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Analysis of biased signaling in the chemokine system

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ABSTRACT

Analysis of biased signaling in the chemokine system.

Chemokines constitute a family of almost 50 small secreted cytokines, recognized by about 20 different 7TM spanning G protein coupled receptors (GPCRs), that activating pertussis toxin sensitive G proteins induce cell migration. These receptor are abundantly expressed by leukocytes and, controlling cell migration, they dictate leukocyte positioning during homeostatic patrolling within peripheral tissues, their maintenance in bone marrow during maturation and in addition mediate their recruitment to inflamed tissues. Upon inflammation in fact a number of chemokines are produced or activated by inflammatory mediators and diffuse within the tissue, generating a chemical gradient along which leukocytes migrate to reach the center of inflammation to contain and remove the insulting factor. This system needs an extremely tight control, since its dysregulation has been demonstrated to be at the basis of different inflammatory diseases, auto-immunity and has also been linked to cancer development. In particular, in this thesis we focused our attention on two regulatory system: post-translational modifications of chemokines, mediated by enzymes specifically released upon inflammation also by immune cells, and on the activity of atypical chemokine receptors, a subfamily of chemokine receptors that despite high structural homology and similar binding properties compared to conventional chemokine receptors, are unable to drive chemotaxis but act instead as key regulators of the chemokine system activity. In detail we looked at the ability of these regulatory mechanisms to modulate chemokine signaling properties generating a biased signaling, an emerging feature of GPCR pharmacology that describes the ability of a given receptor to elicit different or even opposite functional activities depending on the ability of different agonists to stabilize different receptor's active structural conformation, resulting in different phenotypes mediated by the same receptor. In the chemokine system biased signaling has already been described to occur on different receptors upon binding of their different ligands, therefore during our investigation on chemokine regulatory system signaling we maintained our focus on the ability of these systems to bias chemokine signaling properties in order to better understand how this regulation occurs.

To this point we assessed the ability of differently post-translationally modified chemokines to elicit signaling activities on different receptors by measuring in HEK293 cells their potential in inhibiting adenylyl cyclase, a proximal downstream signal of Gα inhibitory proteins activation, and in inducing β-arrestin recruitment to the receptors in energy transfer-based assays. We also compared signaling properties of an atypical chemokine receptor to the ones elicited by a conventional receptor analyzing the phosphoproteome modifications occurring constitutively and after stimulation with the same agonist.

Our results indicate that regulation of CXCL5 and CXCL8 chemokine activity by post-translational modifications is more prone to regulate chemokine activity modifying chemokine potency, rather than generating a bias in their signaling properties. Truncation of chemokine NH_2-terminus increases both

CXCL5 and CXCL8 activity, while citrullination of the most NH_2-terminal arginine results in opposite effects on the two agonists since on CXCL8 increases chemokine potency, while it reduces CXCL5 activity. We investigated the properties of ACKR2 in recruiting β-arrestins, demonstrating that this receptor is able to associate both β-arrestin 1 and 2 in basal conditions while upon agonist stimulation preferentially increases its association with β-arrestin 1, resulting in a completely different agonist-induced outcome of proteome phosphorylation, compared to CCR5, in terms of kinetics, protein phosphorylation modifications, biological function of the regulated proteins and signal mediators activated. Taken together, these results indicate that chemokine system regulation is based not only on chemokine post-translational modifications that modulate chemokine potency, but also on the activity of structurally biased atypical chemokine receptors, as in the case of ACKR2 that interacts with different effectors and kinetics to generate distinct functional outcomes, compared to the conventional chemokine receptor CCR5.

In this thesis it has also been attempted to translate the investigation of chemokine signaling to a clinical intervention for inflammatory diseases. We assessed the modulation of CXCR1 signaling activity exerted by Reparixin, a leukocyte migration inhibitor that blocks cell recruitment to inflamed tissues, that binds to its target receptors in an allosteric binding site, without inhibiting chemokine binding to the receptor. In our assays performed on HEK293 cells we could not detect any inhibition of the signaling pathways assayed, possibly indicating that HEK293 cells are not the best model to assay the activity of this molecule, with the need to assess in this cellular model drug inhibitory activity on read-outs already evaluated in literature on other cell types.

In conclusion, we can say that observations described in this thesis allow to better understand chemokine system regulation, that occurs by biased signaling activity in the case of atypical chemokine receptors that by selected interaction with signaling mediators induce opposite biological outcomes compared to conventional chemokine receptors, while post-translational modifications regulate chemokines activity modulating their potency, rather than biasing their signaling properties.

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1-INTRODUCTION

1.1-CHEMOKINES AND THE CHEMOKINE SYSTEM

Chemokines constitute a family of about 50 low molecular weight (8-12 kDa) proteins that regulate directional cell migration acting on about 20 dedicated seven transmembrane (7TM) G protein coupled receptors (GPCRs) [1]. The migratory events chemokines drive are fundamental to correctly regulate physiological processes such as cell development and leukocyte homing in homeostatic conditions, but also in pathological processes are the main responsible for directed leukocyte migration to inflamed tissues, together with other inflammation-related molecules such as bacterial-derived peptides and activated complement components [1-4]. In adult vertebrates, these small proteins can be distinguished between homeostatic or inflammatory chemokines, based on their constitutive and tissue-restricted expression or their inducible and widespread diffusion, respectively [5]. Chemokine receptors expressed on target cells mediate cell response regulating several signaling pathways responsible for cell activation, adhesion, polarization towards chemokine gradient and finally migration [6]. These activities enable chemokines to orchestrate leukocyte migration, whether that could be during homeostasis maintaining immune cells precursors in the bone marrow or mature leukocyte patrolling in peripheral tissues or during inflammation recruiting leukocytes to inflamed tissues [7]. Regulation of the chemokine system occurs with controlled expression of both chemokines and receptors that determines the specificity of the migratory signal, modifying ligands activity by post-translational modifications affecting chemokine activity and solubility, and by activity of atypical chemokine receptors (ACKRs), that are GPCRs similar to chemokine receptors in terms of structural and binding features, but are devoid of chemotactic activity and instead regulate chemokine activity transporting, presenting or scavenging their ligands [8]. Leukocytes recruitment to sites of infection and inflammation is a fundamental component of host immune defense, and as it needs to be activated only in the case of necessity it also needs to be contained to prevent excessive immune response and to redirect it to tissue repair functions once the inflammatory cause has been removed. To this point dysregulation of the chemokine system has been reported to be involved in several immune diseases [9], spanning from immune deficiency, autoimmunity and inflammation-related diseases [1, 9-11]. Therefore, to selectively regulate leukocyte recruitment within tissues, drug development focused its attention also on chemokine receptors to tackle immune disease, since as GPCRs chemokine receptors are particularly suited to be targeted with small molecules inhibitors, easily synthesizable and deliverable drugs [11]. Unfortunately, drug development for chemokine receptors recorded a high rate of unsuccessful attempts due to the complexity of chemokine system, determined by its redundancy and interplay between different ligands and receptors. Therefore now research in this field is looking forward to understand autonomous chemokine system regulation in order to design drugs mimicking chemokine system's own regulation methods [11].

1.2-CHEMOKINES

Chemokine family has been defined to group almost 50 low molecular weight (8-12 kDa), secreted cytokines with cell migratory activity that share a common structure characterized by the presence of four conserved cysteines [5]. These cysteines form disulfide bonds between them, and in particular the first cysteine interacts with the third and the second with the fourth. Based on the variations in the sequence between the first two NH_2-terminus proximal cysteines, chemokines are divided in four sub-families (Figure 1) [12].

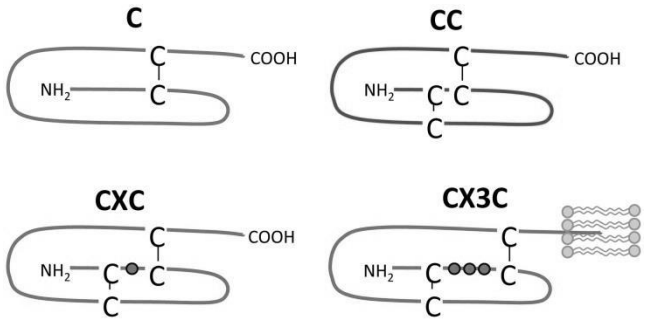


Figure 1: Chemokines classification. Chemokines are divided in 4 subfamilies named C, CC, CXC and CX_3C, based on the relative position of first two NH_2-terminal cysteines, with CX_3C chemokines that have a COOH-terminus that anchors CX_3CL1 to the plasma membrane *[13]*.

CXC chemokines have one amino acid positioned between the first two cysteines, in CC chemokines there are no intervening amino acids and the two cysteines are adjacent, the only member of the CX_3C chemokine subfamily (CX_3CL1) has three amino acids between the two conserved cysteines, while the (X)C chemokines constitute an exception with two members (derived from one alternative spliced mRNA) that have only one amino terminal cysteine [14, 15]. This classification not only allows to define chemokines on the basis of structural properties but also to define families of chemokine receptors, since one single conventional chemokine receptor can bind different chemokines but all its cognate ligands belong to one distinct subclass of chemokines, defining CXC, CC, CX_3C and XC receptors [14]. In humans inflammatory chemokine genes are mainly clustered on chromosomes 4 and 17, indicating that a rapid evolution has been made by gene duplication, while homeostatic chemokines are mainly located alone or in small clusters on different chromosomes. Other than being well conserved across species, in the case of homeostatic chemokines their function is more maintained compared to the inflammatory ones, indicating that their role is more important and strictly conserved due to developmental and physiological functions [5]. Disulfide bridges mediated by the conserved cysteine residues, together with hydrophobic interactions, determine a common three-dimensional structure shared by all the chemokines, consisting of a core of three planar antiparallel β-sheets connected by short loops, a C-terminal α-helix bent compared the β-sheets plan and an extended unstructured N-

terminus preceding the first cysteine [16, 17]. The domains that determine receptor specificity are a region connecting the first cysteines and the first β-sheet called N-loop and the NH_2-terminus, responsible for chemokine binding to receptor extracellular NH_2-terminus and for receptor activation, respectively [15, 18]. The C-terminal α-helix, rich in basic amino acids, is the most important mediator of chemokine interaction with glycosaminoglycans (GAGs), together with other basic residues distributed all over the sequence [15, 19]. In the case of CXC chemokines a further classification can be made on the presence prior to the first cysteine of a three-residue motif of glutamic acid, leucine and arginine, called ELR motif. This motif is present in CXC chemokines recognized by and active on CXCR1 and CXCR2 receptors and is associated to angiogenic properties of these chemokines, while ELR-negative CXC chemokines display an angiostatic activity [20]. Angiogenic activity anyway is not peculiar of ELR-positive CXC chemokines, since has also been associated to CCL2, CCL11 and CCL16, three CC chemokines [20]. Another classification of chemokines can be made on the basis of their function and expression, since it is possible to distinguish between homeostatic and inflammatory chemokines. Homeostatic chemokines are constitutively expressed and regulate physiological migration and homing of various cell types, including leukocytes. On the opposite, inflammatory chemokines are upregulated upon inflammatory events and mainly mediate leukocyte recruitment towards inflamed tissues [5, 21]. This classification is not stringent since there are chemokines with both homeostatic and inflammatory functions, and chemokines that are constitutively present and circulating in plasma or inside platelets in an inactive form but during inflammation are processed or released and sustain an inflammatory behavior [16, 21, 22]. This function-based classification is mostly reflected also by chemokine receptors, since single receptors bind mainly chemokines with similar functions, even if the chemokine system is very redundant and promiscuous, given the fact that in most the cases one receptor can bind different ligands, one chemokine can activate multiple receptors and one leukocyte subset can express different chemokine receptors [3, 21].

1.3-CHEMOKINE RECEPTORS

Three distinct subfamilies of chemokine receptors have been defined based on the function they exert. It is possible to identify conventional chemokine receptors, that drive directional cell migration in response to chemokines, atypical chemokine receptor that do not sustain cell migration but regulate chemokine activity, and microbial chemokine receptors, developed mainly by pathogens as viruses or parasites to escape host immune response [3]. Atypical and microbial chemokine receptors will be reviewed in separated sections to allow a better clarity and understanding of their own molecular and functional features, therefore in this text the generic definition of “chemokine receptors” will refer to conventional chemokine receptors, a definition used only in sections where disambiguation is needed.

Up to date 18 chemokine receptors have been characterized, regardless of splice variants. As already said, chemokine receptors are grouped in 4 classes depending on the bound chemokines, and even if one single receptor recognizes more than one chemokine, all the chemokines recognized belong to the same family (Figure 2A). Due to this characteristic, chemokine receptors have been divided in 4 groups: CXC, CC, CX_3C and XC receptors (CXCR, CCR, CX_3CR and XCR, respectively). CCRs are the most abundant and ten receptors have been described so far, then there are six CXCRs, and only one CX_3CR and one XCR [3, 12]. Many chemokine receptors genes are clustered in chromosome 3, and compared to their agonists, receptors are more conserved across species [5].

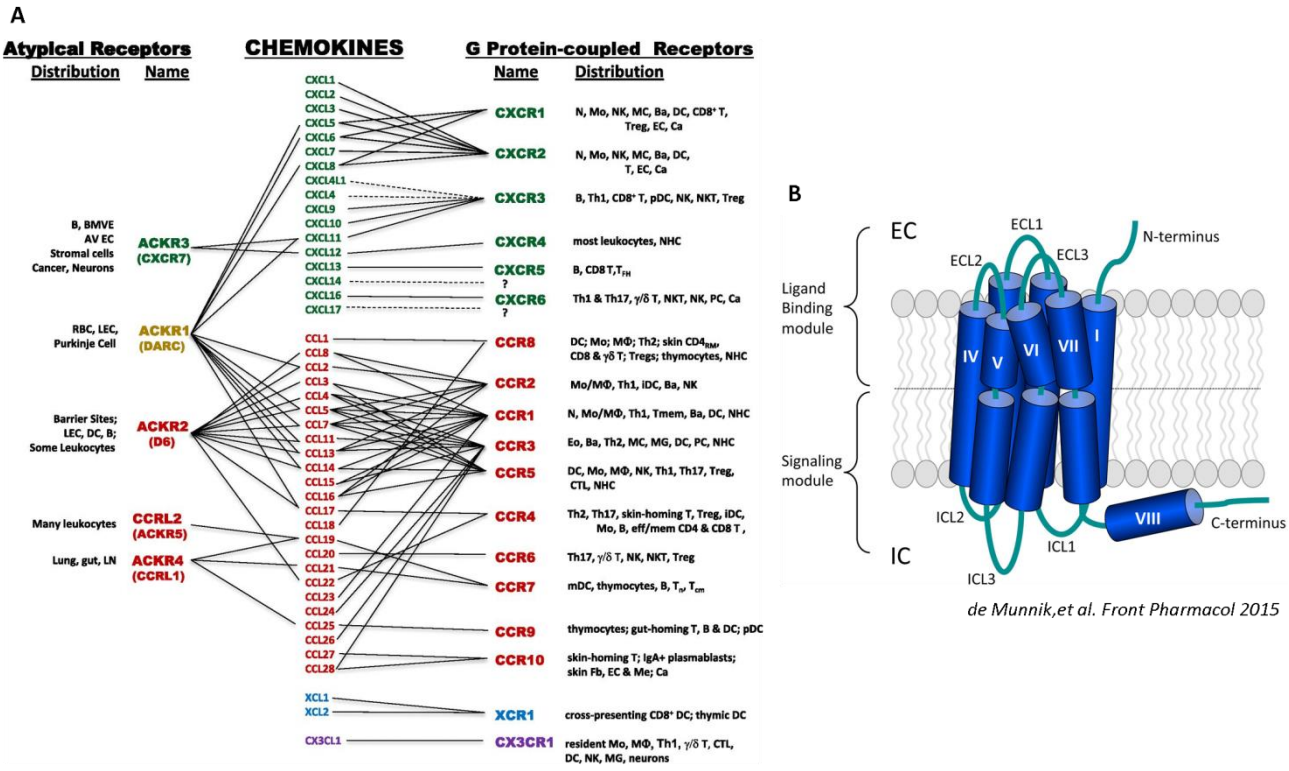


Figure 2: The chemokine system and chemokine receptors structure. A- Chemokines and their receptors are listed with the following color-code: CXC chemokines are in green, CC chemokines in red, XC chemokines in light blue and CX3CL1 is in purple. Cell types expressing each receptor are reported next to each receptor's name. B- 7TM structure of a class A GPCR, with the indication of Extracellular (EC) and Intracellular (IC) sides of the plasma membrane, and the visible TM domains and loops. Adapted from [3, 13].

Chemokine receptors are class A GPCR coupled with pertussis toxin-sensitive Gαi class heterotrimeric proteins. Their structure consists of seven transmembrane domains made of α-helices spanning the plasma membrane, connected by three intracellular and three extracellular loops (Figure 2B) [23]. At the NH_2-terminus, positioned on the extracellular side of the membrane, these receptor have a rather flexible unstructured domain, with no precise crystallographic order, while at the COOH-terminus on cytosolic side there is an unstructured carboxy terminal tail (Figure 2B). On the extracellular side, two disulfide bridges constrain the structure of the 7 TM. In detail, one is made between a cysteine

in extracellular loop (ECL) 1 at the beginning of TM 3 and a cysteine positioned within ECL2, which is conserved in rhodopsin-like class A GPCRs, and the other link is made by one cysteine on the NH_2-terminus with the one at the tip of TM 7, conserved among chemokine receptors [23]. The TM domains define two major binding crevices, one accessible by chemokines from the extracellular side of the transmembrane bundle [19, 24-26], and another cavity on the intracellular side hosts α subunits of G proteins mediating direct signal transduction by ligand-induced structural rearrangement [27, 28]. Fundamental in regulating GPCRs structure and activity are a set of amino acids conserved among GPCRs that organize the structural scaffold around which every GPCR polypeptide assembles to generate receptors with different specificity of ligands and signaling properties [23, 29]. Transmembrane domain 3, due to its central position in the transmembrane bundle, is the most important domain involved in connecting most the domains of the receptor, sensing ligand binding and at its end is placed the DRY motif, highly conserved and involved in G protein coupling and their activation [29, 30]. This motif, placed at the boundary between TM3 and Intracellular Loop (ICL) 2, contains one of the most conserved residues among GPCRs, the Arginine (R) 3.50 (following Ballesteros-Weinstein numbering), and has been shown to regulate receptor activation by interacting with the preceding Aspartic acid (D) in the inactive status, while upon agonist activation interacts with G proteins and another conserved residue on the receptor, at the beginning of ICL3, the Tyrosine (Y) 5.58 [23, 27], acting therefore as a switch for receptor activation. Of notice, among chemokine receptors, this motif is changed to HRY in XCL1 and to DRF in CXCR6, with an histidine replacing the aspartic acid and a phenylalanine replacing the conserved tyrosine, respectively. The substitution in CXCR6 does not result in a change of charge, while in XCL1 the substitution of the acid aspartate with the basic histidine can underlie a constitutive activation of XCR1, as it has been reported for other mutated GPCRs [31, 32]. Other important features of GPCRs are highly conserved residues across all the TM domains that interacting with other residues spatially close to them determine the relative orientation of TM helices, influencing receptor activity state [23, 28, 33]. These residues in fact act as the aforementioned DRY lock that maintain the receptor in an inactive state with strong electrostatic interactions and hydrogen bonds, but at the moment an agonist binds the receptor these switches follow TM rotation and stabilize receptor active conformation, controlling receptor signaling not only in time but also in quality, since these switches can also determine which signaling pathways the receptor activates, contributing to the generation of balanced or biased signaling activities [23, 24, 34, 35]. The motif TxP in TM 2 is highly conserved in chemokine receptors, and plays an important role in sensing chemokines binding, with the proline (P) creating a kink in the transmembrane helix that can be moved upon entering of chemokine N-terminus in the extracellular bundle of the receptor [36, 37]. In TM 6 is present one micro-switch highly conserved in GPCRs, the CWxP motif, in which the cysteine (C) interacts with asparagine (N) 7.45 in TM 7 and the tryptophan (W) forms hydrogen bonds with TM 3 and 7 in inactive state, pushing another micro-switch, the

NPxxY$(x)_{5,6}$F at the end of TM 7, away from the aspartate 2.50 in TM 2. Upon receptor activation this pattern of interactions changes, with tryptophan interacting with phenylalanine 5.47 in TM 5 while cysteine loses its interactions leaving asparagine 7.49 which remains free to interact with aspartate 2.50, inducing a tilt outwards of TM 6 [23, 38, 39]. The already introduced NPxxY$(x)_{5-6}$F located between the end of TM 7 and the putative helix 8 is also important in determining the activity of GPCRs, with tyrosine 7.53 interacting with phenylalanine 7.60 in inactive state and stabilizing receptor's active conformation and TM 6 outwards tilting by its interaction with hydrophobic residues between TM 6 and 7 [23, 40]. However, while the TxP and NPxxY$(x)_{5-6}$F are highly conserved in chemokine receptors, the CWxP motif is not completely conserved across chemokine receptors that may present alternative residues, that can confer differences in terms of constitutive or biased signaling [23, 24, 34]. In GPCRs a putative 8th helix occurs soon after the end of TM 7, usually anchored to the plasma membrane by palmitoylation, that regulates receptor activation, stability, internalization and signaling [41], but among chemokine receptors this motif is present in some molecules, such as CCR5 in which regulates receptor trafficking and signaling [42, 43], while it is absent in receptors like CXCR4 [25]. All these residues mainly conserved across conventional chemokine receptors generate a network of interactions that dictate receptor activity controlling its structural conformation.

1.4-CHEMOKINE RECEPTORS EXPRESSION

Chemokine receptors are expressed by stem cells during development to mediate cell positioning during developing organisms, but in adults are mostly expressed by immune cells [3]. Chemokine receptors have also been described to be expressed by several cancer cells, mediating different functions that can be even opposite one to another. In fact, other than tumor-expressed chemokines that can have angiogenic or angiostatic properties, chemokine receptor expression in tumors influences the aggressiveness of cancer cells, not only by promotion of survival and growth signals but mainly by guiding invasion and metastasis. In particular the major players in this regard are chemokine receptors CXCR4, CCR7 and CCR10 that respond to homeostatic chemokines, constitutively expressed in tissues such as bone marrow, lymph nodes and skin, respectively, that attract cancer cells within the tissue where they can generate metastasis [44, 45]. In physiological conditions leukocytes are the cells mainly expressing chemokine receptors with homeostatic functions as retention of hematopoietic precursors in the bone marrow or guiding leukocyte trafficking and homing in secondary lymphoid tissues [7]. In example, CXCR4 is expressed in hematopoietic stem cells and drives their retention in the bone marrow, where reticular cells produce high levels of CXCL12. During maturation CXCR4 is downregulated to weaken the retention signal, allowing mature immune cells to leave the bone marrow and enter peripheral blood [46]. This receptor can also be upregulated during neutrophil

senescence to drive their reentry and apoptosis in the bone marrow [47]. Another immune cell whose functions are regulated by controlled expression of chemokine receptors for constitutively expressed ligands are Dendritic Cells (DC). DC are present in periphery as immature resident cells, whose positioning can be driven in example by CCR2 in skin-resident DC or by CCR6 in Peyer's patches DC. Upon maturation or inflammation DC increase expression of CCR7, the receptor responding to CCL21 and CCL19, expressed by lymphatic endothelium and lymph nodes respectively, that guide DC in their egression from peripheral tissue towards lymph nodes where they can encounter and activate naïve T cells and exposing antigen-loaded major histocompatibility complex class II (MHCII) [7, 48]. As well, regulation of leukocyte migration towards gradients of inflammatory chemokines relies on the expression of specific receptors on different immune cell populations. Upon inflammatory or infectious stimuli resident innate immune cells such as mast cells, macrophages and DC either release vesicles with preformed chemokines or rapidly start expressing them [49, 50]. Not only particular but a wide range of chemokines is produced, together with inflammatory cytokines that boost inflammatory chemokine production by immune cells and also induce chemokine expression by epithelial cells [51]. As in the case of homeostatic chemokines, specific receptor expression influences the recruitment of determined peripheral blood or tissue resident leukocyte populations rather than others. Receptors for inflammatory chemokines are promiscuously expressed in different leukocytes, even if their abundance dictates cellular responsiveness to determined ligands. In example neutrophils are the first leukocyte population recruited to inflamed tissues thanks to their expression of CXCR1 and CXCR2, whose agonist are highly present in mast cells granules as CXCL1 and CXCL2 or can be found on activated endothelium as CXCL8, and also by formyl peptides and leukotriene B4, released by damaged mitochondria and produced by leukocytes after inflammatory stimulation, respectively [7]. Monocytes as well express formyl peptides receptors and CXCR2, but their arrival within inflamed tissues is mainly dependent on CCR2-ligand CCL2 [52, 53]. Monocytes can also be distinguished between patrolling and inflammatory monocytes on the basis of CX_3CR1 or CCR2 expression, respectively. CX_3CR1 ligand, named CX_3CL1, is in fact expressed as a membrane bound chemokine, and remains mainly localized contributing to act more as an adhesion molecule [7, 52] in contrast to CCL2, which has been shown to form matrix-associated gradients that drive monocyte migration from blood vessels to peripheral inflamed tissues [7, 54]. CCR5 is another chemokine receptor widely expressed on leukocytes, such as mast cells, DC, monocytes, and can be upregulated in activated neutrophils [7]. This wide expression of receptors sensitive to many different chemokines indicates that to support emergency recruitment of leukocytes towards inflamed tissues the chemokine system evolved maintaining a high grade of redundancy and complexity, to be able to robustly support in several ways the immune response [55].

1.5-CHEMOKINE BINDING AND RECEPTOR ACTIVATION

Interactions of chemokines with cognate receptors are considered to follow the model initially proposed for the binding of CXCL12 to CXCR4 [18] that resembles the one proposed for another class A GPCR that binds a peptide with chemotactic properties, the activated complement C5 receptor (C5aR1) [56]. This model based on experimental data proposes that chemokines present in soluble form, bound to GAGs or presented by other receptors such Atypical Chemokine receptors (ACKRs) initially interact with the receptor through their N-loop, binding the NH_2-terminus of the receptor. After this initial contact, the NH_2-terminus of the chemokine that initially is in a disordered structure in solution assumes an order that mediates the correct positioning of all the residues inside the extracellular bundle of the receptor, where these residues can interact with receptor TM helices activating the receptor (see Figure 3) [18].

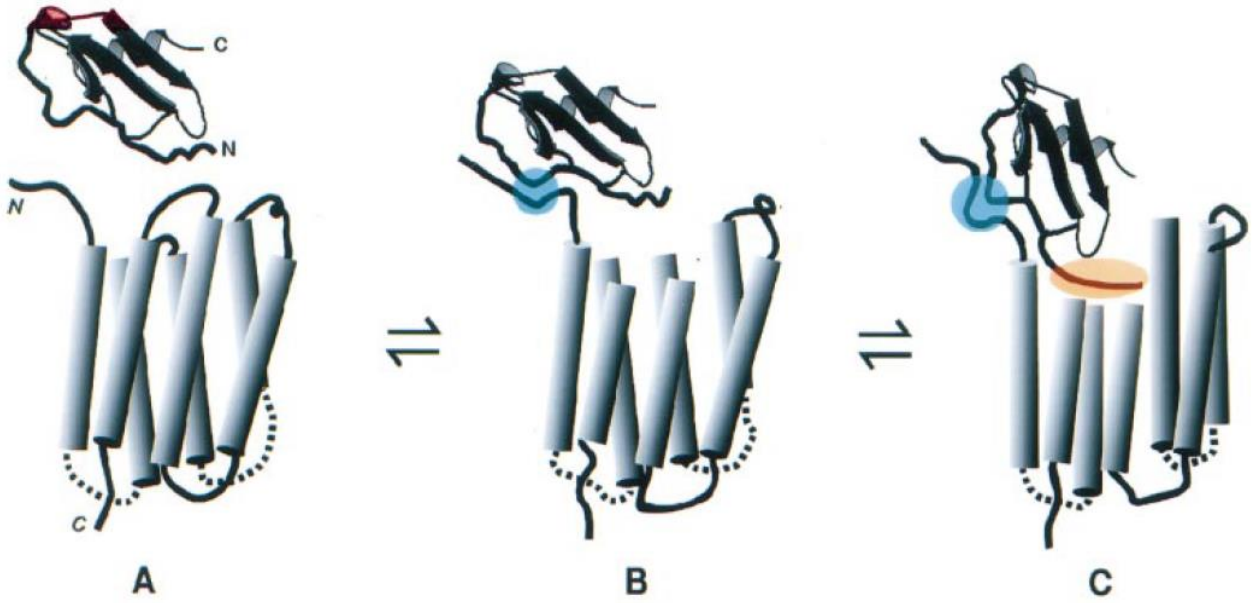


Figure 3: Chemokines bind their cognate receptor in two subsequent steps. In the model proposed for CXCL12 (above, dark grey), after approaching it cognate receptor CXCR4 (below, light grey) (A), the chemokine initially binds the NH_2-terminus of the receptor by its N-loop (B), that causes the receptor to assume a conformation more prone to host the NH_2-terminus of the chemokine that becomes structured thanks to the interaction with the residues present in TM extracellular bundle (C) [18].

This explains the importance of chemokines NH_2-terminus in dictating their activity, since modification of this region more than others affects dramatically their potency but not their binding affinity to the receptor [57-59]. The possibility to modulate chemokine activity by modification of their NH_2-terminus, together with the binding relying on different structural motifs of the chemokines, also explains the ability of different chemokines to bind receptors to exert an antagonistic activity, either by carrying no active NH_2-terminus or by binding chemokine receptors others than their own, occupying the binding site and preventing receptor activation by active chemokines [5, 18, 57]. Furthermore it has been observed that while CC chemokines activate their cognate receptors interacting mainly with

residues belonging to TM helices, deep in the binding crevice, CXC chemokines can also activate receptors acting on more exterior residues, and that different chemokines can bind their cognate receptor in different binding sites, indicating that even if the two step model can be applied to both the chemokine families, differences can be observed between different chemokines in terms of binding conformation that influence receptor occupancy and activation [60].

Chemokine binding to the receptor causes a movement of TM to interact with the chemokine, resulting in a reorientation of the micro-switches, unlocking receptor movement towards an active conformation. In particular, for GPCRs, it has been demonstrated that agonist activation induces, compared to other TM, a greater movement of TM 6 and TM 7, which while moving inward the extracellular segments shift outwards their intracellular half, moving on a fulcrum positioned around the middle of the plasma membrane, that together with other minor movements rearranges the interaction network of all the residues and contacts with signaling partners inducing the activation of signaling cascades [27, 61].

1.6-CHEMOKINE RECEPTOR SIGNALING

1.6.1-HETEROTRIMERIC G PROTEIN-SIGNALING

Responses evoked by binding of chemokines to their respective chemokine receptors rely on the activation of multiple signaling pathways sustained by a plethora of signaling molecules, which in an exemplified view can be attributed to two main signaling transduction modules, one controlled by heterotrimeric G proteins and one by β-arrestins [62, 63]. G proteins are heterotrimeric proteins mostly found just below the plasma membrane, associated to GPCRs, with the Gα subunit, the one with the GTP hydrolyzing activity, that on the one side interacts with the receptor and on the other binds the βγ dimer subunit [27, 62]. The general signaling mechanism adopted by GPCRs consists in an immediate activation of G proteins after agonist binding, which is possible thanks to the structural remodeling that the receptor undergoes. This receptor rearrangement induces in the inactive guanosine diphosphate (GDP)-bound Gα subunit of the G protein associated to the receptor the release of GDP [64]. The Gα subunit in its open conformation, free of guanosine nucleotide, has a high affinity for the GPCR, until it binds guanosine triphosphate (GTP), which activates the Gα subunit that dissociating from both the receptor and the βγ subunit becomes free, as the βγ subunit, to interact with other downstream signaling molecules (Figure 4) [62, 65]. Being constituted by three subunits acting together, G proteins can mediate the activation of different signaling pathways together, with an high grade of complexity and divergence due to the possible combination of different subunits, since up to date in humans have been characterized 21 Gα, 6 Gβ and 12 Gγ different subunits [62]. In particular, possibly due to their

ability to directly interact with GPCRs, Gα subunits differentiated to mediate diverse signaling pathways and can be grouped in four families on the basis of sequence similarities and signaling activity. They can be distinguished between Gαs subunits that stimulate adenylyl cyclase to convert ATP in cAMP, that as second messenger amplifies the signal modulating the activity of protein kinase A (PKA) and the small GTPase Rap1, in example [66]. On the opposite, Gαi subunits inhibit the activity of adenylyl cyclase, leading to a decrease in intracellular cAMP. To this family belong also Gαo proteins, which can, as Gαi do, regulate to the activation of the pathway of c-Src-STAT3 and the small GTPase Rap. Upon activation of Gαi/o subunits the βγ subunit becomes activated and signals as well, leading to the activation of phospholipase C-beta (PLC-β), which mediates the formation of inositol-1,4,5-trisphosphate (IP3) and transient Ca^{2+} fluxes [67]. The PLC-β pathway can also be activated by the Gαq family subunits, resulting in the formation of IP3 and diacilglycerol, that induce the release of Ca^{2+} from intracellular stores and mediate the activation of protein kinase C (PKC), respectively [66]. Gα12/13 subunits regulate different but cross-talking signaling pathway, operating mainly on the activity of small GTPases as Rho or on kinases [66, 68].

Chemokine receptors are mainly coupled to Gαi proteins, since their stimulation with agonists results in a decrease of intracellular cAMP, accumulation of IP3 and generation of transient intracellular Ca^{2+} currents. All these signaling pathways, as chemotaxis, are sensitive to pertussis toxin (PTX) inhibition [67, 69], which catalyzes the ADP-ribosylation of Gαi subunits causing their uncoupling from the receptor [70]. However, emerging evidences suggest that an additional, alternative Gαq-dependent pathway can be engaged by a subset of chemokines and is critically required for regulating chemokine receptor-mediated functions such as inositol trisphosphate (IP3) generation and calcium release and chemotaxis [71].

GPCR activation leads to G protein activation as the first signaling event due to their direct association, but soon after G protein dissociation the receptor remains free in an active conformation that permits the binding of several signal transducers, such as GPCR kinases (GRK) that phosphorylate residues mainly on receptor C-terminal tail, increasing the affinity of this domain for β-arrestins, which prevent further re-association of G proteins to the receptor by steric hindrance, mediate the assembly of the endocytotic machinery to internalize activated receptors desensitizing cells and within the last 15 years have been demonstrated to scaffold a signalosome to sustain several different signaling pathways in a G protein-independent manner [63].

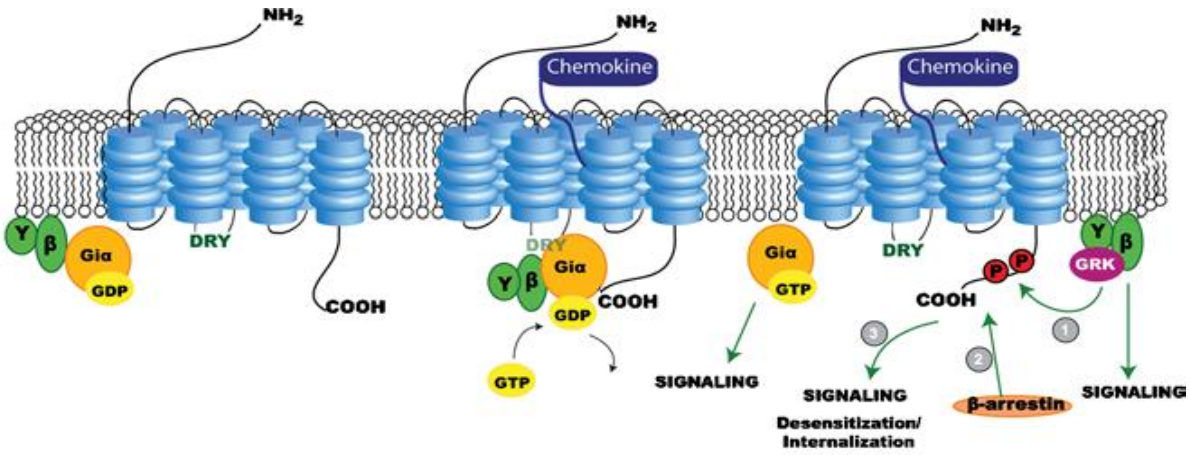


Figure 4: Chemokine receptor activation and signaling pathways. After chemokine binding, G-proteins bind receptor intracellular loops and become active, exchanging GDP into GTP. Activation results in α subunit dissociation from the receptor and from βγ subunits, that subsequently activate a variety of 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 signaling pathways which lead to a variety of cellular responses (Figure derived from [72]).

1.6.2-β-ARRESTIN DEPENDENT SIGNALING PATHWAY

Arrestins constitute a small family of 4 proteins that regulate similar functions interacting with GPCRs. Only two of them, named β-arrestin 1 and β-arrestin 2, are ubiquitously expressed while visual arrestin 1 and arrestin 4 are expressed only in retinal rods and cones, respectively [73]. These proteins have initially been described as able to block GPCR-induced G protein signaling and to desensitize receptors mediating their internalization, but by now it has been well established that they are also able to mediate the activation of several signaling pathways [74, 75]. β-arrestins structure is constituted by one N- and one C-domain consisting mainly of antiparallel β-sheets, with a polar core between the two domains that binds to the GPCR in close proximity of the intracellular bundle, with the N-domain that interacts with receptor C-terminus and the C-domain that can be used as scaffold for the signaling complexes [76]. Upon receptor activation, β-arrestins are recruited to the receptor by increased affinity, that depends on both receptor structural rearrangement that exposes high affinity residues or by phosphorylation of several residues, mainly clustered on receptor C-terminal tail, by different GRK, PKA, PKC or Casein Kinase 2 (Figure 4) [63, 74, 77-79]. β-arrestins not only sterically block any further interaction between G proteins and the receptor, but also act as scaffold to mediate receptor endocytosis via clathrin coated pits, clustering to the receptor different components of the endocytotic machinery [80], and concomitantly contributing to the assembly of a signalosome that mediates many intracellular signaling activities [74, 81]. The first signaling activity described as being regulated by β-arrestins is the one generated by the β2-Adrenergic receptor (β2AR) leading to the phosphorylation of

the MAP kinases ERK 1/2, mediated by β-arrestins scaffolding of tyrosine kinase c-Src to the activated receptor [82]. This evidence lead to the characterization of many other different signaling pathways mediated by β-arrestins, pointing towards the attribution of the role as second alternative signaling pathway mediators, compared to G proteins [78, 83]. Furthermore these evidences disclosed β-arrestins as capable of associating receptors and activating signaling pathways independently from G proteins, raising the idea that a GPCR can possibly activate selected signaling pathways, rather than generating always a balanced signal that equally activates all the available pathways [83, 84]. This initial concept of proof has been demonstrated with experimental evidences showing that GPCRs that conventionally activate both G proteins and subsequently β-arrestins recruitment and signaling can be forced to signal preferentially through only one of these two mediators, inducing different functional outcomes, depending on the agonist bound and therefore this concept has been named functional selectivity, or biased signaling, which will be reviewed in a separate section [84].

