demonstrate that the recycling system to and from the plasma membrane is finely regulated at molecular level by motor proteins such as myosin Vb, which regulates actin-based transport of recycling endosomes, associating multiple endocytic compartments and regulating vesicles exit from perinuclear compartment to the plasma membrane along microtubules network [27, 28]. The expression of the myosin Vb tail, a truncated form of myosin lacking the motor domain, able to displace endogenous myosin Vb and disengaging cargoes from F-actin filaments, inhibits CXCR2 recycling [95].

It is possible that ligand dissociation from the receptor after endosomal pH decrease is important for inducing recycling of some GPCRs. This is not reported for chemokine receptors, as suggested by studies on CCR5, in which ligand dissociation from CCR5 by vesicular acidification is not necessary for receptor recycling [93]. Vesicular acidification has not been completely studied and its role in chemokine receptor trafficking is not well understood. Vesicular acidification could be an important regulatory mechanism, not inducing only ligand dissociation and should be further investigated.

It is not known whether dephosphorylation is necessary for the recycling of all chemokine receptors. It appears that recycling may not require dephosphorylation of the C-terminal residues, but it is required for establishing functionality of recycled receptor on the plasma membrane. Furthermore, the recycling rate of different chemokine receptors varies, but the mechanisms responsible for this discrepancy are poorly understood. It seems that the affinity of β-arrestin interaction may influence the rate of recycling of the receptor, as suggested by recent studies of other GPCRs. In fact, a strong affinity interactions result in slow recycling or degradation, while low affinity interactions may result in rapid recycling [97].

1- D. THE CHEMOKINE SYSTEM REGULATION

The chemokine system can be regulated at various levels. In terms of production, chemokines can be distinguished in homeostatic or inflammatory chemokines. In addition,

chemokines undergo post-translational processing by proteases. Finally, chemokines can be internalized and degraded by chemokine decoy receptors. Taken together, all these events act coordinately to finely regulating the lymph node reaction and the local inflammatory processes (**Figure 9**).

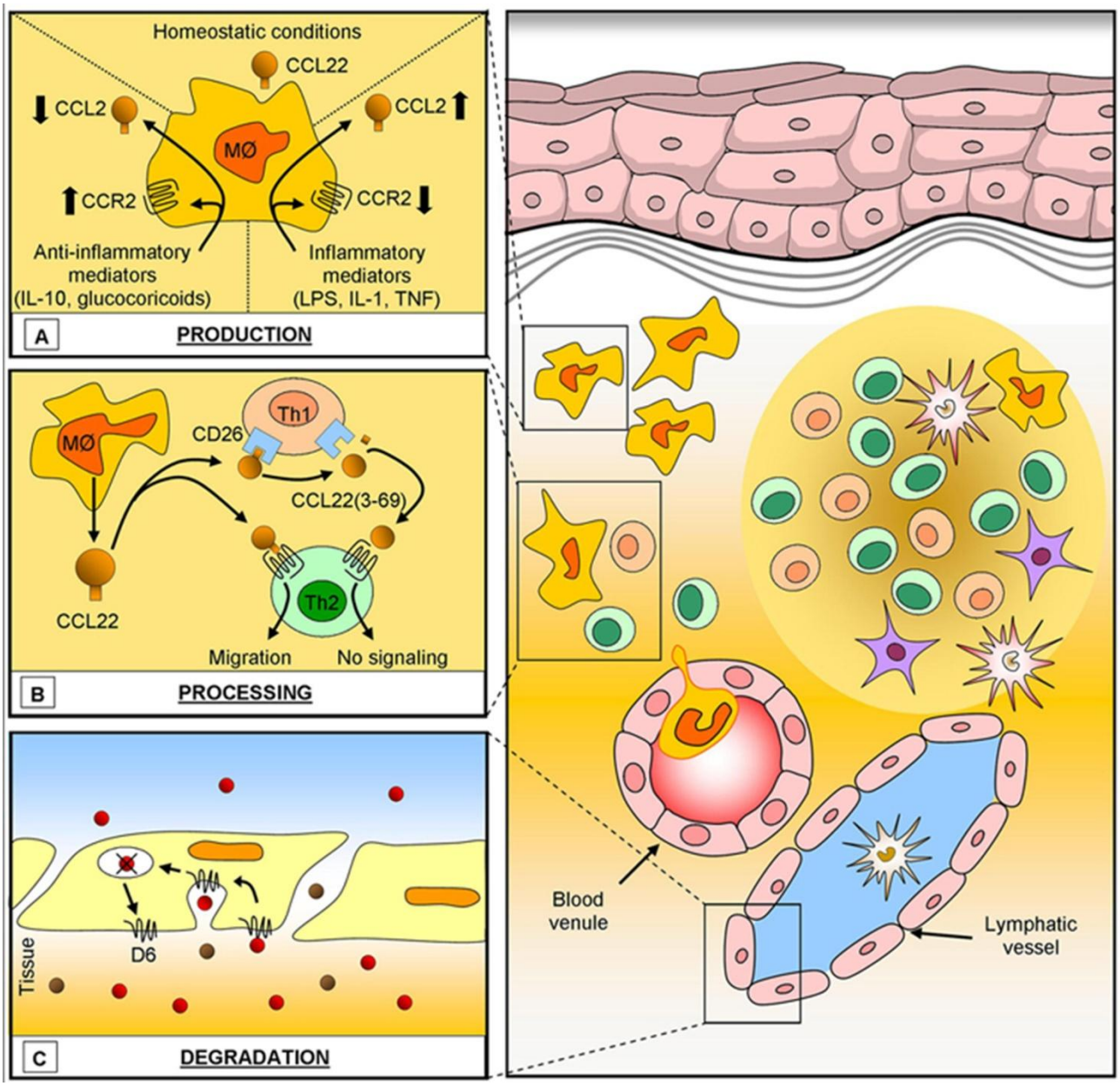

Figure 9: Schematic representation of chemokine regulation. (**A**) Homeostatic chemokines are constitutively expressed and control leukocyte homing in normal conditions. Inflammatory chemokines production is regulated by pro- and anti-inflammatory mediators. (**B**) In certain cases, chemokines undergo post-translational processing by proteases, as CCL22, which is cleaved by the Th1-mediated CD26-dependent protease, and the processed form of CCL22 is biological inactive. (**C**) Chemokines can be internalized and degraded by chemokine decoy receptors, such as ACKR2, previously called D6, expressed on draining lymphatic vessels (Figure derived from [98]).

1- D.1 Regulation at the level of chemokine production

Depending on their regulation and production, chemokines can be distinguished in "homeostatic" chemokines, produced in a constitutive manner in lymphoid tissue, which control leukocyte homing and lymphocyte recirculation in normal conditions, and "inflammatory" chemokines, produced in response to inflammatory stimuli, like TNF or IFN, in order to recruit leukocytes during inflammation (**Figure 9A**) [39]. Inflammatory chemokines generally regulate migration of monocyte/macrophages and T cells. In addition to homeostatic and inflammatory chemokines, there are also "mixed" chemokines, with a mix function, as fraktalkine, highly expressed at cerebral level as homeostatic chemokine, but that could be induced in endothelial cells by TNF-α [40].

Chemokines play a role in the polarization of type I and type II immune responses, supporting selective recruitment of polarized T cells and specific type I and type II effector cells, expressing distinct panels of chemokine receptors [46]. Chemokines are also considered molecular markers of different types of inflammatory macrophages, as a different panel of chemokines characterizes M1 and M2 macrophages, involved respectively in type I and II immune response [43].

1- D.2 Regulation at the level of chemokine processing

Post translational modifications naturally occur for both CXC and CC chemokines and strongly affect their biological activities [99]. Several proteases, including metalloproteinases, thrombin and plasmin, act on different chemokines leading to the production of distinct variants that differ by the extension of the N-terminal or, less frequently, C-terminal region. Several evidences indicate that extracellular processing of chemokines by proteases is an important way to regulate the activity and the function of the chemokine system in different physiological and pathological conditions. As an example, CCL8(6-76), a truncated variant of CCL8, is biologically inactive and acts as a receptor antagonist by blocking the chemotaxis induced by CCL5 and CCL2. Similarly, the Th1-restricted membrane protease CD26 cleaves the CCR4-agonist CCL22, generating two truncated forms of the chemokine, CCL22(3-69) and CCL22(5-69), with reduced capacity to interact with their cognate receptor CCR4, thus blocking Th2 recruitment (**Figure 9B**) [100]. Conversely, the homeostatic chemokine CCL14 circulates in plasma at elevated concentrations as an inactive form CCL14(1-74), and becomes fully active only after amino

terminal processing mediated by proteases associated with inflammatory reactions, generating CCL14(9-74) truncated form. It could be further processed into an inactive form, CCL14(11-74) [101].

1- D.3 Regulation at the level of receptor expression and GAG interaction

The presence of the appropriate receptors in different cell populations dictates the spectrum of action of different chemokines. Chemokine receptors, as their ligands, are subjected to expression control, so that several receptors are detected exclusively in specific cell states. As an example, DCs maturation process is characterized by a complete switch of the chemokine receptors' expression pattern, with down-regulation of receptors for inflammatory chemokines (CCR2 and CCR5 among others) and selective up-regulation of CCR7, which drives the mature cell to the draining lymph node. As a general rule, pro- and anti-inflammatory mediators display divergent effects on agonist production and receptor expression, such as in the case of the CCL2-CCR2 axis on monocytes and DCs [102].

Chemokines are also presented on endothelial cells at the luminal surface by the interaction with glycosaminoglycans (GAG). The positive charge on chemokine leads to the interaction with the negative charge of GAGs, expressed on endothelial cells. In this way, chemokines are not washed out by the blood flux, but leukocytes can recognize and bind them. The site of interaction with GAG can be different for the various chemokines. For example, CXCL8 binds GAG with the α-helix at the C-terminus, while CCL5 interacts with GAG through the domain between N-terminus and the first β-sheet [103]. There are different types of GAGs, and they could vary based on cell type, localization and inflammation state. The selective immobilization of chemokine in a specific site could be a positive feedback mechanism to determine chemokine functionality in different tissues and inflamed states [104].

1- D.4 Regulation by chemokine decoy receptors

Chemokine regulate leukocyte traffic and cell migration through the creation of a chemokine gradient. After receptor binding, chemokines induce receptor activation and second messanger production in order to induce cell migration and cell responses. Chemokine decoy receptor regulates the formation of the chemokine gradient through the

transportation of the ligand through the cytoplasm, in a transcytosis process, or in other cases through internalization and degradation of ligands (**Figure 9C**). D'Amico et al. demonstrated that inflammatory signals lead to a CCR1, CCR2 and CCR5 up-regulation, but they do not elicit cell migration, but are mainly involved in sequestering and scavenging inflammatory chemokines. Thus, in an inflammatory environment, IL-10 generated functional decoy receptors, which act as molecular sinks and scavenger for inflammatory chemokines [105].

Cardona et al. show how signalling chemokine receptors are implicated also in ligand homeostasis. In fact, receptors deficient mice present increased levels of constitutive ligands as CX3CL1, and of inflammatory ligands, as CCL2, CCL3, CXCL1, and CXCL10 in circulation and in brain. The molecular mechanism that couples signalling chemokine receptors and decoy receptor to their scavenging functions is still unknown [106].

In addition to this class of receptor, we can distinguish also the atypical chemokine receptors family, not able to transduce canonical G-protein dependent signalling pathway. This class of receptor competes with canonical receptor, by sequestering chemokines and inducing their degradation, acting as negative regulators of inflammation. These receptors are also considered decoy receptors [11].

2. THE CHEMOKINE DECOY RECEPTORS

The concept of receptor was first formulated by Langley, and subsequently by Ehrlich, at the beginning of the 20th century [107]. The definition of a receptor implies binding of a ligand, usually with high affinity and specificity, and the elicitation of a cellular response to that ligand. In the last few years, receptors capable of recognizing their ligands with high affinity and specificity, but which are structurally incapable of signalling have been described. These receptors are also known as decoy receptors. The first decoy receptor identified in the early 1990s was the type II interleukin-1 receptor (IL-1RII) that binds IL-1 but is not part of a signalling receptor complex. Evidence suggests that IL-1RII, in transmembrane or soluble (sIL-1RII) form, acts as a molecular trap for the agonist, functioning as a negative regulator [108].

2- A.1 Functional decoy receptors

Functional decoy receptors in the chemokine system have recently been described [105]. They are structurally identical to signalling receptors, but under appropriate environmental conditions are uncoupled from the signalling machinery and therefore do not transduce an intracellular signal; nevertheless, they bind and sequester their specific ligands, behaving as decoy receptors. The expression of chemokine receptors that lack the ability to generate an active intracellular signal following ligand-engagement has been described in many cell types. For instance, germinal center B cells express CXCR4 but do not migrate in response to CXCL12 [109], and circulating B cells do not respond to CCL20 in spite of CCR6 expression on their surface [110]. Furthermore, the expression of CXCL8 receptors was reported on DCs [111] and monocytes [112] in the absence of a detectable activation of chemotaxis in response to CXCL8. Interestingly, cell activation can convert these silent receptors into signalling receptors [109, 112]. The particular environmental conditions are required for decoy receptor induction and consist of the simultaneous exposure of the cells to a classical inflammatory stimulus lipopolysaccharide (LPS), LPS and IFN-γ, or TNF and to the anti-inflammatory cytokine IL-10. IL-10 antagonizes the well-known down-modulation of the chemokine receptors CCR1, CCR2 and CCR5 induced by LPS on monocytes and DCs [105]. However, in spite of high expression of these chemokine receptors, the cells are unable to migrate in response to CCL3, CCL4, CCL5 and CCL2. These chemokine receptors on the surface of cells treated with LPS and IL-10 are uncoupled and unable to signal, yet they bind and sequester relevant agonists. Moreover, bone marrow-derived mouse DCs exposed to a pro-inflammatory signal (TNF) and IL-10 failed to migrate in response to CCR1 or CCR2 ligands in vitro, but sequestered CCL2 from sites of inflammation.

2- A.2 Structural decoy receptors

After the initial observation in the IL-1 system, decoy receptors have emerged as a general strategy conserved in evolution to tune the action of cytokines and growth factors [39, 113], through competition with signalling receptors for the ligand [39]. In addition, in some cases, decoy receptors target the agonist for endocytosis and degradation, thus acting as scavengers [114].

A new nomenclature for atypical chemokine receptors (ACKRs) has been recently

proposed [2]. This subfamily includes Duffy antigen receptor for chemokines (DARC, now called ACKR1) [115, 116], D6 (now called ACKR2) [117, 118], CXC chemokine receptor 7 (CXCR7, now called ACKR3) [119], chemoCentryx chemokine receptor (CCX-CKR, now called ACKR4) [120], CC chemokine receptor-like 2 (CCRLs, now called ACKR5) [121] and C5L2 [122] (**Figure 10**).

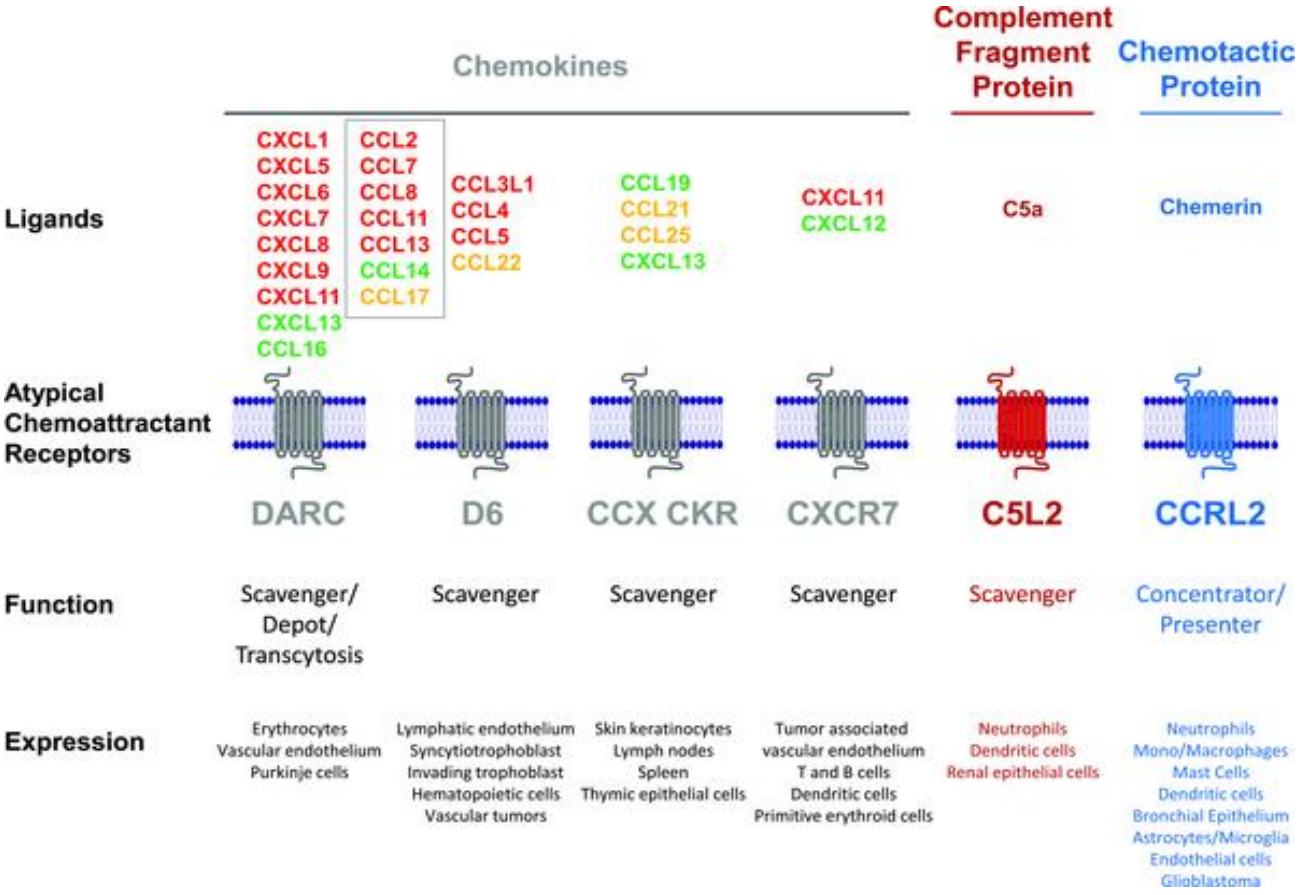

Figure 10: Atypical chemokine receptors ligands specificity, distribution and function. For each receptor is indicated the function and the expression in various tissues. Chemokine are divided in pro-inflammatory, homeostatic and mixed chemokine, reported in red, in green and in yellow. The ligands shared by ACKR1 (DARC) and ACKR2 (D6) are in the grey box [123].

These molecules are referred to as "silent", although this definition has intrinsic limitations. Detailed structure–function analysis of this receptor subfamily is not feasible yet, but it is interesting to note that, although they present high sequences homology, structural determinants that are required for receptor signalling are not conserved in these receptors. They are not involved in cells activation and leukocyte migration, but they play a non-redundant role in inflammatory regulation, by shaping the chemokine gradient.

Their unusual expression patterns and broad chemokine-binding profiles also distinguish this class of receptor from other chemokine receptors (**Figure 11**). ACKR1 is

mainly express on endothelial cells and erythrocytes, suggesting a role in chemokine sequestering and ligand transportation in vassal lumen, through a transcytosis process [124]. ACKR2 is expressed on endothelial cells of afferent lymphatic vessel and has an anti-inflammatory activity by degrading pro-inflammatory chemokine and preventing chemokine transportation to lymph node [5, 125]. ACKR4 is expressed on DC and it is involved in formation of tertial lymphoid structure, controlling agonist levels for CCR7 and CXCR5, through its scavenging activity [11]. ACKR4 can also present antigen on its surface, as demonstrated for immature DCs and T lymphocytes [7]. ACKR5 is expressed on mast cells and it present chemokines to leukocytes [8]. C5L2 is found in granulocytes and immature DCs, and it is mainly involved in C5a removing from the extracellular environment [122].

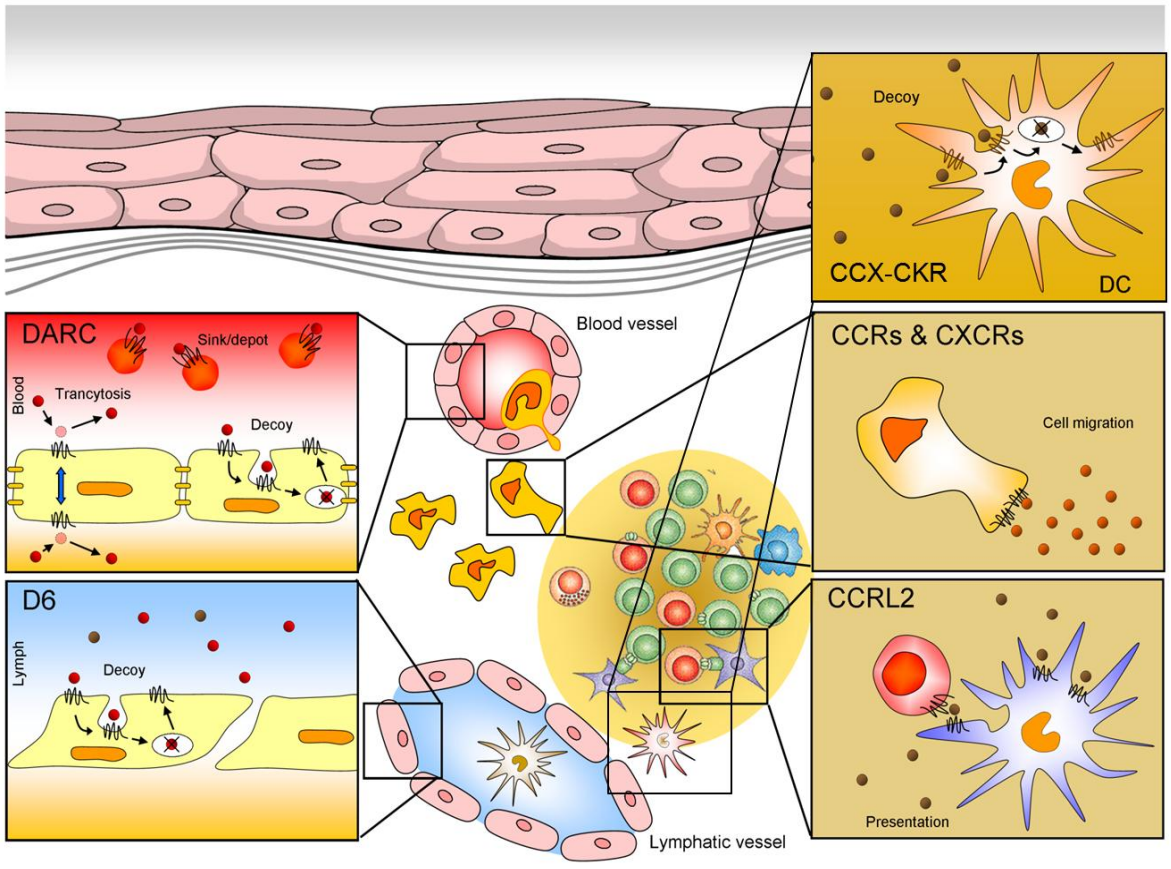

Figure 11: Multiple roles of chemoattractant receptors during leukocyte recruitment. During leukocyte recruitment, conventional chemokine receptors (CCRs and CXCRs) are expressed at the leading edge of migrating leukocytes and induce directional leukocyte migration by G protein-dependent signal transduction pathways. Atypical chemokine receptors play important roles in the leukocyte recruitment process, serving different functions. In particular, ACKR1 (DARC) on vascular endothelial cells transports chemokine to cross biological barriers; ACKR5 (CCRL2) on mast cells presents chemokines to leukocytes, and

ACKR2 (D6) and ACKR4 (CCX-CKR) remove ligands from the extracellular environment, shaping the chemotactic gradient (Figure modified from [126]).

2- A.3 Structural modification of ACKRs

ACKRs act as negative regulator of inflammation and adaptive immunity. Although they present high amino acids identity with canonical chemokine receptors, they present altered structural determinants that impair conventional signalling activities through G protein.

The first structural mutated motif is DRYLAIV, located at the end of the third transmembrane (TM). It is highly conserved in chemokine receptor, as mentioned before, presenting almost 100% of conservation. This motif is important in receptor activation, being involved in GTP exchange of G-proteins. ACKRs lack this motif, showing a modified DRYLAIV sequence, and the alteration presented are highly conserved across the species, suggesting a selective pressure to maintain a specific altered motif, as reported in **Figure 12** [127].

	CXCR7	D6	CCXCKR	CCRL2	DARC	C5L2
Homo sapiens	DRYLSIT	DKYLEIV	DRYVAVT	QRYLVFL	GHRLGAG	DLCFLAL
Mus musculus	DRYLSIT	DKYLEIV	DRYWAIT	QGYRVFS	NPRLNIG	DLFLLAF
Rattus norvegicus	DRYLSIT	DKYLEIV	DRYWAIT	QGYRVFS	---	DLFLLAF
Bos taurus	DRYLSVA	DKYLEIV	DRYWAVT	QRYKEFF	GPQLGAG	DLCLLAL
Canis familiaris	DRYLSIT	DKYLEIV	DRYWAVT	QRYLVFI	GPKLCAG	---
Sus scrofa	DRYLSVA	DKYLEIV	DRYWAVT	QRYQKFF	---	---
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Figure 12: The DRYLAIV motif in ACKRs. Alignment of the DRYLAIV sequences present in the ACKRs, comparing different species. Asterisks indicate conserved acids, colons indicate substitution with similar residues [127].

The correction of DRYLAIV motif is not enough to restore missed G protein signalling activities. In fact, replacement of the second intracellular loop of ACKR3 with the corresponding CXCR4 does not result in a G protein competent signalling receptor [128]. Also modified intracellular motif into DRYLAIV only partially restore signalling competency in ACKR2 [129] or in C5L2 [9]. At the same way, PTX treatment of canonical chemokine receptors CCR5 does not confer ACKRs feature [24]. These clearly demonstrate that in canonical chemokine receptor are present other features that induce receptor activation and G-protein coupling.

Cristallographic analysis of 7TM receptor reveals micro-switch domains important for G-protein coupling, in addition to DRYLAIV motif, including the CWxP motif in the TM6 domain and the NPxxY$^{5\text{-}6}$F motif in the TM7 domain [130], which represent the most likely structural elements important for the functional differentiation between the chemokine receptors and atypical chemokine receptors subfamily [131], as reported in **Figure 13** [127].

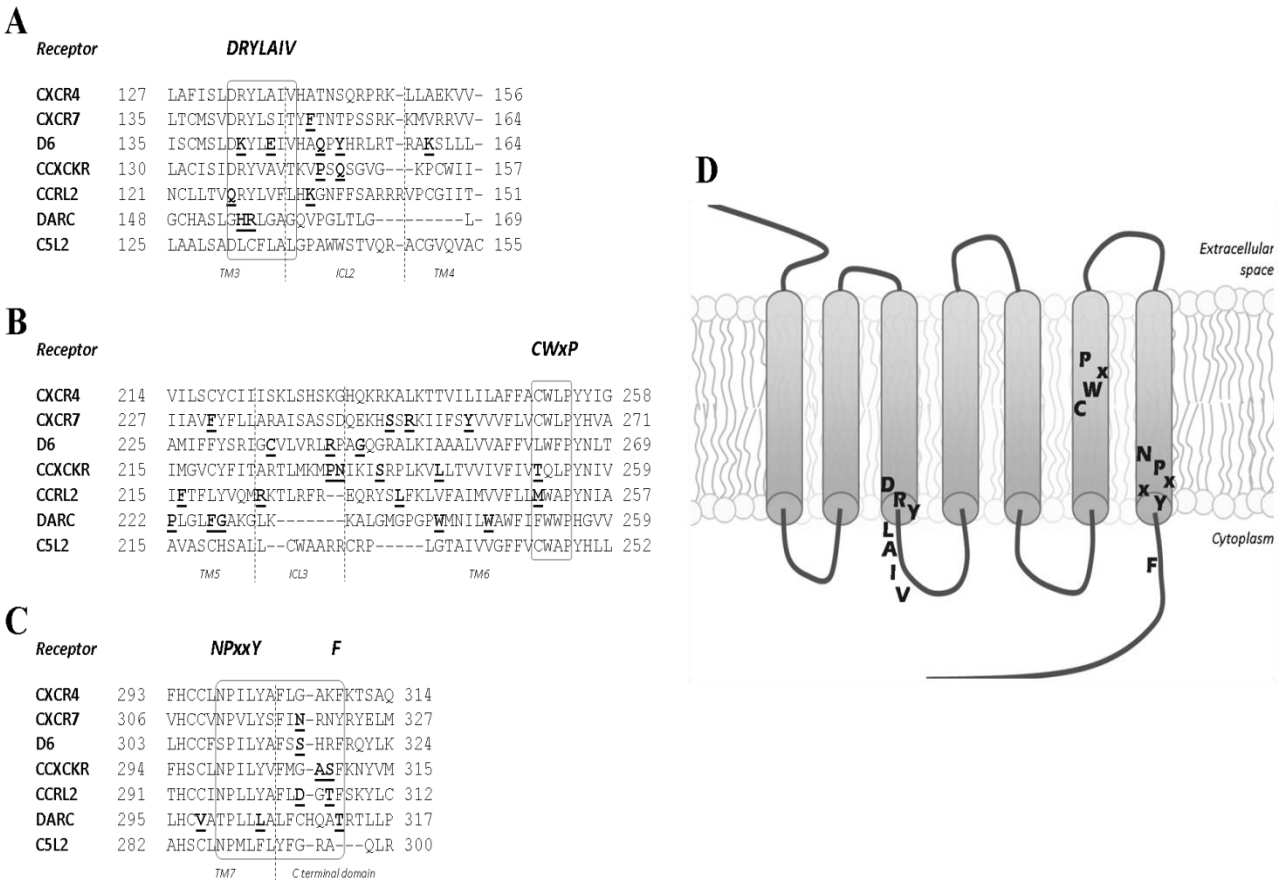

Figure 13: Micro-switch elements in ACKRs. In panel **A**, **B** and **C** are reported the DRYLAIV motif in TM3, the CWxP motif in TM6 and the NPxxY$^{5\text{-}6}$F motif in TM7, respectively. CXCR4 is shown as canonical chemokine receptor and the consensus sequence observed across all conventional chemokine receptors are enclosed by grey boxes. The position of the first and the last residues are indicated. Amino acids residues statistically divergent from the consensus sequence are underlined and in bold, as indicated by Daiyasu et al. [131]. In panel **D** is represented an ACKR with the micro-switch element in evidence (Figure from [127]).

In addition to micro-switch elements, other highly conserved sites in the TM regions of conventional chemokine receptors are not conserved in ACKRs. Several of these residues are located in TM3 or ICL2, which are important in G-protein coupling and receptor

activation. These modifications may be involved in loss of conventional signalling functions by ACKRs.

It is noteworthy that, although ACKR present modifications in intracellular loops required for G-protein coupling, they are also preferentially located in intracellular compartments, maybe reducing their ability to associate and activate a G-protein dependent signalling pathway. The ACKRs lower expression on the cell membrane may result in a reduced number of ligand-receptor binding, reflecting the weak activation of G-protein signalling, not enough to support cell migration and calcium fluxes. Residual G-protein activation reported for some ACKRs, as mentioned below, may sustain some presently unknown biological activities, not related to their classical functions of shaping chemokine gradient.

The ACKRs C-tail is structurally different comparing to canonical chemokine receptors. The β-arrestin relocalization at plasma membrane is induced by conventional chemokine receptors activation upon ligand engagement, in order to promote receptor desensitization and internalization to avoid further stimulation. Some structural features presented in ACKRs provide them to be β-arrestin biased receptors, suggesting that β-arrestins is important in receptor internalization and recycling. The serine/threonine residues present in the ACKRs C-tail seem to be important in β-arrestin interaction. Although the phosphorylation status of these residues is not clear, they are reported to be conserved across different species, as showed in **Figure 14**. Moreover, β-arrestin has been reported to influence receptor stability, trafficking and chemokine uptake in several ACKRs, including ACKR2, suggesting a β-arrestin potential role in ACKRs biology and functions.

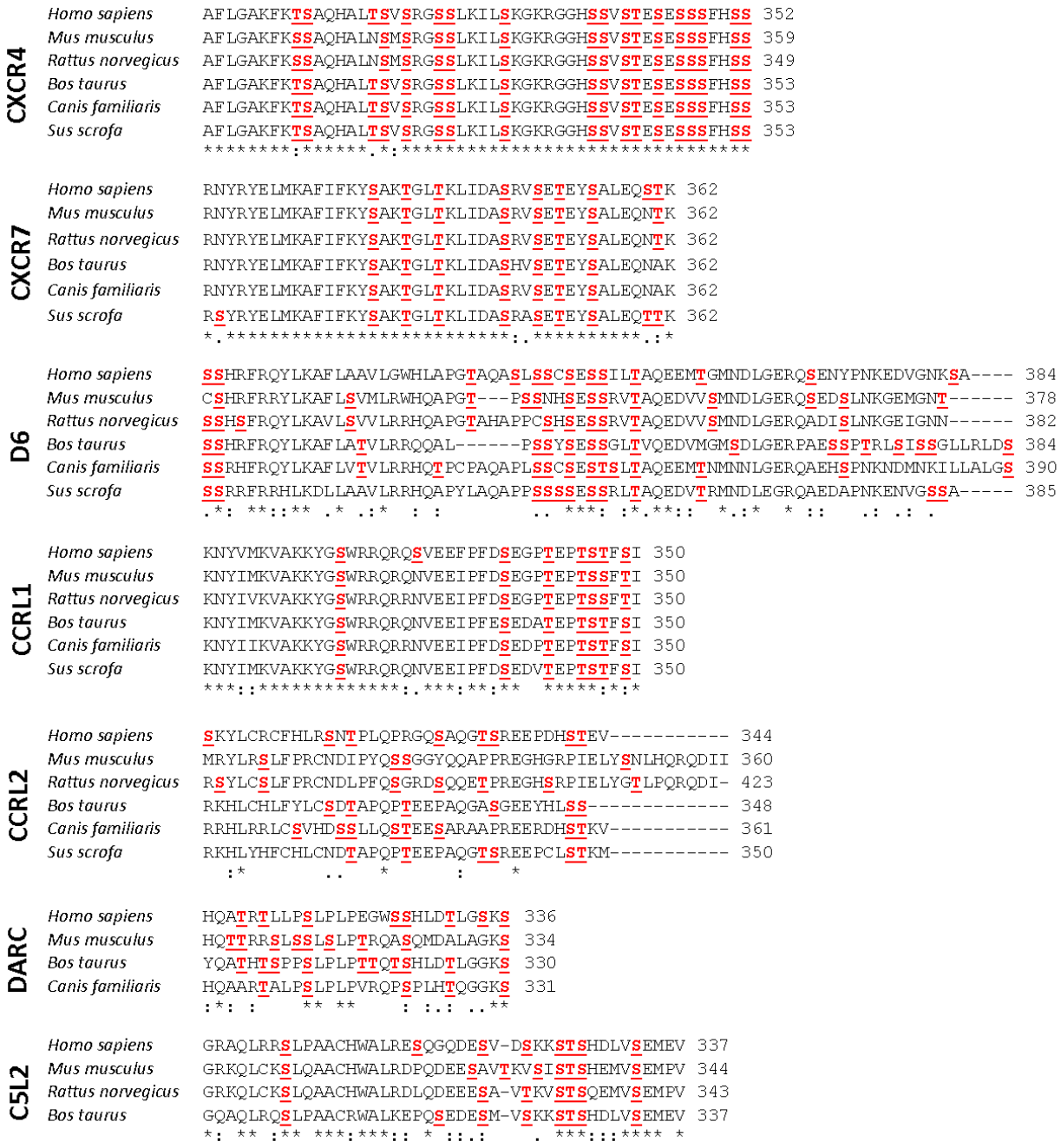

Figure 14: Alignment of ACKRs C-terminus of different species. The position of the last residue of aligned sequences is indicated. Serine/threonine clusters are coloured and highlighted in red. Asterisks indicate conserved amino acids, while colons indicate substitutions with similar residues (Figure from [127]).

