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1. Title page

Title

Atypical chemokine receptor 3 (ACKR3): a comprehensive overview of its expression and potential roles in the immune system.

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2. Running title page

a) Running title

ACKR3 expression and function in the immune system.

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c)

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Number of figures: 1

Number of references: 100

Number of words in *Abstract*: 112

Number of words in *Introduction*: 317

Number of words in *Discussion*: 375

d) Non-standard abbreviations

ACKR3	Atypical chemokine receptor 3
BM	Bone marrow
CXCL11	C-X-C motif chemokine ligand 11
CXCL12	C-X-C motif chemokine ligand 12
CXCR4	C-X-C chemokine receptor type 4
CXCR7	C-X-C chemokine receptor type 7
EGFP	Enhanced green fluorescent protein

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ERK	Extracellular signal–regulated kinase
HSC	Haematopoietic stem cell
MIF	Macrophage migration inhibition factor

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3. Abstract

Atypical chemokine receptor 3 (ACKR3), previously known as C-X-C chemokine receptor type 7 (CXCR7), has emerged as a key player in several biological processes particularly during development. Its CXCL11 and CXCL12 scavenging activity and atypical signalling properties together with a new array of other non-chemokine ligands have established ACKR3 as a main regulator of physiological processes at steady state and during inflammation. Here, we present a comprehensive review of ACKR3 expression in mammalian tissues in search of a possible connection with the receptor function. Besides the reported roles of ACKR3 during development, we also discuss the potential contribution of ACKR3 to the function of the immune system, focusing on the myeloid lineage.

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4. Introduction

This review focuses on the trio formed by CXCL12 and its two receptors, CXCR4 and CXCR7/ACKR3, with a particular emphasis on the latter one, which belongs to the atypical chemokine receptor subfamily. Since the 1990s, with CXCR4 being discovered as a co-receptor for HIV entry (Feng *et al.*, 1996), the CXCL12/CXCR4 axis has been extensively studied in numerous homeostatic and pathological settings including organogenesis, leukocyte trafficking and cancer. ACKR3 was first known as an orphan receptor named receptor dog cDNA 1 or RDC1 (Heesen *et al.*, 1998) and was later adopted into the chemokine receptor family as CXCR7, the second receptor for CXCL12 (Balabanian *et al.*, 2005) and also CXCL11 (Burns *et al.*, 2006), before being renamed ACKR3 due to its atypical non-G protein dependent signalling (Bachelerie *et al.*, 2014). Since then, compelling evidence has underscored the regulatory function of ACKR3 on the CXCL12/CXCR4 signalling axis. Initial studies in zebrafish models revealed that ACKR3 acts as a scavenger receptor that binds and internalises CXCL12, thus indirectly modulating CXCR4 function by modifying chemokine bioavailability (Dambly-Chaudière *et al.*, 2007; Valentin *et al.*, 2007; Boldajipour *et al.*, 2008; Donà *et al.*, 2013). Additionally, ACKR3 may have direct functions in response to CXCL12 as a β-arrestin-biased signalling receptor (Rajagopal *et al.*, 2010), although β-arrestin-mediated signalling downstream of ACKR3 remains to be demonstrated *in vivo*. Furthermore, with the identification of new non-chemokine ligands, including macrophage migration inhibitory factor (MIF) or intermediate opioid peptides, ACKR3 has emerged as a key player in homeostatic processes during embryogenesis and adult life but also in pathological inflammatory and tumour contexts. Here, we first summarise the state of the art on ACKR3 expression with regard to human and rodent tissues and its role in development, before discussing its potential contribution to the function of the immune system. In particular, we focus on myeloid cells, both at homeostasis and in pathological settings, including inflammatory conditions and breast cancer.

5. ACKR3 expression in mammalian tissue

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To address the question of ACKR3 expression, mice have been genetically modified to investigate in which cells and tissues the *Ackr3* promoter is active (Table 1). These mouse models include replacement of the endogenous *Ackr3* coding region by either a β-galactosidase (*LacZ*) reporter (Gerrits *et al.*, 2008) or an enhanced green fluorescent protein (EGFP) sequence (Cruz-Orengo *et al.*, 2011). These models also include the *Ackr3-EGFP* bacterial artificial chromosome (BAC) mouse model, where an *Ackr3* promoter-*EGFP* fusion sequence was inserted into a random location in the genome. In this case, EGFP expression is driven by *Ackr3* promoter activity, while leaving the endogenous *Ackr3* locus intact (Gong *et al.*, 2003; Sánchez-Alcañiz *et al.*, 2011).