Chemokine receptors as well associate β-arrestins after ligand engagement, and this phenomenon has been well studied due to the fact that this interaction has been found to be essential to sustain chemotaxis, with impacts on immune regulation and diseases, and since β-arrestins are involved in internalization of chemokine receptors, that in the case of CXCR4 and CCR5 act as co-receptors for HIV viral particles, sustaining virus internalization during infection [85-87]. The importance of β-arrestins to regulate leukocyte chemokine receptor-dependent activation, adhesion and chemotaxis is sustained by several evidences that not only linked β-arrestins functions to chemokine receptor mediated signaling and chemotaxis [88-91], but also elucidating their role in chemokine receptor activity amenable for leukocyte migration in various immune diseases [92-94]. In particular, β-arrestins have been demonstrated to be able of coupling signaling machineries to chemokine receptors, such as CXCR4, whose activation is fundamental to support cell migration. In fact in the case of CXCR4 it has been shown that β-arrestin 2 mediates CXCL12-induced chemotaxis sustaining receptor internalization [87] and the activation of, among the other proteins involved, p38 MAPK, and that this activation is necessary to induce cell movement towards CXCL12 gradient [95], and when this interaction is affected by receptor structural modification as in CXCR4-mutated WHIM patients, receptor activation leads to defective C-terminal tail phosphorylation and impaired recruitment of β-arrestin to the receptor, resulting in a hyper activation and enhanced chemotaxis, with an increased leukocytes bone marrow retention and defective interaction formation of immunological synapse [92, 93, 96, 97]. β-arrestins are also involved in the desensitization and signaling of CC chemokine receptors, such as CCR2 [98, 99], CCR5 [99, 100] and CCR7 [101], which can activate selective signaling pathways and responses after stimulation with different endogenous ligands, claiming that signaling bias occurs also among chemokine receptors [34, 99, 102]. In the case of CXCL8 receptors CXCR1 and CXCR2, it has been observed that the same chemokine can induce different signaling activities on the two receptors,

correlated to different rates of receptor internalization and recycling, mediated by β-arrestin-recruited adaptor protein 2 (AP-2) to endocytic vesicles [103], and this resulted to be dependent on the activation of different GRKs [104], underlining the importance of biased signaling normally occurring among chemokine receptors [34, 84].

Another important feature that has been observed on GPCR and has been demonstrated on chemokine receptors is the ability to be constitutively active [105, 106]. GPCRs in fact can display ligand-independent activity, which is considered to be dependent on exciting factor present in the environment, that reduce the energy needed to activate the receptor, that could be thermal exchange, receptor density on the plasma membrane, oligomerization or instability of the ionic lock, the interactions between micro-switches that stabilize GPCR conformation, that in fact can be mutated to generate GPCRs blocked in stable conformations allowing for the generation of more precise structural definition and drugs impacting selected conformational status [35, 107, 108]. In the case of CCR1, it has been reported that the receptor can activate cellular migration independently of agonist stimulation, and this mechanism is driven by CCR1 that constitutively forms homo-oligomers, in which receptor molecules can activate PTX-sensitive Gαi proteins, leading to cAMP reduction, is phosphorylated on its C-terminus and undergoes constitutive internalization in a β-arrestin-dependent and G protein-independent manner [106].

1.7-MICROBIAL CHEMOKINE RECEPTORS

Constitutive signaling activity is a characteristic widely diffused among another class of GPCRs that are viral-encoded chemokine receptors [3, 109]. These receptors are able to bind different chemokines in a promiscuous manner, even binding chemokines belonging to different subfamilies, and display a non conventional cellular distribution, since are frequently found to be mainly expressed in intracellular compartments and undergo constitutive internalization and recycling [109-111]. Among these receptors US28 encoded by human cytomegalovirus (CMV) and ORF74 by human herpesvirus 8 (HHV-8) are the best characterized, and have been described as able to activate several signaling pathways not only in response to host chemokine ligands but also in a constitutive, ligand-independent manner [112, 113]. These receptors, other than scavenging host chemokines to suppress immune response, have been found able to directly support infected cells by constitutive activation of several signaling pathways dependent on both G proteins and β-arrestins, among which the phosphorylation of MAPK, activation of NF-kB and STAT3-dependent transcription that sustain cell proliferation, tumoral transformation, angiogenesis and cell migration, that have been accounted for the association to these viruses of carcinogenic properties [109].

1.8-BIASED SIGNALING

Biased signaling, also known as functional selectivity, is a relatively new emerging phenomenon in GPCR biology whereby distinct ligands of the same receptor can selectively activate different signaling pathways [84, 114]. Biased signaling does not simply comprise distinct signaling via either G proteins or β-arrestins, but also includes more subtle differences in the activation of other downstream signaling effectors, as different ligands can preferentially activate different subtypes of G proteins and β-arrestins isoforms, or differently affect signaling events such as ERK activation or Ca2+ mobilization [114]. Signaling bias can occur in three different scenarios, the first of which depends on the observation of the same receptor activity in two distinct tissues, where different cell types can express different signaling machineries to which the receptor, in the absence of its conventional signaling partner, can couple and transduce signals different from its conventional ones, that is the so-called system bias, that can be overcome comparing receptor activities assessed in the same cell type. The observational bias occurs when differences observed are dependent on the assay system exploited to assess agonist activity, since not all the readouts obtained have the same efficacy span, and can be avoided normalizing all the responses towards a single agonist that is reputed to have balanced signaling activity [84]. Given this points, agonist bias can be defined when given agonists, assayed for different signaling activities in the same cell type and compared to a reference agonist assayed in parallel, display different signaling activities on a given receptor, preferentially activating one or more signals compared to the reference ligand (Figure 5) [84]. Accumulating evidence suggest that biased agonists can induce biased signaling mediating GPCRs adoption of a specific ligand-induced conformation, that in row induces distinct conformation of signaling partners resulting in different signalosome scaffolding and signaling activities [115]. In particular, even if a direct comparison has not been made yet, recent data obtained by crystallography models and mutational studies indicate that biased agonists induce structural rearrangements with different positions of several receptor amino acids, among which micro-switches, with newly formed hydrogen bonds and salt bridges between receptor residues, that influence receptor association to downstream signaling modules [24, 63]. This explains also the ability of a given receptor, stimulated with a normally balanced agonist, to elicit only determined signals, due to sequence modifications in its residues important for receptor structure (Figure 5) [118].

Activation of only distinct signaling pathways opened the possibility of fine tuning receptor activation by mediating selective activation with biased ligands or modulating receptor activation by allosteric modulators, compounds that do not activate receptors on their own but bias receptor signaling only upon agonist activation [116]. These possibilities aim at generating drugs with biased signaling activity, selective only for activating receptor-desired beneficial functions avoiding adverse effects than can be generated by the multiplicity of activated pathways [116, 117].

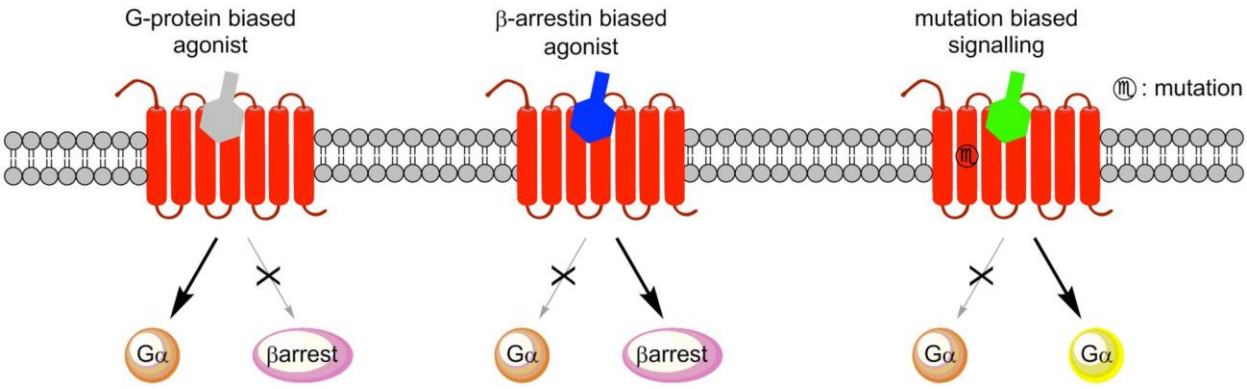


Figure 5: Biased Signaling on GPCR. The same receptor can activate different cellular responses when triggered by different agonists, but can also mediate different responses when mutations occur in its sequence influencing it structural conformation [118].

1.8.1-BIASED AGONISM IN DRUG DISCOVERY

The concept of agonists that activating only selected signaling pathways, producing not only GPCR blockade as antagonists but finely tuning receptor response lead to a re-evaluation of compounds screening, to search among molecule libraries not only compounds able to block a single receptor response assayed in one high throughput screening (HTS) assay, but pushed drug discovery to evaluate compound efficacy assaying receptor activities through different read-outs, searching not only for antagonists but also for selective agonists [119]. An example of the potential benefits of having biased ligands has been given by two well studied receptors, the β-adrenergic receptors and the μ-opioid receptor [120, 121]. Sympathetic nervous system activation is mediated by the release of epinephrine that acts on α- and β-adrenergic receptors, acting mainly on cardiac tissues, kidney and blood vessels. On kidneys and blood vessels the action of α1-adrenergic receptor leads to sodium retention and vasoconstriction, that affect cardiac load since the cardiac muscle needs to work more to face the increase the systolic pressure, meaning it has to increase it work to generate higher pressures to allow blood to reach peripheral vessels. On cardiac tissue α- and β-adrenergic receptors activation cause myocyte hypertrophy and blood vessel dilatation that reduce heart efficiency, resulting in an increase of the heartbeat rate to maintain constant blood diffusion to peripheral tissues [122]. Therefore the ability of adrenergic receptors antagonists to reduce abnormal vascular smooth muscle tissue growth and the induction of electric potentials that induce muscle contraction made of this antagonists the most used drugs to prevent restenosis and controlling atrial fibrillation and tachycardia [122, 123]. In particular, antagonists specific for β-adrenergic receptors (β-blockers) revealed better efficacy compared to α-blockers, and, among β-blockers, Carvedilol became one of the most used drugs thanks to its ability to block vascular smooth muscle cell growth and migration [123] and on cardiomyocytes exhibited antiarrhythmic and anti-ischemic properties [122], resulting in a better clinical outcome compared to patients treated with other β-blockers [124]. Molecular analysis of signaling pathways elicited by β2-

adrenergic receptor revealed that, compared to other β-blockers, carvedilol acts as a biased agonist, acting as an inverse agonist for Gαs stimulation like other β-blockers that antagonize the β2-adrenergic receptor blocking its constitutive signaling activity, while maintains receptor the ability to induce ERK 1/2 phosphorylation in a β-arrestin-dependent manner [120]. Thus, selectively blocking only the β2-adrenergic G protein signaling while maintaining β-arrestin signaling activity results in improved therapeutic effects by carvedilol, compared to other β-blockers that completely abolish receptor activity [120].

Another interesting pharmaceutical application of biased agonism to improve drug efficacy and reduce side effects is the one provided by biased agonists acting on the μ-opioid receptor [125, 126]. The μ-opioid receptor is a GPCR highly expressed in the central nervous system well know for mediating the analgesic effects of opioids such as morphine and its derivatives [126]. The analgesic activity induced by morphine and analogs on the receptor is accompanied by several adverse effects ranging from nausea and constipation to severe respiratory depression, sedation, abuse and tolerance [125, 127] and it has been shown that these adverse effects are directly mediated by the receptor itself, together with analgesy [127]. The only possibility to split these two functions of the receptor has been demonstrated to be possible in the absence of β-arrestin 2, since in mice models knock out for β-arrestin 2 morphine induced a greater analgesic effect compared to wild type animals [128]. The analgesic effects of opioid receptor activation have been shown to rely on the other side of β-arrestin 2 signaling, since Gαi protein activation of potassium channels induces K^+ ions exit and the Gβγ blockade of calcium channels support Ca^{2+} entrance, resulting together in neuron hyperpolarization and impossibility to transmit pain signals, leaving to β-arrestin 2 the task to block these analgesic signals and desensitize opioid receptors [129, 130]. These evidences pushed drug development towards the investigation of G protein biased agonists for the μ-opioid receptor, to selectively elicit signaling activities aimed at blocking nociception without incurring in β-arrestin-dependent desensitization and signaling responsible for typical morphine severe side-effects, and brought to the development of different compounds. In particular, one compound named TRV130 showed in comparison to morphine higher potency in activating G protein signaling while poorly induced β-arrestin 2 recruitment to the μ-opioid receptor, that undergoes reduced phosphorylation and is no longer internalized [126, 131]. These biased agonist activity, together with the evidences that TRV130 induces sustained analgesia with reduced central nervous system depression and gastrointestinal diseases in mice models lead to TRV130 application in clinical trials to evaluate its better efficacy compared to morphine, and is now being tested in different phase 2 clinical trials [125, 131-133].

1.8.2-BIASED AGONISM IN CHEMOKINE SYSTEM

Evidence of biased signaling in the chemokine system is rapidly accumulating, possibly due to the fact that the high degree of promiscuity makes the chemokine system particularly prone to include endogenous ligands that can act on the same or different receptors with different affinities and eliciting different responses [24, 34, 99, 134]. CCR7 in example is activated by CCL19 and CCL21, which elicit equivalent agonism for G protein signaling but differ in their ability to activate GRK and β-arrestins [101, 135], that has been attributed to differences in the recruitment of GRKs to CCR7 by the two agonists [136]. CCR2 activates G protein/β-arrestin balanced signaling when engaged by all ligands except CCL8, which was found biased for signaling to β-arrestin2 versus β-arrestin1 [137], and as well CXCR3 is also subjected to biased signaling when engaged by CXCL11 compared to CXCL9 or CXCL10, but the extend and the nature of the bias reported may have been determined by the amount of receptor expressed in the cell and the cellular assay used [102, 138]. Evidence for biased signaling appears to be even more relevant for chemokine receptors with a large number of ligands. As an example, CCR1 has partial agonists (CCL14, CCL15, CCL23) becoming fully active after processing of their extended N-terminal domain [139], β-arrestin biased ligands (CCL3 and CCL15) and G protein biased agonists (CCL5 and CCL23) [102]. A second type of bias occurring in the chemokine system refers to cases where the same chemokine activates different signaling pathways from a given receptor in a tissue/cell-specific manner or in a species-specific manner [34, 102]. As an example, CCL19 has different roles in inducing chemotaxis depending on the cell type where CCR7 is expressed on [140, 141]. Besides these evidences of ligand bias observed for conventional chemokine receptors, atypical chemokine receptors (ACKRs) represent an example of signaling bias operating at the receptor level, that mediates these receptors activities of chemokine system regulation rather than operating as chemotactic receptors [142, 143], and will be discussed in the following sections.

1.9-PHARMACOLOGICAL TARGETING OF THE CHEMOKINE SYSTEM

Physiological and pathological leukocyte migration to lymphoid organs and inflamed tissues are regulated by chemokines and their receptors during both extravasation and directed migration inside tissues [10]. Dysregulation of the chemokine system can result on the one hand in immune deficiency in the case of poor leukocyte recruitment, but on the other hand, in the case of an exaggerated leukocyte recruitment, the inflammatory response can go from tissue damage after acute injury to inflammatory and auto-immune diseases [1, 10]. In the latter case, since chemokine system characterization, chemokine receptors have been considered an ideal therapeutic target not only because they are expressed with cell specific patterns and drive leukocyte recruitment within tissues, but also because of their GPCR structure. In fact GPCRs are the target of about the 30% of marketed prescription drugs, and

both the ability to target them by orally deliverable small molecules and the possibility to exploit not only their orthosteric binding site but also minor pockets contribute to maintain high interest on their therapeutic potential [144, 145]. Furthermore chemokine receptors activity has been proven to mediate leukocyte recruitment involved in the generation of several clinically relevant diseases, spanning from acute injury and allergy to autoimmune diseases, transplanted organs rejection, HIV and cancer [10]. Many compounds have been described to be able in blocking in vitro chemokine receptor activity and eventually displayed efficacy in mouse models, but only two compounds succeeded to arrive on the market, since most of them failed to progress over all the clinical trials [10, 11]. Common features and recurring problems have been characterized among all these failures, that can be attributed to the complexity and redundancy of the chemokine system, to its pleiotropy and to the lack of a precise correspondence between humans and animal models. In the chemokine system is pretty rare that one given chemokine acts only on one single receptor, and viceversa that one single receptor recognizes one single ligand [1, 5]. This characteristic decreases the chances of success when therapy aims to block one single chemokine or one single receptor, since leukocytes express different chemokine receptors at once. In addition, not only different chemokine receptors can respond to the same ligand but also can be able to be activated by other chemokines expressed together with the therapy target ligand in the inflamed tissue. Redundancy furthermore impedes the attempts to antagonize one single receptor, since agonist accumulation within the tissue can results on the improvement of its cross-reactivity on other receptors, even those that normally respond weakly to that ligand [10]. One chemokine does not only mediate cell migration on one specific cell types but also supports other biological functions on different cellular targets, making of pleiotropy a characteristic of the chemokine system, that increases the difficulty to block one single specific effect of a chemokine [10]. Furthermore speciation constitutes an issue of studying the chemokine system, since there is not complete correlation between human chemokine system and the ones of other animal models, such as murine models [5]. The absence of direct correspondence between human and murine genes and function impedes the translation of experimental evidences observed in mice to humans, and the design of drugs based on the human chemokine system can be not immediately applied to mice [5, 10]. It has also to be kept in mind that leukocyte positioning and recruitment to inflamed tissues are a fundamental component of immune system, and to this point completely blocking even single components of the chemokine system can result in a condition of induced immune deficiency that allows the development of opportunistic infections [10, 146]. To this point classical drug development aimed at targeting chemokine system encountered many more failures than in other fields, therefore with the intriguing opportunities offered by emerging concepts in pharmacology such as biased agonism and targeting of allosteric binding sites, different small molecules have been more or less successfully developed in the last few years, resulting

in the approval for market distribution of three chemokine-receptors targeting drugs, and more successfully translated from research to clinical trial phases [11, 145, 147].

1.9.1-MARAVIROC

Human immunodeficiency virus (HIV) infects cells exploiting its ability to bind with its envelope protein Env cell-expressed CD4 antigen, that can be expressed on different leukocytes as T helper lymphocytes and monocytes. CD4 expression is not the only requirement for HIV fusion to plasma membrane and entry, but for cell infection co-expression of the chemokine receptors CXCR4 and CCR5 are required as well [148, 149]. The discovery that individuals carrying mutations in CCR5 receptor or duplications in CCR5 agonist gene CCL3L1 are more resistant to viral infection and disease progression [150-152], due to the lack of CCR5 expression on cell surface and activity [153] or to increased agonist-induced receptor down regulation [151]. Maraviroc is a small molecule CCR5 antagonist discovered from a HTS of a compound library designed to affect CCR5 functions. This molecule is a competitive CCR5 antagonist, since displaces receptor agonists and impedes agonist induced receptor signaling and internalization [154, 155]. This receptor blockade is worth not only for endogenous receptor agonists but also for glycoprotein (gp) 120 subunit of HIV Env [154]. The inability of the virus to bind to its co-receptor, and the blockade of CCR5 internalization, both necessary for envelope fusion to cell membrane, resulting in reduced infectivity and proliferation of CCR5-tropic virus strains (R5-tropic) [154, 155]. Maraviroc is now available on the market as a drug approved for the treatment of HIV-infected patients, in combination with other anti-retroviral drugs, since it showed in clinical trials to be well tolerated and most importantly to reduce HIV-RNA levels and maintain higher $CD4^+$ cells levels compared to therapies consisting only of background therapies. Furthermore, long term treatment did not produce an increase in incidence of diseases associated to impared CCR5 activity, such as Western Nile virus infection or development of malignancies as lymphomas [155].

1.9.2-PLERIXAFOR – AMD3100

Plerixafor, also known as AMD3100, is a selective CXCR4 antagonist initially developed for the inhibition of CXCR4-mediated HIV infection, on the basis of previous evidences indicating the role of bicyclams in HIV infection inhibition [156, 157]. Plerixafor became highly considered and tested for its efficacy in antagonizing CXCL12 binding to CXCR4, its signaling and internalization, and also for directly preventing gp-120 binding and therefore inhibits HIV T-tropic viruses infection [156]. Proven its ability in reducing HIV infection, plerixafor entered different clinical trials where validated its efficacy as virus entry inhibitor, but as well showed the ability to highly increase the number of circulating leukocytes in peripheral blood [157, 158]. This effect has been shown to depend on the inactivation of CXCR4

interaction with bone marrow CXCL12, that retains immature leukocytes and stem cells in bone marrow niches, resulting in hematopoietic $CD34^+$ stem cell mobilization from bone marrow to peripheral blood [157, 159]. This lead to the application of plerixafor to clinical trials and the approval for its application as an inducer of $CD34^+$ stem cell mobilization to peripheral blood for their collection aimed at their autologous transplantation in patients suffering of non-Hodgkin's lymphoma or multiple myeloma [160]. In addition, plerixafor has been recently used to treat immune deficiency by increasing leukocyte circulation in peripheral blood in patients affected by the WHIM (warts, hypogammaglobulinemia, infections, and myelokathexis) syndrome [161, 162]. This patients suffer of a rare disease caused by different mutations in CXCR4 gene that result in the expression of a receptor truncated in its C-terminal tail, exhibiting an hyperactivated phenotype constraining leukocytes inside the bone marrow, impeding their egress to peripheral blood resulting in immune deficiency, that can be reverted antagonizing CXCR4 effect with long-term low doses of plerixafor [161, 162].

1.9.3-MONOCLONAL ANTIBODY MOGAMULIZUMAB

The only other chemokine receptor-targeted drug already approved for therapy is a modified monoclonal antibody directed against CCR4, named Mogamulizumab, aimed at the treatment of T-cell lymphoma. This antibody binds CCR4 that is mostly expressed by T helper 2 (Th2) and T regulatory (T-regs) cells, mediating antibody dependent cell citotoxicity [163]. Many other molecules are currently under evaluation in clinical trials to demonstrate their ability to improve patients outcome in a series of immune-related pathologies, in example asthma, allergy, psoriasis and transplant rejection or diseases where inflammation has a central role such as multiple sclerosis and cancer [11].

1.10-CXCR1 AND CXCR2, THE TWO CXCL8 RECEPTORS

CXCR1 and CXCR2 are the first described prototypic chemokine receptors mediating inflammatory response, due to their ability to drive neutrophils recruitment to inflamed tissues in response to CXCL8 [164, 165]. These two receptors also mediate chemotaxis in response to other ELR^+ CXC chemokines, with CXCR1 able to be activated by CXCL6 in addition to CXCL8, while CXCR2 can bind and actively recognize all the ELR^+ chemokines[166, 167], and both the receptors can bind the cytomegalovirus (CMV) viral chemokine vCXCL1 [168]. In humans genes encoding these two receptors are clustered on chromosome 2q35, and they are mainly expressed by neutrophils, but also both CXCR1 and CXCR2 are expressed by monocytes, natural killer (NK) cells, mast cells and other leukocytes [169]. Their expression can be down-regulated upon inflammatory stimuli, since metalloproteinases released by cells upon stimulation with lipopolysaccharide (LPS) or tumor necrosis factor α (TNF-α) can cleave

receptor extracellular domains resulting in a loss of cell responsiveness and chemotaxis [170]. CXCR1 structure in the absence of any ligand or associated protein has been solved by nuclear magnetic resonance (NMR) applied to the receptor purified in phospholipid bilayer [171], indicating small differences compared to the crystallized CXCR4 and showing that an 8^{th} helix occurs in CXCR1 C-terminal tail. In primary cells as neutrophils it has been demonstrated that CXCR1 and CXCR2 upon stimulation activate Gαi proteins relying on the interactions with residues at the end of TM3 and the beginning of TM6 [172, 173], and Gβγ subunits that are required for migration [174]. Receptor activation leads to the induction of Ca^{2+} fluxes, PLC activation, MAPK activation and receptor phosphorylation [167, 175] and internalization mediated by β-arrestin recruitment [176, 177]. This signaling results in the activation of several signaling pathways, such as the activation of small GTPases, MAP kinases, focal adhesion kinases and others [178] that together mediate cell movement and sustain cell rolling and adhesion to vascular endothelium, allowing leukocytes to egress bloodstream and reach inflamed tissues [179]. Even though their expression and binding abilities and are overlapping, these two receptors have been proven to mediate the same activities eliciting partially different signaling pathways. In fact the two receptors are phosphorylated upon activation by two different kinases, with CXCR1 that is preferentially phosphorylated by GRK2 while CXCR2 by GRK6, resulting in different kinetics of desensitization and internalization [104], that is reflected too by their ability to induce cytoskeleton rearrangement and focal adhesion kinase activation in different manners [180]. This signaling selectivity is worth not only among the two receptors but also for chemokines, since only CXCL8 can induce phospholipase D activation, even if all CXCR1 agonists CXCL1, CXCL7 and CXCL8 activate Ca^{2+} fluxes [181].

Biological functions of CXCR1 and CXCR2 showed that other than mediating neutrophil response to acute inflammatory events such as bacterial infections and wounds [182, 183], these receptors have a role in mediating leukocyte recruitment and support to different diseases, such as arthritis, septic injury and several pulmonary diseases dependent as asthma and chronic obstructive pulmonary disease (COPD) [184-186]. In addition it has also been described a role for CXCR2 in different neural pathologies, as ischemic injury and multiple sclerosis [187]. CXCR1, CXCR2 and their ligands are also involved in tumor with different shades of complexity, since in certain tumors leukocyte recruitment is beneficial for host since they display an inflammatory phenotype and suppress tumor cells, while in other tumoral contexts they can have pro-tumorigenic phenotype [188]. In addition chemokines can be expressed by both infiltrating leukocytes, endothelial and tumor cells, and as well receptors can [189]. In this context anyhow the role of these ELR^+ and chemokine receptors appears as dangerous to the host, since they seem to induce survival and proliferative signals, and display their angiogenic activity that is necessary to sustain tumor growth [188].

Given the relevance of these two receptors in disease development and support many drugs attempted to modify their response aimed at improving clinical outcomes. CXCL8 neutralization with

antibodies has been attempted but did not reach the market due to lack of efficacy in clinical trials, possibly because of the complexity and the marginal role exerted by neutrophils in psoriasis [3], and an inactive CXCL8 variant has been developed to inhibit the GAG-regulated activation of CXCR1 and CXCR2 in COPD. Different compounds from different companies targeting CXCR1 and CXCR2 instead are now being evaluated in clinical trials to improve patient outcomes in a number of pulmonary diseases with promising results [11]. Among those, the CXCR2 competitive antagonist GSK-1325756B by GlaxoSmithKline is undergoing clinical trials, and also Schering-Plough SCH-527123 [3].

1.10.1-Reparixin, a noncompetitive allosteric inhibitor of CXCR1 and CXCR2

Reparixin is a small molecule non competitive inhibitor of CXCL8-mediated leukocyte migration developed and patented by Dompé with a higher affinity on CXCR1 than on CXCR2 [190, 191]. This molecule has been an under investigation from the evidence that ketoprofen, a non-steroidal antiinflammatory drug, inhibited inflammation not only by inhibiting cyclooxygenase (COX) pathway but also by blocking CXCL8-mediated leukocyte migration [192]. This lead to the analysis of the chemical properties and engineering of this molecule to generate a molecule aimed at specifically blocking CXCL8 receptors, that initially brought to the development of DF1681Y, named Reparixin [190], which showed a preferential affinity for CXCR1 versus CXCR2, and also of other similar molecules with improved affinity for CXCR2 [191, 193]. These molecules are non-competitive inhibitors, they do not affect CXCL8 binding to the receptors, or receptor desensitization after ligand exposure, but are able to block GTPγS binding to neutrophil membrane preparations, they block transient Ca^{2+} fluxes, and suppress Pyk2 focal adhesion kinase phosphorylation [194]. Reparixin has been found to bind to an allosteric hydrophobic binding pocket of CXCR1 and CXCR2, where the inhibitor can interact with residues positioned of TM 1, 3 and 6, allowing chemokine binding to the orthosteric site but impeding receptor structural rearrangement necessary to sustain signaling and chemotaxis [194]. This allosteric inhibitor has proven its efficacy in reducing neutrophil accumulation in inflamed tissues and consequent diseases in different preclinical models, as acute and chronic inflammation models [195, 196], preventing ischemia reperfusion injury [194, 197, 198] and reducing the development of arthritis [199, 200]. Application of Reparixin in the prevention of kidney and lung transplant failed to succeed in clinical trials [3], but is reaching advanced phases for the prevention of pancreatic islets transplantation rejection due to its inhibition of host immune system aggression against intra-hepatic engrafted islets [11, 201, 202].

1.11-CCR5

CCR5 gene is located in humans on chromosome, adjacent to CCR2 with which shares 70% amino acid sequence and several ligands [5]. CCR5 is able to respond to many inflammatory chemokines, all inflammatory and belonging to the CC family, among which CCL2, CCL3, CCL4, CCL5, CCL8 and CCL14 [21]. Among the chemokines CCR5 binds there is also CCL7, which has been reported to act as an antagonist [203], as well as viral-encoded MIP-2 (vMIP-II) from HHV-8 [204]. CCR5 binding properties rely on the interaction of ligands with receptor ECL2 and disulphyde bonds [205-207]. CCR5 is also able to bind nonchemokine ligands, such as gp120 protein from HIV, from which interaction derives its role as co-receptor with CD4 in viral infection [149], and raised interest around this receptor as a potential target for anti HIV therapy, that lead to the first approved chemokine receptor targeting drug, Maraviroc ([154] and previous section) and the definition of CCR5 structure in complex with this drug solved by crystallography [26]. Post-translational modification affect CCR5 biology, since it NH_2-terminal residues can be sulfated and glycosilated, while its C-terminal tail is palmitoylated and this modification impacts its expression on the plasma membrane [208].

Expression of CCR5 has been described on almost all leukocytes, in particular on antigen-presenting cells (APC, that include macrophages and DCs), NK cells, T lymphocytes and $CD34^+$ hematopoietic progenitors [209-211]. The discovery of the human CCR5Δ32 allele in caucasian population occurring in homozygosis, in which a mutation causes the production of a CCR5 receptor constituted of the first 4 TM only and resulting in the lack of functional receptor expression, helped the understanding of CCR5 biology, indicating that it is not fundamental to life since compensatory mechanisms can occur [212]. CCR5 mediates residency and homing into peripheral tissues of APCs, that upon activation and exposure to antigens downregulate CCR5 together with CCR1 to upregulate CCR7 that drives their migration towards lymphatic vessels and lymph nodes [213]. In the lymph nodes APCs encounter $CD4^+$ T helper cells, and this contact induces their production of CCR5 ligands CCL3 and CCL4 inducing the recruitment of $CD8^+$ T cytotoxic lymphocytes to APC for antigen presentation [209]. CCR5 knockout mice models do not display obvious phenotypic alteration in physiological conditions but reveal deficient immune responses in several pathologies, showing reduced infiltrating leukocytes and deficient innate and adaptive immune responses to bacterial and viral infections [214, 215]. The supporting role of CCR5 to immune response also occurs in mediating CCL5 stimulation of anti-apoptotic signal on macrophages in viral infections [216]. On the opposite, CCR5 deficiency has been associated to the prevention of exaggerated immune response, during M. tuberculosis infection or ulcerative intestinal inflammation [217, 218]. CCR5 can have a beneficial role in myocardial wound healing in ischemia reperfusion injury models [219], but as well can have detrimental effects in the support of atherosclerotic plaque formation [220].

CCR5 signaling is characterized by the activation of Gαi proteins, which transduce agonist binding that results in the activation of different signaling pathways [99, 208, 221]. CCR5 activation leads to the reduction of intracellular cAMP, the generation of transient Ca^{2+} fluxes, activation of different Rho-family small GTPases as RhoA and Rac1, that sustain actin polymerization and cytoskeletal rearrangement, focal adhesion kinases (FAK and Pyk2) activation and induces CCR5 C-terminus by GRK and PKC that supports β-arrestin recruitment that drives receptor internalization [24, 99, 208]. Interestingly CCR5 agonist stimulation can sustain MAPK phosphorylation both in a G protein- and β-arrestin-dependent manner, since mutant CCR5 lacking phosphorylable sites on the C-terminus do not recruit anymore β-arrestins after ligand engagement [222], even if it has to be mentioned that it has also been shown that β-arrestins to CCR5 can occur in absence of phosphorylation, not only on receptor C-terminus but also on the second ICL, in the DRY region [85, 100]. Receptor activation leads to β-arrestins recruitment and its internalization mainly through chaltrin-coated vesicles, and also through caveolae and lipid rafts in general in which CCR5 can localize thanks to its palmitoylated C-terminus [208]. After internalization, the receptor is recycled back on the plasma membrane without associating to late endosomes or lysosomes, oppositely to CXCR4 [208, 223]. Biased signaling has been described among CCR5 ligands, that have been evaluated in their ability to induce CCR5 dissociation from different G protein subunits, β-arrestin recruitment, adenylyl cyclase inhibition and Ca^{2+} fluxes generation [99, 102]. In particular, in the paper from Corbisier et al. ligands activity have been compared to CCL3, that was chosen as reference ligand, and results indicated that CCL8 acts as a biased agonist, inducing stronger Gα subunits dissociation rather than adenylyl cyclase inhibition, and also a more pronounced Ca^{2+} fluxes generation compared to cAMP reduction or to β-arrestins recruitment, indicating a preferential activation of Gβγ subunits of CCL8 compared to CCL3 [99].

1.12-CHEMOKINE SYSTEM REGULATION

As already mentioned, the chemokine system is regulated at several levels, beginning with the regulation of chemokines and chemokine receptors expression and limiting it to specific tissue and cell populations or also to specific induction as in the case of inflammatory stimuli as LPS, TNF-α and IL-1β [1, 5]. To this point a distinction between homeostatic and inflammatory chemokines can be made, but evidence indicate that certain chemokines can exhibit a dual behavior acting on different populations or on different receptors, or can be expressed and released in peripheral blood circulation in inactive form to be activated upon inflammation [5, 224]. Fundamental to chemotaxis is the formation of a chemical gradient, that indicates migrating cells the direction to be maintained. Gradient can be influenced by two major factors, one is the passive activity of glycosaminoglycans (GAGs)[54] and the other is mediated by atypical chemokine receptors (ACKRs)[225]. Important as well is the role of peptide-

modifying enzymes that modify chemokines and chemokine receptors, influencing their availability and activity [226].

1.12.1-GAG ROLE IN CHEMOKINE ACTIVITY

Glycosaminoglycans are constituted by long chains of repeated disaccharide units, that can be composed by different saccharides connected by different linkers and different chemical modifications [227]. GAGs are present on almost every cell as soluble or surface bound and the most abundant GAG is heparan sulfate, whose function as for other GAGs is to bind many different proteins, among which proteases and chemokines [227]. The interaction with chemokines is essential to induce correct leukocyte recruitment to inflamed tissues, since chemokines produced in inflamed sites are dispersed in the local area, until they reach endothelial cells of blood and lymphatic vessel where they remain bound to luminal GAGs, which prevent chemokine wash out in the circulation. This immobilization and presentation allows leukocytes rolling on the luminal endothelium to encounter chemokines, activate integrins that mediate firm adhesion to the endothelium and climb up the chemokine gradient in the tissue towards inflamed site [1, 227-229]. Interactions between chemokines and GAGs have been reported to depend on several factors, as electrostatic charges interactions, several residues across the chemokine sequence, their tertiary structure and their oligomerization [19, 54, 227]. All these properties con be modified by enzymes that by post-translational modifications alter chemokine amino acid sequence, structure, affecting GAG interactions [226].

1.12.2-NATURALLY OCCURRING POST-TRANSLATIONAL MODIFICATIONS

Chemokine system can be regulated at the protein level by different enzymes that acting on chemokines can activate, inactivate or modulate chemokine activity with a number of modifications that can be found also in vivo [226, 230]. The most important modification occurring is protein truncation, mediated by several proteases, expressed by several leukocyte populations and stromal cells and also in response to inflammation, indicative of their role in inflammation, and that preferentially cleave N- or C-terminus of chemokines. An example is given by chemokines CXCL16 and CX3CL1, which after synthesis remain bound to cell membranes, where in the case of CX3CL1 acts as adhesion molecule, and after cleavage by a disentegrin and a metalloproteinase of their transmembrane domain are released in their soluble chemotactic form [231, 232]. Another example of such regulation is given by CCL14 and CCL15, that are abundant in human plasma in an inactive form, that can be rapidly activated by urokinase-type plasminogen activator (uPA), plasmin and cathepsin G with NH2-terminal truncation, that activates their inflammatory potential [233, 234]. Matrix metalloproteinases (MMP) are expressed by stromal cells and leukocytes and can be upregulated in inflammation and cancer, and can regulate

activity of different CC and CXC chemokines [235, 236] as cathepsin G and elastase do [230]. NH_2-truncation of chemokines differently affects chemokine activity depending on the chemokine, since in the case of CXCL10 removal of the first three amino acids by CD26 reduces it chemotactic potential [237], while removal of NH_2-terminus of CXCL7 are necessary to its activation [238]. Post-translation modifications can also induce chemokine selectivity for receptors, as it has been observed for CCL5, that in its intact form can activate CCR1, CCR3 and CCR5, but after NH_2-truncation of its first two amino acids increases its potency on CCR5 but becomes an antagonist for CCR1 and CCR3, which is no longer able to activate [239], indicating that post-translational modification can possibly generate biased agonism.