2- A.4 ACKRs intracellular trafficking properties

ACKRs have been described to internalize through both clathrin-coated pits and caveolae (**Figure 15**). ACKR2 is reported to internalized through clathrin-coated pits, in a mechanism that is dynamin, Rab5 [19] and β-arrestin dependent [18]. The same internalization pathway has been observed for ACKR3 (Borroni E. unpublished

observations). Conversely, ACKR4 internalization seems to be caveolae-dependent, as demonstrating by the overexpression of wild-type caveolin-1 [132]. The ACKR4 internalization pathway requires dynamin, but not β -arrestins or clathrin-coated pits. As ACKR4, also ACKR1 is targeted into caveolae after internalization into polarized cells [133].

Like the conventional chemokine receptors CXCR3 and CXCR4, ACKR2 and ACKR4 constitutively internalize, in a ligand-independent manner [18, 132]. Due to its constitutive internalization, ACKR2 is mainly stored at intracellular compartments and only 10% of receptors are detectable on cell surface [125]. Also ACKR3 has been found predominantly in intracellular endosomes and only a small amount of receptor partially colocalizes with the early endosomal marker EEA1 [134]. After CXCL11 and CXCL12 stimulation, ACKR3 internalization is promoted in lymphocytes [135]. Conversely, ACKR5 is predominantly expressed on cell surface and only a small pool is present within the cytoplasm. This receptor is not internalized either in absence or in the presence of ligands [8].

ACKR2 is constitutively associated with both early (Rab4/5 positive) and recycling endosomes (Rab11 positive) [19] but not with lysosomes (Rab7 positive) [17] (**Figure 15**). Differently from canonical chemokine receptors, after ligand stimulation ACKR2 increases its membrane expression through a Rab11-dependent mechanism, optimizing its scavenging activity [19]. Once internalized, chemokines dissociates from the receptor and they are targeted to degradation while the receptor is recycled back to the plasma membrane through both rapid and slow recycling pathways. A similar ligand-dependent up-regulation has been described for ACKR4 [132], though in this case the redistribution mechanism involved has not been elucidated yet. Conversely, ACKR3, mainly localized in early and recycling endosomes, decreased its membrane expression after chemokine stimulation (Borroni EM, unpublished results).

Thus, it is tempting to speculate that some signal events activated upon ligand engagement might regulate receptor cycling events.

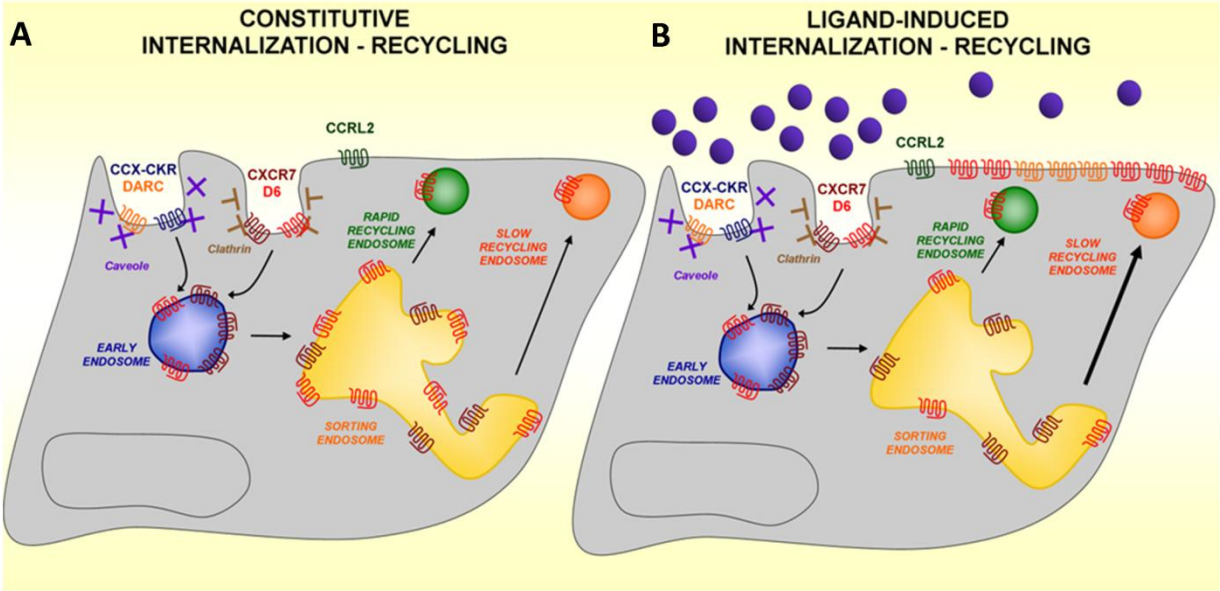

Figure 15: The intracellular trafficking properties of ACKRs. ACKR2 (D6) and ACKR3 (CXCR7) internalize through clathrin-coated pits while ACKR1 (DARC) and ACKR4 (CCX-CKR) through caveolae. ACKR2 and ACKR4 are constitutively internalized and are recycled back to the plasma membrane through the rapid (Rab4) and slow (Rab11) recycling endosomes (panel **A**). ACKR5 (CCRL2) is not internalized even after ligand engagement. ACKR2, after being internalized into clathrin-coated pits vesicles, is transported to Rab5-positive EE through a dynamin-dependent process. After ligand stimulation, ACKR2 and ACKR4 membrane expression is increased (panel **B**) (Figure derived from [10]).

2- B. ACKR1 (DARC)

ACKR1 was originally described as the erythrocyte receptor for chemokines. It binds at least 20 different CC and CXC chemokines [136, 137] and in addition it binds other molecules, present on HIV-1 [138, 139] and malaria parasites (*Plasmodium vivax* and *Plasmodium knowlesi*) [4, 140]. The erythrocytes of most individuals of African descent (>95% Africans in malaria-endemic regions and 70% of African Americans) lack expression of ACKR1, and this loss of ACKR1 expression probably resulted from a selective advantage provided by resistance against infection with these malarial parasites [141]. ACKR1 is also expressed by endothelial cells of postcapillary venules and veins of many organs, including lymph nodes [142], suggesting that this molecule has a role in vascular biology. Venular expression of ACKR1 has been reported in diverse normal tissues, such as the skin, kidneys, lungs, brain, thyroid and spleen, as well as in inflamed tissues, such as the rheumatoid joint synovium, psoriatic skin, various kidney diseases and lungs with supportive pneumonia [143, 144].

At the structural level, ACKR1 presents 40% similarity with chemokine receptors but the DRYLAIV motif in the second intracellular loop completely lack and is mutated into LGHRLGA motif [145]. All four extracellular domains of ACKR1 are involved in its interaction with chemokines, and a sulphated tyrosine residue in the first extracellular domain is essential for the interaction with erythrocyte-binding proteins from malarial parasites [146-148].

ACKR1 has a promiscuous chemokine-binding profile, interacting with 11 pro-inflammatory chemokines of both the CXC- and CC-chemokine subfamilies but not with homeostatic chemokines [137]. Of the pro-inflammatory CXC-chemokines, ACKR1 selectively binds angiogenic chemokines (CXCL1, CXCL3, CXCL5, CXCL6 and CXCL8), which are characterized by the presence of the ELR motif near the N-terminus, but not angiostatic chemokines (CXCL9 and CXCL10), lacking the ELR motif. *In vitro* data show that, after chemokine binding, ACKR1 neither supports ligand-induced signalling nor cell migration [116]. In unstimulated cells, ACKR1 is expressed on the surface membrane. As canonical chemokine receptors, ACKR1 is internalized into intracellular vesicles after ligand binding, in particular into caveolae, and then reappear together with its chemokine cargo on the cell membrane [133].

ACKR1 is not a scavenger receptor and chemokine internalization may facilitate the removal of chemokines from extracellular environment, inducing their translocation. The hypothesis that endothelial cells expressing ACKR1 might mediate facilitated transfer of chemokines across cellular barriers (transcytosis) and present them to underlying leukocytes has been supported by some *in vitro* studies [124, 149]. However, in studies of *ACKR1*-transfected umbilical vein endothelial cells, it was not clear whether these ACKR1-expressing cells could transport CXCL8 more efficiently than control cells lacking ACKR1 expression [124]. The expression of ACKR1 regulates CCL2 levels, if comparing to ACKR1 knockout (KO) mice, indicating that ACKR1 expression on erythrocytes regulates chemokine concentration by sequestering chemokine expressed at high levels [150]. On erythrocytes, ACKR1 do not mediate chemokine internalization and endocytosis, but chemokines remain associated with ACKR1 and can be replaced by other chemokines with higher affinity for the receptor [151]. In addition, ACKR1 has been reported to create heterodimers with CCR5, affecting in this way CCR5 intracellular signalling pathway G-protein dependent, playing an important role in controlling cell migration and inflammation resolution [152].

ACKR1 KO mice develop normally and have normal haematological parameters, but these mice show increased granulocytic infiltration in lungs and liver in a model of endotoxaemia [153]. Chemokines that are bound to ACKR1 on the erythrocyte surface are not accessible to other chemokine receptors, indicating that ACKR1 does not present or transfer bound chemokines to signalling-competent chemokine receptors [154]. Although the fate of ACKR1-bound chemokines has not been investigated, the observation that chemokines injected into ACKR1-deficient mice rapidly disappear from the plasma suggests that ACKR1-expressing erythrocytes could function as a reservoir for chemokines [143, 155]. By protecting blood leukocytes from surplus of plasma chemokines, ACKR1 may also prevent leukocyte desensitization for their subsequent migratory responses [156]. Transgenic mice overexpressing ACKR1 on vascular endothelium show a diminished angiogenic response to CXCL3 in the corneal micropocket assay [157] and two *ACKR1*-transfected tumour cell lines show decreased tumour-associated vasculature and reduced metastatic potential [158, 159]. Consistent with this, in a model of prostate cancer, absence of ACKR1 expression results in enhanced tumour growth that is associated with increased intratumoral concentrations of the ACKR1 angiogenic chemokine ligands CXCL1 and CXCL3 [160]. In a similar context, Bandyopadhyay et al. show that ACKR1 ligates the tetraspanin CD82 on tumour cells, triggering tumour-cell senescence and suppressing metastasis [161]. More recently, Wang et al. show that low expression of ACKR1 protein in human breast cancer samples correlates with reduced expression of oestrogen receptor, increased microvessel density (through increased angiogenesis) and metastasis, and poor prognosis for the patients [158]. These data collectively indicate that ACKR1 seems to function *in vivo* as a negative regulator of inflammation and angiogenesis by clearing chemokines from the tissue microcirculation, although it has also been suggested that ACKR1 facilitates recruitment of cells into the peritoneal cavity [144]. ACKR1 has been recently reported to play a role in modulating the early inflammatory response to bone fracture and subsequent cartilage formation. However, the early cartilage formation was not translated with an early bone formation at the fracture site in ACKR1-KO compared to WT mice [162].

In conclusion, ACKR1 functions as a transcytosis receptor, leading to chemokine immobilization on the apical cell surface [133]. The chemokine transendothelial transport by ACKR1 could be important for chemokine elimination from the tissues, by supporting the placement and function of optimal concentration of chemokines , eliminating the excess [144]. Moreover, it seems to have a complex role in tumour growth and metastasis and in the regulation of chemokine-induced angiogenesis [159, 163].

2- C. ACKR3 (CXCR7)

The decoy receptor ACKR3 is located in the same chromosome of CXCR4 and CXCR2, but as the other decoy receptors, it displays alteration of the canonical DRYLAIV motif, mutated into DRYLSIT motif. It presents more that 40% of sequence homology to CXCR2. Due to the sequence alterations, it is not able to induce GTP binding to heterotrimeric G proteins, indicating the lack of receptor coupling to G proteins [164]. Recently, ACKR3 was shown to retain the ability to control proliferation and migration of primary astrocytes through a CXCL12-dependent Gαi-mediated signalling pathway [165, 166], whereas its signalling activity induced by CXCL11 was completely unaffected by PTX treatment.

ACKR3 binds with high affinity CXCL12, the unique ligand in common with CXCR4, and CXCL11, with 20-fold lower affinity [135, 167]. In zebrafish, ACKR3 has been reported to internalize and sequester CXCL12, shaping in this way chemokine gradient and controlling cell migration. ACKR3 constitutively cycles to and from the plasma membrane, internalizing in endosomes, where it is preferentially localized [168]. The ACKR3 trafficking properties show both a ligand dependent and independent cycling, suggesting an ACKR3 role as a scavenger receptor [6, 169]. Similarly to ACKR1 and CCR5, also ACKR3 has been demonstrated to heterodimerize with CXCR4, influencing in this way its intracellular signalling pathway [164].

ACKR3 is mainly expressed on hematopoietic cells, such as neutrophil, monocytes and B cell, mesenchymal cells and neuronal tissues [170]. Several studies reported ACKR3 mRNA expression also in leukocytes [171], in particular on B cells [172, 173], although no protein levels were detectable in other studies [135, 172, 174]. Hartmann et al. demonstrated the importance of ACKR3, weakly expressed, on rapid CXCR4 signalling on CD4 positive T cells [134]. In human, ACKR3 expression on B cells is associated to their ability to differentiate into plasmablasts and to produce efficiently antibody [172].

Depletion of ACKR3 causes a lethal phenotype, due to heart valve defect, indicating an important role of this receptor in fetal development [175, 176]. The same phenotype was obtained in CXCR4 deficient mice, supporting the idea that ACKR3 lack compromise CXCR4 signalling [33, 177]. In fact, in ACKR3 KO mice, the elevated levels of CXCL12 induce a down-regulation of CXCR4 [168]. This indicates a crucial role of ACKR3 in regulating the availability of CXCL12 for CXCR4. ACKR3 expression on stromal cells is necessary to sustain the CXCL12 gradient required for primordial germ cell migration [119]. ACKR3 has been also reported on tissue barrier as placenta, revealing an important function in acting as a

scavenger receptor on microvessel endothelium. Recently, it has been addressed a ACKR3 expression in tumor cells, in particular on tumors of hematopoietic origin, such as lymphomas [6, 134, 178] and of mesenchymal origin, such as sarcoma, prostate and breast cancer [179-181]. The importance of ACKR3 expression in tumor growth is still unclear. It is known that its tumor expression is accompanied by CXCR4 expression. The ACKR3 up-regulation after CXCL12 engagement, could reduce CXCL12-mediated tumor cell retention, although the dynamics of ACKR3 on cell surface and its ability to degrade CXCL12 in vivo are not so clear.

2- D. ACKR4 (CCX-CKR)

The chemokine receptor ACKR4 is widely expressed in several tissues, T cells and immature DCs. It binds homeostatic CC-chemokines, in particular CCL19 and CCL21, suggestin a role in regulation of chemokine-mediated aspects of the adaptive immune responses, and weakly binds CXCL13, that are constitutively released by DCs and monocytes [7, 120]. ACKR4 has sequence modifications at the N-terminal domain, in particular the DRYLAIV motif in the second intracellular loop is mutated into DRYVAVT motif. This mutation makes ACKR4 unable to transduce intracellular signals after ligand engagement as the other ACKRs [182, 183]. The transfection of human embryonic kidney cell line (HEK293) with the gene encoding ACKR4 allows these cells to take up and degrade extracellular CCL19 with high efficiency, suggesting a scavenging role for this receptor [184].

On binding to their signalling receptors, ACKR4 ligands mediate the trafficking of naive T cells, DCs (CCL19 and CCL21), B cells and follicular B helper T cells (CXCL13) to and within lymphoid organs, and have a major role in the ontogeny of lymphoid organs and extranodal lymphoid tissues, which characterize chronic autoimmune disorders, such as rheumatoid arthritis and multiple sclerosis [185]. In the Experimental Autoimmune Encephalomyelitis (EAE), ACKR4 KO mice show a more rapid onset of the disease, due to the high T cells activation in lymph node as a consequence of increased Th17 responses [186]. Indeed, although CCL19, CCL21 and CXCL13 are secreted constitutively by DCs and monocytes, the production of CCL21 is also augmented by pro-inflammatory signals and CXCL13 production is induced by the anti-inflammatory cytokine IL-10 [183, 187]. No leukocyte expression has been reported yet. Therefore, it is possible that ACKR4 might have a role in homeostatic trafficking and, perhaps more importantly, in regulating traffic

of lymphocytes and DCs in inflammatory and autoimmune conditions in which lymphoid neogenesis occurs [10].

ACKR4- deficient mice are healthy and viable, suggesting no clear role for ACKR4 in development. In ACKR4 KO mice, the levels in the serum and in the lymph node of CCL19 and CCL21 were increased, supporting the ACKR4 role as a scavenger receptor, demonstrating its importance in immune responses [188].

2- E. ACKR5 (CCRL2)

The hypothesis that some orphan receptors with structural similarities to known chemokine receptors could act as chemokine decoy receptors must also be considered. In this respect, the 7TM orphan receptor ACKR5 is an interesting candidate because it shares over 40% amino-acid identity with CC-chemokine receptors, its gene is located in a cluster together with most other CC-chemokine receptors (on chromosome region 3p21–23) [189] and, similar to other decoy receptors, DRYLAIV motif is not conserved but it is mutated into QRYLVFL [121]. ACKR5 expression has been detected on endothelial cells of many lymphoid organs, such as spleen, lymph node, bone marrow, as well as in non-lymphoid organs, such as heart and lung [121]. In particular, it has been detected on mast cells, monocytes, neutrophils and DCs, and, in all cell types, it is highly up-regulated in activated cells, following stimulation with LPS [190]. Bieber et al. reported functional activities (chemotaxis and calcium fluxes) after ACKR5 engagement by CCL2, CCL5, CCL7 and CCL8, although no evidence for a direct ligand–receptor interaction was provided in this report [191]. We failed to confirm ACKR5 recognition of these chemokines or a series of other CC- and CXC-chemokines using *ACKR5*-transfected cells (Bonecchi R., unpublished observations). Leick et al. reported a constitutive ACKR5 internalization and CCL19 binding, but the faith of the internalized chemokines remains unknown [192]. ACKR5 deficient mice are fertile and do not present any problem during development, presenting a normal lifespan [193].

Recently, a new ligand for ACKR5 was found. ACKR5 was shown to bind the N-terminus of chemerin with high affinity, leaving the C-terminus available for interaction with cells expressing ChemR23, the functional chemerin receptor [8]. In addition, ACKR5 expression has been demonstrated both in homeostatic and in inflammatory condition in endothelial cells, supporting a role of ACKR5 in shaping the chemerin chemotactic gradient in vivo [123, 194]. ACKR5 seems to be involved in the regulation and control of both innate

and adaptive immunity. Taken together, these results support different ACKR5 roles, in shaping chemerin-chemotactic gradient, by presenting chemerin to chemR23 positive cells, and in chemokine degradation and down-modulation of cell activation [123]. ACKR5 KO mice present increased levels of circulating chemerin, after systemic administration of inflammatory stimuli, supporting ACKR5 role in shaping chemerin-chemotactic gradient and its ability to immobilized chemerin [194].

2- F. C5L2

Recently, the C5a receptor-like2 receptor (C5L2), which recognizes the chemotactic complement fragment C5a, has been demonstrated to have similar properties to ACKRs. It binds with high affinity C5a, similarly to the canonical C5a receptor (C5aR), but presents an increased affinity for C5a des Arg, compared to C5aR. C5a des Arg is the naturally cleaved form of C5a, which lacks the C-terminal arginine.

As reported before, C5L2 presents mutated DRYLAIV and NPxxY motif, found in the TM3 and in the TM7 respectively. Unlike C5aR, C5L2 does not undergo ligand induced internalization upon stimulation. It is primarily located in intracellular compartments, undergoing constitutive internalization. Its constitutive recycling is not affected by the presence of C5a or C5a des Arg, but is completely inhibited by clathrin inhibitors, suggesting that the constitutive internalization is a clathrin dependent mechanism. After receptor binding, ligands are internalized and they are degraded, supporting the hypothesized scavenging role of C5L2 [122]. Recently, the formation of heteromer has been demonstrated. C5a, but not C5a des Arg, stimulation induces up-regulation of C5aR-C5L2 heteromer formations in a dose dependent manner, suggesting that the arginine residue at the C-terminal is important for heteromer formation and their upregulation [195]. The heteromers induce an unknown C5aR conformational change and modulate its signalling pathway, regulating human physiological responses to C5a. C5L2 is not just a recycling decoy receptor, but plays a novel regulatory role, by which C5L2 may potentiate C5a-induced IL-10 release via a concerted mechanism of heteromer formation between C5aR and C5L2 [195].

C5L2 is expressed by various cell types, in particular neutrophils and DCs. Expression is also found in tissues such as testis and spleen, with weaker expression in the kidney, lung, liver and heart [196]. It has been reported to modulate the signalling activities of C5aR and the related receptor for C3a in mouse neutrophil [197]. Neutrophils stimulated

with C5a and LPS in the presence of C5L2 blocking antibody produce dramatically increased levels of IL-6, compared to the control [198]. Considering neutrophils from C5L2 KO mice, they show increased responses to both forms of C5a, with an increased infiltrate in lung and higher levels of TNF-α and IL-6, compared to control mice in a model of pulmonary immune complex injury [199]. In addition, in sepsis patients, the low C5L2 expression correlates with multi-organ failure induced by sepsis, comparing neutrophils obtain from patients who survived the observation period to patient who did not survived [200]. Moreover, in a sepsis mouse model, C5L2 appears to have an important pro-inflammatory role as C5aR [201]. C5L2 seems to have an important anti-inflammatory role in vivo, in the elimination of C5a des Arg [202], as reported in a mouse model of pulmonary inflammation [199] and also in rat astrocytes [203], but several groups have recently shown a pro-inflammatory role for mouse C5L2, in particular in diseases such as allergic asthma [204], sepsis [205], autoimmune arthritis and metabolic disfunction [206, 207], although its mechanism of action remains uncertain.

2- G. ACKR2

ACKR2 has been identified for the first time in 1997. It belongs to the family of CC chemokine receptors, having the same 7TM structure, presenting the same chromosome localization and 35% of sequence homology with CCR5 [125].

3. THE CHEMOKINE DECOY RECEPTOR ACKR2

ACKR2 is the best-characterized silent chemokine receptor. Originally identified as a CCL3 binding molecule expressed in murine hematopoietic stem cells [208] and soon after in human cells [117, 118], the ACKR2 molecule is an atypical chemokine receptor, that presents alteration in conserved elements that are critical for G-protein coupling and signalling functions of chemokine receptors.

3- A. ACKR2 STRUCTURAL MOTIF

Conventional chemokine receptors, like all other members of the GPCR family, mainly transduce intracellular signals through the activation of heterotrimeric G proteins, and all

chemokine receptors in particular mediate signalling pathway through PTX-sensitive Gαi proteins [209]. Different evidences demonstrated that neither the human nor murine ACKR2 sustains signalling activities typically observed after chemokine receptor triggering, such as calcium fluxes and chemotaxis [14, 100, 118, 210].

ACKR2 binds with high affinity more than 14 pro-inflammatory chemokine, belonging to the CC subfamily. The 7TM domain organization is well conserved, the overall sequence identity to conventional chemokine receptors is in the 30–35% range, similar to the identity rate observed among conventional receptors, and the N-terminal domain presents several charged residues, most likely involved in ligand recognition as for other chemokine receptors, including acidic amino acids and sulphated moieties, but whether receptor sulphation is important for ligand binding remains to be demonstrated [125]. ACKR2 has been found to be glycosylated on the N-linked glycosylation site (**Figure 16**), but this post-transcriptional modification appears not to be relevant for ligand binding and receptor expression [125]. The TxP motif is conserved in ACKR2 and it is supposed to be important for receptor activation, while the aspartic acid and the DRYLAIV motif are mutated into an asparagine and DKYLEIV respectively, as shown in **Figure 16**. The DKYLEIV motif in ACKR2 is highly conserved across the species [211]. Correction of this motif confers weak ligand-induced signalling activities [212], showing how the altered DRY motif is at least in part responsible for the lack of conventional signalling activities in the ACKRs subfamily.

ACKR2 C-tail is longer than canonical chemokine receptor, starting at amino acid 312 till 384, containing a serine/threonine cluster and a putative 8th helix after amino acid 340, as reported in **Figure 16**. The C-tail seems to be important for ACKR2 internalization, allowing receptor recycling to and from the plasma membrane and progressive chemokine depletion [213]. Moreover, the C-tail prevents receptor degradation from entering late endosomes. In fact, complete depletion of ACKR2 C-tail or truncation of the 8th helix or mutation of serine cluster into alanine, strongly reduces ACKR2 stability on cell surface, by targeting ACKR2 to lysosomal compartment through ubiquitination of two lysine residues (142 and 324 amino acids), highly conserved in all mammalian ACKR2 sequences [214].

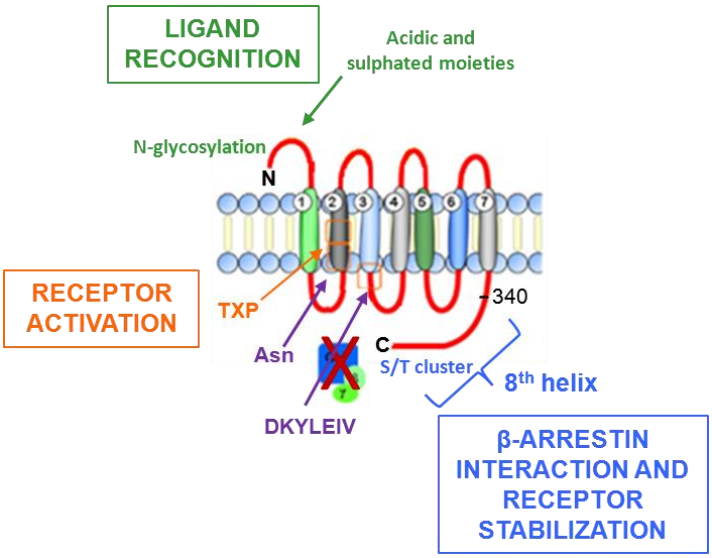

Figure 16: ACKR2 structure-function elements. The 7TM organization is well conserved in ACKR2, as for the other ACKRs. The N-terminal domain presents acidic amino acids, sulphated moieties and N-linked glycosylation sites, which are important for ligand recognition. The TxP motif is conserved in ACKR2 and it is important for receptor activation. The aspartic acid in TM2 and the DRYLAIV motif in TM3 are not conserved in ACKR2. The C-tail of ACKR2 is required to prevent receptor degradation, allowing receptor recycling and progressive chemokine scavenging activities (Figure derived from [53]**)**.

3- B. ACKR2 LIGAND RECOGNITION

Radio ligand binding experiments have demonstrated that ACKR2 recognizes an unusual broad spectrum of ligands, being able to interact with most agonists at inflammatory CC chemokine receptors from CCR1 to CCR5 [100, 118]. Although ACKR2 behaves as a highly promiscuous receptor for CC chemokines, it also expresses some selectivity in ligand recognition. Homeostatic CC-chemokines, agonists at CCR6–CCR10, are not recognized, nor chemokines belonging to other subfamilies [13, 100, 208]. Even among inflammatory CC chemokines, ACKR2 recognition is restricted to the biologically active form. In the case of CCL22, for example, the intact biologically active molecule is efficiently recognized by ACKR2, while N-terminal CD26-processed CCL22 variants, which lose their ability to trigger leukocyte recruitment by acting as CCR4 agonists, are not recognized by ACKR2 [100]. Thus, ACKR2 selectively interacts with biologically active inflammatory CC chemokines. The chemokine binding does not necessary induce ligand degradation, as demonstrated by Savino et al. In fact, ACKR2 is not able to degrade the three cleaved form of CCL14, but only the active one. CCL14(1-74) is a homeostatic chemokine, prevalently presents in inactive form in blood flow, having a reduced agonist activity on CCR1. The

protease CD26 cleave CCL14 into its active form CCL14(9-74), which is recognize and degraded by ACKR2 [23]. This truncated form can be further cleaved into CCL14(11-74), biologically inactive and not degraded by ACKR2. ACKR2 binds with high affinity all the three cleaved form of CCL14, but only the active one is degraded. The degradation seems to be induced by the presence of a proline in position 2 at the N-terminus, as has been observed in other active chemokine, such as CCL3 and CCL8, which exist also in the truncated form lacking proline in position 2 and consequently inactive [23]. For this reasons, chemokine are classified in active and neutral ligand, as reported in **Figure 17**.

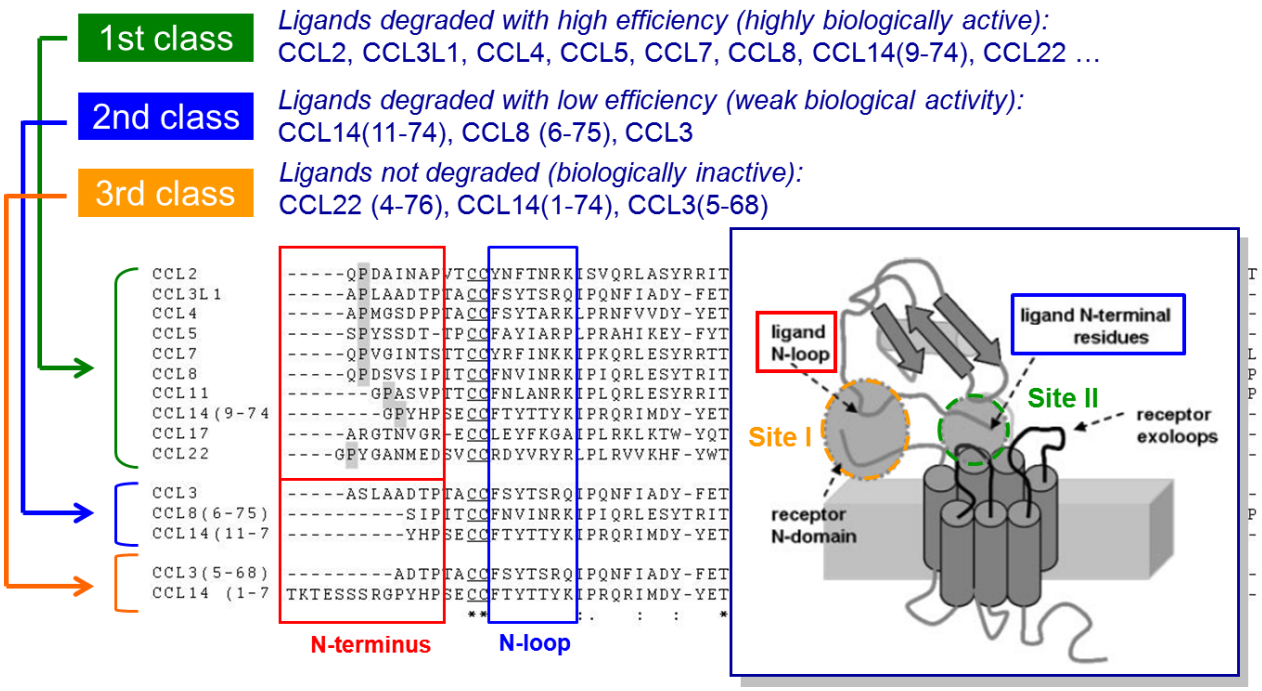

Figure 17: ACKR2 ligand classification. The first class also known as active ligands, having a proline residue in position 2 at the N-terminus, bind ACKR2 with high affinity and are degraded with high efficiency. Neutral ligands, of the second and the third classes, bind ACKR2 with high affinity, but are degraded with low efficiency or are not degraded, respectively (Figure derived from [23].

3- C. ACKR2 EXPRESSION

ACKR2 expression patterns are unusual. The predominant expression sites are "barrier tissues" such as the skin, gut, and lung. Within these tissues, lymphatic endothelial cells (LECs) of afferent lymphatic vessels are the major site of expression (**Figure 18**) [13]. This specific ACKR2 expression confirms its important role in modulating the inflammation

status on lymphatic vessel, contributing in this way to shaping the inflammatory chemokine from inflamed tissue and blocking chemokine influx to lymph nodes [13].

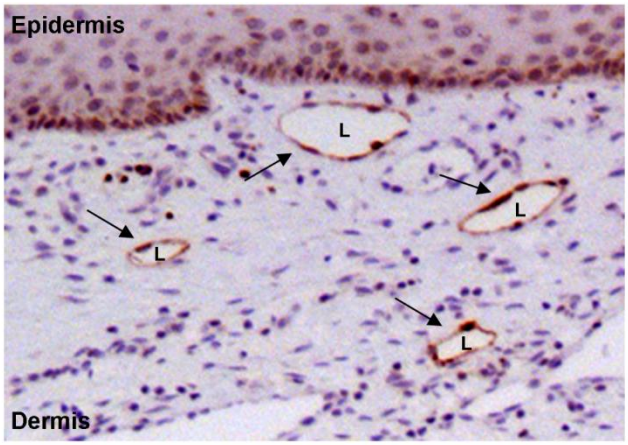

Figure 18: ACKR2 expression on LECs in human skin. Human skin is stained with anti-ACKR2 antibody. "L" marks subcutaneous lymphatic vessels that show ACKR2 immunoreactivity. (Borroni E.M., unpublished data)

In addition, ACKR2 is strongly expressed in the placenta where it is present on invading trophoblast cells, on the apical side of syncytiotrophoblast cells and on decidual macrophages [14]. Chemokines are normally produced by fetal and maternal components and regulate leukocyte traffic at placenta level, important for maintaining the balance between protecting fetal development and tolerance [215]. ACKR2 expression at syncytiotrophoblast between the maternal and fetal blood fluxes suggests an immunosuppressive function at materno-fetal barrier.

ACKR2 has been reported also to be expressed on vascular tumors [13] and at low levels in a range of circulating leukocytes [216, 217], including mast cells, macrophages, neutrophils [218], with most marked expression being evident on subsets of B cells as well as on both plasmacytoid, myeloid DCs and human alveolar macrophages of COPD patients [16]. Its expression has been also observed on astrocytes, at central nervous system [219]. Most recently, strong expression of ACKR2 on murine innate-like B cells has been shown suggesting that these cells are the major vehicles for in vivo ACKR2 function [15, 220].

3- D. ACKR2 TRAFFICKING PROPERTIES

In resting condition, ACKR2 is predominantly located in intracellular compartments at perinuclear levels and only 10% is detectable on the cell surface. ACKR2 is not regulated

or inducible at transcriptional level, but its membrane expression is highly regulated by its intracellular traffic. ACKR2 is constitutively associated to both early (Rab4/5) and recycling endosomes (Rab11) [221], but not with lysosomes (Rab7) [17, 125]. In fact, it is constitutively internalized in a Rab5/Dynamin-dependent mechanism, through clathrin-coated pits, and it is targeted to EE, as previously mentioned. In basal condition, ACKR2 colocalized with EEA-1 [17] and Rab4/Rab11 positive vesicles [19].

Differently from canonical chemokine receptors, after chemokine engagement, ACKR2 does not decrease its membrane expression, thus optimize its degradatory activity by increasing its membrane abundance through a slow Rab11-dependent recycling pathway [19]. Once internalized, chemokines dissociate from the receptor, and are targeted for degradation in Rab7-positive lysosomal compartements, while the receptor is recycled back to the plasma membrane through both rapid (Rab4) and slow (Rab11) recycling pathways, involving ACKR2 transit in the RE, before coming back to the cell surface [19] (**Figure 19**), indicating an important role for Rab4 and Rab11 in maintaining a constant ACKR2 membrane expression. Bonecchi et al. demonstrated how using Rabs dominant negative form influencing the membrane expression of ACKR2, without altering its internalization rate [19]. The receptor internalization rate is not affected by the presence of the ligands, but chemokines induce a dose-dependent ACKR2 up-regulation on plasma membrane by accelerating receptor mobilization from the intracellular pool [19]. Thus constitutive cycling and ligand-dependent receptor up-regulation are mechanisms that allow rapid modulation of ligand uptake and degradation [222]. Receptor up-regulation is dependent on chemokine extracellular concentration, and increasing ligand dose correspond to an increase in ligand degradatory efficiency. Chemokine-dependent mobilization is the only mechanism known to regulate ACKR2 membrane expression and degradatory activity [10]. It is important to note that only active ligands can induce rapid mobilization of the receptor to the plasma membrane [19, 23], suggesting that some signal events activated upon ligand engagement can occur in order to promote receptor cycling. As for canonical chemokine receptor, the trafficking properties of ACKR2 seem also to be modulated by cytoskeleton dynamics [10].

