A narrative review of chemokine receptors CXCR1 and CXCR2 and their role in acute respiratory distress syndrome

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Modulation of neutrophilic chemotaxis and activation through inhibition of the chemokine receptors CXCR1 and 2 may have a therapeutic role in ARDS https://bit.ly/3WONuY4

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Abstract

Acute respiratory distress syndrome (ARDS) is a severe form of acute respiratory failure characterised by extensive inflammatory injury to the alveolocapillary barrier leading to alveolar oedema, impaired gas exchange and, ultimately, hypoxaemia necessitating the use of supplemental oxygen combined with some degree of positive airway pressure. Although much heterogeneity exists regarding the aetiology, localisation and endotypic characterisation of ARDS, what remains largely undisputed is the role of the innate immune system, and in particular of neutrophils, in precipitating and propagating lung injury. Activated neutrophils, recruited to the lung through chemokine gradients, promote injury by releasing oxidants, proteases and neutrophil extracellular traps, which ultimately cause platelet aggregation, microvascular thrombosis and cellular death. Among various neutrophilic chemoattractants, interleukin-8/C-X-C motif ligand 8 and related chemokines, collectively called ELR+ chemokines, acting on neutrophils through the G protein-coupled receptors CXCR1 and CXCR2, are pivotal in orchestrating the neutrophil activation status and chemotaxis in the inflamed lung. This allows efficient elimination of infectious agents while at the same time minimising collateral damage to host tissue. Therefore, understanding how CXCR1 and CXCR2 receptors are regulated is important if we hope to effectively target them for therapeutic use in ARDS. In the following narrative review, we provide an overview of the role of ELR+ chemokines in acute lung injury (ALI) and ARDS, we summarise the relevant regulatory pathways of their cognisant receptors CXCR1/2 and highlight current preclinical and clinical evidence on the therapeutic role of CXCR1 and CXCR2 inhibition in animal models of ALI, as well as in ARDS patients.

Introduction

Chemokines are a superfamily of mostly small (8–14 kDa), basic, structurally related peptides ∼70–80 amino acids long [1] that are critical regulators of not only leukocyte migration to areas of infection and inflammation, but also of homeostatic trafficking of leukocytes into, and out of, peripheral tissues and primary and secondary lymphoid organs. More than 40 chemokines have been identified and classified into one of four subfamilies, C, CC, CXC or CX3C, based on the number and arrangement of conserved cysteine residues in their N-terminal region, *i.e.*, whether the first two (out of four) conserved cysteine residues are adjacent (CC) or separated by one or three amino acids (CXC or CX3C) [2, 3]. The CXC subfamily can be further subdivided into an ELR+ and ELR− group based on the presence or absence of the sequence motif glutamic acid–leucine–arginine (ELR) preceding the conserved most N-terminal cysteine [4]. All ELR+ CXC chemokines attract and activate human neutrophils at low nanomolar concentrations *in vitro* and *in vivo*. Seven human ELR+ CXC chemokines have been described: C-X-C motif ligand (CXCL) 8 (also known as interleukin (IL)-8)), CXCL1, CXCL2, CXCL3, CXCL5, CXCL6

and CXCL7. In humans, CXCL8 is the most potent neutrophil activator and, under inflammatory conditions, one of the most abundantly produced ELR+ CXC chemokines [5].

The biological activities of ELR+ CXC chemokines are mediated through binding to two seven-transmembrane-domain G protein-coupled receptors, CXCR1 and CXCR2, located on the cell surface [6]. Ligand binding results in engagement of intermediate proteins such as phospholipase A, C and D, mitogen-activated protein kinases, phosphatidylinositol-3 kinase, and protein tyrosine kinases [5]. This leads to enhanced intracellular calcium concentration, reduced levels of cAMP, production of diacylglycerol, activation of protein kinase C and B isoforms, and actin polymerisation. The ultimate effects are promotion of cell adhesion, polarisation and directional migration, as well as neutrophil-specific functions such as reactive oxygen species (ROS) production and the release of neutrophil extracellular nets (NETs) [7] (figure 1).

