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Progress and challenges with therapeutic targeting of chemokine receptors

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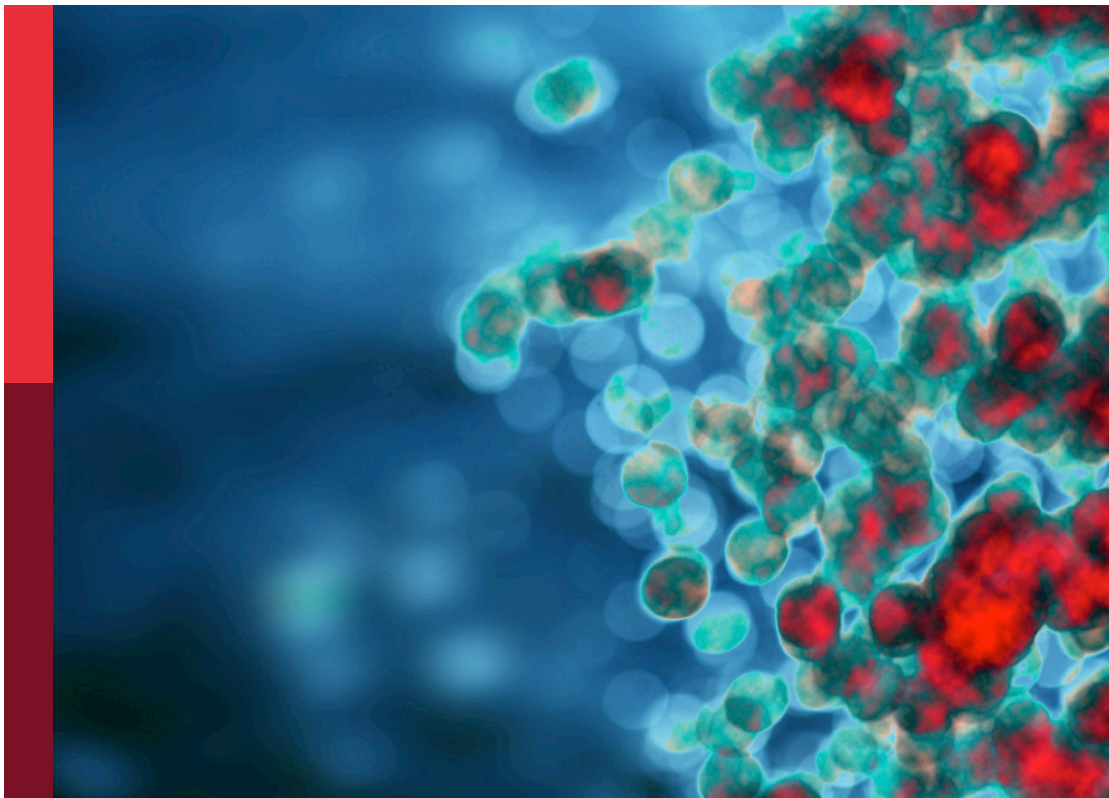

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Progress and challenges with therapeutic targeting of chemokine receptors

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New insights into the analgesic properties of the XCL1/XCR1 and XCL1/ITGA9 axes modulation under neuropathic pain conditions - evidence from animal studies

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Recent studies have indicated the involvement of chemokine-C-motif ligand 1 (XCL1) in nociceptive transmission; however, the participation of its two receptors, canonical chemokine-C-motif receptor 1 (XCR1) and integrin alpha-9 (ITGA9), recently recognized as a second receptor, has not been clarified to date. The aim was to explore by which of these receptors XCL1 reveals its pronociceptive properties and how the XCL1-XCR1 and XCL1-ITGA9 axes blockade/neutralization influence on pain-related behavior and opioid analgesia in the model of neuropathic pain. In our studies we used Albino Swiss mice which were exposed to the unilateral sciatic nerve chronic constriction injury (CCI) as a neuropathic pain model. Animals received single intrathecal (*i.t.*) injection of XCL1, XCL1 neutralizing antibodies, antagonist of XCR1 (vMIP-II) and neutralizing antibodies of ITGA9 (YA4), using lumbar puncture technique. Additionally we performed *i.t.* co-administration of abovementioned neutralizing antibodies and antagonists with single dose of morphine/buprenorphine. To assess pain-related behavior the von Frey and cold plate tests were used. To measure mRNA and protein level the RT-qPCR and Western Blot/Elisa/immunofluorescence techniques were performed, respectively. Statistical analysis was conducted using ANOVA with a Bonferroni correction. Presented studies have shown time-dependent upregulation of the mRNA and/or protein expression of XCL1 in the spinal cord after nerve injury as measured on day 1, 4, 7, 14, and 35. Our immunofluorescence study showed that XCL1 is released by astroglial cells located in the spinal cord, despite the neural localization of its receptors. Our results also provided the first evidence that the blockade/neutralization of both receptors, XCR1 and ITGA9, reversed hypersensitivity after intrathecal XCL1 administration in naive mice; however, neutralization of ITGA9 was more effective. In addition, the results proved that the XCL1 neutralizing antibody

and, similarly, the blockade of XCR1 and neutralization of ITGA9 diminished thermal and mechanical hypersensitivity in nerve injury-exposed mice after 7 days. Additionally, neutralization of XCL1 improves morphine analgesia. Moreover, blockade of XCR1 positively influences buprenorphine effectiveness, and neutralization of ITGA9 enhances not only buprenorphine but also morphine analgesia. Therefore, blockade of the XCL1-ITGA9 interaction may serve as an innovative strategy for the polypharmacotherapy of neuropathic pain in combination with opioids.

KEYWORDS

XCL1, XCR1, ITGA9, CCI, astroglia, chemokine, opioid, microglia

1 Introduction

Neuropathic pain affects 10% of the world's population (1) and is caused by many factors, including mechanical injury to the peripheral or central nervous system. The related complaints require better understanding, diagnosis and treatment because the current therapy is unsatisfactory. Unfortunately, patients with neuropathic pain are less sensitive to opioid drugs, which are the most powerful painkillers currently available in clinics (2). This makes the development of new strategies for pharmacotherapy toward painful neuropathies an urgent need. Such progress requires an extensive understanding of the molecular and cellular mechanisms involved in the development of chronic pain originating from peripheral nerve injury (3). Glia have an undeniable role in the maintenance of homeostasis in the nervous system. Depending on the nature of the stimulus, glial cells can take on a number of activation states, which consequently causes altered gene expression and changes in morphology and function (4). Microglia represent resident immune cells of the central nervous system (CNS), revealing a classically activated phenotype associated with the release of proinflammatory molecules after chronic activation, contributing to neurodegeneration (5). Additionally, astroglia are a population of cells that play an integral role in maintaining CNS homeostasis. Their activation may result in the development of neurodegenerative disorders and is important in the modulation of neuropathic pain (6). This is why the pharmacological modulation of the abovementioned interactions is very effective in relieving painful symptoms in a neuropathic pain model (7). Minocycline (MC), which is one of the most potent substances causing inhibitory effects on the release of pronociceptive factors by glia (8), has the potential to treat the symptoms of neuropathic pain of different etiologies, e.g., in animal models such as streptozotocin (STZ)-induced diabetes (7) and chronic constriction injury (CCI) of the sciatic nerve (9–12). MC was also shown to influence important pain-related intracellular pathways, especially what was well studied, it beneficially influences p38 mitogen-activated protein kinases (MAPK) in an animal model of inflammatory and neuropathic pain (13, 14). Moreover, MC suppresses the increased gene expression of CXCL13, CXCL1, CCL2, CXCL11, and CCL7 after CCI (15). The release of chemokines by neuronal and nonneuronal cells such as microglia/astroglia is an important factor underlying neuroimmune crosstalk during neuropathic pain development and maintenance (4, 16, 17). Our previous studies showed that the neutralization of some chemokines [e.g., CCL1 (18), CCL2 (19), CCL3 (20), CCL7 (19), and CCL9 (20)] and blockade of several receptors [e.g., CCR1 (21), CCR2 (22), CCR3 (23), CCR4 (24), CCR5 (25), CXCR2 and CXCR3 (26)] in animal models of neuropathic pain diminish the development of symptoms; however, the role of XCL1 and its receptors is still unknown.

In our previous study, we demonstrated the spatiotemporal upregulation of XCL1 in several areas of the murine brain (cortex, thalamus, and hippocampus), which began shortly after traumatic brain injury model induction and persisted until up to 5 weeks in the cortex (27). This finding indicates that this chemokine may play a key role in neurodegenerative processes. In 2016, we showed for the first time the important role of XCL1 in diabetic neuropathy (7). To date, it was known that XCL1 is released by some immune cells (28) and it was shown that there is an elevated level of XCL1 protein in primary murine astroglial cells after LPS treatment (27). XCL1 acts through a G-protein coupled receptor, XCR1 (29). For a long time, XCR1 was the only known receptor for XCL1. Recently, Matsumoto et al. showed that XCL1 affects fibroblast migration through the heterodimeric ($\alpha\beta$) transmembrane receptor ITGA9 (30), which opened new research horizons in this field. ITGA9 was proposed as a therapeutic target in autoimmune diseases (31). It remains unknown how XCR1 and ITGA9 are involved in nociceptive transmission; however, their role seems to be extremely important in neuropathy, as our previous research proved the strong pronociceptive properties of their ligand, XCL1, in naive animals (7).

We hypothesized that XCL1 may be significant in neuropathic pain development, acting both through XCR1 and ITGA9. For this purpose, we measured spinal mRNA/protein time-course changes in XCL1, XCR1 and ITGA9 in mice after CCI. Moreover, we performed behavioral tests to evaluate the influence of XCL1 neutralizing antibody (nAb) on mechanical and thermal hypersensitivity and morphine analgesia in CCI-exposed mice. Additionally, we determined the impact of consecutive MC treatment (twice daily, 7 days) on hypersensitivity and the levels of IBA1, GFAP, XCL1, XCR1 and ITGA9 after CCI. An additional aim of this study was to determine whether and how XCR1 and ITGA9 blockade/neutralization influence thermal and mechanical hypersensitivity evoked by intrathecally administered XCL1. We also examined the abovementioned receptor blockade/neutralization effects on hypersensitivity development and opioid analgesia 7 days after CCI. To explain the source of XCL1 and the localization of its receptors in the spinal cord, we performed thorough immunofluorescence studies to determine the cellular localization of XCL1/XCR1/ITGA9 in the spinal cord 7 days after CCI.

2 Materials and methods

2.1 Animals

For our experiments, we used male Albino Swiss adult mice (Charles River, Göttingen, Germany; 9–11 weeks old, weighing 20–25 g). The housing conditions were as follows: 6-10 mice per cage; free access to food and water; temperature of 22 ± 2°C; relative humidity 55 ± 10%; 12-h light/dark cycle. All performed procedures were accomplished with the recommendations of the International Association for the Study of Pain (IASP) and the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and were approved by the Ethical Committee of the Maj Institute of Pharmacology of the Polish Academy of Sciences (permission numbers: 75/2017, 305/2017, 235/2020, 236/2021, 297/2021, 89/2021, 98/2022). The number of animals was reduced to the essential minimum according to the 3R policy.

2.2 Chronic constriction injury

We performed chronic constriction injury (CCI) of the sciatic nerve as a neuropathic pain model, in accordance with Bennett and Xie (1988) (32), modified by Mika et al. (2007) (33). The animals were anesthetized by inhalation of isoflurane (induction, 3%; maintenance, 3%). In brief, there was an incision made below the right hip bone, parallel to the sciatic nerve. The exposed sciatic nerve was loosely tied around the nerve with three ligatures (4/0 silk). The strength of the first knot was dictated by the occurrence of a short contraction in the corresponding hind limb, and the subsequent contractions were performed similarly. All mice developed neuropathic pain-related behaviors (tactile and thermal hypersensitivity).

2.3 Pharmacological studies

2.3.1 Intrathecal and intraperitoneal drug administrations

The intrathecal (*i.t.*) injection was performed according to the method described by Hylden and Wilcox (34) and is a standard procedure in our laboratory (18, 26). A Hamilton syringe with a thin needle (0.3 x 13 mm) was used for administration. The substances used in the experiments were injected in a volume of 5 µl between the L5 and L6 vertebrae (the lumbar region of the spinal cord) until symptoms of correct administration (the tail reflex) were observed. The intraperitoneal (*i.p.*) administered substances were injected with a needle size of 0.45 x 12 mm in terms of body weight and were supposed to be located in the peritoneal cavity.

2.3.2 Single intrathecal administration of an XCL1 neutralizing antibody in mice with chronic constriction injury-induced neuropathy

A single *i.t.* administrations of XCL1 nAb (Mouse XCL1/Lymphotactin Antibody; AF486, R&D Systems; Minneapolis, United States) at doses of 1, 4, 8 and 16 µg/5 µl were performed 7 days after CCI, when mechanical and thermal hypersensitivity had been fully developed. The effect of XCL1 nAb administration on the development of tactile hypersensitivity was measured using the von Frey test, while thermal hypersensitivity was measured using the cold plate test after 1, 4, 24, 48 and 96 hours. XCL1 nAb was dissolved in PBS (Merck; Darmstadt, Germany), and PBS was used as a vehicle (V).

2.3.3 Single intrathecal administration of an XCL1 neutralizing antibody with morphine or buprenorphine in mice with chronic constriction injury-induced neuropathy

The *i.t.* administration of XCL1 nAb (8 µg/5 µl) followed by *i.t.* administration of morphine (M, TEVA; Krakow, Poland) or buprenorphine (B, Polfa S. A; Warsaw, Poland) (2.5 µg/5 µl) was performed 7 days after CCI, when we observed the highest level of XCL1 in the spinal cord and fully developed mechanical and thermal hypersensitivity. XCL1 nAb was administered once, at the dose set up based on previously obtained results. The doses of opioids used for the experiment were set up based on our previous studies (23). First, groups of tested animals received *i.t.* administration of vehicle (PBS) or XCL1 nAb. Next, 2 h after V or XCL1 nAb administration, there was a second *i.t.* injection of vehicle (W, water for injections), M or B. Von Frey and cold

plate tests were performed 0.5 hours after the second administration (of W, M or B) and 2.5 hours after the first administration (of PBS or XCL1 nAb).

2.3.4 Chronic intraperitoneal administration of minocycline in mice with chronic constriction injury-induced neuropathy

Minocycline hydrochloride (MC; Merck) was dissolved in water for injections (W); therefore, the control mice received W according to the same schedule. The MC was first preemptively administered 16 h and 1 h *i.p.* before CCI surgery and then twice daily for 7 days at a dose of 30 mg/kg. The behavioral tests were conducted 30 min after the last MC administration and 7 days after CCI.

2.3.5 Single intrathecal administration of YA4 or vMIP-II preceded by pronociceptive *i.t.* injection of XCL1 in naive mice

Recombinant mouse chemokine-C-motif ligand 1/ lymphotactin protein (XCL1; R&D Systems), recombinant Viral MIP-II protein (vMIP-II, XCR1 antagonist; R&D Systems) and anti-integrin α9 monoclonal antibody (YA4; Fujifilm, Tokyo, Japan) were dissolved in PBS. First, groups of tested animals received *i.t.* administration of vehicle (V; PBS) or XCL1 (X) at a dose of 100 ng/5 µl, which is known to be pronociceptive (7). Next, 2 h after V or XCL1 administration, there was a second *i.t.* administration of V, vMIP-II or YA4 (0.05, 0.5, 1 µg/5 µl). Von Frey and cold plate tests were performed 1, 4, 24, 96 hours after the second administration (V, vMIP-II or YA4), which represents 3, 6, 26 and 98 hours after the first administration (V or XCL1).

2.3.6 Single intrathecal administration of YA4 and vMIP-II in mice with chronic constriction injury-induced neuropathy

vMIP-II and YA4 were dissolved in PBS and administered to mice 7 days after CCI, while the control group received PBS. A single dose of vMIP-II (1 µg/5 µl) or YA4 (1 µg/5 µl), established during the aforementioned experiment, was administered, and behavioral tests were performed after 1, 4, 24, and 96 hours.

2.3.7 Single intrathecal administration of YA4 or vMIP-II with morphine or buprenorphine in mice with chronic constriction injury-induced neuropathy

The experiment aimed to establish the influence of vMIP-II and YA4 on morphine and buprenorphine analgesia 7 days after CCI. First, groups of tested animals received *i.t.* administration of vehicle (V; PBS), vMIP-II or YA4 (1 µg/5 µl, respectively). Next, 3 h after V, vMIP-II or YA4 administration, there was a second *i.t.* administration of vehicle (W; water for injections), M or B (2.5 µg/5 µl). Von Frey and cold plate tests were performed 0.5 hours after the second administration (of W, M or B) and 3.5 hours after the first administration (of V, vMIP-II or YA4).

2.4 Behavioral tests

2.4.1 von frey test

Tactile hypersensitivity was measured using calibrated nylon monofilaments (ranging from 0.6 to 6 g; Stoelting, Wood Dale, USA) to observe reactions to mechanical stimuli as previously described (33). The mice were placed in plastic cages with a wire mesh floor before the experiment. After 5 min of adaptation, von Frey filaments were used in order of increasing pressure [g], and they were applied to the midplantar surface of the ipsilateral (right) hind paw (or both hind paws in case of naive mice) until it was lifted. Control mice were tested in the same way.

2.4.2 Cold plate test

Thermal hypersensitivity was measured using a cold plate/hot plate analgesia meter (Ugo Basile; Gemonio, Italy). The temperature of the plate surface was kept at 2°C, and the maximal time (cutoff) possible for the mouse to be kept on the plate surface was 30 seconds. The animals were placed on a cold plate until the (right) hind paw (or both hind paws in case of naive mice) was lifted as previously described (33). The latency was recorded, and the animals were immediately removed from the plate. In every animal exposed to CCI, the injured foot was the first one to react. Control mice were tested in the same way.

2.5 Biochemical tests

2.5.1 Analysis of gene expression by RT–qPCR

The lumbar (L4–L6) region of the spinal cord was removed from CCI- and naive mice (sacrificed at 1, 4, 7, 14, 35 days). After decapitation, the tissue was dissected, placed into 1.5 ml plastic Eppendorf tubes with RNAlater (Invitrogen; Waltham, USA), frozen and stored at −80°C. For the synthesis of cDNA, 1000 ng of total RNA was reverse transcribed in a total reaction volume of 20 µl with oligo(dT) primer (Fermentas; Warsaw, Poland) using an Omniscript RT Kit (Qiagen; Hilden, Germany). The cDNA was diluted 1:10 with H_2O. For each reaction, 50 ng of cDNA was synthesized from the total RNA template of each individual animal and used for quantitative real-time PCR (RT–qPCR). RT–qPCR was run on a Real-Time PCR iCycler (Bio-Rad; Hercules, USA) using Assay-On-Demand TaqMan probes (Thermo Fisher Scientific; Waltham, USA). The amplification efficiency for each assay was determined by running a standard dilution curve. The following TaqMan primer was used: Mm00434772_m1 (*Xcl1*). The expression of the hypoxanthine guanine phosphoribosyl transferase 1 (Hprt1, Mm00446968_m1) transcript was quantified to control for variation in cDNA amounts. The cycle threshold values were

automatically calculated using iCycler IQ 3.0 software with the default parameters. The abundance of RNA was calculated as $2^{-(\text{threshold cycle})}$.