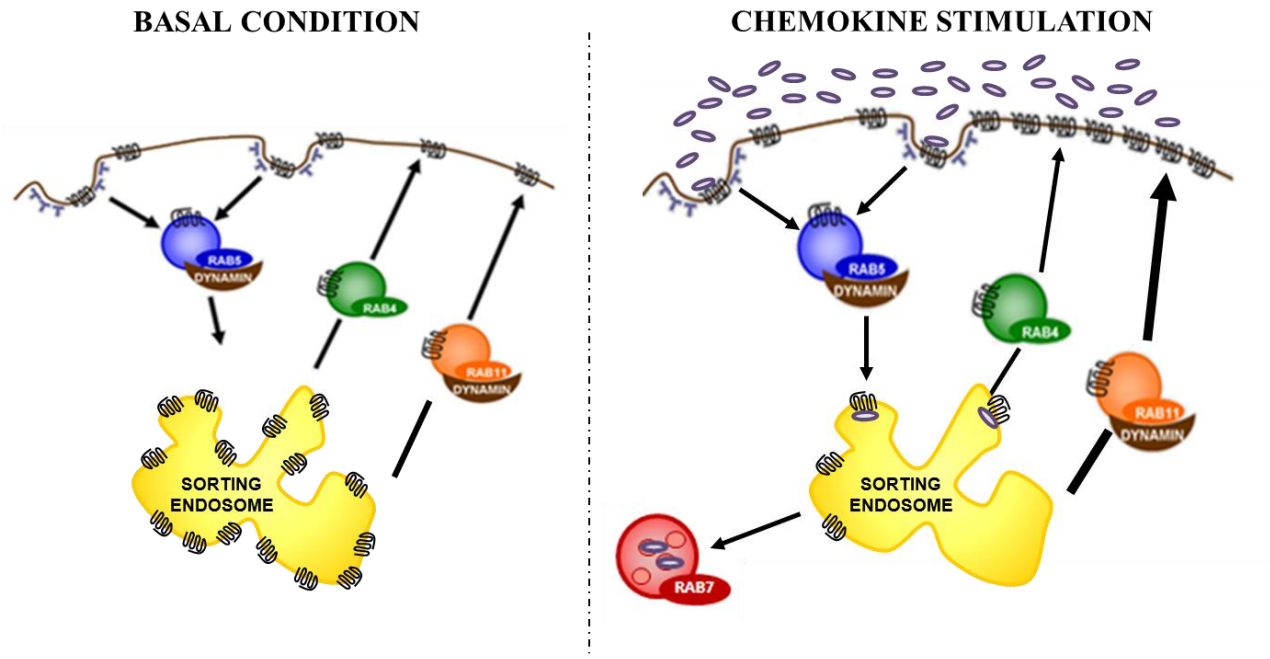

Figure 19: ACKR2 intracellular traffic. In basal condition, ACKR2 is internalized in a Rab5/Dynamin-dependent mechanism and it recycles back to the plasma membrane through a Rab4/Rab11-dependent way. ACKR2 mobilization from intracellular compartments is induced by stimulation only with active ligands, having a proline residue in position 2 at the N-terminus. These ligands are degraded in a Rab7-dependent mechanism. On the other hand, neutral ligands do not induce receptor up-regulation and its distribution, and they are not degraded (Figure modified from [221]).

The constitutive cycling properties, the peculiar expression profile and the absence of signalling activity could suggest a ACKR2 role in mediating chemokine transfer through biological barriers, as previously demonstrated for ACKR1 on vascular endothelium [149]. When ACKR2 is expressed on lymphatic endothelial cell line [223], no evidence for facilitate chemokine transfer through the cell monolayer were obtained.

Conversely, the presence of ACKR2 consistently resulted in the degradation of appropriate ligands. Similar results were obtained in different ACKR2 cell transfectants [14, 222]. Analysis of biochemical properties of ACKR2 indicated that internalized chemokines are rapidly released from the receptor after vesicle acidification, allowing subsequent ligand degradation and leaving ACKR2 free to recycle back to the cell surface [224, 225]. Consistently with this, pre-treatment with ammonium chloride to prevent vesicle acidification resulted in a reduction of chemokine degradation and receptor accumulation in intracellular compartments [223]. Thus, in in vitro settings ACKR2 does not support chemokine transcytosis, but behaves as a decoy receptor that scavenges inflammatory CC chemokines.

3- E. ACKR2 BIOLOGIC ROLES IN VIVO

Murine ACKR2 shares key properties of a chemokine decoy receptor with its human orthologue. ACKR2 KO mice confirm ACKR2 behave as a silent receptor, acting as a promiscuous, efficient scavenger of inflammatory CC chemokines with the expected ligand. ACKR2 KO mice have been generated by D.N. Cook and S.A. Lira [226] and present a highly inflammatory responses and high levels of pro-inflammatory CC chemokines, in particular at skin and lymph nodes level, developing a prolonged inflammatory response [227]. As no functional antibodies against murine ACKR2 are actually available, the investigation of the expression pattern and the biology of the receptor in all the murine models set up are critically limited.

Increased levels of inflammatory CC chemokines were detected in ACKR2 KO mice both locally than in draining lymph node (**Figure 20**), causing lesions development as demonstrating by pre-treatment with chemokine receptors blocking antibodies and the exacerbate inflammatory response is clearly caused by an inefficient control of the chemokine system due to the absence of ACKR2, as demonstrated in different experimental diseases (**Figure 20**) [14, 228]. Different mouse models highlight an ACKR2 non-redundant role in controlling local inflammation, but molecular mechanisms involved are still undefined.

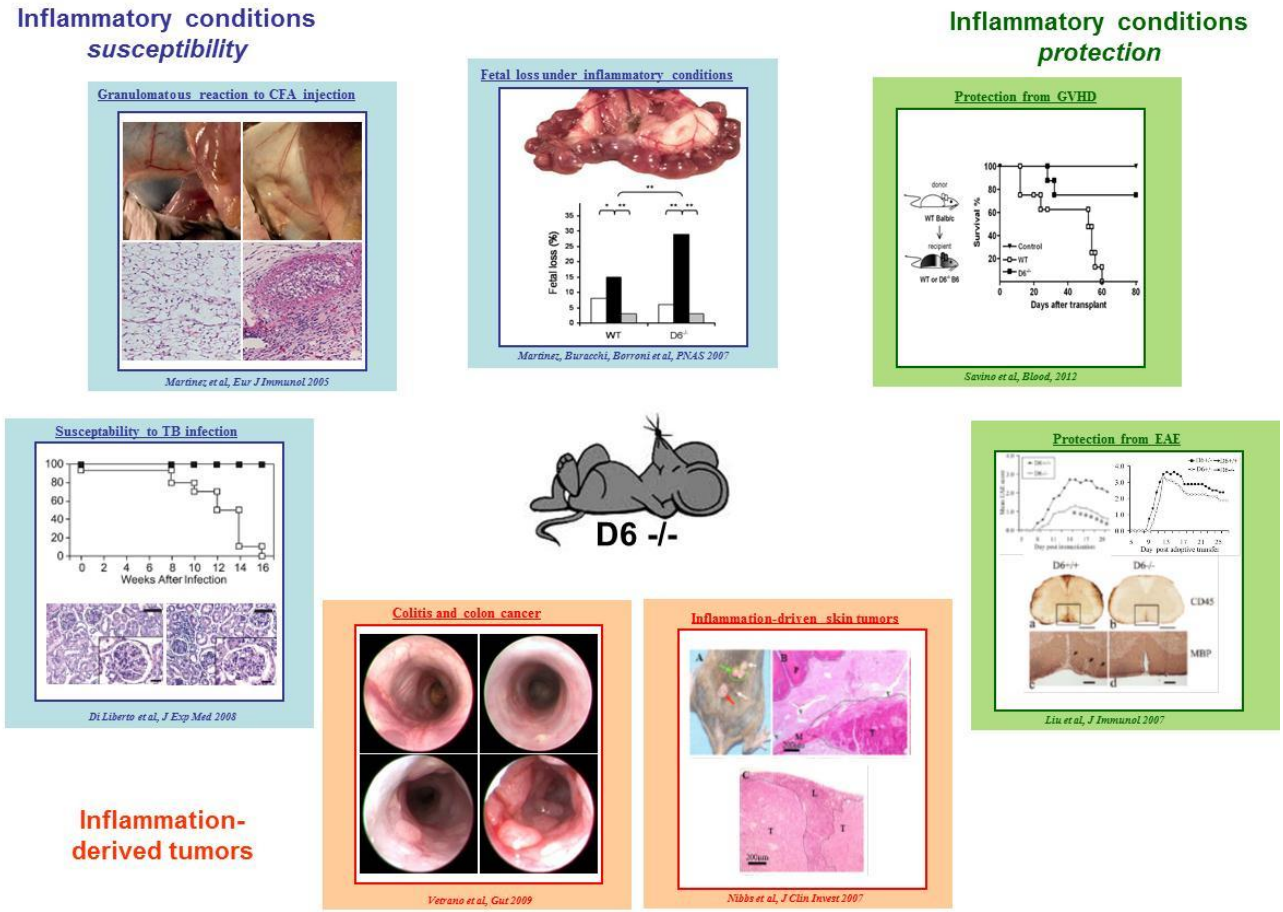

Figure 20: ACKR2 biological role in different pathology.

During the development of an inflammatory reaction, monocyte recruitment has been demonstrated to be driven by chemokine transferred to draining lymph nodes [229, 230]. In the absence of ACKR2, increased concentrations of chemokine were found in draining lymph nodes [231] demonstrating that ACKR2 expression on afferent lymphatic vessel is of strategic relevance, for its inflammatory chemokines scavenging role during chemokine drainage, regulating the amount of chemokines that reach lymph nodes (**Figure 21**), confirming ACKR2 non-redundant role in controlling inflammatory CC chemokines in inflamed tissues. Furthermore, ACKR2 prevents excessive leukocyte adherence on lymphatic surfaces [232]. The defect in the adaptive immune response has been also found in graft versus host disease (GvHD), where ACKR2 KO mice resulted to be partially protected, having enhance immunosuppressive activity [228].

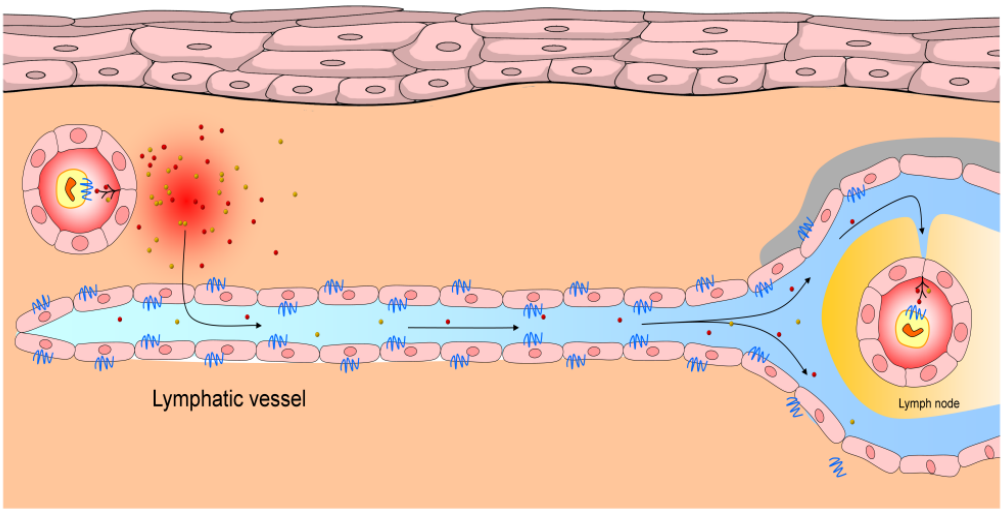

Figure 21: Role of the chemokine decoy receptor ACKR2 in lymphatic vessels. ACKR2 expression on afferent lymphatic vessels plays an important role in regulation of the chemokine gradient, by its scavenging activity of inflammatory chemokines, modulating their concentrations in inflamed tissues and their access to draining lymph nodes [202].

ACKR2 is also expressed in placenta on invading extravillous trophoblasts and on the apical side of syncytiotrophoblast cells. Pregnant mice were treated with LPS, resulting in an increased rate of fetal loss due to higher levels of inflammatory CC chemokines and increased leukocytes infiltrate in placenta [14] (**Figure 20**). Furthermore, ACKR2 has been demonstrated to suppress fetal resorption after embryo transfer into fully allogeneic recipients [233]. These results clearly proposed a role of inflammatory chemokine in fetal abortion and suggest ACKR2 as a possible responsible for controlling chemokines distribution and bioavailability.

In homeostatic condition, ACKR2 KO mice present an increase number of Ly6C^{high} circulating and spleen monocytes, while this population is highly decreased in the bone marrow. In addition, circulating monocytes present an immature phenotype, possibly due to the absence of ACKR2 expression on the non-hematopoietic compartment and lymphatic vessels, which normally express ACKR2 at high levels [20].

Finally, ACKR2 has been reported to have an important role in regulating the inflammation and the leukocyte infiltration in different tumors (**Figure 20**) [234]. In fact, ACKR2 deficient mice present high levels of inflammatory chemokine and infiltrating leukocytes, supporting the ACKR2 role in chemokine scavenging activity and in protection against tumor progression [234]. The ACKR2 expression has been reported in different

tumors, including lymphocytes leukemia cells [235], Kaposi's sarcoma (Savino B, Caronni N et al, 2013, submitted), malignant vascular tumors [13] and breast cancer [236].

4. THE B-ARRESTIN ROLE IN ACKRS BIOLOGY

Arrestins are 48-kDa proteins, first identified as cytosolic proteins required for inducing GPCRs desensitization. In vertebrates, four member of the arrestin family have been cloned. These are the visual arrestin (arrestins1 and 4), which expression is limited to retinal rods and cones, and arrestins2 and 3, also called β-arrestin1 and β-arrestin2, which are ubiquitously expressed in mammalian tissue [237]. Here we focus in particular on β-arrestin1 and β-arrestin2.

β-arrestin1 can be found both in cytoplasm and in the nucleus, while β-arrestin2 is limited to the cytoplasm [238, 239]. They exist in two conformational states, one active and one inactive. The transition between the inactive state to the active one requires conformational changes occurring within the protein itself [240, 241]. The basal conformation of β-arrestins is maintained by multiple intramolecular hydrophobic interactions among highly conserved residues. Upon ligand binding and receptor activation, the receptor-attached phosphates disrupt these intra-molecular forces, promoting a structural reorganization of the arrestin molecule [242, 243]. β-arrestins also contain specific interaction sites with multiple signalling and scaffolding molecules, providing their capacity as regulators of different cellular functions such as membrane, cytosolic and nuclear associated signalling and trafficking.

4- A. β-ARRESTIN AND GPCR

β-arrestins have been firstly identified as terminators of G-protein signalling and mediators of endocytosis, through clathrin-coated pits. β-arrestin is used by many GPCRs as endocytic adaptor (**Figure 22**), but mechanisms involving alternate adaptors also exist [244]. After receptor stabilization and G-protein activation, second messangers signalling pathway are induced, as discussed above. GRKs phosphorylate receptor C-tail at serine/threonine residues, leading to β-arrestin binding which sterically interdict G protein signalling and further coupling between the receptor and G proteins. This limits the G protein signal duration, resulting in receptor desensitization (**Figure 22**) [245]. In addition, β-arrestins scaffold enzymes that degrade G protein-induced second messengers,

providing an additional mechanism for efficient desensitization [246]. Recently, a direct interaction between β-arrestin1 and clathrin promoting receptors endocytosis has been characterized [244]. Clathrin-binding involves a highly conserved stretch of amino acids towards the C-terminal region of β-arrestin1 and β-arrestin2 [247]. It is now understood that β-arrestin functions as an adaptor molecule that binds directly to clathrin via the adaptor protein AP-2 (**Figure 22**) [247, 248]. Upon ligand stimulation, GPCRs induce cytosolic β-arrestin translocation to the plasma membrane, which can occur even in the absence of G protein activation [249]. Interestingly, the internalization processes also require protein kinase activity of PI3K, which acts to phosphorylate cytoskeletal tropomyosin, allowing actin to polymerize [250]. In the unstimulated state, β-arrestin is constitutively phosphorylated by mitogen-activated protein kinase (MAPK), reducing its capacity to interact with clathrin. After agonist stimulation, cytoplasmic β-arrestin is recruited to the plasma membrane, where it is rapidly dephosphorylated, promoting its binding to actin and subsequent endocytosis [251]. Once internalized, receptors could recycle back to the plasma membrane or become targeted for post-endocytotic degradation. Recently, β-arrestin ubiquitination has been demonstrated to determine the stability of the receptor/β-arrestin complexes and consequently the receptor fate [52, 64].

Recent data suggests that GPCR are capable of activating signalling pathway independently of G-protein activation and the signal transduction persists also after receptor internalization. In many cases, β-arrestins have been reported to function as receptor-activated scaffolds to coordinate upstream and downstream components of particular signal transduction pathways, in particular at the early stages of receptor endocytosis. β-arrestin1 and 2 bind various kinases and regulatory proteins. It has been shown to scaffolds members of the MAPK family, creating a signalling receptor complex associated with endosomes (**Figure 22**). Such scaffold assembly is potentiated by receptor activation, providing a ligand-dependent signal transduction event to direct the biological response of the cell. The signalling pathway β-arrestin-dependent is temporally distinct and spatially segregated from the second messenger response G-protein dependent. Although a direct connection has not been demonstrated between endocytosis and β-arrestin-dependent signalling pathways, it is possible that the two processes are linked.

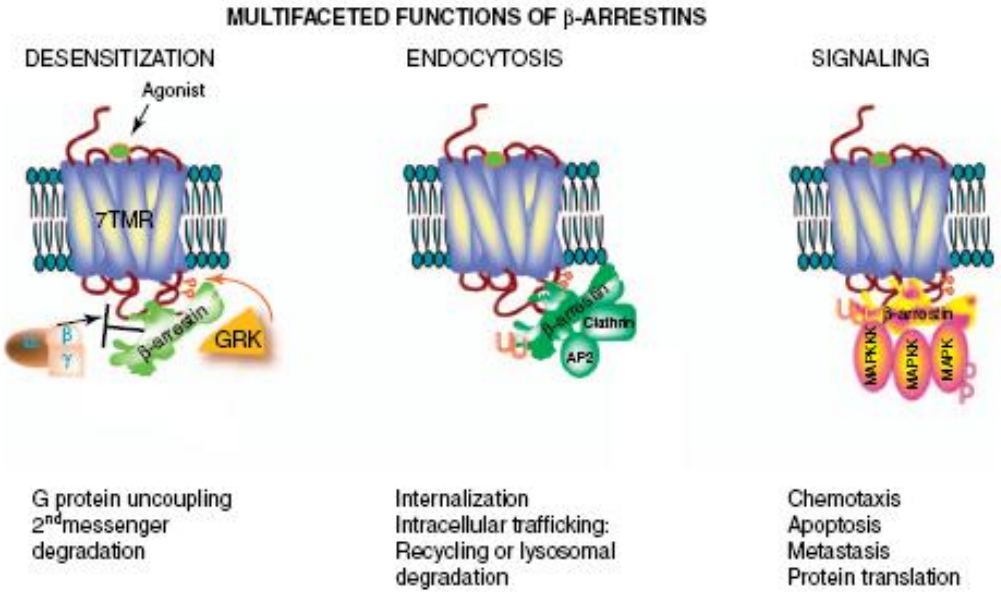

Figure 22: Multifaced functions of β-arrestins. *Desensitization*: agonist stimulation of 7TM receptors leads to G protein coupling and activation, following which receptors are rapidly phosphorylated by GRKs. Phosphorylated receptors present high affinity binding surfaces to recruit the cytosolic adaptors, β-arrestins. Steric binding by β-arrestin interferes with further G protein coupling leading to the desensitization of G protein dependent signalling. *Endocytosis*: agonist stimulation promotes rapid internalization of cell-surface 7TM receptors into clathrin-coated vesicles. This internalization is facilitated by β-arrestin binding which has specific interacting domains for clathrin and AP2. Receptor internalization is followed by post-endocytotic sorting of internalized receptors for recycling or lysosomal degradation. *Signalling*: β-arrestin acquires an active conformation upon forming a complex with agonist-stimulated 7TM receptors and scaffolds MAP kinase family, leading to the robust activation of MAPK, and subsequently targets to distinct subcellular compartments. Such β-arrestin dependent MAPK activity has been shown to regulate cellular chemotaxis, apoptosis, cancer metastasis and protein translation (modified from [68]).

It is now evident that certain ligands can preferentially activate β-arrestins while blocking or minimally activating G-protein or vice versa [67, 252]. This ligand-directed signalling is defined as biased agonism and can elicit different responses. It clearly suggests the existence of distinct activated conformation of the receptor for signalling through G-protein or β-arrestin alternatively [253]. β-arrestin can work in synergy or in opposition to G-protein signals. It clearly demonstrates that the signalling pathway can be switch from a G-protein dependent to G-protein independent one by different factors, for example agonist dose and structure, receptor clustering and perhaps the prevalence of downstream signalling components [67]. Thus, selective targeting of signalling events that contribute to the disease, while preventing other functions, would reduce the negative effects of some therapeutics.

β-arrestins are required in the activation of a variety of downstream signalling pathways, including MAPKs [254], the small GTPase family, including RhoA [255], and the actin filament severing protein cofilin [256]; β-arrestin is also involved in the inhibition of different signalling pathway, such as NFkB [257] and LIMK [256]. In some cases, β-arrestin has been demonstrated to both inhibit and promote some of the same downstream signalling moieties, triggered by different receptors [66]. In fact, β-arrestin can be triggered together with G-protein for activate the same protein, such as MAPK [258], or β-arrestin pathway can synergyze with G-protein-dependent one for having an integrated responses, for example RhoA [255], or the two pathway can work in opposition one the other to the same downstream target, as for PI3K [259, 260]. Of particular interest as target of β-arrestin signalling are proteins that create free actin barbed ends for polymerization, facilitating actin reorganization, important for chemotaxis. β-arrestin has been shown to promote the formation of a complex including cofilin, an actin depolimerizing factor, LIM Kinase, promoting cofilin phosphorylation and inactivation, and the phosphatases slingshot and chonophin [261]. The association through β-arrestin facilitates dephosphorylation of cofilin by its upstream phosphatases, and localizes cofilin activity to the leading edge of the cell to promote actin polymerization and formation of membrane protrusion [262, 263]. The actin cytoskeleton is also controlled by β-arrestin through the activation of small GTPases RhoA family, as demonstrated for the β-arrestin1 dependent MAPK activation of a Rac1-dependent mechanism to elicit actin rearrangement, downstream the β2-adrenergic receptor [264]; or by PAK4 activation through β-arrestin/MAPK dependent mechanism in order to promote LIMK phosphorylation and cofilin inhibition [262, 265, 266].

The different activities of β-arrestin rely on its interaction with different intracellular domain of receptors: interaction with receptor C-tail supports receptor internalization and desensitization, while binding to intracellular loops mediates signalling events [267, 268].

4- B. β-ARRESTIN AND CHEMOKINE RECEPTORS

The chemokine receptor activation after agonist binding induces the C-tail phosphorylation by second messenger-dependent protein kinases and GRKs. This causes β-arrestin binding to ligand-activated/phosphorylated receptor, the inhibition of G-protein-dependent signalling and receptor internalization and desensitization. The β-arrestin1 recruitment is independent of G protein activation and microtubules rearrangement, but

actin reorganization plays an important role in β-arrestin1 relocalization at plasma membrane levels, as demonstrated for CCR5 [269].

Four Serine residues have been identified to be phosphorylated and to be important for β-arrestin binding. An additional β-arrestin1 binding site within a conserved region in the second cytoplasmic loop of CCR5 has been identified to be important for internalization and desensitization [268]. Recently, also the DRY motif of CCR5 has been demonstrated to be involved not only in G-protein activation, but also for β-arrestin mediated chemotaxis and signalling, not for receptor desensitization and internalization [270]. The DRY mutation abrogates CCR5 G-protein activation and reduces receptor stability at plasma membrane as a consequence of its constitutive internalization due to constitutive phosphorylation and interaction with β-arrestins [270]. As for CCR5, β-arrestin2 has been suggested to function not only as a regulator of CXCR4 signalling, but also as a mediator of ligand-induced chemotaxis, probably through a p38/MAPK pathway [271]. In this case, seven serine residues of CXCR4 have been reported to be phosphorylated, having distinct effects on arrestin recruitment and conformation, leading to different effects on calcium mobilization and Erk1/2 activation [272]. Recently, the CWxP microswitch has been reported to be important in β-arrestin recruitment. In fact, after W248A mutation, CCR5 was completely inactive, despite maintained chemokine binding. The replacing of W248 with a smaller aromatic amino acid impairs β-arrestin recruitment, maintaining G protein activity. In addition, the altered positioning of W248 let to a constraint G protein active, but β-arrestin inactive and thus biased CCR5 conformation, demonstrating that the interface of TM6 and TM7 is very important for the activation state of CCR5 and that compounds targeting this area could have biased properties [70].

Recently, also CCR1 has been reported to signal in a β-arrestin dependent manner, as reported by Gilliland et al. CCR1 engages Gαi and β-arrestin2 in a multi-protein complex, through the creation of homo-dimers. The CCR1:G-protein complex functions as a canonical GPCR albeit with high constitutive activity, while the CCR1:β-arrestin2 complex is required for G protein independent constitutive receptor internalization. This complex seems to be important also for a potential scavenging function of the receptor, which may function for maintenance of chemokine gradient and receptor responsiveness to chemokine during inflammation [71].

β-arrestin1 has been recently shown to mediate endosomal sorting of CXCR4 into the degradative pathway, through interaction with C-tail of the receptor, suggesting a β-arrestin interaction at EE levels [273]. β-arrestin1 seems to regulate endosomal

ubiquitination events that are critical for regulating sorting of ubiquitinated CXCR4 into the degradative pathway, controlling in this way the amount of CXCR4 that is degraded [274].

In conclusion, β-arrestins seem to play a non-redundant role not only in chemokine receptor internalization and desensitization, but also in regulating chemokine receptor intracellular trafficking, in continuing and promoting receptor signalling and in inducing chemotaxis.

Recently, chemokine redundancy has been proposed to be a biased process by which different chemokines can play distinct roles. Chemokines targeting the same receptor can display marked differences in their efficiencies for G protein- or β-arrestin-mediated signalling or receptor internalization, suggesting a β-arrestin biased agonism for different chemokines [275], as previously reported for CCR7 [69]. Rajagopal et al. suggested a biased agonism also for CCR10, CXCR1 and CXCR3 ligands [275].

4- C. β-ARRESTIN AND ACKRS

As for canonical chemokine receptors, the association of ACKRs with β-arrestin has been proposed to be important for receptor internalization and recycling. Agonist activation of C5L2 results in relocalization to endocytic vesicles and association with β-arrestin2 [29]. ACKR3 interacts with β-arrestin2 in basal condition and ligand engagement significantly enhances this interaction [30], and ACKR2 relocalized β-arrestins within the cytoplasm even in the absence of ligand [18, 214]. The C-tail of ACKRs is particularly rich of serine residues involved in direct interaction with β-arrestins [214, 276, 277] and these serine residues are well conserved across different species [127]. The interaction of ACKRs C-tail with β-arrestin is a critical event for receptor stability, trafficking, chemokine uptake from the extracellular space and their degradation.

Canonical chemokine receptor has been reported to signal toward β-arrestin and/or G-protein signalling. As previously said for CCR5, replacement of arginine into asparagine in the DRY motif was shown to abrogate G-protein signalling, resulting in receptor constitutive phosphorylation and association with β-arrestins [270]. The observation that ACKRs present similar modification in the DRY motif and associate β-arrestin, raised the hypothesis if these receptors may operate as β-arrestin biased receptors. As no evidence of ACKRs G-protein dependent signalling pathway has been reported, evidence suggests non-conventional signalling activities for these receptors.

4- C.1 β-arrestin and ACKR4

ACKR4 has been previously reported to internalized chemokines in a β-arrestin independent manner and it does not need clathrin-coated pits, but it required dynamin and caveolin-1 [132]. ACKR4-ligand complexes are internalized through caveole and they induce β-arrestin2 translocation at plasma membrane. In addition, after internalization, ligands colocalize with β-arrestin2 in intracellular vesicles, suggesting a possible involvement of β-arrestin in ACKR4 endocytosis in a parallel internalization pathway [31]. Watts et al. clearly demonstrated that ACKR4 recruits both β-arrestins after chemokine engagement and it does not activate typical chemokine receptor-induced β-arrestin- or G-protein-mediated signalling pathway. In fact, it is unable to activate downstream signals to Erk1/2 and Akt upon ligand stimulation, as reported for CCR7 in a β-arrestin dependent manner. ACKR4 is not coupled to Gαi or Gαs protein, but it is able to increase cAMP levels in response to chemokine stimulation, however PTX-sensitive G proteins normally prevent this signalling [31]. The hypothesis proposed by Watts et al. is that in basal condition the receptor is associated to Gαi, preventing signalling of this receptor to cAMP, and it is internalized through β-arrestin1 and β-arrestin2. Pre-treating cells with PTX induces an activation of cAMP through a G-protein dependent manner, in particular Gαs-dependent, as demonstrated after DRY mutation. Chemokines-induced recruitment of β-arrestins to ACKR4 might open avenues yet to be identified to activate G-protein independent signalling pathways, supporting the ideas of ACKRs as β-arrestin biased receptors [31].

4- C.2 β-arrestin and C5L2

The role of β-arrestins for the decoy receptor C5L2 is still controversial. Kalant et al. first demonstrate β-arrestin relocalization at plasma membrane in cells expressing C5L2. In the absence of stimulation in C5L2 positive cells, β-arrestin is distributed at cytosolic levels. After ligands stimulation, β-arrestin relocalizes at plasma membrane through a punctuate distribution, attributed to its association with C5L2, after receptor phosphorylation [9], although the phosphorylation has been reported to be minimal [278]. Van Lith et al. demonstrated that the isoform β-arrestin2 is redistributed at plasma membrane in C5a dose-dependent manner [29], while β-arrestin1 is not translocated after receptor activation [122]. C5L2 is targeting for endocytosis via clathrin-coated pits through a β-arrestin2-mediated mechanism and β-arrestin2 is internalized with the receptor in

endocytic vesicles [279]. These interactions seem not to be involved in C5L2 signalling pathway, not resulting in Erk1/2 phosphorylation.

In addition, Cain and Monk demonstrated that C5L2 couples weakly with Gαi protein as a low level of PTX-sensitive signal transduction can occur following ligand binding [280], although no calcium fluxes have been registered after ligand stimulation. C5L2 has been shown to act as a negative modulator of C5aR signalling, being activated after C5aR activation, resulting in inhibition of C5aR/β-arrestin mediated Erk1/2 activation, with no apparent alteration of G-protein mediated functions [278]. Taken together, all reported data show a C5L2 association with β-arrestin after ligand stimulation, but only β-arrestin2, not the isoform 1. These complexes are not reported to induce a signalling pathway, but they can act as negative regulator of C5aR/β-arrestin2 dependent signalling pathway, resulting in an inhibition of Erk1/2 phosphorylation.

4- C.3 β-arrestin and ACKR3

ACKR3 has been demonstrated to recruit β-arrestin2 in a ligand-dependent G-protein-independent manner, resulting in ligand internalization [281, 282]. Ligand binding to ACKR3 does not result in activation of signalling pathways typical of G protein, but activate MAPKs through β-arrestin2 in endosomes, in particular β-arrestin2, suggesting a role for ACKR3 as a β-arrestin-biased receptor [283]. ACKR3 has been linked also to the activation of Akt, resulting in the regulation of a number of genes associated to the NFkB and MAPK pathways [181], but the involvement of β-arrestin is not still known. The interaction of β-arrestins with ACKR3 has been reported to be dependent on the intracellular tail of the receptor and it is necessary for receptor normal localization, internalization and scavenging activity. The interaction of β-arrestins with intracellular tail of ACKR3 may be critical also for signal transduction and both β-arrestins seem to be involved in ACKR3 activity [284].

As reported by Ray et al., progressive truncation of the C-tail redistributed ACKR3 from intracellular vesicles to plasma membrane, not altering ligand binding capacity, but showing reduced association with β-arrestin2. These lead to less receptor internalization, less chemokine scavenging and less ligand-dependent activation of Erk1/2, demonstrating how the intracellular tail of ACKR3 is a key domain in controlling multiple function of this receptor [276]. Mutation of serine/threonine residues in the C-terminus results in receptor inability to recruit β-arrestins and consequently the internalization is impaired, suggesting

that phosphorylation of the receptor promotes β-arrestin recruitment [284].

ACKR3 in basal condition is ubiquitinated, and this is an essential determinant for regulating intracellular trafficking of the receptor. In order to induce receptor recycling after chemokine binding, β-arrestins play a non-redundant role, controlling the de-ubiquitination of ACKR3 after internalization, rendering the receptor able to recycle to the cell surface [284]. These results have been confirmed also by Mahabaleshwar et al., reported that β-arrestin2 can control the endosomal sorting of ACKR3. The complexes ACKR3-ligands are targeted for degradation to late endosomes, but β-arrestin2 promote ligand separation and receptor recycling to the cell surface. In fact, β-arrestins increase ACKR3 levels in recycling compartment, promoting its proper endosomal sorting [285].

For concluding, ACKR3 recruits both β-arrestins after receptor phosphorylation, although it seems to be a β-arrestin2 biased receptor, and arrestins seem to be important in regulating receptor intracellular trafficking, its scavenging activity and signalling transduction pathways.

4- C.4 β-arrestin and ACKR2

The phosphorylation status and β-arrestin association with ACKR2 are still under debate. McCulloch et al. demonstrated that ACKR2 is constitutively phosphorylated at the serine-rich motif in the C-tail, but the constitutive internalization and receptor recycling are not influenced by the receptor phosphorylation status [125, 214]. ACKR2 expression induces β-arrestin re-localization at the plasma membrane but ACKR2 internalization is not dependent on β-arrestin and even in the complete absence of the C-tail, the receptor still internalizes but presents limited scavenging activity [214]. Conversely, Galliera et al. proposed that ACKR2 retains the ability to associated β-arrestin1 and β-arrestin2 in basal conditions and this interaction is required for its constitutive internalization, as demonstrated by the use of β-arrestin-deficient cells. Colocalization experiments indicate that ACKR2 is constitutively associated with β-arrestin. The receptor is not phosphorylated in the tail and the internalization is phosphorylation-independent [18]. Together, these data indicate that β-arrestin probably regulates ACKR2 recycling from intracellular compartment to the cell surface, as reported for the others ACKRs, and is required for the optimal ACKR2 function [285], and the phosphorylation status of the receptor is not influenced by ligand binding [17, 18]. Despite the differences, the two groups agree that

ACKR2 has the potential to constitutively drive the re-localization of β-arrestins within the cytoplasm through a mechanism that is still unknown.

It is, however, unclear whether a ligand-independent basal level of phosphorylation is present [133], or whether ACKR2 association occurs in the absence of receptor phosphorylation [132]. Moreover, the C-tail of ACKR2 shows acidic residues not detected in other chemokine receptors and seems to be important for β-arrestin association and receptor cycling [132].

AIM

The atypical chemokine receptor ACKR2, previously referred to as D6, is a chemokine scavenger receptor with a non-redundant role in the control of inflammation. The scavenging activity of ACKR2 relies on its trafficking properties, which are supported by constitutive recycling through Rab4/Rab11-positive endosomes and its redistribution from recycling endosomes to plasma membrane after agonist engagement.

Since the trafficking properties of chemokine receptors rely on the cytoskeletal dynamics, the first aim of my thesis was to elucidate the role of actin and microtubules network in regulating the ACKR2 intracellular trafficking, recycling properties and scavenging activity after chemokine engagement. Afterwards, I focused on the signalling molecules and molecular motors reported to regulate the cytoskeletal reorganization, trying to identify the signalling transduction pathway downstream ACKR2 activation.

As the intracellular trafficking and signalling properties of ACKR2 resulted to be a β-arrestin-dependent but G-protein-independent mechanisms, I deeper investigated the role of β-arrestins in ACKR2 activation, evaluating β-arrestins recruitment after ligand stimulation and its association to ACKR2 under basal condition, using a BRET (*Bioluminescence Resonance Energy Transfer*) technology and confocal microscopy analysis.