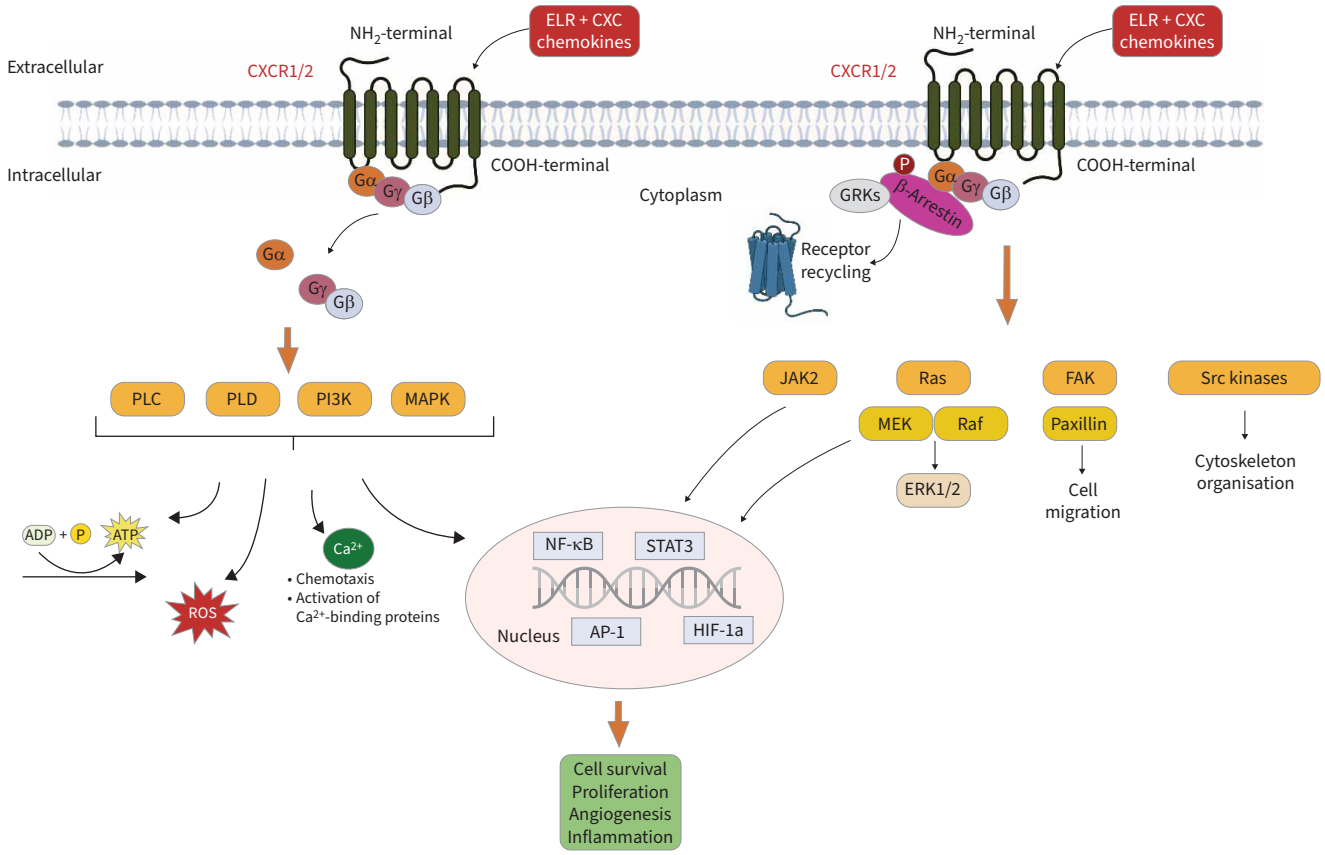

FIGURE 1 CXCR1 and 2 are G-protein coupled receptors containing seven transmembrane domains interconnected by three intracellular and three extracellular loops. The NH_2-terminal and COOH-terminal receptor domains are situated extracellularly and intracellularly, respectively. Binding of chemokine ligands induces conformational changes in the receptors that initiate coupling to primarily pertussis toxin-sensitive heterotrimeric G proteins. The Gα and/or Gβγ-subunits of the G proteins are separated and activated upon receptor ligation leading to downstream regulation of the activity of a series of effector proteins, including phospholipase C (PLC) and phospholipase D (PLD), phosphatidylinositol-3 kinase (PI3K) and mitogen-activated protein kinases (MAPK) (*e.g.*, extracellular signal-regulated kinase (ERK) and p38), linked to such cellular functions as protein phosphorylation, intracellular calcium release and activation of calcium-binding proteins, production of reactive oxygen species (ROS) in the case of neutrophils, as well as the induction of various transcription factors. Such transcription factors include activator protein-1 (AP-1), nuclear factor kappa B (NF-κB), hypoxia-inducible factor-1a (HIF-1a) and signal transducer and activator of transcription proteins (STATs). The result is upregulation of genes controlling progenitor cell survival, proliferation, differentiation, angiogenesis and inflammation. At the same time, CXCR1- and CXCR2-dependent cytoskeletal organisation and migration requires activation of proteins such as Src family kinases, Rho, LIM and SH3 protein-1 (LASP-1), Ras GTPase-activating-like protein 1, and focal adhesion kinase (FAK), and associated proteins such as proline-rich tyrosine kinase 2 and paxillin. Cytoskeletal elements differentially regulate CXCR1- and CXCR2-induced FAK activation and migration. Finally, CXCR1 and CXCR2 are able to form complexes with β-arrestins (upon phosphorylation of the receptors at the COOH terminus) to initiate G protein-independent signalling and/or desensitisation and internalisation of the receptors. GRK: G protein-coupled receptor kinase; JAK2: Janus kinase 2; MEK: mitogen-activated protein/ERK kinase; P: phosphorylated.

The two receptors are expressed in approximately equal numbers on unstimulated neutrophils and share considerable amino acid sequence identity (77%) [8] CXCR1 has two main ligands, CXCL8 and CXCL6 [9, 10]. In contrast to CXCR1, CXCR2 interacts with all ELR+ chemokines [1, 8]. Human CXCR1 and/or CXCR2 also bind nonchemokine ligands, including HIV-1 matrix protein p17, the collagen fragment N-acetyl-proline-glycine-proline and macrophage inhibitory factor, which play a role in neutrophil recruitment in chronic diseases and conditions such as atherosclerosis, COPD and cystic fibrosis [11–13].

Aims

- To introduce the reader to the most important aspects of CXCR1 and 2 regulation, which is pivotal in orchestrating neutrophil chemotaxis to the inflamed lung in ARDS.
- To describe critical interactions between these two receptors and other neutrophilic receptors with significance in the neutrophilic response to inflammation.
- To familiarise the reader with the current research (both in animals and humans) on the efficacy of CXCR1 and 2 inhibitors in acute lung injury (ALI) and ARDS.
- To discuss current uncertainties as to the optimal use of CXCR1 and 2 inhibitors in ARDS and inspire future research on this topic.

Methods

The literature used in this narrative review was selected through searches of the PubMed and Cochrane Library databases (up to February 2024) using variants of the following search terms : “acute lung injury”, “ARDS”, “acute respiratory distress syndrome”, “CXR”, “CXCR1”, “CXCR2”, “animal”, “humans”, “CXCR1 inhibitors and acute lung injury”, “CXCR2 inhibitors and acute lung injury”, “chemoattractant receptors”, “neutrophil receptors”. S.T. and E.G. independently conducted the literature searches and S.T., S.S. and P.P. assessed the eligibility of the identified publications. Both studies using CXCR1 and 2 inhibitors in animal models of lung injury and human studies were included. Studies included for data extraction were restricted to original full-text articles published in English.