1.12.3-POST-TRANSLATIONALLY MODIFIED VARIANTS OF CXCL8 AND CXCL5

CXCL8 is produced in cells as a precursor protein that after different cleavages of the signal peptide becomes a mature protein consisting of 77 amino acids (1-77) that can be released, that is the most abundant variant, even if a variant derived from alternative removal of the signal peptide has been described, with two residual NH_2-terminal amino acids (-2-77) in addition compared to CXCL8 1-77 (Figure 6) [240, 241]. CXCL8 is an ELR^+ CXC chemokine, in which the ELR motif is positioned after 8 NH_2-terminal aminoacids, and is necessary to sustain CXCL8 chemotactic activity. Different proteases can cleave CXCL8 NH_2-terminus generating different variants. CD13 can remove 1 or 2 amino acids to produce 2-77 and 3-77 CXCL8 (Figure 6), while plasmin, thrombin and MMPs can remove up to 8 residues until the ELR motif, producing 6-77, 7-77, 8-77 and 9-77 CXCL8 (Figure 6) [16, 240]. CXCL8 variants activity increases with NH_2-terminal truncation, since -2-77, 1-77, 2-77, 3-77 variants display similar potency that is increased in 6-77 or further truncated variants [16, 240]. CXCL8 NH_2-terminus can also be modified by peptidylarginine deiminases that removes from arginine NH_2^+ groups, replacing them with oxygen changing a positively charged arginine in a neutral citrullne (Figure 6), therefore this process is also known as citrullination [242]. Citrullination of NH_2-terminal arginine in position 5 (Arg^5) results in similar induction of Ca^{2+} fluxes and desensitization on CXCR1 and in stronger activity on CXCR2, compared to the intact 1-77 non citrullinated variant, in similar chemotactic ability in vitro but shows an impared chemotactic activity in vivo, due to a reduced affinity to GAGs [242].

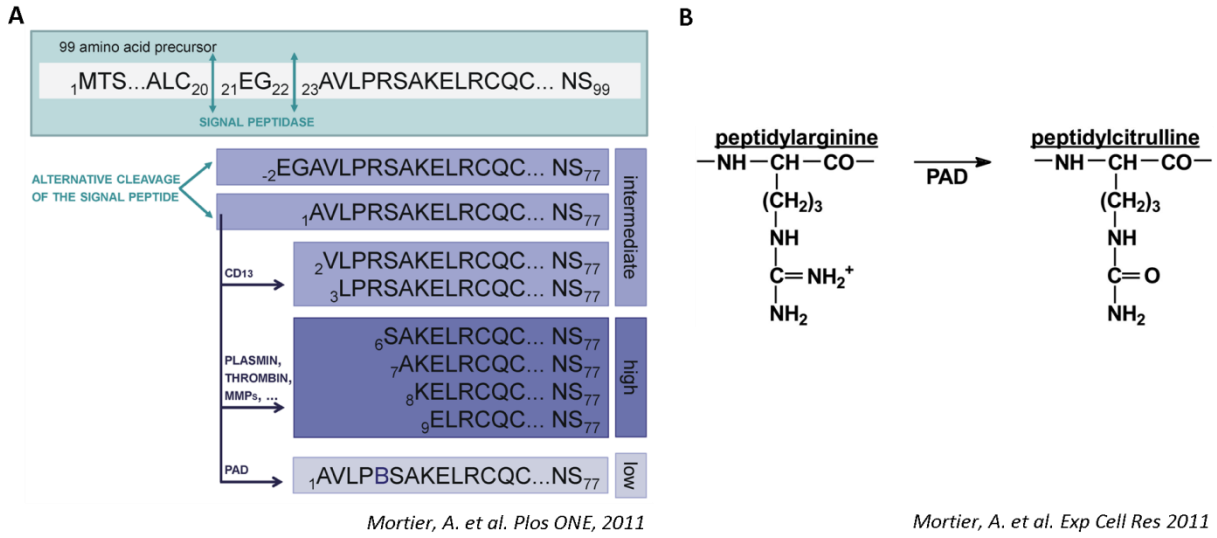


Figure 6: NH_2-terminal sequence of CXCL8 and truncated variants. A-The sequences of CXCL8 precursor, intact, truncated and citrullinated variants are reported, with the indication of the enzyme that can generate that variant [240]. **B-** Chemical modification exerted by peptidyl arginine deiminase that from arginine produces citrulline (B) [228].

CXCL5, agonist of CXCR2 but not of CXCR1, has also been described to undergo different NH_2-terminal processing, leading to generation of variants occurring naturally in extracts from biological samples [243]. Intact CXCL5 (1-78) has been shown to mediate neutrophil chemotaxis in vitro, but in vivo lacks this ability, which instead occurs upon NH_2-terminal cleavage by MMP-8 and -9 and cathepsin G that generate 8-78 and 9-78 CXCL5, increasing its potency both in vitro and in vivo [243-245]. CXCL5 as well can be citrullinated on its NH_2-terminus, on Arg^9, but while for CXCL8 an activity reduction could be observed only in in vivo models, citrullinated 1-78 CXCL5 displayed reduced signaling and chemotactic activity compared to intact CXCL5 already in in vitro assays and evidences were confirmed in vivo [245]. These enzymes are present in inflammatory microenvironments, where many enzymes can act simultaneously, therefore this modifications have also been tested for the ability to prevent excessive truncation that results in chemokine degradation. Intact citrullinated CXCL5 is cleaved by cathepsin G as efficiently as intact CXCL5, while limited truncation of CXCL5 to 2-78 CXCL5 was able to prevent further cleavage [245], while in the case of CXCL8, citrullination prevents its cleavage to thrombin or plasmin processing [242].

1.13-Atypical Chemokine Receptors (ACKRs)

In addition to the above mentioned mechanisms, host chemokine system regulation relies on the activity of atypical chemokine receptors (ACKRs) that constitute a smaller group of chemokine receptors, including at present 4 members named ACKR1 (DARC), ACKR2 (D6), ACKR3 (CXCR7) and ACKR4 (CCX-CKR) and with two other members pending this classification at the moment, CCRL2 and

PITPNM3, waiting for better characterization and functional confirmation [3, 246, 247]. Previously called decoys, interceptors, scavengers, or chemokine-binding proteins, the acknowledged term is now "atypical" as a consequence of their inability to mediate cell migration as conventional chemokine receptors. Evidence indicates that ACKRs exert a non-redundant role in shaping chemokine gradients and dampening inflammation by sequestering chemokines from the microenvironment, thus emerging as crucial regulatory components of chemokine networks in a wide range of developmental, physiological and pathological contexts [225, 246]. Recent increasing evidence have shown that to mediate their function ACKRs use alternative signaling pathways as compared to those observed for conventional chemokine receptors, acting as biased signaling receptors, compared to their conventional counterparts [143, 225, 246]. Consistent with their regulatory activity on a highly promiscuous system, all ACKRs have more than one ligand [225, 248], and in particular ACKR1 and ACKR2 have a large spectrum of ligands, while ACKR3, ACKR4 and CCRL2 are specific for a narrow spectrum of ligands [225]. Interestingly, even if some ACKRs recognize only a limited number of chemokines, this is a very promiscuous receptor family, since while only ACKR2 is specific for inflammatory CC chemokines [249], while the others can bind both CXC and CC chemokines, as ACKR1 and ACKR4 [250, 251]. Evidence on CCRL2 are not clear, but at the moment it is considered able to bind two CC chemokines, inflammatory CCL5 and homeostatic CCL19, other than Chemerin, a non-chemokine migratory protein [252], and ACKR3 is able to bind not only CXCL12 and CXCL11 [253, 254] but also regulates the activity of different peptides, the Macrophage Migration-Inhibitory Factor, a pleiotropic cytokine with chemokine-like functions involved in numerous inflammatory conditions [255], the intermediate opioid peptide BAM22, and adrenomedullin, involved in circadian glucocorticoid oscillation and in cardiac and vascular development, respectively [256, 257].

1.13.1-ACKRs EXPRESSION

According to their intrinsic inability to promote cell migration and their role in shaping the chemokine gradient, ACKRs are mainly expressed on endothelial and epithelial cells at barrier sites (i.e. skin, lung, gut, brain, placenta) rather than on migratory cells as their conventional counterparts [225]. All ACKRs except ACKR4 are expressed at the blood-brain barrier, and all are expressed on endothelial cells. In particular, ACKR1 and CCRL2 are specifically expressed on vascular endothelium [258, 259] whereas ACKR2 and ACKR4 on the lymphatic one [260, 261]. ACKR3 expression on vascular endothelium is well established, while data on its expression on lymphatic endothelium are controversial [256, 262]. Differently from conventional counterparts, ACKRs are usually poorly expressed on cells of hematopoietic origin. ACKR1, ACKR2 and CCRL2 have been reported in some leukocytes subsets, B lymphocytes in particular, whereas data on ACKR3 and ACKR4 are currently controversial due to different techniques and protocol used and to the absence of specific tools [3, 225, 246]. ACKR1 is the

only ACKR expressed on erythrocytes [263], whereas platelets express constitutively ACKR3 [264, 265] and ACKR2 upon systemic sclerosis [266]. Under specific conditions, ACKRs are co-expressed with conventional counterparts on leukocytes and fine tune the chemokine system also interfering with the activation of conventional chemokine receptors through direct competition for cognate ligands. Best example is the CXCL12/CXCR4/ACKR3 "eccentric trio" [267], with ACKR3 co-expressed with its conventional counterpart CXCR4 in several leukocytes, including B and T lymphocytes [268, 269]. Co-expression of ACKR1 and CCR5 has been observed on primary endothelial cells [270], whereas CCRL2 is co-expressed with its conventional counterpart CCR7 on B cells in a maturation stage-dependent manner and on DCs upon stimulation with pro-inflammatory stimuli [271, 272]. ACKRs have also been reported to interfere with the signaling pathways of co-expressed conventional chemokine receptors, indicating their regulatory activity may not be restricted to receptors with shared ligands but also apply to receptors co-expressed on the same cell. Examples are provided by ACKR2 interfering with CXCR5 signaling on B lymphocytes [273] and ACKR4 interfering with CXCR3 signaling on T lymphocytes [274]. Finally, it has to be noted that ACKRs expression on different cell contexts may correspond to different functional properties, as exemplified by ACKR1 which acts as a sink on erythrocytes and as a transporter on endothelial cells [258]. Gene-targeted animal models have been generated for all ACKRs, and while ACKR1-/- and ACKR2-/- animals exhibit normal development and show defects only upon challenge with inflammatory stimuli [275, 276], ACKR3-/- and ACKR4-/- animals exhibit severe phenotypes correlated to their regulation of homeostatic chemokines. ACKR4 deficiency alters thymic homeostasis resulting in the spontaneous development of autoimmune reactions [277], while ACKR3-/- animals suffer early lethality caused by severe cardiovascular defects [278] due to its regulation of adrenomedullin signaling [256]. CCRL2-/- mice develop normally to term, have normal lifespan and do not show an overt phenotype under steady-state conditions, but exert defects in DCs migration to peripheral lymph nodes and mast cells responsible for reduced Th2 responses and cell-mediated contact hypersensitivity, respectively [271].

1.13.2-MECHANISMS OF ACTION OF ACKRS

Though as mentioned the ability to shape the chemokine gradient is a common property, different ACKRs use different mechanisms to fulfill this function, allowing ACKRs to be further classified into three "professional" categories: scavengers, transporters, and presenter [3]. ACKR2, ACKR3, ACKR4 and CCRL2 share the ability to scavenge chemokines [274, 279-283], mediating their clearance to reduce leukocyte migration. ACKR1 and ACKR5 share the ability to transport and present their ligands to other receptors [252, 258, 284, 285], and when expressed on erythrocytes ACKR1 also acts as a sink/reservoir for chemokines, thus working as a chemokine "buffering" receptor [275, 286].

The ability to interfere with conventional chemokine receptors activation represents an additional common theme among ACKRs, though the underlying molecular mechanisms are still largely undefined. All ACKRs but not ACKR4 have been demonstrated to be able to interfere with signaling properties of conventional receptors through direct binding of cognate ligands, and ACKR1, ACKR3 and ACKR4 have also been shown to homo- and heterodimerize with conventional chemokine receptors [270, 274, 281, 287], impacting on the activity of other receptors [271, 272], mainly inhibiting chemokine activity. In the case of ACKR3 though co-expression with CXCR4 improves CXCR4 chemotactic ability [278, 287, 288]. Intracellular trafficking properties of ACKRs are of major relevance for their chemokine gradient shaping functions. Constitutive trafficking has been interpreted as a mechanism allowing to rapidly cope with changes in cellular requirements and is a common feature to most scavenger ACKRs, though information on ACKR1 is not available and currently debated for ACKR5 [225, 248, 289]. Of note however, constitutive trafficking is not a unique feature of scavenger ACKRs, as increasing emerging evidence indicates that several conventional chemokine receptors also undergo constitutive internalization and recycling as CXCR4, CCR7 and CCR1 [106, 290, 291]. Interestingly, constitutive trafficking correlates with ACKR subcellular localization, as scavenger ACKRs are mainly located in recycling endosomes, whereas transporter and presenter ACKRs are preferentially expressed on the cell membrane [225, 248, 289].

1.13.3-ACKRs SIGNALING ACTIVITIES

A distinguishing feature of ACKRs, which initially allowed their classification as “silent” receptors, is their inability to sustain cellular migration [8], which is caused by absent or negligible ability to activate G protein signaling [143, 246, 247, 282, 292]. The only exception is ACKR3, which is constitutively associated to Gαi protein [287] and activates Gαi signaling upon CXCL12, but not CXCL11, stimulation in rodent astrocytes and human glioma cell lines [293]. Noteworthy, although ACKR4 is unable to activate Gαi, Gαs or Gαq subunits, uncoupling of constitutively associated Gαi by pertussis toxin allows the receptor to slightly increase cAMP levels upon ligand engagement [294]. This lack of G protein activity can rely in some alterations ACKRs share in sequence motifs responsible for Gα activation and in some micro-switches [33, 143]. Changes are particularly evident for the DRYLAIV motif located in the second intracellular loop, which is mutated very differently among ACKRs, with ACKR3 retaining the sequence most similar to the canonical one. However, with some exceptions in specific conditions and cell types, ACKRs are usually unable to activate the Gα protein-dependent signaling pathway, which is in any case dispensable for their chemokine regulatory functions [282, 292]. Though chemokines acting on conventional chemokine receptors in most the cases signal in a balanced fashion through the G protein and β-arrestin modules [61, 295], specific ligand or specific conditions can influence receptor signaling, inducing biased signaling [78] and this occurs also in the chemokine system

[34, 99, 134]. In line with this emerging view ACKRs have been proposed as structurally β-arrestin-biased receptors [143], since they are able to associate β-arrestins and this interactions regulate ACKRs intracellular distribution, trafficking, signaling and functions [281, 282, 292, 294, 296, 297]. Exceptions are represented by CCRL2, which has not been investigated in this respect, and ACKR1, which has been shown to induce ERK1/2 phosphorylation in human airway smooth muscle cells whose expression of β-arrestins is debated [270, 298, 299]. In conclusion, β-arrestins recruitment is emerging as a unifying theme among ACKRs, that therefore can be considered as structurally β-arrestin-biased receptors, that could contribute to the fine characterization of GPCR signaling activities [143, 225].

1.14-ACKR2

ACKR2 is the best-characterized atypical chemokine receptor, which shapes the chemokine gradient by acting as scavenger for CC inflammatory chemokines. 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 [300]. 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 [301]. This specific ACKR2 expression confirms its important role in modulating the inflammation and immunosuppressive function status on lymphatic vessel and at materno-fetal barrier, respectively. Moreover, ACKR2 has been reported also to be expressed on vascular tumors [300] and at low levels in a range of circulating leukocytes [302, 303], including mast cells, macrophages, neutrophils [304], with most marked expression being evident on subsets of B cells as well as on both plasmacytoid, myeloid DCs and human alveolar macrophages of chronic obstructive pulmonary disease (COPD) patients [305]. Its expression has been also observed on astrocytes, at central nervous system [306]. 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 [273, 307].

ACKR2 7TM structure is well conserved compared to conventional CCR2 and CCR5 chemokine receptors, with an overall sequence identity that is in the 30–35% range of similarity with these conventional receptors, as same as the N-terminal domain that presents several charged residues, mainly involved in ligand recognition such as acidic amino acids, sulphated moieties and N-linked glycosylation sites, but whether receptor sulphation or glycosylatation are important for ligand binding remains still to be demonstrated [308]. The main structural differences are observed within the transmembrane and C-terminal domains. First, the aspartic acid and the DRYLAIV motif are mutated into an asparagine and DKYLEIV respectively. Interestingly, the DKYLEIV motif is highly conserved across the species [273]. The correction of this motif confers weak G proteins-mediated signaling [297], showing

how the alteration of DRY motif is at least in part responsible for the lack of conventional signaling activities of ACKR2. Conversely, the TxP motif is highly conserved, suggesting that receptor could be activated upon ligand engagement as same as the conventional counterparts. Second, ACKR2 C-tail, starting at amino acid 312 till 384, is longer than conventional receptor, and contains a serine/threonine cluster and a putative 8th helix. The C-tail is involved in ACKR2 internalization both in basal and upon ligand stimulation, allowing receptor recycling to and from the plasma membrane and progressive chemokine depletion [72, 292]. Moreover, this domain prevents ACKR2 degradation protecting the receptor from entrance into late endosomes. In fact, truncation of ACKR2 C-tail or 8th helix, or substitution of serine cluster with alanine, strongly impairs receptor stability on cell surface, by targeting to lysosomal compartment through ubiquitination of 142 and 324 lysine residues, that are highly conserved in all mammalian ACKR2 sequences [309].

Although ACKR2 recognizes a wide spectrum of chemokines, the receptor expresses some selectivity in ligand recognition. Neither homeostatic CC-chemokines, mainly agonists of CCR6–CCR10 receptors, nor chemokines belonging to other chemokine subfamilies (i.e. CXC, XC, CX3C) [249, 300] are recognized. Conversely, ACKR2 is able to interact with most agonists at inflammatory CC chemokine receptors from CCR1 to CCR5 (Figure 7) [249, 300]. Even among inflammatory CC chemokines, ACKR2 recognition is restricted to the biologically active form. For example, the intact biologically active molecule form of CCL22 is efficiently recognized by ACKR2, while N-terminal CD26-processed CCL22 variants, which lose their ability to trigger leukocyte recruitment once bound to CCR4, are not recognized by the receptor [300]. 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 [310]. 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 (Figure 7), 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 [310]. For this reasons, chemokine are classified in active and neutral ligand, as reported in Figure 7.

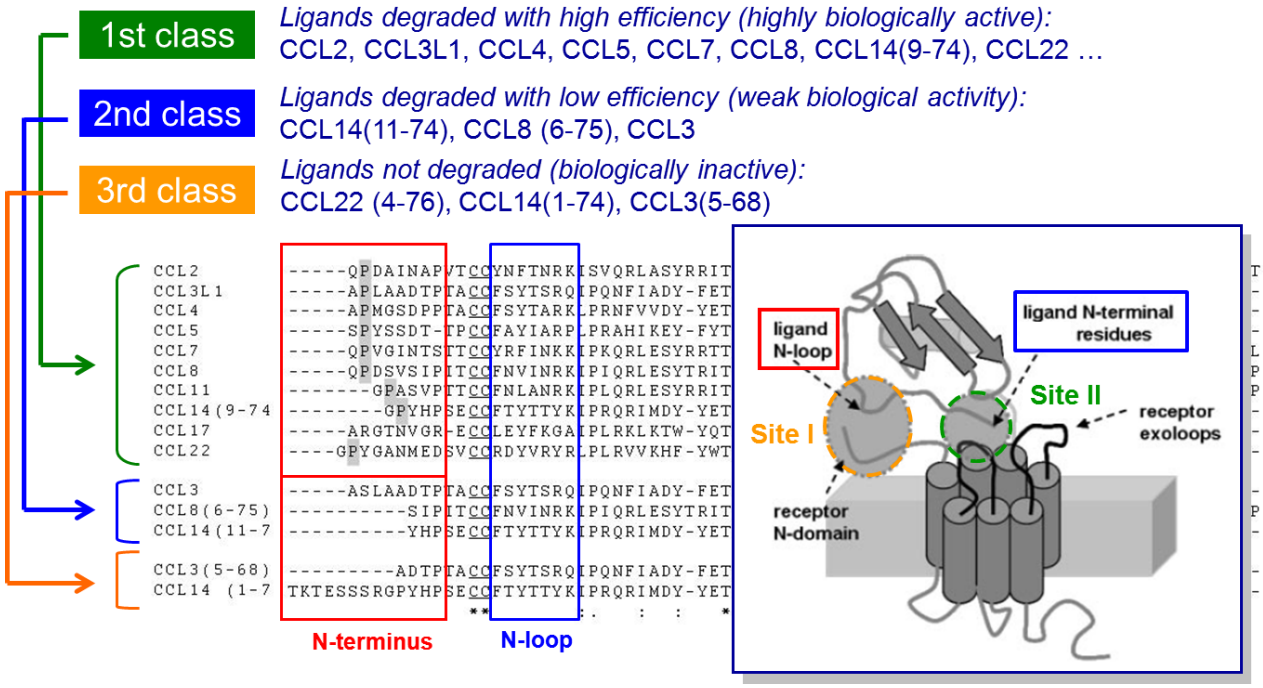


Figure 7: ACKR2 ligand classification. The first class also known as active ligands, having a proline residue in position 2 at the N-terminus, bind ACKR2 with high affinity and are degraded with high efficiency. Neutral ligands, of the second and the third classes, bind ACKR2 with high affinity, but are degraded with low efficiency or are not degraded, respectively (Figure derived from [310]).

ACKR2 scavenger activity relies on its peculiar trafficking properties. In fact, ACKR2 is not regulated or inducible at transcriptional level, but its membrane expression is highly regulated by its intracellular traffic (Borroni E.M., unpublished observation). In resting condition, the receptor is predominantly located in intracellular compartments at perinuclear levels and only 10% is detectable on the cell surface. ACKR2 is constitutively associated to both early (Rab4/5) and recycling endosomes (Rab11) [311], but not with lysosomes (Rab7) [308, 312], which finely control its constitutive internalization and recycling. 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 [313]. Once internalized, ligand dissociate from the receptor, and is targeted into lysosomal vescicles (Rab7) for degradation, while the receptor is recycled back to the plasma membrane through both rapid (Rab4) and slow (Rab11) recycling endosomes [313] (Figure 8). Thus, constitutive internalization and recycling, and ligand-dependent receptor mobilization to cell surface are mechanisms adopted by ACKR2 to allow rapid modulation of ligand uptake and degradation [314].

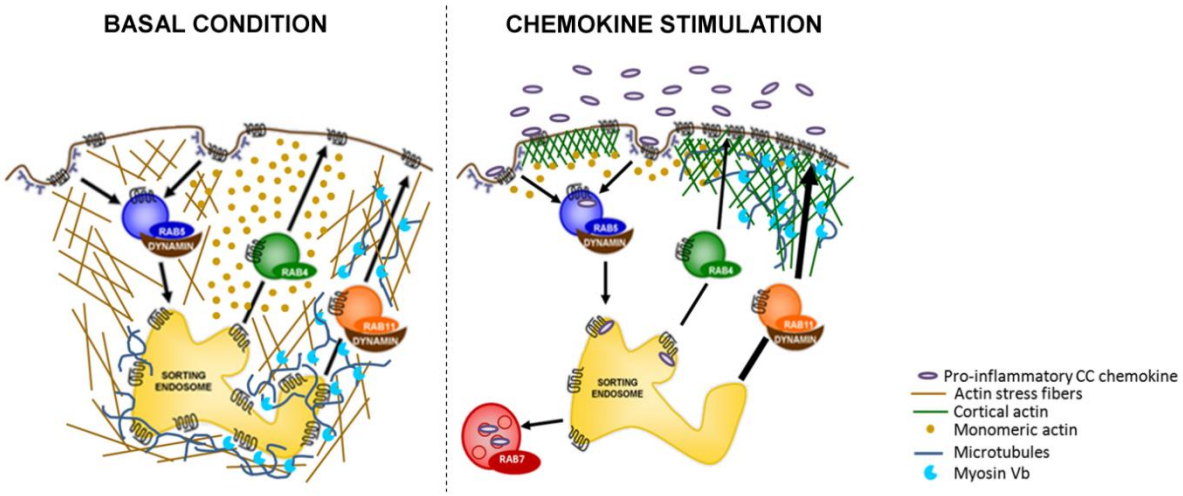


Figure 8: ACKR2 intracellular traffic. In basal condition, ACKR2 is internalized in a Rab5/Dynamin-dependent mechanism and it recycles back to the plasma membrane through a Rab4/Rab11-dependent way. ACKR2 mobilization from intracellular compartments is induced by stimulation only with active ligands, 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 [311]).

To date, chemokine-dependent mobilization is the only mechanism known to regulate ACKR2 membrane expression and degradative activity [248]. It is important to note that only active ligands can induce rapid mobilization of the receptor to the plasma membrane [310, 313], suggesting that some signal events activated upon ligand engagement can occur in order to promote receptor cycling. According to that, a G protein-independent β-arrestin1-Rac1-PAK1-LIMK1-cofilin signaling dependent signaling events downstream ACKR2 controlling the actin cytoskeletal dynamics, have been recently demonstrated that to be required for regulating receptor trafficking properties and scavenger performance [292].

ACKR2 scavenger activity and its role in dampening inflammation have been confirmed in several murine models of inflammatory-based pathologies using ACKR2 KO mice. These mice have been generated by D.N. Cook and S.A. Lira [276] 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 [301]. Interestingly, in homeostatic condition, ACKR2 KO mice present an increased number of Ly6C^{high} circulating and spleen monocytes, while this population is highly decreased in the bone marrow [228]. 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 [315].

2-AIM

Chemokines and chemokine receptors drive leukocytes migration in homeostatic and inflammatory conditions, therefore are considered key regulators of immune response [1]. Chemokines and chemokine receptors are divided into four families (out of which CXC an CC families are the best characterized) depending on chemokine structure which determines their recognition by different receptors, since receptors can bind different ligands but only from the same family, with receptors that can be activated by different ligands and as well chemokines that can activate different receptors [5]. Chemokine system regulates cell migration in different and fundamental activities in controlling cell positioning during development [4], and in adult organism directing immune system activity in both physiological and pathological conditions [3, 55, 316]. Given the complexity and the many functions regulated by this system, its regulation is crucial to increase system potency at defined moments to fight against infections or adverse events, but is also important to turn off the system when the danger has been removed to allow tissue repair prior returning to homeostasis and prevent off-target activity of immune cells. Dysregulation of chemokine system in fact has been associated to immune-deficiency, infections, allergy, auto-immune diseases and cancer, where tumor cells-secreted chemokines recruit leukocytes within the tumor to support tissue remodeling and vascularization and chemokine receptors expressed by tumor cells can promote cells survival [8-11, 225]. Chemokine activity regulation occurs through different mechanisms, among which the most important are chemokine post-translational modifications, mediated by several enzymes that affect chemokines activity and binding specificity, and atypical chemokine receptors (ACKRs) that despite binding chemokines with high affinity do not induce cell migration on the cells they are expressed but rather regulate chemokines activity by their transportation, degradation, or presentation [3, 224-226]. Chemokine receptors belong to the superfamily of G protein coupled receptors (GPCRs), constituted by 7 transmembrane spanning molecules that upon agonist recognition activate several downstream signaling pathways, mainly mediated by G protein, arrestins and kinases, that in recent years have been challenging classical pharmacology with the concept that receptor does not simply switch from an inactive to an active status, but different agonists can produce only selected outcomes on receptor signaling, generating what is now called biased signaling or functional selectivity [84, 115, 317]. In the chemokine system this ability has already been characterized to occur, with different chemokines activating on the same receptor different signaling pathways with different intensities [34, 102, 114, 134], giving biased agoinsm, or with receptors activating different signals depending on the cell they are expressed in [293] resulting in system bias. Another possible bias can depend on the inability of the receptor itself to activate all the signaling pathways conventionally activated by the same ligand on conventional receptors, due to sequence modifications in key signaling motifs that result in structural bias, as in the case of atypical chemokine receptors [282, 292, 294]. To this point the aim of this thesis is to investigate the existence of biased signaling occurring in the chemokine regulation system to provide a better

understanding of mechanism involved in the fine tuning of chemokine activity. Therefore the first aim of this thesis is to assess whether post-translational modification naturally occurring in inflamed tissues on CXCL5 and CXCL8, that regulate activity of this two chemokines by both increasing and decreasing their potency, could underlie the generation of a bias on the signaling properties of CXCR1 and CXCR2 receptors. This has been made in collaboration with professor Paul Proost from KU Leuven in Belgium, who provided us different post-translational chemokine variants. We also evaluated the signaling ability of ACKR2, one of the best characterized atypical receptors, that is a structurally biased receptor unable to activate G proteins but able to signal through a β-arrestin dependent mechanism, to support its activity of inflammation suppressor.

Despite their involvement in many immune diseases and inflammatory-related pathologies and the investments made so far by different companies, only three drugs are currently available on the market targeting the chemokine system, due to the complexity and the redundancy of the chemokine system [10, 11, 125]. Therefore the second aim of this thesis is to translate to a clinical relevance acquired skills and knowledge to provide a possible understanding on the molecular reasons why drug development for chemokine receptors often results in adverse side effects or in therapeutic inefficacy. In particular, we investigated if allosteric non-competitive inhibitors for chemokine receptors could represent a novel mechanism to prevent leukocyte chemotaxis, by blocking only selected signaling pathways and modulating receptor signaling towards a biased signaling. To this point we performed in vitro assays aimed at determining whether signaling bias on CXCR1 can be induced by Reparixin, a small molecule designed by the Italian pharmaceutical company Dompé to target the allosteric binding pocket of CXCR1. This molecule, able to block leukocyte migration and not CXCL8 binding to the receptor, has already demonstrated to be a promising drug to inhibit inflammation in several preclinical models and in advanced clinical trials to be an efficient therapy for preventing rejection of pancreatic islets after transplantation.

3-MATERIALS AND METHODS

3.1-CHEMICALS AND ANTIBODIES

Recombinant human CCL3, CCL3L1, CXCL8 and CXCL5 chemokines were from R&D Systems (Minneapolis, Minnesota, US). Modified variants of CXCL5 and CXCL8 have been synthesized by FMOC technology and kindly provided by professor Paul Proost from KU University of Leuven.

For Western blot analysis, antibodies for phosphorylated cofilin (P-Cofilin, Ser-3) and mouse anti α-tubulin antibody were from Cell Signaling Technologies and Sigma-Aldrich, respectively. Anti-rabbit and anti-mouse IgG-horseradish peroxidase conjugated secondary antibodies were provided by GE Healthcare GmbH (Freiburg, Germany).

For cytofluorimetric analysis, unconjugated anti-HA monoclonal antibody and mouse IgG1 isotype control were from Covance. Goat anti-mouse IgG Alexa® Fluor 647-conjugated secondary antibody was purchased from Molecular Probes. Antibody staining is performed in FACS buffer: PBS 1X with Ca^{2+}/Mg^{2+} + 1% BSA + 0.01% Sodium Azide.

3.2-CELL CULTURES AND TRANSFECTIONS

Human embryonic kidney (HEK293) wild type cells or stably expressing human CXCR1 and CXCR2 were cultured in DMEM (Lonza) supplemented with 10% fetal bovine serum (FBS) (Euroclone, Milan, Italy), 2mM UltraGlutamine (Lonza), 100 U/ml of penicillin/streptomycin (P/S) (Lonza), 25 mM HEPES (Gibco, Life Technologies Ltd). Stable transfectants were generated by lipofection with Lipofectamine2000 (Life Technologies) following manufacturer's instructions, with the HA-CXCR1 pcDNA6 or HA-CXCR2-pcDNA6 plasmids, and selected with 50 µg/ml of Blasticidin (Invitrogen, Life Technologies Ltd) and maintained as bulk populations. HEK293 cells were co-transfected with various combinations of donor and acceptor plasmids with acceptor plasmid used 3 times the amount of donor plasmid used for the BRET using Polyethylenimine (PEI), as described in BRET1 and BRET2 sections.

3.3-PLASMIDS

Plasmids encoding HA-CXCR1, HA-CXCR2, HA-ACKR2 and HA-CCR5 fused to RLuc were generated by amplification with Q5 high fidelity polymerase (New England Biolabs, NEB) cDNA sequences of plasmids already available in the laboratory, with degenerate primers inserting restriction enzyme sites at the extremities, to clone by restriction enzyme digestion and ligation the sequences in pRLuc-N3 (Perkin Elmer). Restriction sites used are HindIII at 5' of all the sequences, while at the 3' we used BamHI for ACKR2 and CCR5 and SmaI for CXCR1 and CXCR2, and these plasmids were used to generate by sub-cloning all the plasmids encoding receptors sequences fused to VENUS, in empty vectors kindly provided by dr. Julie Perroy. Bovine β-arrestins fused to EYFP were kindly gifted by dr. Bice Chini and dr. Marta Busnelli from the Italian National Research Council, Department of Neurosciences, Milan, Italy, and have been used to sub-clone β-arrestins sequences in $pGFP^2$-N3 (Perkin Elmer) to generate β-arrestins-GFP^2 and in RLuc8 empty vector provided by dr. Julie Perroy. Human β-arrestins sequences

have been cloned by human cDNA collection to empty pRLuc-N3 (Clontech) and subsequently sub-cloned with ACKR2 and CCR5 in pEYFP-N1 and pEYFP-C3 (Clontech), and pRLuc-C1 (Perkin Elmer) to generate all the combinations to explore how reporter orientation affected BRET. HA-ACKR2 and HA-CCR5 were sub-cloned in empty pcDNA4/To exploiting HindIII and XhoI restriction sites present in plasmids available in the lab. All the constructs were Sanger-sequenced on both strands to verify correct insertion of the sequence in the vector, absence of mutations and that undesired stop codons or frame-shifts were not intercurring between proteins of interest and reporters.

3.4-CHEMOKINE RECEPTORS EXPRESSION AND CXCR1 INTERNALIZATION

Chemokine receptors membrane expression was evaluated as followed. Cells ($5x10^5$) were labelled with ice-cold FACS buffer containing 5 µg/ml of anti-HA antibody for 1 hour. Cells were then stained with an APC-conjugated goat anti-mouse IgG as secondary antibody for 30 minutes in FACS buffer and fixed in PBS with 1% formalin for 15 minutes. 10^4 events of viable cells identified by FSC-H and SSC-A parameters were 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.

To evaluate CXCR1 cell surface abundance after chemokine stimulation, $2x10^5$ cells were resuspended in 90 µl of DMEM + 1% BSA and incubated 20 minutes at 37°C before stimulation with with or without CXCL8 (100 nM) for indicated time points, then transferred on ice, washed with PBS and stained and analyzed by flow cytometry as previously described.

3.5-COFILIN PHOSPHORYLATION ASSAY

HEK293 T-Rex ACKR2 and CCR5 cells (3×10^5) were seeded onto 12 wells plate, previously coated with 100 µl/well of poly-L-lysine for 30-40 minutes at 37°C and grown in complete culture media without antibiotics at 37°C for 18 hours. Cells were serum-starved by replacing FBS with 0.1% BSA for 18 hours before chemokine stimulation. Cells were subsequently stimulated in pre-warmed DMEM + 1% BSA with 100 nM of CCL3L1 added at the indicated time points. After chemokine stimulation, cells were washed with PBS, lysed and processed as described in western blot paragraph.

3.6-WESTERN BLOT

Cells were lysed for 5 minutes 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), 100 µg/ml PMSF and phosphatase inhibitors: 0.01 M sodium

pyrophosphate, 50 mM sodium fluoride (NaF) and 0.01 M sodium orthovanadate (Na_3VO_4). After centrifugation at 13000 rpm at 4°C for 20 minutes, cell supernatants were quantified by DC Protein Assay (Bio-Rad, Hercules, California, US) following manufacturer's instruction. 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 minutes and then incubated overnight at 4°C with 1:1000 dilution in TBS-T + 5% BSA of the primary antibody against phospho-Ser3 Cofilin (CST). Membranes were washed 5 times for 5 minutes 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 minutes 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 band intensity. α-tubulin staining was performed by using 1:4000 dilution, in TBS-T + 5% BSA of mouse anti-α-tubulin 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) and 19 kDa (cofilin).

3.7-BIOLUMINESCENCE RESONANCE ENERGY TRANSFER (BRET).