MATERIALS AND METHODS

1. CHEMICALS AND ANTIBODIES

Cytochalasin D, latrunculin A, jasplakinolide, nocodazole, paclitaxel, NSC23766, IPA-III, pertussis toxin (PTX) were purchased from Calbiochem (Merck4Biosciences, Darmstadt, Germany). Poly-L-lysine, forskolin and arginine vasopressin (AVP) were from Sigma-Aldrich (St. Louis, Missouri, US). Sodium azide (NaN_3), paraformaldehyde (PFA), Triton X-100, glycine, Tween 20, phenylmethylsulfonyl fluoride (PMSF), EDTA, 3-isobutyl-1-methylxanthine (IBMX) and chemicals for lysis buffer, Tris Buffered Saline (TBS), reducing sample buffer, Tris-Krebs (TK) buffer were from Merck Chemicals (Darmstadt, Germany). Lipofectamine 2000 and RNAiMax were from Invitrogen (Life Technologies Ltd, Paisley, UK). Normal goat serum (NGS) was provided by Dako (Glostrup, Germany). Coelenterazine H was purchased from Molecular Probes (Life Technologies Ltd), while DeepBlueC (Coelenterazine 400a) from Biotium (Hayward, California, US). Polyethylenimine (PEI, linear polyethylenimine, MW 25000) was purchased from Polysciences (Warrington, Pennsylvania, US).

Recombinant human chemokines were from R&D Systems (Minneapolis, Minnesota, US). ^{125}I-CCL2 and ^{125}I-CCL4 were from Perkin Elmer (Waltham, Massachusetts, US).

For Western blot analysis, antibodies for rabbit anti-human β-arrestin 1/2, cofilin and phosphorylated cofilin (P-Cofilin, Ser-3), LIMK1 and phosphorylated-LIMK1 (P-LIMK1, Thr-508), PAK1 and phosphorylated PAK1 (P-PAK, Ser-141) were from Cell Signalling Technology (Danvers, Massachusetts, US). Mouse anti α-tubulin and vinculin antibody were from Sigma-Aldrich. Anti-rabbit and anti-mouse IgG-horseradish peroxidase conjugated secondary antibodies were provided by GE Healthcare GmbH (Freiburg, Germany).

For confocal microscopy analysis and cytofluorimetric analysis, unconjugated anti-human ACKR2 monoclonal antibodies and rat IgG2a isotype controls were from R&D Systems. Antibodies for human myosin Vb, Rab4, Rab11, CCR5 and mouse IgG2a isotype control were from BD Biosciences (San Jose, California, US). Anti α-tubulin and the isotype control IgG1 were from Sigma-Aldrich. Alexa® Fluor 488-conjugated phalloidin, Alexa® Fluor-conjugated secondary antibodies and 4',6-diamidino-2-phenylindole (DAPI) were purchased from Molecular Probes.

2. PLASMIDS

The pRK5 empty vector or expressing human vasopressin receptor 1a (V1aR) and 2 (V2R) were kindly provided by Eric Trinquet (CIS bio international, Bagnols sur Cèze Cedex, Montpellier, France). Human ACKR2 was expressed with pcDNA3.1 vectors (Invitrogen). Human CCR5 was cloned in pcDNA3.1 or pEGFP-N1 vector (Clontech, Mountain View, California, US). The Rab4 S22N-pEGFP plasmid was provided by Robert Lodge (INRS-Institut Armand Frappier and Centre for Host Parasite Interactions, Laval, Canada). The Rab11 S25N-pEGFP construct was produced as previously described [286]. The myosin Vb tail fragment pEGFP plasmid was provided by Ann Richmond (Department of Veterans Affairs, Nashville, USA). The LIMK1, LIMK1 D460A and shRNA LIMK1 plasmid were provided by Kensaku Mizuno (Department of Biomolecular Sciences, Tohoku University, Sendai, Miyagi, Japan). ACKR2 and CCR5 were cloned in pRluc-N3 vector (BioSignal Packard, Packard BioScience Company, Meriden, Connecticut, US), in order to have ACKR2 or CCR5 in frame with *Renilla* luciferase gene, without stopping codon. β-arrestin1 and β-arrestin2 were cloned in frame with Yellow Fluorescent Protein (YFP) in pEYFP-N1 vector (Clontech) or in frame with Green Fluorescent Protein 2 (GFP^2) in $pGFP^2$-N3 vector (BioSignal Packard).

3. CELL CULTURE AND TRANSFECTION

Chinese hamster ovary (CHO-K1) cell line wild type or stably expressing human ACKR2 and CCR5 (untagged or pEGFP-tagged) and choriocarcinoma cell line BeWo were grown in DMEM/F12 (Lonza, Basel, Switzerland). Human embryonic kidney (HEK293 and HEK293T) cell lines wild type or stably expressing human ACKR2 and CCR5 were cultured in DMEM (Lonza). Both culture media were completed by adding 10% fetal bovine serum (FBS) (Euroclone, Milan, Italy), 100 U/ml of penicillin/streptomycin (P/S) (Lonza), 25 mM HEPES (Gibco, Life Technologies Ltd). Cells transfectants were taken under selective pressure by 650 μg/ml of G418 (Invitrogen, Life Technologies Ltd). CHO-K1/ACKR2 transfectants were obtained as previously described [223]. CHO-K1/CCR5 pEGFP, CHO-K1/CCR5, CHO-K1/pcDNA3.1, HEK293/ACKR2 and HEK293/CCR5 were generated by lipofection with the indicated plasmids, selected with 650 μg/ml G418 and cloned by limiting dilution. HEK293T and HEK293/ACKR2 cells were transiently transfected for 24 h with pRK5 empty vector/V1aR/V2R and LIMK WT/D460A respectively according to

Lipofectamine 2000 manufacturer's instructions. Transient transfectants of CHO-K1/ACKR2 cells were generated by lipofection and were collected 24 h later. HEK293T cells were co-transfected with ACKR2/Rluc or CCR5/Rluc with β-arrestin1/YFP or β-arrestin2/YFP for the BRET1 experiments or β-arrestin1/GFP^2 or β-arrestin2/GFP^2 or GFP^2 for BRET2 experiments, using Polyethylenimine (PEI), as described in BRET1 and BRET2 sections.

4. SIRNA TRANSFECTION

HEK293T transfectants (5 x 10^5) were seeded onto poly-L-lysine coated 6 wells plate and grown in complete culture media without G418 and P/S at 37°C for 18 hours. 50-70% confluent cells were transiently transfected for 72 hours with 50 nM of ON-TARGETplus siRNA (Dharmacon, Thermo Scientific Pierce, Rockford, Illinois, US) for human cofilin, PAK1, β-arrestin1 and control pool (scrambled) according to Lipofectamine RNAiMax manufacturer's instructions. Knocking-down of indicated genes was checked for each experiment by Western blot analysis.

5. ACKR2 INTERNALIZATION AND CELL SURFACE EXPRESSION

Antibody feeding experiments were performed to evaluate ACKR2 internalization. When indicated, CHO-K1/ACKR2 cells were pre-treated for 1 hour with vehicle (DMSO) or with 1 μM cytochalasin D, latrunculin A, jasplakinolide, paclitaxel, 10 μM nocodazole; for 30 min with vehicle (water) or 200 μM NSC23677, 5 μM IPA-III, or for 16 hours with PTX 100 ng/ml. Cells were washed in PBS (Phosphate Buffered Saline- Biosera, East Sussex, United Kindom) + 1% Bovine Serum Albumin (BSA- Sigma Aldrich) and stained on ice with 5 μg/ml of anti-ACKR2 antibody for 1 hour. Labelled cells were then incubated for the indicated times at 37°C in DMEM-F12 with 1% BSA to allow ACKR2 internalization. Samples were then returned to ice, washed and incubated with Alexa® Fluor 647 anti-rat IgG for 30 min in FACS buffer (PBS supplemented with 1% BSA and 0.01% NaN_3).

ACKR2 cell surface abundance after chemokine stimulation was evaluated as followed. Cells were incubated at 37°C with or without CCL3L1 (100 nM) for indicated time points in DMEM-F12 + 1% BSA. Cells were then transferred on ice, washed and labelled with ice-cold FACS buffer containing 5 μg/ml of anti-ACKR2 antibody for 1 hour. Cells were then stained with secondary antibody Alexa® Fluor 647 anti-rat IgG for 30 minutes in

FACS buffer. When CHO-K1/ACKR2 transient transfectants were used, cell surface expression of ACKR2 was evaluated in two distinct gates, referred to the viable $pEGFP^{neg}$ and $pEGFP^{pos}$ cells, respectively.

For each experiment, 3 x 10^5 events of viable cells identified by FSC-H and SSC-A parameters are acquired by BD FACSCanto flow cytometer and analyzed by BD FACSDiva software (BD Biosciences). Data were calculated as percentage of mean fluorescence intensity (MFI) of stimulated over unstimulated cells or treated over untreated cells.

6. CHEMOKINE SCAVENGING ASSAY

Cells (5 x 10^4) were incubated in complete culture media without G418 in 96-wells plate at 37°C for 18 hours. When HEK293T transfectants or siRNA-treated cells were used, wells were previously coated with 100 µl/well of poly-L-lysine for 30-40 min at 37°C. When indicated, BeWo or CHO-K1/ACKR2 cells were incubated for 1 hour with vehicle or with 1 µM cytochalasin D, latrunculin A, jasplakinolide, paclitaxel, 10 µM nocodazole; or for 30 min with vehicle or with 200 µM NSC23677, 5 µM IPA-III, or for 16 hours with 100 ng/ml of PTX. Cells were then incubated at 37°C for 3 up to 24 hours in 60 µl of culture media + 1% BSA + 25 mM HEPES + 0.1 nM of ^{125}I-CCL2 or ^{125}I-CCL4, and the indicated concentrations of unlabelled CCL2 or CCL4. Proteins in the supernatants were precipitated with 20% tricholoracetic acid (TCA) (Carlo Erba Reagents, Milano, Italy) at 4°C for 15 min and both soluble and insoluble fractions were considered. The radioactivity present in each fraction was measured by using a Wizard Automatic γ Counter (Perkin Elmer). Data refer to radioactivity present in the TCA insoluble phase that is representative of the degraded chemokine. Degradation rate curves were obtained by data fitting with nonlinear regression and interpolation with Michaelis-Menten equation using the Prism4 software (GraphPad Software; San Diego, California, US).

For non-radioactive degradation assay, cells were incubated at 37°C in culture media + 1% BSA + 25 mM HEPES supplemented with 1 nM of CCL3L1. When the myosin Vb tail fragment was used, chemokine scavenging was evaluated on sorted $pEGFP^{neg}$ and $pEGFP^{pos}$ CHO-K1/ACKR2 transient trasfectants cells, after 18 hours. Cells were incubated with 1nM CCL3L1 for the indicated time points. Chemokine concentration in the supernatant was measured by ELISA (R&D Systems).

7. IMMUNOFLUORESCENCE AND CONFOCAL MICROSCOPY ANALYSIS

Cells (1 x 10^5) were seeded onto poly-L-lysine coated 14 mm diameter glass dishes (Menzel-Gläser, Thermo Fisher Scientific, Waltham, Massachusetts, US) in 24 wells plate and grown in complete culture media without G418 at 37°C for 18 hours. When indicated, CHO-K1/ACKR2 cells were incubated for 1 hour with vehicle (DMSO) or with 1 μM cytochalasin D, latrunculin A, jasplakinolide, paclitaxel, 10 μM nocodazole; or for 30 min with vehicle (water) or with 200 μM NSC23677, or for 16 hours with 100 ng/ml of PTX. Cells were untreated or treated with CCL3L1 (100 nM) in pre-warmed culture media + 1% BSA + 25 mM HEPES, fixed with 4% PFA for 15 min, permeabilized with 0.3% Triton X-100 in PBS + 2% BSA + 0.1% Glycine + 5% NGS for 1 hour. β-arrestin1/YFP or β-arrestin2/YFP transfected cells were permeabilized when indicated. Fixed cells were incubated with 1 μg/ml of anti-ACKR2 or anti-CCR5 primary antibody or rat IgG2a or mouse IgG2a isotype controls for 2 hours at room temperature (RT). To detect microtubules, cells were incubated for 1 hour at RT with 1 μg/ml anti α-tubulin. To visualize myosin Vb, Rab4 and Rab11, cells were incubated for 1 hour at RT with 2,5 ug/ml anti myosin Vb, anti-Rab4 and anti-Rab11, respectively. The coverslips were washing 3 times with washing buffer (PBS + 0.2% BSA + 0.05% Tween 20) and then were incubated with 2 μg/ml of secondary antibody for 1 hour at RT, extensively washed, and incubated with 0,5 μM of Alexa® Fluor 488-conjugated phalloidin for 30 min, when indicated. Following 10 min incubation at RT with 300 nM of DAPI (Invitrogen), specimens were mounted on glass slide (Menzel-Gläser) with 20 μl FluoSave reagent (Calbiochem, Merck4Biosciences, Darmstadt, Germany) and kept in dark at RT for 24 hours. High-resolution images (1024 x 1024 pixels) were acquired sequentially with a 60X 1.4 N.A. Plan-Apochromat oil immersion objective by using a FV1000 laser scanning confocal microscope (Olympus; Hamburg, Germany). Differential Interference Contrast (DIC- Nomarski technique) was also used. Images were assembled, cropped by Photoshop software (Adobe Systems; San Jose, CA). Quantitative colocalization and statistical analysis were performed by Imaris Coloc 4.2 (Bitplane AG, Zurich, Switzerland) software and FV1000 1.6 colocalization software (Olympus). Quantification of Pearson's Coefficient of Correlation (PCC) was performed inside a selected region of interest per image, representative of the analysed cell. For statistic, colocalization analysis was performed on at least 30 cells representative of each experimental condition.

8. COFILIN/LIMK1/PAK1 PHOSPHORYLATION ASSAY

Cells (3 x 10^5) were seeded onto 12 wells plate, previously coated with 100 µl/well of poly-L-lysine for 30-40 min at 37°C and grown in complete culture media without G418 at 37°C for 18 hours. Cells were serum-starved by replacing FBS with 0.1% BSA for 18 hours before chemokine stimulation and when indicated BeWo or CHO-K1/ACKR2 cells were pre-treated for 1 hour with vehicle (DMSO) or with 1 µM cytochalasin D, latrunculin A, jasplakinolide, paclitaxel, 10 µM nocodazole or for 30 minutes with vehicle (water) or 200 µM NSC23766, 5 µM IPA-III, or for 16 hours with 100 ng/ml of PTX. Then, cells were stimulated in pre-warmed culture media + 1% BSA + 25 mM HEPES with 100 nM of the indicated chemokines added at the indicated time points. After chemokine stimulation, cells were lysed and processed as described in western blot paragraph.

9. WESTERN BLOT

Cells were lysed for 5 min with ice-cold lysis buffer (50 mM Tris HCl pH 8 + 150 mM NaCl + 5 mM EDTA + 1.5 mM $MgCl_2$ + 1% Triton X-100 + 10% glycerol) freshly prepared and supplemented with protease inhibitors EDTA-free (Roche, Basel, Switzerland) and 100 µg/ml PMSF. When protein phosphorylation level was checked, lysis buffer was supplemented with phosphatase inhibitors: 0.01 M sodium pyrophosphate, 50 mM sodium fluoride (NaF) and 0.01 M sodium orthovanadate (Na_3VO_4). After 4°C centrifugation at 13000 rpm for 20 min, cell supernatants were quantified by DC Protein Assay (Bio-Rad, Hercules, California, US). Equal protein amounts of total cell lysates (20-50 µg) were diluted in Laemmli Sample Buffer (Bio-Rad), containing 0.71 M β-mercaptoethanol, loaded in 12% or 4-20% polyacrylamide pre-casted minigels (Bio-Rad) and then transferred polyvinylidene fluoride (PVDF) membrane (Bio-Rad). Membranes were blocked in 5% non-fat dry milk (Nestlé, Vevey, Switzerland) in TBS-T (TBS + 0.1% Tween 20. TBS: 25mM Tris HCl pH 7.5 + 150 mM NaCl) for 1-2 hours at RT, rinsed with TBS-T for 5 min and then incubated overnight at 4°C with 1:1000 dilution in TBS-T + 5% BSA of the indicated primary antibody. Membranes were washed 5 times for 5 min with TBS-T and incubated for 1 hour at RT with 1:2000 dilution in TBS-T + 5% BSA of anti-rabbit IgG-horseradish peroxidase conjugated secondary antibody. Membranes were washed 5 times for 5 min with TBS-T and incubated with chemiluminescent HRP substrate (Immobilon Western-Millipore, Billerica, Massachusetts, US). Blots were acquired by ChemiDoc XRS Imaging

System (Bio-Rad). Densitometric analysis was performed by Quantity One software (Bio-Rad) and protein band intensity was calculated by normalization over α-tubulin or vinculin band intensity, except for P-LIMK1 which was normalized over total LIMK1. α-tubulin or vinculin staining was performed by using 1:4000 or 1:10000 dilution, respectively, in TBS-T + 5% BSA of mouse anti-α-tubulin or anti-vinculin primary antibody for 2 hours at RT, followed by incubation at RT for 1 hour with 1:2000 dilution in TBS-T + 5% BSA of anti-mouse IgG-horseradish peroxidase conjugated secondary antibody. Bands were detected as approximately 50 kDa (α-tubulin), 125 kDa (vinculin), 19 kDa (cofilin), 72 kDa (LIMK1), 68 kDa (PAK1) and 50 kDa (β-arrestin).

10. RAC1 ACTIVATION ASSAY

2.5 x 10^6 HEK293/ACKR2 cells or HEK293/ACKR2 cells treated for 48 hours with control siRNA (scrambled) or β-arrestin1 specific siRNA were plated onto poly-L-lysine coated T75 flask and grown at 37°C for 18 hours complete culture media without G418. Cells were serum-starved by replacing FBS with 0.1% BSA for 18 hours and then stimulated in pre-warmed culture media + 1% BSA + 25 mM HEPES with 100 nM CCL3L1 added at the indicated time points. Rac1 activation was measured according to the manufacturer's instructions (Thermo Scientific Pierce). Briefly, stimulated cells were lysed with ice-cold lysis buffer for 5 min and total cell lysates were quantified by DC Protein Assay (Bio-Rad). For positive control sample, 10 mM of EDTA pH 8 and 0.1 mM of GTPγS are added to total cell lysates and incubated at 30˚C. After 15 min, reaction was stopped by placing sample on ice and adding 60 mM of $MgCl_2$. 20 µg of GST-human PAK1-PBD were added to glutathion-agarose resin previously washed with lysis buffer, into which 500 µg up to 1 mg of total cell lysates and the positive control sample were immediately transferred, before incubating the reaction mixture at 4°C for 1 hour. After column washing with lysis buffer, samples were then incubated at RT for 2 min with reducing sample buffer (125 mM Tris HCl pH 6.8, 2% glycerol, 4% SDS (w/v) + 0.05% bromophenol blue + 0.71 M of β-mercaptoethanol), centrifuged and supernatants containing Rac1-GTP immunoprecipitated lysates were collected. 40 µl/lane of Rac1-GTP immunoprecipitated lysates and 25 µg/lane of total cell lysate were loaded into 12% polyacrylamide-precasted mini gel to detect Rac1-GTP pulled down and total Rac1, respectively. Proteins were transferred to PVDF membrane. Membrane was blocked with TBS + 3% BSA at RT for 1-2 hours, incubated overnight at 4°C with 1:2000 dilution in TBS-T + 3% BSA of mouse anti-

Rac1 primary antibody followed 1 hour at RT with 1:20000 dilution in TBS-T + 5% non-fat dry milk of anti-mouse IgG-horseradish peroxidase conjugated secondary antibody. Blot was acquired as previously described (see *Western blot* paragraph). A GST-PBD band at 35 kDa was detected without interfering with Rac1 band at 22kDa. Densitometric analysis is performed by Quantity One (Bio-Rad) software and results are obtained by Rac1-GTP normalization over total amount of Rac1 measured in total cell lysate.

11. G PROTEIN ACTIVATION ASSAY

G protein activation was assessed by using homogenous time-resolved fluorescence (HTRF) technique developed by CisBio International according to the manufacturer's instructions (Camarillo, California, US). Briefly, to analyze Gαi and Gαs activation CHO-K1/pcDNA3.1, ACKR2 and CCR5 were seeded into poly-L-lysine coated 384-well plates (Greiner Bio-One, Frickenhausen, Germany) at a density of 3 x 10^4 cells per well in complete culture media without G418. After 18 hours, cells were rinsed with TK buffer (136 mM NaCl + 5 mM KCl + 1.2 mM $MgCl_2$ + 2.5 mM $CaCl_2$ + 10 mM glucose + 20 mM Trizma base, pH 7.4) and incubated for 1 hour with the indicated chemokines (200 nM) in C-buffer (TK buffer + 0.5 mM IBMX). For Gαi activation, 20 μM of forskolin was prepared in C-buffer and added together with chemokines. HEK293T transiently transfected with V2R and stimulated with 100 nM of AVP were used as positive control for Gαs activation. Cells were subsequently lysed by adding cAMP/cGMP conjugate and lysis buffer and incubated with cAMP-d2 and anti-cAMP cryptate to measure cAMP formation. To analyze Gαq activation, cells were seeded as previously described. After 18 hours, cells were rinsed with stimulation buffer and incubated for 1 hour with the indicated chemokines (200 nM). HEK293T transiently transfected with V1aR and stimulated with 100 nM of AVP were used as positive control for Gαq activation. Cells were subsequently lysed by adding IP-One Tb conjugate and lysis buffer and incubated with IP1-d2 and anti-IP1 cryptate Tb conjugate to measure IP1 formation. Plate was read on the compatible HTRF reader Mithras LB 940 (Berthold Technologies). Results were calculated from the 665 nm/620 nm ratio x 104 (R) and expressed in Delta F% obtained by the following equation: (standard or sample R - negative R)/(negative R) x 100. Data were analyzed and showed as follow: Delta F% of each data points of both CHO-K1/ACKR2 and CCR5 were normalized over the corresponding data points of CHO-K1/pcDNA3.1 and after, values obtained were normalized over values corresponding to unstimulated conditions. Same analysis was

performed for transient transfected HEK293T/V1aR and V2R normalized on HEK293T/pcDNA3.1.

12. BIOLUMINESCENCE RESONANCE ENERGY TRANSFER 1 (BRET1)

The Bioluminescence Resonance Energy Transfer (BRET) assay is hugely used for studying protein-protein interactions. The assay is based on the energy transfer between fusion proteins containing *Renilla* luciferase (Rluc) and a mutant of the Green Fluorescente Protein GFP (YFP in BRET1 assay and GFP^2 in BRET2 assay). Rluc is the bioluminescent donor and GFP mutants are the fluorescent acceptors. The energy transfer efficiency is highly dependent on the distance between the donor and the acceptor moieties and their relative orientation with respect to each other. The typical effective distance between the donor and the acceptor is up to 100 Å (**Figure 23**) [287]. This range correlates well with most of biological interactions, making this technology an excellent tool for monitoring macromolecular interaction [287].

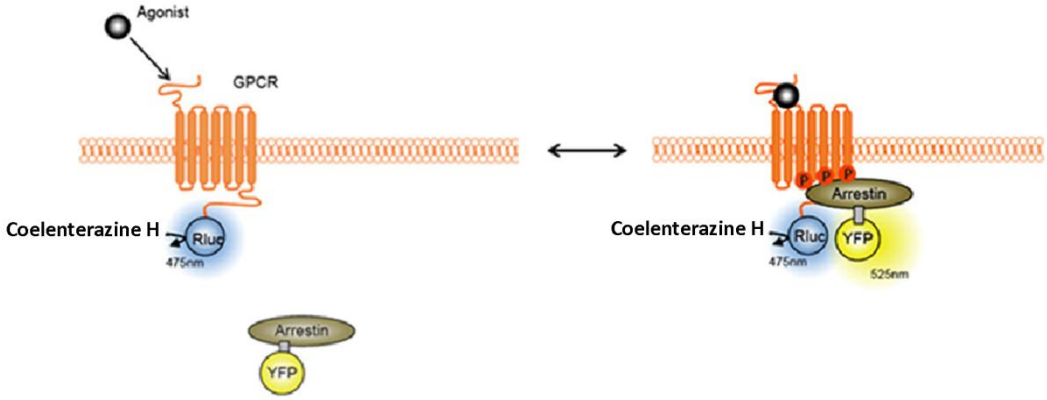

Figure 23: Bioluminescence Resonance Energy Transfer (BRET). BRET technology is based on the energy transfer between fusion proteins containing *Renilla* luciferase (Rluc) and a mutant of the Green Fluorescent Protein (GFP). In figure are represented the postulated molecular events following activation of a GPCR by agonist that lead to recruitment of arrestin to phosphorylated receptor causing related BRET signal. Arrestins can be tagged with different versions of GFP (YFP or GFP^2) and GPCRs are tagged with Rluc to monitor intermolecular interaction by BRET (Figure derived from [287]).

The BRET1 signal is generated by the catalytic oxidation of Coelenterazine H by Rluc resulting in the emission of light at 475 nm. When an appropriate energy acceptor is present within BRET-permissive distance from Rluc, part of the energy can be transferred to the acceptor. This process leads to the excitation of YFP and subsequent emission of light with a characteristic spectrum having a maximum peak at 535 nm.

HEK293T cells (1.5x10^6 cells in a T25 plate, in DMEM without P/S) were transiently co-transfected with PEI in defined stoichiometric ratios of each vector, usually 1.5 µg of receptor-Rluc (ACKR2/Rluc or CCR5/Rluc) constructs and 5 µg of β-arrestins-YFP (β-arrestin1/YFP or β-arrestin2/YFP) constructs or pcDNA3 empty vector. 48 hours post-transfection, cells were washed, harvested in PBS supplemented with 0.5% (w/v) glucose and quantified with DC protein assay (Bio-rad). For BRET1 experiments, we considered 80 µg of cells for each condition, in a 96 wells plate (Perkin Elmer, black frame and white well). Cells were incubated for 8 min at RT in dark with 5 µM of Coelenterazine H. After incubation, cells were stimulated with PBS or with the indicated chemokines (100 nM), and plate was immediately read with spectrophotometer Infinite F500 (Tecan, Mannerdorf, Switzerland), in order to measure luminescence and fluorescence value. These values were collected at room temperature with 1 second exposure time at repeating time intervals, for a resulting kinetics of 20 min, using 540/35 nm and 440/40 nm filters, respectively for fluorescence and luminescence. The BRET ratio is calculated as the ratio of YFP (550/40 nm) emission to Rluc (470/30 nm) emission:

$$\boldsymbol{BRET\ ratio} = \frac{\boldsymbol{Em\ YFP}}{\boldsymbol{Em\ Rluc}}$$

To evaluate protein-protein interactions after chemokine stimulation, we considered the BRET ligand effect, the difference between the BRET ratio of cells treated with chemokine and the BRET ratio of untreated cells.

$$\boldsymbol{BRET\ ligand\ effect} = \frac{\boldsymbol{BRET\ ratio\ (ligand - treated\ cells)}}{\boldsymbol{BRET\ ratio\ (PBS - treated\ cells)}}$$

The BRET ligand effect was then plotted in a graph against time, expressed in seconds. Receptor/β-arrestins interaction curves were obtained by data fitting with one-site nonlinear regression and interpolation with Michaelis-Menten equation using the Prism4 software (GraphPad Software).

13. BIOLUMINESCENCE RESONANCE ENERGY TRANSFER 2 (BRET2)

In the original BRET system, Rluc and YFP emit light between 475-480 nm and 525-530 nm, respectively, resulting in a poor spectral resolution, with an emission wavelength difference between donor and acceptor of ~45-55 nm [287]. The BRET2 assay uses a proprietary coelenterazine derivative called DeepBlueC that displays different spectral properties when oxidized by Rluc. The reaction produces light at 395 nm, a much shorter wavelength than that of the BRET1 system, and excites GFP^2 that re-emits light at 510 nm, providing a broad spectral resolution between the donor and acceptor emission (~115 nm). This larger spectral resolution increases the robustness of the detection (**Figure 24**).

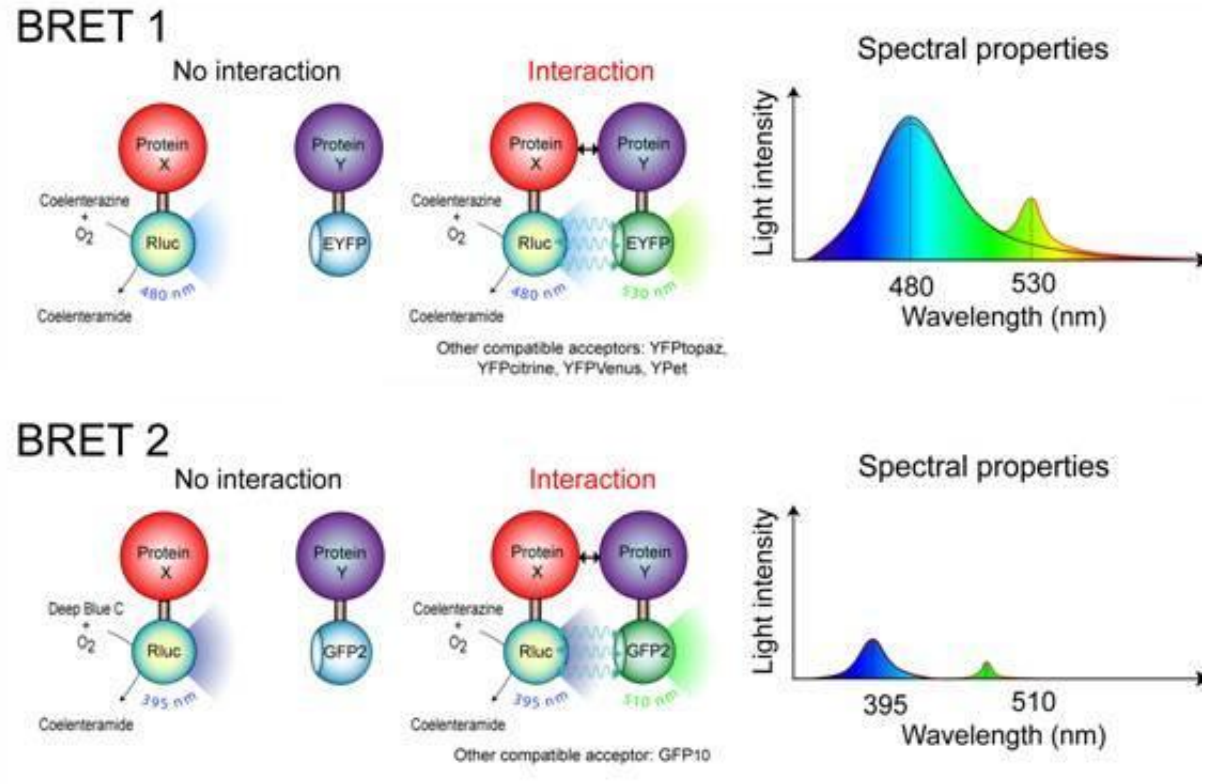

Figure 24: Differences between BRET1 and BRET2, referring to the used substrate, the acceptor molecule used and the spectral properties to each interacting couple (Figure derived from [288]).

In BRET2 assay, the substrate catalyzed by Rluc is DeepBlueC (DBC). In the presence of oxygen, Rluc catalyzes the transformation of DBC into coelenteramide with concomitant light emission peaking at 395 nm. The acceptor in BRET2 assay is a GFP variant (GFP^2) that is engineered to maximally absorb the energy emitted by the Rluc/DBC reaction. Excitation of GFP^2 results in an emission at 510 nm [288].

HEK293T cells (4x10^5 cells in a 6 well plate, in DMEM without P/S) were transiently co-transfected using PEI with 0.2 µg of ACKR2/Rluc, and with decreasing quantity of β-arrestin1/GFP^2, β-arrestin2/GFP^2 or GFP^2: 1.6 - 1.2 - 0.8 - 0.4 - 0.2 - 0.1 - 0.05 - 0.025 - 0.0125 - 0.006 - 0.0028 - 0 µg, the last one used to measure the background in cells transfected with receptor/Rluc construct alone. In order to have the same quantity of DNA in each condition, we added also pcDNA3 empty vector to reach 1.8 µg of total DNA. After 48 hours, cells were washed, suspended in PBS supplemented with 0.5% (w/v) glucose and quantified with DC protein assay (Bio-Rad). To evaluate GFP^2 and Rluc quantity in each condition, we measured the luminescence and fluorescence value with spectrophotometer Infinite F500 (Tecan). The fluorescence value of GFP^2 was measured in duplicate at 510 nm, after excitation of GFP^2 with a monochromator at 400 nm. The fluorescence value for each sample is obtained after mean fluorescence subtraction of background fluorescence, obtained from cells transfected only with receptor/Rluc vector.

FLUORESCENCE value = Mean Fluorescence – Background Fluorescence

The same cells in which fluorescence was evaluated, have been considered for luminescence measurement. Cells have been incubated with 5 µM of Coelenterazine H for 8 min at dark, as for the BRET1 assay. Then the luminescence value has been collected at room temperature with 1 second exposure at 475 nm. The use of coelenterazine H as substrate leads to an emission peak of 475-480 nm, in order to prevent the energy transfer between Rluc and GFP^2 that is excited only at lower wavelengths. The mean values of luminescence and fluorescence are considered to obtain the following ratio:

$$FLUORESCENCE\ \ LUMINESCENCE = \frac{Fluorescence\ value\ at\ 510\ nm}{Luminescence\ value\ at\ 475\ nm}$$

This ratio is important to quantify and normalize the amount of fluorescent protein versus the amount of luminescent protein.

The BRET signal of ACKR2/β-arrestins interaction is measured after cells incubation with DeepBlueC at 5 µM. The plate is immediately read with spectrophotometer Infinite F500 (Tecan), in order to measure luminescence and fluorescence value. The luminescence value of Rluc and the fluorescence value of GFP^2 are measured with 410/80 nm and

515/30 nm filters, respectively. As for the BRET1 assay, the BRET ratio is calculated as the ratio of GFP^2 (515/30 nm) emission to Rluc (410/80 nm) emission.

The BRET ratio data are then plotted in a graph against Fluorescence/Luminescence ratio. ACKR2/β-arrestins interaction curves were obtained by data fitting with one-site nonlinear regression and interpolation with Michaelis-Menten equation using the Prism4 software (GraphPad Software). The curve obtained with GFP^2 alone, considered as negative control, should have a linear behavior. If ACKR2 associates β-arrestin, we should obtain a non-linear curve with a hyperbole-like behavior.

14. STATISTICAL ANALYSIS

Data were analyzed by unpaired Student's t test and, where indicated, with Mann-Whitney test (GraphPad software, Prism 4).

RESULTS

1. ACKR2 AND THE ACTIN CYTOSKELETON

1- A. THE SCAVENGING ACTIVITY OF ACKR2 REQUIRES CHEMOKINE-DEPENDENT ACTIN REARRANGEMENT.

The cytoskeleton and its regulatory and motor proteins are involved in the transport of intracellular vesicles along network of microtubules and actin filaments [289] and play a major role in receptor signalling, endocytosis and trafficking [290, 291]. As ACKR2 mobilization from intracellular stores to the plasma membrane after chemokine engagement is required to optimize its chemokine-scavenging properties [19], we decided to investigate the role of actin cytoskeleton in the regulation of ACKR2 trafficking and chemokine scavenging function. CHO-K1/ACKR2 cells were untreated or treated with 100 nM CCL3L1 for 1 hour and then stained for ACKR2 and phalloidin, an actin marker. Confocal microscopy analysis of CHO-K1/ACKR2 cells showed that in basal conditions ACKR2 did not colocalize with actin filaments, either in intracellular compartments or on the cell surface (**Figure 25A** and **25B**). Interestingly, CCL3L1 induced a time-dependent reorganization of actin cytoskeleton that resulted in increased ACKR2 colocalization with actin filaments, mostly evident at plasma membrane (**Figures 25A** and **25B**). Opposite to ACKR2, in basal conditions the canonical chemokine receptor CCR5 was preferentially expressed at plasma membrane, as previously described [292], where it partially colocalized with actin filaments (**Figure 25A** and **25C**). Treatment with CCL3L1 induced a massive reorganization of actin cytoskeleton also after engagement with CCR5. However, opposite to ACKR2, CCR5 triggering by CCL3L1 resulted in its internalization and decreased the colocalization level of the internalized receptor with actin filaments in endocytic compartments (**Figures 25A** and **25C**).

As we previously demonstrated, chemokine engagement increased ACKR2 expression on the cell membrane by accelerating receptor recycling routes and this adaptive up-regulation allowed the receptor to increase its scavenging efficiency [19]. To evaluate whether ligand-induced actin rearrangements have a functional relevance on ACKR2 scavenger function, we verified the ability of CHO-K1/ACKR2 to degrade chemokines in scavenging assays performed in the presence or absence of cytochalasin D and jasplakinolide, which depolymerize and stabilize actin filaments, respectively. CHO-K1/ACKR2 cells were pre-treated with 1 μM of the indicated inhibitors for 1 hour, then the scavenging function of ACKR2 was evaluated. As shown in **Figure 25D**, both agents exerted

a significant inhibitory effect on ACKR2-mediated chemokine degradation properties, indicating that dynamic actin cytoskeleton rearrangement was functionally important for the chemokine scavenging activity of ACKR2, sustaining ACKR2-mediated uptake and targeting of ligands to the degradative pathway.