ELR+ chemokines in ARDS

ARDS is a heterogeneous syndrome of acute respiratory failure characterised by pulmonary oedema due to excessive alveolocapillary permeability associated with inflammatory injury to the alveolocapillary barrier [14]. The classic pathological finding, identified however in only approximately 45% of post-mortem lung specimens [15], is diffuse alveolar damage characterised by neutrophilic alveolitis and hyaline membrane deposition. ARDS is a significant medico–social problem in terms of both short-term (*i.e.*, in-hospital) mortality of 35–45% [16] and long-term morbidity manifesting as impairments in physical and cognitive function, as well as mental health [17], for which no effective pharmacologic treatment has been identified.

The animal experimental counterpart of human ARDS is ALI. Animal models of ALI aim to reproduce either sepsis, which is a known major cause of human ARDS, through systemic administration of lipopolysaccharide (LPS), inoculation of live bacteria or precipitation of endogenous infection (caecal ligation) or through induction of other forms of direct (inhalation of bleomycin) or indirect (ischaemia/reperfusion) lung injury [18]. Notwithstanding the irrefutable fact that none of these models adequately reproduces the full characteristics of human ARDS, they do demonstrate, in variable degrees depending on the model, a pathologic hallmark of the syndrome which is the acute neutrophilic inflammatory response within the alveolar and interstitial spaces accompanied by fibrin-rich proteinaceous alveolar exudates containing an array of pro- and anti-inflammatory cytokines.

The vital role of neutrophils in ARDS is not surprising given that the lung, as a barrier organ, is heavily patrolled by neutrophils, which aggregate in the pulmonary capillaries in higher concentrations than in the systemic circulation and can rapidly enter lung tissues in response to infectious and inflammatory stimuli [19]. In response to such stimuli, alveolar epithelial and endothelial cells as well as resident macrophages release a multiplicity of inflammatory chemokines which not only entice neutrophil migration to the inflammatory site, but also ensure their progressive activation as they migrate through the interstitium. At the inflammatory nidus, activated neutrophils employ an armamentarium of mechanisms to combat pathogens, including synthesis of ROS by NADPH oxidase and nitric oxide synthase pathways, release of soluble factors such as antimicrobial peptides and proteinases prestored in neutrophil granules and, occasionally, generation of NETs, all of which can cause bystander injury to host cells and loss of lung function [20]. In humans, the concentration of neutrophils in the bronchoalveolar lavage fluid (BALF) of patients with ARDS correlates with both disease severity and clinical outcomes [21, 22]. Neutrophils isolated from the peripheral blood of patients with new-onset ARDS live longer than neutrophils from healthy controls, with those isolated from patients with moderate to severe ARDS having the lowest

spontaneous apoptosis rate. Furthermore, these long-living neutrophils produce more NETs, directly correlating with disease severity [23]. Although NETs are important in host defence by trapping and killing micro-organisms [24], they can also contribute to target organ injury, as demonstrated by the fact that animals which partially lack peptidylarginine deiminase 4 (PAD4), an enzyme involved in NET formation [25], are afforded protection from bacterial acute lung injury, whereas homologous *Pad4* knockout mice demonstrate reduced NET formation and lung injury at the expense of increased bacterial load and inflammation [26]. In humans, NET inhibition seems to be the predominant mechanism explaining the protective effect of enoxaparin in coronavirus disease 2019 (COVID-19) related ARDS [27], whereas increased plasma levels of NET components are associated with ARDS severity and mortality [26].

All ELR+ chemokines have been implicated in neutrophil migration in the acutely inflamed lung. Among them, CXCL8 plays a predominant role [28]. There is a significant correlation between CXCL8 levels in the systemic circulation and BALF of patients at risk of developing ARDS and the likelihood of subsequent disease [29], as well as with disease severity and outcome [28]. Indeed, high pulmonary oedema fluid levels of CXCL8 (>4000 $pg \cdot mL^{-1}$) are associated with impaired alveolar fluid clearance in patients with ARDS [30]. In COVID-19 patients who require mechanical ventilation due to severe ARDS, CXCL1, CXCL5 and CXCL8 concentrations are extremely upregulated in the lungs and are up to 1000-fold higher compared to levels in lungs of severe influenza patients that also require mechanical ventilation [31].

Compared with mock-infected animals, neutrophils isolated from mice infected with lethal doses of influenza A virus show greater susceptibility to NETotic death when stimulated with CXCL8, which is probably a reflection of the higher CXCR2 expression or activation in infected animals [32]. Similar findings have been observed in humans. Neutrophils isolated from patients with trauma who later develop ARDS demonstrate enhanced *ex vivo* neutrophil migratory activity in response to CXCL8 at early time points which precede the clinical manifestations of ARDS. This priming of neutrophils is associated with elevated pulmonary concentrations of CXCL8 [33].

This correlation between the presence of CXCL8-primed neutrophils and ARDS development has led to the evaluation of the role of CXCL8 in stratifying patients at risk for developing ARDS due to various aetiologies including both trauma and medical causes. Indeed, CXCL8, in combination with other biomarkers and clinical predictors of disease severity, can accurately differentiate between patients at high *versus* lower risk of ARDS development [34, 35]. Furthermore, CXCL8 is one of three biomarkers which, when used in isolation, have nearly equivalent prognostic value for ARDS to a more extensive list of laboratory and clinical prognosticators [35].

Besides CXCL8, the other ELR+ chemokines appear to play a role in pulmonary neutrophilic chemotaxis, although most of the data is derived from animal models in which CXCL8 is absent. CXCL1 and CXCL2 are important chemokines for lung neutrophil recruitment in rodents where they are produced by alveolar macrophages and type II alveolar epithelial cells [36–38]. CXCL1 is extremely elevated in the BALF [39, 40] and, less so, the peripheral blood of patients with severe COVID-19-related ARDS [41] as well as in the BALF and plasma of mice exposed to intratracheal LPS instillation [42]. A systemically administered neutralising antibody against CXCL1 prevents neutrophil sequestration in the alveoli of mice exposed to *Streptococcus pneumoniae* intranasal inoculation [43]. Interestingly, the same antibody administered intranasally does not prevent alveolar neutrophil accumulation, possibly because it has no effect on the formation of CXCL1 gradients between the lung and the blood. Influenza A pneumonia is also associated with increased CXCL1 and CXCL2 expression by alveolar epithelial cells, which intensifies as the latter cells become senescent and may be a factor contributing to the higher influenza mortality in older individuals [44].