2.5.2 Western blot analysis

The lumbar (L4–L6) regions of the spinal cord were removed from CCI- and naive mice (sacrificed at 1, 7, 35 days) and used for the study. Selected time points represent different phases in injury development – very early, developed and fully established, basing on mRNA analysis. The tissues were placed into 2 ml plastic Eppendorf tubes with RIPA buffer with a protease inhibitor cocktail (inhibitors with broad specificity for various proteases; Merck) and homogenized. Then, the samples were centrifuged (14.000 rpm) for 30 min at 4°C (in the case of time course studies). In the case of the tissue collected from animals chronically treated with minocycline, the lumbar (L4–L6) regions of the spinal cord were placed in tubes with RIPA buffer with protease inhibitor cocktail, homogenized and fractionated in accordance with available protocols, slightly modified by us (35–37). Firstly, the nuclear fraction was separated by centrifugation (2750 rpm) for 5 min at 4°C. The obtained supernatant was then re-centrifuged (8900 rpm) for 5 min at 4°C, after that the pellet contained mitochondria. The supernatant was then centrifuged once again in an ultracentrifuge (28.700 rpm) for 60 min at 4°C resulting in the separation of the membrane (pellet) and cytosolic (supernatant) fractions, which were used for further analyses. The study conducted on two fractions of protein homogenates aimed to differentiate the presence of receptors inside the membrane, which may be changed by the possible internalization - a rapid decrease in the number of cell-surface binding sites in activated cells. The bicinchoninic acid (BCA) method was used to measure the total protein concentration. The samples of protein (10 µg) were then heated for 8 min at 98°C with the addition of loading buffer (4 × Laemmli Buffer; Bio-Rad). Then, the samples were loaded in 4–15% Criterion TGX precast polyacrylamide gels (Bio-Rad) and transferred to Immune-Blot PVDF membranes (Bio-Rad) with the semidry transfer system (30 min, 25 V). Then, the membranes were blocked (5% bovine serum albumin; Merck) in TBST (Tris-buffered saline with 0.1% Tween 20) for 1 h, washed with TBST (4 × 5 min), and incubated overnight with the following commercially available primary antibodies: rabbit anti-XCR1 (1:5000, Lifespan Biosciences; Seattle, USA), rabbit anti-ITGA9 (1:3000, Abcam; Cambridge, Great Britain), mouse anti-β-actin (1:1000; Merck), rabbit anti-IBA1 (1:500, Novus Biologicals; Centennial, USA), and rabbit anti-GFAP (1:10000, Novus Biologicals) at 4°C. Then, the membranes were incubated in anti-rabbit or anti-mouse secondary antibodies (Vector Laboratories; Burlingame, USA) conjugated with horseradish peroxidase at dilutions of 1:5000 for 1 h at room temperature. The primary and secondary antibodies were dissolved in a SignalBoost Immunoreaction Enhancer Kit (Merck). Then, the membranes were washed in TBST (again 4 × 5 min). The detection of immune complexes was attained by the Clarity Western ECL Substrate (Bio-Rad) and visualized with the Fujifilm LAS-4000 Fluor Imager system. The immunoreactive bands obtained in Western blot analysis were quantified using Fujifilm Multi Gauge software.

2.5.3 Enzyme-linked immunosorbent assay analysis

The lumbar (L4–L6) regions of the spinal cord were removed from naive and CCI-exposed mice (sacrificed at 1, 7, 35 days) and used for Enzyme-Linked Immunosorbent Assay (ELISA) as stated in the manufacturer's protocol. The tissue homogenates were fixed in RIPA buffer with a protease inhibitor cocktail (Merck) and incubated at -20°C. The level of XCL1 was measured in the tissue homogenates using the Mouse XCL1/Lymphotactin ELISA Kit (Sandwich ELISA, LS-F53223; LifeSpan Biosciences) with the following detection ranges: 6.25–400 pg/ml. The manufacturer provided the positive controls for each assay.

2.5.4 Immunofluorescence analysis by confocal microscopy

Seven days after CCI, the mice were sacrificed, and their spinal cords were removed and postfixed in 4% paraformaldehyde (PFA) overnight at 4°C. After dehydration, the tissues were paraffin embedded and sectioned (7 µM) on a microtome (Leica, RM45). Adjacent coronal sections from corresponding regions of the lumbar (L4 to L6) spinal cords of naive and CCI mice were incubated overnight at 4°C with the following primary antibodies: rabbit anti-XCL1 (1:50, Novus Biologicals), rabbit anti-ITGA9 (1:50, Abcam), rabbit anti-XCR1 (1:50, Lifespan Biosciences), mouse anti-NeuN (1:250, Merck), rat anti-IBA1 (1:1000, Abcam), and chicken anti-GFAP (1:10000, Merck). Antigen-bound primary antibodies were visualized with appropriate Alexa Fluor 488/594–conjugated donkey secondary antibodies (1:100, Invitrogen). Hoechst 33342 (Invitrogen) was used to stain cell nuclei. Stained sections were examined and acquired under a high-class confocal microscope (Leica TCS SP8 WLL) equipped with HyD, PMT and TLD detectors. The ipsilateral part of the lumbar spinal cord was visualized on representative images.

2.5.5 Statistical analysis

The behavioral studies (*in vivo*) are presented as the means ± SEMs. The biochemical studies (*ex vivo*) are presented as fold changes relative to the controls (naive) ± SEM. The RT–qPCR results are presented as the normalized averages derived from the threshold cycle. The results of i.t. administration of YA4/vMIP-II in CCI-induced neuropathy (mean ± SEM) were statistically evaluated using a t test with Welsh correction. The other results (mean ± SEM) were evaluated using one-way ANOVA (F value) followed by Bonferroni's *post hoc* test for comparison of intergroup differences (p value). Additionally, the results were evaluated using two-way ANOVA (F value) to determine the time × drug interaction. All of the statistical analyses mentioned above were

performed with GraphPad Prism ver. 8.1.1 (330) (GraphPad Software, Inc., San Diego, USA).

3 Results

3.1 Spatiotemporal changes in the mRNA and/or protein levels of XCL1, its receptors and pain-related behavior after chronic constriction injury of the sciatic nerve in mice

Chronic constriction injury led to the development of mechanical [$F = 70.90$; $p < 0.0001$] (Figures 1A, B) hypersensitivity. These pain-related changes were observed until the last time point tested, as shown using the von Frey test. The mRNA level of *XCL1* was significantly elevated 4 days after CCI [$F = 9.492$; $p < 0.0001$], and this elevated level was maintained until the 35th day after nerve injury [$F = 9.492$; $p = 0.0033$] (Figure 1C). In the protein study, the elevated level of XCL1 protein was maintained from 1 day after CCI [$F = 70.26$; $p = 0.0011$] up to day 35 [$F = 70.26$; $p < 0.0001$] (Figure 1D). The protein level of XCR1 increased significantly 1 day after surgery [$F = 9.88$; $p = 0.0031$] and remained elevated until day 7 in the spinal cord [$F = 9.88$; $p = 0.0102$] (Figure 1E). The protein level of ITGA9 was significantly reduced compared to that in naive animals on day 7 after damage to the sciatic nerve [$F = 2.37$; $p = 0.0153$] (Figure 1F).

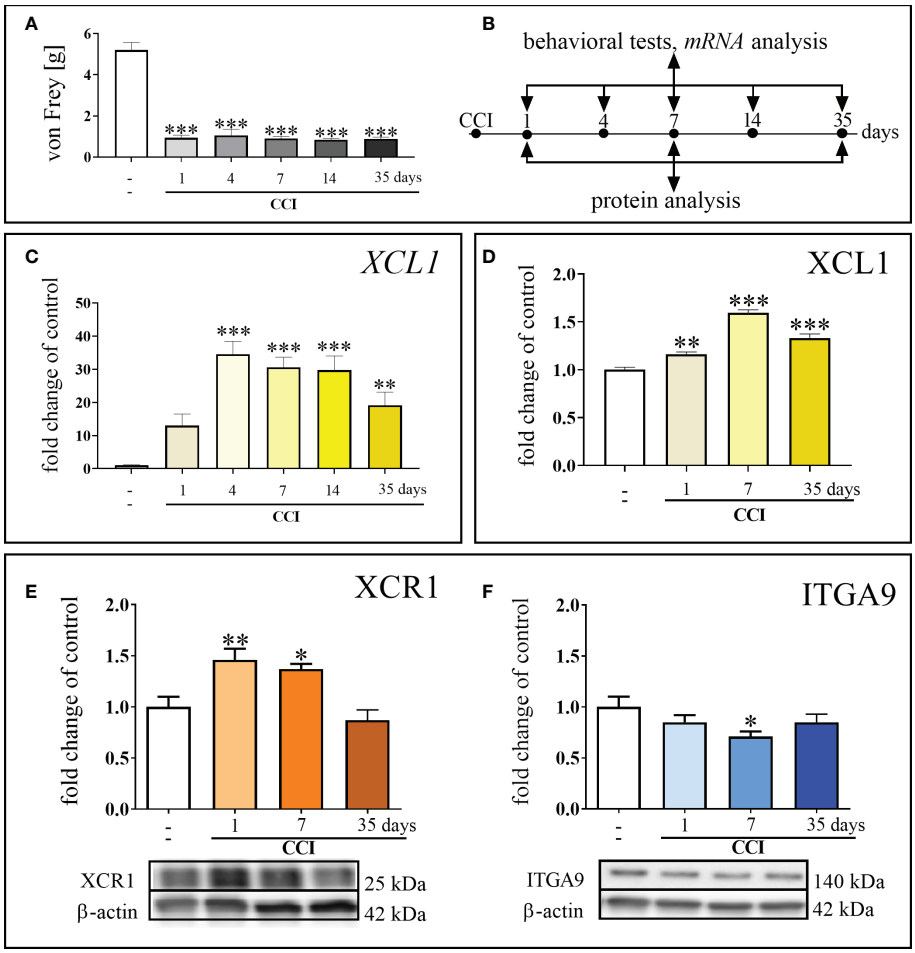

FIGURE 1
Development of mechanical hypersensitivity after chronic constriction injury (CCI) of the sciatic nerve in mice (1, 4, 7, 14, 35 days) **(A)**. Scheme of tissue collection at the indicated time points for behavioral tests and *mRNA*/protein analyses **(B)**. Time-dependent changes in the expression of XCL1 *mRNA* by RT–qPCR **(C)** and protein by ELISA **(D)**; XCR1 protein by Western blot **(E)** and ITGA9 protein by Western blot **(F)** in the spinal cord of naive and chronic constriction injury-exposed mice (1, 4, 7, 14 and/or 35 days). The data are presented as the mean fold changes relative to the control ± SEM (n =5–10). The results were evaluated using one–way ANOVA followed by Bonferroni's *post hoc* test for comparisons of selected pairs. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ indicate significant differences between the naive vs. CCI-exposed groups at each of the investigated time points: 1, 4, 7, 14, 35 days. "-"– naive.

3.2 Effects of a single intrathecal XCL1 nAb administration on pain-related behavior measured 7 days after chronic constriction injury of the sciatic nerve in mice

The analgesic effect of XCL1 nAb administration (Figure 2A) in CCI-exposed mice was observed for doses of 1, 4, 8, and 16 µg/5 µl in the von Frey (Figure 2B) and/or cold plate (Figure 2C) tests. The dose of 1 µg/5 µl was effective 4 hours after administration [cold plate: $F = 15.04$; $p = 0.0025$]. The doses of 4, 8, 16 µg/5 µl showed their analgesic properties 1 hour after administration (4 µg/5 µl [von Frey: $F = 20.93$; $p = 0.0117$; cold plate: $F = 26.51$; $p = 0.0003$], 8 µg/5 µl [von Frey: $F = 20.93$; $p < 0.0001$; cold plate: $F = 26.51$ $p < 0.0001$], 16 µg/5 µl [von Frey: $F = 20.93$; $p = 0.0015$; cold plate: $F = 26.51$ $p < 0.0001$]), and their effects were elevated until 48 hours (4 µg/5 µl [von Frey: $F = 40.02$; $p < 0.0001$; cold plate: $F = 14.47$; $p = 0.0019$], 8 µg/5 µl [von Frey: $F = 40.02$ $p < 0.0001$; cold plate: $F = 14.47$; $p < 0.0001$], 16 µg/5 µl[von Frey: $F = 40.02$ $p < 0.0001$; cold plate: $F = 14.47$; $p < 0.0001$]). Two-way ANOVA confirmed a significant interaction between the treatment and the analyzed time points [von Frey: $F = 14.39$; cold plate: $F = 6.56$].

3.3 Effects of a single intrathecal XCL1 nAb administration on morphine and buprenorphine analgesia 7 days after chronic constriction injury of the sciatic nerve in mice

For the co-administration with opioids (Figure 3A) we have chosen the dose of 8 µg/5 µl, basing on its the effectiveness as shown in time-/dose- dependency study (Figure 2). Selected dose of XCL1 nAb (8 µg/5 µl) and morphine (2.5 µg/5 µl) similarly significantly reduced mechanical [$F = 20.02$; $p = 0.0004$] (Figure 3B) and thermal [$F = 66.85$; $p < 0.0001$] hypersensitivity (Figure 3C). Buprenorphine at a dose of 2.5 µg/5 µl also diminished both mechanical [$F = 13.07$; $p < 0.0001$] (Figure 3D) and thermal [$F = 31.43$; $p < 0.0001$] (Figure 3E) hypersensitivity in CCI-exposed mice.

The influence of XCL1 nAb on morphine analgesia was significant and reduced both mechanical [$F = 20.02$; $p = 0.0020$] (Figure 3B) and thermal [$F = 66.85$; $p < 0.0001$] (Figure 3C) hypersensitivity compared to morphine administered alone. Otherwise, there was no observable impact of the XCL1 nAb on buprenorphine analgesia (Figures 3D, E).

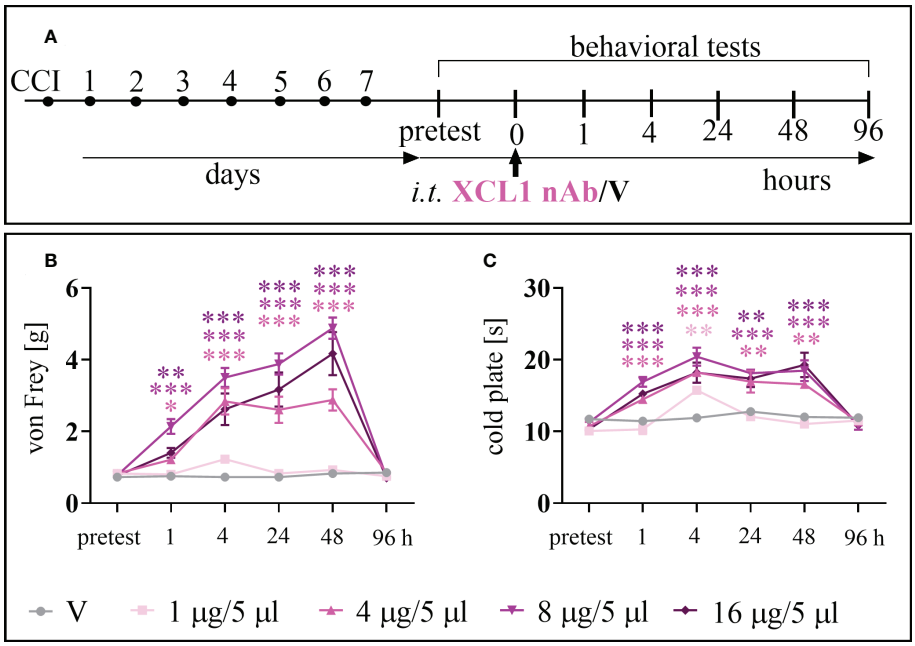

FIGURE 2
The effects of chemokine-C-motif ligand 1 (XCL1) neutralizing antibody (nAb) administered according to scheme **(A)**, at a dose of 1, 4, 8, or 16 µg/5 µl, on mechanical **(B)** and thermal **(C)** hypersensitivity 7 days after chronic constriction injury (CCI) of the sciatic nerve in mice. The data are presented as the mean ± SEM (n =6–8). The results were evaluated using one–way ANOVA followed by Bonferroni's *post hoc* test for comparisons of selected pairs. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ indicate significant differences between the V– vs. nAb–treated groups at each of the investigated time points: 1, 4, 24, 48 and 96 h. "V"– vehicle (PBS).

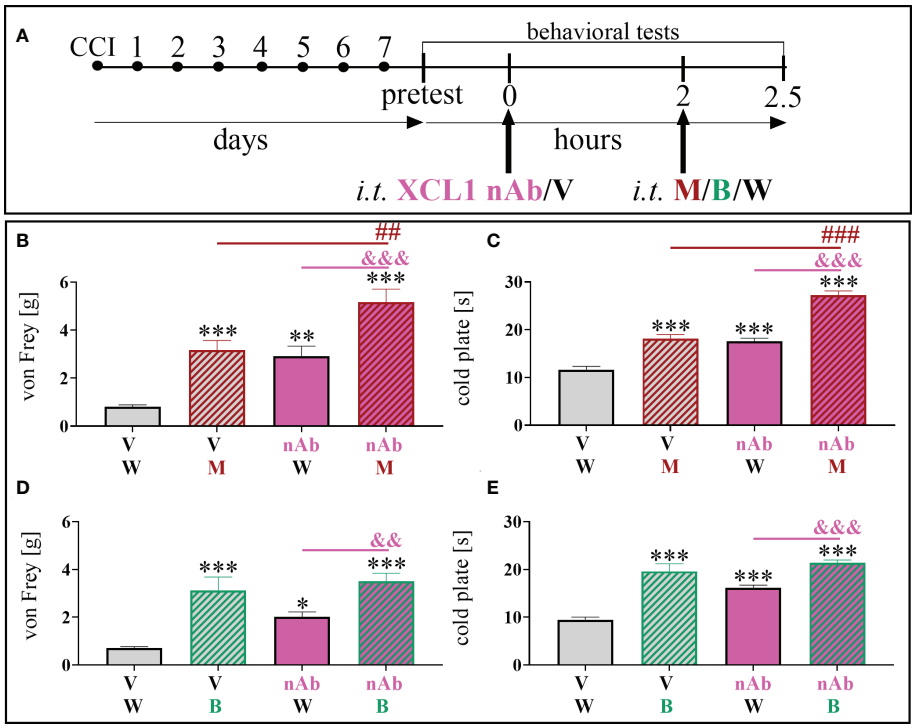

FIGURE 3
The influence of a chemokine-C-motif ligand 1 (XCL1) neutralizing antibody (nAb) **(B–E)** at a dose of 8 µg/5 µl on morphine (M) 2.5 µg/5 µl **(B, C)** and buprenorphine **(B)** 2.5 µg/5 µl **(D, E)** effectiveness, administered according to scheme **(A)**, 7 days after chronic constriction injury (CCI) of the sciatic nerve in mice. The data are presented as the mean ± SEM (n =6). The results were evaluated using one–way ANOVA followed by Bonferroni's *post hoc* test for comparisons of selected pairs. *p < 0.05; **p < 0.01; ***p < 0.001 indicate significant differences between the V +W– and M–/B–/nAb–treated groups; $^{\#\#}$p < 0.01; $^{\#\#\#}$P < 0.001 indicate significant differences between the M– and M+nAb–treated groups; $^{\&\&}$p < 0.01; $^{\&\&\&}$P < 0.001 indicate significant differences between the nAb– and M+nAb–/B+nAb–treated groups. "V"– vehicle (PBS); "W" – vehicle (water for injections).