In parallel, several groups have investigated *Ackr3* expression at the transcriptional level by means of northern blot, real time polymerase chain reaction, or *in situ* hybridization (Table 2) and at the protein level by means of immunofluorescence, immunohistochemistry or flow cytometry (Table 2). *Ackr3* mRNA is mostly detected in mouse heart, kidney, spleen, lung and brain (Table 2) and is transiently expressed during embryogenesis, in accordance with different reporter mouse models (Table 1). To summarise, by combining various techniques, ACKR3 mRNA and protein have been detected i) in mesenchymal stromal cells, ii) in brain-resident cells including astrocytes, glial and neuronal cells, iii) in cells of the vascular system and more specifically cells from vascular smooth muscle and venous endothelium and of particular interest, iv) in immune cell populations. In the immune system, ACKR3 mRNA is detected using transcriptomic approaches in haematopoietic lineages, in both lymphoid (e.g. B cells) and myeloid (e.g. macrophages) cells. However, in EGFP reporter mouse models, the *Ackr3* promoter-dependent signal cannot be distinguished from background in any of the studied immune cell subsets in steady state (i.e. $CD45^+$, $CD19^+$ B cells, $CD4^+$ and $CD8^+$ T cells, $CD11b^+$ and $CD11c^+$ myeloid cells) (Cruz-Orengo *et al.*, 2011). This was confirmed in LacZ mouse models (Berahovich *et al.*, 2014). Furthermore, assessing ACKR3 protein expression in native conditions poses a technical challenge due to its constitutive recycling between the membrane and the endosomal compartment, leading to a predominant intracellular localisation. This is a limitation for antibody generation and validation (Berahovich *et al.*, 2010)

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and a potential source of discrepancies reported in studies related to ACKR3 expression and function. For instance, ACKR3 protein was not detected either in human and mouse leukocyte subsets in peripheral blood (Berahovich *et al.*, 2010). However, ACKR3 protein was detected in human secondary lymphoid organ-derived B cells and dendritic cells (Infantino *et al.*, 2006).

6. ACKR3 function: from genetically modified mice to non-chemokine ligands

Determining when and where ACKR3 is expressed has led to greater understanding of the functions that ACKR3 might be exerting. In particular, the expression pattern of ACKR3 may hint towards a functional role in such cells or tissues. These functions could have a direct effect on ACKR3-expressing cells either through non-canonical signalling pathways or modulation of CXCR4 functions, or in a paracrine way via the modulation of CXCL12 and CXCL11 levels, impacting nearby-cell function through CXCR4 and CXCR3 respectively. Valuable information about the function of ACKR3 was first provided by an analysis of the effects following constitutive and cell-type conditional *Ackr3* gene deletion in various mouse models (Table 1). Subsequently, the identification of other non-chemokine ligands has broadened our understanding of ACKR3 biology, particularly in terms of the role of ACKR3 beyond the chemokine system. In the following section, we discuss how the study of knockout mouse models and non-chemokine ligands has led to further insights into the functions of ACKR3.

6.1. Lessons from constitutive and conditional knock-out models

Most *Ackr3*$^{-/-}$ mice develop normally in early embryonic stages, but die either perinatally or *in utero* in late developmental stages, usually from embryonic gestation day E17.5, due to cardiovascular complications (Sierro *et al.*, 2007; Yu *et al.*, 2011; Trousse *et al.*, 2015). Accordingly, it seems that ACKR3, similarly to CXCR4 and CXCL12, is essential to normal mouse development and physiology and that it plays a complementary or non-redundant role with regard to CXCR4. However, the lethal phenotype obtained in the C57Bl/6 background is less severe on a mixed genetic background (129 Sv/Evbrd x C57Bl/6) with a survival rate of

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approximately 30% (Gerrits *et al.*, 2008). This might be linked to CXCL11-associated ACKR3 functions, because the expression of this chemokine ligand is absent in C57Bl/6 mice (Sierro *et al.*, 2007). Considering the cardiovascular and cerebral defects observed in *Ackr3*-deficient mice, a large body of work has focused on ACKR3 contribution to heart and brain physiology. During both heart and brain development, *Ackr3* undergoes a change in expression pattern after E14.5, which coincides with the onset of mouse death (Sierro *et al.*, 2007; Gerrits *et al.*, 2008; Sánchez-Alcañiz *et al.*, 2011; Wang *et al.*, 2011; Yu *et al.*, 2011).

Two studies suggest that a link may exist between *Ackr3* expression and the control of cell proliferation in heart tissue. For example, in constitutive *Ackr3*$^{-/-}$ mice, an increased cell proliferation prevented heart valve thinning that led to a lethal cardiovascular phenotype (Yu *et al.*, 2011). Moreover, in another study, 25% of surviving adult *Ackr3*$^{-/-}$ mice suffered from cardiac hyperplasia (Gerrits *et al.*, 2008). Of note, migration and apoptosis of semilunar valve mesenchymal cells remained normal from E14 to E18.5 in *Ackr3*$^{-/-}$ mice. In contrast, during brain development, constitutive and conditional loss of *Ackr3* in GABAergic neurons (Table 1) led to an abnormal distribution of interneurons in the cortex, suggesting a link between *Ackr3* expression and neuron migration (Sánchez-Alcañiz *et al.*, 2011; Wang *et al.*, 2011; Trousse *et al.*, 2015). ACKR3 could have a cell intrinsic function as suggested by ERK1/2 phosphorylation in cultured neurons, likely downstream of ACKR3 (Wang *et al.*, 2011). However, it remains to be determined whether ACKR3-mediated ERK1/2 phosphorylation occurs *in vivo* and whether it is relevant during brain development. Alternatively, the role of ACKR3 in interneuron positioning could occur through cell-extrinsic effects as a scavenger receptor. *Ackr3*-deficiency results in increased CXCL12 protein levels but unchanged *Cxcl12* mRNA levels in cortical homogenates (Sánchez-Alcañiz *et al.*, 2011). Failure to maintain a CXCL12 gradient leads to accumulation of migrating interneurons in inappropriate locations in the cortex. Moreover, the abnormal distribution of interneurons was rescued when *Ackr3*$^{-/-}$ interneurons were transplanted into *Ackr3*$^{+/+}$ brain, suggesting that ACKR3 expression in other cells can rescue the phenotype (Sánchez-Alcañiz *et al.*, 2011). Furthermore, a recent report has provided mechanistic insights into ACKR3-mediated CXCL12 endocytosis in interneurons by

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demonstrating that receptor phosphorylation was required for this process, whereas β-arrestins were dispensable (Saaber *et al.*, 2019). Altogether, these findings in *Ackr3*-deficient mice support a role for ACKR3 as a scavenging receptor, in particular in the brain. Even though no obvious defects in the immune system were found in these mouse models, this aspect was not fully explored in the studies, implying that a knowledge gap might exist in this field (discussed in section 7).