CXCL3 is produced by human alveolar epithelial and pulmonary endothelial cells in response to LPS and mediates LPS-induced activation of downstream inflammatory pathways in these cells [45].

CXCL5 is upregulated in the lung and expressed by alveolar type II epithelial cells in response to LPS stimulation [46]. Anti-CXCL5 antibodies attenuate LPS-induced neutrophil accumulation in the lung [47]. CXCL5 may, in fact, be the dominant effector of neutrophil influx to the lung in a mild and self-limited inflammatory model, such as that of LPS, due to prolonged expression (compared to CXCL1 and CXCL2) in the lung. Interestingly, however, in inflammatory conditions such as *Escherichia coli* pneumonia characterised by very high concentrations of CXCL1 and CXCL2 in the plasma, CXCL5 may actually moderate lung neutrophil influx by inhibiting scavenging of CXCL1 and 2 by decoy receptors such as atypical chemokine receptor 1 or the Dufy antigen receptor for chemokines on red blood cells in the

peripheral circulation, thus desensitising CXCR2 and impairing formation of chemokine gradients to the lung [48].

CXCL6 levels in BALF increase 24 h after bleomycin-induced ALI in mouse lungs and a monoclonal antibody against CXCL6 attenuates acute inflammation by reducing pulmonary neutrophil influx [49].

CXCL7 is abundantly produced by platelets [50] but also by neutrophils, megakaryocytes, natural killer cells and lymphocytes [51]. *Cxcl7*$^{-/-}$ mice are protected from ALI through preservation of endothelial/epithelial barrier function combined with impaired neutrophil transmigration, whereas transgenic expression of CXCL7 restores sensitivity to ALI [52].

Given the contribution of ELR+ chemokines in ARDS/ALI pathogenesis, it is important to consider how their cognisant receptors, *i.e.*, CXCR1 and CXCR2 are expressed and regulated, especially within the context of acute inflammation.

Ligand-dependent CXCR1/2 regulation

Understanding the regulatory mechanisms of CXCR1/2 signalling upon exposure to ELR+ chemokines is essential if we hope to target them therapeutically (table 1). The cell surface expression of the receptors is a dynamic process tightly linked to their ligation status. Ligation of CXCR1/2 typically induces a refractory period during which the receptor cannot transduce signals when stimulated a second time with the same or other agonists, a phenomenon known as desensitisation [53]. Desensitisation is typically associated with receptor internalisation, an event that requires G protein-coupled receptor kinase (GRK)-mediated phosphorylation of the intracellular domain of the agonist-occupied receptor followed by β-arrestin binding, which uncouples the receptor from the G protein and leads to dissociation of the Gα (mostly Gαi) from the Gβ/γ subunits. Interaction of β-arrestin with clathrin and the AP-2 adapter facilitates receptor endocytosis which is usually followed by cell surface recycling [54–56].

The two receptors differ in their recycling kinetics. CXCR2 becomes internalised much faster than CXCR1 in response to CXCL8 (95% CXCR2 is internalised *versus* 10% CXCR1 in the first 5–10 min) and recovers more slowly (35% CXCR2 is recycled *versus* 100% of CXCR1 after 90 min) at the cell surface than CXCR1 [57–59]. The reasons behind the faster internalisation of CXCR2 are not entirely clear but may be related to the higher affinity of CXCL8 for CXCR2 [60] and the differential phosphorylation and dephosphorylation (by involved phosphatases such as the wild-type p53-induced phosphatase 1 [61, 62]) of the cytoplasmic tails of the two receptors.

Furthermore, the ligand concentrations required for receptor internalisation differ between the two receptors. CXCR2 is readily downregulated at picomolar concentrations of CXCL8 (50% at 0.17 nM) while 10-fold higher levels of CXCL8 are required to reach the same effect for CXCR1 [4].

The above suggests that neutrophils are more likely to respond, at least initially, to one or more of the several CXCR2 ligands initiating migration distant from the site of inflammation, whereas CXCR1 responds to higher ligand concentrations closer to the inflammatory nidus. CXCR1 may in fact be the dominant ELR+ CXC receptor mediating CXC chemokine-related neutrophil signalling in acute inflammatory conditions due to its stability on the cell surface. Indeed, in sepsis, CXCR2 expression, as

TABLE 1 Differences in ELR+ cytokines-induced responses between CXCR1 and CXCR2

CXCR1	CXCR2
Internalises slowly (relative to CXCR2)	Internalises quickly (relative to CXCR1)
Resurfaces quickly (relative to CXCR1)	Resurfaces slowly (relative to CXCR1)
Responds to high ligand concentrations likely to be found close to the inflammatory source	Responds to very low concentrations of ligand
Primarily responsible for ROS production and neutrophil degranulation	Required for trans-endothelial neutrophil migration
Primarily responsible for NET production	Involved in reverse neutrophil migration

Most notable differences between CXCR1 and 2 based on current literature. References in text. NET: neutrophil extracellular trap; ROS: reactive oxygen species.

well as the chemotactic responses to CXCR2-related chemokines, is reduced by 50% on neutrophils of septic patients compared to normal volunteers, whereas CXCR1 expression is preserved [63].

The multiplicity of ligands for both receptors, particularly for CXCR2, controls and diversifies receptor functionality. This is because the downstream pathways activated upon receptor ligation differ depending on the ligand, a phenomenon known as biased agonism [64]. Such differences may at least partly explain the well-described differential functionality of the two receptors. As examples, CXCR1-dependent responses include pathways downstream of phospholipase D [65] as well as ROS production and degranulation in response to pathogens [66]. Inhibition of CXCR1, but not CXCR2, results in a decrease in superoxide anion production by neutrophils, which underlines the importance of CXCR1 in mounting an oxidative burst [8]. Indeed, patients carrying the genetic variant *CXCR1–T276* are more susceptible to bacterial infections because their neutrophils demonstrate impaired degranulation and blunted fungi-killing ability [67]. The release, by neutrophils, of NETs, which are networks of extracellular fibres consisting of neutrophil DNA, histones and bactericidal enzymes that can trap and incapacitate pathogens [68], is probably related to CXCR1 activation as it occurs by maximally activated neutrophils. NET formation follows other killing processes such as phagocytosis and ROS generation [69], in close proximity to the inflammatory epicentre and can be inhibited in humans by an allosteric inhibitor of both receptors, which however demonstrates much higher affinity for CXCR1 [70].