3.4 Effects of chronic intraperitoneal minocycline administration on mechanical hypersensitivity and changes in the protein levels of XCL1-, XCR-1-, ITGA9-, IBA1-, and GFAP-positive cells 7 days after chronic constriction injury of the sciatic nerve in mice

Chronic minocycline administration (Figure 4A) significantly reduced mechanical hypersensitivity, which had been fully developed in W–treated animals 7 days after CCI [F = 87.26; p < 0.0001] (Figure 4B). The study revealed that after chronic MC treatment, there was a significant reduction in the protein levels of XCL1 [F = 6.43; p = 0.0326] (Figure 4C) and IBA1 [F = 31.09; p = 0.0082] (Figure 4D) compared to CCI-exposed mice. There were no changes in the level of GFAP (Figure 4E).

The level of the XCR1 protein in the cytoplasmic fraction was diminished [F = 3.37; p = 0.0252] in the W–treated group, and MC did not influence this effect (Figure 4F). The protein level of XCR1 in the membrane fraction was significantly elevated (Figure 4G) in the W–treated group [F = 3.63; p = 0.0159]. It was different in the group receiving MC, where the level of the XCR1 protein in the membrane fraction was not changed compared to naive animals (Figure 4G). Regarding the ITGA9 protein, the expression levels were not changed between the W– and MC–treated groups in the cytoplasmic fraction (Figure 4H). The protein level of ITGA9 in the membrane fraction in the CCI-exposed group was significantly diminished [F = 3.96; p = 0.0115] (Figure 4I). This was not the case in the group receiving MC, in which the level of the ITGA9 protein in the membrane fraction was not changed compared to that in naive animals (Figure 4I). Additionally, the minocycline treatment diminished the levels of p38, ERK, JNK and AKT (Supplementary File – Figure 1).

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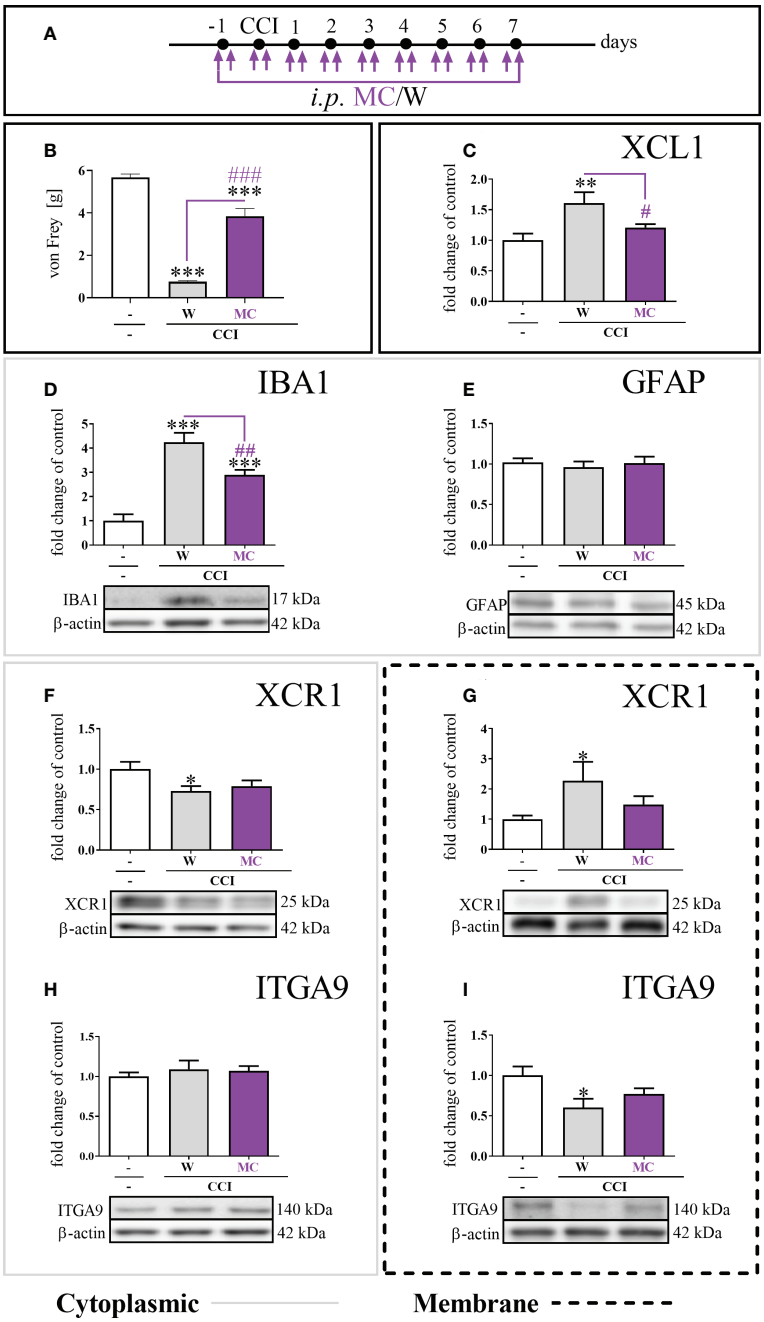

FIGURE 4
The influence of repeated (preemptive and then twice daily for 7 days) minocycline (MC) administration **(A)**, at a dose of 30 mg/kg, on mechanical hypersensitivity **(B)**; XCL1 protein level in the cytoplasmic fraction by ELISA **(C)**, IBA1 **(D)**, GFAP **(E)**, XCR1 **(F)**, and ITGA9 **(H)** protein levels in the cytoplasmic fraction by Western blot; XCR1 **(G)** and ITGA9 **(I)** protein levels in the membrane fraction by Western blot, seven days after chronic constriction injury (CCI) of the sciatic nerve in mice. The data are presented as the mean fold changes relative to the control ± SEM (n =5–14). The results were evaluated using one-way ANOVA followed by Bonferroni's *post hoc* test for comparisons of selected pairs; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ indicate significant differences between the naive vs. W–/MC–treated groups; $^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$ indicate significant differences between the W– vs. MC–treated groups. "-"– naive; "W" – vehicle (water for injections).

3.5 Effects of a single intrathecal ITGA9 nAb (YA4) and XCR1 antagonist (vMIP-II) administration preceded by XCL1 injection in naive mice on mechanical and thermal hypersensitivity

ITGA9 neutralization by YA4 diminished mechanical (Figure 5B) and thermal (Figure 5C) hypersensitivity developed after pronociceptive XCL1 administration (XCL1 was injected 2 hours before YA4) in naive animals (Figure 5A). The effect was observed for the XCL1+YA4-treated groups compared to the XCL1+V-treated group in both behavioral tests since 1 hour (3 hours after XCL1 administration) for doses of 0.05 µg/5 µl [von Frey: $F = 9.94$; $p = 0.0018$; cold plate: $F = 5.90$; $p = 0.0298$], 0.5 µg/5 µl [von Frey: $F = 9.94$; $p = 0.0402$; cold plate: $F = 5.90$; $p = 0.0011$], and 1 µg/5 µl [von Frey $F = 9.94$; $p = 0.0002$]. It was still effective until the 4th hour (6th hour after XCL1 administration) for doses of 0.05 µg/5 µl [von Frey: $F = 11.95$; $p < 0.0001$; cold plate: $F = 6.85$; $p < 0.0001$], 0.5 µg/5 µl [von Frey: $F = 11.95$ $p < 0.0001$; cold plate: $F = 6.85$; $p = 0.0041$], and 1 µg/5 µl [von Frey: $F = 11.95$ $p < 0.0001$; cold plate: $F = 6.85$; $p = 0.0135$]. Twenty-four hours after administration of YA4 (26 hours after XCL1 administration), there was still an observable antinociceptive effect in the cold plate test [dose of 0.05 µg/5 µl: $F = 3.82$; $p = 0.0164$] (Figures 5B, C).

The analgesic effect of XCR1 blockade by vMIP-II injection was more dose-dependent and diminished mechanical (Figure 5D) and thermal (Figure 5E) hypersensitivity - the pronociceptive effect of XCL1 administration (XCL1 was administered 2 hours before vMIP-II) in naive animals (Figure 5A). The effect was observed for the XCL1+ vMIP-II-treated groups compared to the XCL1+V-treated group in both behavioral tests after 1 hour (3 hours after XCL1 administration) for doses of 0.05 µg/5 µl [cold plate: $F = 12.10$; $p = 0.0046$], 0.5 µg/5 µl [von Frey: $F = 13.72$; $p = 0.0139$; cold plate: $F = 12.10$; $p < 0.0001$], and 1 µg/5 µl [von Frey: $F = 13.72$; $p < 0.0001$; cold plate: $F = 12.10$; $p < 0.0001$]. It was still effective until the 4th hour (6th hour after XCL1 administration) for doses of 0.05 µg/5 µl [cold plate: $F = 6.72$; $p = 0.0494$], 0.5 µg/5 µl [von Frey: $F = 5.59$; $p = 0.0293$; cold plate: $F = 6.72$; $p = 0.0119$], and 1 µg/5 µl [von Frey: $F = 5.59$; $p = 0.0051$; cold plate: $F = 6.72$; $p < 0.0001$] (Figures 5D, E).

3.6 Effects of a single intrathecal ITGA9 nAb (YA4) and XCR1 antagonist (vMIP-II) administration on mechanical and thermal hypersensitivity 7 days after chronic constriction injury of the sciatic nerve in mice

The neutralization of ITGA9 by YA4 (Figure 6A) at a dose of 1 µg/5 µl started to influence mechanical hypersensitivity and simultaneously was the most effective 4 hours after administration [$t = 5.58$; $p = 0.0006$] and lasted until 24 hours [$t = 3.22$; $p = 0.0097$] (Figure 6B). Similarly, in the case of thermal hypersensitivity, neutralization started to be effective 1 hour after administration [$t = 2.72$; $p = 0.0190$], was the most powerful 4 hours after administration [$t = 6.17$; $p < 0.0001$], and similarly ceased but was still effective at 24 hours [$t = 2.35$; $p = 0.0366$] (Figure 6C).

The blockade of XCR1 by vMIP-II injection (Figure 6A) at a dose of 1 µg/5 µl also effectively diminished mechanical and thermal hypersensitivity in the CCI model, but only 4 hours after administration, as revealed both by the von Frey test [$t = 3.06$; $p = 0.0207$] (Figure 6D) and the cold plate test [$t = 3.56$; $p = 0.0042$] (Figure 6E).

3.7 Effects of a single intrathecal ITGA9 nAB (YA4) and XCR1 antagonist (vMIP-II) administration on morphine and buprenorphine analgesia 7 days after chronic constriction injury of the sciatic nerve in mice

The influence of YA4 on morphine analgesia (Figure 7A) was significant and more effectively reduced both mechanical [$F = 13.27$; $p = 0.0239$] (Figure 7B) and thermal [$F = 15.54$; $p = 0.0025$] (Figure 7C) hypersensitivity compared to the administration of substances alone. The influence of YA4 on buprenorphine effectiveness (Figure 7A) was also observable. Strong reduction of both mechanical [$F = 50.06$; $p < 0.0001$] (Figure 7D) and thermal [$F = 23.16$; $p < 0.0001$] (Figure 7E) hypersensitivity was demonstrated compared to separately injected compounds.

After administration of vMIP-II with buprenorphine (Figure 7A), attenuation of thermal [$F = 24.00$; $p < 0.0001$] (Figure 7I) and mechanical [$F = 42.60$; $p < 0.0001$] (Figure 7H) hypersensitivity was observed and was the strongest compared to single substance action. Otherwise, there was no observable impact of vMIP-II on morphine analgesia in either behavioral test (Figures 7F, G).

3.8 The cellular localization of XCL1, XCR1 and ITGA9 in the spinal cord 7 days after chronic constriction injury of the sciatic nerve in mice revealed by immunofluorescence staining

Results of the immunofluorescence analysis from CCI-exposed mice are shown in Figures: 8A-Z, 9A-Z, 10A-Z and from naive animals in the Supplementary File: Figures 2A-Z, 3A-Z, 4A-Z. For robustness of the visualization, two independent corresponding regions of the spinal cord of each section were compared, as shown in Figures 8a–c, Figures 9a–c and Figures 10a–c.

Fluorescence immunohistochemical staining revealed clear colocalization of XCL1 with GFAP-positive cells (Figures 8D, H). No colocalization was observed between XCL1 and IBA1 (microglia marker) or NeuN (neuronal marker) (Figures 8L, P, U, Z; respectively).

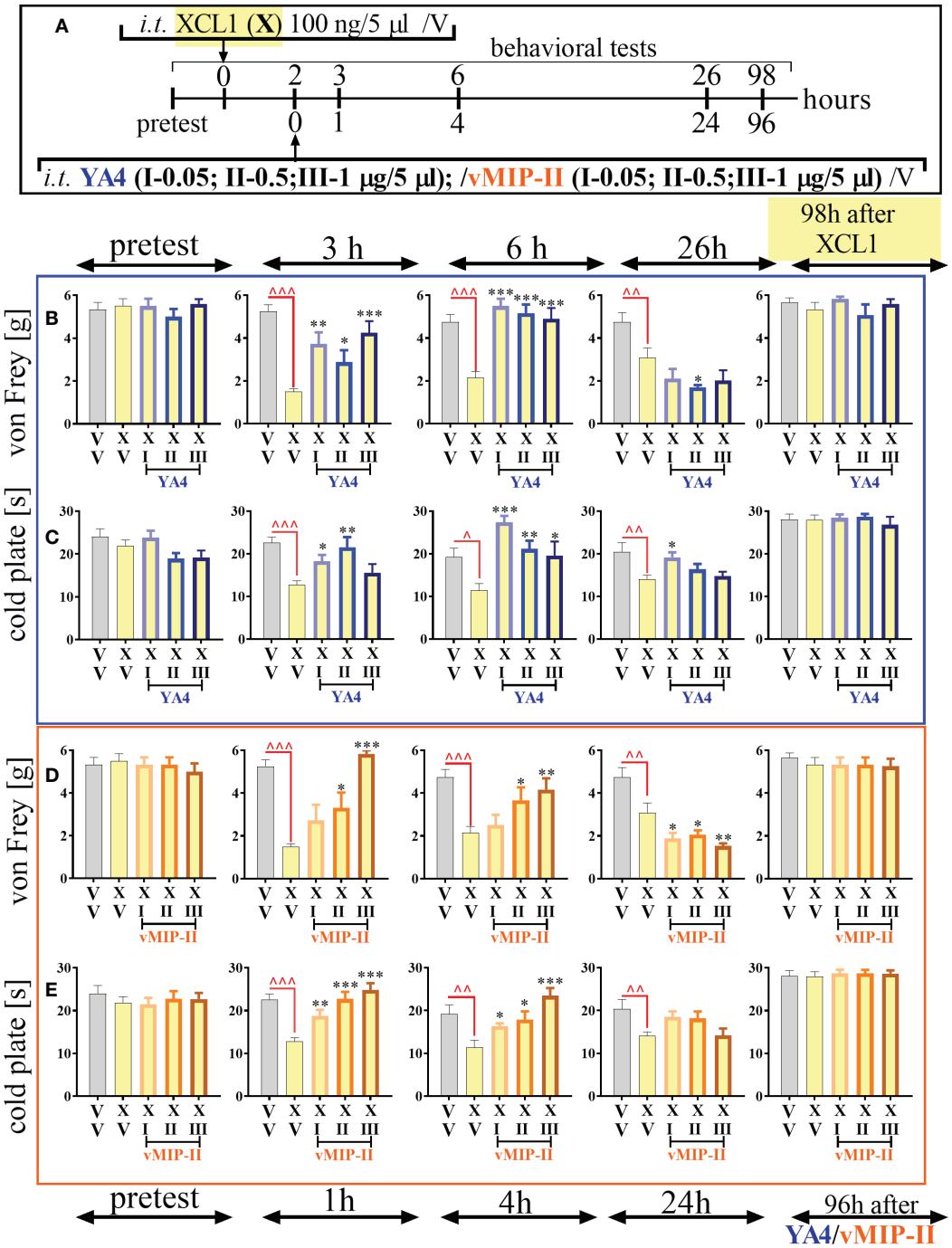

FIGURE 5
The influence of ITGA9 neutralization by YA4 **(B, C)** or XCR1 blockade by vMIP-II **(D, E)**, according to scheme **(A)**, at a dose of 0.05 (I), 0.5 (II), 1 (III) µg/5 µl on mechanical **(B, D)** and thermal **(C, E)** hypersensitivity in naive mice after pronociceptive XCL1 100 ng/5 µl (X) administration. Data are presented as the mean ± SEM (n =4–6). The results were evaluated using one-way ANOVA followed by Bonferroni's *post hoc* test for comparisons of selected pairs; ^p < 0.05; ^^p < 0.01; ^^^p < 0.001 indicate significant differences between the veh-(V+V) vs. XCL1(X+V)–treated group; *p < 0.05; **p < 0.01; ***p < 0.001 indicate significant differences between the XCL1(X+V)– vs. XCL1+YA4–/XCL1+vMIP-II–treated groups. Abbreviations: "V"– vehicle (PBS).

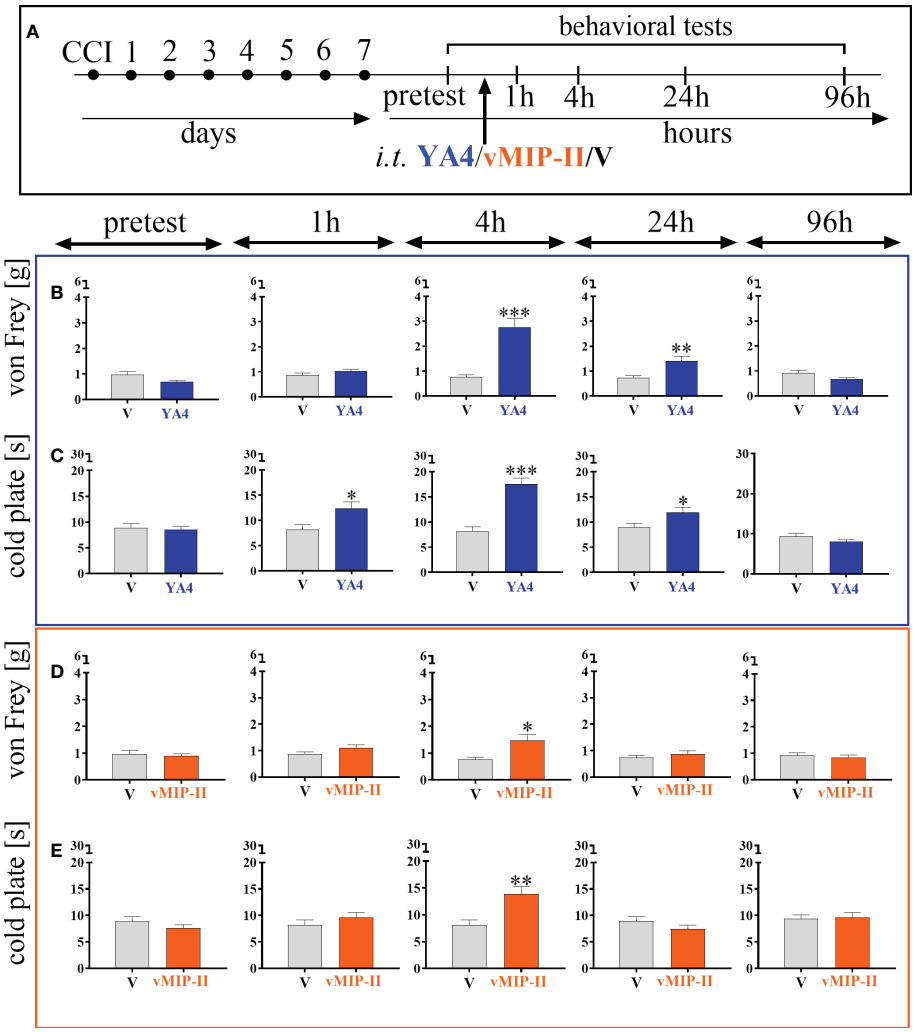

FIGURE 6
The influence of ITGA9 neutralization by YA4 **(B, C)** or XCR1 blockade by vMIP-II **(D, E)**, administered according to scheme **(A)**, at a dose of 1 µg/5 µl on mechanical **(B, D)** and thermal **(C, E)** hypersensitivity 7 days after chronic constriction injury (CCI) of the sciatic nerve in mice. Data are presented as the mean ± SEM (n =5–8). The results were evaluated using t tests for comparisons of selected pairs; *p < 0.05; **p < 0.01; ***p < 0.001 indicate significant differences between the V– vs. YA4–/vMIP-II–treated groups. "V"– vehicle (PBS).