6.2. Microenvironment-dependent functions of ACKR3 and non-chemokine ligands

Differences observed in heart and brain tissues in *Ackr3*$^{-/-}$ mice can, at least partially, be explained by the microenvironment having an impact on the biological effects exerted by ACKR3. ACKR3 tissue-dependent functioning might be related to its capacity to interact with several ligands and therefore could be dependent on the surrounding cells producing or processing such ligands. Firstly, within the chemokine system, CXCL12 displays six isoforms in humans and three in mice due to alternative splicing. These isoforms have different C-terminal extensions, and have different expression patterns and functions (discussed in Janssens *et al.*, 2018). Secondly, CXCL12 isoforms are processed post-translationally by the microenvironment producing forms of CXCL12 with different binding and signalling properties on CXCR4 and ACKR3 (Peng *et al.*, 2012; Janssens *et al.*, 2017, 2018; Szpakowska *et al.*, 2018b). Additionally, the microenvironment changes during disease conditions. For example, CXCL11, which is a ligand for CXCR3 as well as for ACKR3, is normally not detectable in physiological conditions but an inflammatory context, in which cytokines such as interferon are produced, can induce CXCL11 expression (Flier *et al.*, 2001; Müller *et al.*, 2010; Van Raemdonck *et al.*, 2015; Singh *et al.*, 2016). This implies that ACKR3 function may vary in pathological conditions compared to steady state (Figure 1), adding another layer of complexity. Lastly, ACKR3 tissue-dependent functions likely depend on the presence of other reported endogenous ligands of ACKR3 outside the chemokine system, including MIF (Alampour-Rajabi *et al.*, 2015), intermediate opioid peptides (Ikeda *et al.*, 2013) and possibly proteins in the adrenomedullin pathway (Klein *et al.*, 2014).

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MIF is an inflammatory cytokine that lacks the structural requirements to qualify as a chemokine. However, a previous study has suggested it should be considered as a pseudo-CXC chemokine (Bernhagen *et al.*, 2007) as it binds with high affinity to CD74 (K_d ≈ 9 nM), CXCR2 (K_d ≈ 1.4 nM), CXCR4 (K_d ≈ 19.8 nM) and to ACKR3 in the nanomolar range (K_d not determined) (Leng *et al.*, 2003; Alampour-Rajabi *et al.*, 2015; Bernhagen, 2018). MIF has a physiological role as a chemoattractant (Bernhagen *et al.*, 2007), and is involved in innate and adaptive immune responses by promoting macrophage activation and B cell survival (Gore *et al.*, 2008). Moreover, MIF is a mediator in several inflammatory conditions and cancers in an autocrine and paracrine manner by promoting tumour growth (Nobre *et al.*, 2017) and inducing metastasis through CXCR4 (Dessein *et al.*, 2010). Furthermore, MIF/ACKR3 signalling has been studied in platelets, where it prevents apoptosis (Chatterjee *et al.*, 2014), providing evidence for a role of ACKR3 in the haematopoietic system.

Intermediate opioid peptides such as BAM22 are produced in the adrenal cortex by subcapsular cell hyperplasia cells and BAM22 has been shown to displace CXCL12 from ACKR3 (IC_{50} = 32.2 nM) (Szpakowska *et al.*, 2018a). The BAM22/ACKR3 signalling axis has a critical role in the modulation of circulating glucocorticoids. This occurs through the increase of the amplitude of adrenocorticotropic hormone (ACTH)-induced glucocorticoid diurnal oscillation in females (Ikeda *et al.*, 2013). ACKR3 is highly expressed in the adrenal glucocorticoid-producing cells especially in female mice compared to males in support of the sex differences of the BAM22/ACKR3-dependent glucocorticoid oscillations.

Finally, adrenomedullin is a peptide hormone involved in angiogenesis and is implicated in cardiovascular diseases. A link between adrenomedullin and ACKR3 pathways may exist on the basis that haploinsufficiency of adrenomedullin partially rescued the lethal defects in *Ackr3*$^{-/-}$ mice by normalising the cardiac hyperproliferation (Klein *et al.*, 2014). ACKR3 was suggested as an adrenomedullin scavenger, but a recent paper showed that adrenomedullin does not displace CXCL12 from ACKR3 within the 6pM to 1μM range (Szpakowska *et al.*, 2018a). However, the presence of ACKR3 inhibited canonical adrenomedullin signalling (Klein

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et al., 2014), suggesting a crosstalk between adrenomedullin and ACKR3 pathways that remains to be fully explored.