CXCR2 ligation, on the other hand, is indispensable for efficient trans-endothelial migration, *i.e.* the initial tethering and crawling of neutrophils on the endothelium followed by breaching of the endothelial barrier through the endothelial junctions [71]. Interestingly, however, CXCR2 is also involved in the reverse process. Although the primary mode of resolution of inflammation has traditionally been thought to involve neutrophil apoptosis and macrophage phagocytosis, newer evidence in animal models proposes that neutrophils can also resolve local inflammation through reverse migration (moving away from the inflammatory focus) and return into the vasculature [72], which is orchestrated through CXCR2. Live imaging of neutrophil migration in a zebrafish tail transection model revealed that, in *Cxcr2* mutant animals or upon the use of a selective antagonist of CXCR2, neutrophils were normally recruited to the wound site but persisted there, leading to a dramatic accumulation of neutrophils at the wound at later time points and suggesting a defect in resolution of inflammation [73].

CXCR1/2 interaction with other neutrophil receptors relevant in ARDS

Another layer of receptor regulation that needs to be considered in the context of acute inflammation and ARDS is that of the interaction of CXCR1/2 among themselves and with other receptors for neutrophilic chemokines and cytokines that are known to have a role in ARDS pathophysiology (table 2). In fact, the two receptors can both homodimerise and heterodimerise among themselves even in the absence of ligand and with equal apparent affinities [74]. The presence of ligand increases the stability of the homodimers whereas it disrupts heterodimers [75].

TABLE 2 Schematic representation of the interactions between CXCR1/2 and other neutrophil cell surface receptors

Primary effect	Secondary effect
↑FPR	↓CXCR2 ↓BLT1/2 ↓C5aR
↑S1PR ↑LPAR	↓CXCR1
↑TLR	↓CXCR1/2
↑CXCR2	↑BLT1 ↑CXCR1
↑CCR	↑CXCR1

In most instances ligand-induced activation of one neutrophil surface receptor (depicted in the left-hand column as ↑) leads to desensitisation of other neutrophil receptors (depicted in the right-hand column as ↓). However, reciprocal upregulation has also been described. References in text. BLT: leukotriene B4 receptor; C5aR: complement component 5a receptor; CCR: chemokine receptor; FPR: formyl peptide receptor; LPAR: lysophosphatidic acid receptor; S1PR: sphingosine-1-phosphate receptor; TLR: Toll-like receptor.

Irrespective of oligomerisation, CXCR1/2 can be regulated by other CXCR receptors. A characteristic example is the negative regulatory loop between CXCR2 and CXCR4. The two receptors have opposite roles in regulating neutrophil trafficking from the bone marrow with CXCR4 activation retaining neutrophil precursors in the bone marrow and CXCR2 ligands counteracting this effect and promoting neutrophil mobilisation [76].

In addition to CXCR1 and CXCR2, human neutrophils harbour receptors for three additional subfamilies of chemoattractants, namely chemotactic lipids (*e.g.*, leukotriene B4 or LTB4 with its receptors BLT1 and BLT2; sphingosine 1-phosphate or S1P; lysophosphatidic acid or LPA), complement anaphylatoxins (C3a and C5a with their receptors C3aR and C5aR) and formyl peptides (*e.g.*, N-formyl-Met-Leu-Phe or fMLF with the receptors FPR1 and FPR2), all of which function by activating dedicated receptors that are coupled to G proteins [77].

Chemoattractants are hypothesised to cooperate spatiotemporally, in a hierarchical manner, to orchestrate neutrophil migration. CXCR1/2 receptors can undergo cross-desensitisation by the other two major neutrophil chemoattractants, fMLF and C5a [78]. In most cases, this interaction is not reciprocal. Agonist ligation of the fMLF receptor takes precedence over other chemoattractants by desensitising CXCR1/2, as well as the complement and leukotriene receptors *in vitro* [77, 79]. However, protracted signalling through CXCR1, but not CXCR2, has also been shown to desensitise fMLF and C5a receptors [59, 80]. In some instances, chemoattractant receptors such as the receptors for S1P and LPA can form heterodimers with CXCR1 and downregulate CXCL8-induced chemotaxis. This effect is at least partially due to reduced transcription of *cxcr1* upon neutrophil exposure to the lipid chemoattractants [81].

Examples of coordinated action between separate chemoattractant receptors also exist. *In vitro*, the CXCR2 ligands CXCL1 and CXCL2 augment leukotriene B4 production by murine neutrophils, which, in turn, amplifies chemokine-mediated neutrophil chemotaxis *via* BLT1 in an autocrine and/or paracrine manner [82].

Receptors not traditionally linked to neutrophil chemotaxis such as CCR may also interact with CXCR in ways that are disease- and tissue-specific. Chemokines CCL2 and CCL7 can induce neutrophil chemotaxis towards BALF from patients with ARDS and they do so in synergy with CXCL8 [83]. Neutrophils isolated from BAL of the same patients express lower levels of CXCR1 compared to blood neutrophils, but higher levels of CCR2, hence their ability to respond to both chemokines. In this manner, the relative depletion of CXCR1 in the neutrophils that have reached the nidus of inflammation is compensated for by upregulation of CCR2, thus renewing the ability of these cells to respond to chemotactic signals and particularly CXCL8. This was confirmed in BALF neutrophils from ventilated COVID-19 patients with ARDS, in which downregulation of CXCR1, CXCR2 and C5aR was observed together with upregulation of FPR1, FPR2, CXCR4 and CCR1 [31].