In contrast, in the case of XCR1, there was clear colocalization with NeuN-positive cells (Figures 9U, Z). However, it is worth emphasizing that a strong XCR1-positive signal was observed in other cells but not in cells expressing GFAP (astroglia), IBA1 (microglia, macrophages), or NeuN (neurons) (Figure 9).

ITGA9 was also shown to colocalize with a neuronal marker (NeuN) (Figures 10U, Z) but similarly to XCR1, not with astroglia (Figures 10D, H) and IBA1-positive cells in the spinal cord (Figures 10L, P).

Because the activation of IBA1 and GFAP was expected to be visible in CCI-treated animals, the experiments designed to show colocalization were focused on these groups. Nevertheless, to maintain good laboratory practice, we also collected the stainings performed on naive animals (Supplementary File – Figures 2–4). As shown, after nerve injury, there was much stronger activation of IBA1-positive (microglia/macrophages) and GFAP-positive cells in the ipsilateral dorsal and ventral parts of the spinal cord (Figures 8a, b, 9a, b, 10a, b) than in the respective supplementary data (Supplementary File – Figures 2a, b, 3a, b, 4a, b).

4 Discussion

Our results demonstrated for the first time the upregulation in the level of XCL1 mRNA/protein during neuropathy development (already one day after CCI), which was maintained for up to 35 days, and as revealed by confocal analysis of immunofluorescent staining, these chemokines are produced mainly by spinal astroglial cells. Importantly, our study also

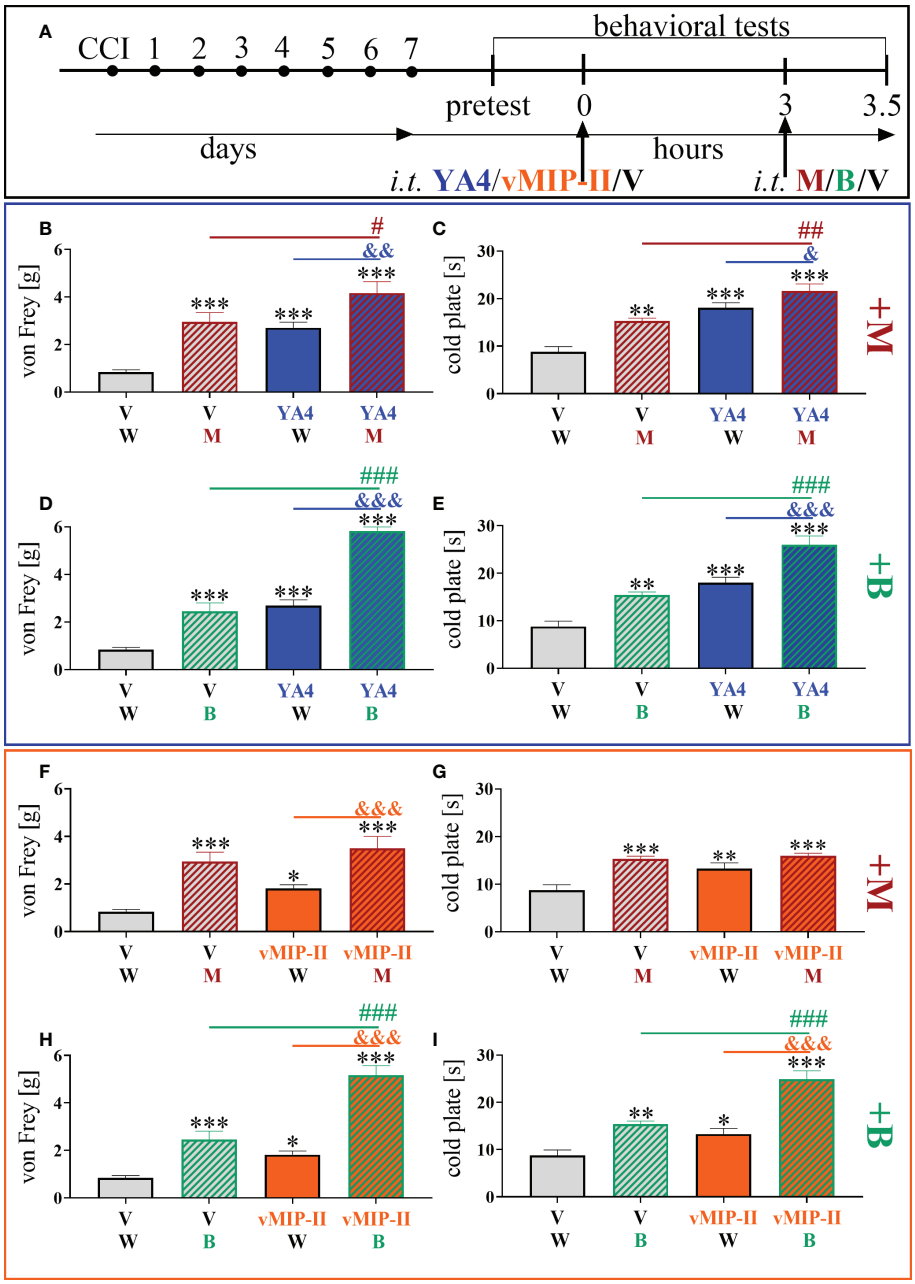

FIGURE 7
The influence of ITGA9 neutralization by YA4 **(B–E)** or XCR1 blockade by vMIP-II **(F–I)** at a dose of 1 µg/5 µl on morphine (M) 2.5 µg/5 µl **(B, C, F, G)** and buprenorphine **(B)** 2.5 µg/5 µl **(D, E, H, I)** effectiveness, administered according to scheme **(A)**, 7 days after chronic constriction injury (CCI) of the sciatic nerve in mice. Data are presented as the mean ± SEM (n =5–12). The results were evaluated using one-way ANOVA followed by Bonferroni's *post hoc* test for comparisons of selected pairs; *p < 0.05; **p < 0.01; ***p < 0.001 indicate significant differences between the V– vs. YA4–/vMIP-II–/M–/B–treated groups; $^{\#}$p < 0.05; $^{\#\#}$p < 0.01; $^{\#\#\#}$P < 0.001 indicate significant differences between the M–/B–treated vs. M+YA4–/B+YA4–/B+vMIP-II–treated groups; $^{\&}$p < 0.05; $^{\&\&}$p < 0.01; $^{\&\&\&}$p < 0.001 indicate significant differences between the YA4– vs. M+YA4–/B +YA4– or vMIP-II– vs. M+vMIP-II–/B+vMIP-II–treated groups. "V"– vehicle (PBS); "W" – vehicle (water for injections).

unprecedentedly showed that blockade/neutralization of both XCL1 receptors, XCR1 by vMIP-II and ITGA9 by YA4, reverse XCL1 nociceptive properties. In addition, we proved that the XCL1 neutralizing antibody reduces mechanical and thermal hypersensitivity and improves morphine analgesia in CCI-exposed mice. Additionally, our results indicated that the mechanisms of minocycline analgesic action in neuropathy may also involve the decrease in the pronociceptive XCL1. Moreover, behavioral studies provided the first evidence that vMIP-II and

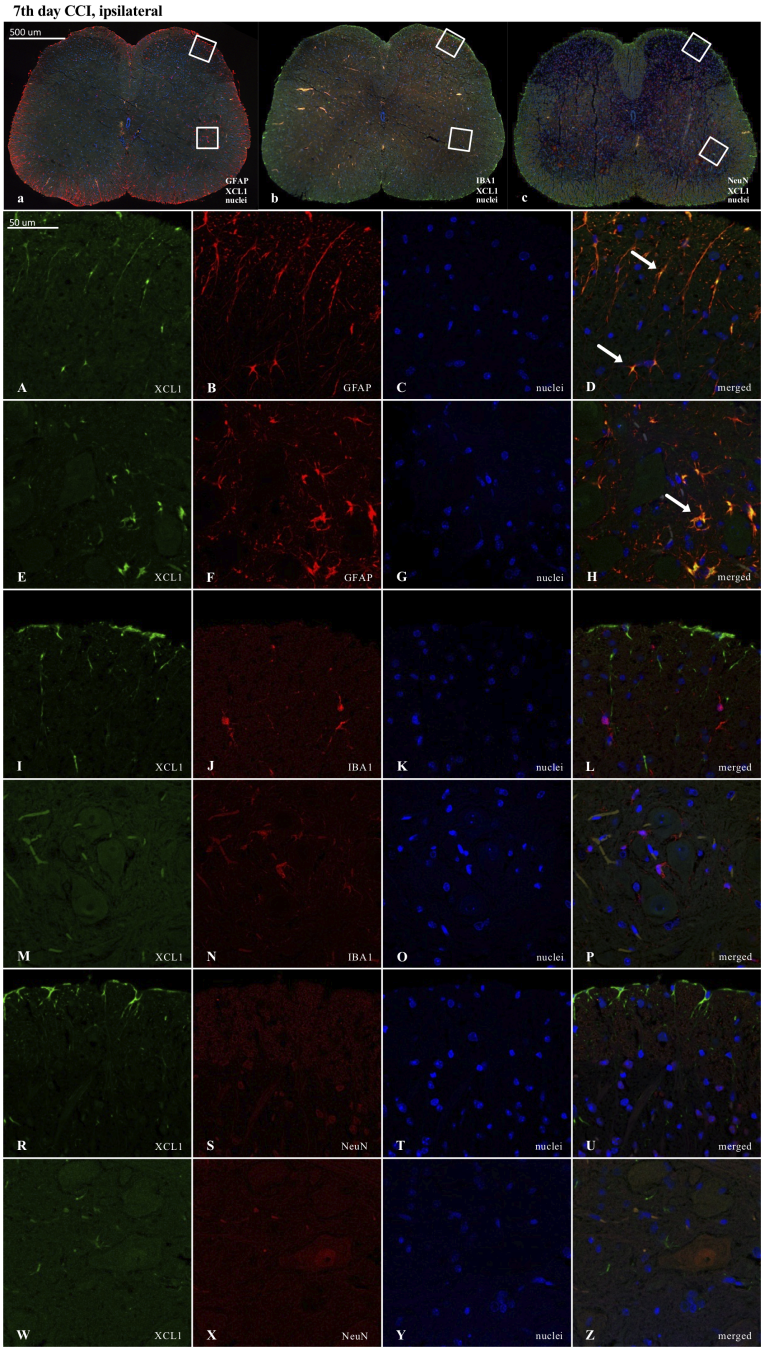

FIGURE 8
Immunofluorescence analysis of chemokine-C-motif ligand 1 (XCL1) localization in the lumbar (L4 to L6) spinal cord 7 days after chronic constriction injury (CCI) of the sciatic nerve in mice. Dorsal **(A–D, I–L, R–U)** and ventral **(E–H, M–P, W–Z)** parts of lumbar spinal cord (**a,b,c**) were shown as an approximate fragments of selected images. Representative immunofluorescence images from colocalization analysis performed on spinal cord, paraffin-embedded 7 µM microtome slices: XCL1 (green: **A, E, I, M, R, W**) with astroglia marker glial fibrillary acidic protein; (GFAP, red: **B, F**); microglia marker ionized calcium-binding adaptor molecule 1; (IBA1, red: **J, N**); and with neuronal marker neuronal nucleus; (NeuN, red: **S, X**), Nuclei are in blue (**C, G, K, O, T, Y**). High magnification, three-dimensional image rendering shows XCL1 localization inside GFAP-positive cells (yellow: **D, H**). Scale bars: 50 µm (**a, b, c**), 500 µm **(A–Z)**.

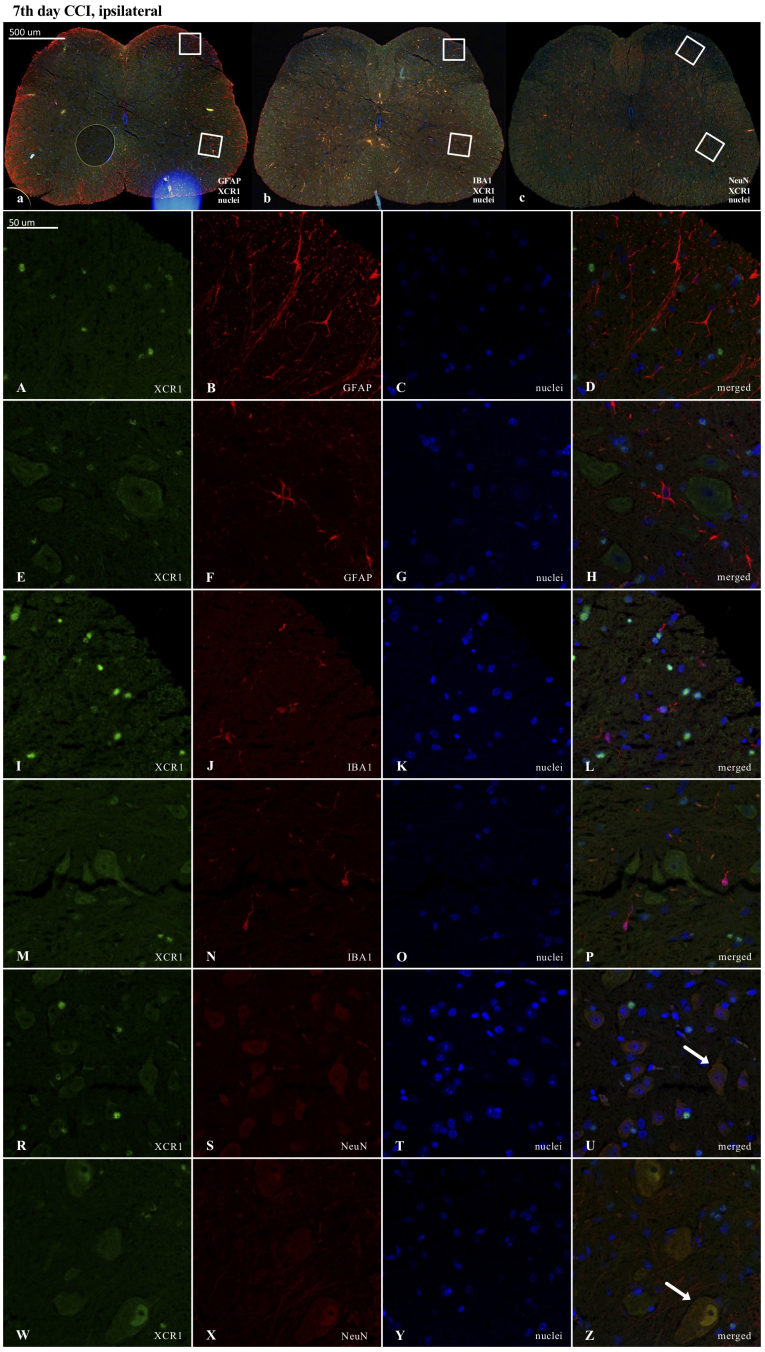

FIGURE 9
Immunofluorescence analysis of chemokine-C-motif receptor 1 (XCR1) localization in the lumbar (L4 to L6) spinal cord 7 days after chronic constriction injury (CCI) of the sciatic nerve in mice. Dorsal **(A–D, I–L, R–U)** and ventral **(E–H, M–P, W–Z)** parts of lumbar spinal cord (**a,b,c**) were shown as an approximate fragments of selected images. Representative immunofluorescence images from colocalization analysis performed on spinal cord, paraffin-embedded 7 µM microtome slices: XCR1 (green: **A, E, I, M, R, W**) with astroglia marker glial fibrillary acidic protein; (GFAP, red: **B,F**); microglia marker ionized calcium-binding adaptor molecule 1; (IBA1, red: **J, N**); and with neuronal marker neuronal nucleus; (NeuN, red: **S, X**), Nuclei are in blue (**C, G, K, O, T, Y**). High magnification, three-dimensional image rendering shows XCR1 localization inside NeuN-positive cells (yellow: **U, Z**). Scale bars: 50 µm (**a, b, c**), 500 µm **(A–Z)**.

YA4 diminish thermal and mechanical hypersensitivity in CCI-exposed mice and enhance buprenorphine and, in the case of YA4, also morphine analgesia. Importantly, immunofluorescence staining indicated that XCR1 and ITGA9 are expressed on neurons. Remarkably, our results clearly showed that blocking the XCL1-ITGA9 interaction appears to be more potent in relieving neuropathic pain.

XCL1 is known to play an essential role in the classical immune response (28). It is produced by subsets of T and NK cells during the course of inflammation, leading to chemotaxis of these cells by binding to XCR1 (28). However, in the course of neuropathy, the CD^{4+} and CD^{8+} T helper cells are unchanged in the spinal cord, in contrast to strongly activated glial cells (21). Recently, it was shown that the mRNA and protein level of XCL1 is increased in primary astroglia but not microglia in LPS-stimulated mouse cell cultures (27). Moreover, importantly, XCL1 stimulation of primary microglial and astroglial cells does not directly induce the production of pronociceptive interleukins (IL-1β, IL-18, IL-6) and chemokines (CCL3, CCL4, CCL9) (27). Therefore, we hypothesized that in neuropathy, XCL1 acts through neuronally localized receptors. Our immunofluorescence analysis proved that XCR1 and ITGA9 are located on spinal neurons, not micro- and astroglial cells. In the present study, we demonstrated for the first time the quick and strong upregulation of spinal XCL1, which lasts up to 5 weeks. Importantly, intrathecal injection of XCL1 in naive mice evoked thermal and mechanical hypersensitivity after 1 h, which lasted up to 24 h (7). Moreover, our studies provided the first evidence that neutralization of XCL1 results in reductions in thermal and mechanical hypersensitivity in CCI-exposed mice. In turn, XCL1-evoked hypersensitivity in naive mice is abolished by pretreatment with vMIP-II and YA4, suggesting that both receptors are responsible for its pronociceptive properties. Many studies have demonstrated that glial cells activated under neuropathic pain conditions produce many pronociceptive cytokines, including interleukins (23, 38, 39) and chemokines (21–24, 40–44). We have followed that concept, and based on our results, we propose that XCL1 is produced by astroglia and activates neuronal XCR1 and ITGA9, which are both strongly engaged in neuropathic pain development. It was already shown by an *in vitro* study that minocycline, a glial inhibitor, treatment before LPS stimulation prevented *XCL1* mRNA upregulation in primary astroglial cells (7). Our present results confirmed that consecutive minocycline treatment (twice daily, 7 days) attenuates CCI-evoked neuropathic pain by inhibiting microglia/macrophages and diminishing the levels of p38, ERK, JNK and AKT, which is congruent with literature data (13, 45–48). It was also demonstrated that minocycline diminished hypersensitivity of spinal neurons after traumatic spinal cord injury (49). Importantly, we observed for the first time that minocycline treatment also lowered the protein level of XCL1 in the CCI-induced neuropathy model, which might be considered behind the additional mechanism of its beneficial effects in neuropathy.