7. Potential role of ACKR3 within the immune system at steady state and during inflammation

The absence of obvious immune-haematopoietic defects in the available *Ackr3*$^{-/-}$ mouse models does not exclude a role for ACKR3 in the immune system. Evidence suggests that the CXCL12/CXCR4 signalling axis can regulate the function of the immune system, notably by controlling immune cell subset migration and compartmentalisation (Wei *et al.*, 2006; Balabanian *et al.*, 2012), or haematopoietic stem cell (HSC) homing, retention, and quiescence in bone marrow (BM) (Sugiyama *et al.*, 2006). ACKR3 mRNA and protein are also expressed in certain immune cell subsets such as B cells and myeloid cells, as reported by several groups (Table 2). ACKR3 might also be involved in the circadian oscillation of CXCL12 expression levels, which regulate immune cell trafficking from and to BM (Figure 1). In a similar manner to glucocorticoids, CXCL12 transcript and protein levels rhythmically oscillate in BM with light-dark cycles (Katayama *et al.*, 2006; Méndez-Ferrer *et al.*, 2008, 2010). This oscillation regulates retention in and mobilisation from BM of CXCR4-expressing HSCs, which are released during sleep when CXCL12 levels are low and return to BM when CXCL12 levels have increased again (Méndez-Ferrer *et al.*, 2010). CXCL12 is produced by osteoblasts in the bone fraction, endothelial cells around both endosteal and vascular niches, and perivascular mesenchymal stromal cells in the marrow fraction, with the latter representing a major contributor to the CXCL12 pool (Itkin *et al.*, 2016). Depending on the production site, CXCL12 regulates either HSC maintenance or retention (Itkin *et al.*, 2016; Asada *et al.*, 2017). Considering that the ACKR3 scavenging function shapes CXCL12 gradients, the contribution of ACKR3 to the circadian oscillation of CXCL12 levels remains an open question (Figure 1). Although most of this evidence relates to CXCL12/CXCR4 function, they indirectly point towards a role for ACKR3 in some processes within the immune system, both at steady state

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and in inflammatory settings. In the following sections we explore this apparent knowledge gap focusing on myeloid cells.

7.1. Potential role for ACKR3 in the myeloid compartment at steady state

Myeloid cells, such as neutrophils, dendritic cells or monocytes are key players in innate immunity and CXCL12/CXCR4 tightly regulate their homeostasis (De Filippo and Rankin, 2018). In particular, this includes their retention in BM and functioning in peripheral tissues (Chong *et al.*, 2016; Evrard *et al.*, 2018). CXCL12 promotes the extravasation of monocytes and their *in vitro* differentiation (Sánchez-Martín *et al.*, 2011; Chatterjee *et al.*, 2015) as well as the egress of plasmacytoid dendritic cells from BM (Chopin *et al.*, 2016). Among myeloid cells, neutrophils are the most abundant type in peripheral blood. They are produced and released from BM following daily oscillations and consequently, neutrophil numbers in circulation vary during light-dark cycles (Ella *et al.*, 2016). Neutrophils have a short lifespan in circulation (~12 hours) and when senescent, "aged" neutrophils express high levels of CXCR4 allowing them to migrate back to BM to be eliminated in a process called clearance (Figure 1) (Casanova-Acebes *et al.*, 2013). Their egress from BM might be partly due to changes in CXCL12 levels with the circadian rhythms (Méndez-Ferrer *et al.*, 2008; Ella *et al.*, 2016), as described for HSCs. However, other mechanisms may account for neutrophil release as it precedes CXCL12 oscillations (Casanova-Acebes *et al.*, 2013). Recently, clock genes have been described as intrinsic aging regulators in neutrophils in combination with CXCR2 and CXCR4 (Adrover *et al.*, 2019). Disruption of the aging process has consequences on immune cell trafficking at steady state and immune defence against infection. While ACKR3 is detected in neutrophils, further studies are needed to determine its expression levels during neutrophil maturation and its possible contribution to this process. There is also a case for exploring whether cells of the microenvironment (e.g. cells in BM niches) express ACKR3, and how these factors could be related to neutrophil biology.

7.2. ACKR3 and the myeloid compartment in inflammatory settings

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ACKR3 expression is usually faint or undetectable at steady state in the endothelium and in myeloid cells, but can be up-regulated during inflammation, for instance by proinflammatory cytokines such as IL-8 (Singh and Lokeshwar, 2011) or IL-1β *in vitro* (Watanabe *et al.*, 2010) and by environmental cues such as lipopolysaccharide (LPS) (Cao *et al.*, 2016; Konrad *et al.*, 2017; Ngamsri *et al.*, 2017) or during infection by oncoviruses (reviewed in Freitas *et al.*, 2014). Along this line, ACKR3 is highly upregulated during monocyte-to-macrophage differentiation *in vitro*, switching to a more pro-inflammatory cell phenotype (Wanshu *et al.*, 2013; Chatterjee *et al.*, 2015). Another example can be found during central nervous system inflammation, where ACKR3 is upregulated in endothelial cells of the blood-brain barrier (Cruz-Orengo *et al.*, 2011). Antagonizing the scavenging activity of ACKR3 using small molecule CCX771 blocked leukocyte infiltration in the parenchyma, including that of $CD11b^+$ myeloid cells, preventing chronic inflammation and therefore improving disease recovery (Cruz-Orengo *et al.*, 2011). This could be associated with a restoration of the CXCL12 polarity along the blood-brain barrier, which is essential for its integrity and for preventing infiltration of $CXCR4^+$ cells (McCandless *et al.*, 2008).