ACKR2 degrades different ligands with different efficiency [23]. In particular, we have recently identified a set of neutral ligands which bind ACKR2 with high affinity but are unable to induce receptor redistribution and are not degraded [23], suggesting that ACKR2 trafficking and scavenging activities are functionally related and that signalling events induced by active but not by neutral ligands may control ACKR2 trafficking. Active ligands (in our case CCL2, CCL4 and CCL14(9-74)) are efficiently degraded and are known to induce receptor redistribution, while neutral ligands, in this case CCL14(1-74), CCL3, CCL3(5-68) are unable to induce receptor redistribution and are poorly degraded. Consistent with this hypothesis, confocal microscopy analysis of CHO-K1/ACKR2 cells engaged to different ligands showed that active ligands increased colocalization of the up-regulated ACKR2 with actin filaments at plasma membrane, while neutral ligands were completely inactive, not inducing actin reorganization nor increasing the colocalization of ACKR2 with actin. The same was observed for CXCL8, a non-ligand of ACKR2, used as negative control (**Figure 25E** and **25F**).

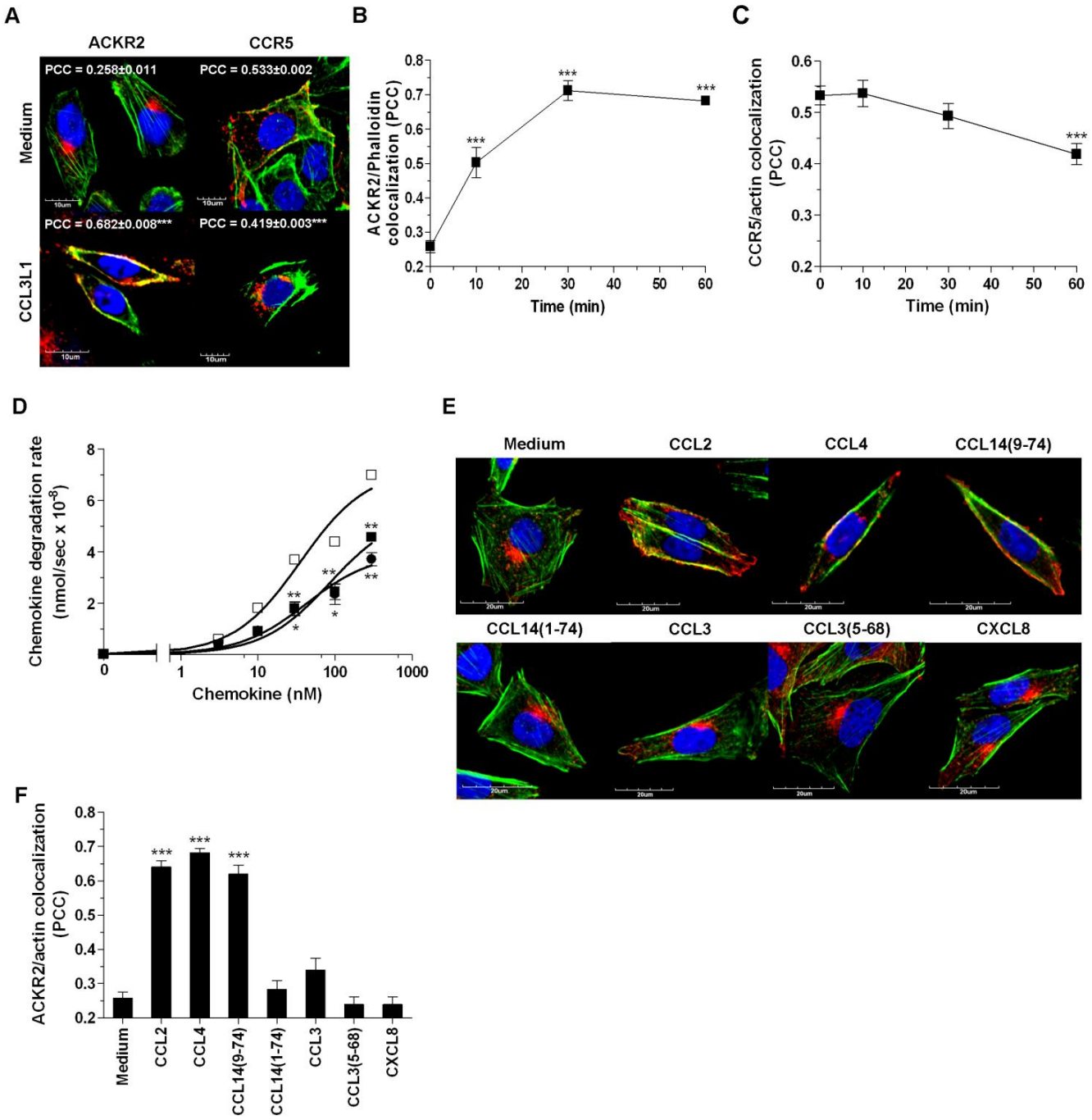

Figure 25: The chemokine-degradatory function of ACKR2 requires ligand-induced actin reorganization. (**A**) Confocal analysis of CHO-K1/ACKR2 and CHO-K1/CCR5 cells untreated or treated with 100 nM CCL3L1 for 1 hour. Each panel shows images of nuclear staining with DAPI (in blue), merged with double staining for ACKR2 or CCR5 (in red) and F-actin (phalloidin staining, in green). (**B** and **C**) Quantification of the amount of colocalization of F-actin with (**B**) ACKR2 and (**C**) CCR5 in response to chemokine stimulation at indicated time points. *** P≤ 0,005 for stimulated versus untreated cells. (**D**) Chemokine scavenging by CHO-K1/ACKR2 cells treated for 30 min with 1 μM cytochalasin D (■), 1 μM jasplakinolide (●), or vehicle (□) and then incubated with 0.1 nM ^{125}I-CCL2 and the indicated concentrations of unlabeled CCL2. Data are the means ± SEM of 6 experiments. * P≤ 0.05 and ** P≤ 0.01 for treated versus

control cells. (**E**) Confocal analysis of CHO-K1/ACKR2 cells stimulated with the indicated chemokines (all at 100 nM) for 1 hour. Each panel shows images of nuclear staining with DAPI (in blue), merged with double staining for ACKR2 (in red) and F-actin (phalloidin staining, in green). (**F**) Quantification of the extent of ACKR2 colocalization with F-actin in response to treatment with the indicated chemokines (all at 100 nM) for 1 hour. *** P≤ 0.005 for stimulated versus untreated cells. All colocalization results are indicated as Pearson's coefficient of correlation (PCC) and are shown as the means ± SEM of 100 cells analysed in 3 independent experiments. Data were analysed by unpaired Student's T test [24].

As also reported in our recently published work [24], the existence of a set of neutral ligands that are not able to support receptor redistribution at plasma membrane nor actin reorganization and are not degraded by ACKR2, led us to hypothesize the requirement of an agonist-induced signalling event downstream ACKR2.

1- B. LIGAND-INDUCED PHOSPHORYLATION OF COFILIN SUSTAINS INCREASED CELL-SURFACE ACKR2 ABUNDANCE AND CHEMOKINE DEGRADATION

As the functionality of actin cytoskeleton depends on a dynamic equilibrium between filamentous and monomeric actin, we speculated that ACKR2 might regulate its trafficking and scavenging properties by affecting actin dynamics. The dynamics of actin assembly are finely regulated by several factors, one of which is cofilin, a major actin depolymerizing factor [293]. As many GPCR [294, 295], including chemokine receptors, are reported to utilize cofilin to sustain their trafficking properties and signalling pathways [263, 296, 297], we decided to investigate the role of cofilin in ACKR2 trafficking by a Western blot analysis using a specific antibody against the phosphorylated form of cofilin at the Serine-3 residue, after CHO-K1/ACKR2 and CHO-K1/CCR5 stimulation with 100 nM of CCL3L1 at the indicated time points. Data were normalized on tubulin, as indicated on Materials and Methods section. We observed that both ACKR2 and CCR5 sustained cofilin phosphorylation, with different kinetics but comparable efficiency (F**igure 26A**). Interestingly, ACKR2 induced cofilin phosphorylation was evident after engagement by active, CCL4 and CCL14 (9-74), but not neutral, CCL14 (1-74) and CCL3, ligands (**Figure 26B**).

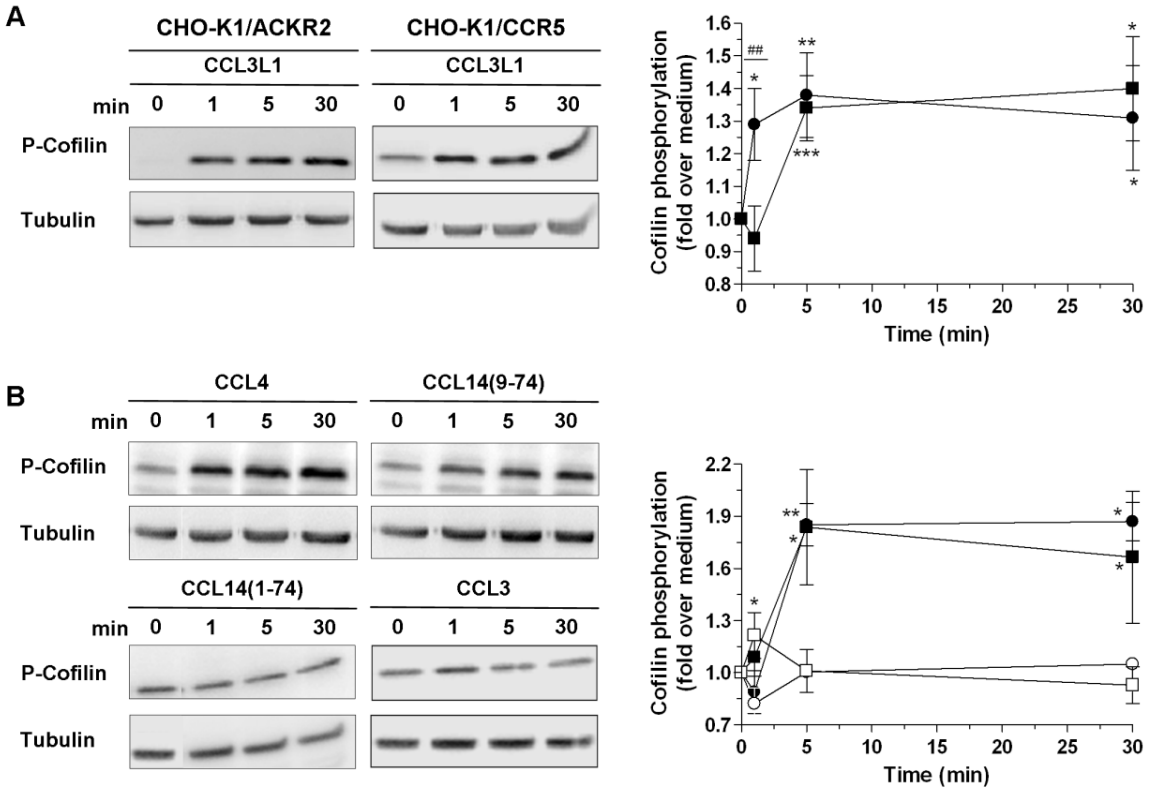

Figure 26: ACKR2 and CCR5 promote ligand-induced cofilin phosphorylation. (**A**) CHO-K1/ACKR2(■) and CHO-K1/CCR5 (●) cells were treated with CCL3L1 (100 nM) and analysed for cofilin phosphorylation (P-Cofilin). (**B**) CHO-K1/ACKR2 cells were treated with active ligand, CCL4 (■) and CCL14(9-74) (●), or with neutral one, CCL14(1-74) (○) and CCL3 (□), all at 100 nM for the indicated time points. Tubulin is shown as a loading control. Representative Western blots are shown on the left, and quantification of the relative amounts of phosphorylated cofilin are shown on the right and is indicated as the fold increase in phosphorylated cofilin levels compared to that in untreated cells. Results are the means ± SEM of 6 experiments. * P≤ 0.05, ** P≤ 0.01, *** P≤ 0.005 for stimulated versus untreated cells; # P≤ 0.05 for ACKR2-expressing versus CCR5-expressing cells [24].

Similar results were obtained in experiments performed with the choriocarcinoma cell line BeWo, which endogenously expresses ACKR2. Cells were treated with 100 nM of CCL3L1 for the indicated time and cofilin phosphorylation levels were analysed with a Western blot assay (**Figure 27**).

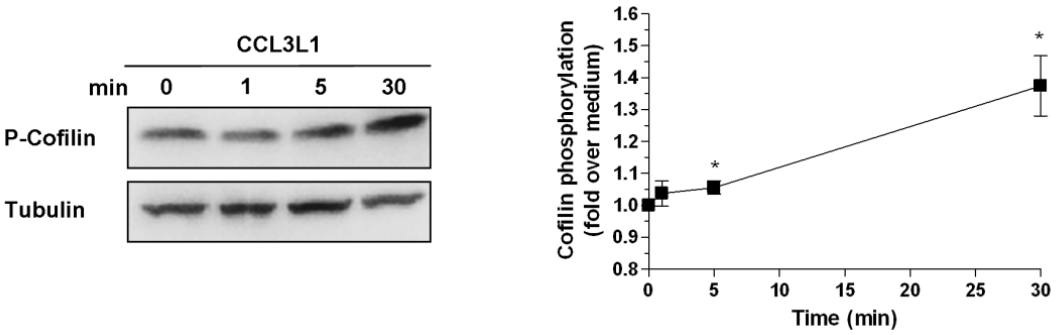

Figure 27: ACKR2 induces cofilin phosphorylation in BeWo cell line. Representative blots of BeWo cells stimulated with CCL3L1 (100 nM, 1 hour) and harvested at the indicated time points. Amount of phosphorylated cofilin is calculated as fold over untreated cells from densitometry measurements of blots followed by normalization to tubulin levels, as reported on the right panel. Results are representative of 3 different experiments performed. * P≤ 0.05 for stimulated versus untreated cells [24].

As these results suggested the involvement of cofilin in ACKR2 up-regulation and chemokine degradation, we performed knockdown of cofilin in HEK293T/ACKR2 cells by small interfering RNA (siRNA). Cells were treated for 72 hours with 50 nM of control siRNA scrambled and with a specific siRNA for cofilin. The silencing efficiency after 72 hours was evaluated with Western blot, measuring the amount of protein after siRNA treatment, normalized over tubulin (**Figure 28A**). Cofilin knockdown cells significantly impaired ligand-dependent ACKR2 up-regulation (**Figure 28B**) and the rate of chemokine degradation (**Figure 28C**). Conversely, cells knockdown for cofilin had no effect on the CCR5-mediated rate of CCL4 degradation (**Figure 28D**).

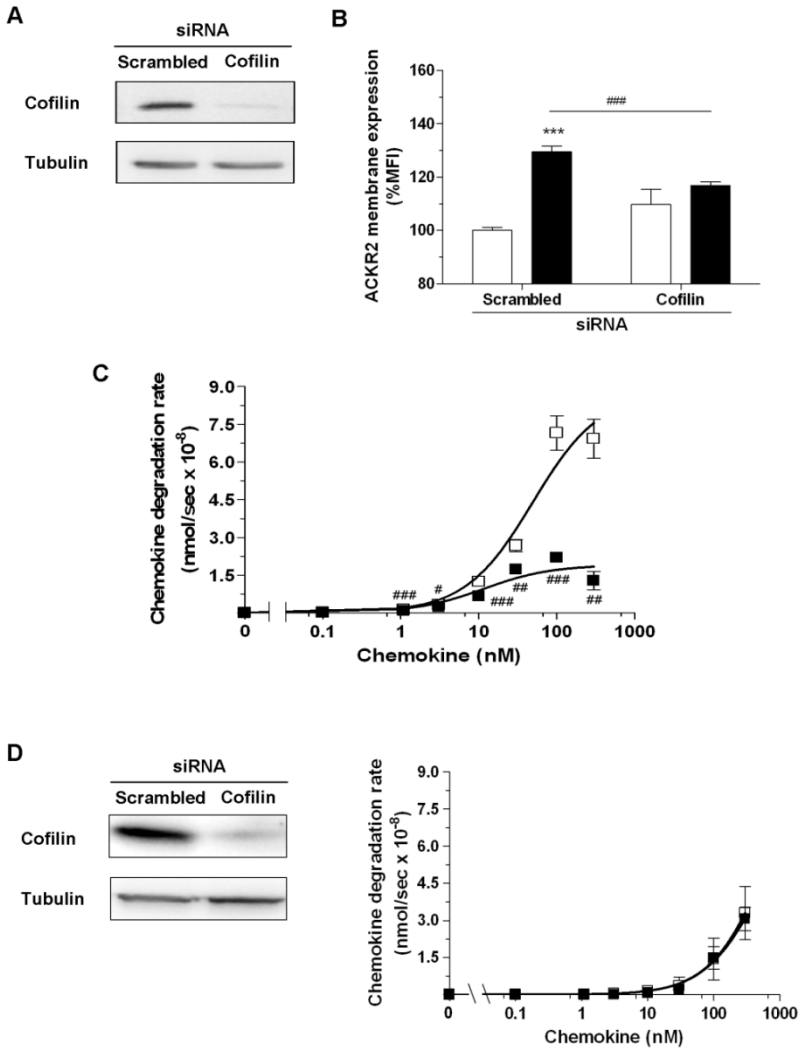

Figure 28: Ligand-induced cofilin phosphorylation sustains increased ACKR2 membrane abundance and its chemokine scavenging function. HEK293T/ACKR2 (**A** to **C**) and HEK293T/CCR5 (**D**) cells were treated with 50 nM control (scramble) or cofilin specific siRNAs for 72 hours and analysed by Western blot to determine their cofilin and tubulin content (**A**). Cells were analysed for (**B**) the amount of cell-surface ACKR2 before and after treatment with 100nM CCL3L1 for 1 hour (open bar; untreated cells, black bar: stimulated cells), and for the chemokine-scavenging ability of (**C**) ACKR2 and (**D**) CCR5 (□: control siRNA; ■: cofilin-specific siRNA) after incubation with 0.1 nM ^{125}I-CCL4 and the indicated concentrations of unlabeled CCL4. Results are the means ± SEM of 6 experiments. Data were analysed by unpaired Student's T test. *** P≤ 0.005 for stimulated versus unstimulated cells. # P≤ 0.05, ## P≤ 0.01, ### P≤ 0.005 for cofilin knockdown versus control cells [24].

These results clearly indicate that ACKR2 engagement after ligand binding activates a specific cofilin-dependent signalling pathway required to sustain the adaptive up-regulation and the scavenging function of ACKR2, as reported in [24].

1- C. ACKR2-MEDIATED COFILIN PHOSPHORYLATION OCCURS THROUGH A PAK1- AND LIMK1-DEPENDENT PATHWAY

Cofilin activity is mainly regulated by its phosphorylation/dephosphorylation status at the critical Ser-3 residue, which promotes actin assembly and disassembly, respectively [298, 299]. In particular, inhibition of cofilin actin-severing function by phosphorylation at Ser-3 is maintained through the basal activity of LIM kinase, whose the only known substrate is cofilin [300, 301]. As LIMK1-dependent cofilin inactivation through phosphorylation is critical for chemokine-induced actin reorganization [297, 302], Western blot analysis with an antibody specific for LIMK1 phosphorylated at Thr-508 (a marker of its activation) was performed, after HEK293T/ACKR2 cell stimulation with 100 nM of CCL3L1 at the indicated time points, revealing that LIMK1 was rapidly phosphorylated (**Figure 29A**), with a kinetic similar to cofilin phosphorylation (**Figure 26A**). The kinase-death dominant negative form LIMK1 D460A abrogated cofilin phosphorylation, demonstrating LIMK1 direct involvement in cofilin inactivation after ACKR2 engagement (**Figure 29B**) and in the increase of receptor plasma membrane expression after chemokine stimulation (**Figure 29C**).

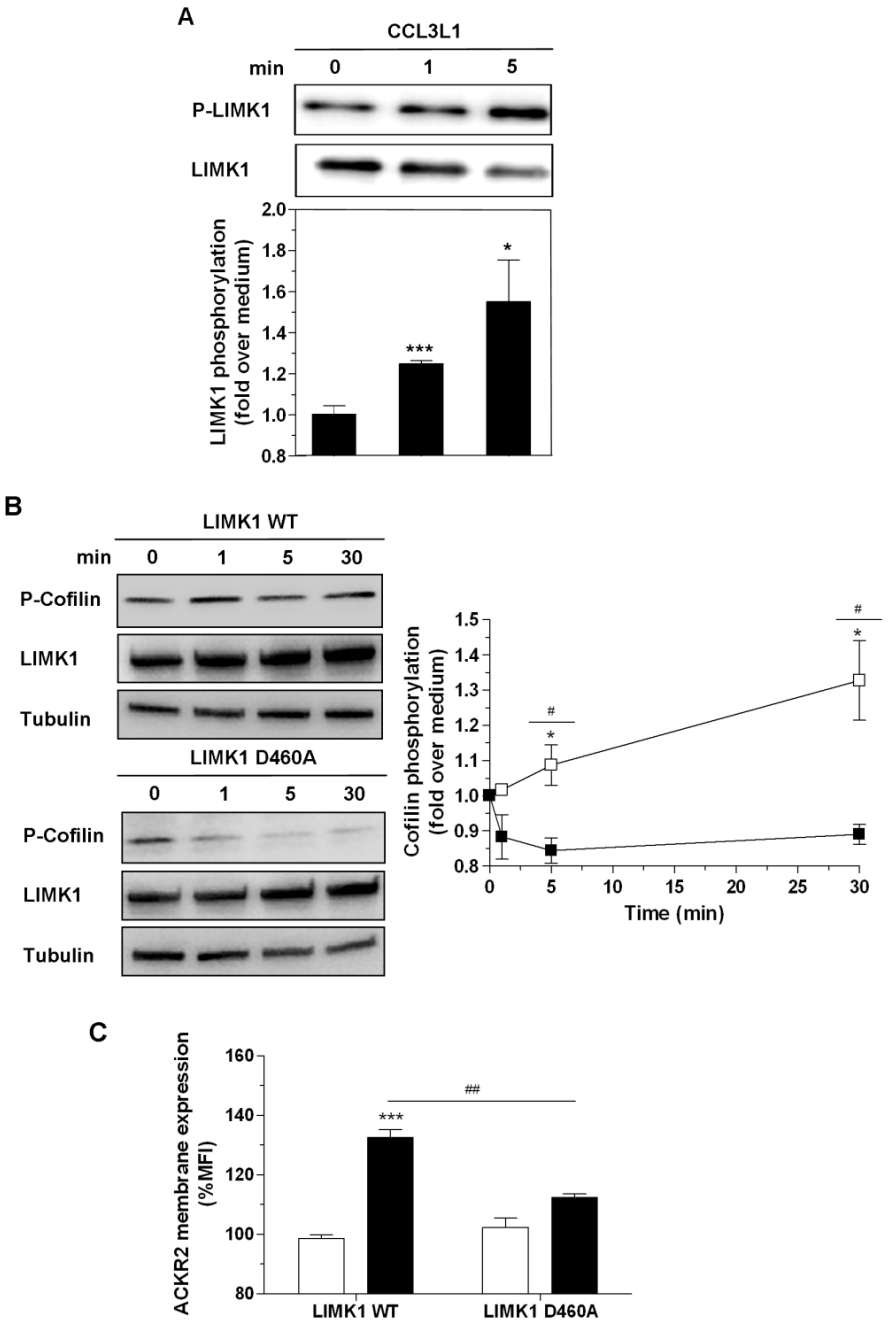

Figure 29: LIMK1 activation induces cofilin phosphorylation and increases ACKR2 membrane expression. Representative Western blot and densitometric analyses of the amounts of (**A**) phosphorylated LIMK1 (P-LIMK1) in HEK293T/ACKR2 cells treated with CCL3L1 (100 nM) at the indicated times; (**B**) phosphorylated cofilin and total LIMK1 in cells expressing LIMK1 WT (□) or LIMK1 D460A mutant (■) that were treated with CCL3L1 (100 nM) for the indicated time point. Amount of phosphorylated cofilin is shown as fold over untreated cells, and is calculated from densitometry measurements of blots followed by normalization over tubulin levels. Amount of phosphorylated LIMK1 is shown as fold over untreated cells, and is calculated from densitometric measurements of blots followed by normalization to total LIMK1. (**C**) HEK293T/ACKR2 cells were treated for 72 hours with LIMK1 WT or with LIMK1 D460A expressing plasmid and were analysed for cell-surface ACKR2 expression under basal conditions and after treatment with

CCL3L1 (100 nM) for 1 hour (empty bars: untreated cells; filled bars: stimulated cells). Results are the mean ± SEM of 4 experiments. * P≤ 0.05, *** P≤ 0.005 for stimulated versus untreated cells; # P≤ 0.05, ## P≤ 0.01 for LIMK1 D460A- versus WT LIMK1-expressing cells [24].

LIMK1 is activated through phosphorylation by several kinases, including PAKs [303]. To assess the potential involvement of a PAK-dependent signalling pathway in ACKR2-mediated cofilin phosphorylation, we first determined the effect of ligand stimulation on the activation through phosphorylation of PAK kinases, performing a Western blot assay, using a specific antibody against the phosphorylated form of PAK. We found that PAK1 was rapidly phosphorylated after ACKR2 engagement (**Figure 30A**). Furthermore, we confirmed the involvement of PAK1 in the LIMK1-cofilin pathway by showing that PAK1 activity was required for LIMK1 and cofilin phosphorylation (**Figures 30B** and **30C**, respectively). In fact, the effect of the PAK1 inhibitor IPA-III demonstrated that PAK1 activity was required for ACKR2-induced phosphorylation of LIMK1 and cofilin. Finally, we demonstrated that ligand-induced ACKR2-dependent PAK1 activation was relevant for ACKR2 biology by showing that ACKR2 abundance at the cell surface and the ability of ACKR2 to degrade chemokines was significantly impaired in PAK1 knockdown cells, treated with a specific PAK1 siRNA (**Figure 30D** and **30E**).

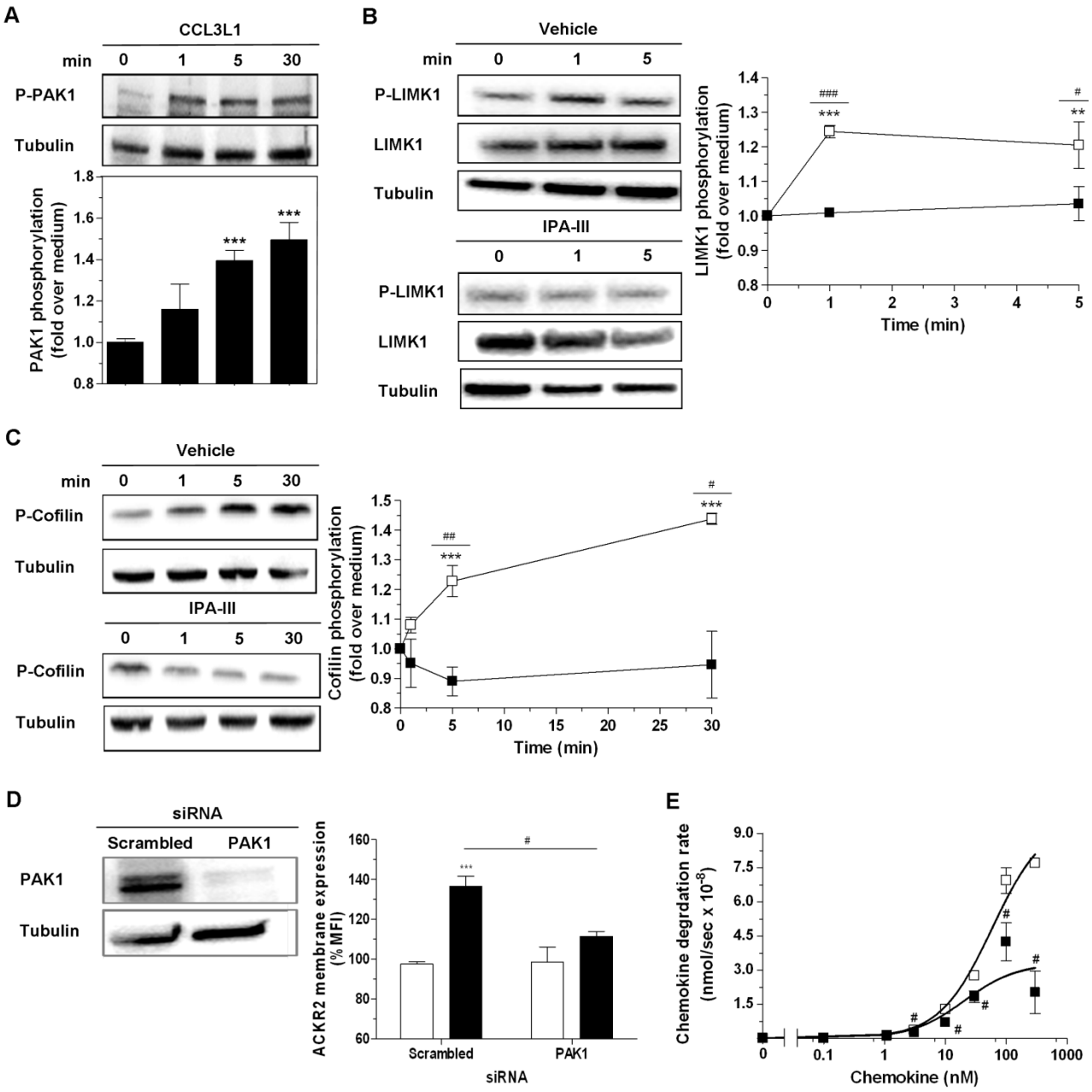

Figure 30: ACKR2-mediated cofilin phosphorylation occurs through the PAK1-LIMK1 pathway. Western blot and densitometric analyses of the amounts of (**A**) phosphorylated PAK1 (P-PAK1) in HEK293T/ACKR2 cells treated with 100 nM CCL3L1 and (**B**) phosphorylated LIMK1 and (**C**) phosphorylated cofilin in cells pre-incubated for 30 min with 5 μM IPA-III (■) or vehicle (□). Representative Western blot and quantified densitometric data are shown. Amount of phosphorylated cofilin or phosphorylated PAK1 is shown as fold over untreated cells, and is calculated from densitometry measurements of blots followed by normalization to tubulin levels. Amount of phosphorylated LIMK1 is shown as fold over untreated cells, and is calculated from densitometry measurements of blots followed by normalization to LIMK1 total levels. Results are the mean ± SEM of 4 experiments. ** P≤ 0.01 and *** P≤ 0.005 for stimulated cells versus untreated cells; # P≤ 0.05, ## P≤ 0.01 and ### P≤ 0.005 for IPA-III-treated versus vehicle-treated cells. (**D** and **E**) HEK293T/ACKR2 cells treated with 50 nM control (scramble) or PAK1-specific siRNA for 72 hours were analysed for PAK1 and tubulin content by Western blot. Cells were also analysed for (**D**) cell-surface ACKR2

expression under basal conditions and after treatment with 100 nM CCL3L1 for 1 hour (empty bars: untreated cells, filled bars: stimulated cells), as well as for (**E**) chemokine-scavenging activity (□: control siRNA; ■: Pak1-specific siRNA) after incubation with 0.1 nM ^{125}I-labeled CCL4 and the indicated concentrations of unlabeled CCL4. Data were analysed by unpaired Student's t test. Results are the mean ± SEM of 3 experiments. *** P≤ 0.005 for stimulated versus untreated cells; # P≤ 0.05 for PAK1- knockdown versus control cells [24].

Similar results were obtained in experiments performed using BeWo cells (**Figure 31**). Cells were pre-treated for 1 hour with 5 μM of IPA-III, a specific inhibitor of PAK1, or were pre-treated for 72 hours with control siRNA (scrambled) or with PAK1-specific siRNA. Cells were subsequently analysed by Western blot for cofilin phosphorylation (**Figure 31A**) and for ACKR2-dependent degradation of CCL4 (**Figure 31B**).

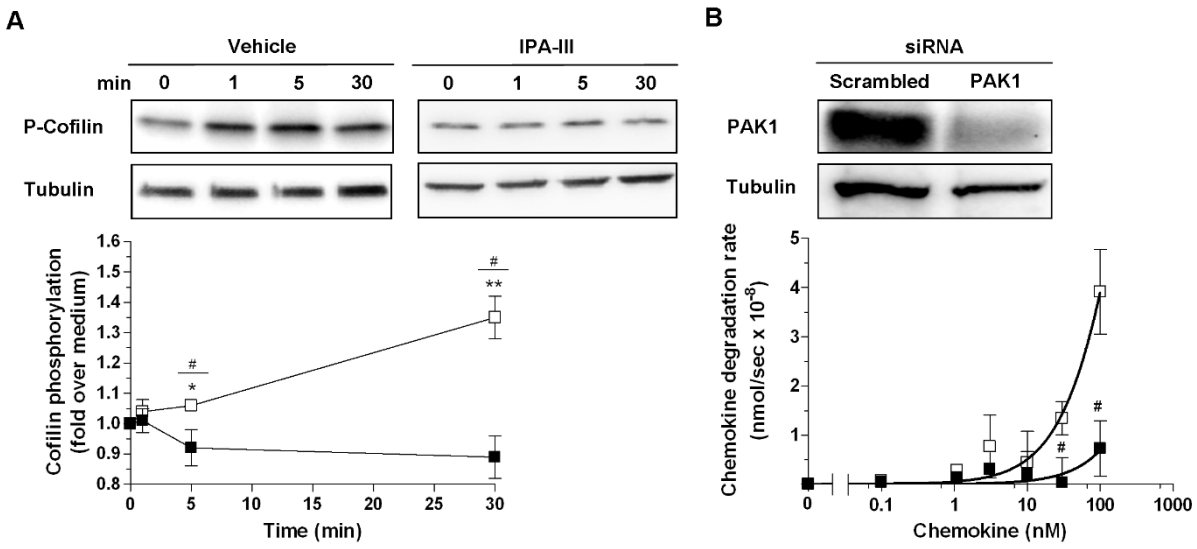

Figure 31: ACKR2 induces cofilin phosphorylation through PAK1 to sustain chemokine degradation in BeWo cell line. (**A**) Representative blots of BeWo cells pre-incubated (37°C; 1 hour) with vehicle (water, □) or IPA-III (5 μM, ■), stimulated with CCL3L1 (100 nM) and harvested at the indicated time points. Amount of phosphorylated cofilin is shown as fold over untreated cells, and is calculated from densitometry measurements of blots followed by normalization to tubulin levels. (**B**) BeWo cells treated with 50 nM control (scramble, □) or PAK1-specific siRNA (■) for 72 hours were analysed for PAK1 and tubulin content by Western blot. Cells were also analysed for chemokine-scavenging activity after incubation with 0.1 nM ^{125}I-labeled CCL4 and the indicated concentrations of unlabeled CCL4. Data were analysed by unpaired Student's T test. Results are the mean ± SEM of 3 experiments. *= p≤0.05, **= p≤0.01 of stimulated versus unstimulated cells; #= p≤0.05 of IPA-III-treated versus vehicle-treated cells or PAK1- knockdown versus control cells [24].

Taken together, these data suggest that ACKR2 activation upon chemokine engagement lead to cofilin inactivation through a PAK1-LIMK1-dependent pathway in order to promote actin network reorganization that is required to sustain receptor scavenging activity [24].