BRET1. In this assay the receptor is fused at its C-terminal tail with the donor RLuc and coexpressed with a fusion protein between EYFP and β-arrestins (β-arrestin1 or β-arrestin2). Variations in the BRET1 signal were monitored kinetically or in a concentration-dependent manner after ligand addition. To co-express the two fusion proteins, $3x10^6$ HEK293 cells were seeded in 10 ml of complete DMEM into 100 mm tissue culture dish and after 18 h at 37°C, cells were transiently co-transfected with 1,5 µg of RLuc fusion-donor plasmid and 5 µg of EYFP fusion-donor plasmid or empty pcDNA3 vector, mixed in 1,5 ml of Optimem (Life Technologies), to which 19.5 µg of PEI (from a 1 mg/ml stock solution) were added, gently vortexed for 15 seconds and incubated 20 minutes at room temperature before being transferred onto cells. 48 hours later, cells were washed twice with PBS, detached with Versene, and resuspended in BRET assay buffer (PBS, pH 7.4 + 0.5 mM $MgCl_2$ + 0.1% (w/v) glucose). Cells were quantified as protein content by DC Protein Assay and 80 µg of proteins/well were distributed in a 96-wells microplate (polystyrene Black Frame & White Well; PerkinElmer). Kinetic measurements were done assaying at most 4 wells at once to measure luminescence emission values of each sample every 13 seconds. Measurements were made by incubating cells with 5 µM of Coelenterazine h (Life Technologies) for 8 min before stimulation with 100 nM of chemokines or PBS as vehicle and then luminometer readings were started. All reagents were previously diluted in BRET assay buffer. Alternatively, dose-response curves were generated by incubating 80 µg cells with various concentrations of chemokines and after 20 minutes Coelenterazine h was added for 8 additional minutes. BRET1 signal between RLuc and YFP was measured using a Synergy H4 reader (BioTek) that allows the

sequential integration of signals detected with RLuc filter (440/40 nm) and YFP filter (540/35 nm). BRET ratio was calculated as follows:

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

The changes in BRET induced by the ligands reflect the amount of β-arrestins recruitment to the receptor. These were monitored in real-time up to 20 minutes and expressed on graphs as Δ BRET or BRET ligand effect using the formula:

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

For dose-response curves, BRET values obtained by cells co-transfected with donor plasmids and empty pcDNA3, stimulated with different concentrations of chemokine as other samples, were subtracted from BRET values obtained by cells co-expressing donor and acceptor proteins and stimulated with the corresponding concentrations of chemokines, to obtain a BRET value devoid of background aspecific signal, called Net BRET.

$$\boldsymbol{Net\ BRET\ = BRET\ ratio\ (RLuc\ and\ EYFP\ cells) - BRET\ ratio\ (RLuc\ only\ cells)}$$

Kinetic curves can be generated fitting data with one phase exponential association function, dose response curves by non-linear fitting calculated with GraphPad Prism 5 software, to obtain BRETmax and LogEC50 parameters.

BRET2. Cells were seeded in 1 ml of complete DMEM at a density of $4x10^5$ into P12 multiwell plate. After 18 hours at 37°C, cells were transiently transfected with 200 ng of CCR5-RLuc and 10 serial 1 to 2 dilutions starting from 1 µg of empty $pGFP^2$ vector, β-arrestin1-GFP^2 or β-arrestin2-GFP^2, comprising of a blank sample transfected with donor plasmid only. To each transfection was added sufficient amount of pcDNA3 or any empty vector to bring the final DNA amount to 1.2 µg, and to plasmids diluted in 150 µl of Optimem and 3.6 µg of PEI were added. 48 hours after transfection, cells were washed twice with PBS, detached with Versene, and resuspended in BRET assay buffer. Cells were quantified as previously described and 80 µg of proteins/well were distributed in a 96-wells microplate. Using a Tecan F500 reader, fluorescence was measured through filters with excitation peak at 340/35 nm and excitation at 535/25 nm and refers to the amount of GFP^2 (β-arrestins) transfected. Subsequently, Coelenterazine h was added to the final 5 µM concentration, incubated 8 minutes in the dark and luminescence detected to obtain luciferase quantification. Background fluorescence measured from the blank sample was subtracted from fluorescence derived from all the samples (1), and fluorescence of each transfectant was normalized over its own luminescence (2) to obtain a fluorescence/luminescence ratio (acceptor/donor):

$$(1)\ \boldsymbol{FLUORESCENCE\ value\ =\ Mean\ Fluorescence - Background\ Fluorescence}$$

$$(2)\ \boldsymbol{FLUORESCENCE/LUMINESCENCE} = \frac{\boldsymbol{Fluorescence\ value\ at\ 510\ nm}}{\boldsymbol{Luminescence\ value\ at\ 475\ nm}}$$

80 µg of cells were plated in each well, Coelenterazine 400a/Deep Blue C (5 µM, Gentaur) was added and immediately BRET2 readings were collected using the same reader. BRET2 ratio was calculated as previously described for BRET1. The measured BRET was plotted as a function of the Fluorescence/Luminescence ratio for each donor-acceptor couple, resulting in a linear trend in the case of non-specific interactions and in a hyperbolic curve indicating specific association of the donor molecule with the acceptor.

3.8-BRET IMAGING

BRET imaging experiments have been performed in the laboratory of dr. Julie Perroy at the Institut de Genomique Fonctionnelle, in Montpellier (France), following already published protocols [318, 319]. Briefly, HEK293 cells transfections were carried out by calcium phosphate technique, seeding $3x10^6$ cells onto each 100mm diameter cell culture dish 24 hours before transfection, performed diluting 4 µg of donor plasmid (β-arrestin 1 or 2-RLuc8) and 4 µg of acceptor plasmid (ACKR2 or CCR5-Venus) or empty pDsRedMono in sterile water with 250 mM $CaCl_2$, mixed with 2x Hepes Buffered Saline (2x HBS, 280 mM NaCl, 50 mM Hepes pH 7 and 1,5 mM Na_2HPO_4) and immediately transferred on cells. After 16 hours cells were harvested, seeded on glass-bottom culture dishes and cultured for an additional 24 hours. All images were obtained using a bioluminescence-dedicated Axiovert 200 M inverted fluorescence microscope with a Plan-Apochromat 63×/1.40 Oil M27 objective and images were collected with an EMCCD camera at room temperature. We identified transfected cells with a monochromatic light and appropriate filters to image VENUS or DsRed (Excitation 480/40 nm- Emission 525/50 nm or Ex 540/40 nm- Em 600/50 nm, respectively). We then applied to cells Coelenterazine H (CoelH) to the final concentration of 20 µM and we performed sequential acquisitions with emission filters for Luciferase emission at 480/60 nm and VENUS emission at 535/50 nm of 20 seconds each, using the Metamorph software (Molecular Devices). After collecting 3 to 6 images of unstimulated cells we added 100 nM ligands (recombinant human CCL3L1 or CCL3) and resumed image collection up to 20 minutes. For each timepoint we calculated the pixel-by-pixel 535 nm/480 nm ratios (BRET Ratio) by dividing the absolute light intensities per pixel of images obtained at 535 nm over 480 nm. The ratios obtained (comprised between 0,3 and 1,2) were converted to a continuous 256 pseudo-color look-up table and visualized as displayed in the figures. After defining areas of interest on plasma membrane and cytoplasm, we calculated the mean intensity and standard deviation of pixels to determine the average intensity and distribution of the 535 nm/480 nm ratio within the cell of interest using Image J software (NIH). Constitutive association was calculated by subtracting from the BRET ratio obtained from cells with β-arrestins-RLuc8 and ACKR2/CCR5-Venus the BRET ratio obtained by cells expressing the β-arrestins-RLuc8 only, positive for DsRed expression. Ligand-induced association of β-arrestins to receptors at different timepoints was measured as percentage of ligand-induced BRET ratio over the mean of BRET ratio measurements in unstimulated cells. Statistical analysis of the data

obtained from at least 7 replicates for each condition ±SEM were performed with two-ways ANOVA with Bonferroni post-hoc test.

3.9-cAMP MEASUREMENT ASSAY BY ALPHASCREEN TECHNOLOGY

Quantification of intracellular cAMP in HEK293 cells stably expressing CXCR1 or CXCR2 was performed with the AlphaScreen cAMP assay kit (Perkin Elmer) as follows: 70% confluent cells were washed twice with PBS, detached with Versene (PBS + 0.5mM EDTA, Gibco/LifeTechnologies) and resuspended at 10^7 cells/ml in stimulation buffer (StimB: Hanks Balanced Salt Solution (HBSS; Lonza) containing 0.5 mM 3-isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich), 0.1% w/v Bovine Serum Albumin (BSA; Sigma-Aldrich) and 5 mM HEPES (Lonza), pH 7.4. 5 µL of cells/anti-cAMP AB mix solution containing StimB (30%) + 10^7 cells/mL (20%, corresponding to 104 cells final) + anti-cAMP AB working solution (50%, prior 1 to 25 dilution of AB stock solution in StimB) were plated in triplicate on a white opaque 384 well microplate (PerkinElmer). Chemokine serial dilutions were prepared 2X concentrated in StimB containing 2X concentrated forskolin (20 µM) and finally 5 µL of each dilution was added in triplicate to the wells. In addition, vehicle was performed in triplicate by adding to the cells 5 µL of StimB mixed 1 to 2 with 20 µM forskolin. Control points were performed in triplicate as follows: 5 µL cells/anti cAMP AB mix with 5 µL StimB without forskolin and chemokine to measure the maximal recordable AlphaScreen signal in resting cells, 5 µL of a 1 to 2 dilution of anti-cAMP AB working solution in StimB with either 5 µL of StimB or 5 µL of cAMP (5 µM, prior 1 to 10 dilution of 50 µM cAMP stock solution in StimB), to detect the maximal and the minimal AlphaScreen signal, respectively. Plate was incubated 30 minutes at room temperature (RT) in the dark. Streptavidin DB/biotinylated cAMP detection mix was prepared in lysis/detection buffer (prior 1 to 10 dilution of 10X stock solution in MilliQ H2O) by 1 to 150 dilution of the Streptavidin DB stock and 1 to 24 dilution of the biotinylated cAMP solution (1 µM, prior 1 to 10 dilution of 10 µM biotinylated cAMP stock solution in PBS). This solution has to be prepared 30 minutes before use and incubated at RT in the dark to allow biotinylated cAMP binding to streptavidin DB. Chemokine stimulation was blocked by adding to each well 15 µL of Streptavidin DB/biotinylated cAMP detection mix to each well. Plate was incubated for at least 1 hour at RT in the dark. The measurement of AlphaScreen signal was performed by a Synergy H4 Microplate Reader (BioTek) equipped with the following filter setting: excitation was provided by a tungsten lamp through a 680/30 nm filter placed on the excitation wheel and plug on emission wheel, whereas emission arising from the sample was subsequently acquired after filter switching through a 570/100 nm filter placed on the emission wheel with a plug on excitation wheel. Dose-response curves can be generated plotting the AlphaScreen signal measured against agonist concentration expressed as logarithm of molarity and analyzed with nonlinear curve fitting equations (GraphPad Prism 5 software). Statistics can be obtained by ANOVA and Bonferroni post-hoc test.

3.10-BIAS CALCULATION

To determine bias between the ability of the receptors to inhibit adenylyl cyclase and to induce β-arrestin recruitment to the receptors, we stepwise-approached the method described by van der Westhuizen E.T. et al. on Molecular Pharmacology in 2014 [117]. Briefly, we used the formula they provide to calculate with Graphpad Prism 6 the Transduction Coefficient (TC) = $\log(\tau/K_A)$, where the term τ incorporates ligand efficacy, receptor density and coupling within the system, and the dissociation constant K_A is the reciprocal of the conditional affinity of the ligand in the functional system. TC value is calculated for each ligand in each assay by fitting dose-response curves obtained for pathway A and B with the Black-Leff operational model [320]. For each assay, TC values of tested ligands undergo the subtraction of the one generated by the reference ligand chosen arbitrarily (intact chemokine variants or vehicle treated cells) to obtain a relative effectiveness of the ligand for each signaling pathway [$\Delta\log(\tau/K_a) = \log(\tau/K_a)_{ligand} - \log(\tau/K_a)_{reference}$), that can be subsequently compared across the signaling pathways A and B analyzed [$\Delta\Delta\log(\tau/K_a) = \Delta\log(\tau/K_a)_A - \Delta\log(\tau/K_a)_B$]. Statistical analysis was performed using a one-way Anova on the $\Delta\log(\tau/K_a)$ to make pairwise comparisons between two pathways activated by a given ligand, where $P < 0.05$ was considered statistically significant.

3.11-SILAC

HEK293 T-Rex cells are HEK293 stably expressing the tetracycline repressor, generated and sold by Life Technologies. HEK293 T-Rex cells were maintained in complete DMEM with 25 μg/ml Blasticidin, were transfected with Lipofectamine2000 and HA-ACKR2 or HA-CCR5 pcDNA4/To plasmids and maintained in the presence of 500 μg/ml Zeocin (Life Technologies) to generate bulk populations of HEK293 T-Rex ACKR2 or CCR5. For SILAC experiments, SILAC-DMEM medium lacking glutamine (Glu), arginine (Arg) and lysine (Lys) (Life Technologies) was supplemented with 2mM UltraGlutamine (Lonza), and with [$^{12}C_6$,$^{14}N_4$]arginine and [$^{12}C_6$,$^{14}N_2$]lysine or [$^{13}C_6$,$^{15}N_2$]lysine and [$^{13}C_6$,$^{15}N_4$]arginine (Cambridge Isotope Laboratories, Tewksbury, MA, USA) we prepared light or heavy medium, respectively, with final concentrations of 21 mg/l for Arg and 48 mg/l for Lys. To colture cells, complete medium was obtained adding to light and heavy media 10% dyalized FBS (Life Technologies) and 100 U/ml PenStrep (Lonza) (complete SILAC-DMEM). During the adaptation phase, HEK T-Rex ACKR2 or CCR5 were grown in light or heavy SILAC medium for five passages to achieve complete amino acid incorporation, prior to further manipulation of the cells. Cells in complete SILAC-DMEM were seeded in plates for 24 h in presence or absence of 1 μg/ml tetracycline (Life Technologies). After 24 h, complete SILAC-DMEM was replaced with heavy or light SILAC-DMEM with 0.1% BSA, with or without tetracycline for 18 hours. The day of the experiment and 10^7 of all cell types were resuspended in 1 ml of heavy or light SILAC DMEM with 0.1% BSA and incubated at 37°C with gentle shaking for 30 minutes, at the end of which non-induced cells were transferred to ice, while induced cells were stimulated with 100nM of CCL3L1 for the indicated time points at the end of which a small amount of cells was kept to analyze cofilin phosphorylation, while the rest of cells were washed with ice-cold PBS and stored at -80 for further mass spectrometry analysis performed by Prof. Gabriella Tedeschi at Filarete Institute (Milan, Italy).

Data analysis was performed mainly with excel-formatted databases, Gene Ontology (GO) analysis was performed with the Panther GO Enrichment analysis tool available on the Gene Ontology Consortium website (http://geneontology.org/). Venn diagrams and pie charts were realized with Excel software (Microsoft Office suite) and Histograms with Graphpad Prism 5 software.

3.12-STATISTICAL ANALYSIS

All statistical analysis were performed with GraphPad Prism 5 software, assaying statistical significance with Student's t-test for comparing one single parameter between two distinct samples, One-way analysis of variance with Dunnett's multiple comparison for analysis of one single parameter between different samples, and Two-ways Anova with Bonferroni post-hoc test to compare different parameters between multiple samples. The test used in each assay with p values considered statistically significant and reported in figure legends.

4-RESULTS

4.1-Effects of post-translational modifications on CXCL5 and CXCL8 signaling

Chemokines are small soluble mediators that once released from cells diffuse within tissues forming a chemical gradient that drives migrating cells towards the highest concentration of chemokine [1], directing immune cells recruitment to inflamed tissues and their activation and interaction [1, 4, 7]. Chemokine activity is finely regulated in several ways, among which one consists of chemokine post-translational modification, mediated by a number of peptide-modifying enzymes. In fact in serum not only active chemokines or their degradation products can be found, but also many naturally modified variants [16, 226, 228, 230]. These modified chemokine variants exhibit truncated N- or C-terminus, an altered glycosylation pattern or arginine residues modified to citrulline compared to their intact or natural or predominant variant [226, 228]. All these modifications are made by different enzymes, which have been found to be expressed by leukocytes and different cell types inside inflamed tissues, in tumors, by bacteria or are induced during viral infection [16]. Once modified, chemokines exhibit differences in potency, stability and GAG binding affinity compared to their unmodified variants and these properties are reflected by alteration in leukocyte recruitment, modulating inflammatory responses [16, 226, 230]. Among chemokines, modified variants of the CXC chemokines are the best characterized, with CXCL5, CXCL8, CXCL10 and CXCL11 that can also undergo specific citrullination on their arginine residues close to the N-terminus, the chemokine region mostly involved in receptor signaling activation [228].

Chemokine receptors, as other GPCRs, upon binding of their cognate chemokines activate multiple signaling pathways elicited by both G proteins and β-arrestins, which respectively on the one hand immediately activate calcium release from intracellular stores and inhibit adenylyl cyclase, and on the other hand uncouple G proteins from the receptor, sustain delayed signaling and mediate receptor internalization [114]. Conventionally chemokines equally activate all the signaling pathways, but in some cases, defined by the cellular context or by ligand specificity, chemokines can preferentially activate one signaling pathway rather than the other [34, 99, 134]. This can be the case of CCR5, which, upon binding of CCL8 preferentially signals inducing Ca^{2+} release rather than inhibiting adenylyl cyclase or mediating β-arrestin recruitment to the receptor, compared to CCL3 [99]. Another example is the activity of CXCL11 on CXCR3, which induces receptor internalization better than G protein or β-arrestin recruitment [102]. This phenomenon, known as functional selectivity or biased signaling, dictates ligand activity and can influence the chemotactic potential of one agonist compared to another one.

Post-translational modification of two chemokines specific for CXCR1 and CXCR2 receptors, CXCL5 and CXCL8, have already been described to modify chemokine activity both in vitro and in vivo [228]. In particular, N-terminal truncation of both chemokines increases their signaling and chemotactic activity, while citrullination of their most N-terminal arginine reduces or increases signaling activity of CXCL5 and CXCL8, respectively [240, 242, 245]. Given the role of these two chemokines in neutrophils

and monocytes recruitment, the role of these cells in inflammation and that these modifying enzymes are released in inflamed tissues regulating chemokine activity, we decided to investigate in vitro how post-translational modifications affect G protein activation and β-arrestin recruitment of chemokines CXCL5 and CXCL8 signaling elicited through their receptors CXCR1 and CXCR2, and whether possible differences observed between differently modified chemokines could underlie a signal bias.

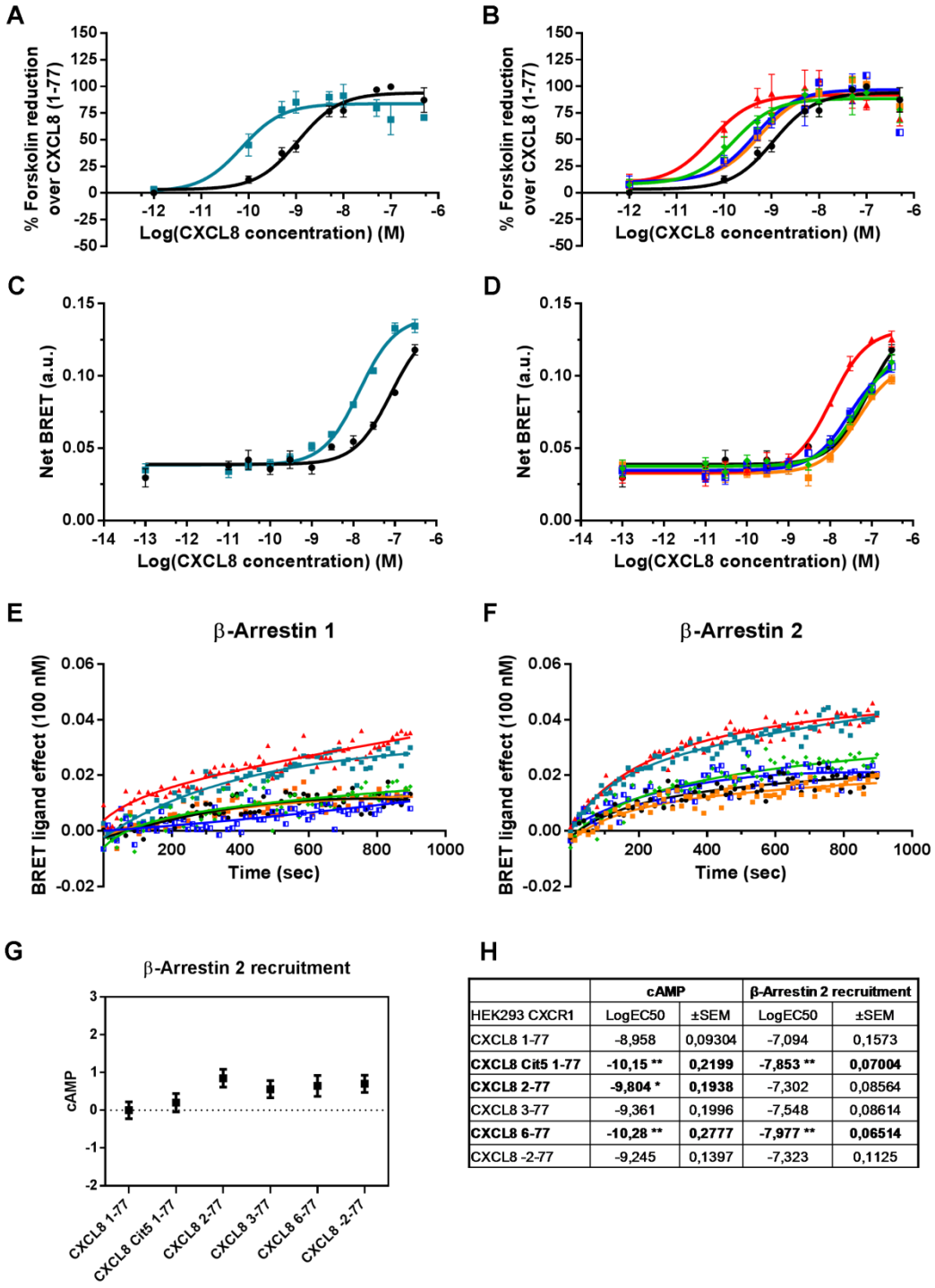
4.1.1-POST-TRANSLATIONAL MODIFICATIONS OF CXCL8 ALTER CXCR1 SIGNALING ABILITY

Modifications of NH_2-terminus of CXCL8 have been demonstrated to modify chemokine binding and activity on its receptors CXCR1 and CXCR2 [240]. In particular evidences reported that while citrullination of arginine in position 5 does not produce major changes in *in vitro* chemokine activity, truncation of the most NH_2 residues up to the 9^{th}, which leave an intact ELR motif, improve chemokine binding and *in vitro* ability to signal and mediate chemotaxis on CXCR1 and CXCR2. To understand whether these differences observed among CXCL8 variants in terms of activity underlie a biased activation of CXCL8 receptors, we evaluated the ability of several CXCL8 variants (intact CXCL8 1-77, truncated CXCL8 2-77, 3-77, 6-77 and the citrullinated CXCL8 on arginine 5 in intact 1-77 $–cit^5$ CXCL8 1-77) to activate G proteins reducing intracellular cAMP and to induce β-arrestins recruitment to both CXCR1 and CXCR2, by Alphascreen technology and BRET, respectively.

Measurement of CXCL8 variants ability to activate G proteins by Alphascreen assay in presence of forskolin results in an increase in Alphascreen signal, indicating that increasing chemokine concentration results in a stronger energy transfer between donor and acceptor beads. This assay is in fact based on the formation of a complex consisting of donor beads coated with streptavidin, biotinylated cAMP and acceptor beads coated with an antibody against cAMP and the necessity for the energy transfer to occur between donor and acceptor beads in close proximity. This assay is based on the production upon excitation with light at 680 nm by donor beads of oxygen singlets that diffuse within 200 nm at most before decaying, and if acceptor beads are in close proximity can be excited by oxygen singlets and emit light between 520 and 620 nm. The close proximity and the energy transfer between donor and acceptor beads is achieved only when a biotinylated cAMP is bound on one side by the streptavidin coated donor bead, and on the other side by the antibody against cAMP on the acceptor bead, therefore the maximal luminescent signal can be recorded when all the beads are complexed together by biotinylated cAMP, and any interference by non-biotinylated cAMP that is bound by acceptor beads cannot bring donor beads in close proximity to the acceptor and results in a loss of energy transfer and signal. The assay is designed to provide a robust signal in response to endogenous cAMP, which can be generated by stimulated adenylyl cyclases (as in the case of stimulatory Gαs proteins), resulting in a great displacement of biotinylated cAMP from donor-acceptor beads complexes and signal reduction, while in the case of adenylyl cyclases inhibition (as by Gαi proteins activation) the

resolution of the assay is reduced because it would have to measure minimal difference of cAMP concentrations. To overcome this problem we tested the ability of forskolin to induce adenylyl cyclase activation and cAMP accumulation in HEK293 cells in a dose-dependent manner, to measure forskolin concentration necessary to induce cAMP increase in at least 90% of the cells (EC90), and we found out that 10 μM of forskolin was sufficient. To this point we performed the assay adding to cells not only different concentrations of chemokines but also simultaneously constant 10 μM forskolin to increase our sensitivity range. When we measured the ability of the different CXCL8 variants to reduce forskolin activity, we normalized the values recorded to the ones assayed for intact CXCL8 1-77, to compare all the differences observed to the same reference chemokine. Results obtained indicate that citrullination on Arg^5 of CXCL8 increase chemokine potency measured as G protein activation of more than 10 fold, compared to CXCL8 1-77 (Figure 9, A and H), and truncation of NH_2-terminus of CXCL8 as well progressively increases chemokine potency, with only a trend measurable for CXCL8 3-77 but a statistically significant increase for CXCL8 2-77 and even more important increase for CXCL8 6-77, that displays a potency comparable to the citrullinated variant (Figure 9, B and H). Notably, the variant derived from the alternative CXCL8 precursor -2-77, appeared to be slightly more potent than the 1-77 variant (Figure 9, B and H).

G protein activation by GPCR is conventionally followed by receptor desensitization, that prevents excessive cellular responses and regulates agonist activity. Desensitization is initiated both by G protein-activated kinases that phosphorylate GPCR and by structural rearrangement of the GPCR itself, that exposes its carboxy-terminal intracellular tail to kinases and also to β-arrestins, that binding the GPCR on the one side sterically occupy GPCR intracellular site preventing inactive G proteins association to the receptor, and on the other side scaffold the assembly of different proteins and mediate their interaction with the receptor. These proteins are mainly components of the endocytotic machinery that induces receptor internalization to dampen cellular responsiveness to agonist, but also are components of different signaling machineries that can be activated by the receptor. Both CXCL8 receptors have been demonstrated to undergo agonist-induced desensitization and internalization in a β-arrestin-dependent manner [176, 177], therefore we decided to assess if post-translational modifications of CXCL8 can affect its ability to induce β-arrestins translocation and association to CXCR1 and CXCR2.



	cAMP		β-Arrestin 2 recruitment	
HEK293 CXCR1	LogEC50	±SEM	LogEC50	±SEM
CXCL8 1-77	-8,958	0,09304	-7,094	0,1573
CXCL8 Cit5 1-77	**-10,15 ****	**0,2199**	**-7,853 ****	**0,07004**
CXCL8 2-77	**-9,804 ***	**0,1938**	-7,302	0,08564
CXCL8 3-77	-9,361	0,1996	-7,548	0,08614
CXCL8 6-77	**-10,28 ****	**0,2777**	**-7,977 ****	**0,06514**
CXCL8 -2-77	-9,245	0,1397	-7,323	0,1125

Figure 9: Effects of CXCL8 modifications on CXCR1 signaling. CXCL8 variants are reported as CXCL8 1-77 (●, black circle), CXCL8 cit[5] 1-77 (■, petrol square), CXCL8 2-77 (◆, green rhombus), CXCL8 3-77 (▯, half clear, blue square), CXCL8 6-77 (▲, red triangle) and CXCL8 -2-77 (■, orange square). **A-B)** Alphascreen measurement of intracellular cAMP in HEK293 CXCR1 cells stimulated with 10 µM forskolin and different concentrations of CXCL8 variants, with the result reported as percentage of inhibition of forskolin-induced cAMP increase normalized over intact CXCL8 1-77 ±SEM; N=3. **C-D)** Measurement of CXCL8 variants-induced β-arrestin 2 recruitment to CXCR1 in living HEK293 cells by BRET. Results are expressed as Net BRET calculated subtracting the BRET ratio obtained by cells expressing CXCR1-RLuc only stimulated for 30 minutes with different concentrations of commercial CXCL8 from the BRET ratio obtained from cells co-transfected with CXCR1-RLuc and β-arrestin 2-EYFP and stimulated for 30 minutes with corresponding concentrations of CXCL8 variants ±SEM; N=3. **A** and **C** are the comparison of citrullinated CXCL8 versus intact CXCL8, and **B** and **D** report the effects of NH_2-terminus truncation on CXCL8. **E-**

F) Effect of citrullination and truncation on CXCL8-induced β-arrestin 1 (panel **E**) and β-arrestin 2 (panel **F**) recruitment to CXCR1. HEK293 cells co-transfected with CXCR1 and β-arrestin 1 or 2 were first incubated with 5 μM Coelenterazine H for 8 minutes at room temperature and subsequently CXCL8 variants were added at the final concentration of 100 nM before beginning recording luminometer readings up to 15 minutes. One representative experiment out of 4 performed is shown for each condition. **G**) Bias plot indicates the ΔΔlog(τ/KA) ±SE for cAMP signaling over β-arrestin 2 recruitment. **H)** Table reports the mean LogEC50 ±SEM obtained by nonlinear regression curve fitting in the cAMP and β-arrestin 2 recruitment assays. Values statistically different from CXCL8 1-77 are reported in bold, with *=p<0.05, **=p<0.01 calculated by one-way Anova with Dunnett's multiple comparison.

To investigate interactions between chemokine receptors and β-arrestins we used BRET, a technique based on the energy transfer occurring between Renilla Luciferase (RLuc), that catalyzes its substrate Coelenterazine H emitting light peaking at 480 nm and EYFP, a modified GFP variant that is excited by the light produced by RLuc and emits light peaking at 530 nm, only if it is found in close proximity (100 nm) to the donor. BRET exploits this properties of RLuc and EYFP to measure interactions between two proteins in live cells by the co-expression of the protein of interest fused to the donor or to the acceptor by co-transfecting plasmids encoding the two fusion proteins in cells and measuring the energy transfer by assessing luminescence at 480 and 530 nm generated by samples [321].

To measure β-arrestins recruitment to CXCR1 after stimulation with the different CXCL8 variants we initially performed kinetic BRET measurements assessing interactions between CXCR1-RLuc and β-arrestins-EYFP overexpressed in HEK293 cells by co-transfection of the two plasmids. Therefore we recorded luminescence generated by coelenterazine H and transferred to CXCR1-RLuc and β-arrestins-EYFP arising from samples stimulated with PBS or 100 nM of each CXCL8 variant over 15 minutes time, collecting luminescence from each sample every 13 seconds. Results obtained indicate that energy transfer expressed as BRET ratio (Emission 530 nm/ Emission 480 nm) increases over time in cells stimulated with CXCL8 variants compared to cells stimulated with PBS, assessed subtracting BRET ratio obtained in cells stimulated with PBS from BRET ratio obtained in cells stimulated with chemokines, in curves with a trend towards positive values (Figure 9 E, F). Apparently the same chemokines induce a stronger increase in association between CXCR1 and β-arrestin 2 than β-arrestin 1, but anyway both β-arrestins increase their association to CXCR1 after chemokine stimulation, and similarly between the two β-arrestins CXCL8 6-77 and citrullinated CXCL8 induce a stronger increase compared to CXCL8 1-77 (Figure 9 E, F), while the other truncated variants induce comparable β-arrestins recruitment to CXCR1 to CXCL8 1-77 (Figure 9 E, F). Since all the variants generate a similar efficacy ranking in the recruitment between β-arrestin 1 and 2 to CXCR1, but apparently β-arrestin 2 is recruited better than β-arrestin 1, we decided to characterize how CXCL8 variants affect only β-arrestin 2 recruitment to CXCR1 in a dose-dependent manner, to avoid generating experimental bias dependent on poor signal output. Dose-response curves generated stimulating HEK293 cells co-expressing CXCR1-RLuc and β-arrestin 2-EYFP show how citrullination (Figure 9 C) and truncation of the first 5 amino acids in CXCL8 6-77 (Figure 9 D)

increase CXCL8 potency in terms of CXCR1 association to β-arrestin 2 almost ten times (Figure 9 H). More modest truncation on NH_2-terminal residues of CXCL8 and the elongated -2-77 variants demonstrate to be able to minimally increase the β-arrestin 2 recruitment ability of CXCR1, compared to CXCL8 1-77 (Figure 9 C and H).

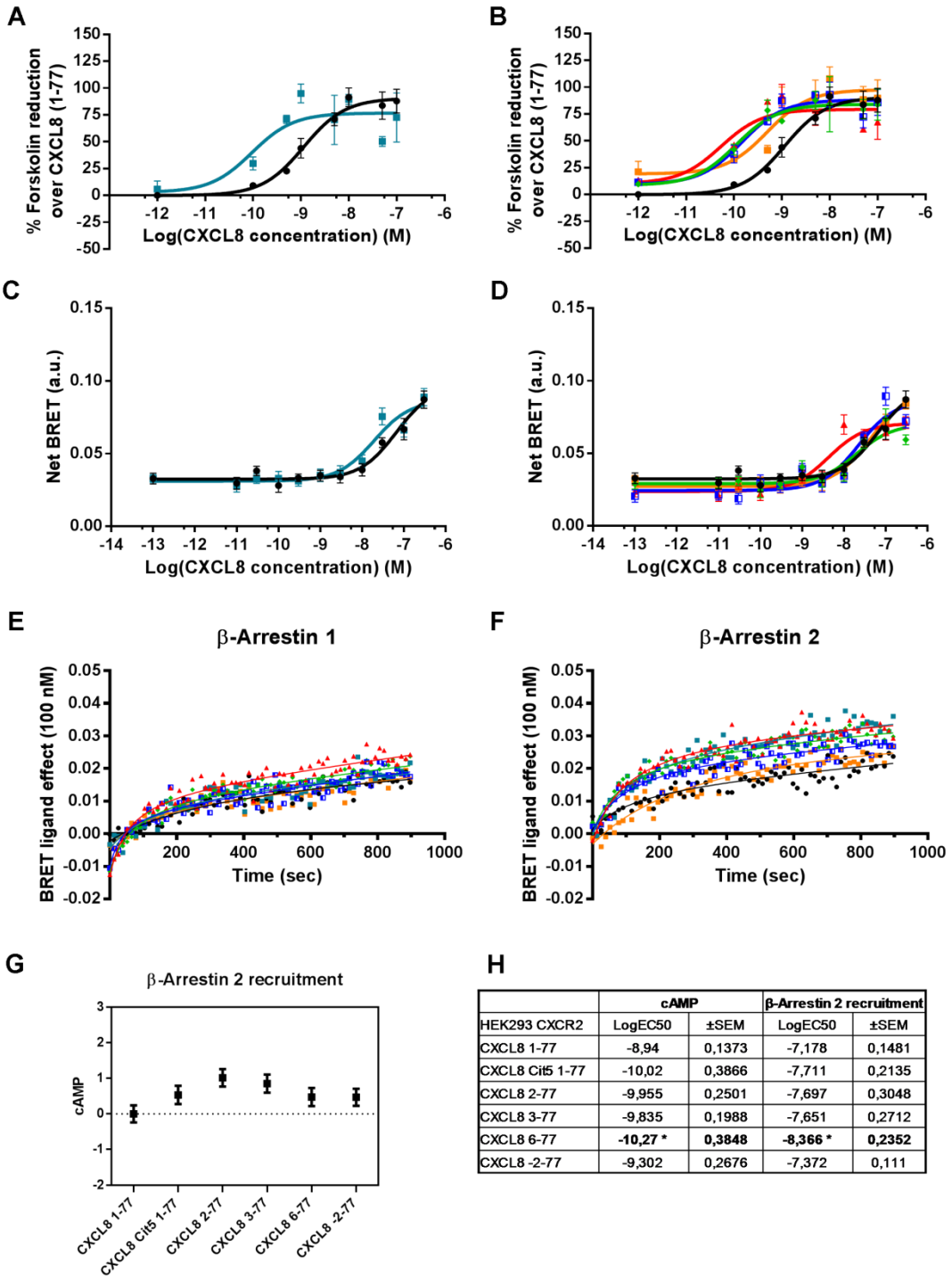
To determine whether post-translational modifications of CXCL8 could induce a preferential signaling of CXCR1 between cAMP or β-arrestin 2-recruitment, we calculated the bias occurring between these two signaling pathways comparing for the two assays the activity of each CXCL8 variant, compared to the activity of intact CXCL8 1-77. To quantify bias we recurred to the operational model of bias, that allows to compare the activity of different ligands in different pathways normalizing all the signals recorded from all the ligands regardless their agonistic activity is full or partial, obtaining a single parameter that is called transduction coefficient (TC, =log(τ/KA)) [117, 322, 323]. In this valure are included several parameters of the system assayed, since τ incorporates ligand efficacy, receptor density and coupling with the system used for the assay, while KA is the reciprocal of the conditional affinity of the ligand in the assay system [322], and this value can be calculated by fitting the Black-Leff operational model to dose-response curves in all the pathways assayed. This system allows to calculate bias in a way preventing the interference of system or observational bias that could occur due to experimental settings [84]. Transduction coefficients measured have been for each assay have been compared for all the CXCL8 variants, resulting in no statistically significant variation in bias factors measured on CXCR1.

4.1.2-Post-translational modifications of CXCL8 modify CXCR2 signaling

We next sought to address the effects of post-translational modifications of CXCL8 on the signaling activities of its second receptor, CXCR2. To evaluate the impact of CXCL8 modifications on CXCR2 signaling we used HEK293 cells stably expressing CXCR2 to measure cAMP modulation and transiently transfected HEK293 cells to monitor β-arrestins recruitment to CXCR2 by BRET.