XCR1 is the well-known receptor for XCL1. It is expressed in T cells, B cells and neutrophils (50), Schwann cells, oligodendrocytes (51) and neurons (7, 51). Previous immunofluorescence studies have shown that in the nervous system, XCR1 is expressed in nonpeptidergic and non-IB4 binding terminals of A-delta and C-fiber afferents and/or within excitatory interneurons (51). Our immunofluorescence analysis of lumbar spinal cord sections showed that XCR1 is expressed by neurons and other cells but not microglia and astroglia. Importantly, the membrane, but not cytoplasmic, fraction of spinal protein level XCR1 increased after CCI but not in the group of animals receiving minocycline, which is probably related to its ability to prevent glial activation (52). Since minocycline causes a decrease in the level of XCL1, that is produced by activated astroglial cells, regulation of the membrane level of XCR1 may results from the ligand-receptor interactions favorably altered by it. The pharmacological blockade of XCR1 by vMIP-II was previously shown to inhibit the expression of pERK and p38MAPK in tissue exposed to XCL1 (51), which is a similar effect to that caused by minocycline treatment, as shown by our study and others (13, 14). Moreover, it lowers spontaneously hyperactive neuronal discharges, which are also characteristic of central sensitization (51). The behavioral analysis performed in our studies provided the first evidence that pharmacological blockade of XCR1 by vMIP-II may evoke neuropathic pain relief. These findings indicate that the XCL1/XCR1 axis can participate in many aspects of neuro-glial interactions and play a significant role in nociceptive transmission.

Importantly, XCL1 can also act through ITGA9 (30), an extracellular matrix component, which was shown to participate in the pathophysiology of some intractable disorders by the regulation of the cell physiological state and acts through a diversity of signaling pathways. This adhesion molecule exerts a crucial function to regulate multistep processes, including migration, proliferation, and metastasis (31, 53–55). Integrins consist of two subunits, α and β, so they are heterodimers. On cell membranes, they can interact with extracellular ligands and serve as receptors to mediate intracellular signals. As adhesion proteins, integrins are engaged in a variety of cellular functions (56). If the local responses are disturbed, integrin activation may occur, leading to tissue damage and inflammation (57). ITGA9, which is formed of α9 and β1 subunits, is one of the less known. Its role in nociceptive transmission needs to be studied, which is why we focused on this topic in our work. The results of our research revealed for the first time that after CCI, the protein level of ITGA9 remains at a similar level in the cytoplasmic fraction and is lowered in the membrane fraction, which may be caused by the internalization of the receptor after its activation by XCL1 in the course of neuropathy. Our immunofluorescence staining indicated the presence of ITGA9 in spinal neurons but not in microglial and astroglial cells. The importance of this receptor in nociceptive transmission was proven for the first time by our pharmacological research. The spinal blockade of ITGA9 by YA4 significantly diminished both thermal and mechanical hypersensitivity evoked by CCI. The results are promising because it was already shown that blocking ITGA9 has

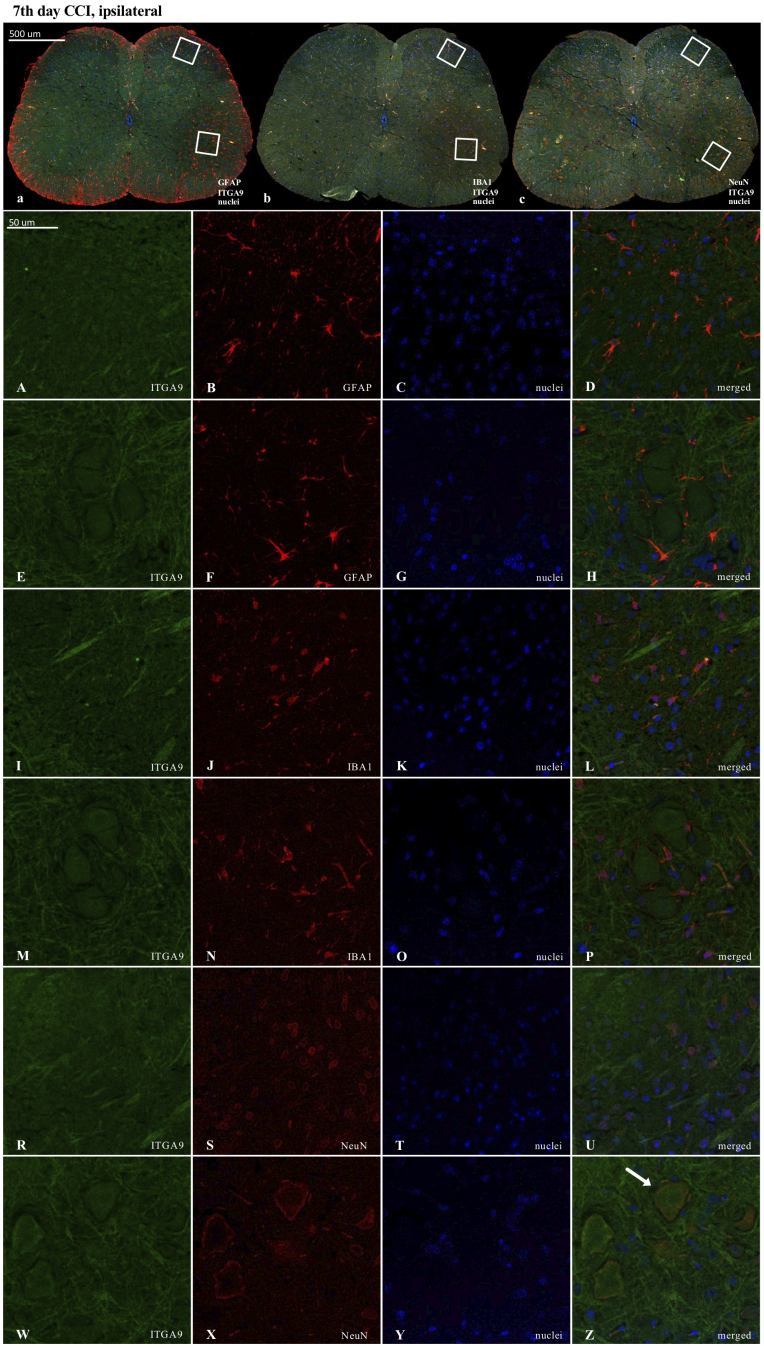

FIGURE 10
Immunofluorescence analysis of integrin alpha-9 (ITGA9) localization in the lumbar (L4 to L6) spinal cord 7 days after chronic constriction injury (CCI) of the sciatic nerve in mice. Dorsal **(A–D, I–L, R–U)** and ventral **(E–H, M–P, W–Z)** parts of lumbar spinal cord (**a,b,c**) were shown as an approximate fragments of selected images. Representative immunofluorescence images from colocalization analysis performed on spinal cord, paraffin-embedded 7 µM microtome slices: ITGA9 (green: **A, E, I, M, R, W**) with astroglia marker glial fibrillary acidic protein; (GFAP, red: **B, F**); microglia marker ionized calcium-binding adaptor molecule 1; (IBA1, red: **J, N**); and with neuronal marker neuronal nucleus; (NeuN, red: **S, X**), Nuclei are in blue (**C, G, K, O, T, Y**). High magnification, three-dimensional image rendering shows ITGA9 localization inside NeuN-positive cells (yellow: **U, Z**). Scale bars: 50 µm (**a, b, c**), 500 µm **(A–Z)**.

beneficial effects in mouse models of experimental autoimmune encephalomyelitis (31) and arthritis (58). According to some literature data from *in vitro* studies, XCL1 signaling *via* ITGA9 may be neuroprotective by increasing the number of neurospheres, promoting neuronal differentiation and positively affecting neurogenesis (59); it also potentiates the regeneration of axons (60). On the other hand, some researchers have suggested that its blockade can reverse the outcomes of autoimmune diseases (31). Therefore, in our opinion, ITGA9 is an interesting target for pharmacotherapy, and it is definitely tempting to look for new pharmacological tools for its modulation.

Opioids are used in chronic pain treatment, but in neuropathy, they exhibit lower effectiveness (16, 61). Many studies have proven that pharmacological inhibition of glial activation in neuropathy provides beneficial effects on opioid analgesic efficacy (4, 62). Initially, the reason for this phenomenon was the reduced release of cytokines (including chemokines) by these cells (16, 61). Recent data in the literature confirmed this theory, showing that intrathecal administration of CCL2- and CCL7 neutralizing antibodies enhanced the analgesic effects of morphine and buprenorphine in CCI-exposed mice (19). Moreover, in cancer-induced bone pain, inhibition of CXCL10, CXCL11 and CXCL13 enhanced morphine analgesic properties in rats (63–65). Moreover, in diabetic neuropathic pain, intrathecal administration of CCL1-, CCL3- and CCL9 neutralizing antibodies enhanced morphine effectiveness (18, 20). Our results provide the first evidence that an XCL1 neutralizing antibody improves morphine (a strong agonist of MOR, with lower affinity to DOR, KOR), but not buprenorphine (an agonist of MOR/NOR; antagonist of KOR/DOR), analgesia in CCI-exposed mice. Apart from the fact that these opioids acting through different receptors, which might be the explanation for why neutralization of XCL1 differently affects their analgesic properties, it is important to keep in mind that they also showed different pharmacokinetics and pharmacodynamics. Additionally it is known that chemokine receptors may create heterodimers with the opioid receptors MOR and DOR, which are involved in morphine analgesia (66–68), however, no such data are available for NOR. Some chemokines (e.g., CCL2, CCL5, CXCL12, CX3CL1) have already been known to interfere with the analgesic effects induced by morphine, DAMGO (selective ligand of MOR) and/or DPDPE (selective ligand of DOR) due to heterologous desensitization (67, 68). To date, there are no reports that have documented heterologous desensitization of NOR and chemokine receptors, but this mechanism could be one of the reasons why buprenorphine analgesia is improved by the blockade of XCR1. However, this topic requires further in-depth investigation. We previously proved that the blockade of other typical chemokine receptors, such as CCR1 by J113863 (21), CCR2 by RS504393 (22) and CCR5 by maraviroc (69), enhanced the analgesic properties of morphine and buprenorphine under neuropathy. What may be surprising is that a blockade of ITGA9 acts similarly, which enhances the analgesic effects of morphine and – to a greater extent – the latter buprenorphine. However, this is not a typical chemokine receptor. It was already shown that some integrins play an important role in the modulation of opioid signaling in trigeminal ganglion neurons. The β1 integrin subunit (present in ITGA9) was shown to have a high degree of colocalization with MOR in these neurons (70). These are particularly significant results since they indicate for the first time that ITGA9 may be an important potential target for the pharmacotherapy of neuropathy. Therefore, our results are particularly important because ITGA9 is not a typical chemokine receptor, which indicates the complexity of neuroimmunological processes occurring in neuropathy. We assume that one of the mechanisms underlying the beneficial properties of chemokine receptor blockers/antagonists is to prevent the anti-opioid effects of chemokines. The inhibition of neuroimmune imbalance may contribute to a potential therapeutic mechanism based on increasing the efficacy of opioids in neuropathic pain treatment.

5 Conclusion

Based on the current results, we can confirm that XCL1/XCR1 and XCL1/ITGA9 signaling play important roles in CCI-induced neuropathy; however, ITGA9 seems to be a more potent neuronal target and may serve as an innovative strategy for the polypharmacotherapy of neuropathic pain in combination with opioids. Moreover, our data suggest that minocycline, a widely used antibiotic that affects many intracellular pathways, can reveal high analgesic potential in neuropathy by influencing more immune factors than was previously thought, including XCL1. In view of the obtained data and current literature, we suggest that modulation of XCL1 signaling may serve as a promising target for combined therapy with opioids and indicates minocycline repurposing potential in the treatment of neuropathic pain. Moreover, both XCL1 receptors (XCR1 and ITGA9), seem to be important novel targets with beneficial properties for pharmacological intervention after nerve injury. Both used pharmacological tools (MIP-II protein, YA4) are available for experimental studies, but as far as we know, they are not drugs used in the clinics. That is why we need more studies, taking into consideration neuropathic pain of different etiologies, especially in the light of the knowledge that both, chemokines neutralization and neutralization/blockade of their receptors are successfully used as a treatment of varied diseases.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by Ethical Committee of the Maj Institute of Pharmacology of the Polish Academy of Sciences (LKE: 75/2017, 305/2017, 235/2020, 236/2021, 297/2021, 89/2021, 98/2022). According to the 3R policy, the number of animals was reduced to the necessary minimum.

Author contributions

AC, ER, AP, JB, KP, KC, GK and JM substantially contributed to the conception and design of the study and to the analysis and interpretation of the data. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.1058204/full#supplementary-material

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Chemokines and chemokine receptors as promising targets in rheumatoid arthritis

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Rheumatoid arthritis (RA) is an autoimmune disease that commonly causes inflammation and bone destruction in multiple joints. Inflammatory cytokines, such as IL-6 and TNF-α, play important roles in RA development and pathogenesis. Biological therapies targeting these cytokines have revolutionized RA therapy. However, approximately 50% of the patients are non-responders to these therapies. Therefore, there is an ongoing need to identify new therapeutic targets and therapies for patients with RA. In this review, we focus on the pathogenic roles of chemokines and their G-protein-coupled receptors (GPCRs) in RA. Inflamed tissues in RA, such as the synovium, highly express various chemokines to promote leukocyte migration, tightly controlled by chemokine ligand-receptor interactions. Because the inhibition of these signaling pathways results in inflammatory response regulation, chemokines and their receptors could be promising targets for RA therapy. The blockade of various chemokines and/or their receptors has yielded prospective results in preclinical trials using animal models of inflammatory arthritis. However, some of these strategies have failed in clinical trials. Nonetheless, some blockades showed promising results in early-phase clinical trials, suggesting that chemokine ligand-receptor interactions remain a promising therapeutic target for RA and other autoimmune diseases.

KEYWORDS

rheumatoid arthritis, chemokine, chemokine receptor, migration, leukocyte, blockade

1 Introduction

Chemokines are a family of small chemotactic cytokines (approximately 8-15 kDa). Chemokine ligand-receptor interactions control leukocyte migration during inflammation, promoting migration from the circulation into the extravascular space in inflamed tissues (1, 2). Nearly 50 chemokines have been identified in mammals (3), commonly formed by four conserved cysteine residues—the first and third and the second and fourth forming disulfide bridges. Chemokines are divided into four subclasses according to the position of the first two conserved N-terminal cysteine residues: CC-chemokines (β-chemokines), having adjacent

cysteine residues; CXC-chemokines (α-chemokines), having two cysteine residues separated by another amino acid; CX_3C-chemokines (δ-chemokines), having two cysteine residues separated by three other amino acids; and C-chemokines (γ-chemokines), with only the second and fourth cysteine residues (4, 5). The glutamate-leucine-arginine (ELR)-positive (CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, and CXCL8) but not ELR-negative CXC chemokines (CXCL4, CXCL4L1, CXCL9, CXCL10, and CXCL11) have three amino acid residues (Glu-Leu-Arg) before the first conserved cysteine residue. The ELR motif is important for angiogenesis (6, 7). Some chemokines are activated by matrix metalloproteinase-mediated (MMPs)-mediated proteolysis (8).

Chemokine receptors are expressed on the surface of immune cells. "Classical" chemokine receptors are G-protein-coupled transmembrane receptors (GPCRs) and induce cell migration, whereas "atypical" chemokine receptors (ACKRs) are not coupled to G proteins and regulate cell migration (9, 10). ACKRs scavenge chemokines to regulate chemokine gradients and dampen inflammation in a G protein-independent manner (3, 11, 12). Chemokine ligand-receptor interactions are presented in Table 1 (13).

The chemokine system may play a central role in rheumatoid arthritis (RA) pathogenesis. Several chemokines are highly expressed in the blood and inflammatory tissues, such as arthritic joints, of patients with RA. Furthermore, some genes encoding chemokine ligands and receptors have been reported as risk factors for RA development (14–42), and their expression is associated with clinical disease activity and severity (43–69). The regulation of immune cell recruitment into joints represents a major hallmark for therapeutic intervention, as the inhibition of the chemokine system can suppress the characteristic inflammation of RA, thereby halting its pathogenesis.

In this review, we summarize the pathogenic roles of chemokines and their receptors in RA. In addition, we provide evidence from recent human clinical trials using inhibitors of the chemokine system in RA and discuss the potential clinical benefits of chemokine blockade in patients with RA.

2 Rheumatoid arthritis

RA is an autoimmune disease characterized by autoantibody production, leading to the settlement of inflammatory processes with cytokine and chemokine production. This results in synovial inflammation, hyperplasia and swelling, cartilage and bone destruction and deformity, and systemic features, such as cardiovascular, pulmonary, and skeletal disorders (70).

Inflammatory cytokines, including interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α, play important roles in RA development. Biological agents, such as TNF-α and IL-6 inhibitors, have revolutionized RA therapies (71). However, approximately 50% of patients with RA are non-responders to these therapeutic approaches (72). Therefore, there is an ongoing need to identify novel targets and treatment strategies for RA.

Animal models of inflammatory arthritis have provided determinant information for the understanding of RA pathogenesis and development of RA therapeutics. Models such as type II collagen-induced arthritis (CIA) (73), collagen antibody-induced arthritis (CAIA) (74), K/BxN arthritogenic serum transfer model of arthritis (K/BxN) (75), and adjuvant-induced arthritis (AIA) (76) show RA-like arthritic phenotypes, including synovial hyperplasia with leukocyte infiltration and bone destruction. Furthermore, models of inflammatory arthritis and RA also show upregulated expression of chemokine ligands and their receptors in the serum, immune cells, and synovium (77–84). Thus, these animal models are useful for elucidating the pathogenic role of chemokines in RA.

2.1 Chemokines in RA

Various chemokines are highly expressed in the serum, synovial fluids (SFs), and synovial tissues (STs) of patients with RA compared with those of healthy donors (HD) (Table 2). For instance, the CC-chemokines CCL2, CCL5, CCL11, CCL13, CCL18, CCL19, CCL20, CCL22, CXC-chemokine CXCL2, CXCL5, CXCL8, CXCL9, CXCL10, CXCL11, CXCL12, CXCL13, and CXCL16 were increased in the serum and/or plasma of patients with RA compared with those of HD (43, 44, 46, 47, 54, 57, 85–90).