1- D. LIGAND-INDUCED COFILIN PHOSPHORYLATION DEPENDS ON RAC1 ACTIVATION

PAKs are downstream effectors of the small GTPase Rac1 [304]. To evaluate the potential involvement of Rac1 in the ACKR2-dependent PAK1/LIMK1 pathway, we determined the effect of ACKR2 stimulation on the level of active Rac1. Active GTP-bound Rac1 was pulled down through a GST-PAK1 column from cell lysates of CCL3L1-stimulated HEK293T/ACKR2 cells, as described in the Material and Methods section. Samples were then eluted and immunoblotted for total Rac1, together with total lysate. As shown in **Figure 32A**, ACKR2 engagement resulted in marked Rac1 activation that began at 1 min and peaks at 5 min, in agreement with the kinetics of LIMK1 activation (**Figure 29A**). The role of Rac1 in the signalling pathway leading to cofilin phosphorylation was then revealed by the marked inhibition of both PAK1, LIMK1, and cofilin activation following treatment with the Rac1-specific inhibitor NSC23766 (used at 200 μM for 1 hour) [305] (**Figures 32B** to **32D**, respectively), These results suggested that the PAK1/LIMK1/cofilin pathway was controlled by the ability of ACKR2 to promote Rac1 activation. Consistent with this, confocal microscopy analysis showed that following CCL3L1 stimulation, ACKR2 was retained in intracellular compartments and its colocalization with actin filaments at plasma membrane was impaired in the presence of NSC23766 (**Figure 32E**), indicating that Rac1 activity downstream ACKR2 was required to induce actin rearrangements, mobilize the receptor from intracellular stores, and sustain its up-regulation. Finally, we demonstrated that ligand-induced ACKR2-dependent Rac1 activation had a functional relevance on ACKR2 activity as HEK293T/ACKR2 cells treated with NSC23766 showed a significant inhibition of chemokine-dependent receptor up-regulation (**Figure 32F**) and ACKR2-mediated chemokine degradation (**Figure 32G**).

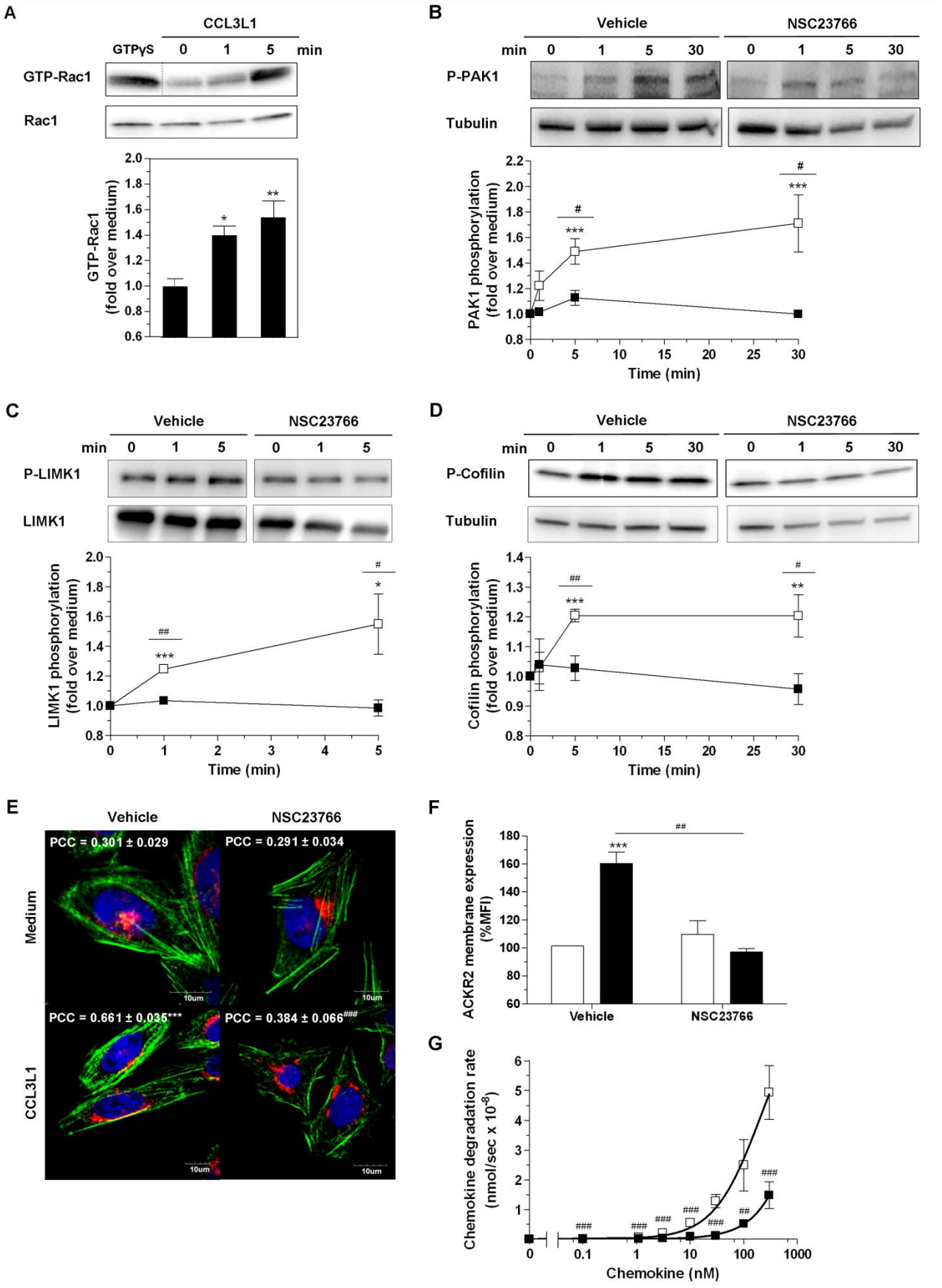

Figure 32: Ligand-induced cofilin-phosphorylation and actin reorganization depends on Rac1-activation. (**A**) HEK293T/ACKR2 cells were treated with 100 nM CCL3L1 for the indicated time point and tested for Rac1 activity. GTPγS was used as a positive control. (**B** to **D**) HEK293T/ACKR2 cells were pre-

incubated for 30 min with 200 μM NSC23766 (■) or vehicle (□), stimulated with 100 nM CCL3L1 and then analysed by Western blot to detect: (**B**) phosphorylated PAK1 (P-PAK1), (**C**) total LIMK1 and phosphorylated LIMK (P-LIMK1), (**D**) phosphorylated cofilin (P-Cofilin), and tubulin as a control. Representative Western blots and quantified densitometric data are shown. (**E**) Confocal analysis of NSC23766-pre-treated (200 μM for 1 hour) CHO-K1/ACKR2 cells in the presence or absence of 100 nM CCL3L1 for 1 hour. Each panel shows nuclear staining (DAPI, in blue) merged with the double staining for ACKR2 (in red) and F-actin (phalloidin, in green). Quantification of the extent of ACKR2 colocalization with F-actin as PCC is shown as the mean ± SEM from 100 cells analysed in each experiments performed. (**F** and **G**) CHO-K1/ACKR2 cells were analysed for (**F**) cell-surface abundance of ACKR2 under basal conditions and after treatment with 100 nM CCL3L1 for 1 hour (empty bars: untreated cells; filled bars: stimulated cells), as well as for (**G**) chemokine-scavenging activity (□: vehicle-treated; ■: NSC23766-treated cells) after incubation with 0.1 nM ^{125}I-labeled CCL4 and the indicated concentrations of unlabelled CCL4. Results are the means ± SEM of 5 experiments. Data were analysed by unpaired Student's t test. *P≤ 0.05, **P≤ 0.01 and ***P≤ 0.005 for stimulated versus untreated cells; $^{\#}$P≤ 0.05, $^{\#\#}$P≤ 0.01 and $^{\#\#\#}$P≤ 0.005 for NSC23766-treated versus vehicle-treated cells [24].

Similar results were obtained using BeWo cells. Cells were pre-treated with 200 μM for 1 hour and then analysed for cofilin phosphorylation (**Figure 33A**) and for ACKR2 scavenging activity, as reported in **Figure 33B**.

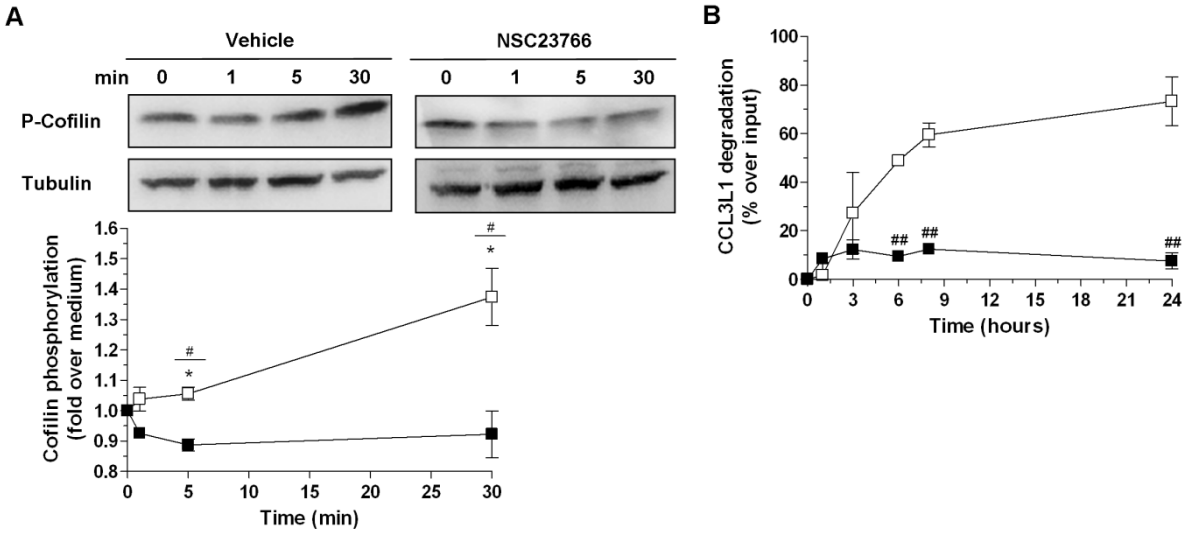

Figure 33: ACKR2 induces cofilin phosphorylation through Rac1 to sustain chemokine degradation in BeWo cell line. (**A**) Representative blots of BeWo cells pre-incubated (37°C; 1 hour) with vehicle (water, □) or NSC23766 (200 μM, ■), stimulated with CCL3L1 (100 nM) and harvested at the indicated time points. Amount of phosphorylated cofilin is shown as fold over untreated cells, and is calculated from densitometry measurements of blots followed by normalization to tubulin levels. (**B**) BeWo cells pre-incubated (37°C; 1 hour) with vehicle (water, □) or NSC23766 (200 μM, ■) were analysed for chemokine-scavenging activity with test ELISA after incubation with 1 nM of CCL3L1 for the indicated time

points, Data were analysed by unpaired Student's T test. Results are the mean ± SEM of 3 experiments. *= p≤0.05 of stimulated versus unstimulated cells; #= p≤0.05 and ##= p≤0.01 of NSC23766-treated versus vehicle-treated cells [24].

Taken together these results clearly demonstrate that, upon ligand stimulation, ACKR2 activates Rac1 to promote cofilin phosphorylation through a PAK1-LIMK1-cofilin signalling pathway, required to mobilize ACKR2 from intracellular compartments, to support its up-regulation on the cell membrane and ultimately its scavenging activity [24].

1- E. ACKR2 PROMOTES COFILIN PHOSPHORYLATION INDEPENDENTLY OF G PROTEIN ACTIVATION

Upon chemokine stimulation, canonical chemokine receptors promote leukocyte adhesion, calcium fluxes and directional migration activating signal transduction cascades downstream heterotrimeric Gαi proteins [58, 306, 307]. ACKR2 is structurally unable to couple to and signal through G proteins, lacking sequences motifs highly conserved in conventional chemokine receptors, reported to be involved in the coupling of GPCRs to G proteins [12]. ACKR2 is unable to promote activities mediated by Gαi-dependent signalling, as chemotaxis and calcium fluxes [14, 100]. We performed a homogeneous time-resolved fluorescence (HTRF) experiments on CHO-K1/ACKR2 and CHO-K1/CCR5 cells to evaluate the capacity of ACKR2 to activate a G protein dependent response, after chemokine engagement. We showed that upon engagement by various ligands, neither ACKR2 nor CCR5 were able to elicit Gαq (**Figure 34A**) or Gαs (**Figure 34B**) activation, differently from the vasopressin receptors V1b and V2 used as positive controls for Gαq and Gαs proteins, respectively. Surprisingly, a weak activation of Gαi was detected after ACKR2 stimulation by its ligands, although at considerably lower levels comparing to the conventional chemokine receptor CCR5 (**Figure 34C**).

As Gαi activation is required by several chemokine receptors to promote chemotactic response by actin reorganization induced after cofilin phosphorylation [297, 308], we investigated whether ACKR2 promoted the phosphorylation of cofilin with this signalling pathway G-protein dependent. CHO-K1/ACKR2 and CHO-K1/CCR5 cells were pre-treated with the Gαi inhibitor pertussis toxin (PTX) for 16 hours, stimulated with 100 nM CCL3L1 at the indicated time point and analysed for Western blot. Opposite to signalling of the conventional chemokine receptor CCR5 (**Figure 34D**), PTX pre-treatment had no effect on

cofilin phosphorylation in ACKR2-expressing cells after CCL3L1 stimulation (**Figure 34E**). Furthermore, cells with inactivated Gαi showed no impairment in ligand-dependent increase in ACKR2 membrane expression (**Figure 34F**) and on chemokine degradation efficiency in CHO-K1/ACKR2 cells (**Figure 34G**).

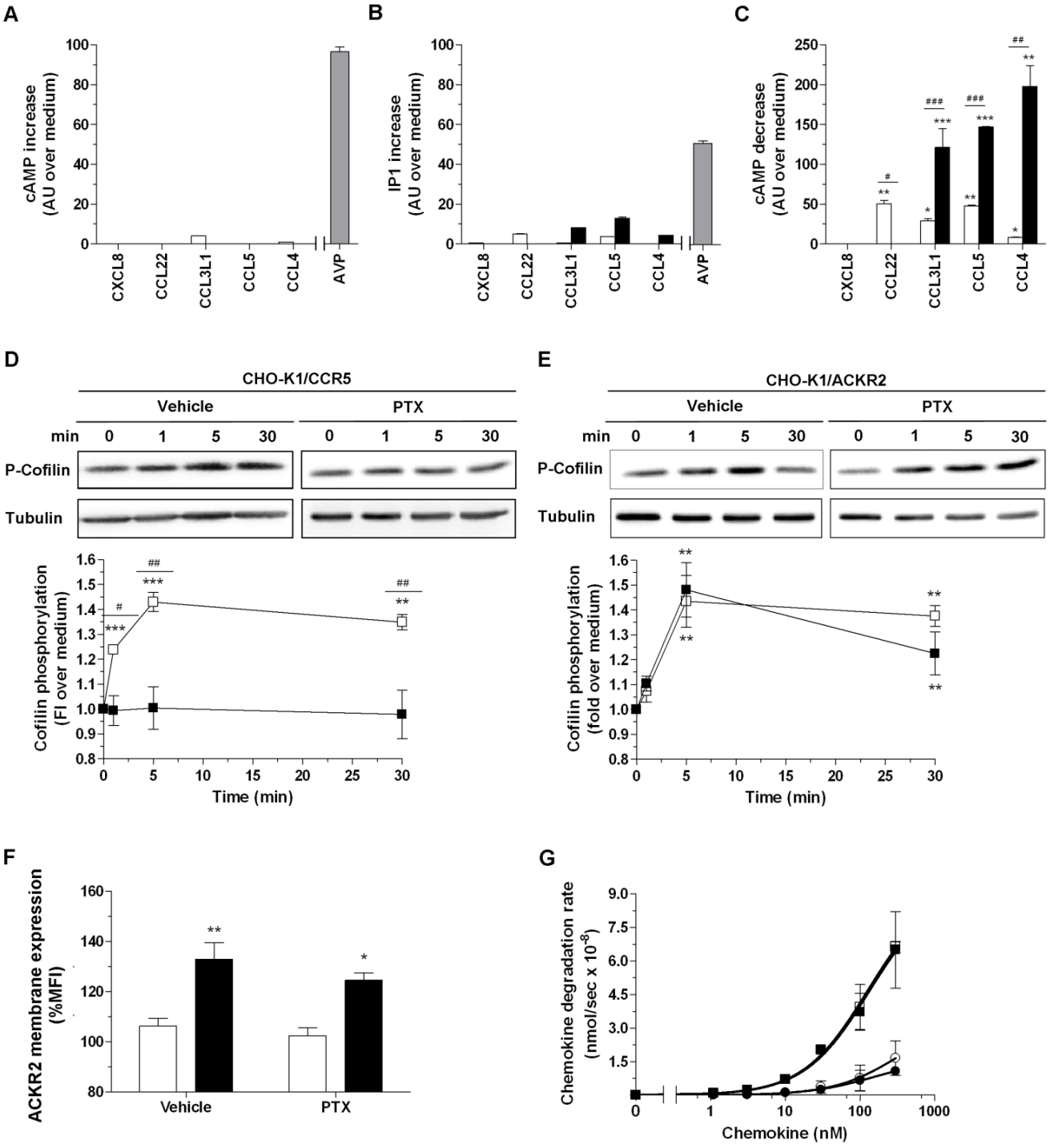

Figure 34: ACKR2 promotes cofilin phosphorylation independently of G proteins. (**A** to **C**) Quantification of the extend of activation of (**A**) Gαs, (**B**) Gαq and (**C**) Gαi subunits in CHO-K1/ACKR2 cells

(empty bars) and CHO-K1/CCR5 cells (filled bars), stimulated with the indicated chemokines (all at 200 nM) for 1 hour. HEK293T/V1a and HEK293T/V2 cells stimulated with 100 nM AVP (gray bars) for 1 hour were used as positive controls for the activation of (**B**) Gαq and (**A**) Gαs, respectively. (**D** and **E**) CHO-K1/CCR5 and CHO-K1/ACKR2 cells pre-treated for 16 hours with vehicle or pertussis toxin (PTX; 100 ng/ml) and stimulated with 100 nM CCL3L1 were then analysed by Western blot with antibodies against phosphorylated cofilin and tubulin. Representative Western blot and quantification of the amounts of phosphorylated cofilin in cells treated with vehicle (□) or PTX (■) are shown. (**F**) CHO-K1/ACKR2 cells were analysed for their amounts of cell surface ACKR2 expression under basal conditions and after treatment with 100 nM CCL3L1 for 1 hour (empty bars: untreated cells; filled bars: stimulated cells). (**G**) Cells were incubated with 0.1 nM ^{125}I-labeled CCL4 and the indicated concentrations of unlabeled CCL4. Measurement of the rate of degradation of CCL4 in CHO-K1/ACKR2 cells pre-treated for 16 hours with vehicle (□) or PTX (100 ng/ml; ■) and CHO-K1/CCR5 cells pre-treated for 16 hours with vehicle (○) or PTX (100 ng/ml; ●). Results are the means ± SEM of 4 experiments. Data were analysed by unpaired Student's t test. *P≤ 0.05, **P≤ 0.01 and ***P≤ 0.005 for stimulated versus untreated cells; #P≤ 0.05, ##P≤ 0.01 and ###P≤ 0.005 for ACKR2-expressing cells versus CCR5 expressing cells in (**C**) or for PTX-treated versus vehicle-treated CHO-K1/CCR5 cells in (**D**) [24].

The same results were obtained in BeWo cell lines. Cells were pre-treated for 16 hours with PTX and then analysed for cofilin phosphorylation, after 100 nM CCL3L1 stimulation at the indicated times (**Figure 35A**), and for ACKR2 scavenging activity, as reported in **Figure 35B**.

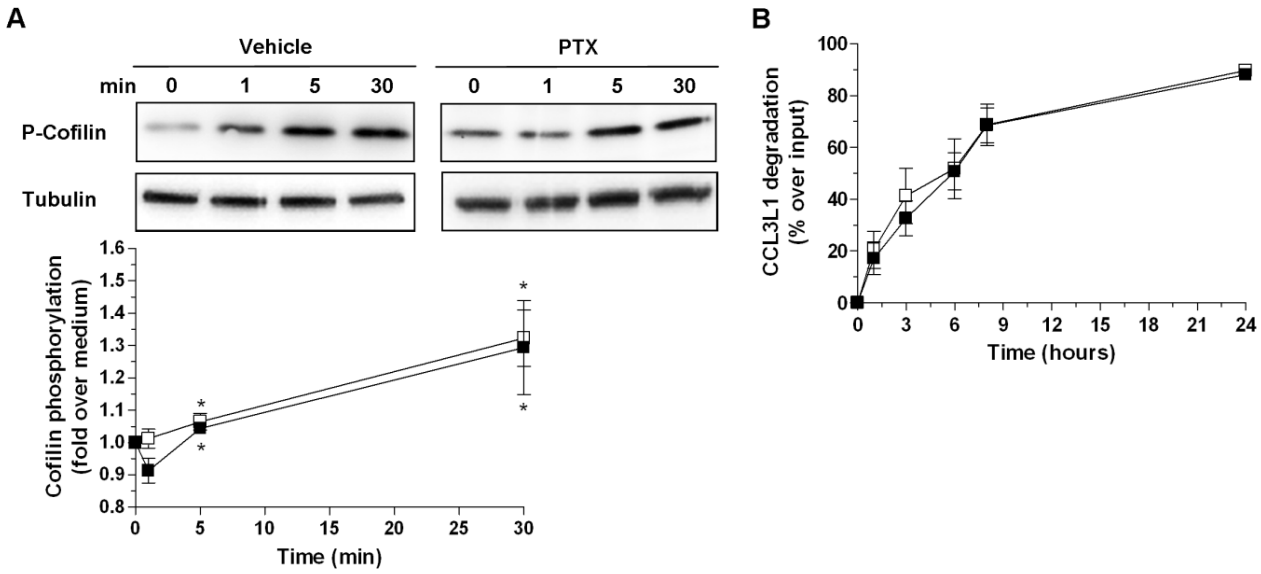

Figure 35: ACKR2 induced cofilin phosphorylation to sustain chemokine degradation in BeWo cell line is not Gαi-depedent. (**A**) Representative blots of BeWo cells pre-treated for 16 hours at 37°C with

vehicle (water, □) or PTX (100 ng/ml, ■), stimulated with CCL3L1 (100 nM) and harvested at the indicated time points. Amount of phosphorylated cofilin is shown as fold over untreated cells, and is calculated from densitometry measurements of blots followed by normalization to tubulin levels. (**B**) BeWo pre-treated for 16 hours at 37°C with vehicle (water, □) or PTX (100 ng/ml, ■) were analysed for chemokine-scavenging activity with test ELISA after incubation with 1 nM CCL3L1 for the indicated time points. Results are the mean ± SEM of 4 experiments. Data were analysed by unpaired Student's t test. *= p≤0.05 of stimulated versus unstimulated cells [24].

These results clearly indicate that ACKR2 does not require Gαi coupling to trigger cofilin phosphorylation and sustain its scavenging function, as demonstrated both in CHO-K1/ACKR2 and BeWo cells [24]. The residual G-protein activation reported here suggests a new insight in the atypical chemokine receptor ACKR2, but more information are needed to investigate new possible functions.

1- F. ACKR2-MEDIATED COFILIN PHOSPHORYLATION IS SUSTAINED BY A β-ARRESTIN1-DEPENDENT PATHWAY

β-arrestins are not only involved in receptor desensitization and internalization but recently has been proposed a β-arrestins role as multifunctional scaffold proteins that recruit and assemble signalling molecules to GPCRs [64, 309]. Interestingly, β-arrestins have been reported to exert a spatial control activity over the actin cytoskeleton, acting as an adaptor protein able to scaffold cofilin together with its regulatory molecules and kinases [261, 263], promoting signalling events through small GTPases [255, 310]. As the role of β-arrestins in ACKR2 biology remains controversial [17, 18], we tested whether β-arrestin1 could be upstream of ACKR2-dependent cofilin phosphorylation. We performed experiments in HEK293T/ACKR2 cells treated with 50 nM of scrambled control or a β-arrestin1-specific siRNA for 72 hours. Cells were then analysed for cofilin phosphorylation after 100 nM CCL3L1 stimulation. Immunoblotting analysis clearly indicated that ligand-induced cofilin phosphorylation was completely abrogated in β-arrestin1 knockdown cells compared to scrambled-treated cells (**Figure 36A**). Similar results were obtained when Rac1 activity was investigated (**Figure 36B**), showing that cells knockdown for β-arrestin1 failed to exhibit Rac1 activation after CCL3L1 stimulation. Interestingly, confocal microscopy analysis of β-arrestin1 knockdown cells showed a widely diffused expression of ACKR2 into the cytoplasm, with increased colocalization with actin filaments, under basal conditions. After 100 nM CCL3L1 stimulation for 1 hour, ACKR2 was still retained in

intracellular compartments, strictly associated to actin filaments, and actin cytoskeleton reorganization and receptor redistribution at plasma membrane were abrogated (**Figures 36C** and **36D**). According to these results, flow cytometric analysis revealed that β-arrestin1 knockdown did not modify ACKR2 constitutive endocytosis (**Figure 36F**) but resulted in significantly reduced receptor membrane expression and chemokine-dependent upregulation (**Figure 36E**), with a marked impairment in the ACKR2 chemokine degradation efficiency (**Figure 36G**).

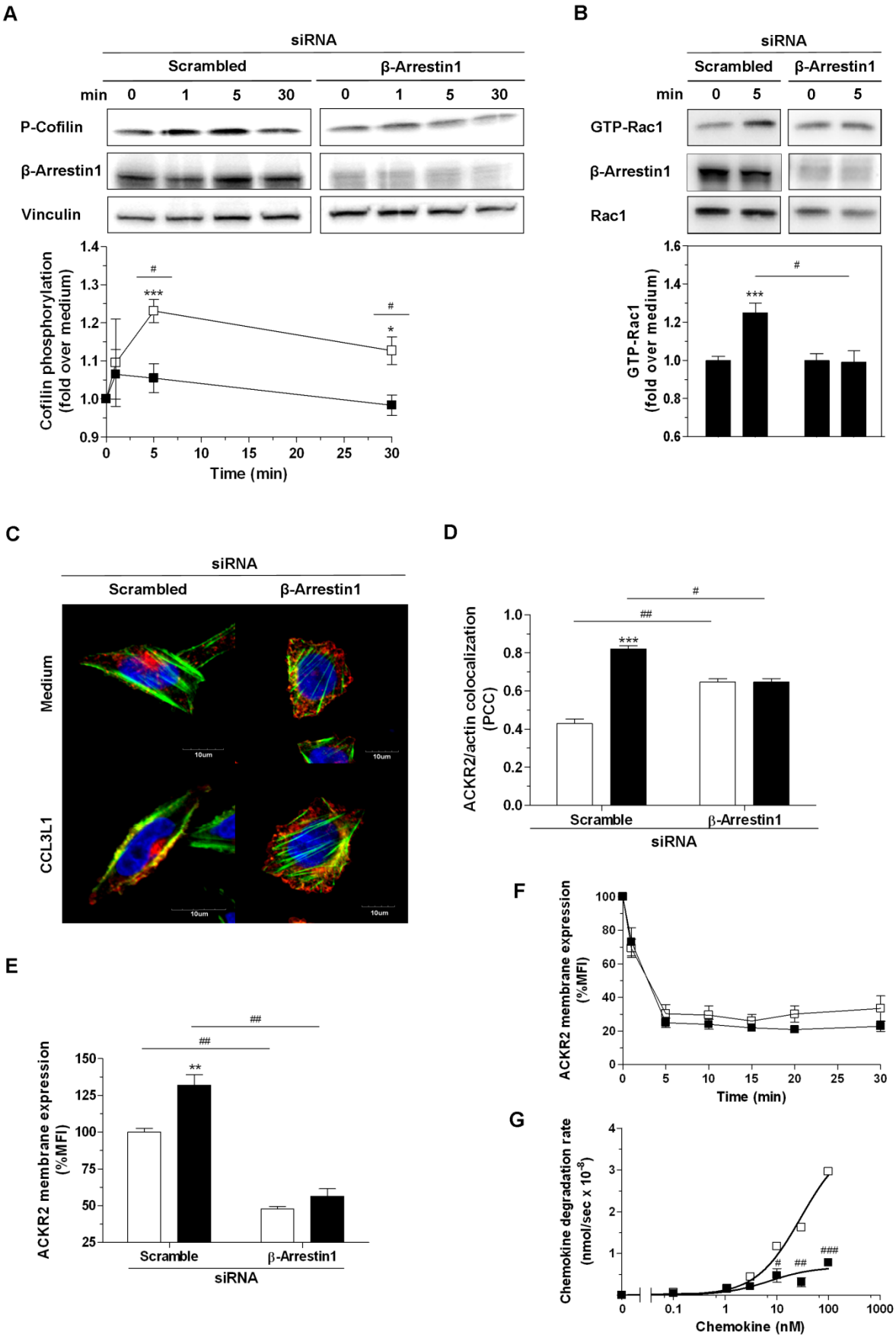

Figure 36: Ligand-induced cofilin phosphorylation and actin reorganization depends on β-arrestin1. (**A**) HEK293T/ACKR2 cells were treated with 50 nM scrambled siRNA (□) or β-arrestin1-specific

siRNA (■) for 72 hours, stimulated with 100 nM CCL3L1 for the indicated time points and then analysed by Western blot to determine the amounts of phosphorylated cofilin, β-arrestin1 and vinculin proteins. Representative Western blots and quantified densitometric data are shown. (**B**) HEK293T/ACKR2 cells were treated with 50 nM scrambled siRNA or β-arrestin1-specific siRNA for 72 hours, stimulated with 100 nM CCL3L1 for the indicated time points and analysed for the extent of Rac1 activity. Quantifications of these results are shown beneath the blots for each panel, normalized over total Rac1. (**C**) Confocal analysis of CHO-K1/ACKR2 cells treated with 50 nM control or β-arresin1-specific siRNA and then treated with or without 100 nM CCL3L1 for 1 hour. Each panel shows nuclear staining (DAPI, blue) merged with double staining for ACKR2 (red) and F-actin (phalloidin, in green). (**D**) Quantification of the colocalization of ACKR2 with F-actin as PCC is given as the mean ± SEM of 100 cells analysed in each of 3 experiments performed (empty bars: untreated cells; filled bars: stimulated cells). (**E** to **G**) Control and β-arrestin1-knockdown cells were analysed for (**E**) their cell-surface abundance of ACKR2 under basal conditions and after treatment with 100 nM CCL3L1 for 1 hour (empty bars: unteated cells; filled bars: stimulated cells), as well as for (**F**) constitutive internalization and (**G**) their chemokine–scavenging activity (□: untreated cells; ■ β-arrestin1-specific siRNA) after incubation with 0.1 nM ^{125}I-labeled CCL4 and the indicated concentrations of unlabelled CCL4. Results are the means ± SEM of 5 experiments. Data were analysed by unpaired Student's T test. *P≤ 0.05, **P≤ 0.01 and ***P≤ 0.005 for stimulated versus untreated cells; $^{\#}$P≤ 0.05, $^{\#\#}$P≤ 0.01 and $^{\#\#\#}$P≤ 0.005 for β-arrestin1 knockdown versus control cells[24].

Taken together, these results clearly demonstrate that β-arrestin1 is the upstream adaptor molecule required to sustain the ACKR2-dependent signalling pathway leading to cofilin phosphorylation, receptor trafficking and chemokine degradation, as reported in our published work [24].

2. ACKR2 AND THE MICROTUBULE CYTOSKELETON

2- A. ACKR2 CONSTITUTIVE RECYCLING IS REGULATED BY CYTOSKELETAL DYNAMICS

As we previously mentioned, actin and microtubules cytoskeleton and their motor proteins are involved in the transport and trafficking of intracellular vesicles and in receptor signalling and endocytosis [290, 291]. In the previous section, we investigated the role of actin cytoskeleton in the regulation of ACKR2 trafficking and its ability to degrade chemokines, showing an ACKR2-dependent signalling pathway that lead to the actin reorganization, in order to sustain the scavenging activity of the receptor. We decided to deeper investigate the ACKR2 association with cytoskeleton elements.

CHO-K1/ACKR2 cells were pre-treated with modulator of actin cytoskeleton, in particular two actin-depolymerizing factors, cytochalasin D (1 μM) and latrunculine A (1 μM), and one polymerizing factor, jasplakinolide (1 μM), for 1 hour at 37°C. In agreement with our previous observations, confocal microscopy analysis of CHO-K1/ACKR2 cells showed that in resting conditions ACKR2 mainly localized in perinuclear compartments and did not colocalize with actin, but its colocalization with actin was increased by pharmacological modulation, as clearly reported by PCC values (**Figure 37A**). Conversely, a strong colocalization of ACKR2 with microtubules at perinuclear compartment was observed at resting conditions. Pharmacological alteration of microtubules dynamics with nocodazole (microtubules depolymerizing factor, 10 μM) or with paclitaxel (stabilizing agent of microtubules network, used at 1 μM), both used for 1 hour at 37 °C, promoted ACKR2 cytoplasmic rather than perinuclear distribution (**Figure 37B**).

To understand the role of cytoskeleton in ACKR2 constitutive cycling, its membrane expression levels and constitutive internalization were quantified by flow cytometry after cell treatment with cytoskeletal stabilizing and destabilizing agents. Both actin stabilizing and destabilizing agents induced a significant increase of ACKR2 surface expression levels (**Figure 37C**). While cytochalasin D and jasplakinolide affected ACKR2 constitutive internalization, latrunculin A treatment was ineffective, indicating that this drug might increase ACKR2 surface expression acting specifically on receptor recycling pathway (**Figure 37D**). Inhibition of microtubule dynamics by nocodazole also resulted in increased ACKR2 surface expression (**Figure 37E**), but no significant effects on ACKR2 constitutive internalization were detectable (**Figure 37F**), suggesting that disruption of microtubule network was responsible for alteration of ACKR2 recycling properties.

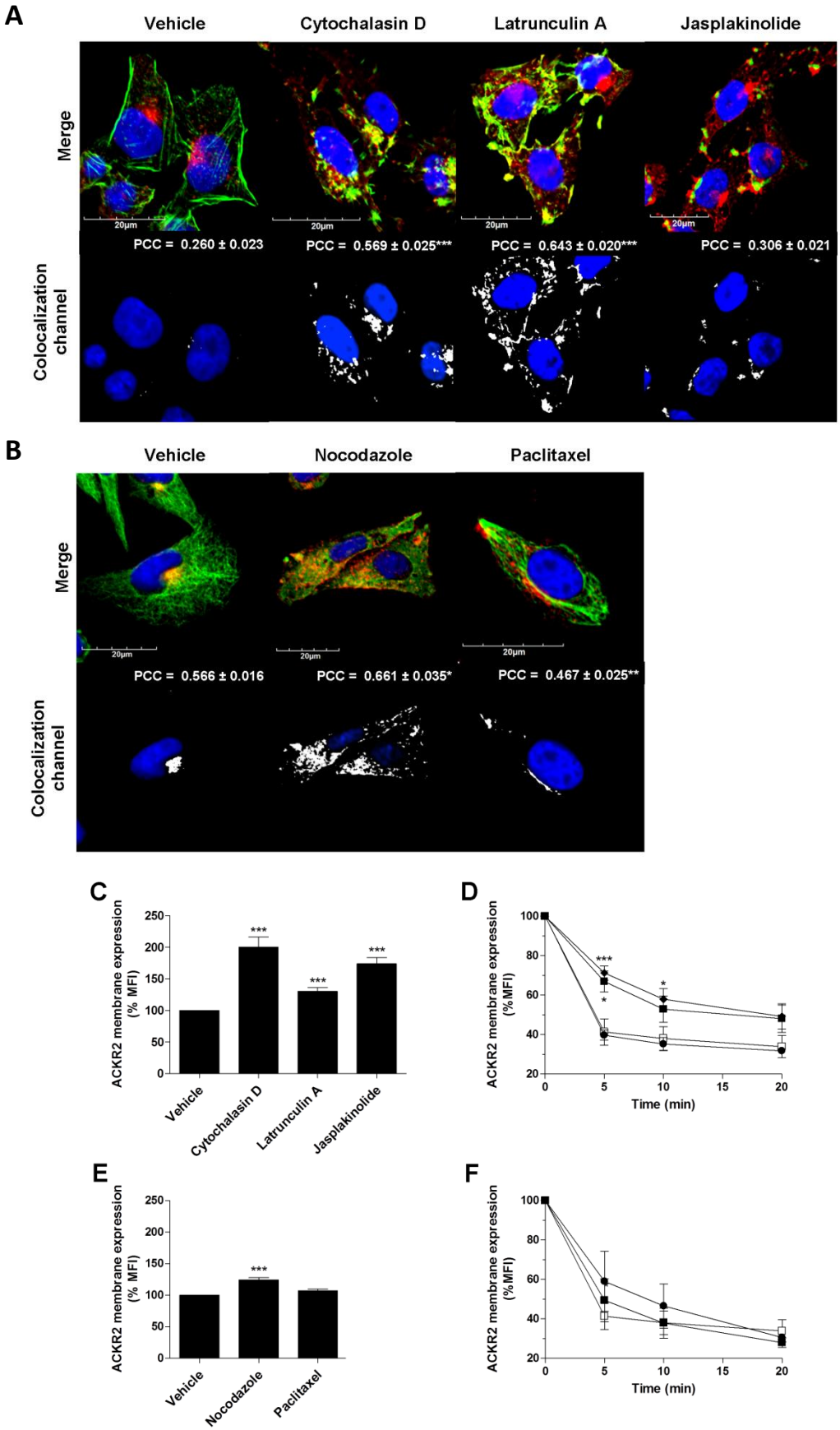

Figure 37: ACKR2 constitutive recycling is regulated by cytoskeleton dynamics. Confocal analysis of CHO-K1/ACKR2 cells incubated for 1 hour with the indicated inhibitors of (**A**) actin and (**B**) microtubules dynamics. Nuclear staining (DAPI, blue) is merged to double staining of ACKR2 (red) and F-actin (phalloidin

staining, green) or microtubules (α-tubulin staining, green) on upper panels, and to colocalization channel on lower panels. Quantification of ACKR2 colocalization with actin or microtubules expressed as PCC is the mean ± SEM of at least 20 cells analysed in 3 different experiments and is reported in each colocalization panel. CHO-K1/ACKR2 cells were incubated for 1 hour with vehicle (□, DMSO), cytochalasin D (■, panel **D**, 1 μM), latrunculin A (●, panel **D**, 1 μM), jasplakinolide (♦, panel **D**, 1μM), or with nocodazole (■, 10 μM, panel **F**) and paclitaxel (●, panel **F**, 1 μM) and then analysed for (**C** and **E**) ACKR2 membrane expression and (**D** and **F**) ACKR2 constitutive internalization, as described in Material and Methods section. Results are representative of mean ± SEM of at least 3 experiments. Data were analysed by unpaired Student's T test. *P≤ 0.05, **P≤ 0.01 and ***P≤ 0.005 for inhibitor-treated versus vehicle-treated cells.