Dose response curves generated by Alphascreen cAMP assay indicate that both citrullination of CXCL8 and removal of its NH_2-terminal residues induce an increase in CXCL8 potency (Figure 10 A, B and H), with curves shifted to the left and EC50 concentrations decreasing, but only for CXCL8 6-77 this increase in potency is statistically relevant, even if for citrullinated CXCL8 has been calculated a potency 10 times higher than CXCL8 1-77 (Figure 10 H).

Measurement of the ability of the different variants to induce β-arrestins association to CXCR2 over time showed that after agonist activation both β-arrestin 1-EYFP and β-arrestin 2-EYFP are recruited to CXCR2-RLuc (Figure 10 E and F), with a higher trend for β-arrestin 2 than β-arrestin 1. Dose response curves indicate that CXCL8 6-77 can induce a strong increase of more than 10 times in β-arrestin 2 recruitment to CXCR2 compared to CXCL8 1-77 (Figure 10 D and H), while all the other variants only tend to increase CXCL8 activation of this signaling pathway (Figure 10 C, D and H).



	cAMP		β-Arrestin 2 recruitment	
HEK293 CXCR2	LogEC50	±SEM	LogEC50	±SEM
CXCL8 1-77	-8,94	0,1373	-7,178	0,1481
CXCL8 Cit5 1-77	-10,02	0,3866	-7,711	0,2135
CXCL8 2-77	-9,955	0,2501	-7,697	0,3048
CXCL8 3-77	-9,835	0,1988	-7,651	0,2712
CXCL8 6-77	**-10,27 ***	**0,3848**	**-8,366 ***	**0,2352**
CXCL8 -2-77	-9,302	0,2676	-7,372	0,111

Figure 10: Effects of CXCL8 modifications on CXCR2 signaling. CXCL8 variants are reported as CXCL8 1-77 (●, black circle), CXCL8 cit[5] 1-77 (■, petrol square), CXCL8 2-77 (◆, green rhombus), CXCL8 3-77 (▯, half clear, blue square), CXCL8 6-77 (▲, red triangle) and CXCL8 -2-77 (■, orange square). **A-B)** Alphascreen measurement of intracellular cAMP in HEK293 CXCR2 cells stimulated with 10 µM forskolin and different concentrations of CXCL8 variants, with the result reported as percentage of inhibition of forskolin-induced cAMP increase normalized over intact CXCL8 1-77 ±SEM; N=3. **C-D)** Measurement of CXCL8 variants-induced β-arrestin 2 recruitment to CXCR2 in living HEK293 cells by BRET. Results are expressed as Net BRET calculated subtracting the BRET ratio obtained by cells expressing CXCR2-RLuc only stimulated for 30 minutes with different concentrations of commercial CXCL8 from the BRET ratio obtained from cells co-transfected with CXCR2-RLuc and β-arrestin 2-EYFP and stimulated for 30 minutes with corresponding concentrations of CXCL8 variants ±SEM; N=3. **A** and **C** are the comparison of citrullinated CXCL8 versus intact CXCL8, and **B** and **D** report the effects of NH_2-terminus truncation on CXCL8. **E-**

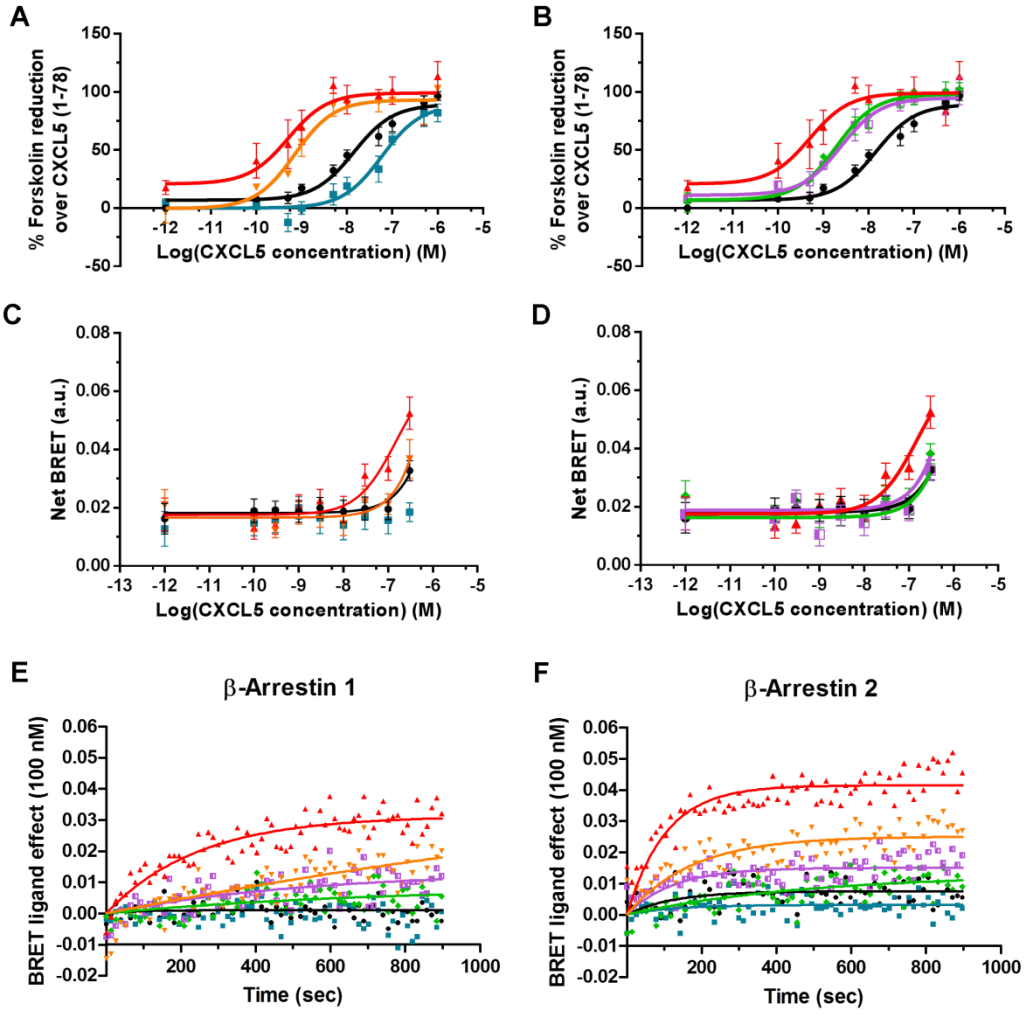
F) Effect of citrullination and truncation on CXCL8-induced β-arrestin 1 (panel **E**) and β-arrestin 2 (panel **F**) recruitment to CXCR2 monitored over 15 minutes time in presence of PBS or 100 nM of CXCL8 variants. One representative experiment out of 4 performed is shown for each condition. **G**) Bias plot indicates the ΔΔlog(τ/KA) ±SE for cAMP signaling over β-arrestin 2 recruitment. **H)** Table reports the mean LogEC50 ±SEM obtained by nonlinear regression curve fitting in the cAMP and β-arrestin 2 recruitment assays. Values statistically different from CXCL8 1-77 are reported in bold, with *=p<0.05 calculated by one-way Anova with Dunnett's multiple comparison.

Bias analysis has been performed to assess the existence of a bias in signaling activities elicited by CXCL8 on CXCR2 after post-translational modifications. The results obtained indicate that neither citrullination of arginine 5 on cit^{5}CXCL8 nor truncation of the NH_2-terminus of CXCL8 1-77 induce a preferential activation of β-arrestin 2 recruitment to CXCR2 over adenylyl cyclase inhibition (Figure 10 G).

Taken together, these results indicate that post-translational modifications of CXCL8 NH_2-terminus affect chemokine signaling properties on both CXCR1 and CXCR2, with more evident effects on CXCR1, increasing agonist potency in the cases of citrullination of arginine 5, cleavage of the first amino acids by different proteases. Notably, the elongated variant CXCL8 -2-77 also displays an increased activity on CXCR1 and a trend towards increase on CXCR2.

4.1.3-POST-TRANSLATIONAL MODIFICATIONS ALTER CXCL5 SIGNALING ACTIVITY ON CXCR2

We also investigated the ability of post-translational modifications to unbalance CXCR2 signaling properties modifying CXCL5. As CXCL8, CXCL5 can be truncated on its NH_2-terminus and can also undergo the action of peptidyl deiminases, that citrullinate arginine in position 9, resulting in modifications of CXCL5 signaling properties in vitro and chemotactic ability in vivo. To investigate the effects of these modifications on CXCR2 signaling we assayed the ability to activate G proteins and β-arrestins recruitment to CXCR2 of CXCL5 citrullinated on arginine 9 in both intact CXCL5 1-78 (cit^{9}CXCL5 1-78) and truncated 9-78 CXCL5 (cit^{9}CXCL5 9-78) and of truncated CXCL5 4-78, 8-78 and 9-78 variants.
Results indicate that citrullination of CXCL5 decreases chemokine potency to activate Gα inhibitory proteins of about 4 times (Figure 11 A and H) both in the case of cit^{9}CXCL5 1-78 compared to intact CXCL5 1-78 and also in the case of cit^{9}CXCL5 9-78 compared to the non citrullinated truncated CXCL5 9-78. Interestingly, cit^{9}CXCL5 9-78 displays an increased potency compared to CXCL5 1-78 of more than 10 fold (Figure 11 A and H). CXCL5 truncation of NH_2 residues increases chemokine potency, ranging from more than 10 times in the case of CXCL5 4-78 and CXCL5 8-78 to about 50 times for CXCL5 9-78 (Figure 11 B and H).



H

	cAMP		β-Arrestin 2 recruitment	
HEK293 CXCR2	LogEC50	±SEM	LogEC50	±SEM
CXCL5 1-78	-7,781	0,0715	~ -2,854	0,1877
CXCL5 Cit9 1-78	**-7,088** *	**0,08547**	N.D.	
CXCL5 4-78	**-8,791** ***	**0,06976**	~ -3,347	0,1748
CXCL5 8-78	**-8,699** **	**0,09265**	~ -3,171	0,1695
CXCL5 9-78	**-9,54** ***	**0,1513**	-6,82	0,1266
CXCL5 Cit9 9-78	**-9,057** ***,#	**0,08845**	~ -3,616	0,1578

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	β-Arrestin 1		β-Arrestin 2	
CXCR2	BRETmax	±SEM	BRETmax	±SEM
CXCL5 1-78	0,0009611	0,0005429	0,007530	0,0006852
CXCL5 Cit9 1-78	N.D.		0,003141	0,0008704
CXCL5 4-78	0,008510	0,005739	0,01301	0,003013
CXCL5 8-78	0,01361	0,004377	**0,01508** **	**0,0008235**
CXCL5 9-78	0,03133	0,001447	**0,04150** ***	**0,0007620**
CXCL5 Cit9 9-78	0,05819	0,07681	**0,02509** ***, ###	**0,0008092**

<u>Figure 11</u>: Effects of CXCL5 modifications on CXCR2 signaling. CXCL5 variants are reported as CXCL5 1-78 (●, black circle), CXCL5 cit[9] 1-78 (■, petrol square), CXCL5 4-78 (◆, green rhombus), CXCL5 8-78 (◨, half clear, purple square), CXCL5 9-78 (▲, red triangle) and cit[9] CXCL5 9-78 (▼, orange down-facing triangle). **A-B)** Alphascreen measurement of intracellular cAMP in HEK293 CXCR2 cells stimulated with 10 µM forskolin and different concentrations of CXCL5 variants, with the result reported as percentage of inhibition of forskolin-induced cAMP increase normalized over intact CXCL5 1-78 ±SEM; N=3. **C-D)** Measurement of CXCL5 variants-induced β-arrestin 2 recruitment to CXCR2 in living HEK293 cells by BRET. Results are expressed as Net BRET calculated subtracting the BRET ratio obtained by stimulated for 30 minutes with different concentrations of commercial CXCL5 from the BRET ratio obtained from cells co-transfected with CXCR2-RLuc and β-arrestin 2-EYFP and stimulated for 30 minutes with corresponding concentrations of CXCL5 variants ±SEM; N=2. **A** and **C** are the comparison of citrullinated CXCL5 versus non

citrullinated variants, and **B** and **D** report the effects of NH_2-terminus truncation on CXCL5. **E-F)** Effect of citrullination and truncation on CXCL5-induced β-arrestin 1 (panel **E**) and β-arrestin 2 (panel **F**) recruitment to CXCR2 monitored over 15 minutes time in presence of PBS or 100 nM of CXCL5 variants. One representative experiment out of 4 performed is shown for each condition. **G**) Bias plot indicates the ΔΔlog(τ/KA) ±SE for cAMP signaling over β-arrestin 2 recruitment. **H)** Table reports the mean LogEC50 ±SEM obtained by nonlinear regression curve fitting in the cAMP and β-arrestin 2 recruitment assays. **I)** Table reporting the mean BRETmax ±SEM obtained by one phase exponential association curve fitting in the kinetics of β-arrestins recruitment assays. N.D.=not determined. Values statistically different calculated by one-way Anova with Dunnett's multiple comparison are reported in bold, with *,#=p<0.05, **,##=p<0.01 and ***,###=p<0.001 indicating statistically relevant differences compared to CXCL5 1-78 (*) or CXCL5 9-78 (#).

We next assessed the activity of the different CXCL5 to induce β-arrestins recruitment to CXCR2 by BRET, and we observed that CXCL5 1-78 and its citrullinated variant did not induce over time an increase compared to PBS treated cells and CXCL5 4-78 shows only a tendency to increase over time with BRET ligand effect values oscillating above zero (Figure 11 E, F and I). Only variants truncated of more than 7 amino acids induce a significant increase in energy transfer between β-arrestin 1 and 2-EYFP and CXCR2-RLuc compared to PBS stimulated cells (Figure 11 E, F), with progressive truncation that progressively increases CXCL5 activity (Figure 11 panel I). In addition, citrullination of CXCL5 9-78 results in a significant decrease in BRETmax between CXCR2 and both β-arrestin 1 and 2, compared to CXCL5 9-78 (Figure 11 E, F and I).

We also monitored BRET between CXCR2 and β-arrestin 2 in cells stimulated with different concentrations of CXCL5 variants, but all these chemokines, at the concentrations assayed, induced an increase in BRET signal only at high concentration, far from reaching a point of signal saturation in all the cases (Figure 11 C and D). This lead to ambiguous fitting of nonlinear regression curves to all the curves, except for cit^{9}CXCL5 1-78, for which it has been impossible to calculate the fitting and for CXCL5 9-78 that generated a precise fitting but anyway it has to be taken into account that the result is influenced by the poor output recorded. This also affects bias calculation, since even if the operational model of bias generated a fitting for each condition, it resulted in values with high standard errors and that would anyway be influenced by the inability of CXCL5 to induce a strong β-arrestin 2 recruitment to CXCR2 at the concentrations tested.

These results indicate that citrullination of CXCL5 affects chemokine ability to activate CXCR2-dependent G protein signaling while on the opposite truncation improves chemokine potency. In terms of β-arrestin recruitment we cannot do precise quantitative conclusions of the ability of CXCL5 variants to induce a dose-dependent increase in β-arrestin 2 recruitment, but from kinetics measures we can say qualitatively that, as we observed for G protein signaling, β-arrestins recruitment to CXCR2 is induced by CXCL5 only after NH_2-terminus truncation, and that this effect can be reduced by CXCL5 citrullination of arginine 9.

4.2-Atypical chemokine receptor 2 (ACKR2) interactions with b-arrestins

Atypical chemokine receptors are fundamental regulators of chemokine activity that instead of directly promoting cellular migration, support system activity by transporting, presenting or scavenging their ligands, actively regulating chemokine activity or preventing excessive ligand accumulation [248]. Since their discovery and deorphanization, ACKRs have long been considered as decoy molecules unable to activate intracellular signaling, because high affinity binding of chemokines did not elicit Ca^{2+} fluxes generation or chemotaxis as the same ligands did on cognate conventional receptors. Recent evidences indicate indeed that chemokine binding on these receptors activates cellular responses, but these signaling events are more prone to occur in a biased fashion, since these receptor elicit only selected signals, compared to the activities of the same chemokines on conventional chemokine receptors [3, 143, 225]. This differences are believed to depend on mutation of key residues in motifs involved in GPCR signaling, generating seven transmembrane receptors that on the one hand lost G protein-dependent signaling and on the other retained the ability to signal towards different pathways, many of which mediated by β-arrestins, making of ACKRs structurally biased receptors that evolved from conventional chemokine receptors to play an important regulatory role in chemokine biology [33, 143, 225]. Among ACKRs, ACKR2 is one of the best characterized in terms of signaling, trafficking activity and biological function. ACKR2 is expressed more prominently in cells of barrier tissues such as trophoblasts in placenta and endothelial cells in afferent lymphatic vessels, where it binds with high affinity a broad range of inflammatory CC chemokines and mediates their degradation [142, 297]. Efficient chemokine degradation is possible thanks to ACKR2 peculiar trafficking properties and is supported by its biased signaling activity. In fact, upon active ligand stimulation, ACKR2 activates a G protein-independent and β-arrestin 1-dependent signaling pathway that improves receptor scavenging ability [292, 310]. This biased signaling activity allows the receptor to actively modify its trafficking properties increasing its recycling rate to upregulate its expression on plasma membrane and offer more binding sites to chemokines, that once bound to the receptor are continuosly internalized with ACKR2 that targets them to degradation [292, 313]. Given ACKR2 characteristics of structurally biased signaling receptor and its role in the regulation of the chemokine system, with an impact on inflammation and tumor biology, we decided to better investigate ACKR2 signaling activities focusing on the characterization of molecular mechanisms underlying the differences between ACKR2 and CCR5 activity that allow this atypical receptor to control chemokine activity dampening inflammation rather than mediating cell migration. To this points we wanted to better define ACKR2 interactions with β-arrestins, since the signaling machineries controlled by these proteins are fundamental to regulate ACKR2 trafficking and activity [292, 309, 324].

4.2.1-ACKR2 IS ASSOCIATED TO B-ARRESTINS IN BASAL CONDITIONS

ACKR2 colocalization to β-arrestin has already been described to occur constitutively in cells transfectants analyzed by confocal microscopy [309, 324]. Published evidences show that ACKR2 overexpression is able to induce relocalization of β-arrestin in basal conditions in HEK293 cells [309] and that in β-arrestin knock out mouse embryonic fibroblasts overexpressed ACKR2 loses its subcellular localization and trafficking properties [324]. To confirm this evidence we decided to assess ACKR2 constitutive interactions with β-arrestins by BRET^2, a BRET variant that compared to BRET has a broader emission peaks resolution and therefore has a better quality of detection [321]. To this point we transfected HEK293 cells to express constant amount of ACKR2 or CCR5 fused to RLuc and increasing amounts β-arrestin 1 or β-arrestin 2 fused to GFP^2 or free GFP^2 to measure non-specific interactions. As it can be observed in panel A of Figure 12, energy transfer between ACKR2 and both β-arrestin 1 and β-arrestin 2 rapidly increases from low ratios of fluorescence over luminescence, meaning that luciferase fused to ACKR2 can easily transfer exciting light to β-arrestins-GFP^2 also in conditions of poor relative abundance of donor protein compared to acceptor. In contrast, in the case of co-transfection of ACKR2-RLuc with free GFP^2, BRET ratio increases linearly due to spontaneous collision between donor and acceptor (Figure 12 A), differently from energy transfer occurring between ACKR2-RLuc and β-arrestins-GFP^2, which increases as a hyperbolic function, reaching a plateau at saturating concentrations of acceptor. This is indicative of a strong specific constitutive association between ACKR2 and β-arrestins. Differently from ACKR2, when CCR5-RLuc association with β-arrestins-GFP^2 is investigated, saturation curves generated by CCR5-RLuc with β-arrestins-GFP^2 do display an hyperbolic behavior ending with a plateau, but are poorly separated by the curve generated by CCR5-RLuc and free GFP^2 (Figure 12 B), meaning that only a minimal association between CCR5 and β-arrestins occurs in basal conditions.

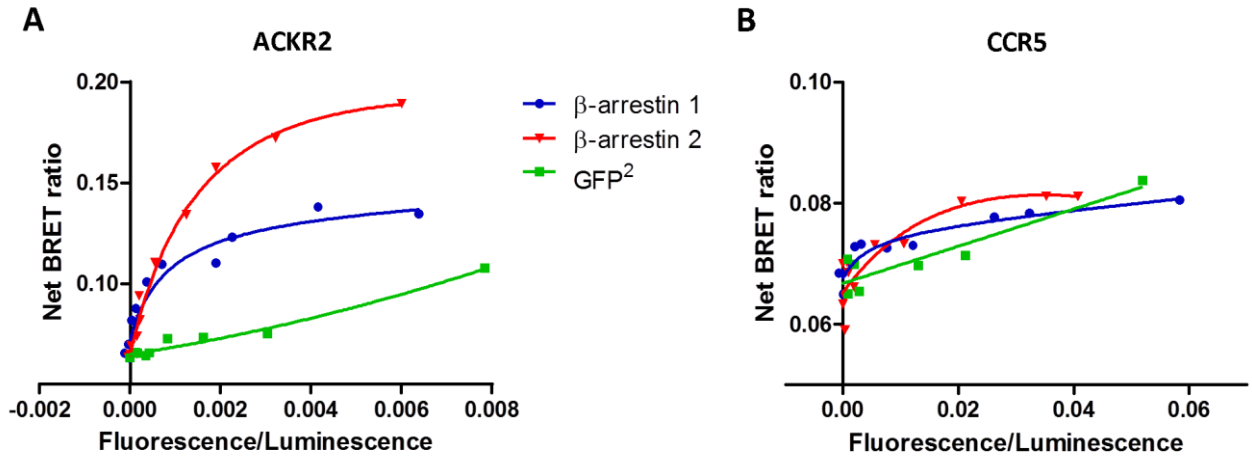
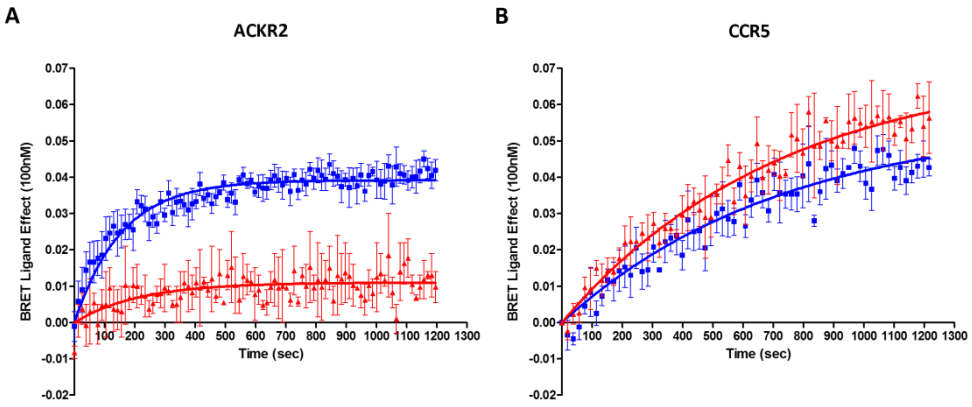


Figure 12: ACKR2 strongly associates β-arrestins in unstimulated conditions. Saturation binding curves obtained plotting Net BRET^2 values, measured after addition of 5 μM DeepBlueC, as functions of the amount of donor and acceptor molecules present in each transfectant, expressed as ratio between GFP^2 quantification, measured after direct fluorescent excitation, and RLuc quantification obtained as measurement of luminescent signal after addition of 5 μM Coelenterazine H. Curves were obtained by coexpression of ACKR2-RLuc (**A**) or CCR5-RLuc (**B**) together with β-arrestin 1-GFP^2 (blue circle, ●), β-arrestin 2-GFP^2

(red triangle, ▼), or free GFP[2] (green square, ■). Data reported are representative of 1 experiment out of three independent replicates.

4.2.2-Active ligands stimulation increases preferentially β-arrestin 1 recruitment to ACKR2

Activation of CCR5 by agonists results in the modulation of several signaling pathways sustained by G proteins and β-arrestins, with the latter that also occupy receptor intracellular domains to block further G protein coupling and mediate its internalization through clathrin-dependent endocytosis [86]. Conversely, high affinity binding to ACKR2 of many CC inflammatory chemokines does not result in Ca^{2+} fluxes generation and cell migration but in the activation of a β-arrestin-dependent signaling pathway. This signaling pathway exploits β-arrestin 1 to activate the small GTPase Rac1, that activating different downstream effectors as PAK1, LIMK1 and inducing Cofilin phosphorylayion controls actin cytoskeleton rearrangement, that sustains an increase in receptor expression on the cell surface and improves ACKR2 scavenging activity [292, 310, 325]. To better elucidate the relationship existing between ACKR2 and β-arrestins we decided to investigate their association in presence of ACKR2 ligands by BRET, overexpressing in HEK293 cells ACKR2-RLuc and β-arrestins-EYFP by transient transfection. Experiments demonstrate that in presence of CCL3L1 the energy transfer between ACKR2-RLuc and β-arrestin 1-EYFP rapidly increases compared PBS-stimulated cells, while stimulation of cells expressing ACKR2-RLuc and β-arrestin 2-EYFP results in a weak increase of energy transfer (Figure 13 A and C). In contrast, stimulation of CCR5 with the same ligand CCL3L1 results in a strong increase in energy transfer between CCR5-RLuc with both β-arrestin 1- and β-arrestin 2-EYFP (Figure 13 B and C). These results indicate that ACKR2 stimulation with its active ligand CCL3L1 induces preferentially β-arrestin 1 recruitment to ACKR2, while in the case of CCR5, CCL3L1 induces comparable recruitment to both β-arrestin 1 and β-arrestin 2.



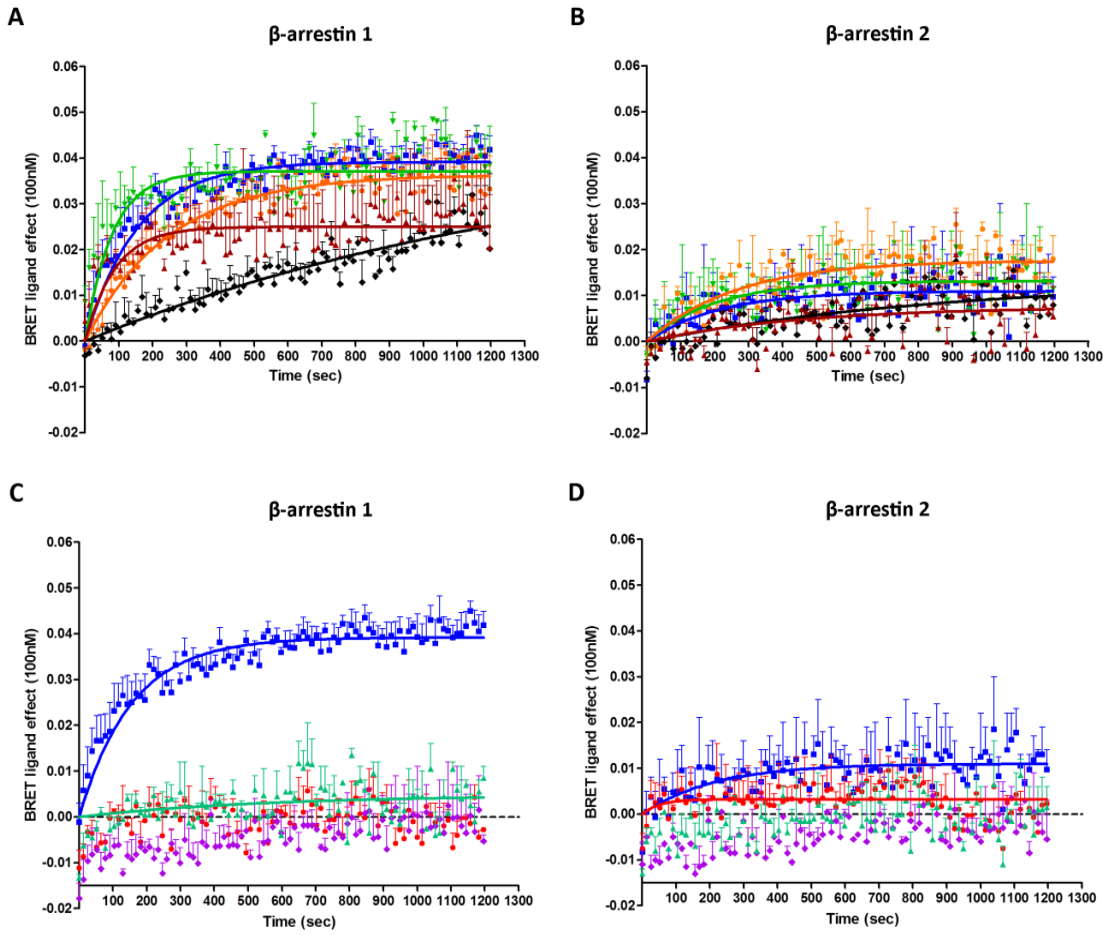
C

	β-arrestin 1		β-arrestin 2	
	BRETmax	±SEM	BRETmax	±SEM
ACKR2	**0,03915** ***	**0,0005093**	0,01097	0,0008032
CCR5	0,05611	0,004881	0,07117	0,005012

Figure 13: ACKR2 stimulation with CCL3L1 strongly increases receptor association preferentially with β-arrestin 1. BRET ligand effect kinetic curves were generated measuring up to 20 minutes BRET occurring in cells coexpressing ACKR2-RLuc (**A**) or CCR5-RLuc (**B**) together with β-arrestin 1-EYFP (■) or β-arrestin 2-EYFP (▲), stimulated with 5 µM coelenterazine H for 8 minutes and, prior to beginning luminescence recordings, with PBS or 100nM CCL3L1. Curves are obtained by fitting one phase exponential association function to BRET ligand effect plotted as function of time **C**) Table reports the BRETmax ±SEM values obtained by fitting one phase exponential association equation to BRET ligand effect plotted as function of time. Data reported are the results from at least 4 independent experiment, in bold are reported BRETmax values statistically different between β-arrestin 1 and β-arrestin 2 recruitment for each receptor. **=p<0.01 and ***=p<0.001 calculated by two-tailed unpaired Student's t-test comparing the results obtained for each receptor with the two β-arrestins.

Based on the degradative efficiency of the receptor, ACKR2 ligands can be distinguished between active ligands (agonists), the ones efficiently degraded by the receptor and able to activate ACKR2 signaling activity increasing ACKR2 expression on the plasma membrane, and neutral ligands, chemokines that bind ACKR2 with high affinity but are poorly or not degraded, do not activate the signaling cascade that leads to cofilin phosphorylation and receptor upregulation [292, 310]. We decided to evaluate the ability of some active and neutral ACKR2 ligands to induce β-arrestins recruitment, comparing their activity to CXCL8, a chemokine that does not bind to ACKR2. Stimulation of ACKR2 with its neutral ligand CCL3 and its NH_2-terminally truncated variant CCL3(-4) or the chemokine CXCL8 that does not bind ACKR2 results in BRET ligand effect values oscillating around zero in both β-arrestin 1 and β-arrestin 2 recruitment assay, where it has been possible to fit one phase exponential association curve only to CXCL8 and CCL3, respectively. (Figure 14 C,D and E). On the opposite active ligands such as CCL2, CCL4, CCL5 and CCL17 significantly induce energy transfer increase between ACKR2-RLuc and β-arrestin 1-EYFP compared to CXCL8 (Figure 14 A,E), while when tested on their ability to increase ACKR2 association to β-arrestin 2 all active ligands produce a trend towards the increase of BRET signal, but only CCL4 and CCL5 stimulation resulted in significantly different BRETmax, that we had to compare to CCL3 due to the inability of the equation to fit a curve to CXCL8 in this assay (Figure 14

B,E). These results indicate that active agonists induce an increase in ACKR2 association to β-arrestin 1, an effect that occurs better than with β-arrestin 2, and these increases occur only with ACKR2 active ligands and not with neutral or irrelevant ligands.



E

	β-arrestin 1		β-arrestin 2	
ACKR2	BRETmax	±SEM	BRETmax	±SEM
CCL3L1	**0,03915 *****	**0,0005093**	0,01097	0,0008032
CCL3	N.D.		0,003196	0,0004643
CCL3(-4)	N.D.		N.D.	
CCL2	**0,03710 *****	**0,0005612**	0,01317	0,000751
CCL4	**0,03632 ****	**0,0006755**	**0,01758 #**	**0,0007508**
CCL5	**0,04496 *****	**0,01036**	**0,01356 #**	**0,004694**
CCL17	**0,02497 ***	**0,0007902**	0,007582	0,001603
CXCL8	0,004779	0,002147	N.D.	

Figure 14: Only active ligands induce an increase in ACKR2 association with β-arrestin 1. BRET ligand effect kinetic curves were generated measuring up to 20 minutes BRET occurring in cells co-expressing ACKR2-RLuc together with β-arrestin 1-EYFP (**A, C**) or β-arrestin 2-EYFP (**B, D**) stimulated with PBS or with 100nM of active ligands (**A, B**) CCL3L1 (■), CCL2 (▼), CCL4 (●), CCL5 (◆) and CCL17 (▲); or stimulated with neutral ligands or chemokines not bound by ACKR2 (**C, D**) as CCL3 (●), CCL3(-4) (◆), CXCL8 (▲). Reported results are the mean of at least 3 independent replicates ±SEM, curves are obtained by fitting one phase exponential association equation to BRET ligand effect plotted as function of time. **E**) Table reports the BRETmax ±SEM values obtained and compared by one-way Anova with Dunnett's multiple comparison test, in bold are reported values statistically

different compared to CXCL8 (*) in the case of β-arrestin 1 recruitment assay, and to CCL3 (#) in β-arrestin 2 recruitment assay. *,#=p<0.05, **,##=p<0.01 and ***,###=p<0.001.

Even if CCR5 activation induces recruitment of both β-arrestins with only minimal differences in terms of magnitude in our assay, our results indicate that stimulation with active ligands increases ACKR2 association preferentially to β-arrestin 1. To exclude that observed differences in energy transfer magnitude are due to differences in terms of reporter proteins expression, we decided to assay by flow cytometry expression of β-arrestins and ACKR2 in parallel with each BRET experiment. We decided to quantify proteins expression by flow cytometry instead of assessing EYFP fluorescence and RLuc luminescence on plate-reader luminometer since ACKR2 is mainly expressed in intracellular compartments and interaction with β-arrestins regulate its subcellular distribution [292, 309, 324], so we wanted to avoid differences that can occur in receptor localization after β-arrestins overexpression since only receptor molecules present on plasma membrane are accessible to chemokine stimulation. Because of this, rather than assessing ACKR2 expression by measuring luminescence arising from RLuc that arises also from intracellular ACKR2 molecules, we quantified ACKR2 expression as the ability of an antibody directed against hemoagglutinin (HA) tag, expressed by our receptors, to bind to differently transfected cells. Therefore receptor expression has been determined on the flow cytometer as cell associated fluorescence recorded on the Allophycocyanin (APC) channel thanks to the recognition of cell-bound anti-HA antibody generated in mouse of a secondary antibody directed against mouse IgG1 and conjugated to APC, while β-arrestins-EYFP have been quantified by EYFP fluorescence recorded on the FITC channel. Our results indicate that ACKR2 is similarly expressed between β-arrestin 1 and β-arrestin 2 transfectants, when measuring APC mean fluorescence intensity (MFI) in these cells normalized to cells expressing ACKR2-RLuc alone (Figure 15 A), and also β-arrestin 1 and β-arrestin 2-EYFP have the same relative abundance when normalized to ACKR2 (Figure 15 B), indicating that both ACKR2 and β-arrestins expression is similar in both co-transfectants, meaning that differences observed in ACKR2 / β-arrestins energy transfer are not dependent on differences in reporter protein expression.

Different cell types have evolved to exert different functions, therefore can express different sets of molecules which regulate specific cellular responses in terms of intracellular signaling and phenotype. This differences can be also reflected by differences in the ability of one molecule to activate cell responses in different cell types, that depending on the ability of the signaling molecule to couple its downstream effectors in determined cell types, can result in a biased signaling defined as system bias [84]. Therefore to assess bias downstream a molecule, all the assays have to be performed in the same cell type, to avoid insurgency of system bias. HEK293 cells are considered as a model system to study intracellular signaling because of their high rate of proliferation, the easiness by which they can be transfected, and in the field of chemokine receptors because they endogenously express relevant amounts of CXCR4 receptor only, responding only to CXCL12. To be confident that differences observed

in HEK293 cells by BRET on ACKR2 recruitment to β-arrestins are not dependent on an intrinsic inability of ACKR2 to recruit β-arrestin 2 in HEK293 cellular system, we decided to repeat the experiments of β-arrestins recruitment to ACKR2 induced by CCL3L1 in the cell line HTR8. This cell line is derived from a malignant choriocarcinoma originated from placental trophoblasts, endogenously expressing ACKR2, and therefore can represent a system where ACRK2 can find a set of signaling partner more similar to the ones it has when endogenously expressed in vivo, compared to HEK293 cells. As it can be observed in panel C of Figure 15, in presence of CCL3L1 ACKR2-RLuc preferentially interacts with β-arrestin 1, compared to β-arrestin 2, in both the independent replicates we performed, indicating that preferential recruitment of β-arrestin 1 to activated ACKR2 is not an artifact dependent on the HEK293 cellular system used to assess these interactions.