Furthermore, the role of ACKR3 has been explored in pulmonary inflammation with regard to the lung epithelial barrier function and the recruitment of myeloid cells (Figure 1). In an acute inflammation mouse model induced by LPS inhalation, ACKR3 protein is upregulated in the lung tissue, both in epithelial and endothelial cells (Ngamsri *et al.*, 2017). However, in chronic lung injury mouse models induced upon repeated bleomycin injection or hydrochloric acid inhalation, ACKR3 mRNA and protein levels are decreased in endothelial cells (Cao *et al.*, 2016). These findings indicate that ACKR3 may play a role in the early stages of inflammation. Interestingly, in acute inflammation, CXCL12 mRNA and total protein levels in lung homogenates were increased (Cao *et al.*, 2016; Konrad *et al.*, 2017; Ngamsri *et al.*, 2017) and at least mRNA levels remained high in chronic inflammatory settings (Cao *et al.*, 2016). Regarding immune cell recruitment, neutrophils were recruited to the lung tissue in acute inflammation (Konrad *et al.*, 2017; Ngamsri *et al.*, 2017), whereas macrophages were recruited in chronic inflammation (Cao *et al.*, 2016). However, these studies did not explore neutrophil

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and macrophage recruitment in both short and long-term inflammatory processes. Pharmacological modulation of ACKR3 with either CCX771 (Ngamsri *et al.*, 2017) or TC14012 (Cao *et al.*, 2016) prevented microvascular permeability and further alveolar epithelial damage. Although both molecules induce β-arrestin recruitment to ACKR3, the downstream signalling pathways have not been assessed to our knowledge (Zabel *et al.*, 2009; Montpas *et al.*, 2015). These molecules can be considered as functional antagonists due to their capacity to displace CXCL12 from ACKR3, thus inhibiting the decoy activity of the receptor. While the potential therapeutic benefit of targeting ACKR3 is promising, it cannot be claimed so far which function of ACKR3 contributes to disease improvement, i.e. as a signalling or scavenging receptor. It will be essential to determine where ACKR3 is being expressed using reporter mouse models in order to decipher its potential protective or pro-inflammatory role in central nervous system and pulmonary inflammatory diseases.

Lastly, chronic inflammation can promote the progression of cancer (Hanahan and Weinberg, 2011) and is often initiated and maintained by infiltrating immune cells that secrete cytokines and chemokines in the tumour microenvironment (Nagarsheth *et al.*, 2017). For example, in breast cancer, the microenvironment likely induces myeloid-derived suppressor cells that contribute to immune evasion and consequently sustain tumour growth (reviewed in Markowitz *et al.*, 2013). CXCL12 also plays an important role in breast cancer. Indeed, CXCL12 production by cancer-associated fibroblasts (CAFs) enhances proliferation and survival of cancer cells as well as tumour growth and angiogenesis (Orimo *et al.*, 2005). Additionally, CXCL12 facilitates tumour cell intravasation by affecting vasculature integrity (Ahirwar *et al.*, 2018) and contributes to immune evasion by recruitment of T cells, which differentiate into immunosuppressive regulatory T cells (Tregs) in the tumour (Su *et al.*, 2017; Costa *et al.*, 2018). Furthermore, sites in the body that constitutively display high concentrations of CXCL12 such as lung or BM are common metastatic destinations for breast cancer cells (Müller *et al.*, 2001). Interestingly, blockade of CXCR4 with antagonist AMD3100 in breast cancer mouse models reduced the number of Tregs and neutrophils in the tumour, improving the immunosuppressive microenvironment (Chen *et al.*, 2019). CXCR4 blockade

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further reduced activated CAF numbers and increased the transcription of genes associated with anti-tumour immunity such as *Ifng* and *Gzma* in the tumour mass.

ACKR3 is upregulated in several types of cancer including breast cancer, frequently in tumour-associated vasculature as well as in the primary tumours (Freitas *et al.*, 2014). The clinical relevance of ACKR3 in breast cancer has been discussed elsewhere as a part of the mini-review series 'From insight to modulation of CXCR4 and ACKR3 (CXCR7) function' (Neves *et al.*, 2019). Studies using mouse orthotopic xenografts suggest that ACKR3 might play a role in maintaining proliferation in the primary tumour, while decreasing intravasation of tumour cells and thus reducing metastasis (Hernandez *et al.*, 2011). Furthermore, ACKR3 endothelial expression is likely involved in preventing breast cancer metastasis (Stacer *et al.*, 2016). Collectively, these findings highlight the dual role of ACKR3 either expressed in the primary tumour or in the tumour-associated vasculature. Further research is warranted to understand the underlying mechanisms of ACKR3 that impact the different stages of breast cancer progression and whether this occurs through ACKR3 itself or via modulation of the CXCL12/CXCR4 axis. ACKR3 could shape CXCL12 (and likely CXCL11) availability within the tumour due to its scavenging activity. This could decrease tumour cell intravasation and metastasis as reported (Hernandez *et al.*, 2011; Stacer *et al.*, 2016; Ahirwar *et al.*, 2018) and potentially immune cell infiltration. In essence, decreasing CXCL12 availability could inhibit CXCR4 responses and thus reverse the immunosuppressive microenvironment in breast cancer, including reduced Treg and increased cytotoxic T cell numbers.