Peripheral blood mononuclear cells (PBMCs) derived from patients with RA highly express CCL2, CCL3, CXCL2, and CX_3CL1 compared to those derived from HD (91–93). These chemokines are differentially produced by different immune cells in patients with RA: T cells produce CCL3, CCL4, CCL5, and CXCL13 (93–96); B cells express CXCL9/10 (97); monocytes generate CCL2, CCL18, CCL19, and CX_3CL1 (93, 98, 99); macrophages express CCL25, CXCL4, CXCL7, and CX_3CL1 (93, 100, 101); dendritic cells (DCs) produce CCL17, CCL18, and CCL19 (102–104); and neutrophils generate CCL3 and CCL18 (103, 105, 106).

CC-chemokines are expressed in RA synovial endothelial cells (ECs) in different concentrations (high-abundance: CCL7, CCL8, CCL14, CCL16, CCL19, and CCL22; low-abundance: CCL1-3, CCL5, CCL10, CCL11, CCL12, CCL13, CCL15, CCL17, CCL18, CCL20, CCL21, CCL23, CCL24, CCL25, CCL26, CCL27, and CCL28 (107), whereas ELR^+ CXC-chemokines (CXCL1, CXCL2, CXCL3, CXCL5, and CXCL6) are expressed in the SFs of patients with RA (108). Additionally, various CC-chemokines (CCL1, CCL2, CCL3, CCL4, CCL5, CCL7, CCL11, CCL13, CCL15, CCL17, CCL18, CCL19, CCL20, CCL21, CCL25, and CCL28), CXCL8, CXCL9, and CXCL10 are also expressed in SFs, STs, and/or fibroblast-like synoviocytes (FLSs) derived from patients with RA (86, 91, 100, 102, 109–121).

Cartilage and chondrocytes from patients with RA express CCL2, CCL5, CCL13, CCL18, CCL25, CXCL1, CXCL8, CXCL10, and XCL1 (109, 118, 122, 123). In addition, osteoclasts (OCs) and OC progenitors (OCPs) from patients with RA produce CCL2, CCL3, CCL4, CCL5, CXCL9, CXCL10, and CX_3CL1 (124, 125).

Several chemokines (CCL3, CCL4, CCL5, CCL3L1, CCL21, CCL26, CXCL8, CXCL9, CXCL10, CXCL12, and CXCL13) have been reported as risk factors for RA development (11–25). Certain chemokines (CCL2, CCL5, CCL20, CCL23, CCL25, CXCL2, CXCL5, CXCL7, CXCL8, CXCL9, CXCL11, CXCL12, and CXCL13) are associated with disease activity and/or severity (40–58). Moreover, CCL23, CXCL9, CXCL10, CXCL11, and CXCL13 may be potential biomarkers for RA (48, 56).

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TABLE 1 The chemokines and chemokine receptors.

Name	Other names	Receptors
CC chemokine (β chemokine)		
CCL1	I-309, TCA3	CCR8
CCL2	MCP-1	CCR2, CCR4, ACKR1, ACKR2
CCL3	MIP-1α	CCR1, CCR5, ACKR2
CCL3L1	LD78β	CCR1, CCR3, CCR5, ACKR2
CCL4	MIP-1β	CCR5, ACKR2
CCL4L1	LAG-1	CCR5
CCL5	RANTES	CCR1, CCR3, CCR4, CCR5, ACKR2
CCL6	C-10, MRP-1	Unknown
CCL7	MARC, MCP-3	CCR2, CCR3, ACKR1, ACKR2
CCL8	MCP-2	Human: CCR1, CCR2, CCR3, CCR5, ACKR1, ACKR2 Mouse: CCR8, ACKR1, ACKR2
CCL9/10	MIP-1γ, MRP-2, CCF18	Unknown
CCL11	Eotaxin-1	CCR3, ACKR2
CCL12	MCP-5	CCR2
CCL13	MCP-4, NCC-1, Ckβ10	CCR2, CCR3, CCR5, ACKR1, ACKR2
CCL14	HCC-1, MCIF, Ckβ1, NCC-2, CCL	CCR1, ACKR1, ACKR2
CCL15	Leukotactun-1, HCC-2, MIP-5, NCC-3	CCR1, CCR3
CCL16	HCC-4, NCC-4, LEC (human only)	CCR1, CCR2, CCR5, ACKR1
CCL17	TARC, dendrokine, ABCD-2	CCR4, ACKR1, ACKR2
CCL18	PARC, DC-CK1, AMAC-1, Ckβ7, MIP-4	CCR8, ACKR6
CCL19	MIP-3β, ELC, Exodus-3, Ckβ11	CCR7, ACKR4
CCL20	MIP-3α, LARC, Exodus-1, Ckβ4	CCR6
CCL21	SLC, 6Ckine, Exodus-2, Ckβ9, TCA-4	CCR6, CCR7, ACKR4
CCL22	MDC, DC/β-CK	CCR4, ACKR1, ACKR2
CCL23	MPIF-1, Ckβ8, MIP-3, MPIF-1	Unknown
CCL24	Eotaxin-2, MPIF-2, Ckβ6	CCR3
CCL25	TECK, Ckβ15	CCR9, ACKR4
CCL26	Eotaxin-3, MIP-4α, IMAC, TSC-1	CCR3, CX3CR1
CCL27	CTACK, ILC, Eskine, PESKY, skinkine	CCR10
CCL28	MEC	CCR3, CCR10
CXC chemokine (α chemokine)		
CXCL1	Gro-α, GRO1, NAP-3	CXCR2, ACKR1
CXCL2	Gro-β, GRO2, MIP-2α	CXCR2, ACKR1
CXCL3	Gro-γ, GRO3, MIP-2β	CXCR2, ACKR1
CXCL4	PF-4	Unknown
CXCL4L1	PF4V1	Unknown
CXCL5	ENA-78	CXCR2, ACKR1
CXCL6	GCP-2	CXCR1, CXCR2, ACKR1
CXCL7	NAP-2, CTAPIII, β-Ta, PEP	CXCR2, ACKR1
CXCL8	IL-8, NAP-1, MDNCF, GCP-1	CXCR1, CXCR2, ACKR1

(Continued)

TABLE 1 Continued

Name	Other names	Receptors
CXCL9	MIG, CRG-10	CXCR3
CXCL10	IP-10, CRG-2	CXCR3
CXCL11	I-TAC, β-R1, IP-9	CXCR3, ACKR1, ACKR4
CXCL12	SDF-1, PBSF	CXCR4, ACKR3
CXCL13	BCA-1, BLC	CXCR5, ACKR1, ACKR4
CXCL14	BRAK, bolekine	Unknown
CXCL15	Lungkine, WECHE	Unknown
CXCL16	SRPSOX	CXCR6
CXCL17	DMC, VCC-1	Unknown
CX3C chemokine (δ chemokine)		
CX_3CL1	Fractalkine, Neurotactin, ABCD-3	*CX_3CR1*
C chemokine (γ chemokine)		
XCL1	Lymphotactin α, SCM-1α, ATAC	XCR1
XCL2	Lymphotactin β, SCM-1β	XCR1

This Table is modified from Miyabe Y et al., Targeting the Chemokine System in Rheumatoid Arthritis and Vasculitis. JMA J. 2020;3(3):182-192 (13). The authors have the right to use the original Table 1 in Reference 13 and got the permission from Japan Medical Association.

2.2 Chemokine receptors in RA

Multiple chemokine receptors as well as chemokines contribute to RA pathogenesis (Table 3). Polymorphisms in CCR2, CCR5, CCR6, and CCR7-encoding genes are considered risk factors for RA development (29–42). $CD4^+$ cells expressing CCR5 are increased in the blood of patients with active RA compared with that of patients with inactive RA patients and HD. Furthermore, $CD4^+$ cells expressing CX_3CR1 are decreased in patients with RA, and the $CD4^+$ $CD95^+$ T cell subset expressing CCR7 is associated with disease activity (63). In addition, CXCR4 and CXCL12 show higher expression in the serum and joints of patients with active RA than in those of HD and patients with RA remission. Moreover, the expression of these chemokines in the synovium has been correlated with disease score in patients with RA treated with TNF-α inhibitors (54, 55).

Chemokine receptors on T cells [CCR2, CCR4, CCR5, CCR6, CCR7, CXCR3, CXCR4, CXCR6, and CX_3CR1 (111, 126–129)], B cells [CCR5, CCR6, CCR7, CXCR3, CXCR4, and CXCR5 (130–132)], monocytes [CCR1, CCR2, CCR5, CCR9, CXCR4, and CX_3CR1 (33, 100, 133–137)], macrophages [CCR7, CCR9, and CXCR3 (100, 138)], and neutrophils [CCR1, CCR5, CXCR1, and CXCR2 (79, 106, 139)] were more highly expressed in patients with RA than in HD.

Stromal cells of patients with RA also express chemokine receptors. For instance, ECs express CCR7, CCR10, CXCR2, CXCR4, CXCR5, CXCR6, CXCR7, and ACKR1 (6, 140–147),

TABLE 2 The chemokine production in RA patients.

Source	Chemokine
Blood	CCL2, CCL5, CCL11, CCL13, CCL18-20, CCL22, CXCL2, CXCL5, CXCL8-13, CXCL16
PBMC	CCL2, CCL3, CXCL2, CX_3CL1
T cell	CCL3, CCL4, CCL5, CXCL13
B cell	CXCL9, CXCL10
Moncyte	CCL2, CCL18, CCL19, CX_3CL1
Macrophage	CCL25, CXCL4, CXCL7, CX_3CL1
Dendritic cell	CCL17, CCL18, CCL19
Neutrophil	CCL3, CCL18
Endothelial cell	CCL7, CCL8, CCL14, CCL16, CCL19, CCL22
Fibroblast-like synoviocytes	CCL1-5, CCL7, CCL11, CCL13, CCL15-21, CCL25, CCL28, CXCL1-3, CXCL5, CXCL6, CXCL8-10
Chondrocyte	CCL2, CCL5, CCL13, CCL18, CCL25, CXCL1, CXCL8, CXCL10, XCL1
Osteoclast	CCL2-5, CXCL9, CXCL10, CX_3CL1

TABLE 3 The expression of chemokine receptors in RA patients.

Cell	Chemokine receptor
T cell	CCR2, CCR4, CCR5, CCR6, CCR7, CXCR3, CXCR4, CXCR5, CXCR6, CX_3CR1
B cell	CCR5, CCR6, CCR7, CXCR3, CXCR4, CXCR5
Monocyte	CCR1, CCR2, CCR5, CCR9, CXCR4, CX3CR1
Macrophage	CCR7, CCR9, CXCR3
Neutrophil	CCR1, CCR5, CXCR1, CXCR2
Endothelial cell	CCR7, CCR10, CXCR2, CXCR4, CXCR5, CXCR6, CXCR7, ACKR1
Fibroblast-like synoviocytes	CCR2, CCR3, CCR5, CCR6, CCR9, CXCR2, CXCR4, CXCR6, ACKR6
Osteoclast	CCR1, CCR2, CCR4, CCR7, CCR9, CXCR2, CXCR3, CXCR4, CX_3CR1

whereas FLSs express CCR2, CCR3, CCR5, CCR6, CCR9, CXCR2, CXCR4, CXCR6, and ACKR6 (86, 100, 115, 148–150). OCs and OCPs express CCR1, CCR2, CCR3, CCR4, CCR7, CCR9, CXCR2, CXCR3, CXCR4, and CX_3CR1 (124, 125).

2.3 The pathological function of chemokine receptors in RA

Chemokines and their receptors control lymphocyte recruitment to inflamed joints in RA patients and animal models (Figure 1). In RA patients, the recruitment of T cells into the synovium is controlled by CCR4, CCR5, CXCR3, CXCR4, and CXCR6 (95, 97, 102, 126, 127, 129, 151–154). Inhibition of CCL2, CCL5, or CXCL12 suppresses Th1 cell migration *in vitro*, suggesting that these chemokines might promote Th1 cell recruitment to the RA synovium (129). $CD4^+$ T cells of patients with RA treated *in vitro* with anti-CCL22 antibodies differentiate into regulatory T cells (Tregs) *via* STAT5 activation (85). In SCID mice implanted with human RA synovium, recruitment of $CD4^+$ $CD28^-$T cells, resembling effector memory T cells, is controlled by CCL5 and CXCL12 (127). CCR6 promotes Th17 cell recruitment into the inflamed joint in SKG arthritic mice (155), myostatin-deficient (KO) mice, TNF-α transgenic (Tg) arthritic mice (156), and chemotactic ability of Th17 cells derived from patients with RA *in vitro* model (155, 157). In addition, the CCR4 blockade suppresses Th17 cell migration to the arthritic joints in CIA mice (84). The CIA model also shows joint infiltration of $CCR6^+$ type 3 innate lymphoid cells (iLC3s), which highly express IL-17A and IL-22. Furthermore, the number of $CCR6^+$ iLC3s in the SF of patients with RA is correlated with disease activity (158).

Follicular helper T (Tfh) cells contribute to the formation and maintenance of germinal centers (GC). $CXCR5^+$ Tfh cells are increased in the blood of patients with RA and CIA mice. Furthermore, the number of these cells correlates with the levels of clinical RA markers, such as C-reactive protein, rheumatoid factor, and erythrocyte sedimentation rate (159). In transwell experiments, the CXCL13/CXCR5 axis showed chemotactic activity in B cells of patients with RA (130). CXCR5 KO mice are resistant to CIA development; B cell-specific CXCR5 deficiency leads to mild arthritis with impaired germinal center (GC) response and antibody production, whereas T cell-specific CXCR5 deficiency promotes resistance to arthritis development by impaired GC response, antibody production, and inflammatory cytokine response (160).

CCL19, CXCL12, and CXCL13 levels in the serum of patients with RA are associated with the clinical response to rituximab (89). In addition, these chemokine levels in the SFs of patients are also correlated with both the number of $CD19^+$ $CD24^{hi}$ $CD27^+$ B cells and disease activity and severity (161). The CCL20/CCR6, CXCL12/CXCR4, and CXCL13/CXCR5 axes regulate B cell migration into RA SFs (130, 161, 162), whereas the CCL19/CCR7, CCL20/CCR6, CCL21/CCR7, and CXCL12/CXCR4 axes regulate B cell recruitment into the RA synovium (130, 131).

CCL2 and CXCL8 enhance neutrophil chemotactic ability in cells from patients with RA, and CCR2 KO mice are resistant to AIA model through the suppression of CCL2/CCR2-induced neutrophil recruitment (163). CCL3 expression is associated with the neutrophil number in the SFs from patients with RA (106). Furthermore, the chemotactic activities of CCL4 and CCL5 are also correlated with the number of neutrophils in the SFs from patients with RA (94). An amino-terminal-modified methionylated form of CCL5 (Met-RANTES) antagonized the binding of CCL3 and CCL5 to their receptors CCR1 and CCR5, respectively, and the blockade inhibited arthritis in AIA rats *via* the suppression of neutrophil and macrophage migration into the joints (164).

ELR-positive CXC chemokines (CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, and CXCL8) regulate neutrophil migration and angiogenesis *via* the receptor CXCR2 (6). CXCL5 expressed in RA SFs promotes neutrophil recruitment to EC *in vitro* (165). CXCL1 and CXCL5 induce neutrophil migration into the articular cavity of AIA mice, and chemotaxis is inhibited by the blockade of CXCR1/CXCR2 with repertaxin (79). CXCL1 and CXCL8 induce neutrophil chemotaxis *in vitro*, which is also inhibited by the blockade of CXCR1/CXCR2 and DF 2162, the later inhibiting neutrophil recruitment in zymosan-induced arthritis in mice and AIA in rats (166, 167). Furthermore, *in vitro*, the ligand for CXCR1, CXCR2, CXCL2 enhances murine neutrophil migration, and the CXCL2-neutralizing antibody inhibits migration (139). Both CXCR2 and CCR1 are expressed in mice neutrophils, and their abrogation attenuates inflammatory arthritis in K/BxN mice (168). Recent *in vivo* imaging of joints showed that CCR1 promotes neutrophil crawling on the joint endothelium, whereas CXCR2 amplifies late neutrophil recruitment and survival in the joint (169).

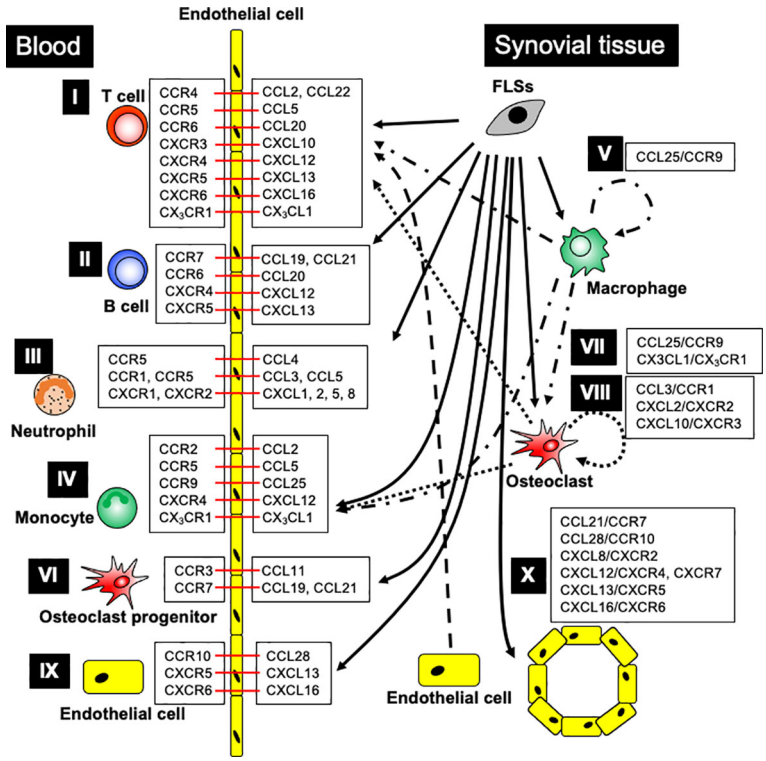

FIGURE 1
The major contribution of chemokine ligand-receptor interactions in RA patients. I. T cell recruitment: FLSs generate CCL2, CCL5, CCL20, CXCL13, and CXCL16; OCs produce CXCL10; ECs release CCL22; monocytes, macrophages, and OCs produce CX_3CL1, promoting T cell recruitment into the arthritic joints through the indicated chemokine receptors. II. B cell recruitment: FLSs generate CCL19-21, CXCL12, and CXCL13, enhancing B cell recruitment into arthritic joints through the respective chemokine receptors. III. Neutrophil recruitment: FLSs generate CCL3-5, CXCL1, CXCL2, CXCL5, and CXCL8, leading to neutrophil recruitment into arthritic joints through the indicated chemokine receptors. IV. Monocyte recruitment: FLSs generate CCL2, CCL5, CCL25, and CXCL12; synovial macrophages produce CCL25 and CX_3CL1; OCs produce CCL2, CCL5, and CX_3CL1, promoting monocyte recruitment into the arthritic joints through chemokine receptor signaling. V. Synovial macrophage development: FLSs and macrophages generate CCL25, which promotes monocyte differentiation into macrophages. VI. Osteoclast progenitor recruitment: FLSs generate CCL11, CCL19, and CCL21, while ECs generate CCL19, leading to OCP recruitment into arthritic joints through the indicated chemokine receptors. VII. Osteoclast differentiation: FLSs and macrophages generate CCL25, and synovial macrophages and OCs generate CX_3CL1, promoting osteoclast differentiation through chemokine receptors. VIII. Osteoclastogenesis: FLSs, T cells, and neutrophils generate CCL3; FLSs generate CXCL2; and OCs generate CXCL10, ensuring osteoclastogenesis through the indicated chemokine receptors. IX. Endothelial cell recruitment: FLSs generate CCL28, CXCL13, and CXCL16, stimulating endothelial cell recruitment into arthritic joints through the indicated chemokine receptors. X. Angiogenesis: FLSs generate CCL21, CCL28, CXCL8, CXCL12, CXCL13, and CXCL16, supporting angiogenesis through indicated chemokine receptors. Black arrow indicates chemokine production, and red bar shows chemokine ligand-receptor interaction.