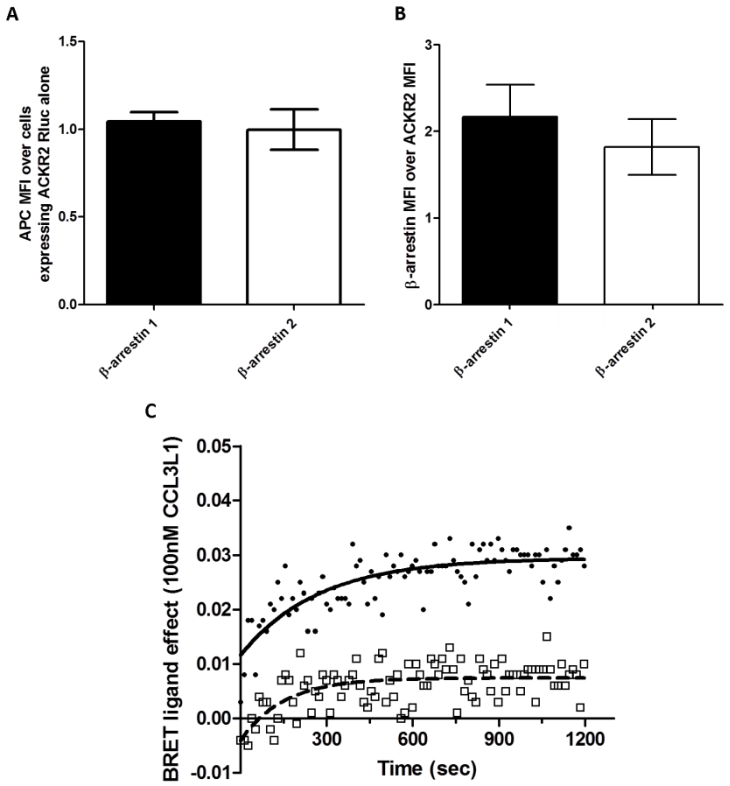


Figure 15: Differences between β-arrestin 1 and β-arrestin 2 recruitment by activated ACKR2 are not dependent on reporter expression or on the cellular system used for the assay. A-B) Flow cytometric analysis of anti-HA and APC-anti-mouse antibodies staining and EYFP-associated fluorescence on HEK293 cells co-transfected with HA-tagged ACKR2-RLuc and empty pcDNA3, β-arrestin 1-EYFP or β-arrestin 2-EYFP. Mean results obtained in 4 independent experiments are expressed as fold increase in APC mean fluorescence intensity (MFI) over cells co-transfected with HA-ACKR2-RLuc and pcDNA3 (**A**), or as relative β-arrestins-EYFP-dependent FITC fluorescence normalized over anti-HA antibody fluorescence (**B**). Statistical analysis was performed with paired Student's t-test and gave no significativity. **C)** Kinetic measure of 100 nM CCL3L1 effect on BRET in HTR8 cells co-transfected with ACKR2-RLuc and β-arrestin 1-EYFP (●, black circle and line) or β-arrestin 2-EYFP (□, empty square and disconnected line), over 20 minutes. Curves were generated by one-phase exponential association fitting, N=2.

Energy transfer techniques as BRET rely on the close proximity between donor and acceptor molecules, therefore energy transfer signals are extremely sensitive to distance between reporter proteins which, in the quaternary complex of proteins of interest and reporters, is influenced by their spatial orientation [321, 326]. Thus we investigated how reporter protein orientation and species-specificity affects BRET signal between ACKR2 and β-arrestins. To this point we cloned from human peripheral blood monocytes cDNA available in the laboratory β-arrestin 1 and β-arrestin 2 sequences in pEYFP-N1, pEYFP-C3, pRLuc-N3 and pRLuc-C1 vectors, to generate plasmids encoding for human β-arrestins fused to EYFP or RLuc by their C- or N-termini that we named *hs*β-arrestin (1 or 2)-EYFP, EYFP-*hs*β-arrestin, *hs*β-arrestin-RLuc and EYFP-*hs*β-arrestin, respectively, to distinguish them from plasmids encoding bovine β-arrestins previously used. We also generated plasmids to express ACKR2 and CCR5 fused by their intracellular C-termini to EYFP by subcloning their sequences from ACKR2-RLuc and CCR5-RLuc to pEYFP-N1. BRET measurements indicate that the combination of receptors fused to RLuc and *hs*β-arrestins to EYFP allows to record best increases in BRET signal, compared to the combination of receptors fused to EYFP and *hs*β-arrestins to RLuc, in cells stimulated with CCL3L1 (Figure 16 A-C versus B-D). In particular, for both ACKR2-RLuc and CCR5-RLuc the strongest energy transfer induced by CCL3L1 can be recorded when RLuc-fused receptors are co-transfected with EYFP-*hs*β-arrestins, indicating that, compared to EYFP fused to *hs*β-arrestin C-terminus, N-terminus-fused EYFP is brought to closer proximity to receptor C-terminus-fused RLuc, when β-arrestins are recruited to the receptor (Figure 16 A and C). Data indicate that in the case of activated CCR5-RLuc energy transfer occurs with a hierarchy that reflects data obtained with bovine β-arrestins-EYFP, with EYFP-*hs*β-arrestin 2 that is recruited better than EYFP-*hs*β-arrestin 1, and following EYFP-*hs*β-arrestin 1 is recruited better than *hs*β-arrestin 2-EYFP and *hs*β-arrestin 1-EYFP (Figure 16 C). CCL3L1-stimulated ACKR2 instead recruits *hs*β-arrestins with an energy transfer hierarchy that is EYFP-*hs*β-arrestin 1 > *hs*β-arrestin 1-EYFP > EYFP-*hs*β-arrestin 2 > *hs*β-arrestin 2-EYFP, meaning that, differently from CCR5 and despite EYFP orientation in the ACKR2-β-arrestin complex, ACKR2 preferentially increases its association to β-arrestin 1 rather than β-arrestin 2 (Figure 16 A).

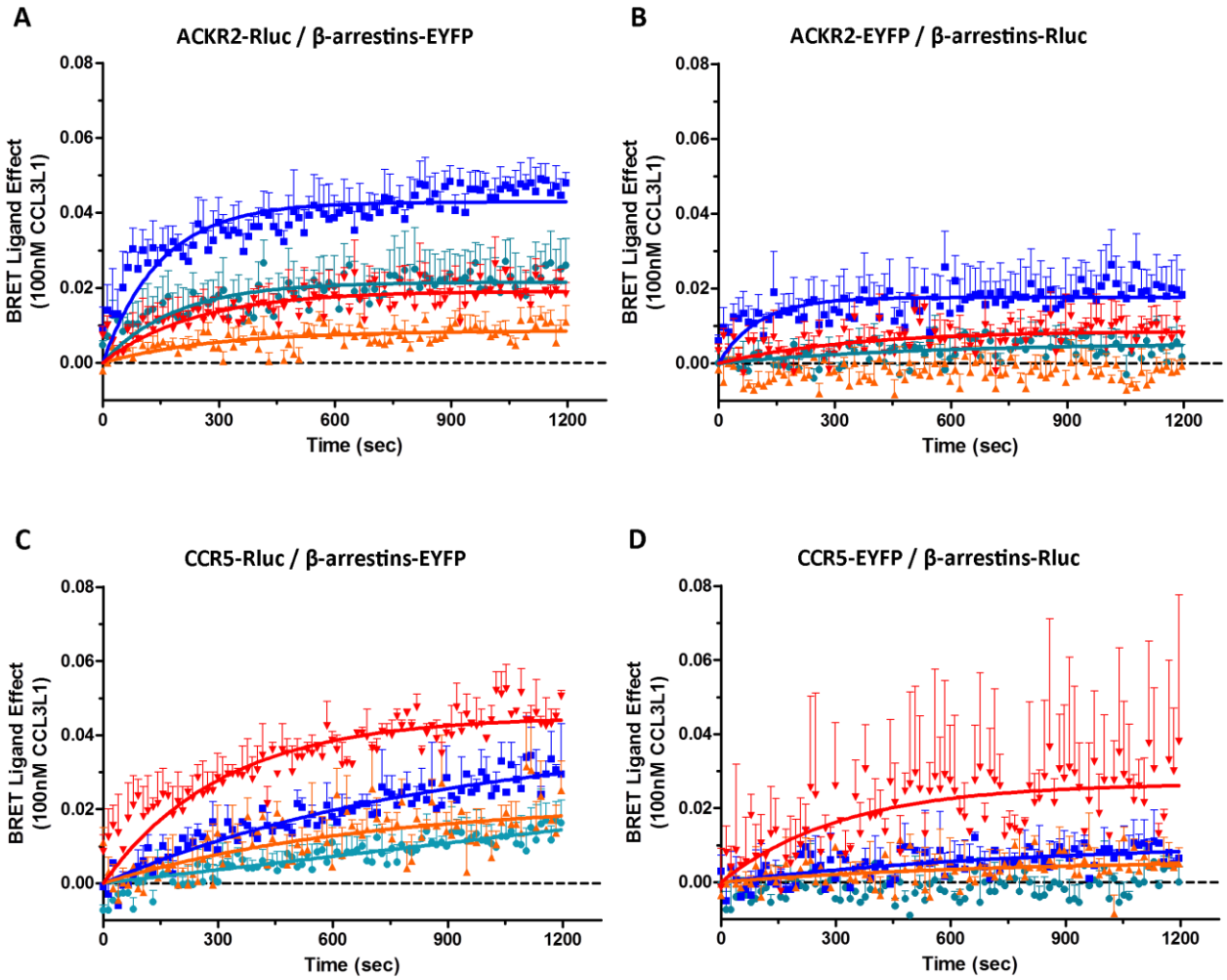


Figure 16: Energy transfer between ACKR2 and β-arrestin 1 occurs better than with β-arrestin 2 independently of reporter orientation. BRET ligand effect kinetic curves were generated measuring up to 20 minutes BRET occurring in cells stimulated with 100 nM CCL3L1 co-expressing ACKR2 fused to RLuc or EYFP (**A, B**) or CCR5-RLuc or EYFP (**C, D**) together with EYFP-human β-arrestin 1 (■), human β-arrestin 1-EYFP (●), EYFP- human β-arrestin 2 (▼) or human β-arrestin 2-EYFP (▲). Results are expressed as mean ± SEM of three independent replicates with curves were generated by one-phase exponential association equation.

4.2.3-ACKR2 IS DIFFERENTLY ASSOCIATED TO BOTH B-ARRESTIN1 AND B-ARRESTIN2 IN BASAL CONDITIONS

To efficiently scavenge inflammatory CC chemokines, ACKR2 exploits its ability to be constitutively internalized in Rab5-positive vesicles formed by clathrin-coated pits to reach intracellular compartments [312] that occurs in a β-arrestin-dependent manner [309, 324]. From intracellular stores ACKR2 is continuosly recycled back on the plasma membrane with Rab4- and Rab11-positive vesicles, while chemokines that have been internalized by ACKR2 are driven to degradation [313]. This peculiar trafficking affects receptor cellular distribution, in fact ACKR2 is mainly localized in perinuclear compartments, with only a minor portion of total molecules distributed on plasma membrane [312]. This distribution can be actively altered by ACKR2 upon ligand binding, activating G protein-independent and β-arrestin1-dependent signaling that sustains mobilization of intracellular receptor reserve from

perinuclear compartments towards cell surface [292]. To better understand our results obtained by BRET on cell populations, we decided to deeper investigate whether the differential association of β-arrestins to ACKR2 can be correlated to ACKR2 trafficking or localization. Therefore, to investigate whether this interactions occurred in defined cellular compartments, such as the cell surface or intracellular compartments, we decided to monitor interactions of both ACKR2 and CCR5 with β-arrestins by Image BRET, a technique based on BRET that allows to visualize protein-protein interactions in single live cells by a CCD camera-equipped microscope [319].

For this technique we generated plasmids encoding for bovine β-arrestins fused to RLuc8, a *Renilla Luciferase* engineered to produce a brighter signal compared to wild type RLuc, and we also fused ACKR2 and CCR5 sequences to VENUS, a brighter variant of EYFP with comparable spectral properties. In HEK293 cells we separately co-transfected plasmids encoding one β-arrestin fused to RLuc8 together with ACKR2-Venus, CCR5-Venus or an empty pDsRedMono vector, generating different cell lines transiently expressing either a combination of β-arrestin-RLuc8 and a receptor-Venus, or β-arrestin-RLuc8 with DsRed, a fluorophore irrelevant for the coelenterazine H-based BRET assay. The day after transfection, cells were harvested and each combination of cells expressing β-arrestins with receptors was mixed 1 to 1 with β-arrestin and DsRed-transfected cells before being seeded onto glass-bottomed culture dishes and 24 hours later images were acquired. Seeding together cells expressing β-arrestins-RLuc8 and DsRed together with cells expressing both β-arrestins-RLuc8 and one receptor fused to VENUS allows to discriminate on microscope field cells in which is expressed the BRET donor and not the acceptor (β-arrestins-RLuc8/DsRed) from cells expressing both the donor and the acceptor (β-arrestins-RLuc8/receptor-VENUS) by different fluorescence positivity. This allows to measure BRET ratio derived from donor emission overflow in the acceptor emission channel or from specific protein-protein interactions on the same visual field, at the same time. When we measure in HEK293 cells the interaction between ACKR2 and β-arrestins in absence of chemokine stimulation, ACKR2-VENUS strongly interacts with both β-arrestin 1- and β-arrestin 2-RLuc8 (Figure 17 A-K and U), as previously shown by BRET saturation assay. In particular, while there are only, even if statistically different, minimal differences in the localization of ACKR2-VENUS interaction with β-arrestin 2-RLuc8 (Fig. R9 and U), β-arrestin1-RLuc8 association occurs more prominently with ACKR2-VENUS present at the level of plasma membrane (Fig. R9 and U). By Image BRET we can also reproduce data obtained on CCR5 by BRET on cell population, in fact we can record a weak energy transfer occurring between β-arrestins-RLuc8 and CCR5-VENUS (Fig. R9 I-T and U).

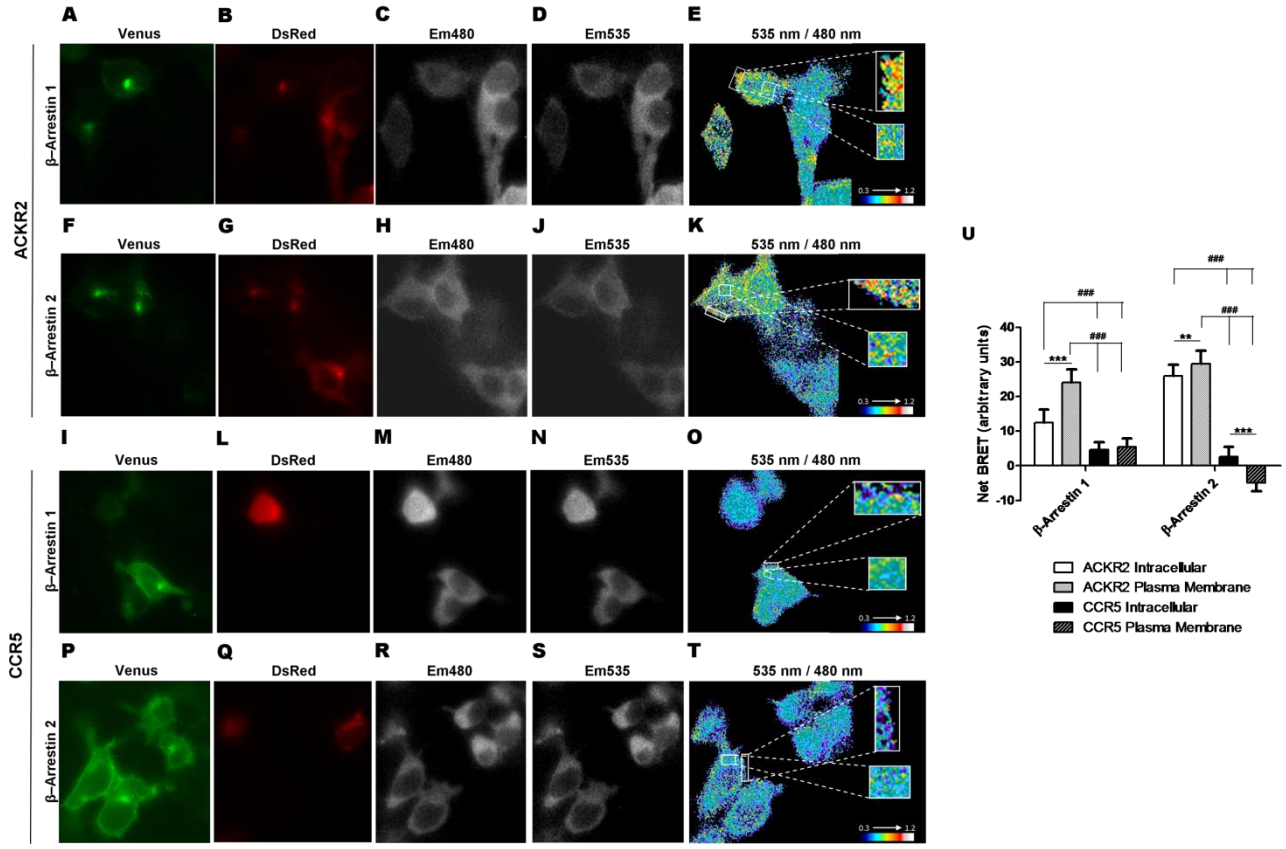


Figure 17: **Constitutive association of ACKR2 and CCR5 to β-arrestins**. Representative images of HEK293 cells co-transfected with either ACKR2-Venus and β-arrestin1-RLuc8 (A-E) or β-arrestin2-RLuc8 (F-K), or with CCR5-Venus and β-arrestin1-RLuc8 (I-O) or β-arrestin2-RLuc8 (P-T). Images were generated acquiring from the same optical field fluorescence filtered for Venus (A, F, I, P) and DsRed (B, G, L, Q), or collecting luminescence filtered through the 480/60 nm filter for RLuc (C, H, M, R) and 535/50 filter for Venus (D, J, N, S). Ratios obtained from Em480 and Em535 images are reported in figures (E, K, O, T), where pixel values between 0,3 and 1,2 are pictured as 256 pseudo colors reported on the scale bar. Net BRET obtained by subtraction of mean BRET ratios in intracellular or cell surface ROI of DsRed-positive cells from respective mean values measured on Venus-positive cells are reported in graph (U), and were obtained from at least 10 replicates. **= P<0,01; ***= P<0,001 for values in intracellular versus plasma membrane ROI. ###= P<0,001 for values obtained for ACKR2 versus CCR5.

4.2.4-CCL3L1 STIMULATION SELECTIVELY INCREASES ACKR2 ASSOCIATION TO B-ARRESTIN 1 INDEPENDENTLY ON CELLULAR LOCALIZATION

Since upon agonist stimulation ACKR2 preferentially increases β-arrestin 1 association and is rapidly mobilized from intracellular compartments to the plasma membrane [313], while on the opposite CCL3L1 stimulation of CCR5 induces its internalization [86], we assessed whether a distinct cellular distribution of interactions between ACKR2 or CCR5 and β-arrestins could correlate with these opposite behaviors, by monitoring image BRET after ligand stimulation. It can be observed a strong increase in ACKR2 interaction with β-arrestin 1 after cell stimulation with the active ligand CCL3L1, and in particular this interaction occurrs both inside the cell and on plasma membrane (Figure 18, A and F), whereas β-arrestin 2-RLuc8 interaction with ACKR2-VENUS statistically increases rapidly only on plasma

membrane, appearing weaker and transient compared to β-arrestin 1 (Figure 18 B and K). On the contrary, CCR5-VENUS stimulation with CCL3L1 results in a rapid and strong increase in BRET signals with both β-arrestins-RLuc8 (Figure 18 A, B, N and R), with a stronger interaction all over the cells with β-arrestin 2 (Figure 18 B and R), while in the case of β-arrestin 1 this increase is statistically relevant on the cell surface and shows only a trend in intracellular compartments (Figure 18 A and N).

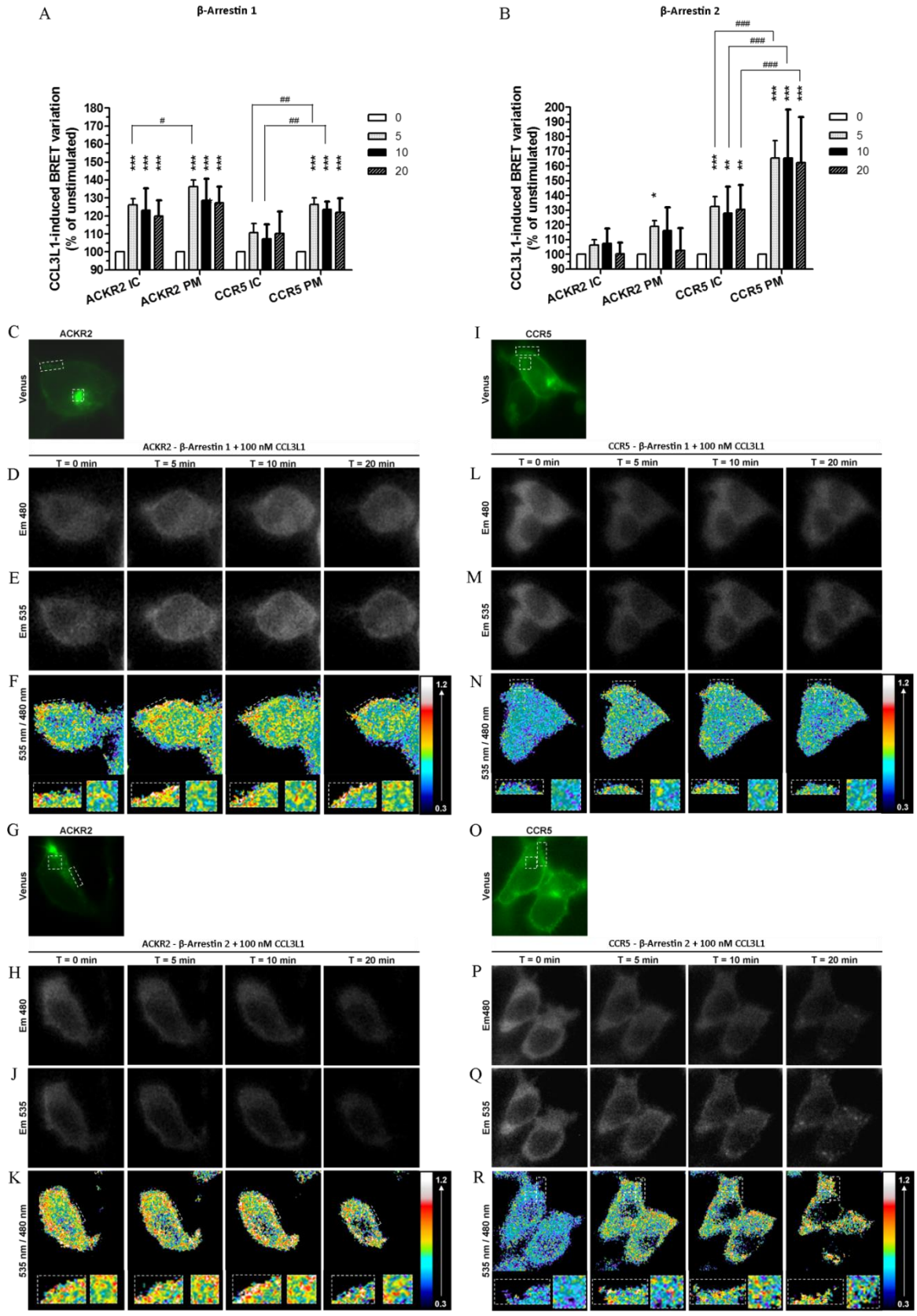


Figure 18: CCL3L1 induces different β-arrestins recruitment to ACKR2 and CCR5. A, B: Graphs reporting the CCL3L1-induced β-arrestin 1 (**A**) and β-arrestin 2 (**B**) recruitment to ACKR2 and CCR5 at different timepoints in intracellular (IC) or on plasma membrane (PM) ROI, expressed as increase over unstimulated conditions. **C-R**: Representative images of transfected HEK293 cells obtained at different timepoints after stimulation with 100nM CCL3L1. Images of cells expressing ACKR2-VENUS and β-

arrestin 1-RLuc8 (**C-F**) or β-arrestin 2-RLuc8 (**G-K**) or CCR5-VENUS in combination with β-arrestin 1-RLuc8 (**I-N**) or β-arrestin 2-RLuc8 (**O-R**) are reported, with indications of the filter used to acquire luminescent images. Dotted lines define the ROI considered for analysis on each cell. *= P<0,05, **= P<0,01 and ***= P<0,001 indicate values statistically different from unstimulated conditions (0 min) by two-way Anova with Bonferroni post-hoc test. #= P<0,05, ##= P<0,01 and ###= P<0,001 indicate differences between BRET signal recorded in IC or PM compartments.

To verify that ACKR2 recruitment of β-arrestins occurrs only after active ligands stimulation as in BRET on cell populations, we stimulated cells also with CCL3. As expected, CCL3 does not induce any significant change in ACKR2 association to β-arrestins (Figure 19 A, B, F and K). In the case of CCR5, CCL3 induces preferentially on the plasma membrane a β-arrestin 2 recruitment that is stronger than the one with β-arrestin 1 (Figure 19 A, B, N and R), similarly to CCL3L1 stimulation but with less intensity, as could be expected by published data indicating that CCL3L1 is a stronger CCR5 agonist compared to CCL3 [327].

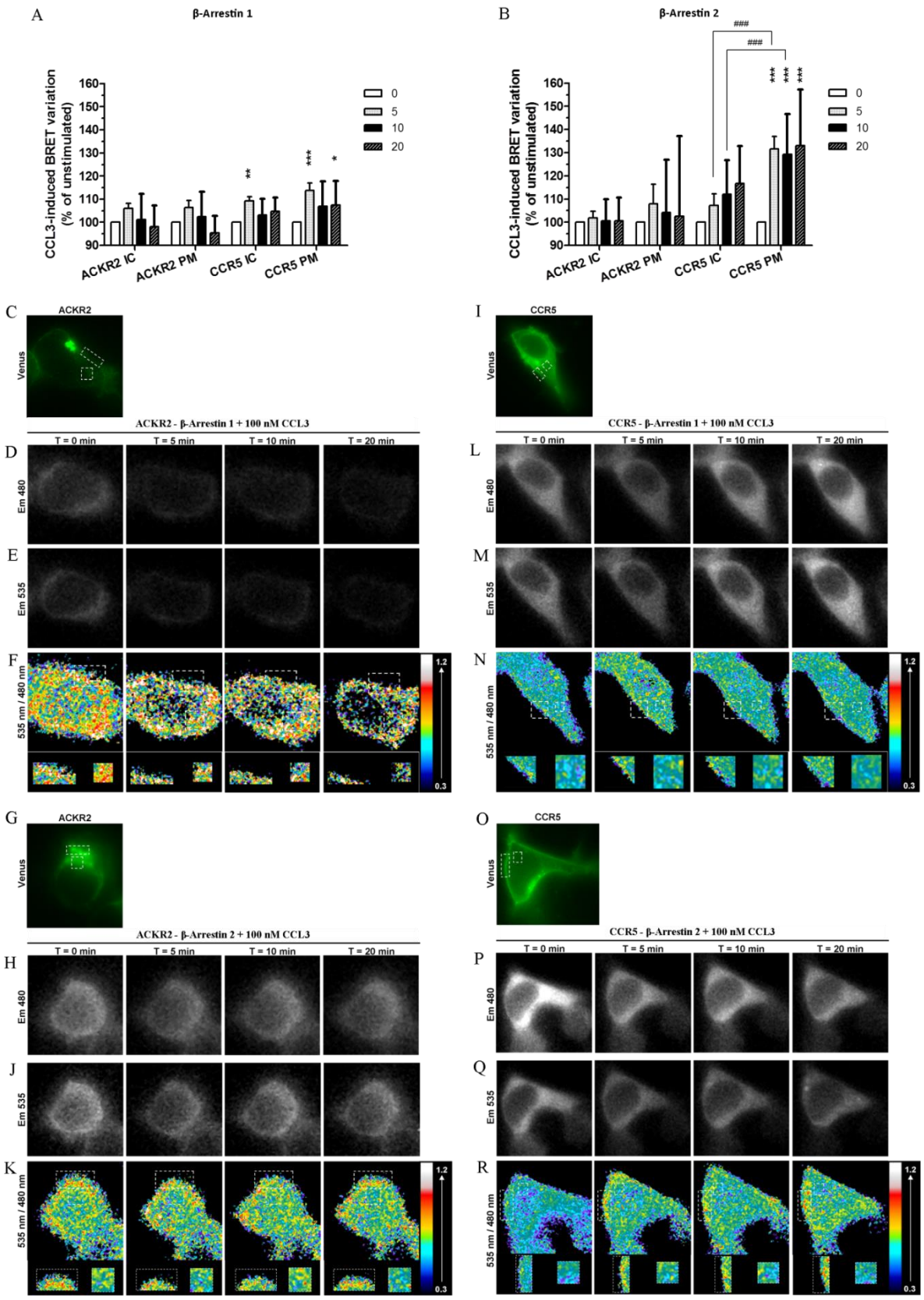


Figure 19: **CCL3 induces β-arrestins recruitment to CCR5 and not to ACKR2**. **A, B**: Graphs reporting the CCL3-induced β-arrestin 1 (**A**) and β-arrestin 2 (**B**) recruitment to ACKR2 and CCR5 at different timepoints in IC or on PM ROI, expressed as increase over unstimulated conditions. **C-R**: Representative images of transfected HEK293 cells obtained at different timepoints after stimulation with 100nM CCL3. Images of cells expressing ACKR2-VENUS and β-arrestin 1-RLuc8 (**C-F**) or β-arrestin 2-RLuc (**G-K**) or CCR5 in combination with β-arrestin 1 (**I-N**) or β-arrestin 2 (**O-R**) are reported, with indications of the filter used to acquire

images. Dotted lines define the ROI considered for analysis on each cell. *= P<0,05, **= P<0,01 and ***= P<0,001 indicate values statistically different from unstimulated conditions (0 min) and ###= P<0,001 indicates differences between BRET signal recorded in IC or PM compartments, determined by two-way Anova with Bonferroni post-hoc test.

4.3-Analysis of ACKR2 signaling by global phosphoproteome analysis

The idea that ACKRs are signaling GPCRs rather than decoy chemokine receptors that silently contribute to chemokine system activity became consistent only in the last few years. In fact, since their characterization, ACKRs have been shown to be unable to activate typical chemokine signaling events that are G proteins activation and Ca^{2+} fluxes generation to promote cell migration, and evidences demonstrated that chemokine binding induced certain activity, such as receptor trafficking modification, but the formal demonstration of receptor signaling arrived with the observation that ACKR3, the second receptor for CXCR4 ligand CXCL12, is able to activate several signaling pathways in a G protein-independent and β-arrestin-dependent manner [282], and these signaling not only regulate chemokine degradation and gradient formation [4] or antagonize CXCR4 activity competing for β-arrestin binding [328], but are also involved in tumor cell survival and proliferation [329]. From our results and published observations, ACKR2 as well is able to be activated by its agonists, to recruit β-arrestin 1 that mediates its signaling for which up to date the only regulated functions are receptor trafficking and ligand degradation [292, 313], which explain ACKR2 involvement in different inflammation and tumor models only in part or by it chemokine degradative activity [142, 315, 330], and not by its ability to directly sustain signaling events. To explore the possibility that ACKR2 can regulate different signaling pathways we decided to investigate ACKR2 signaling activity measuring by mass spectrometry after Stable Isotope Labeling by Amino acids in Cell colture (SILAC) differences in the phosphoproteome of cells after ACKR2 expression and stimulation with its agonist CCL3L1; aimed at characterizing not only ACKR2 signaling events impacting on different cell functions, but also with the goal to understand differences in signaling events underlying a structurally biased receptor. This approach is based on the differential labeling of proteins in different samples adding to colture media amino acids with natural carbon and nitrogen or with their heavier isotopes, that allow to discriminate protein peptides derived from different sample preparations in mass spectrometry, which allows to quantify the abundance of one peptide inside a mixture, making possible to compare the amount of one peptide in one sample to another, assessing simultaneously the whole cell proteome [331]. When this analysis is performed on peptide fractions enriched for phosphorylation the result is a comparison between the phosphorylation status of all the peptides (and by bioinformatics all the proteins they belong) present in one sample versus samples differently treated [332]. This approach has already been used also to assess the implications of biased agonists acting on a GPCR on the global phosphoproteome of cells and compare it to a balanced agonist [333] and has also been successfully applied to study CXCR4 signaling events in breast cancer stem cells

[334], therefore we decided to look at all the modifications in phosphorylation of the whole proteome that can be generated by ACKR2, comparing them to what happens in the case of CCR5 both in a constitutive and ligand-induced manner.

4.3.1-ACKR2 AND CCR5 EXPRESSION AND ACTIVATION CAN BE INDUCED IN HEK293 T-REX SYSTEM

To investigate proteome phosphorylation differences induced by CCL3L1 on ACKR2 and CCR5, we had to prepare 100 millions of cells for each experimental condition to generate enough material for lysate preparation, protein separation and phosphoprotein enrichment, tryptic digestion of proteins and mass spectrometry analysis; therefore we had to use cell lines transfected to express exogenous ACKR2 and CCR5. To this point we used HEK293 T-Rex, a HEK293 cell line engineered to stably express the tetracycline repressor protein, which occupies tetracycline-responsive operons positioned between any promoter and its downstream gene, inhibiting gene expression. In presence of tetracycline the repressor protein binds tetracycline and releases DNA operon, allowing RNA polymerase to transcript the tetracycline regulated gene. To this purpose we subcloned from plasmids already present in the laboratory DNA sequences encoding HA-tagged ACKR2 and CCR5 in pcDNA4/To vector, in which 2 repetitions of tetracycline operator sequence have been positioned immediately downstream the CMV promoter sequence and upstream the gene insertion site, to allow constitutive recruitment of polymerase on the CMV promoter but gene expression only in response to tetracycline stimulus, that induces the dissociation of tetracycline repression when it is present, as in HEK293 T-Rex cells. We generated HEK293 T-Rex stably expressing HA-tagged ACKR2 (HEK T-Rex ACKR2) and HA-tagged CCR5 (HEK T-Rex CCR5) by plasmid transfection and antibiotic selection of cells that incorporated the plasmid adding to colture medium 100 µg/ml Zeocin, to whom resistance is conferred by a resistant transgene present in pcDNA4/To plasmid. Stable transfectants were maintained as bulk populations, and after testing different effective concentrations of tetracycline at different time points (data not shown), we decided that induction of receptor expression with 1 µg/ml of tetracycline for 24 hours resulted in efficient induction of receptors expression, and began preparing samples for SILAC.

In detail, we generated 4 different samples for each receptor-transfected cell, to make by SILAC 3 different paired comparisons, since this kind of analysis is based on the direct and paired comparison of differences in quantification of peptides labelled with different isotopes. Our first aim of this analysis is to investigate if these two receptor have constitutive signaling activities, therefore to assess which signaling events are activated upon receptor expression induction by tetracycline we generated cells coltured in light medium, maintained without tetracycline, and compared this condition to cells coltured with heavy medium, in which receptor expression was induced by 1 µg/ml tetracycline for 24 hours. We also wanted to investigate globar receptor signaling activities after their stimulation with 100 nM CCL3L1 at short and long time points, stimulating for 3 or 30 minutes cells coltured with light medium in

which receptor expression was induced, comparing their phosphorylation to unstimulated cells expressing receptors, labeled with heavy aminoacids. To this point the same populations of HEK T-Rex ACKR2 or CCR5 were split to be coltured with mediums additioned with light or heavy amino acids. Manipulation of 100 million of cells at once could have represented an issue in terms of homogeneity of receptors expression and cells stimulation, therefore we decided to prepare all the samples in 10 different replicates of 10 million cells each, which have been assessed every time by flow cytometry for receptor expression induction and for effective responsiveness to chemokine evaluating by western blot the phosphorylation of Cofilin, a signaling pathway that we already described to be activated in ACKR2 stimulation [292]. As it can be observed in panels D and G of Figure 20, only a minimal percentage of HEK293 T-Rex ACKR2 or CCR5 cells coltured in light medium and in absence of tetracycline are positive to anti-HA antibody staining, that can be reconduced to signal background or aspecific binding of the antibody on cell surface, as occurs on cells stained with an irrelevant mouse IgG1 used as isotype control (Figure 20 C). Induction of receptor expression by 24 hours stimulation with 1 μg/ml of tetracycline induces expression of HA-tagged ACKR2 or CCR5 in at least 90% of analyzed cells, with only minimal differences between cells coltured in light (Figure 20 E,H) and heavy medium (Figure 20 F,I), indicating that expression of ACKR2 and CCR5 in HEK293 T-Rex cells has been efficiently achieved prior to generating samples for SILAC analysis. To preserve sample quality and avoid loss of protein phosphorylation before performing SILAC analysis cells were collected after stimulation as intact cells, transferring them to ice, adding ice-cold TBS to block stimulation and centrifuging them to collect the cell pellet that has been stored at -80°C before analysis performed by an external service. To collect intact cells, we performed stimulation on cells in 1 ml suspension, at 10 million cells/ml concentration and incubated for 30 minutes before chemokine stimulation at 37° C, with constant gentle shaking to prevent cell precipitation. In detail, we used cells coltured with heavy amino acids and receptor expression induced to generate samples representative of basal conditions of stimulation. After sample cooling, before centrifugation, 5% of the samples were collected to be lysed and analyze cofilin phosphorylation on Ser3 to assess efficacy of CCL3L1 stimulation. Western blots show that on both ACKR2 and CCR5 induction minimally affect cofilin phosphorylation compared to cells unstimulated with tetracycline, and that stimulation over time with 100 nM CCL3L1 induces an increase in cofilin phosphorylation compared to the initial time point (Figure 20 J,K). These results indicate that in the samples we prepared, CCL3L1 response is elicited in a comparable manner with respect to what we already described in other cellular models [292], therefore their proteome phosphorylation can be assayed to generate, at the best of our knowledge, reliable databases to interrogate ACKR2 and CCR5 biology.