8. Discussion

After being de-orphaned in 2005, CXCR7 was proposed to act as an atypical chemokine receptor, ACKR3, with a primary role as a decoy receptor for CXCL12 and CXCL11, whose function was to merely internalise the ligands. Over the years, accumulating evidence has supported the concept of ACKR3 being a major regulator of the CXCL12/CXCR4 axis and possibly CXCL11/CXCR3. Furthermore, in some cell lines ACKR3 displays β-arrestin-biased signalling capacity *in vitro* in response to its chemokine and non-chemokine ligands, an

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observation mainly supported by β-arrestin recruitment and ERK1/2 phosphorylation studies (Zabel *et al.*, 2009; Rajagopal *et al.*, 2010; Wang *et al.*, 2011; Alampour-Rajabi *et al.*, 2015). However, ERK1/2 phosphorylation has not been formally demonstrated to be β-arrestin-mediated for ACKR3 and particularly in *in vivo* settings. Moreover, this concept has recently been challenged, as a panel of known β-arrestin-biased receptors unexpectedly required functional G proteins to elicit ERK1/2 phosphorylation activity, whereas β-arrestins were not essential (Grundmann *et al.*, 2018). This can be highly relevant to ACKR3, which is already known to engage with, but not activate, G proteins (Levoye *et al.*, 2009). In addition, studies on CRISPR-Cas9-mediated β-arrestin knockout cell lines have shown that β-arrestins might not be necessary for ERK1/2 phosphorylation (Luttrell *et al.*, 2018). Instead, they may act as regulators that fine-tune ERK1/2 phosphorylation depending on the cell type and its strength in potentiating G-protein or β-arrestin-mediated signalling (Luttrell *et al.*, 2018). Altogether, these recent findings raise questions about the molecular mechanisms by which ACKR3 is exerting its still underappreciated functions in homeostatic processes, e.g. in hormonal and neuronal systems and potentially in the haematopoietic system.

To conclude, ACKR3 expression and function in immune cells remain poorly understood, and future research should focus on i) unambiguously characterising the ACKR3 expression patterns in physiological and pathological contexts, ii) clarifying the mechanisms by which ACKR3 acts as a signalling or a scavenging receptor and iii) understanding its function in homeostatic processes, such as the circadian oscillation of CXCL12 levels or neutrophil trafficking, as well as in pathological conditions (Figure 1). Importantly, inflammation-related pathological conditions can highly dysregulate ACKR3 expression. Thus, mechanistically deciphering the precise contribution of ACKR3 to immune cell recruitment in inflammatory context should identify ACKR3 as a novel therapeutic target in various diseases and cancer.

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9. Authorship Contributions

Wrote or contributed to the writing of the manuscript: Koenen, Bachelerie, Balabanian, Schlecht-Louf, Gallego.

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11. Footnotes

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12. Legends for figures.

Figure 1. Potential roles of ACKR3 in steady state and inflammation within the myeloid cell compartment.

Neutrophils are produced in bone marrow (BM) from haematopoietic stem cells during granulopoiesis. They are released into the bloodstream following circadian oscillations and increase their surface CXCR4 expression while aging over time. After approximately 12 hours in circulation, at the end of the dark phase, aged CXCR4^{high} neutrophils migrate back to BM to be eliminated. As part of their patrolling function, they can migrate into healthy tissues. The role of ACKR3 is unknown at steady state, but it could potentially contribute to either the circadian oscillations of CXCL12 within BM via its scavenging activity or to the rhythmic release of neutrophils. When circulating neutrophils encounter inflammatory signals, they can adhere and roll on endothelium and extravasate from the bloodstream to infiltrate inflamed tissue, where they accumulate. Inflammation leads to an upregulation of CXCL12 within the tissue as a cue to attract immune cells. Furthermore, during inflammation, CXCR4 and ACKR3 are upregulated on endothelium as shown in lung inflammatory conditions. In addition, ACKR3 is reported to be upregulated in inflamed tissue (for example in lung alveolar epithelium upon lung inflammation) but its role with regard to CXCL12 level regulation and subsequent immune cell recruitment is not completely understood.

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13. Tables.

Table 1. Genetically modified mouse models to study *Ackr3* expression *in vivo* and associated phenotypes.

Model	Genetic background	Description	References
Constitutive deletion			
Ackr3$^{-/-}$	C57Bl/6	Mice carrying loxP-flanked *Ackr3* exon 2 crossed with *Deleter*-Cre mice Phenotype: Perinatal death of >95% *Ackr3*$^{-/-}$ mice. Thickened semilunar valves. Normal development of B cells and granulocytes. Altered neuron migration during embryonic development.	(Sierro *et al.*, 2007; Sánchez-Alcañiz *et al.*, 2011; Wang *et al.*, 2011; Yu *et al.*, 2011; Trousse *et al.*, 2015)
Ackr3$^{-/-}$	129 Sv/Ev x C57Bl/6	Knock-in of LacZ reporter in *Ackr3* exon 2 (IRES-LacZ/PGK- Neo cassette) Phenotype: Perinatal death of 70% *Ackr3*$^{-/-}$ mice. Non-viable mice: Myocardial degeneration, fibrosis, and cardiac hyperplasia. No defects in semilunar valves. Surviving mice: Cardiac hyperplasia in 25%. Normal lifespan, no haematopoietic or haematological defects, no reproductive defects.	(Gerrits *et al.*, 2008)
Conditional (tissue or cell type specific) deletion			
Tie2-Cre; *Ackr3*$^{flox/-}$	C57Bl/6	Endothelium-specific deletion. Phenotype: Mice survive to adulthood and are fertile. Cardiac hypertrophy, thickened ventricular walls, and thickened semilunar valves in 40% of the mice.	(Sierro *et al.*, 2007; Yu *et al.*, 2011)
Dlx5/6-Cre; *Ackr3*$^{flox/flox}$	C57Bl/6	GABAergic neuron-specific deletion. Phenotype: Mice survive to adulthood. Altered migration of neurons during embryonic development (E16.5).	(Sánchez-Alcañiz *et al.*, 2011)