CXCL9 blocking peptide decreases neutrophil recruitment into the joints of AIA mice (170).

In vitro approaches further clarified the role of some of these chemokines in RA samples. CCL2/CCR2 and CCL5/CCR5 enhance monocyte chemotaxis (171). CCL3, highly expressed in RA SFs, enhances macrophage chemotaxis (172). CCL25 induces the chemotactic activity of monocytes and differentiation into macrophages (100). CCR9 abrogation suppressed $CD11b^+$ cell migration into joints in a CIA model (82). The CXCL12/CXCR4 axis promotes monocyte migration into the joints of RA ST-transplanted SCID mice (173). Furthermore, increased CX_3CL1 expression in SFs of RA patients induced monocyte chemotaxis *via* CX_3CR1 *in vitro* (93).

Increased OC differentiation and activity lead to bone loss and joint destruction in patients with RA. CCL3 enhanced osteoclastogenesis *via* OC migration and activation in the AIA rat model (174). CCL11/CCR3 induced OCP migration and bone resorption *in vitro* (175). CCL19 and CCL21, increased in RA SFs and serum, and their receptor CCR7, expressed in murine OCPs. These chemokines did not affect OC differentiation but promoted OC migration and increased OC resorption activity *in vitro* and *in vivo* (176). The CCL25/CCR9 axis initiates the transformation of OCPs into mature OCs *in vitro* (100). CXCL2 promotes monocyte recruitment and osteoclastogenesis in RA samples *in vitro*, as well as in mouse bone marrow-derived macrophages (90, 177). CXCL10 KO and CXCR3 KO in mice ameliorated arthritis in CAIA model by suppressing macrophage and T cell accumulation in arthritic joints. In addition, CXCL10 and CXCR3 inhibition decreased osteoclastogenic cytokine levels in the serum and spleen of CAIA (154). Furthermore, *in vitro*, CX_3CL1/CX_3CR1 regulates monocyte, DC, and OCP differentiation into osteoclasts (125, 178).

Several chemokines contribute to cartilage damage in arthritic joints. For instance, interferon-γ (IFN-γ) enhances CCL13 expression, inducing RA FLS proliferation in the cartilage of patients with RA *in vitro* (123). CXCL12, which induces MMP-3 production in chondrocytes *in vitro*, is also highly expressed in the SFs of patients with RA (179). CCL5 induces both MMP-1- and MMP-13-mediated collagen degradation in the SFs of patients with RA (180). In addition,

CXCR4-CXCL12 signaling increased both MMP-9 and MMP-13 production in human chondrocytes *in vitro* (181).

Chemokine receptors are also expressed in stromal cells, although their functions remain unknown. Angiogenesis is determinant for RA pathogenesis, namely for synovial proliferation and pannus formation (182). CCL21, *in vitro*, induces human microvascular ECs angiogenesis and migration *via* CCR7, suggesting that the CCL21/CCR7 axis may contribute to angiogenesis in RA (140). The CXCL12/CXCR4 axis also showed angiogenic activity in RA SFs in Matrigel *in vivo* (145) and the CXCL13/CXCR5 axis facilitated EC migration and angiogenesis in CIA mice (142). CCL28 and CCR10, highly expressed in RA synovium, regulate angiogenesis by EC recruitment, and CCL28 blockade inhibits EC migration and capillary formation (141). The CXCL16/CXCR6 axis promoted chemotactic and angiogenic activity in human umbilical vein ECs (HUVEC), which is a cell line (147). The CXCL12/CXCR4 and CXCL12/CXCR7 axes promote angiogenic activity in HUVEC, contributing to RA angiogenesis. CXCR7 is also expressed on ECs in the RA synovium. Furthermore, CXCR7 blockade ameliorated arthritis in CIA mice by suppressing angiogenesis (183).

FLS-producing inflammatory cytokines and degenerative enzymes initiate synovial inflammation and joint damage in RA (184). Several chemokines (CCL11, CCL25, CXCL4, CXCL7, CXCL10, and CX_3CL1) mediate the FLS chemotactic activity in RA *in vitro* models (82, 86, 100, 185, 186). In addition to this chemotactic activity, some chemokines (CCL2, CCL5, CCL18, CCL20, and CXCL12) increase the production of IL-6, CCL2, CXCL8, MMP-3, and COX-2 from FLS of patients with RA *in vitro* models (100, 115, 149, 150). The CX_3CL1/CX_3CR1 axis enhances MHVEC migration *in vitro* and angiogenesis in Matrigel *in vivo* (187). The CCL21/CCR7 axis induces VEGF and angiotensin 1 (Ang1) production in RA fibroblasts and CXCL8 and Ang1 production in macrophages (119).

3 Targeting the chemokine system in RA

In general, the signaling of "classical" G protein-coupled chemokine receptors is mediated by activating pertussis toxin-sensitive Gi-type G proteins. Activated G proteins regulate multiple downstream signaling cascades, such as the JAK/STAT pathway and PI3K phosphorylation (188). In contrast, signaling of "atypical" chemokine receptors is independent of G proteins and remains somewhat unclear. In this section, we provide an update on arthritis animal models and clinical trials using drugs targeting chemokines and their receptors while discussing their potential as therapeutic targets.

3.1 Targeting the chemokine system in animal models of inflammatory arthritis

Animal experiments are useful in the testing and development of new therapeutic agents and treatment approaches. Some chemokine ligands and receptors in KO, Tg, and naturally mutant mice are used as arthritic models (Table 4). For instance, CCL3 KO mice showed milder clinical and histopathological scores in the CAIA model (189), whereas *plt*/*plt* mice, a naturally occurring CCL19 and CCL20 mutant strain, also showed mild arthritis in CIA model (190). CXCL10 KO mice showed mild arthritis in CAIA model through the inhibition of macrophage and T-cell migration into the synovium (154). CXCL14 Tg mice showed exacerbated autoimmune arthritis in a CIA model, caused by an excessive immune response against type II collagen (191).

CCR2 KO in the DBA/1J background exacerbated the CIA model because of the enhanced Th17 cell response and increased autoantibody production (192, 193). CCR2 deficiency in IL-1Ra KO mice enhanced neutrophil migration (194). Furthermore, CCR2 deficiency in DBA/1J caused severe arthritis in CIA with cutaneous *M. avium* infection (195). In contrast, CCR2 KO in C57BL/6 mice showed decreased neutrophil infiltration into arthritic joints in AIA model (163). CCR4, CCR6, CCR7, CCR9, CXCR5, and CXCR6 deficiency ameliorated arthritis in CIA mice by suppressing the migration of Th17 cells (CCR4), DC (CCR7), and $CD11b^+$ splenocytes (CCR9) (82, 84, 160, 190, 196, 197). CCR5 KO mice showed conflicting results, with a reduced clinical score in CIA model in one study (198) and no changes in others (193). Although CCR6 KO mice were resistant to CIA model, the deficient CCR6 did not improve in an animal model of K/BxN and TNF-α Tg mice (196). In addition, CCR7 inhibition decreased autoantibody production and T cell proliferation in AIA mice (199). CXCR3 KO mice showed mild arthritis in CAIA model *via* the inhibition of both macrophage and T cell migration into the synovium (154). CXCR4-conditional KO in T cells reduced arthritic symptoms in CIA mice by inhibiting T cell migration (200). T cell- or B cell-specific CXCR5 KO mice, as well as fully CXCR5 KO mice, were resistant to both CIA and AIA models (160). CXCR6 KO mice showed resistance to K/BxN serum-induced arthritis and CIA model (147).

The blockade of a single chemokine (CCL2, CCL5, CCL24, CXCL8, CXCL9, CXCL10, and CXCL16) or chemokine receptor (CCR2, CCR5, CCR9, CXCR1, CXCR2, CXCR3, and CXCR4) demonstrated preventive and/or therapeutic effects in distinct animal models (Table 5). For instance, monomeric mutant CCL2, but not CCL5 mutant (^{44}AANA^{47}), ameliorated arthritis in AIA rats (201). Met-RANTES, which antagonizes the binding of CCL5 to CCR1 and CCR5, reduced the arthritic score and decreased macrophage infiltration into STs in CIA mice and AIA rats (83, 164). The anti-CCL5 antibody, but not the anti-CCL3 antibody, reduced the arthritic score in AIA rats (202). CCL24 blockade ameliorated arthritic symptoms in rats with AIA model (203). Anti-CXCL5 antibody ameliorated arthritis in the AIA rat model by inhibiting neutrophil migration (204). CXCL8-based decoy proteins prevented CXCR1 and CXCR2 signaling in neutrophils and ameliorated arthritis in AIA mice (205). The CXCL9 blocking peptide, which competes with CCL3 and CXCL6 binding, reduced neutrophil migration in AIA mice (170). Monoclonal bispecific antibodies against TNF-α and CXCL10 attenuated arthritis symptoms in mice by inhibiting CXCL10-mediated $CD8^+$ T cell migration (206). Anti-CXCL16 antibody attenuated arthritis in CIA mice by suppressing T cell recruitment (126). Anti-CX_3CL1 antibody decreased arthritic symptoms by inhibiting osteoclast migration into the synovium of CIA mice (207).

Regarding chemokine receptors, CCR1 antagonist J-113863 decreased the arthritic score but did not affect auto-antibody production in CIA mice (208). Small-molecule inhibitors of CCR2 combined with Methotrexate (MTX) treatment reduced both the arthritic score and bone destruction *via* the suppression of OC activity

TABLE 4 The phenotypes of chemokine ligands and receptors gene-modified mice in RA models.

Gene	RA model and phenotypes
CCL3	CCL3 KO mice (C57BL/6 background) showed a mild arthritis and decreased serum amyloid P level in CAIA
CCL19, CCL21	*plt*/*plt* mice, a naturally occuring CCL19 and CCL21 mutation strain (B6N.DDD-plt/NknoJ), showed a mild arthritis in CIA
CXCL10	CXCL10 KO mice (C57BL/6 mice) showed mild arthritis, and decrease of macrophage and T cell accumulation in arthritic joints in CAIA
CXCL14	CXCL14 Tg mice (C57BL/6 background) showed severe arthritis and increased T cell and B cell response in CIA
CCR2	CCR2 KO mice (C57BL/6 background) showed decrease of neutrophil recruitment into the joints in AIA
	CCR2 KO mice (DBA/1J background) showed severe arthritis in CIA and increase of Th17 cell population, autoantibody production, and neutrohpil infiltration into joints in CIA
	CCR2 KO mice (DBA/1J, but not BALB/c background) developed arthritis than WT mice in CIA with cutaneous *M. avium* infection
	CCR2 KO mice (DBA/1J background) showed severe arthritis and elevated autoantibody production in CIA
	CCR2 KO mice (DBA/1J background) showed severe arthritis in CAIA and enhanced protease activation from monocytes and neutrophils in CAIA
	CCR2 deficiency promoted spontaneous arthritis development and neitrophil infiltration into joints in IL-1R antagonist KO mice (BALB/c background)
CCR4	CCR4 KO mice (C57BL/6 background) showed mild arthritis via inhibition of Th17 cell expansion in CIA
CCR5	CCR5 KO mice (DBA/1J background) showed mild arthritis and decrease of autoantibody production in CIA
	CCR5 KO mice (DBA/1J background) showed comparable severity with WT mice in CIA
CCR6	CCR6 KO mice (C57BL/6 background) showed mild arthritis and decrease of autoantibody production in CIA
	CCR6 KO mice (C57BL/6 background) showed comparable severity with WT mice in K/BxN
	CCR6 deficiency did not affect the arthritis development in spontaneous RA model, human TNF-α Tg mice (C57BL/6 background)
CCR7	CCR7 KO mice (C57BL/6 background) showed a completely resistance to arthritis and decrease of autoantibody production in CIA, via inhibition of DC chemotactic ability
	CCR7 KO mice (C57BL/6 background) showed mild arthritis, decrease of autoantibody production and T cell proliferation in AIA
CCR9	CCR9 KO mice (C57BL/6 background) showed mild arthritis and inhibition od CD11c-positive splenocyte migration in CIA
CXCR3	CXCR3 KO mice (C57BL/6 mice) showed mild arthritis, and decrease of macrophage and T cell accumulation in arthritic joints in CAIA
CXCR4	CXCR4 KO mice (DBA/1 background) showed resistance to arthritis in CIA
CXCR5	CXCR5 KO mice (C57BL/6 background) showed mild arthritis, decrease of autoantibody production and T cell proliferation in AIA
	CXCR5 null KO mice (C57BL/6 background) showed completely resistance to arthritis and decrease of autoantibody production, but did not affect leukocyto migration into joints in CIA
	B cell-specific CXCR5 KO mice (C57BL/6 background) showed mild arthritis and decrease GC formation in CIA
	T cell-specific CXCR5 KO mice (C57BL/6 background) showed completely resistance to arthritis and decrease GC formation in CIA
CXCR6	CXCR6 KO mice (C57BL/6 background) showed resistance to arthritis and decrease leukocyto recruitment in K/BxN
	CXCR6 KO mice (C57BL/6 background) showed resistance to arthritis and impaired cytokine polarization in T cells in CIA

in CIA mice (209). Compound 22, a CCR4 inhibitor, ameliorated arthritis by reducing Th17 cell migration into the joints of CIA mice (84). A CCR5 antagonist (maraviroc) decreased the arthritic score and $CD8^+$ T cell activation in CIA mice (210); however, other CCR5 antagonists (MCC22) did not change the arthritic score in K/B.g7 arthritic mice (211). In CIA monkeys, a CCR5 antagonist (SCH-X) reduced arthritic score but did not change biomarker expression (212). CCR9 antagonist (CCX8037) reduced the arthritic score by inhibiting $CD11b^+$ splenocyte recruitment into joints in CIA mice (82). The CXCR1/CXCR2 antagonist (SCH563705), but not the CCR2 antagonist (MK0812), reduced the arthritic score in CAIA mice (213). Furthermore, the blockade of CXCR1 and CXCR2 (DF 2162) ameliorated arthritis by inhibiting neutrophil migration in AIA rats (167). Anti-CXCR3 antibody reduced the arthritic scores and T cell influx into joints in adaptive transfer-induced arthritic rats (214). The CXCR3 antagonist (AMG487) contributed to the modulation of the Th17/Treg cell balance in CIA mice (215). Other CXCR3 antagonists, such as SCH 546738 and JN-2, also treated arthritis in CIA mice (216, 217). A CXCR4 antagonist (T140) reduced the arthritic score and auto-antibody production in CIA mice (218).

3.2 Clinical trials of chemokine-targeted therapy in human RA

Based on valuable animal research, various therapeutic agents against chemokine ligands or their receptors have been developed and tested in patients with RA (219). However, several chemokines or

TABLE 5 The therapeutic effect of chemokine-targeted agents in RA models.

Target	Therapeutic effect
CCL2	Recombinant monomeric mutant CCL2 (p8A-MCP-1) protein reduced arthritic score and cytokine production in AIA rat
CCL3	Anti-CCL3 antibody did not affect arthritic score in AIA rat
CCL5	Met-RANTES reduced arthritic score in CIA mice
	Met-RANTES reduced arthritic score and macrophage infiltration into STs in AIA rat
	Recombinant CCL5 mutant (^{44}AANA^{47}) protein did not affect arthritic score in AIA rat
	Anti-CCL5 antibody reduced arthritic score in AIA rat
CCL24	Anti-CCL24 antibody reduced arthritic score in AIA rat
CXCL5	Anti-CXCL5 antibody reduced arthritic score and inflammatory cytokine production in AIA mice
CXCL8	CXCL8-based decoy protein reduced arthritic score and neutrophil recruitment in AIA mice
CXCL9	Antagonistic CXCL9 fragment (74–103) reduced arthritic score, neutrophil influx and cytokine production in AIA mice
CXCL10	Bispecific antibody against CXCL10 and TNF-α reduced arthritic score and CD8+ T cell migration in TNF-α Tg mice and K/BxN mice
CXCL16	Anti-CXCL16 antibody reduced arthritic score in CIA mice
CX_3CL1	Anti-CX_3CL1 antibody decreased arthritic symptoms by inhibition of osteoclast migration into synovium in CIA mice
CCR1	CCR1 antagonist (J-113863) reduced arthritic score, but not autoantibody production in CIA mice
CCR2	Small-molecular inhibitor of CCR2, comnined with MTX treatment reduced arthritic score and bone loss in CIA mice
	Anti-CCR2 antibody (MC) reduced arthritic score and monocyte population in blood in CIA mice
	CCR2 antagonist (MK0812) did not affect arthritic score in CAIA mice
CCR4	CCR4 antagonist (Compound 22) reduced arthritic score and decrease Th17 cells in joints in CIA mice
CCR5	CCR5 antagonist (maraviroc) decreased arthritic score and CD8+ T cell activation in CIA mice
	CCR5 antagonist (SCH-X) reduced arthritic score,but did not affect biomarkers expression in CIA monkey
	CCR5 antagonist (MCC22) did not affect arthritic score in K/B.g7 arthritic mice
CCR9	CCR9 antagonist (CCX8037) reduced arthritic score and inhibited CD11b-positive splenocyte influx into joints in CIA mice
CXCR1/ CXCR2	CXCR1/CXCR2 antagonist (SCH563705) reduced arthritic score, inflammatory cytokine production and neutrophil frequency in blood inCAIA mice
	CXCR1/CXCR2 inhibitor (DF 2162) reduced arthritic score, cytokine production and neutrophil influx in AIA rat
CXCR3	Anti-CXCR3 antibody reduced arthritic score and T cell influx into joints in adaptive transfer-induced arthritic rat
	CXCR3 antagonist (AMG487) reduced arthritic score and modulated Th17/Treg cell balance in CIA mice
	CXCR3 antagonist (SCH 546738) reduced arthritic score in CIA mice
	CXCR3 antagonist (JN-2) reduced arthritic score and cytokine production in CIA mice
CXCR4	CXCR4 antagonist, 14-mer peptide T140 reduced arthritic score and autoantibody production in CIA mice

chemokine receptor inhibitors have failed to show positive results in clinical trials (Table 6). For instance, the CCL2-blocking antibody (ABN912) did not promote clinical improvements in patients with RA (220). In addition, the CCR2 antibody (MLN1202) failed at phase IIa of the clinical trial due to the reduction of monocyte levels and no changes in synovial biomarkers (221).