Taken together, these results indicate that ACKR2 trafficking properties require intact actin and microtubules networks. The dynamic turnover of actin filaments is required to sustain ACKR2 constitutive endocytosis, while both actin and microtubules networks are involved in processes regulating ACKR2 internalization and recycling.

2- B. ALTERATION OF CYTOSKELETAL DYNAMICS CAUSES ACKR2 MISSORTING TO RECYCLING COMPARTMENTS

ACKR2 is sorted to the plasma membrane through the rapid Rab4-dependent and the slow Rab11-dependent pathways [19]. As reported previously, ACKR2 cycling properties were altered by pharmacological disruption of cytoskeletal networks, in particular by latrunculin A (**Figure 37C** and **37D**) and by nocodazole (**Figure 37E** and **37F**). Both of them cause altered ACKR2 membrane expression without affecting the internalization rate of the receptor, suggesting that receptor recycling pathways were altered. To investigate the molecular mechanisms of the altered trafficking properties induced by pharmacological inhibitors, pEGFP-tagged Rab4 and Rab11 dominant negative mutants (Rab4-S22N and Rab11-S25N, respectively) were transiently transfected in CHO-K1/ACKR2 cells. Rab4-S22N, but not Rab11-S25N, completely abolished latrunculin A-dependent increase of ACKR2 expression on cell surface (**Figure 38A**), indicating that its effect was mediated by Rab4 activity. Conversely, the effect of nocodazole on ACKR2 membrane levels was inhibited by transfection with Rab11-S25N but not Rab4-S22N (**Figure 38B**), indicating that the increase of receptor membrane expression was mediated by Rab11 activity. Interestingly, confocal microscopy analysis revealed that latrunculin A treatment resulted in slight increase of ACKR2 colocalization with the early endosome marker Rab4 preferentially at plasma membrane, where F-actin aggregates and actin filaments were lost

(**Figure 38C**). Conversely, in nocodazole-treated cells the disruption of microtubule cytoskeleton impaired recycling endosome formation causing loss of ACKR2 perinuclear distribution and colocalization with Rab11 (**Figure 38D**).

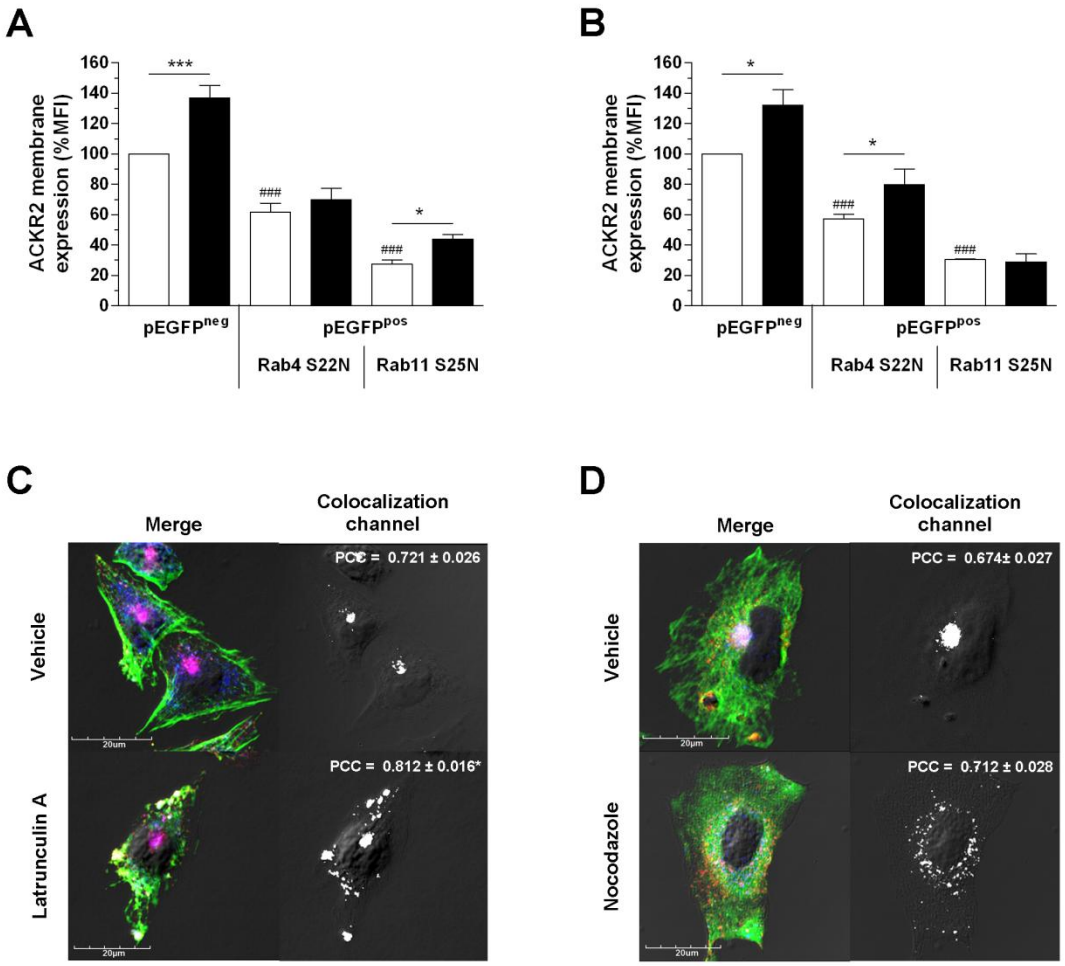

Figure 38: Alteration of cytoskeletal dynamics causes ACKR2 missorting to recycling compartments. (**A** and **B**) CHO-K1/ACKR2 cells were transiently transfected with the indicated pEGFP-tagged plasmids and incubated for 1 hour with vehicle (DMSO), latrunculin A (panel **A**, 1 μm) and nocodazole (panel **B**, 10 μm) (open bars: vehicle-treated cells; filled bars: inhibitor-treated cells). ACKR2 membrane expression was evaluated in two distinct gates referred to the viable pEGFP^{neg} and pEGFP^{pos} cells, respectively. Data are expressed as percentage of MFI of pEGFP^{neg}/pEGFP^{pos} cells. Results are representative of mean ± SEM of 3 experiments. (**C** and **D**) Confocal analysis of CHO-K1/ACKR2 cells incubated for 1 hour with vehicle (DMSO), latrunculin A (panel **C**, 1 μm) and nocodazole (panel D, 10 μm). Left panels show ACKR2 staining (red) merged to (**C**) Rab4 (blue) and F-actin (phalloidin staining, green) or (**D**) Rab11 (blue) and microtubules (α-tubulin staining, green). Nomarski interference contrast (DIC) is merged to colocalization channel on right panels. Quantification of ACKR2 colocalization with Rab4 or Rab11 as PCC is the mean ± SEM of at least 100 cells analysed in 3 different experiments performed and is indicated into each panel. Data were analysed by unpaired Student's T test. *P≤ 0.05 and ***P≤ 0.005 for inhibitor-treated versus vehicle-treated cells. ###P≤ 0.005 for pEGFP^{pos} versus pEGFP^{neg} cells.

Altogether, these results demonstrate that dynamic actin turnover and intact microtubules are required for ACKR2 constitutive sorting to rapid (Rab4-positive) and slow (Rab11-positive) recycling pathways, respectively.

2- C. THE SCAVENGER FUNCTION OF ACKR2 REQUIRES LIGAND-INDUCED REARRANGEMENT OF MICROTUBULES

We previously reported that agonist-induced mobilization of ACKR2 from intracellular endosomes to the plasma membrane is required to optimize its chemokine-scavenging properties [19]. As previously mentioned, the trafficking of intracellular vesicles relies on the regulatory and motor proteins of the cytoskeleton [290] and we reported that actin reorganization through cofilin is functionally relevant for the scavenger function of ACKR2, supporting the idea that cytoskeleton may play an important role in regulating ACKR2 intracellular trafficking.

We here investigated the role of microtubules in receptor trafficking. CHO-K1/ACKR2 cells were stained for ACKR2 and microtubules, after treatment with medium or with 100 nM CCL3L1. Confocal microscopy analysis revealed that ACKR2 activation by chemokines stimulates microtubules reorganization and increases the extent of receptor colocalization with microtubules from perinuclear compartment to plasma membrane, reported in each panel and expressed as PCC (**Figure 39A**). Conversely, the conventional chemokine receptor CCR5 was preferentially found at the plasma membrane under basal conditions, where it colocalized with microtubules, and treatment with CCL3L1 stimulated its internalization into perinuclear compartments without affecting its colocalization with microtubules (**Figure 39B**). Treatment with the microtubules depolymerizing agent nocodazole (10 μM for 1 hour at 37°C) substantially impaired agonist-dependent increase in ACKR2 membrane expression whereas stabilization of microtubules by paclitaxel had no significant effect (**Figure 39C**). Interestingly, while nocodazole inhibited ACKR2 chemokine degradadation rate, stabilization of microtubules by paclitaxel resulted in increased chemokine scavenging, suggesting that the integrity of the microtubule network was functionally relevant for the degradatory function of ACKR2 (**Figure 39D**). ACKR2 colocalization with microtubules at the plasma membrane was significantly increased only by ACKR2 active agonists (CCL3L1, CCL4, CCL22, CCL2), while neutral ligands (CCL3(5-68), CCL3) were as ineffective as CXCL8, which is not recognized by ACKR2 and was used here as a negative control (**Figure 39E** and **39F**).

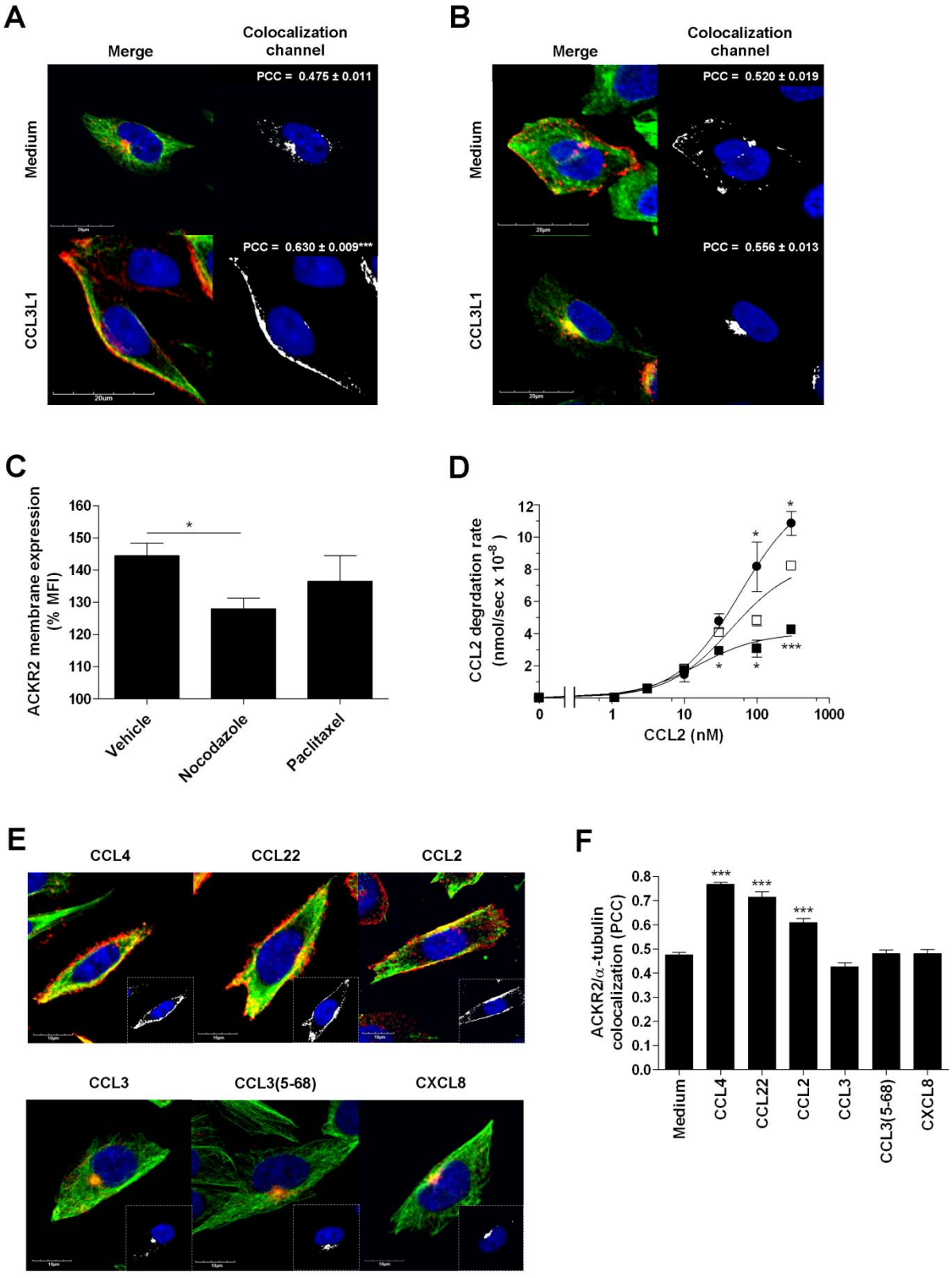

Figure 39: ACKR2 chemokine scavenger function requires ligand-induced microtubules rearrangement. Confocal analysis of (**A**) CHO-K1/ACKR2 or (**B**) CHO-K1/CCR5 cells stimulated with CCL3L1 (100 nM, 1 hour). Nuclear staining (DAPI, blue) is merged to double staining of ACKR2 or CCR5 (red) and

microtubules (α-tubulin staining; green) on left panels, and to colocalization channel on right panels. Quantification of ACKR2 or CCR5 colocalization with microtubules in response to treatment with CC3L1 is reported into each panels and expressed as the mean PCC ± SEM of at least 20 cells analysed in 3 different experiments. CHO-K1/ACKR2 cells are pre-treated (1 hour) with vehicle (DMSO), nocodazole (10 μM) and paclitaxel (1 μM) and analysed for ACKR2 (**C**) upregulation after stimulation with CCL3L1 (100 nM, 1 hour) and (**D**) scavenging activity (□ vehicle, ■ nocodazole, ● paclitaxel) following incubation with ^{125}I-CCL2 (0.1 nM) and indicated concentrations of CCL2. Results are shown as mean ± SEM of at least 3 experiments. (**E**) Confocal analysis of CHO-K1/ACKR2 upon stimulation with indicated chemokines (100 nM, 1 hour). Nuclear staining (DAPI, blue) is merged to double staining of ACKR2 (red) and microtubules (α-tubulin staining; green) on left panels and colocalization channels are enclosed in the corresponding panels. (**F**) Quantification of ACKR2 colocalization with microtubules in response to treatment of the indicated chemokines. The colocalization results are indicated as the mean PCC ± SEM of at least 30 cells. Data were analysed by unpaired Student's T test. * = p≤0.05, *** = p≤0.005 stimulated versus unstimulated cells (panels **A** and **F**) or inhibitor-treated versus vehicle-treated cells (panels **C** and **D**).

All together, these results indicate that, ACKR2 requires a signalling activity to promote not only the reorganization of the actin cytoskeleton but also of the microtubules network, in order to sustain its chemokine scavenger activity.

2- D. MYOSIN Vb SUSTAINS ACKR2 UPREGULATION AND CHEMOKINE DEGRADATION

Membrane recycling events are finely regulated at molecular level by motor proteins, including myosin Vb, an actin-based transporter which interacts with endocytic compartments and regulates vesicles traffic to the plasma membrane along microtubule network [26, 27]. Interestingly, myosin Vb is shown to regulate trafficking and receptor-mediated chemotaxis of the chemokine receptor CXCR2 [95]. We decided to investigate the role of myosin Vb in regulating ACKR2 intracellular trafficking. We performed a confocal microscopy analysis of CHO-K1/ACKR2 cells, stained for endogenous myosin Vb. Under basal conditions, myosin Vb colocalized with ACKR2 preferentially at perinuclear compartments (**Figure 40A**), while after stimulation with 100 nM CCL3L1, myosin Vb was redistributed and increased its colocalization with ACKR2 mostly at plasma membrane level (**Figure 40A**), though ACKR2 association with myosin Vb was also observed in Rab11-positive recycling vesicles at perinuclear compartments and in Rab11-negative early endosome diffused within cytoplasm and at periphery (**Figure 40B**). After chemokine stimulation, ACKR2 association with myosin Vb was still observed at plasma membrane,

but not in Rab11-positive vesicles. In contrast, myosin Vb did not colocalize with CCR5, neither under basal condition nor after CCL3L1 engagement (**Figure 40C**).

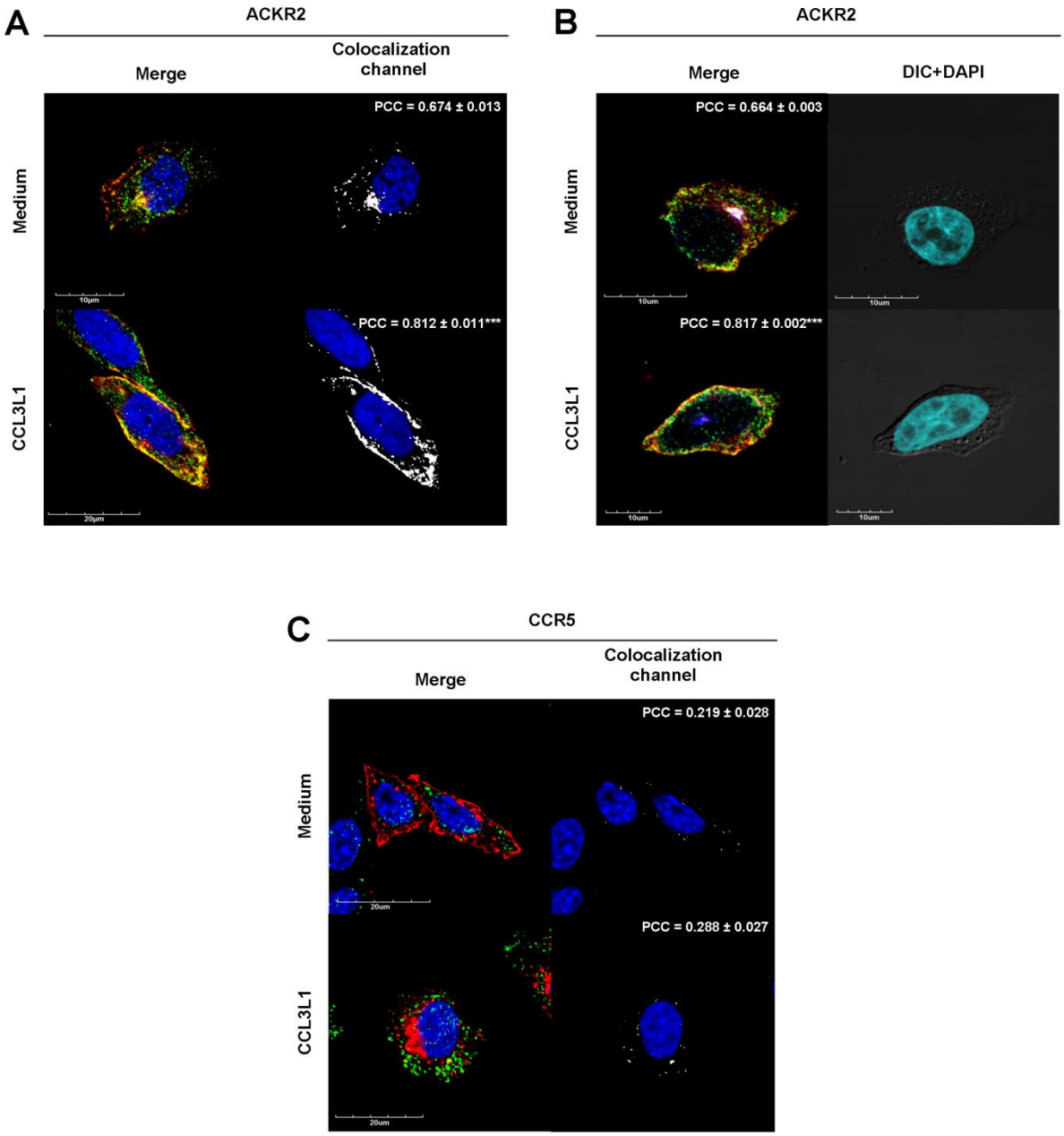

Figure 40: Myosin Vb regulates ACKR2 intracellular trafficking and distribution. Confocal analysis of (**A** and **B**) CHO-K1/ACKR2 stimulated with CCL3L1 (100 nM, 1 hour). (**A**) Nuclear staining (DAPI, blue) is merged to double staining of ACKR2 (red) and myosin Vb (green) on left panels, and to colocalization channel on right panels. Quantification of ACKR2 colocalization with myosin Vb as PCC is the mean ± SEM of at least 30 cells analysed. (**B**) ACKR2 staining (red) is merged to myosin Vb (green) and Rab11 staining (in blue) on left panels, and nuclear staining (in cyan) is reported with Nomarski interference contrast (DIC) on right panels. Quantification of ACKR2 colocalization with myosin Vb as PCC is the mean ± SEM of at least 30 cells analysed. (**C**) Confocal analysis of CHO-K1/CCR5 stimulated with CCL3L1 (100 nM, 1 hour). Nuclear

staining (DAPI, blue) is merged to double staining of CCR5 (red) and myosin Vb (green) on left panels, and to colocalization channel on right panels. Quantification of CCR5 colocalization with myosin Vb as PCC is the mean ± SEM of at least 30 cells analysed. *** = p≤0.005 stimulated versus untreated cells.

To investigate the role of myosin Vb in ACKR2 trafficking, CHO-K1/ACKR2 cells were transiently transfected with pEGFP-tagged myosin Vb tail, which operated as a myosin Vb dominant negative displacing endogenous myosin Vb and disengaging cargos from F-actin filaments [28]. Confocal microscopy analysis showed a similar intracellular distribution pattern of ACKR2 in cells expressing or not myosin Vb tail under basal conditions, with the receptor maintaining its colocalization with both microtubules (**Figure 41A**) and Rab11 (**Figure 41B**) at perinuclear compartments. After CCL3L1 stimulation, microtubule cytoskeleton reorganization with increased ACKR2-microtubules colocalization at plasma membrane (**Figure 41A**) and receptor mobilization from Rab11-positive recycling vesicles (**Figure 41B**) were completely abrogated in myosin Vb tail-positive cells. Flow cytometric analysis revealed that abrogation of myosin Vb activity did not affect the constitutive endocytosis of ACKR2 (**Figure 41C**), but resulted in substantially reduced receptor expression at the plasma membrane and inhibition of the chemokine-dependent increase in ACKR2 expression (**Figure 41D**), which was accompanied by a marked impairment in the ability of ACKR2 to degrade chemokines (**Figure 41E**).

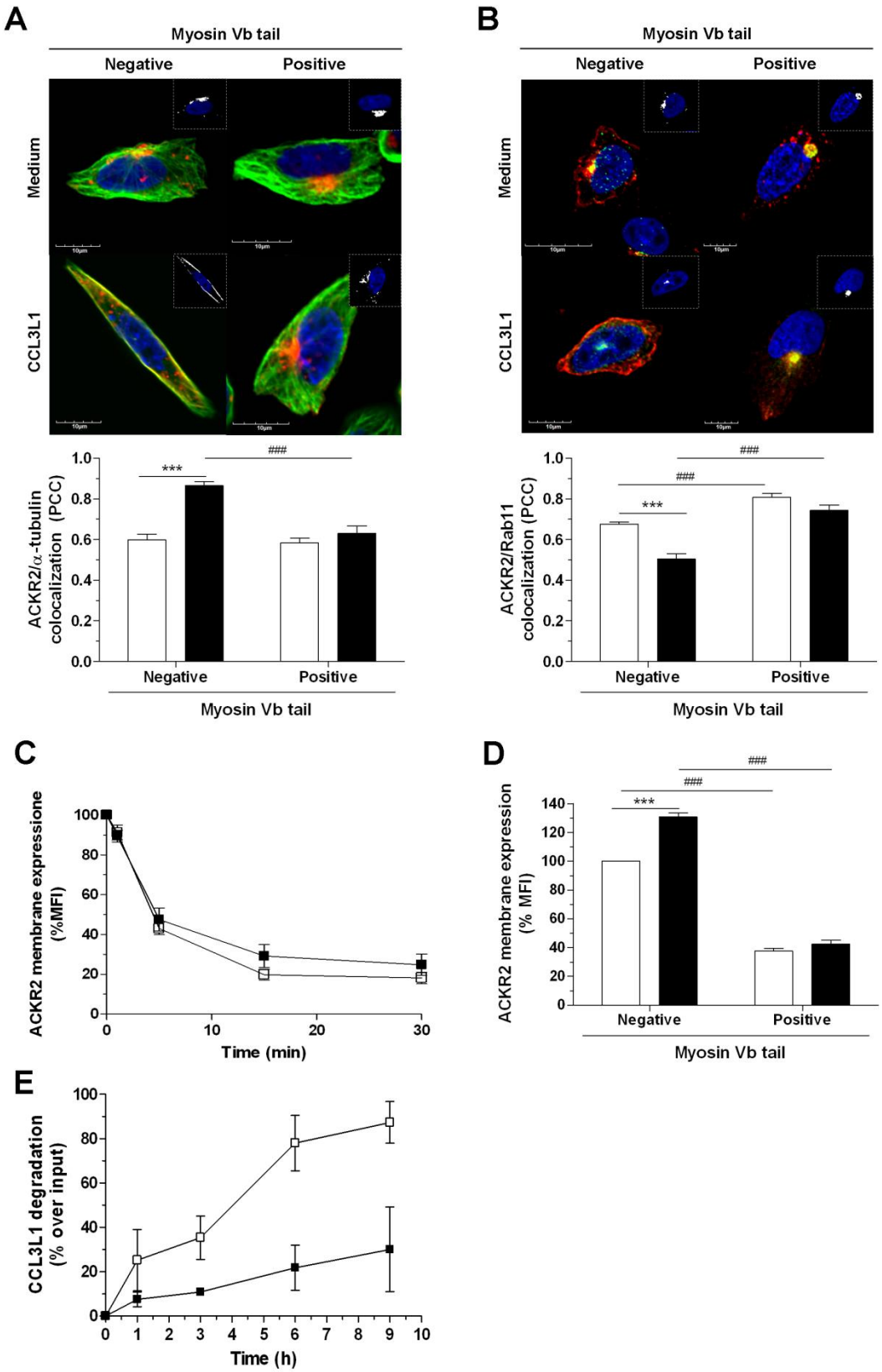

Figure 41: Myosin Vb sustains ACKR2 upregulation and chemokine degradation. (**A** and **B**) Confocal analysis of CHO-K1/ACKR2 transiently transfected with pEGFP-tagged myosin Vb tail and stimulated or not with CCL3L1 (100 nM, 1 hour). Nuclear staining (DAPI, blue) is merged to double staining of ACKR2 (red) and (**A**) microtubules (α-tubulin staining; green) or (**B**) Rab11 (green). Colocalization channels merged to DAPI are enclosed in the corresponding panels. Quantification of ACKR2 colocalization

with microtubules and Rab11 as PCC is the mean ± SEM of at least 20 cells (open bar: untreated cells, black bar: stimulated cells) and is shown below the corresponding panels. Myosin Vb tail^{neg} (□) and myosin Vb tail^{pos} (■) cells are analysed for ACKR2 (**C**) constitutive internalization; (**D**) membrane expression and upregulation after CCL3L1 (100 nM, 1 hour; open bars: untreated cells, closed bars: stimulated cells) and (**E**) scavenging activity by test ELISA following incubation with CCL3L1 (1 nM) at indicated time points. Results are representative of mean ± SEM of 3 experiments. *** = p≤0.005 stimulated versus untreated cells; ### = p≤0.005 myosin Vb tail^{pos} versus myosin Vb tail^{neg} cells.

Taken together, these results indicate that myosin Vb activity is required for the correct trafficking of ACKR2 from Rab11 recycling endosome to the plasma membrane.

2- E. ACKR2 PROMOTES MICROTUBULES REARRANGEMENT THROUGH A G PROTEIN-INDEPENDENT β-ARRESTIN1-DEPENDENT PATHWAY

In the previous section, we demonstrated that ACKR2 is a β-arrestin1-biased signalling receptor whose trafficking and scavenging activities require actin cytoskeleton rearrangements induced by the activation of a Rac1-PAK1-LIMK1 signalling pathway. As components of this signalling cascade are also involved in microtubules organization and dynamics [311, 312] and LIMK functions as a signalling node controlling the crosstalk between actin and microtubule networks [313], we assessed the involvement of this signalling pathway in ligand-induced ACKR2 dependent microtubules rearrangement. Consistently with our previous observations, ACKR2 did not require Gαi protein activity to induce microtubules reorganization, as PTX treatment had no effect on CCL3L1-induced ACKR2-microtubules colocalization at plasma membrane (**Figure 42A**). On the contrary, inhibition of each individual element of the β-arrestin dependent signalling cascade identified previously to be important in actin reorganization, inhibited either pharmacologically or at molecular level, did not affect ACKR2 colocalization with microtubules under basal conditions but prevented agonist-dependent ACKR2 redistribution at the plasma membrane and its colocalization with microtubules. CHO-K1/ACKR2 cells were treated for 72 hour with 50 nM control siRNA (scrambled), or with β-arrestin specific (**Figure 42B**), or with Pak1 specific siRNA (**Figure 42D**), or with LIMK1-specific siRNA expressing vector, comparing to the control one (**Figure 42E**), or cells were treated with vehicle or with the specific Rac1 inhibitor NSC23766 (**Figure 42C**).

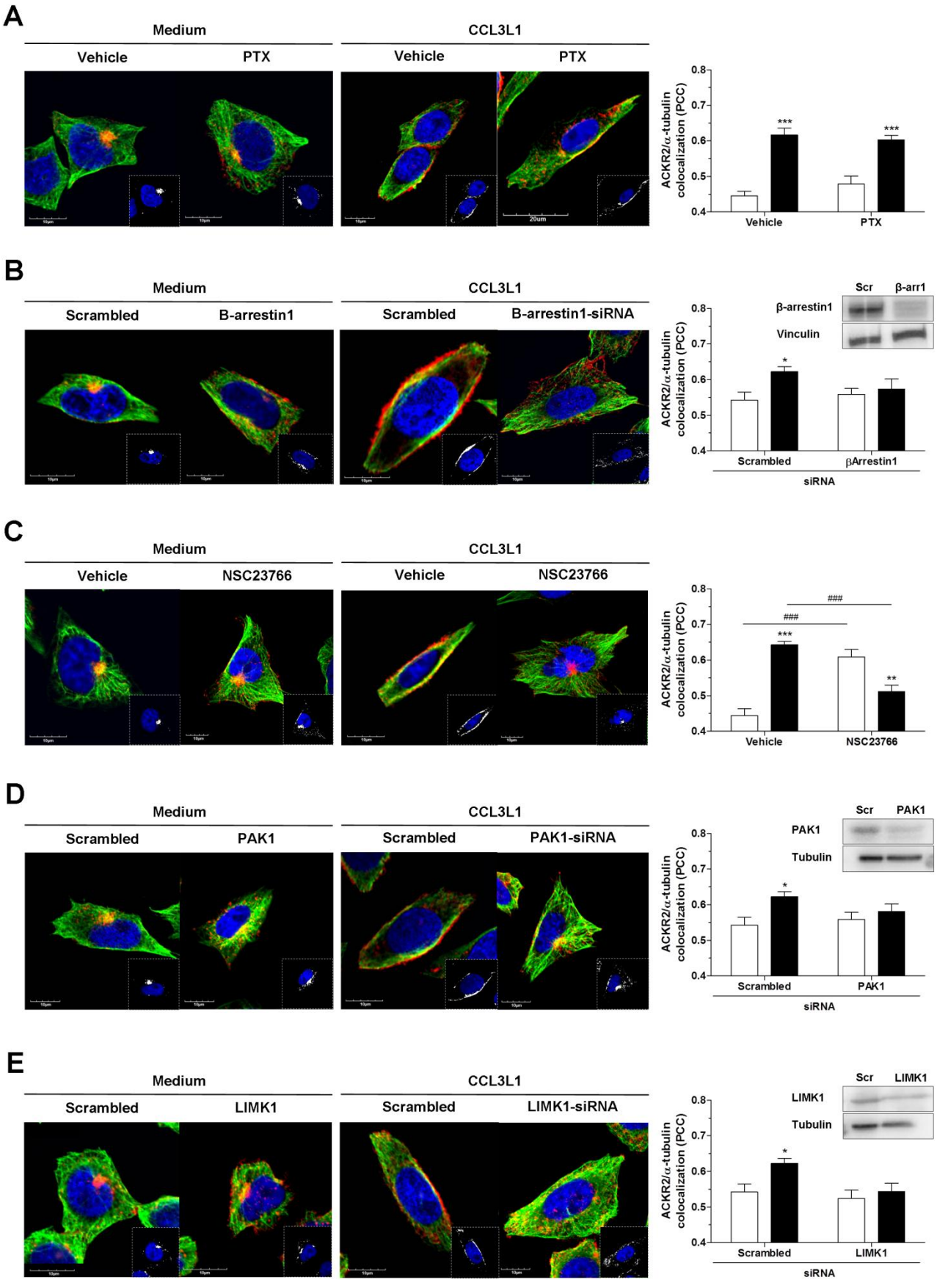

Figure 42: ACKR2 promotes microtubules rearrangement through a G protein-independent β-arrestin1-dependent pathway. Confocal analysis of CHO-K1/ACKR2 cells pre-treated with (**A**) PTX (100

ng/ml, 16 hours) and (**C**) NSC23766 (200 μM, 1 hour), or transfected with (**B**, **D**) scrambled and (**B**) β-arrestin1-specific and (**D**) PAK1-specific siRNA (50 nM, 72 hours) or (**E**) LIMK shRNA-expressing plasmid (72 hours), followed by stimulation with CCL3L1 (100 nM, 1 hour). SiRNAs transfected cell were analysed by Western blot for β-arrestin1, PAK1 and LIMK1 content, respectively, as shown on the right of corresponding panels, normalized over tubulin or vinculin levels. Each panel shows nuclear staining (DAPI, blue) merged to double staining for ACKR2 (red) and microtubules (α-tubulin staining; green). Quantification of ACKR2 colocalization with microtubules as PCC is the mean ± SEM of at least 20 cells and is shown on the right of corresponding panels. *= p≤0.05, **= p≤0.01 and *** = p≤0.005 stimulated versus unstimulated cells. ### = p≤0.005 NSC23766-treated versus vehicle-treated cells.