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Dlxl12b-Cre; Ackr3$^{flox/-}$	C57Bl/6 CD1	GABAergic neuron-specific deletion. Phenotype: Neuron positioning defects similar to those of constitutive *Ackr3* $^{-/-}$ mice.	(Wang *et al.*, 2011)
Emx1-Cre; Ackr3$^{flox/-}$	Not given	Glutamatergic neuron-specific deletion. Phenotype: No effect on the position of cortical projection neurons.	(Wang *et al.*, 2011)
Tbr2-Cre; Ackr3$^{flox/flox}$	Not given	Glutamatergic neuronal progenitor-specific deletion. Phenotype: Altered migration of neurons in adulthood.	(Abe *et al.*, 2018)
Dbx1-Cre; Ackr3$^{flox/flox}$	C57Bl/6	Cajal-Retzius neuron progenitor-specific deletion. Phenotype: Defects in the positioning of a subpopulation of Dbx1-expressing neurons during embryonic development (E14.5).	(Trousse *et al.*, 2015)
Conditional-inducible (cell type-specific inducible) deletion			
Scl-Cre^{ERT}; Ackr3^{flox}	C57Bl/6	Tamoxifen-inducible deletion of *Ackr3* from Scl-expressing cells (HSC, myeloid lineage, endothelium and regions in the central nervous system) in adult mice. Findings: ~35% increase in CXCL12 plasma levels. No other apparent phenotype described.	(Stacer *et al.*, 2016)
Reporter gene			
Ackr3$^{+/EGFP}$	C57Bl/6	Replacement of *Ackr3* exon 2 by EGFP. No phenotype is described.	(Cruz-Orengo *et al.*, 2011)
Ackr3-EGFP	CD1	BAC insertion of the *Ackr3* promoter fused to an *EGFP* coding region. Endogenous *Ackr3* locus remains intact. No secondary effects.	The Gene Expression Nervous System Atlas (GENSAT) (Gong *et al.*, 2003)

Table 2. ACKR3 mRNA and protein detection in mammalian tissue.

			mouse/rodent		*human*		
System	**Cell type**	**Origin**	**mRNA**	**Protein**	**mRNA**	**Protein**	**References**
Haemato-poietic system	B cells	Peripheral blood, BM, and/or lymphoid organs	RNAseq	FC	Transcriptomics, RT-PCR	FC, IF	(Infantino *et al.*, 2006; Sierro *et al.*, 2007; Heng *et al.*, 2008; Tarnowski *et al.*, 2010; Biajoux *et al.*, 2012; Wang *et al.*, 2012; Melo *et al.*, 2014; Alampour-Rajabi *et al.*, 2015)
	T cells (CD4+, regulatory, helper, memory)		RNAseq		Transcriptomics, RT-PCR	FC, IF	(Balabanian *et al.*, 2005; Infantino *et al.*, 2006; Sierro *et al.*, 2007; Heng *et al.*, 2008; Tarnowski *et al.*, 2010; Biajoux *et al.*, 2012; Melo *et al.*, 2014)
	Innate lymphoid cells		RNAseq				(Heng *et al.*, 2008)
	NK cells				Transcriptomics, RT-PCR		(Infantino *et al.*, 2006; Sierro *et al.*, 2007)
	Dendritic cells		RNAseq		Transcriptomics, RT-PCR	FC, IF	(Infantino *et al.*, 2006; Sierro *et al.*, 2007; Heng *et al.*, 2008)
	Monocytes				RT-PCR	FC, IF	(Infantino *et al.*, 2006; Tarnowski *et al.*, 2010; Chatterjee *et al.*, 2015)

	Neutrophils		RNAseq			(Heng *et al.*, 2008)
	Macrophages		RNAseq		FC	(Heng *et al.*, 2008; Chatterjee *et al.*, 2015)
	Basophils			RT-PCR	FC	(Infantino *et al.*, 2006)
	Mesenchymal stem cells	BM	RT-qPCR	Transcriptomics, RT-PCR		(Kim *et al.*, 2016; Liu *et al.*, 2018; Kuçi *et al.*, 2019)
Circulatory / Lymphatic system	ND	Whole (heart)	Lac-Z reporter, northern blot	Northern blot		(Burns *et al.*, 2006; Gerrits *et al.*, 2008; Berahovich *et al.*, 2014)
	Venous endothelium	Digestive tract, heart, kidney, lung, liver, lymphoid organs	Lac-Z reporter (except liver)		IHC	(Berahovich *et al.*, 2014)
	Vascular smooth muscle cells	Heart, nervous system, kidney, digestive tract, skeletal muscle, lymphoid organs	RT-PCR		IHC	(Neusser *et al.*, 2010; Rajagopal *et al.*, 2010; Berahovich *et al.*, 2014)
	Sinusoidal cells	Spleen	IHC		IHC	(Berahovich *et al.*, 2014)
	Endothelial cells	Kidney			IHC	(Neusser *et al.*, 2010)