The interaction of CXCR1/2 with neutrophil innate immune receptors, such as Toll-like receptors (TLRs), is of particular interest in the context of the importance of the latter in ALI/ARDS [84, 85]. Several studies demonstrate that TLR-mediated signalling generally leads to downregulation of CXCR1 and CXCR2 because of activation-induced internalisation. *In vitro* stimulation of neutrophil populations with TLR2 and TLR4 agonists leads to downregulation of CXCR2 and, to a lesser extent, CXCR1 [86]. The same effect was noted *in vivo* in a mouse model of polymicrobial sepsis induced by caecal ligation and puncture [87]. TLR2 appears to be critical for the downregulation of CXCR2 on circulating neutrophils during severe sepsis since this event can be prevented in *Tlr2*$^{-/-}$ mice. In accordance, *Tlr2*$^{-/-}$ mice show sustained neutrophil migration into the infectious focus and, consequently, experience lower bacteraemia, less systemic inflammation and an improved survival rate. The underlying mechanism for TLR2-induced downregulation of CXCR2 appears to be a TLR2 ligand-induced enhanced expression of GRK-2 that promotes CXCR2 internalisation [87], a process that is inhibited by IL-33 [88]. In contrast, the TLR4-induced CXCR2 downregulation appears to be multifactorial and is related to the role of TLR4 signalling in inducing the secretion, in a nuclear factor κB dependent manner, of CXCR2 ligands such as CXCL1 [89, 90] and CXCL2 [91], as well as the upregulation, in a glycogen synthase kinase-3 beta controlled process [92] of the anti-inflammatory cytokine IL-10. Both these processes lead to downregulation of CXCR2 either *via* promoting receptor internalisation [57] or by inhibiting gene transcription and protein expression [93].

Granulocyte colony stimulating factor (G-CSF), signalling through its receptor, which is amply expressed in mature neutrophils, negatively regulates CXCR2-induced mobilisation of neutrophils from the bone marrow in response to inflammation. Although possibly counterintuitive, given its prototypical role as a neutrophil-mobilising cytokine, G-CSF appears to regulate and moderate neutrophil mobilisation from bone marrow, which, upon CXCR2 ligation, tends to be very rapid and potentially exorbitant. Such a burst of

mobilised neutrophils, although physiologically necessary for an efficient host defence, if unchecked can lead to disproportionate neutrophil accumulation into tissues resulting in organ damage. Indeed, exposure of mice to *E. coli* or to LPS in the presence of G-CSF blockade leads to massive neutrophil accumulation in the lung and severe lung injury. G-CSF limits CXCR2-induced neutrophil migration by promoting phosphorylation of signal transducer and activator of transcription protein (STAT) 3, which negatively regulates Erk1/2 signalling downstream of CXCR2 [94]. In humans, plasma G-CSF levels are strongly correlated with the presence of emergently mobilised immature neutrophils in hospitalised patients with COVID-19-related ARDS [95]

Finally, LPS and the inflammatory cytokine tumour necrosis factor-α, of pivotal importance in ARDS pathophysiology [96, 97], decrease the surface expression of CXCR1 and CXCR2 on human neutrophils in a dose- and time-dependent manner. This downregulation is caused mainly by enzymatic cleavage and shedding of the receptors from the cell surface, possibly through the activity of serine proteases and/or metalloproteinases [98, 99] and not through receptor internalisation.

CXCR1/2 blockade in ALI/ARDS

Mice with genetic deletion of CXCR1 or CXCR2 are protected against ALI induced by LPS or hyperoxia [100, 101]. Blocking of CXCR1/2 signalling, *via* use of combined CXCR1 and 2 antagonists [102–106], ELR+ chemokine analogues or antibodies [49, 107–109], and isolated CXCR2 antagonists [32, 110–112] has been tested as a therapeutic modality in preclinical models of ALI with positive effects on gas exchange, capillary vascular permeability, lung oedema and neutrophil accumulation in BALF, as well as reduction of inflammatory cytokines, severity of lung injury, morbidity and mortality (table 3).

TABLE 3 Inhibition of CXCR1/2 pathway in animal models of acute lung injury (ALI)

Inhibitor	Model	Results	Reference
CXCR1/CXCR2 antagonist	Guinea pig models infected with *Escherichia coli*	↓Neutrophil recruitment ↓Pleural haemorrhagic effusion ↓Fever	[102]
CXCR1/CXCR2 antagonist	Mouse models with ALI	↑Gas exchange ↓Neutrophil recruitment ↓Vascular permeability	[103]
CXCR1/CXCR2 antagonist	Agricultural occupational dust mouse models exposed to swine barn dust extract	↓Neutrophil recruitment ↓Pro-inflammatory cytokines	[104]
CXCR1/CXCR2 antagonist	Mouse models infected with influenza A virus or *Streptococcus pneumoniae*	↓Morbidity ↓Neutrophil recruitment ↓Pulmonary viral titres	[105]
CXCR1/CXCR2 antagonist	Pulmonary fibrosis mouse models exposed to bleomycin+particulate matter	↓Severity of pulmonary fibrosis	[106]
Anti-CXCL8 antibody	Rabbits with endotoxaemia-induced acute respiratory distress syndrome	↓Pulmonary oedema ↓Neutrophil recruitment ↑Gas exchange	[107]
Humanised anti-CXCL8 monoclonal antibody	Rabbits with ALI	↓Neutrophil recruitment	[108]
Anti-CXCL6 monoclonal antibody	Mouse models with bleomycin-induced ALI	↓Neutrophil recruitment ↓Pro-inflammatory cytokines ↓Fibrosis markers	[49]
Amino-truncated CXCL8 molecule with arginine and proline substitutions at K11 and G31	Guinea pig models infected with aspiration pneumonia	↓Neutrophil recruitment ↓Pleural haemorrhagic effusion ↓Vascular permeability	[109]
CXCR2 antagonist	Mouse model of VILI	↓VILI severity ↓Neutrophil recruitment ↓Microvascular permeability	[110]
CXCR2 antagonist	Rat orthotopic single lung transplantation model of cold ischaemia–reperfusion	↓Neutrophil recruitment ↓Severity of lung injury ↓Microvascular permeability	[111]
CXCR2 antagonist	Mouse models exposed to cigarette smoke	↓Neutrophil recruitment	[112]
CXCR2 antagonist and oseltamivir	Mouse models and piglets infected with viral pneumonia	↑Survival ↓Neutrophil recruitment ↓NETosis	[32]

List of animal studies on the efficacy of inhibitors of the CXCR1/2 pathway in various models of ALI. CXCL: C-X-C motif ligand; NETosis: excessive production of neutrophil extracellular traps; VILI: ventilator induced lung injury.