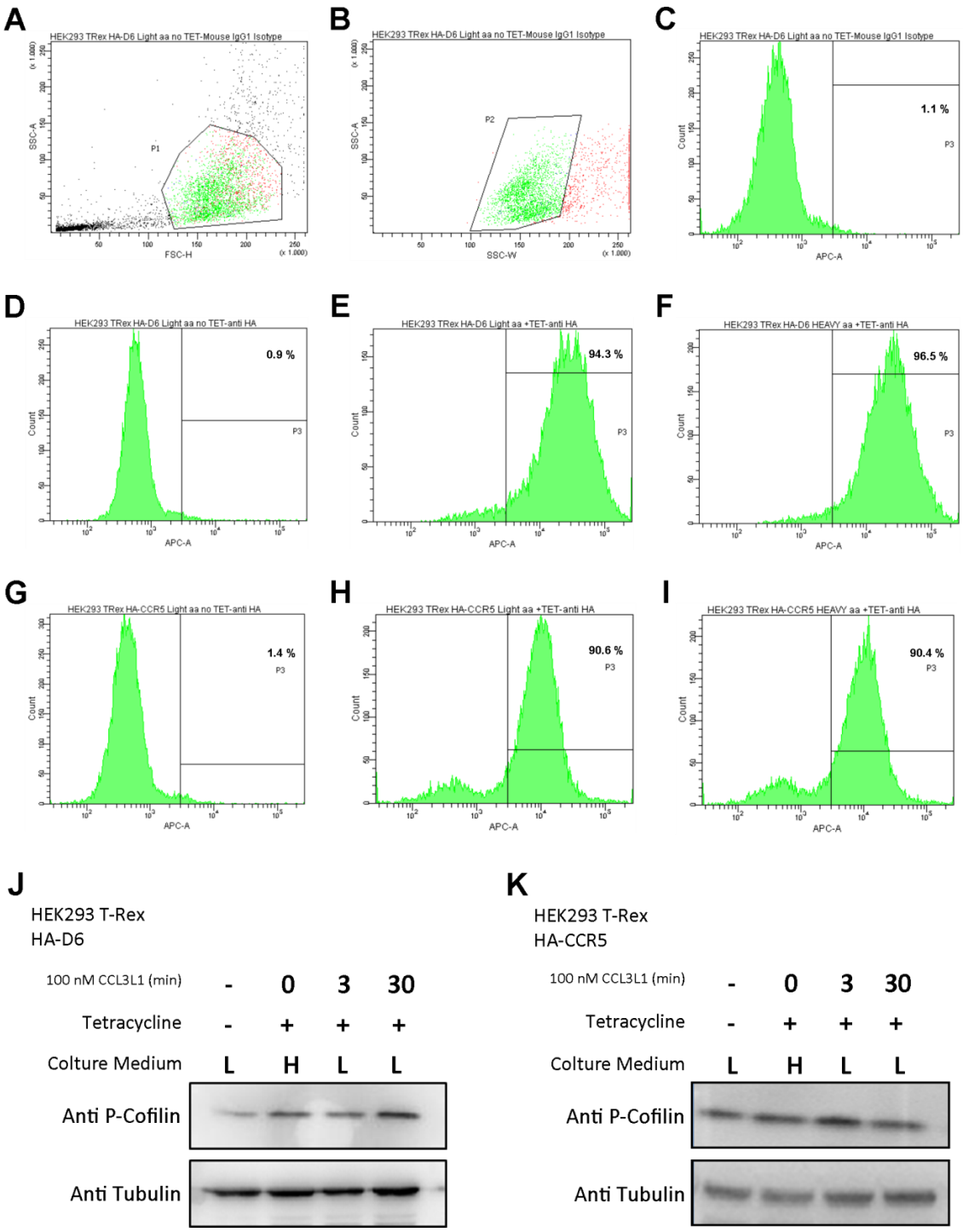


Figure 20: ACKR2 and CCR5 are functionally expressed in HEK293 T-Rex cells upon induction with tetracycline. A-I: Flow cytometric analysis of receptors expression after anti-HA and APC-anti-mouse antibodies staining of HEK T-Rex ACKR2 and CCR5, one experiment is reported as representative of all the experiments performed. **A-B**: dot plots reporting forward scatter height (FSC-H) versus side scatter area (SSC-A) (**A**) or side scatter width (SSC-W) versus area (SSC-A) fluorescences (**B**), used to discriminate live cells from debris and macroscopic agglomerates and singlets of cells from doublets, respectively. Panels from **C** to **I** show histograms of APC fluorescence intensity distribution in different cell samples. Panel **C** reports fluorescence the mouse IgG1 antibody stained cells that allows to define P3 gate, which will include all events with higher fluorescence considered as positive for HA expression, whose parental percentage of events reported represents the percentage of positive cells included in P2, defined as cell singlets. **D-F** and **G-I** report APC FI of HEK T-Rex ACKR2 and CCR5 cells, respectively, gated on

P2 population, coltured with light (**D, E, G, H**) or heavy medium (**F, I**), after 24 incubation with (**E, F, H, I**) or without (**D, G**) 1 µg/ml of tetracycline. In panels **J** and **K** are shown representative western blots for HEK T-Rex ACKR2 (**J**) and CCR5 (**K**) cells in absence and presence of 1 µg/ml tetracycline and after the addition of 100 nM CCL3L1 for the indicated time points, probed with anti-phospho-Cofilin (p-Ser3) or anti-Tubulin antibodies.

4.3.2-PHOSPHOPROTEOME ANALYSIS OF ACKR2 AND CCR5 REVEALS CONSTITUTIVE RECEPTOR ACTIVITY

Sample processing, protein separation and phosphorylated peptide enrichment have been performed by an external service cared by professor Gabriella Tedeschi in Fondazione Filarete (Milano, Italy), that returned to us databases containing protein fragments identified by mass spectrometry and their relative abundance in heavy medium-coltured cells expressing the receptor and unstimulated, expressed as fold increase compared to each condition of light medium coltured cells, that are cells without receptor expression induction (that will be referred to as basal condition) and cells expressing ACKR2 or CCR5 stimulated for 3 or 30 minutes with 100 nM CCL3L1 (referred to as T3 or T30, respectively). We here compare the categories of protein modulated differentially and similarly between ACKR2 and CCR5 to investigate the possible signaling implications underlying structural signaling bias of ACKR2.

A first level of analysis can be performed evaluating the number and the functional classification by gene ontology of proteins whose expression is increased between cells in which receptors expression was not induced and cells where receptors where expressed, to evaluate whether expression of these molecules can impact on expression levels of other proteins. Among 9858 proteins identified, only 283 proteins are quantitatively different between cells not induced and induced for receptors expression (Figure 21 A), and of proteins differentially expressed only the 1.83% percent is common between ACKR2 and CCR5 transfected cells, meaning that these two receptors can modulate expression of different proteins. It has to be mentioned that even if the number of different proteins whose expression is modulated by the expression of ACKR2 or CCR5 is similar (135 vs 133), and they are similarly divided between proteins that are up- and down-regulated (Figure 21 B and C), these proteins that change their levels of expression after receptor induction constitute more than 25% percent of total proteins identified and specific for ACKR2, while only less than 10% of total proteins identified specifically in HEK293 T-Rex CCR5 cells change their level of expression after CCR5 induction (Figure 21 B and C). This results indicate that induction of ACKR2 expression induces more important modifications in the proteome regulating expression levels of more proteins compared to CCR5.

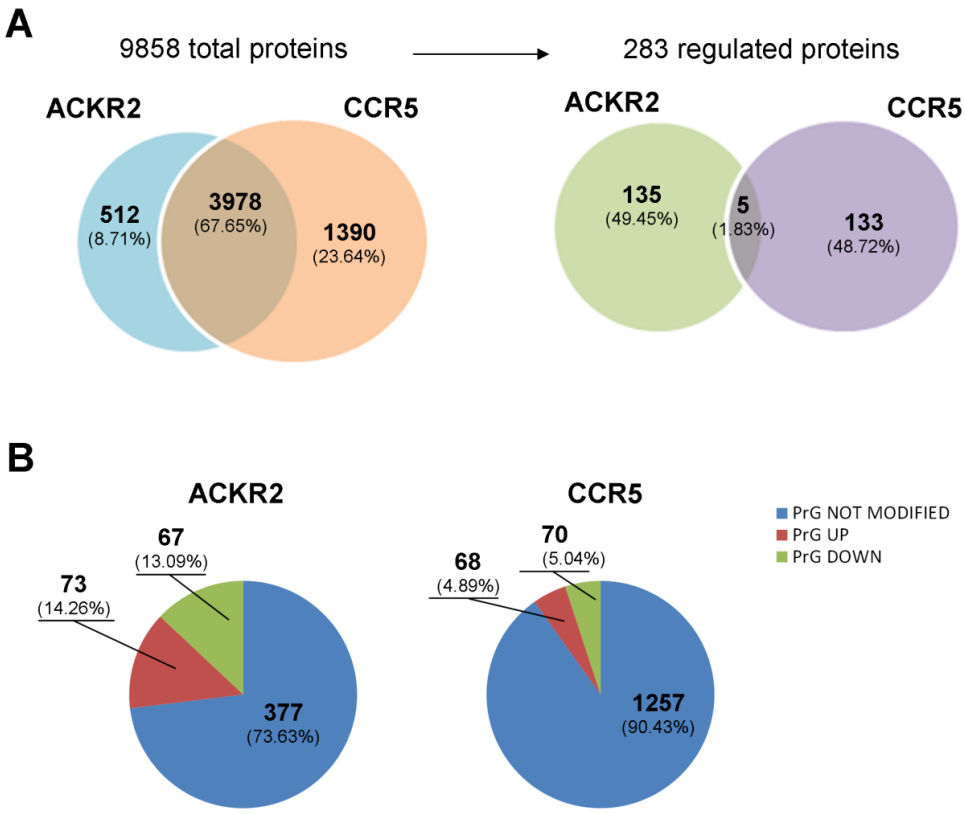


Figure 21: Modifications on protein expression levels modulated by ACKR2 and CCR5 expression. A: Venn diagrams represent the amount of total proteins (on the left) and the amount of protein with different abundance (on the right) identified by mass spectrometry by ratiometric comparison of signals generated by peptides with heavy amino acids (HEK T-Rex cells treated with tetracycline) over peptides with light amino acids (cells untreated with tetracycline). The diagrams show the overlap between the two datasets of proteins derived by cells transfected with plasmids encoding tetracycline-inducible ACKR2 and CCR5. Pie charts in panel **B** show the amount of proteins (and their proportion over total identified proteins) not modified (in blue), up-regulated (in red) and down-regulated (in green) by ACKR2 (**on the left**) and CCR5 (**on the right**) expression induction.

We next analyzed if receptor expression can induce modifications of proteome phosphorylation, indicative of a consititutive signaling activity of receptors that has already been described to occur in chemokine receptors [106, 290, 291]. If prior to SILAC analysis is performed an enrichment of lysate for phosphoresidues, by mass spectrometry can be visualized the differences in phosphopeptide abundance between the two different conditions discriminated by incorporation of light or heavy amino acids [332]. Our analysis detected 3005 phosphopeptides in HEK T-Rex cells induced and not for receptor expression by tetracycline, representative of phosphosites belonging to 1238 different proteins (Figure 22 A). Only 38.77% of proteins has been detected in samples derived from both cell transfectants, and with a minor absolute abundance of phosphoproteins in ACKR2-transfected cells (20.19% of all phosphoproteins detected) compared to CCR5 (41.03%). Among the proteome detected, only 280 phosphosites belonging to 178 different proteins (14.38% of total) are differentially phosphorylated after receptor expression induction, and more than 60% out of these phosphorylation events occur only in ACKR2 expressing cells, while CCR5 induction specifically activates 30% of these proteins (Figure 22 A). Specifically, tetracycline

induction of ACKR2 expression modifies phosphorylation of almost 17% (126 proteins) of the cell proteome (Figure 22 C), with most of the phosphosites of these proteins being de-phosporylated (Figure 22 B), while CCR5 expression changes the phosphorylation status of less than 7% (69 proteins) of the proteome specific for each cell type (Figure 22 C). This indicates that ACKR2 can constitutively regulate phosphorylation levels of more proteins compared to CCR5.

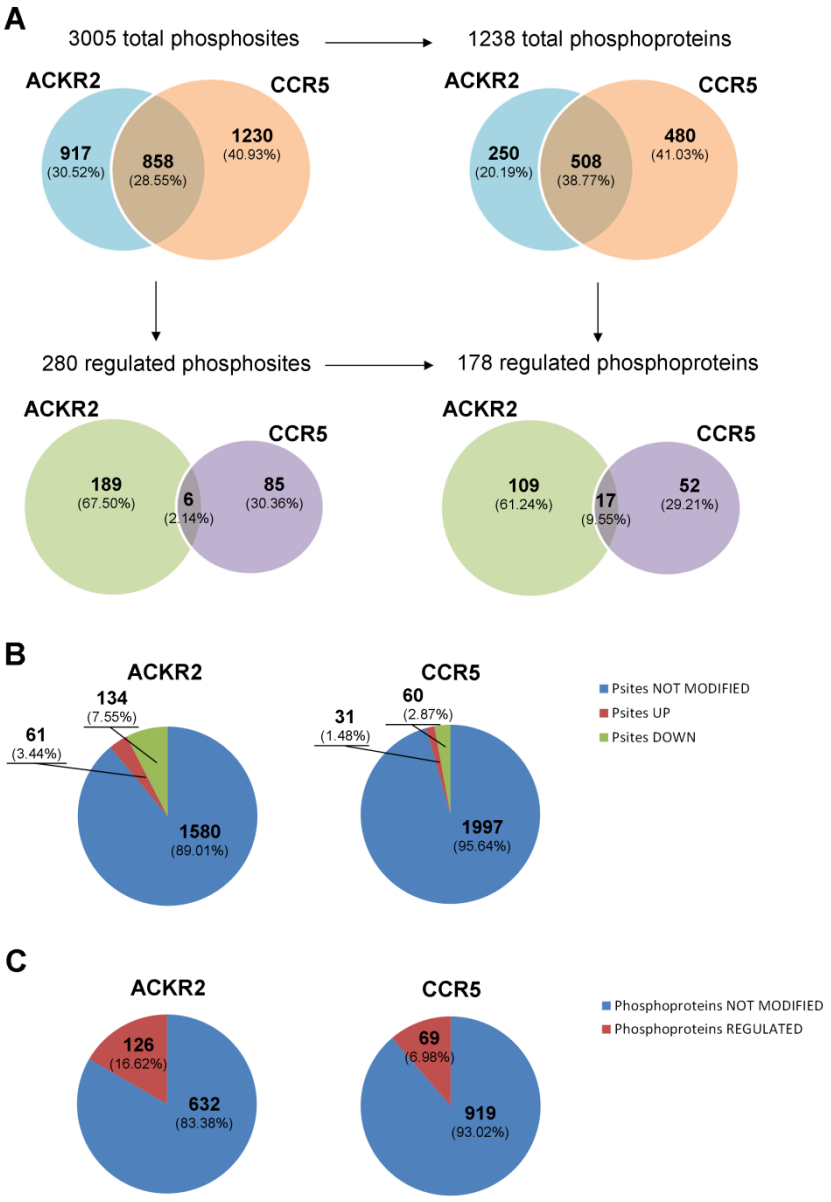


Figure 22: Changes in proteome phosphorylation levels modulated by ACKR2 and CCR5 expression. A: Venn diagrams represent the amount of phosphosites detected (on the left) and the protein to which phosphosytes belong (on the right) and their sharing between generated datasets by HEK T-Rex ACKR2 and CCR5 cells. The diagrams show the overlap between the two datasets of phosphopeptides and phosphoproteins derived by cells in which receptor expression was induced by tetracycline, above are reported all the phosphopeptides and proteins detected and below only peptides who revealed differences in phosphorylation after tetracycline stimulation. Pie charts in panel **B** show the amount of phosphopeptides (and their proportion over total identified phosphopeptides) not modified (in blue), up-regulated (in red) and down-regulated (in green) found specifically in ACKR2 (**on the left**) and CCR5 (**on the right**) datasets obtained after receptor expression induction, while panel **C** shows receptor-specific differences in protein phosphorylation levels.

4.3.3-Phosphoproteome analysis of ACKR2 and CCR5 after CCL3L1 stimulation reveals differences in receptor signaling activities

To assess how agonist-induced signaling activity of ACKR2 and CCR5 affects proteome phosphorylation, indicative of the multiple activation of kinases and downstream effectors, we analyzed datasets obtained from cells stimulated for 3 or 30 minutes, representative of immediate and long-term responses of agonist stimulation. To this point we analyzed proteome phosphorylation of cells with receptor expression was induced and stimulated for 3 or 30 minutes with 100 nM CCL3L1, coltured with light amino acids additioned to the medium, comparing them to cells in which receptor expression was induced as well but coltured with heavy amino acids and stimulated with agonist-free medium.

Dataset analysis reveals that only 45% of 932 phosphoproteins analyzed (to which belong 2072 different peptides detected) can be found in both ACKR2 and CCR5 datasets, and among those proteins 3 minutes of CCL3L1 stimulation can modify phosphorylation of almost 30% of these proteins, indicating that ACKR2 and CCR5 activation leads to a great number of signaling events soon after stimulation (Figure 23 A). Among these protein phosphorylation events only a minor proportion is common to the two receptors (13,04%), with CCR5 that specifically regulates phosphorylation of more than a half of the proteins (51.09%). In detail, the vast majority of these proteins become phosphorylated after CCL3L1 stimulation, while only about 10% of the modified phosphoproteins are de-phosphorylated (Figure 23 B), and together these phosphorylation-modified proteins constitute almost 30% of all phosphoproteins detected in CCR5 expressing cells (Figure 23 C). Regarding ACKR2 activation, at 3 minutes it is able to modulate phosphorylation of about 18% of the phosphoproteins detected in HEK T-Rex ACKR2 cells (Figure 23 C), and differently from CCR5 only 60% of modifications are increases in protein phosphorylation, while the remaining 40% are events of de-phosphorylation (Figure 23 B), indicating a massive activation of phosphatases downstream of ACKR2 rapidly after CCL3L1 stimulation.

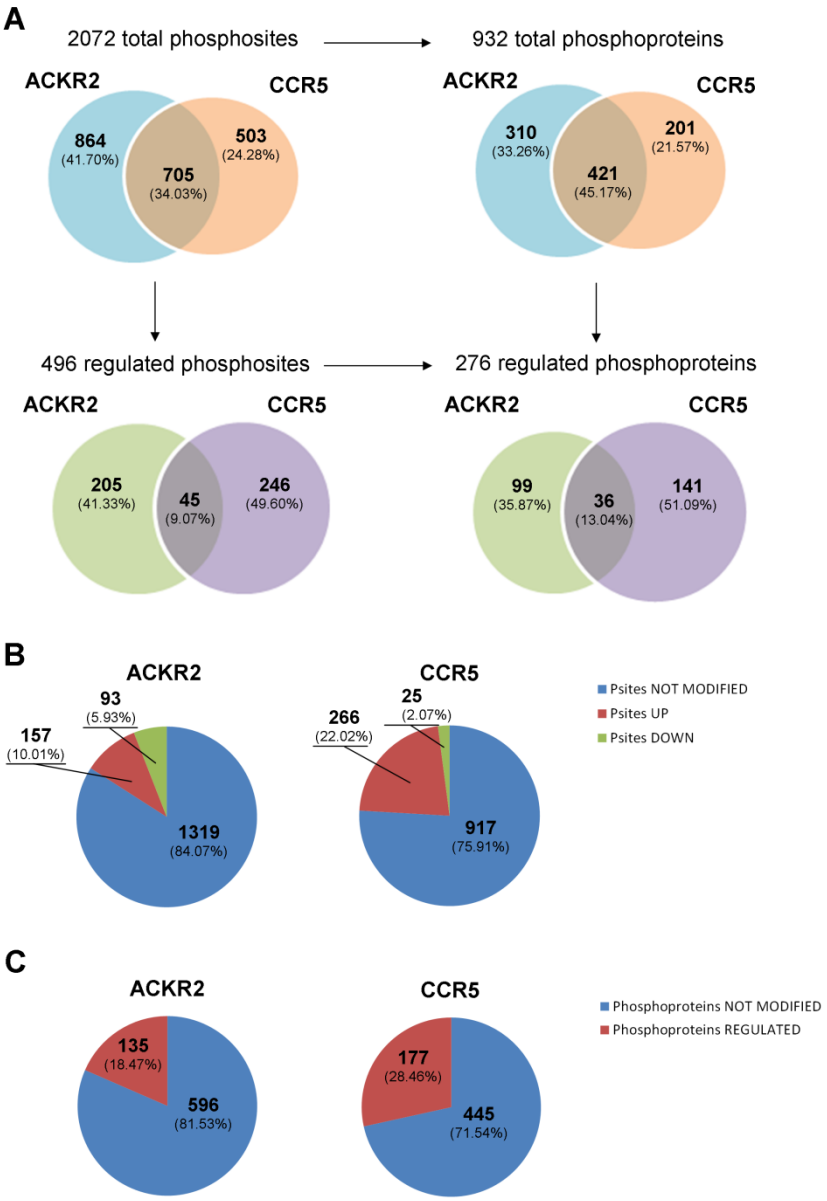


Figure 23: Proteome phosphorylation changes induced after 3 minutes CCL3L1 stimulation of ACKR2 and CCR5. A: Venn diagrams represent the amount of phosphosites detected (on the left) and the protein to which phosphosytes belong (on the right) and their sharing between datasets derived from HEK T-Rex ACKR2 and CCR5 cells. The diagrams show the overlap of datasets obtained by cells expressing receptors and stimulated with 100 nM CCL3L1, compared to unstimulated cells. Above are reported all the phosphopeptides and proteins detected in both the conditions, and below only peptides and proteins who revealed differences in phosphorylation after chemokine stimulation. Pie charts in panel **B** show the amount of phosphopeptides (and their proportion over total identified phosphopeptides) not modified (in blue), up-regulated (in red) and down-regulated (in green) by CCL3L1 stimulation found only in ACKR2 (**on the left**) and CCR5 (**on the right**) datasets, while panel **C** shows receptor-specific differences in protein phosphorylation regulation.

Analysis of phosphorylation events induced by receptor at 30 minutes after stimulation, indicative of distal downstream effectors, show that at later time points receptor stimulation results in a bigger number of phosphorylated proteins detected, compared to 3 minutes stimulation. Among the 513 phosphoproteins differentially phosphorylated between unstimulated cells and cells stimulated with CCL3L1, only 19.49% occur in both ACKR2 and CCR5 datasets (Figure 24 A), while opposite to 3 minutes stimulation 51.07% of these modifications occur specifically downstream of ACKR2. This is reflected by the fact that in ACKR2 dataset 37.47% of proteins are modified compared to basal levels (Figure 24 C), among which 25% of phosphosites display higher levels of phosphorylation and 5% are less phosphorylated than in basal condition (Figure 24 B). CCR5 has instead approximately the same percentage of modulated phosphoproteins that it showed at 3 minutes, but in this case the unbalance between phosphosites up and down-regulated is less prominent (11% phosphosites with increased and 6% with decreased phosphorylation over total phosphosites detected, compared to basal level) (Figure 24 B).

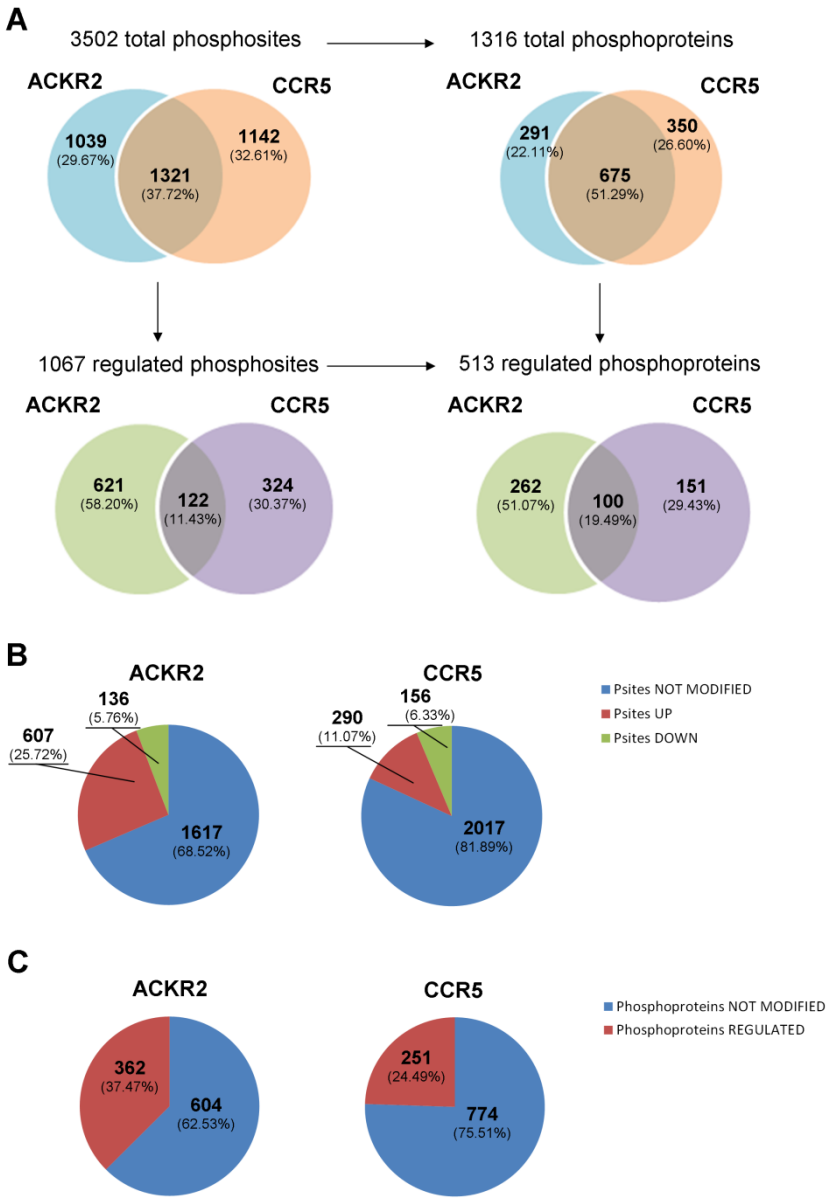


Figure 24: Proteome phosphorylation changes induced after 30 minutes CCL3L1 stimulation of ACKR2 and CCR5. A: Venn diagrams represent the amount of phosphosites detected (on the left) and the protein to which phosphosytes belong (on the right) and their sharing between datasets derived from HEK T-Rex ACKR2 and CCR5 cells. The diagrams show the overlap of datasets obtained by cells expressing receptors and stimulated for 30 minutes with 100 nM CCL3L1, compared to unstimulated cells. Above are reported all the phosphopeptides and proteins detected in both the conditions, and below only peptides and proteins who revealed differences in phosphorylation after chemokine stimulation. Pie charts in panel **B** show the amount of phosphopeptides (and their proportion over total identified phosphopeptides) not modified (in blue), up-regulated (in red) and down-regulated (in green) by CCL3L1 stimulation found only in ACKR2 (**on the left**) and CCR5 (**on the right**) datasets, while panel **C** shows receptor-specific differences in protein phosphorylation regulation.

Taken together, these results indicate differences between ACKR2 and CCR5 activation in terms of kinetics, with CCR5 rapidly mediating changes in protein phosphorylation that are maintaned up to 30 minutes, that mainly consist in phosphorylation events, while ACKR2 has a delayed kinetic activation,

with changes in protein phosphorylation that can increase in time up to doubling at 30 minutes, and changes in terms of modification, since a prominent portion of the modifications are residue dephosphorylation, indicative of a significant activation of phosphatases and not only of kinases.

4.3.4-GENE ONTOLOGY ANALYSIS OF DATABASES

To make a general distinction of the functions regulated by ACKR2 and CCR5 signaling events elicited upon their expression and activation we performed a gene ontology analysis, to group differently phosphorylated proteins between different conditions in classes depending on their biological functions. This allowed us to obtain an indications on the biological meaning of receptors signaling, identifying functional classes that were more represented in modificated proteins.

After induction of receptor expression, are modulated proteins involved in the regulation of cytoskeleton activity, cell organization and transcription (Figure 25 A). In particular, the most different class between ACKR2 and CCR5 is the one involved in the regulation of transcriptional processes. Interestingly this class of proteins are most abundantly regulated by both ACKR2 and CCR5 after CCL3L1 stimulation, but in the case of ACKR2 represent the 68% and 70% of total modified proteins at 3 and 30 minutes, respectively (Figure 25 B and C), while in the case of CCR5 increase over time going from 53% to 65% of total regulated proteins. The second most regulated class is the one including proteins involved in cell organization, required to promote cell adaptation functional to cell response. These proteins are the double in terms of percentage in CCR5 compared to ACKR2, which may represent the reorganization cells incur to support CCR5 induced cell migration. Of notice, there are differences in terms of kinetics in regulation of ontology classes, in example in the case of CCR5 proteins associated to chemokine receptor signaling belonging to the MAP kinase family are mainly regulated at 3 minutes after stimulation, and disappear at 30, while downstream ACKR2 are present as a small percentage after 3 minutes and increase at 30 minutes after stimulation, possibly underlying a delay in activation of the same molecules. In the case of ACKR2 instead a component of signaling functions related to macromolecules organization appears at 3 minutes and disappears at 30, possibly indicating an undergoing preparation of the signalosome required by ACKR2 to generate stronger signals at later time points.

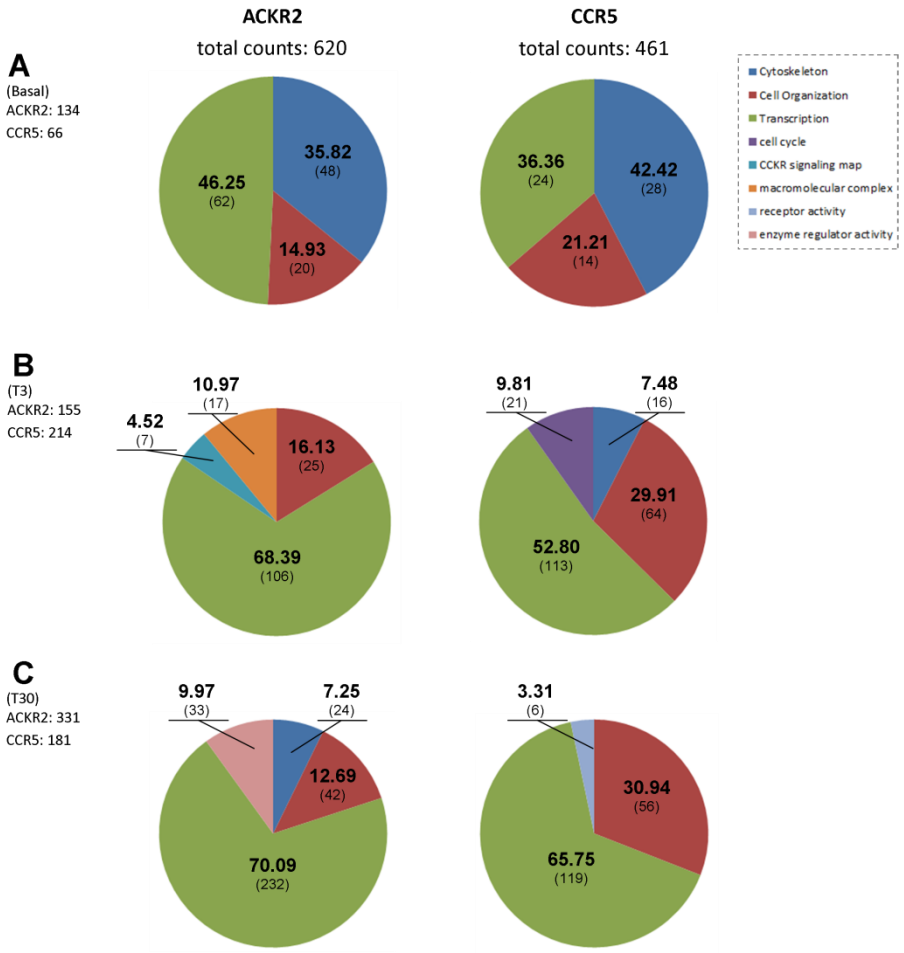


Figure 25: Gene ontology of phosphoproteins modulated by ACKR2 and CCR5. Pie charts show the distribution among gene ontology classes of differently phosphorylated proteins between datasets analyzed, with datasets generated with HEK T-Rex ACKR2 on the left and CCR5 on the right. Panel **A** shows distribution of regulated proteins between cells in which receptor expression was induced and cells where it has not (Basal), in panels **B** and **C** are grouped proteins differentially phosphorylated in cells stimulated with 100 nM CCL3L1 for 3 minutes (T3, **B**) or 30 minutes (T30, **C**) compared to unstimulated cells. Gene ontology classes are reported in the legend.

4.3.5-Regulated kinase motifs differ between ACKR2 and CCR5

Trying to understand what are the signaling events more proximal to the receptors, the ones regulated upstream all the signaling cascades that regulate cell function, we analized the phosphorylation sites regulated in all the conditions, looking at recurring motifs recognized by kinases and phosphatases on target proteins, to find possible proteins responsible for ACKR2 or CCR5 distinct signaling events. Since phosphorylation domains are not univocally defined but are more addressed by conserved residues positions, it could happen that one phosphosite can be recognized by more than one kinase or phosphatase, therefore we measured the fold enrichment of each kinase domain in each dataset and compared it abundance with the ones found in the corresponding dataset of CCR5 by

subtraction, obtaining values representative of kinase motif more abundantly regulated by ACKR2 and by CCR5.

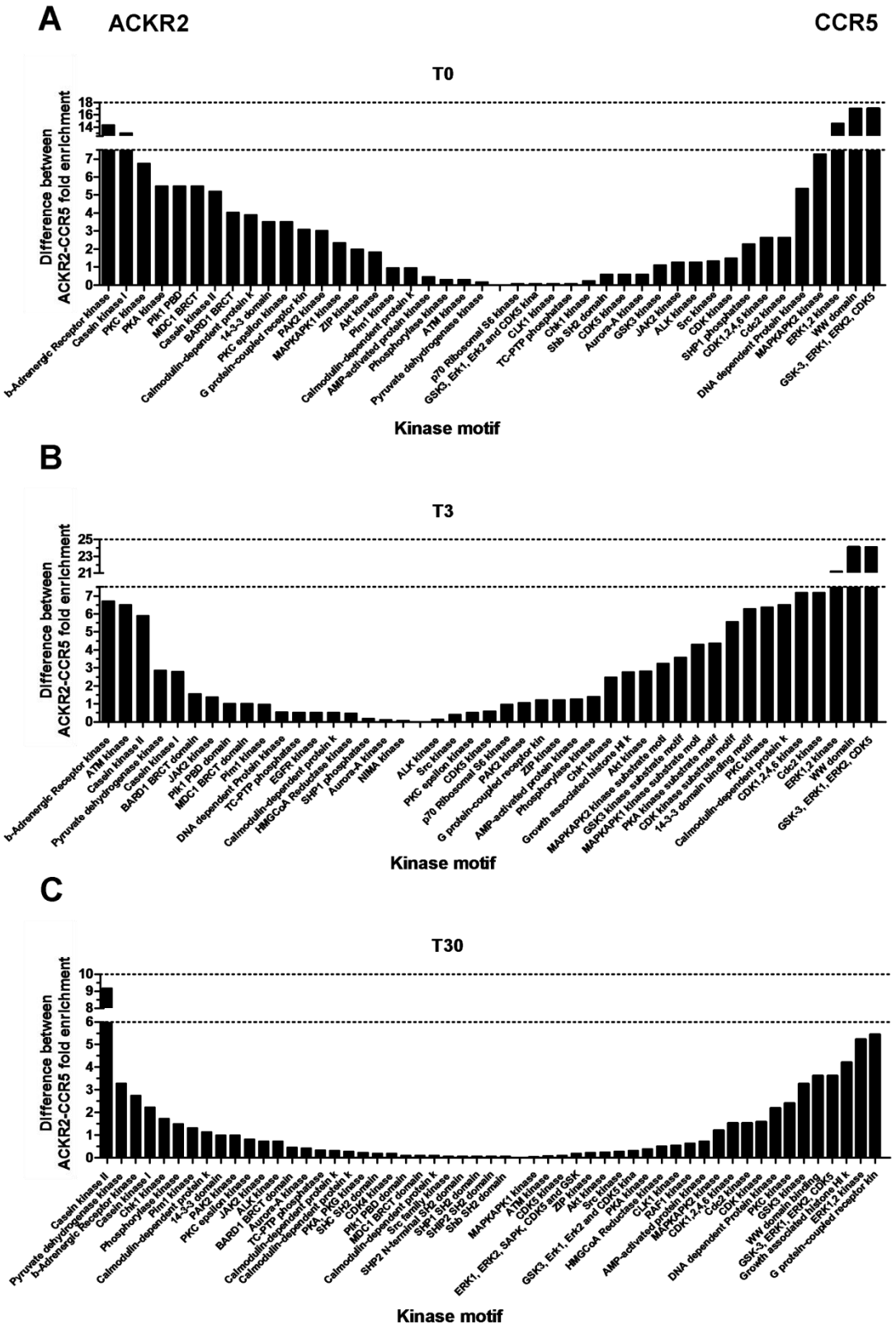


Figure 26: Kinase motifs distribution between ACKR2 and CCR5. Histograms show the distribution of relative enrichment of ACKR2 (left) and CCR5 (right) specific kinase motifs in phosphosites modulated in T0 (A, induction of receptor expression by tetracycline), T3 (B, 3 minutes stimulation with 100 nM CCL3L1) and T30 (C, 30 minutes stimulation with 100 nM CCL3L1), expressed as differences between ACKR2 fold enrichment and CCR5 fold enrichment (ACKR2 F.E.-CCR5 F.E). Motifs were

distributed from the left, with motifs more abundant in HEK T-Rex ACKR2 cells, decrease towards right with motifs similarly recurring between ACKR2 and CCR5 datasets, and end on the right with motifs more specific for CCR5.