	ND	Kidney lymphatic vessels			IF	(Neusser *et al.*, 2010)
	ND	Vena cava	RT-qPCR			(Klein *et al.*, 2014)
	ND	Thoracic duct	RT-qPCR			(Klein *et al.*, 2014)
Nervous system	ND	Brain (whole)	Lac-Z reporter, ISH, northern blot		Northern blot	(Burns *et al.*, 2006; Gerrits *et al.*, 2008; Thelen and Thelen, 2008; Yu *et al.*, 2011; Berahovich *et al.*, 2014)
	ND	Brain vasculature	EGFP reporter, ISH			(Schönemeier *et al.*, 2008; Cruz-Orengo *et al.*, 2011)
	Astrocytes	Brain		IHC		FC (intracellular) (Calatozzolo *et al.*, 2011; Puchert *et al.*, 2017)
Respiratory system	ND	Lung (whole)	RT-PCR, northern blot	IF	Northern blot	(Burns *et al.*, 2006; Berahovich *et al.*, 2014; Cao *et al.*, 2016; Konrad *et al.*, 2017; Ngamsri *et al.*, 2017)
	ND	Lung vessels	Lac-Z reporter			(Gerrits *et al.*, 2008)
Secretory system	ND	Kidney (whole)	Northern blot		RT-qPCR, northern blot	IHC (Burns *et al.*, 2006; Neusser *et al.*, 2010; Maishi *et al.*, 2012; Berahovich *et al.*, 2014)
	Renal multipotent progrenitors	Patient-derived healthy kidney tissue			RT-qPCR	IF (Mazzinghi *et al.*, 2008)

ND	Kidney tubules	Lac-Z reporter	IHC	IHC	(Neusser *et al.*, 2010; Berahovich *et al.*, 2014)
ND	Kidney glomeruli	Lac-Z reporter			(Gerrits *et al.*, 2008)

ND not determined; NK Natural killer; BM bone marrow; RNAseq RNA sequencing; RT-qPCR Real time quantitative polymerase chain reaction; RT-PCR Reverse transcription polymerase chain reaction; ISH In situ hybridization; FC flow cytometry; IHC Immunohistochemistry; IF Immunofluorescence.

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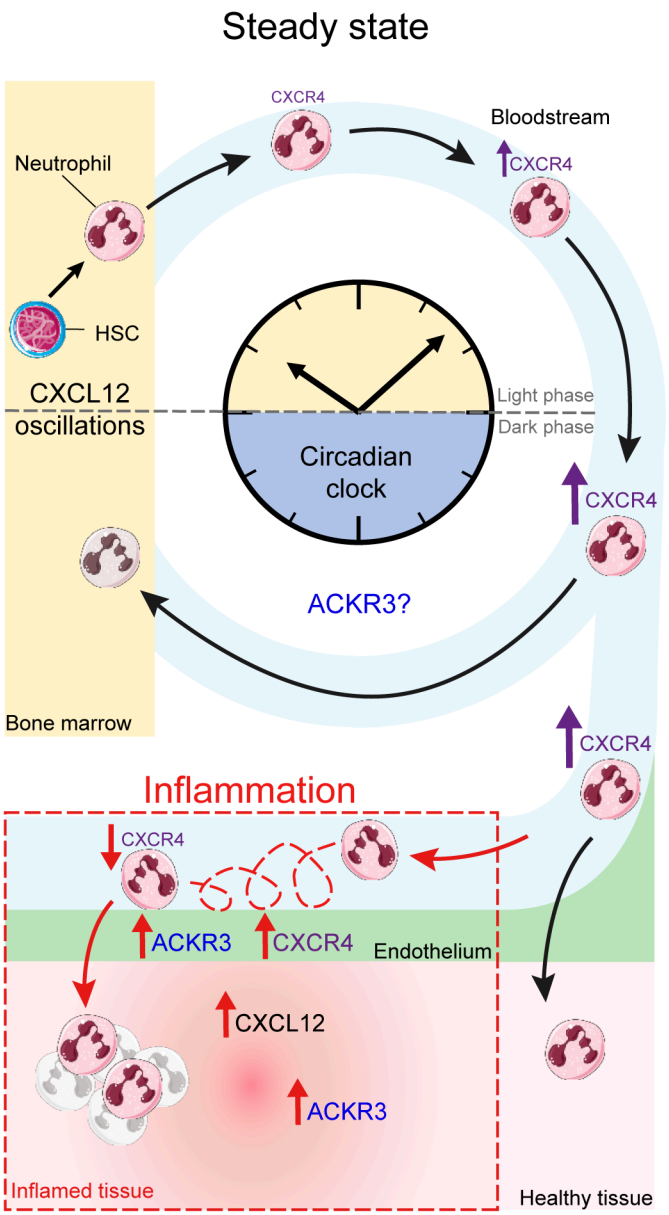

Figure 1.