In humans, inhibition of IL-8/CXCL8 and CXCR1/2 has been tested in only a few studies in the context of acute noncardiogenic hypoxemic respiratory failure fulfilling current criteria for ARDS. An anti-CXCL8 monoclonal antibody (BMS-986253) was tested in hospitalised patients with severe COVID-19 in a single-centre, randomised, open-label, phase 2 trial (NCT04347226). The end-point was time-to-improvement in the National Institute of Allergy and Infectious Diseases seven-point ordinal scale following treatment with anti-CXCL8 therapy compared to standard of care. The study was terminated after enrolling 43 patients as the interim analysis indicated that the futility boundary was reached [113]. A competitive and selective CXCR2 antagonist (Danirixin, GSK1325756), in combination with the antiviral oseltamivir, was tested in patients hospitalised for influenza in a phase 2b, double-blind, three-arm study (NCT02927431). Influenza-positive participants were randomised 2:2:1 to receive *intravenous* (*i.v.*) danirixin 15 mg twice daily+oral oseltamivir 75 mg twice daily (OSV), *i.v.* danirixin 50 mg twice daily+OSV or *i.v.* placebo twice daily+OSV, for up to 5 days with the primary end-point being time to clinical response. The study was terminated early due to low enrolment, thus preventing any conclusions [114].

A noncompetitive allosteric inhibitor of both CXCR1 and CXCR2 (reparixin [115], Dompé Farmaceutici SpA, Milan, Italy) was tested in two studies involving patients with acute pulmonary infection complicated by acute respiratory failure (arterial partial pressure of oxygen/fraction of inspired O_2) >100 to <300 mmHg). In a phase 2 clinical trial [116] of 56 patients with acute respiratory failure due to COVID-19, reparixin was compared to standard of care for its efficacy in preventing deterioration of respiratory failure, need for admission to the intensive care unit, mechanical ventilation or death. The rate of these events was significantly lower in the reparixin group compared with the group receiving standard therapy (16.7% (95% CI 6.4–32.8%) *versus* 42.1% (95% CI 20.3–66.5%), p=0.02). A subsequent phase 3 study [117] enrolled 278 COVID-19 patients admitted to the hospital with respiratory failure. The proportion of patients alive and free of respiratory failure (primary end-point) was numerically greater in the reparixin group, but the difference did not reach statistical significance, possibly due to lower than anticipated mortality in both groups due to changes in population immunity and advancements in therapeutic options for COVID-19, primarily steroids and remdesivir.

Unanswered questions and future research directions

The discrepancy between the encouraging preclinical data and the sparse clinical data on CXCR1/2 inhibition in ALI/ARDS is puzzling and may very well be related to knowledge gaps in the various ways that these receptors are regulated in humans in the context of acute lung inflammation, a topic that this review attempted to succinctly broach. There remain several yet unanswered questions that will need to be addressed as a prerequisite for successful further development of CXCR1/2 inhibitors in this clinical context (table 4). The ideal timing (with time being a proxy for severity of disease) for administering an inhibitor is one such question. Overwhelming lung inflammation may be accompanied by a "protease storm" which may lead to cleavage of CXCR from neutrophils [118] and adversely affect the effectiveness of any anti-CXCR therapeutic regimen, especially if this regimen is initiated later in the disease course or in extremely sick patients when proteolytic activity may be at its height. Furthermore, hyper-inflammatory states such as severe COVID-19 are characterised by emergency granulopoiesis or demand-adapted granulopoiesis [95, 119, 120], which results in the emergence of low-density neutrophils that do not express mature neutrophil markers such as CD10 and CXCR2 and demonstrate dysfunctional behaviour

TABLE 4 Proposed future directions for research on the field of CXCR1 and 2 inhibition in the context of acute respiratory distress syndrome and unanswered questions on CXCR1/2 inhibitors

Future research focus	Unanswered questions
Timing	Early *versus* late disease administration
Preferential focus on type of receptor	CXCR1 *versus* CXCR2 *versus* CXCR1/2 inhibitors
Severity of disease	Mild/moderate *versus* severe disease administration
Heterogeneity of disease	"Hyper" *versus* "hypo" inflammatory phenotype and how to define them (*i.e.* systemic *versus* alveolar inflammatory markers)
Mode of administration	Systemic *versus* localised to the lung *versus* combined administration
Duration of treatment	Short term *versus* prolonged treatment and preservation of balance between harmful and beneficial neutrophilic effects
Effect on other cell types	Focus on endothelial and epithelial cell specific CXCR1/2 inhibition
Treatment combinations	Combinations with other neutrophilic receptor blockers or pulmonary oedema clearing drugs