As it can be observed in Figure 26 A, in basal conditions ACKR2 and CCR5 show activation of pretty distinct kinases and phosphatases, that regulate proteome phosphorylation acting on different motifs between ACKR2 and CCR5, since most of the motifs have a fold increases higher than 1, compared to the other receptor, indicating that constitutive receptor activity regulates most of the phosphoproteome residues differentially between HEK T-Rex ACKR2 and CCR5 cells. After 3 minutes of stimulation with CCL3L1 the distribution of regulated phosphomotifs shows that motifs specifically regulated by CCR5 are more abundant than for ACKR2 (Figure 26 B), resembling the differential kinetic of phosphosites enrichment observed by signaling activation in Figure 23. Conversely specific phosphomotifs tend to converge to the center and be regulated similarly at 30 minutes, possibly indicating that even if at 30 minutes can be found most phosphorylated peptides and proteins differentially regulated between analyzed conditions (Figures R14, R15 and R16 panels A. 1067 regulated phosphosites in T30 versus 496 and 280 sites in T3 and T0, respectively), most of the differences between ACKR2 and CCR5 occurring in terms of proteome phosphorylation occur at shorter timepoints. Interestingly, among kinase motifs specifically regulated by ACKR2 can be found bot in basal conditions and after stimulation the β-adrenergic receptor kinase motif and casein kinase 1 and 2 motifs, possibly indicating strong interactors with ACKR2, mediating receptor signaling in both constitutive conditions and after ligand stimulation.

4.4-CXCR1 AND CXCR2 SIGNAL MODULATION BY NONCOMPETITIVE ALLOSTERIC INHIBITORS

Among inflammatory chemokines, CXCL8 is a ELR^+ CXC chemokine whose expression is induced by inflammatory stimuli as LPS, TNF-α and IL-1β [335] and exerts its functions recruiting several leukocyte populations to inflamed tissues acting on CXCR1 and CXCR2 receptors [3]. In particular these two receptors are highly expressed on neutrophils and monocytes, the most important players of innate immunity and acute inflammation, regulating their influx in damaged tissues in many models of inflammatory diseases, not only during acute inflammation but also in auto-immune diseases, transplantation and cancer-related inflammation [10, 11]. Therefore, since chemokine system characterization, chemokine receptors as CXCR1 and CXCR2 have been considered an ideal therapeutic target to block leukocyte infiltration and inflammation, not only because they are expressed with a cell specific pattern and they drive leukocyte recruitment within tissues, but also because of their GPCR structure, which allows them to be targeted by orally deliverable small molecules [10, 11]. Over the years, many drugs have been developed and described to be specific and effective in vitro on chemokine receptors, but only two compounds are actually available on the market, since most of them failed to

progress over all the clinical trials, due to adverse side effects as immune deficiency [146] or therapy inefficacy due to the complexity and redundancy of the chemokine system, since the same cell can be recruited through different overlapping stimuli this system is hard to be tackled by conventional pharmacological antagonists [10, 11, 55]. Drug development is now taking into account all the possibilities given by biased agonism to generate molecules able to activate only distinct signals and activities of a given GPCR. As well drug development addressed to chemokine receptors is considering the option of molecules that can bind receptors in their minor hydrophobic binding pocket to allow the receptor to bind its ligands reducing the risk of cross activation of other receptors but block chemotaxis [11, 145, 147]. In particular, the Italian company Dompé developed small molecules that are allosteric inhibitors which have already been shown to block leukocyte infiltration in various models of inflammation. These molecules bind to CXCR1 and CXCR2 without displacing CXCL8, that despite binding to the receptors no longer mediates its migratory activity [194]. Unfortunately these molecules effects on CXCR1 signaling have been investigated only to functional cell aspects, as cell migration or adhesion molecules activation, and at the molecular level only by their ability to block GTPγS binding to cell membrane, Ca^{2+} fluxes generation and chemokine binding displacement. To better understand how these inhibitors interfere with the conventional CXCR1 signaling activities elicited by CXCL8, and whether this better therapeutic efficacy underlies a modulation of receptor signaling towards a biased signaling, we decided to investigate different receptor activities induced by CXCL8 on CXCR1 after the treatment with Reparixin (DF1681Y), an allosteric modulator that is actually in phase 3 clinical evaluation, which has a higher specificity for CXCR1 compared to CXCR2, and DF3053Y, an orthosteric inhibitor designed by Dompé that occupies chemokine binding site impeding CXCL8 binding to CXCR1.

4.4.1-Reparixin does not block CXCR1 internalization

To understand the mechanism by which Reparixin blocks cell migration even if allows CXCR1 to bind CXCL8, we wondered what macroscopically happens to CXCR1 expressed in HEK293 cells upon CXCL8 binding together with Reparixin, measuring CXCL8 ability to induce HA-tagged CXCR1 internalization over time after stimulation, looking by flow cytometry at cell-associated fluorescence.

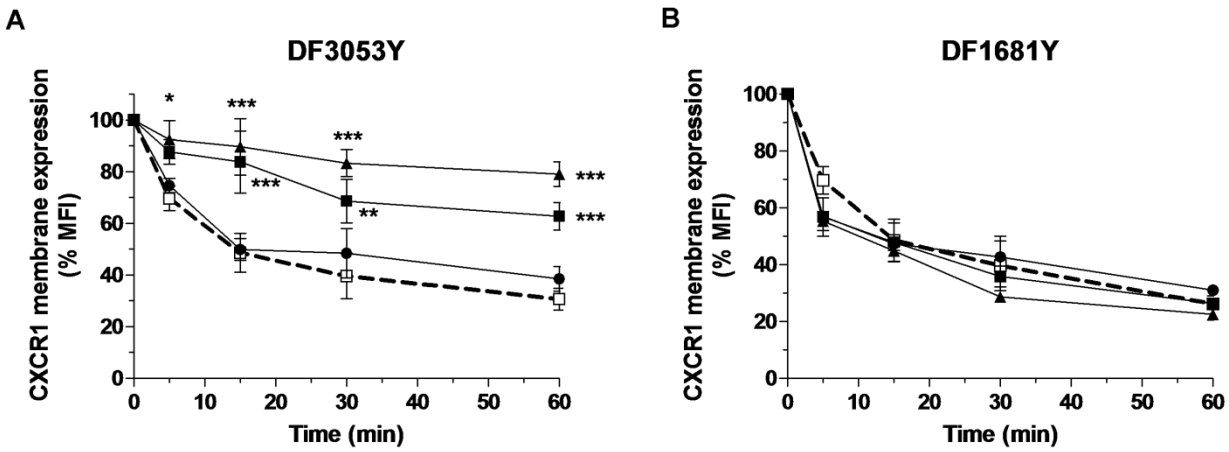


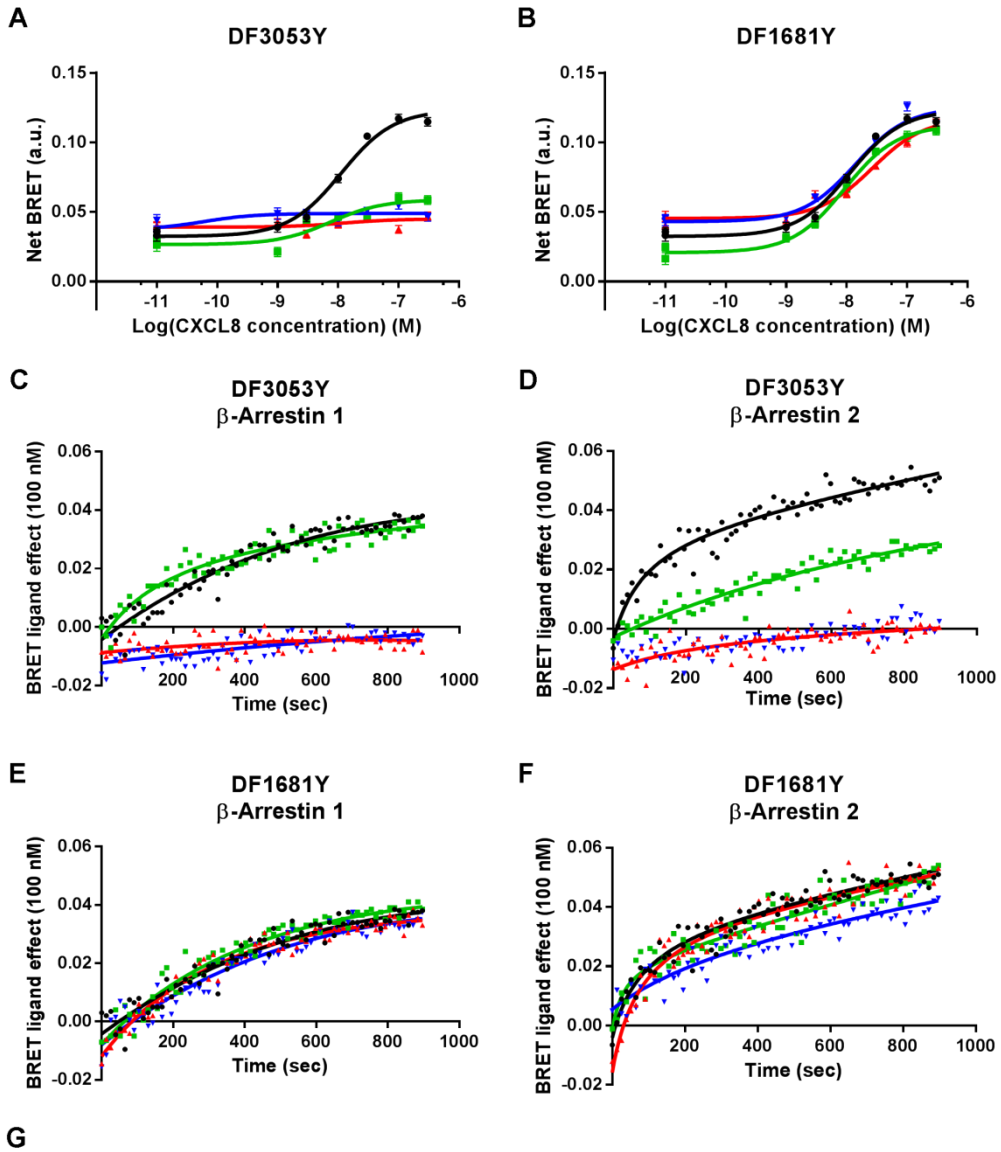
Figure 27: CXCL8-induced internalization of CXCR1 is not affected by Reparixin. Graphs show variations over time of HA-CXCR1 expression on HEK293 cells surface after cell stimulation with 100 nM CXCL8, measured by flow cytometry after cell staining with a mouse anti-HA antibody and a secondary goat anti-mouse IgG conjugated to APC. Treatment with vehicle (DMSO) is plotted with empty squares and thick dotted line (□, **- -**), while concentrations of DF3053Y (**A**) and Reparixin-DF1681Y (**B**) are represented as follows: 0.1 µM (●), 1 µM (■) and 10 µM (▲). Variations in fluorescence of each sample are reported as percentage of mean cell fluorescence over basal fluorescence at the initial time point of each treatment. Statistical analysis of three independent experiments was performed by two-way Anova with Bonferroni post-hoc test, with *=p<0.05, **=p<0.01 and ***=p<0.001 compared to DMSO-treated cells.

Results indicate that CXCL8 induces a decrease over time in CXCR1 expression on the cells surface, as expected, in cells treated with vehicle (DMSO) (Figure 27) and receptor internalization is blocked when cells are treated with at least 1 µM DF3053Y (Figure 27 A), that avoid CXCL8 binding and activation of CXCR1. This effect is more prominent at 10 µM, which significantly blocks CXCR1 internalization even at 5 minutes after stimulation. On the contrary, HEK293 CXCR1 cells treatment with Reparixin does not block receptor internalization, resulting in a similar behavior of the receptor in cells treated with DMSO (Figure 27 B). Apparently Reparixin even induces on CXCR1 a trend to be internalized faster, compared to vehicle treatment.

4.4.2-Reparixin does not block CXCR1 interaction with β-arrestins after agonist stimulation

Since CXCR1 is internalized after ligand exposure in a β-arrestin-dependent manner [176], we decided to assess the effect of these inhibitors on CXCL8-induced β-arrestin recruitment to CXCR1.
Our results indicate that the orthosteric inhibitor DF3053Y efficiently blocks β-arrestin recruitment to CXCR1 in a dose-dependent manner, since 0.1 µM treatment still allows CXCR1 to partially recruit both β-arrestin 1 and 2 (Figure 28 C and D) even if significantly reducing CXCL8 efficacy (Figure 28 A and G), but already at 1 µM DF3053Y completely blocks receptor response compared to vehicle-treated cells (Figure 28 A, C and D). On the opposite Reparixin did not induce any significant modification to agonist-

induced β-arrestin interaction with CXCR1, and even at the highest dose there is no reduction in CXCL8 potency or efficacy (Figure 28 B, E, F and G).



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	β-Arrestin 2 recruitment			
CXCR1	LogEC50	±SEM	BRETmax	±SEM
DMSO	-7,947	0,09621	0,1233	0,004196
DF3053Y 10uM	-7,914	1,74	**0,04497 *****	**0,004996**
DF3053Y 1uM	-10,22	4,777	**0,04892 *****	**0,002585**
DF3053Y 0,1uM	-8,127	0,3678	**0,05885 *****	**0,005289**
DF1681Y 10uM	-7,576	0,1586	0,1175	0,006575
DF1681Y 1uM	-7,857	0,1548	0,1252	0,006358
DF1681Y 0,1uM	-8,034	0,06234	0,1124	0,002643

Figure 28: Effects of Reparixin on CXCL8-induced β-arrestin recruitment to CXCR1. Treatments are indicated as DMSO (●, black circle), 0.1 µM of drug (■, green square), 1 µM of drug (▼, blue facing down triangle) and 10 µM of drug (▲, red triangle). Prior to each experiment cells were pretreated for 30 minutes with the indicated concentration of inhibitor or the corresponding dilution of DMSO. **A-B)** Measurement of different concentration of inhibitors on dose-response curves of β-arrestin 2 recruitment to CXCR1 in living HEK293 cells by BRET. Results are expressed as Net BRET calculated subtracting the BRET ratio obtained by cells expressing only CXCR1-RLuc preincubated with DMSO and stimulated for 30 minutes with different

concentrations of commercial CXCL8, from the BRET ratio obtained from cells co-transfected with CXCR1-RLuc and β-arrestin 2-EYFP and stimulated for 30 minutes with corresponding concentrations of CXCL8 in presence of vehicle or different concentrations of DF3053Y (**A**) or DF1681Y (**B**) ±SEM; N=5. **C-F)** Effect of inhibitors on CXCL8-induced β-arrestin 1 (panel **C, E**) and β-arrestin 2 (panel **D, F**) recruitment to CXCR1 monitored over 15 minutes time in presence of PBS or 100 nM of CXCL8 variants and different concentrations of inhibitors or DMSO. One representative experiment out of 3 performed is shown for each condition. **G**) Table reports the mean LogEC50 and BRETmax ±SEM obtained by nonlinear regression curve fitting in the cAMP and β-arrestin 2 recruitment assays, with values statistically different reported in bold, calculated by one-way Anova with Dunnett's multiple comparison with ***=p<0.001 compared to DMSO-treated cells.

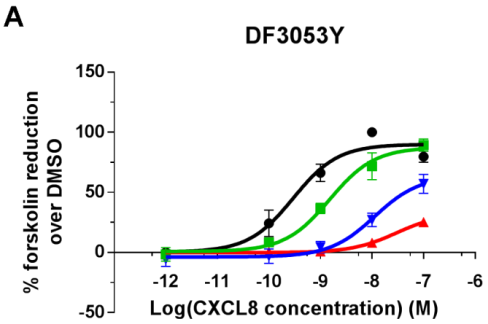
4.4.3-REPARIXIN DOES NOT BLOCK CXCR1 ADENYLYL CYCLASE INHIBITION

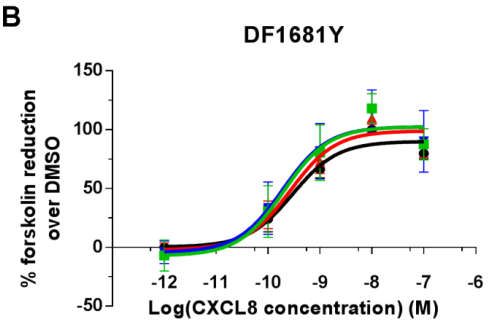
Reparixin has already been described as able to block efficently G protein activation, generation of Ca^{2+} fluxes and phosphorylation of focal adhesion kinases on CXCL8-stimulated polymorphonuclear leukocytes (PMN) [194]. Given our results indicating that CXCR1 treatment with this inhibitor still results in receptor internalization and β-arrestin recruitment after CXCL8 stimulation, we wanted to assess its ability to block CXCR1-dependent G protein signaling, therefore we measured the ability of increasing concentrations of CXCL8, in presence of different concentrations of Reparixin and DF3053Y, to reduce forskolin-induced intracellular cAMP increase in HEK293 CXCR1 cells.

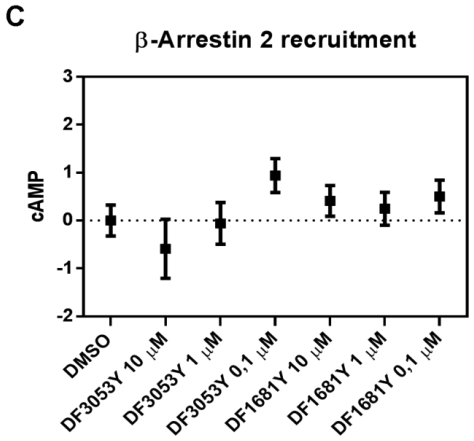
Our results confirm the inhibitory effect of DF3053Y, that is able to significantly reduce CXCL8 activity in terms of potency and efficacy, avoiding chemokine binding to CXCR1 (Figure 29 A, D), but also show that Reparixin treatment of HEK293 CXCR1 cells is ineffective in blocking adenylyl cyclase inhibition by CXCL8 (Figure 29 B, D). This result is unexpected since intracellular cAMP decrease is considered to be mainly mediated by α inhibitory subunits of G proteins, whose activation downstream CXCL8 stimulation of CXCR1 was demonstrated to be inhibited by Reparixin [194]. This raises open questions that require more investigations to confirm the ability to block in HEK293 cells CXCR1 signaling activities that have already been described in other cell types as PMN, to verify the reliability of HEK293 cells to assess Reparixin inhibition of CXCR1 and eventually confirm our data regarding receptor internalization and β-arrestin association.

We performed bias calculation to compare signaling modulation of at least DF3053Y, on which inhibition we can rely, to vehicle treated cells and it reported no significant changes in CXCR1 activation balance (Figure 29 C).

Taken together, these results indicate that inhibition of CXCR1 by occupation of its orthosteric binding pocket is an effective method to prevent CXCR1 activation by CXCL8 in vitro, while on Reparixin we can only say that it does not block CXCL8-induced CXCR1 G protein activation, β-arrestin recruitment or internalization in HEK293 cells, pending on the confirmation of inhibitor activity in at least one assay already reported in literature to be blocked by this inhibitor.







D

	cAMP			
CXCR1	LogEC50	±SEM	cAMPmax	±SEM
DMSO	-9,529	0,1923	90,02	5,396
DF3053Y 10uM	**-7,522 ****	**0,1951**	**32,91 ****	**4,439**
DF3053Y 1uM	**-7,95 ***	**0,2116**	63,66	8,175
DF3053Y 0,1uM	-8,821	0,1627	87,49	5,599
DF1681Y 10uM	-9,57	0,2322	98,93	7,282
DF1681Y 1uM	-9,719	0,3967	102,5	13,09
DF1681Y 0,1uM	-9,7	0,353	102,5	11,94

Figure 29: Effects of Reparixin on CXCL8-induced CXCR1 inhibition of forskolin. Treatments with inhibitors are indicated as DMSO (●, black circle), 0.1 μM of drug (■, green square), 1 μM of drug (▼, blue facing down triangle) and 10 μM of drug (▲, red triangle). **A-B)** Alphascreen measurement of intracellular cAMP in HEK293 CXCR1 cells pretreated for 30 minutes with the indicated concentration of inhibitor before being stimulated with 10 μM forskolin and different concentrations of CXCL8, with the result reported as percentage of inhibition of forskolin-induced cAMP increase normalized over DMSO treatment ±SEM; N=3. **C**) Bias plot indicates the ΔΔlog(τ/KA) ±SE for cAMP signaling over β-arrestin 2 recruitment. **D)** Table reports the mean LogEC50 and cAMPmax ±SEM obtained by nonlinear regression curve fitting in the cAMP assay. Values statistically different from DMSO-treated cells are reported in bold, with *=p<0.05 and **=p<0.01 calculated by one-way Anova with Dunnett's multiple comparison.

5-DISCUSSION

Chemokine system is a highly complex system comprising about 50 ligands that can interact more or less promiscuously with about 20 chemokine receptors, that inducing cell migration towards chemical gradients of chemokines sustain accumulation of cells on which receptors are expressed at sites where cognate chemokine is more abundant [1, 5]. Specific cell positioning regulated by chemokines is a fundamental process to coordinate different biological functions, among which in adult organisms are leukocyte trafficking within peripheral tissues and their homing in lymphoid organs in homeostatic conditions [7]. Due to their abundance of expression on leukocytes, chemokine receptors also drive immune cells recruitment to inflamed tissues in response to pathological processes [1-5, 7], dependent on the fact that chemokines expression is usually induced upon inflammatory stimuli, with many different chemokines up-regulated at once to warn about a dangerous situation and recruit leukocytes in inflamed tissues to face the offense [1, 7]. This mechanism of chemokine activity regulation is generally devoid of stringent control, since most chemokine receptors can be expressed by more than one leukocyte, respond to different chemokines and most chemokines can activate more than only one receptor, giving rise to a redundant and complex system that can be hardly regulated by an on/off mechanism simply based on gene expression [55]. In the case of immune response control, misleading signals can result in immune deficiency, for defective leukocyte recruitment to inflamed tissues or maintenance of hematopoietic progenitors in bone marrow niches, or on the opposite can result in excessive immune response that causes tissue injury, auto-immunity, inflammation related diseases and even support cancer development [1, 9-11]. Therefore, given the relevance of chemokine system regulation, fine tuning of chemokine activity is exerted at multiple levels, with different steps of regulation that determine chemokine fate, modulating their activity and controlling leukocyte recruitment to prevent immune system dysregulation [7]. Chemokine activity on target cells is mediated by chemokine receptors, that belong to the superfamily of 7 TM GPCRs, and upon chemokine recognition receptors undergo a conformational rearrangement that activates heterotrimeric pertussis toxin-sensitive Gαi proteins [23, 28, 69, 171] leading to the activation of many intracellular signaling events, among which occur also receptor phosphorylation and β-arrestins recruitment, that desensitize the receptor, mediate its internalization and simultaneously support the activation of many other signaling cascades [74, 336]. A relatively new concept in pharmacology has provided the idea and evidences that GPCRs do not act as bimodal switches that shift from an inactive to an active state, but they can be activated with different grades to activate in particular circumstances only selected signaling pathways and not indiscriminately all the signals they can conventionally elicit, on the basis of which agonist they bind, the cellular type in which they are expressed [84] or their structural properties that regulate receptor conformation [24, 221]. These evidences have been found to apply also to the chemokine system, with receptors like CXCR3, CCR2, CCR5 and CCR7 that can generate different responses upon binding of different ligands or by manipulation of their structure [24, 99, 101, 102, 135,

136, 221], that impact on their chemotactic ability, inducing a preferential signaling towards signaling pathways mainly involved in the regulation of other biological functions of chemokines rather than promoting cell migration [77, 114]. To this point one of the aim of this thesis has been to investigate the existence of bias signaling in two key components of the chemokine system regulation, post-translational modifications of chemokines and the regulatory activity of atypical chemokine receptors.

Post-translational modifications are enzymatic reactions that modify proteins, regulating their structure, stability and activity. Several evidences demonstrated that post-translational modification naturally occur also on the chemokine system, affecting both receptors [337] and chemokines [226]. In particular these enzymes can be released during infection, deriving from bacterial origin to support infection establishment interfering with host immune system, but can also be released by immune cells of the host itself to achieve pathogen or host pathologic cells degradation and clearance. These enzymes act on the most different proteins, among which chemokines, that have been identified in vivo to be occurring with post-translational modifications, that can be generated by pathogens or by host response to pathogens [226]. Most of the modifications on chemokines are made by proteases, that cleave chemokines affecting their biological activity that either reducing it, by chemokine inactivation and degradation, or on the opposite increasing it, depending on the chemokine modified and on the modifications exerted [16, 226]. In this thesis the attention was focused on the post-translational modifications occurring on two ELR^+ CXC chemokines, CXCL5 and CXCL8, that acting on CXCR1 and CXCR2 constitute an important signal for the activation and recruitment to inflamed sites of neutrophils, a key component of innate immune response. Both CXCL5 and CXCL8 can be cleaved by different proteases on their NH_2-terminus, generating chemokine variants differing in the exposure of their ELR^+ motif, fundamental for receptor activation and chemotaxis [186, 226]. Another modification occurring on these two chemokines is substitution of NH_2-terminal arginine with citrulline, by the removal of the imino moiety present on arginine and its replacement with an oxygen. All these modifications have been described to appear on CXCL5 and CXCL8 in different immune models and diseases [226]. Proteases cleaving CXCL8 and CXCL5 NH_2-terminal residues have already been shown to improve their chemotactic activity, increasing it in the case of CXCL8 and activating CXCL5 which is a poor chemoattractant in its intact form [240, 243-245, 338, 339]. These modification enhance CXCL8 and CXCL5 signaling activity measured by induction of Ca^{2+}, increase receptor desensitization and improve chemotaxis both in vitro and in vivo, proving themselves as strong regulators of the chemokine system by amplifying chemokine activity in the first phases of inflammation, where neutrophils begin to accumulate and release these enzymes in inflamed tissue where have been recruited by chemokines, that are modified to recruit more and more immune cells at the inflammatory site [226, 228]. Citrullination has been shown to be able to generate in the case of CXCL8 a more potent agonist compared to intact CXCL8 in in vitro assays, but in vivo, even if it is still able to induce stronger neutrophil mobilization within the blood, it lacks

chemotactic ability within the tissue due to its deficient binding to GAGs that retain chemokines close to inflamed tissues [340, 341]. In the case of CXCL5, citrullination dampens its activity, reducing CXCL5 signaling and chemotactic ability.

In our experiments, aimed at evaluating the ability of these post-translational modification to induce CXCL8 and CXCL5 biased agonism, we tested different chemokine variants on their ability to activate Gαi proteins and to recruit β-arrestins to CXCR1 and CXCR2. We observed that in the case of CXCL8 no bias occurs after chemokine modification, compared to the signaling induced by intact CXCL8 on both CXCR1 and CXCR2 receptors in HEK293 cells, in terms of selectivity between receptor inhibition of adenylyl cyclase and β-arrestins recruitment. Anyway, we have been able to reproduce evidences already published on the behavior of these chemokine variants, with truncation of NH_2-terminal residues that progressively increases chemokine potency in signal activation on both CXCR1 and CXCR2. In our hands citrullination of arginine 5 of CXCL8 increases chemokine potency assayed on HEK293 cell transfectants, in part different with published data reporting that this modification only affects receptor binding [340] and not its signaling properties [341] on neutrophils, possibly due to differences in the cellular system. We also evaluated the effects of post-translational modifications acting on CXCL5 NH_2-terminus, recording that these modifications alter chemokine potency, with progressive cleavage that increases chemokine potency and citrullination of arginine in position 9 that reduces it signaling ability, in agreement with previously published data [245]. We also calculated the ability of these modifications to bias CXCL5 agonism, resulting in no reliable data due to the poor ability of CXCL5 to induce β-arrestin recruitment to CXCR2 at the concentration we tested, limited by the amount of variants availability and partly by the reflection of a biological relevant setting, in which is almost impossible to find elevated concentrations of chemokines. Therefore we can only conclude that these modifications for sure modulate CXCL5 potency, but their ability to bias CXCL5 signaling have still to be assessed as soon as new amounts of different variants will be synthesized. Taken together, these results allow us to conclude that NH_2-terminal truncation mediated by different proteases acting on both CXCL8 and CXCL5 increase chemokine activity, while citrullination has divergent effects on CXCL8 and CXCL5, where increases and decreases chemokine potency, respectively. Therefore we can say that chemokine system regulation by post-translational modifications occurring on CXCL8 do not induce any selective activation between G proteins and β-arrestins recruitment on CXCR1 and CXCR2 in HEK293 cells, but only have a role in globally regulating chemokine potency. On CXCL5 modifications instead more experimental evidences are needed before accounting any effect on functional selectivity, while it can be said that truncation and citrullination of CXCL5 NH_2-terminal residues act in opposition to modulate chemokine activity.

Chemokine system regulation is mediated also by atypical chemokine receptors, that despite sharing conserved sequence and structural homology to chemotactic chemokine receptors [3], possess

several differences in structural motifs involved in GPCR conformational change that underlies receptor activation and coupling to signal transducers [23, 33, 143] that allows them to regulate the chemokine system rather than promoting cell migration. These receptors therefore are thought to have evolved from chemokine receptors by modifying structural features important to activate migratory signals into motifs that still regulate their activation but induce structurally biased signaling activities that allow them to control chemokine functions by transport, present or degrade their ligands in barrier tissues, where they are preferentially expressed compared to conventional chemokine receptors [3, 143, 225, 246, 282, 292, 294]. ACKR2 is one of the most characterized ACKRs and due to its ability to efficiently mediate degradation of its ligands, it dampens inflammation and reduces leukocyte recruitment [142]. Its activity relies on both a constitutive trafficking, by which ACKR2 is continuously internalized and recycled back to the plasma membrane, and on its G protein independent and β-arrestin dependent signaling activity, induced after agonist binding to increase receptor recycling rate improving its degradative performance [292, 312]. To better elucidate this ACKR2 structurally biased signaling activity and to understand the relevance of this bias in the regulation of chemokine system activity, we decided to assess different aspects of ACKR2 signaling properties, comparing them to the signals elicited by the conventional chemokine receptor CCR5, which mediates chemotaxis upon recognition of many ACKR2 ligands. Therefore we first assessed the ability of these receptors to interact with β-arrestins and after that we investigated the most relevant signaling activities elicited by these receptors in constitutive conditions and after chemokine stimulation by quantitative measurement of modifications in the phosphorylation status of HEK293 cell transfectants whole proteome.

Previously published evidences obtained by confocal microscopy, which only allows to measure colocalization of proteins, indicated that ACKR2 can drive β-arrestins relocalization in basal conditions, and that this association regulates receptor intracellular localization [292, 309, 324]. Our results obtained by BRET, that allows to monitor physically interacting molecules, are in agreement with these data and indicate that ACKR2 is able to associate both β-arrestin 1 and β-arrestin 2 in basal conditions, with β-arrestin 1 preferentially associated to molecules expressed on the cell surface, rather than to ACKR2 present in intracellular stores, and this is in contrast to CCR5 that is only weakly associated to β-arrestins in basal conditions. ACKR2 binds different inflammatory CC chemokines, that are degraded by ACKR2 with different efficacy, depending on the presence of a proline in position 2 from the NH_2-terminus of the chemokine, and can be distinguished between active ligands, the ones efficiently degraded, and neutral ligands, the ones bound but poorly or not degraded [310]. Active ligands are considered as ACKR2 agonists, since we already published our evidences indicating that these active ligands induce the activation by ACKR2 of a G protein-independent and β-arrestin 1-dependent signaling pathway. This pathway, impacting on Cofilin phosphorylation, regulates actin cytoskeleton rearrangement necessary to improve ACKR2 scavenging ability, increasing its recyling rate and its

expression on the cell surface [292, 313]. To link this agonist-induced signaling activity to β-arrestin interaction, we assessed by BRET the ability of chemokines to modify ACKR2 association to β-arrestins. Our results demonstrate that, regardless of ACKR2 subcellular localization, agonist stimulation induces an increase of β-arrestin 1 recruitment to ACKR2, but not of β-arrestin 2, which remains associated to the receptor with only minimal differences between agonist and PBS stimulation. Furthermore, this ACKR2 preference occurs not only in HEK293 cells but also in a choriocarcinoma cell line originated from throphoblasts, that endogenously express ACKR2, indicating that this preferential recruitment of β-arrestin 1 compared to β-arrestin 2 is a specific characteristic of the receptor and not of cell type used for the experiment. On the opposite, CCR5 stimulation induces recruitment of both β-arrestin 1 and 2, as previously reported [86, 100], and in our hands this occurs preferentially on the plasma membrane after 20 minutes stimulation. These results indicate that agonist-induced β-arrestin recruitment occurs differentially between CCR5 and ACKR2, and in the case of CCR5 the association with both β-arrestin 1 and 2 is initially mainly induced on cell surface and at later time points increases also in the intracellular compartments, possibly due to the fact that CCR5 is internalized after ligand exposure, resulting in a cytosolic localization of endocytic vesicles in which CCR5 remains associated to β-arrestins. CCL3L1 stimulation instead increases ACKR2 interactions preferentially with β-arrestin 1, and since these proteins regulate receptor trafficking [309, 324], and is β-arrestin 1 the essential regulator of ACKR2 up-regulation on the plasma membrane in response to active ligands [292], we can speculate that after agonist exposure ACKR2 increases its association preferentially to β-arrestin 1 because this protein and not β-arrestin 2 may be more suited to associate ACKR2 molecules present in intracellular stores and direct their mobilization to plasma membrane. In addition, this preferential increase in ACKR2 association to β-arrestin 1 occurs only in presence of agonists and not with neutral ligands, meaning that ACKR2 is a chemokine receptor structurally biased to signal through β-arrestins and not G proteins, but is also biased in the selective association of β-arrestin 1 for modulating its agonist-induced signaling activity. To explore the signaling implications of such a great ability of ACKR2 to interact with β-arrestins in basal conditions and after ligand stimulation, we decided to analyze the signaling activities that ACKR2 regulates and the differences with CCR5-mediated signals, in terms of phosphorylation modifications on the whole proteome, assessed by SILAC. Our analysis describes that ACKR2 expression is sufficient to modify expression levels of different proteins inside the whole proteome, and to modulate their phosphorylation more than occurring after CCR5 expression. In addition, stimulation with the same agonist induces in the whole proteome very different changes between CCR5 and ACKR2 both in terms of activation kinetics, with ACKR2 that is more prone to induce changes in phosphorylation at later time points, and signal transducers, since ACKR2 is modulates a significant quote of phosphorylations and also dephosphorylations, that in the case of CCR5 represent only a minor portion of the modifications. These differences are also reflected by the kinase motifs regulated,

indicative of the fact that ACKR2 and CCR5 exploit different kinases to modulate proteome phosphorylation, which is reflected by the classification of these modifications by gene ontology, that describe ACKR2 as able to induce stronger modulation of proteins whose functions are involved in the regulation of transcription and macromolecular complex formation, compared to CCR5.

These results tell us that signaling activities elicited by ACKR2 are extremely different compared to CCR5, in terms of both kinetics of regulation of the same molecules, as MAP kinases, and in the regulation of proteins with different functions by the activation of different sets of kinases. Differences in functions and association to β-arrestin 1 or β-arrestin 2 have already been reported to occur for other GPCRs [342] and also for ACKR3 [281], therefore in our opinion ACKR2 evolved to constitutively interact with both β-arrestins, that can eventually regulate its constitutive trafficking ability and support its constitutive signaling, but to respond to agonist stimulation ACKR2 is able to increase its association preferentially to β-arrestin 1 rather than β-arrestin 2. Up to date, needing a direct experimental demonstration, we can only speculate that this preferential association results in the differences we observed in proteome phosphorylation, but for sure it is possible that these differences can underlie the regulation of different activities downstream ACKR2 regulated by the two β-arrestins. To this point we consider that a deeper investigation of ACKR2 signaling activities could help unveiling not only the mechanisms involved in chemokine system regulation exerted by ACKRs, but could also improve the current knowledge existing on GPCRs and biased signaling, defining ACKR2 as a structurally biased receptor that can selectively engage β-arrestins to regulate its functions.

We consider that a better understanding of the mechanisms underlying biased signaling in chemokine receptors, and on how this occurs due to structural modifications in ACKRs, would help drug design aimed at targeting the chemokine system, that up to now, despite all the efforts invested, lead only to three commercialized drugs targeting the chemokine system [10, 11, 55]. In detail, since in the case of ACKRs the lack of G protein activation but the retained interaction with β-arrestins allow these receptors to internalize chemokines and dampen inflammation, we tried to characterize the migration inhibitory activity of Reparixin, a small molecule inhibitor of CXCR1 that does not inhibit CXCL8 binding to the receptor, but prevents leukocytes migration within inflamed tissues in different preclinical model of inflammatory diseases [194, 197-199]. This inhibitor showed promising results in advanced clinical trials for the prevention of rejection of transplanted pancreatic islets [201]. In particular our aim has been to characterize if this promising chemokine receptor drug relies on the modulation of CXCR1 signaling towards a biased activity, blocking G protein signaling and maintaining β-arrestins recruitment as ACKRs do, mediating chemokine degradation, since it could represent a more effective therapeutic approach compared to conventional antagonistic approaches [10, 11, 119].

Our results indicate that differently from an ortosteric inhibitor, that preventing CXCL8 binding to CXCR1 prevents its activation and signaling on all the functional readouts observed, by binding CXCR1

at the level of an allosteric binding site Reparixin allows CXCL8 to induce receptor internalization, β-arrestins recruitment and adenylyl cyclase inhibition, assessed on HEK293 cells. These results are, at least regarding the adenylyl cyclase inhibition, unexpected in our mind and contradictory to what has already been published on this inhibitor, since it is able to block G proteins activation in PMN, that are considered to be the main responsible for cAMP decrease observed after chemokine receptors activation. This results therefore raise different possibilities, the most valuable of which is that this inhibitor, assayed on HEK293 cells, is not effective, and therefore the activity of this inhibitor should be assayed in our HEK293 cell model reproducing data obtained on other signaling pathways in other cell types as PMN, such as GTPγS binding to membrane preparations, focal adhesion kinases activation or Ca^{2+} fluxes generation [194, 343], to verify that HEK293 cells represent a reliable model to study allosteric modulators activity.

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