These results clearly indicate that agonist-induced activation of the β-arrestin1-dependent signalling cascade, including a Rac1-PAK1-LIMK1 pathway is required not only to induce actin reorganization, but also to rearrange the microtubules cytoskeleton, in order to sustain ACKR2 intracellular trafficking and optimized its scavenger activity.

3. ACKR2 AND B-ARRESTIN

3- A. ACKR2 RECRUITS β-ARRESTIN1, BUT NOT β-ARRESTIN2, AFTER CHEMOKINE STIMULATION

The scavenger activity of ACKR2 has been associated to the activation of the Rac1-PAK1-LIMK1-cofilin signalling cascade via a G-protein-independent, β-arrestin1-dependent pathway, leading to actin cytoskeleton reorganization, receptor up-regulation and chemokine degradation [24]. In addition, also the microtubule network had a functional relevance in ACKR2 scavenger activity, as we previously shown [Cancellieri C et al, 2013, submitted]. Thus, it is tempting to speculate that ACKR2 behaves as a β-arrestin1-biased receptor as demonstrated by the activation of the Rac1-PAK1-LIMK1 pathway finely coordinating cytoskeletal network rearrangements, required to sustain its scavenging activity.

To better clarify the recruitment of β-arrestin after ACKR2 stimulation, we performed a quantitative analysis through a Bioluminescence Resonance Energy Transfer (BRET1) assay, in order to analyse if chemokine engagement promotes ACKR2 and β-arrestin association. HEK293T were transiently co-transfected with the following couples of vectors: ACKR2/Rluc and β-arrestin1/YFP; ACKR2/Rluc and β-arrestin2/YFP; CCR5/Rluc and β-arrestin1/YFP; CCR5/Rluc and β-arrestin2/YFP. After 48 hours, cells were incubated

with Coelenterazine H and stimulated with 100 nM CCL3L1. The Luminescence and Fluorescence values had been measured as described in Material and Methods section, in order to obtain the BRET ligand effect, reported in each figure. After chemokine stimulation, ACKR2 was able to recruit β-arrestin1 with the same efficiency of the canonical chemokine receptor CCR5 (**Figure 43A**). On the contrary, ACKR2 recruited β-arrestin2 with a lower efficiency compared to CCR5 (**Figure 43B**).

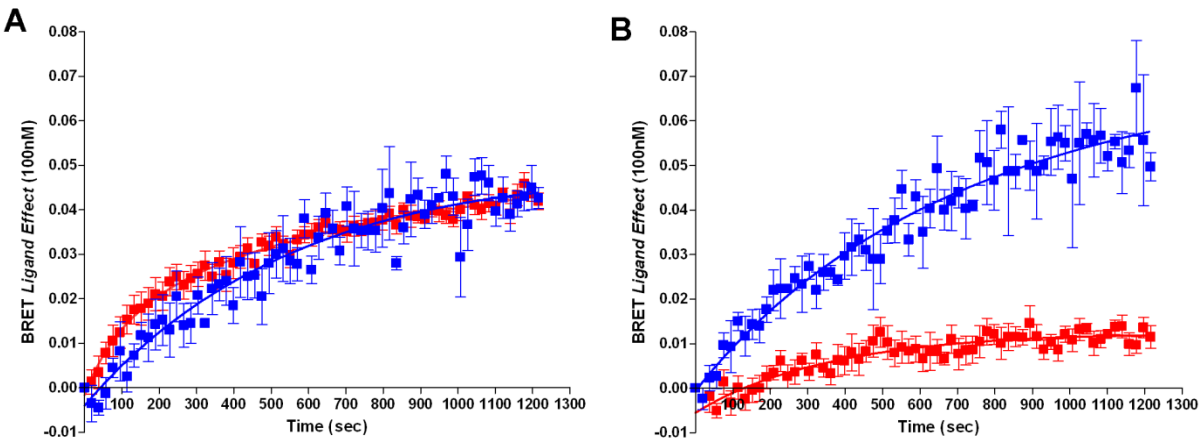

Figure 43: ACKR2 recruits β-arrestin1 but not β-arrestin2 after chemokine stimulation. (**A** and **B**) HEK293T cells were co-transfected with ACKR2/Rluc and β-arrestin1/YFP or CCR5/Rluc and β-arrestin1/YFP (**A**) or with ACKR2/Rluc and β-arrestin2/YFP or CCR5/Rluc and β-arrestin2/YFP (**B**). After 48 hours, the BRET ligand effect was evaluated after incubation with Coelenterazine H (5 μM for 8 min) and stimulation with CCL3L1 (100 nM for 20 min), as described in Material and Methods section. In panels (**A**) and (**B**) are reported the β-arrestin1 and β-arrestin2 recruitment, respectively, for ACKR2 (■) and for CCR5 (■). Results are the mean.± SEM of 6 independent experiments.

These results clearly indicate that ACKR2 recruits β-arrestin1 but not β-arrestin2 upon chemokine engagement, differentially from the canonical chemokine receptor CCR5 which recruits the two β-arrestins with a similar kinetic.

3- B. ACKR2 RECRUITS β-ARRESTIN1 ONLY AFTER STIMULATION WITH ACTIVE LIGANDS, NOT WITH NEUTRAL ONES.

The β-arrestin1 dependent signalling pathway that induces actin reorganization [24], receptor mobilization from intracellular compartments [19] and ACKR2 scavenging activity

is activated only after receptor engagement with active ligands [23]. As we previously demonstrated, after chemokine stimulation ACKR2 recruits β-arrestin1, but not β-arrestin2. Here, the specific β-arrestin1 recruitment was evaluated with different chemokines, distinguishing between active and neutral one. HEK293T cells were co-transfected transiently with ACKR2/Rluc and β-arrestin1/YFP for 48 hours and then cells were incubated with Coelenterazine H and stimulated with 100 nM of reported ligands. The Luminescence and Fluorescence values were measured, as described in Material and Methods section, in order to obtain the BRET ligand effect, reported in each figure. In **Figure 44A** are shown active ligands, CCL4, CCL2, CCL5 and CCL17, while in **Figure 44B** are reported neutral ligands, CCL3 and CCL3 (5-68).

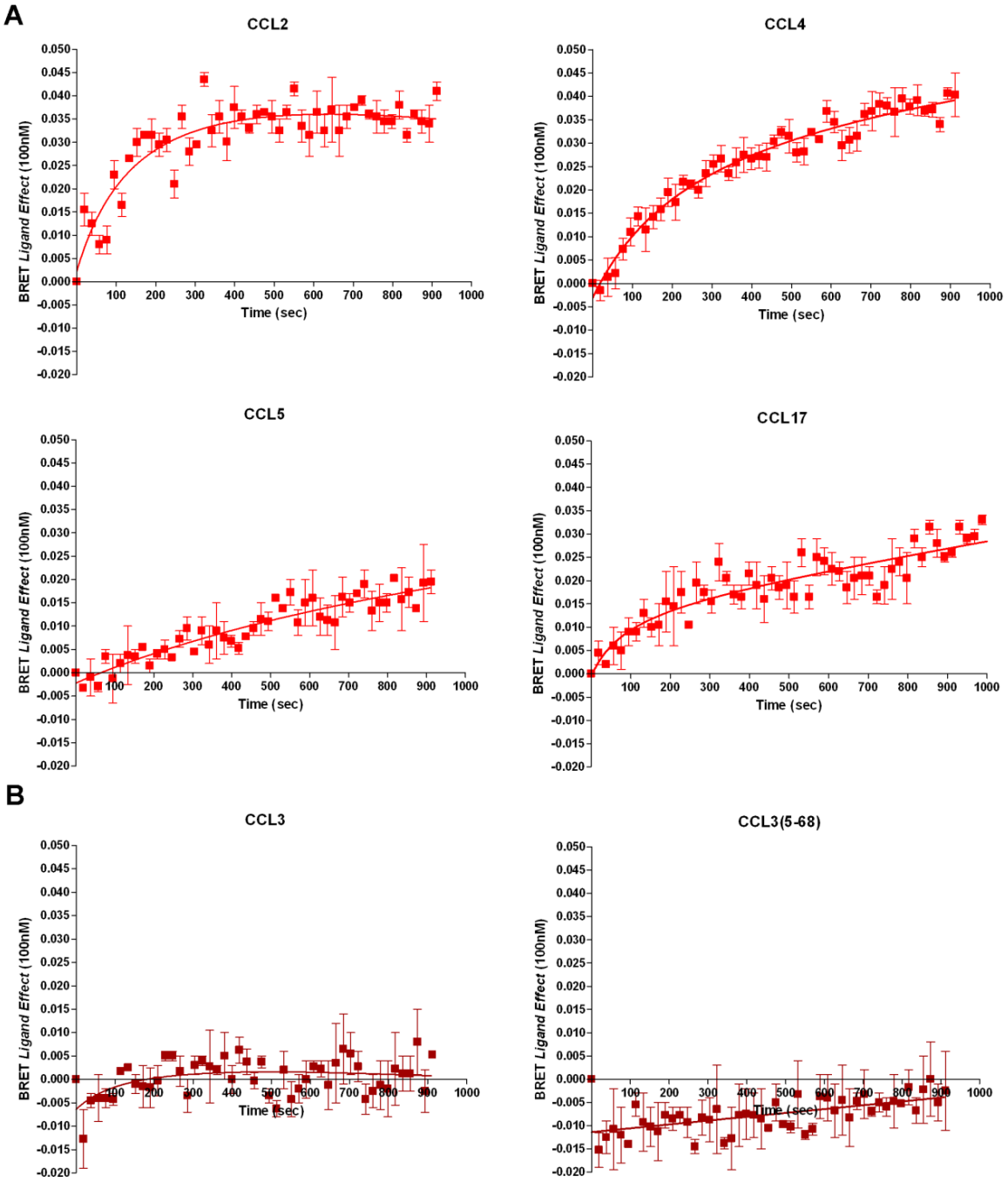

Figure 44: ACKR2 recruits β-arrestin1 only after active ligands stimulation, not with neutral ones. (**A** and **B**) HEK293T cells were co-trasfected with ACKR2/Rluc and β-arrestin1/YFP. After 48 hours, cells were incubated with coelenterazine H (5 μM for 8 min) and then was measured the BRET ligand effect, as described in Material and Methods section. In panel (**A**) is reported the β-arrestin1 recruitment with active ligands CCL2, CCL4, CCL5 and CCL17 (■, all used at 100 nM). In panel (**B**) is reported the β-arrestin1 recruitment with neutral ligands CCL3 and CCL3 (5-68) (■, all used at 100 nM). Results are the mean.± SEM of 3 different experiments.

These data clearly demonstrate that active ligands but not neutral ones promotes selective recruitment of β-arrestin1 to ACKR2, confirming the role of β-arrestin1 in ACKR2 scavenging function, as previously reported.

3- C. ACKR2 CONSTITUTIVELY ASSOCIATES β-ARRESTIN1 AND β-ARRESTIN2

β-arrestin1 is clearly important in ACKR2 signalling, inducing a specific transduction pathway leading to actin and microtubules reorganization. Cytoskeletal networks dynamics as actin turnover and intact microtubules are also involved in ACKR2 constitutive cycling for the correct sorting of receptor to both rapid and slow recycling compartments. Interestingly, studies on other GPCRs suggest that their accumulation in the recycling endosome is dependent upon the phosphorylation status of serine/threonine residues in the cytoplasmic tail of the receptor or its association with β-arrestin [314]. Although the phosphorylation status of the receptor at the cytoplasmic tail is still debated, it has been reported that ACKR2 expression under basal condition modifies the intracellular distribution of β-arrestin, leading to cluster formation with ACKR2, in particular at plasma membrane, suggesting that β-arrestin is important for the regulation of the constitutive cycling of ACKR2 [213, 214]. The C-tail of the receptor is important in β-arrestin relocalization [18, 214], suggesting a constitutive interaction. As previously reported, β-arrestin1 knockdown cells show a widely diffused pattern of ACKR2 in the cytoplasm and loss of receptor recycling routes [24], supporting that a cross-talk between ACKR2 and β-arrestin is required to ensure receptor constitutive cycling.

To evaluate the constitutive association of ACKR2 with β-arrestin1 and β-arrestin2, we performed a BRET2 assay, as described in Material and Methods section. HEK293T cells were co-transfected with ACKR2/Rluc and β-arrestin1/GFP^2 or ACKR2/Rluc and β-arrestin2/GFP^2 or ACKR2/Rluc and soluble GFP^2, the last one used as a negative control. After 48 hours, the fluorescence values of GFP, the luminescence value of Rluc, the BRET ratio of ACKR2/β-arrestin association were measured. The BRET ratio and Fluorescence/Luminescence ratio were reported in each graph, in particular for soluble GFP^2 (**Figure 45A**), for β-arrestin1 (**Figure 45B**) and for β-arrestin2 (**Figure 45C**).

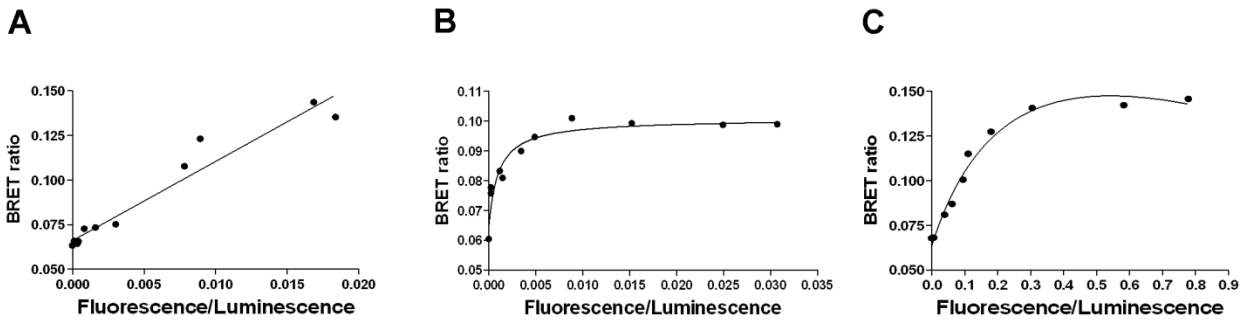

Figure 45: ACKR2 constitutively associates β-arrestin1 and β-arrestin2. HEK293T cells have been transiently co-transfected with (**A**) ACKR2/Rluc and GFP^2, (**B**) ACKR2/Rluc and β-arrestin1/GFP^2 or (**C**) ACKR2/Rluc and β-arrestin2/GFP^2. After 48 hours, cells have been incubated with coelenterazine H (5 μM for 8 min) or with DeepBlueC (5 μM), in order to measure the luminescence value and the BRET ratio, respectively, as described in Material and Methods section. In addition, the fluorescence value has been measured, in order to obtain the Fluorescence/Luminescence ratio, reported in each graph with the BRET ratio, for (**A**) GFP^2, used as control, (**B**) β-arrestin1 and (**C**) β-arrestin2. The graphs are representative of 3 different experiments.

ACKR2 constitutively associates both β-arrestins, but after chemokine engagement, only β-arrestin1 induce a specific signalling pathway that lead to cytoskeletal reorganization in order to sustain receptor up-regulation and scavenging activity.

3- D. ACKR2 ASSOCIATES B-ARRESTINS BOTH AT PLASMA MEMBRANE AND AT INTRACELLULAR COMPARTMENTS LEVELS.

ACKR2 is associated at β-arrestin1 and β-arrestin2 under basal condition, and after active ligand engagement, β-arrestin1 is preferentially recruited and induce a signalling pathway to sustain ACKR2 scavenging activity. As the intracellular ACKR2 distribution was maintained by its constitutive recycling capability, able to ensure the degradatory function of the receptor, as previously demonstrated, we performed a confocal microscopy analyses in order to clarify the intracellular or plasma membrane distribution of D6:β-arrestins complexes. As reported also for CXCR7 [285], β-arrestins are hypothesised to be involved in receptor regulation of intracellular trafficking, as β-arrestin1 knockdown cells demonstrated a loss of intracellular distribution. To evaluate if the ACKR2 association with β-arrestins was limited to the plasma membrane, HEK293T/ACKR2 cells were transiently co-trasfected with β-arrestin1/YFP or β-arrestin2/YFP. The association between the

receptors and β-arrestins was evaluated with confocal microscopy, considering fluorescence of YFP and ACKR2 staining with a specific antibody. We considered cells not permealized and cells permealized, in order to evaluate the ACKR2/β-arrestins association at plasma membrane or at intracellular compartments, respectively (**Figure 46**).

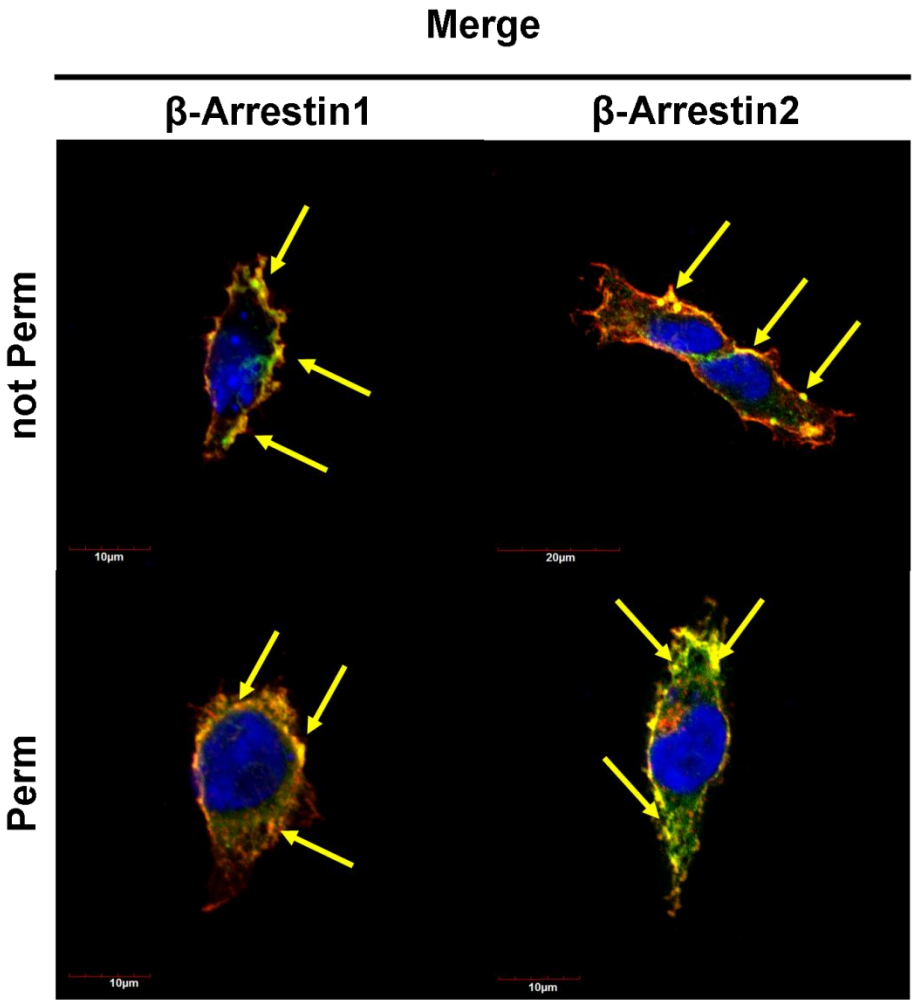

Figure 46: ACKR2 colocalized with β-arrestin1 and β-arrestin2 both at plasma membrane and at intracellular levels. Confocal analysis of HEK293T/ACKR2 cells transfected with β-arrestin1/YFP (on left panel) or β-arrestin2/YFP (on right panel). Nuclear staining (DAPI, blue) is merged to ACKR2 staining (red) and β-arrestins/YFP (green). In the upper panels are reported cells not permeabilized. while in the lower panels are reported cells permeabilized, as described in Material and Methods section. Harrows indicate ACKR2:β-arrestins complexes localization. The images are representative of 20 different images acquired in 2 different experiments.

These results indicate that ACKR2 colocalized with β-arrestin1 and β-arrestin2 at plasma membrane and at perinuclear levels. It would be interesting to better clarify in which compartments this association is achieved, and new experiments using specific markers of intracellular vesicles are ongoing.

DISCUSSION

ACKR2, previously referred to as D6, is a member of a subfamily of chemokine receptors, including ACKR1 (DARC) [133], ACKR3 (CXCR7) [6], ACKR4 (CCX-CKR) [132], ACKR5 (CCRL2) [8] and C5L2 [122], which are also called "atypical" chemokine receptors (ACKRs) for their inability to activate the Gαi-mediated signalling pathway and to induce directional cell migration in contrast to conventional chemokine receptors, presumably as a consequence of naturally occurring mutations in domains involved in G protein coupling [127]. Based on *in vitro* and *in vivo* studies, ACKRs have been suggested to act as negative regulators of innate and adaptive immunity [315] and their biological functions may be supported by their ability to generate and shape chemokine gradients through different biochemical processes, including sequestration and degradation, transcytosis, or presentation of the ligands, depending on the different receptors [11, 316]. As no evidence of G-protein dependent signalling activity has been actually described to promote chemotaxis activity as in canonical chemokine receptors, ACKRs are presently considered "silent" receptors.

ACKR2 is the best-characterized atypical chemokine receptor, able to bind with high affinity pro-inflammatory CC chemokine [12]. The predominant expression sites are lymphatic endothelial cells (LECs) of lymphatic vessels of "barrier tissues" such as the skin, gut, and lung [13]. The selective ACKR2 expression confirms its important role in fine tuning inflammatory responses, by shaping the chemokine gradient through the sequestration and degradation of chemokine from inflamed tissues [13]. ACKR2 seemed not ti be regulated or induced at transcriptional level, but recent evidence suggests that ACKR2 expression is negatively regulated at transcriptional level by KRas signalling cascade in Kaposi's Sarcoma derived cells (Savino B, Caronni N et al, 2013, submitted). ACKR2 membrane expression and scavenging activity is finely regulated by its intracellular traffic properties. ACKR2 is predominantly located in intracellular compartments at perinuclear levels and only 10% is detectable on the cell surface. ACKR2 is not regulated or induced at transcriptional level, but its membrane expression is finely regulated by its intracellular traffic [19]. In fact, it is constitutively associated to both early (Rab4/5) and recycling endosomes (Rab11) and after ligand engagement, ACKR2 is mobilized from intracellular compartment to the plasma membrane, without altering the constitutive internalization rate, in order to optimize its scavenging function [19]. Protease-inactivated chemokines, considered neutral ligands, are not degraded and are unable to induce receptor up-regulation, while chemokine efficiently degraded by ACKR2 are also called active ligands and can induce receptor mobilization [23]. These neutral ligands do not have

a proline residues in position 2 and do not affect the constitutive cycling properties of ACKR2 suggesting that the scavenging activity of the receptor is not a passive event accounted for by internalization of the ligand associated with the constitutively cycling receptor, but seems to be supported by ACKR2 ability to activate specific signalling pathway after receptor engagement [19, 23].

GPCR signal transduction properties rely on G protein-mediated signalling pathways that promote cell migration integrated with a β-arrestins-dependent signalling module that functionally uncouples G proteins from the receptor [72]. Recently, it has been proposed a role for β-arrestins not only in receptor desensitization and internalization, but also as adaptor proteins, able to provide and regulate different signalling activities [68]. In this complex signalling network, molecular pathways leading to cytoskeleton reorganization are among the early cellular events following chemokine stimulation and are important for regulating their chemotactic function [317] and the intracellular trafficking properties of several chemokine receptors [21, 22, 318-320]. A major target these signalling events converged at is the actin-binding protein cofilin, which represents a key phosphoregulatory mechanism controlling actin reorganization [293]. The only known kinase able to finely regulate cofilin activity is LIMK1 [300, 301], which is under the control of the Rho family small GTP-binding proteins [304]. The activation of the Rac1-PAK1-LIMK1-cofilin pathway is required for the regulation of chemokine receptor intracellular trafficking properties [22] and its chemotactic activity [297] under β-arrestin and G-protein control [68, 261], demonstrating that the two modules cooperate in the activation of signalling cascades downstream chemokine receptors.

I here provide the first evidence for a signalling activity of an ACKR demonstrating that the "silent" chemokine receptor ACKR2 activates the Rac1-PAK1-LIMK1-dependent signalling pathway through a β-arrestin1-dependent G protein-independent mechanism that finely coordinates both actin rearrangements by cofilin phosphorylation and microtubules network reorganization. I also demonstrate that microtubules and actin have a functional relevance in ACKR2 correct intracellular sorting and recycling to the cell membrane and that the scavenger activity of ACKR2 is associated to the activation of this signalling pathway.

Myosin Vb is a motor protein which finely regulates membrane recycling system interacting with active forms of several Rab proteins to regulate transfer of receptors from sorting endosomes to the recycling compartment along microtubules network [26, 27]. Here I describe a role of myosin Vb as an adaptor molecule cooperating with Rab11 to

sustain both ACKR2 constitutive and ligand-induced trafficking properties, thus supporting receptor function and allowing ACKR2 to develop its scavenger activity. Myosin Vb is also required for the chemotactic activity of the conventional chemokine receptors [95, 221, 315] and also in controlling their intracellular trafficking having a role in receptor re-sensitization after ligand stimulation [95]. It raises the question of how the same molecule may support divergent biological activities downstream atypical and conventional chemokine receptors. Myosin Vb activation may be triggered either by calcium-dependent mechanism or by cargo proteins, which pull the globular domains away from the motor domains allowing the molecule to extend out into its active state [321]. Interestingly, conventional chemokine receptors activate calcium transients fluxes through the G protein-dependent signalling modules [315], while ACKR2 activation does not sustain neither the Gi pathway nor calcium transits, and requires β-arrestin signaling module to sustain its scavenger activity [24]. We hypothesize that signalling pathway downstream β-arrestin or β-arrestin itself could be responsible for myosin Vb activation through a calcium-independent mechanism possibly involving its scaffolding function for adaptor proteins.

The actin reorganization has been reported to be required for the chemotactic activity of the canonical chemokine receptor CCR5 [21] and in this thesis and in our recently published work we demonstrated that this is achieved through cofilin phosphorylation, but it is not important for its residual chemokine-scavenging activity [24]. In this way, the same signalling pathway seems to support completely divergent biological activities downstream an atypical and a conventional chemokine receptor, ACKR2 and CCR5, respectively. In fact, in most cases, GPCRs signals are a balanced fashion through the G proteins and β-arrestins modules for the fine regulation of biological functions [59]. Mutations in key residues have been shown to generate G protein- or β-arrestin-biased receptors [68]. Interestingly, β-arrestin-biased chemokine receptors have been generated after mutations in key residues involved in G protein coupling, such as the highly conserved DRYLAIV motif in the second intracellular loop [322] and some conserved residues in 2, 3 and 5 transmembrane domains [323]. Similar mutations within these regions crucial for G protein-coupling are endogenously observed in ACKR2. While Gαi proteins are partially activated by ACKR2, but are not involved in the reported signalling pathway and in the functionality of ACKR2 [24], here we provide evidence of the ACKR2 recruitment of β-arrestin1, but not β-arrestin2, after chemokine engagement, using a BRET1 technology. In particular, β-arrestin1 recruitment is specific after active, but not neutral, ligand stimulation, supporting the idea that β-arrestin1 regulates the Rac1-PAK1-LIMK1

signalling pathway and the receptor intracellular distribution and trafficking, suggesting a role of ACKR2 as a β-arrestin-biased receptor.

Obviously, these observations raise the intriguing question whether modifications in regions crucial for G protein-coupling are sufficient to convert a chemotactic chemokine receptors into a scavenger decoy receptor and vice versa. In this respect, Lagane B. et al showed that arginine replacement by asparagine in the DRY motif of the canonical receptor CCR5 (R126N-CCR5) impairs G protein coupling efficiency and chemotaxis, reducing receptor stability at plasma membrane. R126N-CCR5 is constitutively phosphorylated, promoting its association with β-arrestins and receptor internalization. As reported in our recently published work and in this thesis, CCR5-positive cells treatment with PTX and G protein uncoupling is not enough to convert a chemotactic receptor into a chemokine scavenger but other specific signalling features seems to be required [24]. In addition, correcting the mutation in the DRY motif in ACKR2 do not activate G-protein-dependent transduction pathway, without promoting a chemotactic activity [212]. Recent studies have reported that the DRY motif is not the only motif involved in G-protein coupling, but other micro-switch domains highly conserved in chemokine receptor and mutated in ACKRs, are involved [127].

Studies on other GPCRs suggest that their accumulation in the recycling endosome is dependent upon the phosphorylation of serine/threonine residues in the cytoplasmic tail of the receptor or its association with β-arrestin [314]. The ACKR2 C-tail is rich of serine/threonine residues and, although the phosphorylation status of ACKR2 cytoplasmic tail is still debated, it has been demonstrated that ACKR2 expression mediates β-arrestins re-localization exclusively to cell periphery [214], and β-arrestin1 knockdown cells showed a widely diffused pattern of ACKR2 in the cytoplasm and loss of receptor recycling routes [24], indicating that a cross-talk between ACKR2 and β-arrestins is required to ensure receptor stability and constitutive cycling. ACKR2 C-tail truncated form are degraded [18, 214] and, according to all these structural considerations, ACKR2 seems to act as a β-arrestin-biased receptor, which has been naturally engineered from canonical chemokine receptors by mutation in key residues, unbalancing the G-protein and β-arrestin signalling modules. ACKR2 unbalanced signalling behaviour could be responsible of the lack of migratory activity and the gain of scavenging function, indicating that β-arrestin module selective activation allows ACKR2 to shift from a chemotactic to scavenger receptor.

Constitutive cycling and ligand-dependent receptor up-regulation represent unique mechanisms used by scavenger receptors, including ACKRs [221], to rapidly modulate

ligand uptake and degradation depending on the immediate needs of the tissue. Several features distinguish conventional chemokine receptor from ACKR2, which appears to be structurally adapted to perform chemokine scavenging, being constitutively internalized, recycled and not down-regulated after chemokine engagement [53]. Although ACKR2 constitutive cycling which sustains ligand concentration-dependent optimization of its scavenger performance, represents a rapid and unique mechanism allowing ACKR2 to control inflammation, the precise molecular mechanisms involved in ACKR2 sorting to the different recycling pathways are presently unknown [19]. Here I provide the first evidence that ACKR2 constitutive cycling is finely regulated by cytoskeletal networks dynamics as actin turnover and intact microtubules are required for the correct sorting of the receptor to both rapid and slow recycling compartments. ACKR2 constitutive cycling cannot be simply explained as a consequence of a passive transport along pre-existing intracellular trafficking routes as conventional chemokine receptors do not exerted the same behaviour once expressed in the same cellular context [17], but the ACKR2 constitutive cycling could require specific signals which are constitutively activated downstream ACKR2 to cytoskeleton. In this respect, we demonstrated that ACKR2 is able to constitutively recruit β-arrestin1 and β-arrestin2 under basal condition. The ACKR2 association with β-arrestins is reported to be both at plasma membrane and at intracellular compartments levels, supporting the constitutive activation of ACKR2, as same as it has been recently reported for CCR1 by Gilliland et al. [71]. In fact, the CCR1 constitutive activity bring to a basal phosphorylation of the receptor and a constitutive recruitment of β-arrestin2, leading to receptor internalization [71]. They also demonstrated a constitutive CCR1 association with both G-protein and β-arrestin2, through the creation of homo-oligomers, and while the CCR1:G-protein complex is involved in chemotactic function of receptor, the receptor:β-arrestin2 complexes may be related to a potential scavenging function of CCR1, supporting the two modules signal model of chemokine receptors. It is easy to speculate that also ACKR2 could constitutively form homo-oligomers with the two isoform of β-arrestins, and after ligand stimulation, the conformational changes in activated ACKR2 could lead to a selective association with β-arrestin1, in order to support the ACKR2 functional activity. Additional experiments are needed to clarify this aspect. While β-arrestin1 has been reported to regulate the ACKR2 cycling in order to optimize the degradatory efficiency of the receptor after chemokine stimulation, β-arrestin2 do not influence receptor constitutive recycling and scavenging activity (Cancellieri C, unpublished results), suggesting a β-arrestin2 possible involvement in other function of ACKR2, not yet

identified. In fact, β-arrestins are reported to regulate transcriptional molecules and ACKR2 seems to activate the transcription of genes involved in inflammation, as CXCL8, and in protein stability, as HSPA6 (Borroni EM, unpublished results), prompting that β-arrestin2 constitutive recruitment could have a role in the transcriptional regulation induced by ACKR2 expression.

In conclusion, I here provide evidence of G protein-independent β-arrestin-dependent signalling events downstream ACKR2 controlling the actin cytoskeletal dynamics and microtubules network via myosin Vb, and demonstrate that this signalling pathway is required for regulate ACKR2 trafficking properties and its scavenger performance, because inhibition of each component of the β-arrestin1-Rac1-PAK1-LIMK1-cofilin signalling cascade abrogates ACKR2 ability to degrade chemokines. In addition, I demonstrate β-arrestin1 recruitment after active ligand stimulation, and a constitutive association of ACKR2 with both β-arrestin1 and β-arrestin2, that occurs at plasma membrane and at intracellular compartment. Our results and recent evidences support a role for G-protein-independent, β-arrestin-dependent signalling events in the biological activities of ACKRs [278, 283] and the unbalances activation of these two signalling modules could be the first conceptual element in our understanding of the molecular mechanisms underlying the loss of chemotactic activity and the gain of the chemokine gradient-shaping functions characteristic of ACKRs. Thus, the atypical chemokine receptor subfamily could be defined as endogenous, β-arrestin-biased signalling receptors.

Recently, the association of inflammation and tumor biology is ascertain, as the immune system have a key role in promoting the neoplastic progression. ACKR2 has been demonstrated to act as a negative regulator also in some tumor biology. In fact, the ACKR2 overexpression is reported to reduce CCL5 and CCL2 chemokine levels and tumor-associated macrophages infiltration in breast cancer cell line [236]. In addition, ACKR2 role has been recently proposed also in Kaposi's sarcoma tumor, as the chemokine levels regulation mediated by ACKR2 lead to a less inflammatory chemokine bioavailability and inhibition of monocyte differentiation and VEGF production at tumor site, resulting in reduced tumor growth (Savino B, Caronni N et al., 2013, submitted). Because of its increased relevance in inflammation and tumor biology [10], insights into ACKR2 signalling properties may lead to the identification of new therapeutic approaches acting at the regulation of innate and adaptive immune responses.

Finally, a better understanding of ACKR2 signalling properties may provide new insights in the pharmacology of the conventional chemokine receptors. In fact, the intrinsic

unbalances signalling through the β-arrestin module makes ACKRs prototypic molecules to design allosteric modulators of conventional chemokine receptors aimed at impair their Gi-dependent chemotactic activity preserving the receptor-mediated β-arrestin-dependent scavenger activity which allows chemokine removal and reduced bioavailability. The receptor antagonists currently present and commonly used can bind the receptor, blocking all receptor activities and signalling pathway, and ligands continue to circulate and are able to bind other receptors, due to the promiscuity of the chemokine system and bringing to other collateral effects. The identification of selective allosteric modulators able to induce a specific signalling pathway will resolve this problem.

In future, we propose to analyse in a deeper way the β-arrestins role in ACKR2 activity, evaluating in which compartments β-arrestins are recruited, with an ImageBRET technology, and evaluating which ACKR2 structural determinants are important in this recruitment, using a new cloning methodology of swapping mutants generation. Through silent mutation introduction in ACKR2 and CCR5 sequences, we can create chimeric receptors through the reciprocal substitution of domain containing micro-switch. In addition, we propose to better understand the role of β-arrestin2 in the constitutive activation of ACKR2, investigating its role in the transcriptional profile of ACKR2 expressing cells. Moreover, ACKR2 mediate not only cofilin phosphorylation but also phosphorylation of other proteins such as Erk1/2 and Akt that are not involved in the scavenging activity of the receptor (Cancellieri C et al, unpublished observation). It should be interesting to investigate other ACKR2 functions and its involvement in the activation and control of other signalling pathways. We have generated a Tet-on inducible system, by which we could evaluate the phosphoproteomic panel comparing ACKR2-expressing cells, stimulated or not with chemokine, to cells not induced or to CCR5-expressing cells, by a SILAC analysis (*Stable Isotope Labeling by Amino acids in Cell culture*). Finally, it would be interesting understanding if this signalling pathway is shared by other ACKRs, in order to identify common characteristics to better classify ACKRs as β-arrestin biased receptors.

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