such as impaired oxidative burst [121]. Another 1–2% of peripheral blood neutrophils in hyperinflammatory states have undergone reverse transmigration and are characterised by low cell surface expression of CXCR1 [122]. These neutrophils may be less likely to respond to CXCR1/2 inhibitors, which might therefore be more useful when given to patients at the very first indication of systemic inflammation as opposed to those with full-blown multi-organ failure. This quandary is further compounded by the indisputable fact of ARDS heterogeneity. Cluster-based [123] and latent class analysis-based methods [124] have identified "hyper" and "hypo" inflammatory ARDS subtypes which are not only phenotypic but appear to have prognostic and therapeutic implications with differential response to ARDS treatment interventions [125, 126]. Subtype stratification has been based mainly on plasma inflammatory markers [123]. However, the main or, at least initial, site of inflammation in ARDS is the alveolus and the concentration of inflammatory mediators there either does not closely correlate with systemic levels [127, 128] or is consistently higher than in the systemic circulation [127, 129, 130]. In fact, CXCL8 is one of the chemokines more prominently compartmentalised in the alveolus with concentrations 20 times as high in BALF than in serum [129]. As the severity of localised lung inflammation progresses accompanied by shock and multi-organ failure, these sharp chemokine gradients decline in reverse correlation with mortality and probably as a consequence of the disruption of the integrity and ultimate failure of the alveolocapillary barrier [129]. The presence of such chemokine gradients early on in the disease process may represent a window of opportunity to maximise the efficacy of systemically administered CXCR1/2 blockers as these drugs moderate lung sequestration of neutrophils chemotactically driven to the lung, and/or mitigate persistent alveolar inflammation which has been shown to be positively correlated with mortality in ARDS patients [128]. Conversely, it may explain the failure of such drugs at later and more advanced disease stages when an equilibrium has been created between the lung and the periphery, thus limiting the rationale of using an anti-chemoattractant pathway therapeutic approach.

Another unanswered question regarding timing is the optimal duration of treatment with a CXCR1/2 inhibitor. In recent years, we have become aware that neutrophils inhabit all native tissues and organs independent of inflammation or commensal microbiota in the tissue. In the lung, these tissue-specific neutrophils are mature cells characterised by high expression of CXCR2 that carry out angiogenic functions and contribute to vascular repair after tissue injury [131]. Inhibition of their function through continued blocking of CXCR2 signalling after the critical period of dysregulated inflammation may hinder lung repair and regeneration.

Additionally, although CXC receptors are usually considered in relation to their expression on neutrophils, they are also expressed in other types of cells, albeit at lower levels, including human bronchial epithelial cells [132] and endothelial cells where they are upregulated in response to intermittent hypoxia [133]. These receptors are functional as the endothelial cells exhibit chemotactic responses to CXCL8 in proportion to the cell surface expression of the receptors, which is inhibited by anti-CXCR1 and anti-CXCR2 antibodies [134]. The presence of CXCR2 in mouse endothelial and epithelial cells is pivotal for successful migration of neutrophils to sites of inflammation. *Cxcr2* knockout mice cannot recruit neutrophils in the lungs after exposure to aerosolised LPS inhalation and this defect cannot be corrected upon their reconstitution with wild-type bone marrow [135]. The above may suggest that aerosolised administration of CXCR1/2 inhibitors, which can topically target bronchial epithelial cells and/or endothelial specific CXCR1/2 blockers, may be viable therapeutic options to consider, possibly in combination with systemic drug administration.

The best route of administration of CXCR1/2 targeting drugs is in itself a source of contention. As discussed earlier, an inhaled CXCL1 blocker does not prevent early neutrophil infiltration in the alveoli when administered concurrently with an inhaled pathogen because although it may partially suppress local CXCL1 there still remains a CXCL1 gradient from the blood to the locally inflamed alveoli [43]. Inhaled CXCR1/2 blockers may not work when administered for localised pulmonary inflammation such that caused, *e.g.*, by aspiration of gastric contents, especially at the early stages of the disease when systemic inflammation is absent, as they cannot completely irradicate chemokine gradients towards the localised lung injury. They may, however, be useful to prevent lung involvement in patients at risk for ARDS, whereas a combination of localised and systemic therapy may be preferable in cases of established lung infection and may conceivably enhance the effectiveness of systemic-only therapy by moderating the efflux of CXCL8 from the alveolus to the systemic circulation and mitigating rapid downregulation of CXC1 and 2 in peripheral neutrophils. Further research is needed to address this question.

Another largely unanswered question pertains to the relative importance, in humans, of the inhibition of one receptor compared to the other. As stated above, most preclinical studies on CXCR1 and CXCR2 were conducted in rodents, where information on CXCR1 function compared to its human homologue is scarce

given that the primary human CXCR1 ligand CXCL8 does not exist. As a result, our knowledge base is skewed towards CXCR2 and may not adequately represent human neutrophil physiology.

Finally, the additive or even synergistic effect of combining CXCR1/2 inhibitors with inhibitors against other ARDS-relevant receptors has not been explored and may have a significant therapeutic potential. As an example, although preclinical and small clinical studies have shown that β2-adrenergic receptor (β2AR) agonists enhance alveolar fluid clearance *via* a cAMP-dependent mechanism, multicentre phase 3 clinical trials failed to show that β2AR agonists provide a survival advantage in patients with ARDS. This was later shown to be due to an antagonistic effect of CXCL8 to the alveolar epithelial response to β2AR agonists mediated through heterologous β2AR desensitisation and downregulation (50%) *via* the GRK2/PI3K signalling pathway [30]. The obvious question that follows this observation is whether the combined use of a CXCR1/2 inhibitor or a CXCL8 blocker and a β2AR agonist would be beneficial in sustaining alveolar fluid clearance. Along the same line of thought, the combination of CXCR1/2 inhibitors with inhibitors of other CXC receptors or other types or neutrophilic receptors makes not only intuitive sense but is supported by some evidence. As indicative examples, inhibition of CXCR4 would potentially enhance efficacy of CXCR2 blockade by preventing CXCR2 sepsis-induced downregulation, promoting release from the bone marrow of reserve neutrophils enriched in CXCR2 [136], enhancing inhibition of neutrophil influx in inflamed tissue [137] and preventing $CD4^+$ T-cell sepsis-induced exhaustion [138], whereas TLR4 blockade may conceivably have the same effect through the previously discussed prevention of downregulation of CXCR1 and 2. It is largely undisputed that, when addressing a highly complex pathologic syndrome, such as ARDS, single pathway therapies are unlikely to be a panacea. Pleiotropic therapeutic modalities based on intimate insight of the complex interactions of closely intertwined pathways will be the best way to move forward. Development of sensitive and specific neutrophil activation markers available at the point of care for longitudinal assessments will be an invaluable tool to further advance this goal and may allow for more precise and individualised taming of the untoward effects of inflammation in ARDS.

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