Animal experiments have suggested CCR5 as a good RA therapeutic candidate (198, 210–212, 222). However, reports showed that CCR5 is not determinant for RA development (223–225), and all clinical trials using CCR5 antagonists failed (226–228).

A phase II clinical trial with a CCR1 antagonist (CCX354-C) showed good efficacy in the ACR20 response in patients with abundant CCX354-C in plasma but not in those with poor CCX354-C plasma concentration. However, ACR responses did not significantly vary between placebo- and CCX354-C-treated patients (229). CCR1 antagonist (MLN3897, 10 mg, once, daily) combined with MTX had no discernible effects on the disease, despite high MLN3897 plasma concentrations and receptor occupancy of the therapeutic target (230). Another trial using a CCR1 antagonist (CP-481,715) and MTX also failed in phase II (231). CCR1 ligands, CCL3 and CCL5, can bind to other chemokine receptors, CCR3, CCR4, and CCR5 (Table 1). Therefore, even though CCR1 on leukocytes might be inhibited, other chemokine receptors can still promote leukocyte recruitment into inflamed joints in RA. This could explain the failures in the use of CCR1 as a therapeutic target.

In contrast, the combination of CXCL10 blocking antibody (MDX-1100) and MTX showed a mild therapeutic effect on the ACR20 response; however, ACR50, ACR70, and EULAR were not

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TABLE 6 The chemokine ligands and receptors-targeted therapy in RA patients.

Target/ Drug type	Drug name/ Synonym	Released year StudyEfficacy	Study outcome	Adverse event	Inhibitory mechanism
CCL2/ Antibody	ABN912/Not Available	2006Pgase Ib Not effective	There was no detectable clinical benefit of ABN912 compared with placebo.	There were no differences in the number of nature of Aes between ABN912-treated and placebo-treated patients.	The neutralizing anti-CCL2 monoclonal antibody prevents binding of the CCL2 and its receptorCCR2.
CXCL10/ Antibody	MDX1100/ Eldelumab, BMS-936557	2012Phase II Effective	The ACR20 response was 54% (MDX-1100 and MTX) and 17% (placebo and MTX) at weeks 12. However, ACR50, ACR70 and EULAR good responses were not significantly difference between MDX-1100- and placebo-treated patients.	51.5% of MDX-1100-treated and 30.3% of placebo-treated patients experienced AE. Serious AEs were not reported in MDX-1100-treated patients.	This neutralizing anti- CXCL10 monoclonal antibody binds to CXCL10, but not other CXCR3 ligands, CXCL9 or CXCL11.
CX_3CL1/ Antibody	E6011/ Quetmolimab	2023Phase III Effective	The ACR20 response rates in E6011 200 mg and 400/200 mg were maintained 50-70% during the extension phase, and the ACR20 response rates in 100 mg were fluctuated but were maintained >45% at most time points. The ACR50 response rates in 200 mg and 400/200 mg ware maintained 25-45% during extension phase, and the ACR20 response rates in 100 mg were fluctuated but were maintained >20% at most time points. The ACR70 response rates in 400/200 mg ware maintained 15-35% during extension phase, and the ACR20 response rates in 100 mg and 200 mg were fluctuated but were maintained >10% at most time points.	The incidence of AE and TEAEs were similar across the four treatment groups (AE, 97.9% in placebo, 100.0% in E6011 200 mg, 100% in 200 mg, and 98.8% in 400/200 mg groups, and TEAE, 55.3% in placebo, 57.7% in 100 mggroup, 58.0% in 200 mg group, and 54.3% in 400/200 mg group). The incidence of serious AE was 10.7% overall.	This neutralizing anti- CX_3CL1 monoclonal antibody prevents binding of the CX_3CL1 and its receptor CX_3CR1.
CCR1/ Small molecule	CCX354-C/ Not Available	2013Phase IINot Effective	The ACR responses were not significantly difference between placebo and CCX354-C at week 12. Only CCX354-C abundant patients in plasma showed good ACR20 response.	39% of CCX354-C (200 mg once daily)- treated, 57% of CCX354-C (100 mg twice daily) and 49% of placebo-treated patients experienced TEAR. The drug-related serious TEAE was not reported.	This orally-active small molecule is a potent and selective antagonist of CCR1.
	CP-481,715/ Not Available	2010Phase IINot Effective	The ACR20 response was 34.0% (CP- 481,715 with MTX) and 47.9% (placebo with MTX) at week 6. Not significantly difference.	Not shown.	This small molecule binds CCR1 and inhibits chemotaxis activity of CCL3, CCL5, CCL7, CCL8, CCL14, CCL15 and CCL23.
	MLN3897/ AVE-9897, GSK2941266	2009Phase IIa Not effective	The ACR20 response was 35% (MLN with MTX) and 33% (placebo with MTX).	The rates of drug-related AEs (12% of both groups) and serious AEs (1% of MLN3897 and 2% of placebo) were no notable differences between MLN3897- and placebo treated patients.	This oral small molecule is CCR1 antasonist.
CCR2/ Antibody	MLN1202/ Plozalizumab, hu1D9	2008Phase IIa Not effective	Monocyte levels was decreased, but not synovial biomarkers (clinical response rates were similar between MLN1202 and placebo).	One patients (0.5 mg/kg MLN1202) experienced a serious AE (pericarditis) at day 42 after the last dose of study drug.	Anti-CCR2 antagonistic antibody prevents binding of the CCL2 and its receptor CCR2.

(Continued)

TABLE 6 Continued

Target/ Drug type	Drug name/ Synonym	Released year StudyEfficacy	Study outcome	Adverse event	Inhibitory mechanism
CCR5/ Small molecule	Maravinoc/ Celsentri, Selzentry, UK 427857	2012Phase IIa Not effective	Maravinoc(UK-427,857) showed no significant difference in ACR20 responders (23.7%: maraviroc and 23.8: placebo) atweek 12.	55% of Maraviroc-treated patients showed TEAE such as constipation (7.8%), nausea (5.2%) and fatigue (3.9%). The serious AEs were none.	This orally bioavailable small molecule is a potent and selective antagonist of CCR5.
	SCH351125/ Ancriviroc, SCH-C	2010Phase Ib Not effective	No improvement was observed by medication (3 patients did not complete, 9 patients caused serious phenotype).	20 patients received SCH351125, and 3 patients did not complete the study due to AE.	This orally bioavailable small molecule is an antagonist of CCR5.
	AZD5672/Not Available	2010Phase IIb Not Effective	The ACR response was 35% (AZD5672) and 38% (placebo).	23% of AZD5672-treated and 12% of placebo- treated patients experienced infection-related AE.	This orally bioavailable small molecule is a potent and selective antagonist of CCR5.

significantly different between the treatment and placebo groups. The frequency of adverse events (AEs) in MDX-1100-treated patients was higher than that in placebo-treated patients; but any MDX-1100-treated patients experienced serious AEs (232). Phase III of the clinical trial using MDX-1100 has not yet been launched.

The clinical trial using CX3CL1 blocking antibody (E6011, 200-400 mg) was effective for ACR20, ACR50 and ACR70 responses in RA patients with an inadequate response to MTX. The incidence of AEs and treatment-related AEs (TEAEs) were similar across the four treatment groups (placebo, E6011 100 mg, 200 mg, and 400/200 mg groups). Nonetheless, the incidence of serious AEs was similar between E6011- and placebo-treated patients. AEs such as nasopharyngitis, upper respiratory tract infections, bronchitis, pharyngitis, stomatitis, and back pain occurred in over 5% of the overall patients (233). However E6011 was no clear benefit in the ACR20 response rate was observed in RA patients with an inadequate response to biological DMARDs (234).

Chemokine-targeted therapy encompassed several AEs; however, the overall incidence of AEs was 40-50%, and the incidence of serious AE was 0-5% in chemokine-targeted therapies (Table 6). These numbers increased to an AE incidence of 60-80% and serious AEs of 5-25% in patients treated with anti-IL-6R antibody, tocilizumab (235–237). Furthermore, AE incidence was 50-70%, and serious AE incidence was 5-10% in trials using anti-TNF-α antibody, infliximab (238–240). These clinical findings suggest that chemokine-targeted therapy is safer for patients with RA than cytokine-targeted therapy.

In addition to the above-mentioned blockade agents, other inhibitors of chemokine ligands or their receptors have demonstrated therapeutic effects on arthritis in RA models. Thus, these chemokine ligands and respective receptors may be promising targets for new RA therapies.

4 Conclusion

In this review, we summarize the functional roles of chemokine ligand–receptor interactions in arthritic joints of animal models and RA patients. Although several inhibitors of chemokines and/or their receptors have shown therapeutic effects in animal models of arthritis and clinical trials of patients with RA, limited therapeutic effects have been reported, suggesting that chemokine-targeted therapy still requires improvement. In targeting chemokine receptors, the choice of the most relevant receptor and ensuring high receptor occupancy at all times might be the key to therapeutic effects. In addition, inhibition of a single chemokine alone may not be sufficient to completely suppress leukocyte migration due to the functional overlap between chemokine systems. Therefore, the combined targeting of multiple chemokines and/or their receptors may be a more effective approach for human RA. Our previous study in animal models demonstrated that broadly cross-reactive chemokine-blocking antibodies for CXCR2 ligands dramatically ameliorated inflammatory arthritis compared with inhibition with antibodies against a single chemokine (241).

Further understanding of the importance of different chemokines at different stages of RA is required for the development of drugs that effectively target the system. We have previously developed an *in vivo* imaging technique to fully dissect the functional roles of chemokines and their receptors in inflamed joints in animal models (242). Interestingly, CXCR2 and ACKR1 are required for neutrophil apoptosis in the joint space, whereas the classical C5aR1 and atypical C5a and C5aR2 receptors are required for neutrophil apoptosis in the joint (146, 169). Altogether, the development of effective inhibitors of chemokines and their receptors has untapped therapeutic potential in RA.

Author contributions

MM and YM conceived the manuscript; MM prepared the draft manuscript; MM, JS, CM, KY and YM approved the published version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Therapeutic targeting of HCMV-encoded chemokine receptor US28: Progress and challenges

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The pervasive human cytomegalovirus (HCMV) causes significant morbidity in immunocompromised individuals. Treatment using the current standard-of-care (SOC) is limited by severe toxic adverse effects and anti-viral resistance development. Furthermore, they only affect HCMV in its lytic phase, meaning viral disease is not preventable as latent infection cannot be treated and the viral reservoirs persist. The viral chemokine receptor (vCKR) US28 encoded by HCMV has received much attention in recent years. This broad-spectrum receptor has proven to be a desirable target for development of novel therapeutics through exploitation of its ability to internalize and its role in maintaining latency. Importantly, it is expressed on the surface of infected cells during both lytic and latent infection. US28-targeting small molecules, single-domain antibodies, and fusion toxin proteins have been developed for different treatment strategies, e.g. forcing reactivation of latent virus or using internalization of US28 as a toxin shuttle to kill infected cells. These strategies show promise for providing ways to eliminate latent viral reservoirs and prevent HCMV disease in vulnerable patients. Here, we discuss the progress and challenges of targeting US28 to treat HCMV infection and its associated diseases.

KEYWORDS

HCMV (human cytomegalovirus), US28, targeting, drug development, small molecule, single-domain antibodies (sdAb), fusion toxin protein, viral chemokine receptor

1 HCMV disease and vCKR US28

The G protein-coupled receptor (GPCR) family comprises a vast number of receptors, which are involved in diverse aspects of cell signaling in the body. Some viruses encode homologs of GPCRs, including viral chemokine receptors (vCKRs), with distinct roles in infection, cardiovascular disease, and various types of cancers (1). The herpesvirus family is particularly adept at chemokine mimicry with several members carrying and maintaining viral chemokines, receptors, and chemokine-binding proteins (2). Human cytomegalovirus (HCMV, or HHV-5) is a pervasive herpesvirus that infects more than half the population on a global scale (3). Infection is typically transmitted during early childhood and leads to life-long latency from where HCMV sporadically reactivates throughout its host's lifetime, thus

maintaining and further transmitting the infection (4). While HCMV infection is largely subclinical in immune-competent individuals, both primary infection and reactivation of latent virus reservoirs cause significant morbidity and mortality in immunocompromised individuals (5). Because of its omnipresence and clinical significance in vulnerable patient groups, the adverse impact of HCMV disease is substantial. Congenital CMV disease, affecting 0.5-1% of live births and causing a wide range of developmental disorders (6, 7), including sensorineural hearing loss, vision impairment, and intellectual disability, has been ranked as one of the highest priority target diseases for vaccine development (8). In transplant recipients, HCMV is the most common and impactful viral infection causing debilitating and difficult-to-manage disease post-transplantation, increasing the risk of graft rejection and mortality (9). Furthermore, HCMV has been linked to cancers, the most well-supported being glioblastomas (GBM) (10–12), and cardiovascular disease (13, 14). The current standard-of-care (SOC) treatment (Table 1) consists of DNA synthesis inhibitors such as ganciclovir and foscarnet (15), but their use is limited by significant toxic adverse effects and can be impaired by viral resistance development when used in long-term regimens (16). Furthermore, the HCMV-specific terminase inhibitor letermovir was recently approved for prophylaxis in recipients of allogeneic stem cell transplants (17). Treatment of HCMV infection is further challenged by these replication inhibitors not affecting the virus in its latent stage where viral transcriptional activity is silenced, and replication is halted (18, 19). This implies that reactivation is not preventable as only lytic infection is treatable and the latent virus reservoirs persist (20).

HCMV carries a large genome of ~235 kb linear double-stranded DNA comprising more than 750 translated open reading frames (ORFs) (21). Although most have unknown functions, more than 40 interact with the immune system (22, 23). In this vast genetic landscape, several genes with homology to components of the chemokine system has been identified (2). These include viral chemokines (UL146 and UL147), chemokine-like envelope proteins (UL128 and UL130), secreted chemokine binding proteins (UL21.5), and chemokine receptor homologs (US27, US28, UL33, and UL78). The best studied of these is the vCKR US28. This broad-spectrum receptor is expressed on the surface of HCMV infected cells, both in the lytic and latent phase (24, 25), and was initially recognized as a chemokine scavenging protein due to its promiscuous binding of many endogenous chemokines (26). Chemokine binding results in fast internalization of the ligand-receptor complex in a dynamin-dependent but arrestin-independent manner (27, 28), the internalized chemokine undergoes lysosomal degradation, and US28 is

TABLE 1 Overview of current standard-of-care drugs and the novel US28-targeting strategies under development, including modes of action (MOA), treatment effects and therapeutic applications.

	Drug/ Modality	MOA	Effect	Approved for[1]/ Therapeutic potential	Infection stage
Current therapeutics	Ganciclovir/ valganciclovir	Viral DNA polymerase inhibitor, activated by HCMV protein kinase UL97[2]	Replication inhibition	Prophylaxis and treatment of HCMV diseases in immunocompromised adults	Lytic
	Foscarnet	Viral DNA polymerase inhibitor	Replication inhibition	HCMV retinitis in people living with HIV/ AIDS[3]	Lytic
	Cidofovir	Viral DNA polymerase inhibitor	Replication inhibition	HCMV retinitis in people living with HIV/ AIDS[3]	Lytic
	Letermovir	HCMV terminase complex inhibitor (encoded by HCMV genes UL56, UL51 and UL89)	Replication inhibition	HCMV prophylaxis following allogeneic hematopoietic stem cell transplantation (allo-HSCT)	Lytic
	Maribavir	HCMV protein kinase UL97 inhibitor[2]	Replication inhibition	Treatment refractory post-transplant HCMV disease	Lytic
Novel US28-targeting strategies	Small molecules	Inhibition of vGPCR US28 constitutive signaling	Viral reactivation ➔ exposure to targeted killing by the immune system	Reducing latent HCMV load prior to immunosuppression (e.g. cancer patients and transplant recipients) Anti-proliferative treatment of US28+ GBM tumors	Latent
	Single-domain antibodies[4]	Inhibition of vGPCR US28 constitutive signaling	Viral reactivation ➔ exposure to targeted killing by the immune system	Reducing latent HCMV load prior to immunosuppression (e.g. cancer patients and transplant recipients) Anti-proliferative treatment of US28+ GBM tumors	Latent
		Photosensitizer-conjugate	Targeted killing of infected cells	*Ex vivo* clearance of HCMV infection in donor organs Treatment of US28+ cancers	Lytic and latent
	Fusion toxin proteins	Molecular trojan horse for intracellular toxin delivery through vGPCR US28	Targeted killing of infected cells	*Ex vivo* clearance of HCMV infection in donor organs Treatment of US28+ cancers	Lytic and latent

[1]FDA approved applications of the drugs.
[2]Combination of ganciclovir and maribavir is contraindicated as ganciclovir requires activation by UL97 that maribavir inhibits.
[3]Traditionally also used off-label as second-line treatment for ganciclovir-resistant HCMV infection.
[4]Also envisioned fused to a toxin moiety as an FTP (US patent US 2022/0324947 A1 (2022) 2020/08/05).

recycled to the cell surface where the process can repeat (24). This cycle theoretically removes pro-inflammatory chemokines from the extracellular environment at infection sites and promotes viral immune evasion, however, its biological significance has not been clearly established. It has been suggested that this effect is more pronounced during latency as the overabundance of extracellular chemokines in the lytic phase exceeds the scavenging capacity of US28 expressing cells (29). A well-established role of US28 during the latent phase is maintaining latency by subduing expression of the major immediate early promotor (MIEP). This effect is in part mediated by suppression of the mitogen-activated protein kinase (MAPK) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathways (25). Attenuation of MAPK signaling was recently shown to be the result of US28 interacting with the ephrin receptor A2 (EphA2) (30), whereas NF-κB signaling is subdued through rapid downregulation of interferon gamma inducible protein 16 (IFI16) by US28 (31). These functions underline an importance for HCMV immune evasion.

From a structural point of view, US28 overall resembles other class A GPCRs (1, 32–34). Of note, recent years' advancements in structural biology of membrane proteins using for instance cryo-EM, have resulted in a multitude of structures across class A (and class B1) GPCRs. Often more than one structure for each receptor is defined, thereby capturing the receptors in various conformational states (35, 36). For US28, an apo-structure as well as complexes with CX_3CL1 and a G protein-biased CX_3CL1 variant have been solved (1, 32–34). Together, these have shed light on helical connectivity and the role of various receptor domains and microswitches for US28 activity. Overall, the structural alterations result in a differentiation of US28 from its homologous endogenous chemokine receptors (CX_3CR1 and CCR1, CCR2, and CCR5) in terms of i) a broader chemokine recognition pattern (26, 37–39); ii) a broader activation profile, not only including Gαi like the endogenous receptors, but also other G proteins such as Gαq (40, 41); iii) a fast and constitutive internalization (24, 27, 28, 42, 43); and iv) a robust ligand-independent signaling (1, 44).

In search of novel therapeutics targeting HCMV infection, a new approach has emerged in recent years where US28's role as a surface protein during both lytic and latent infection is exploited through its ability to internalize and role in viral reactivation. The strategies for therapeutic targeting of US28 so far encompass three distinct modalities (Figure 1 and Table 1): small molecules, single-domain antibodies (sdAbs, so-called nanobodies), and fusion toxin proteins

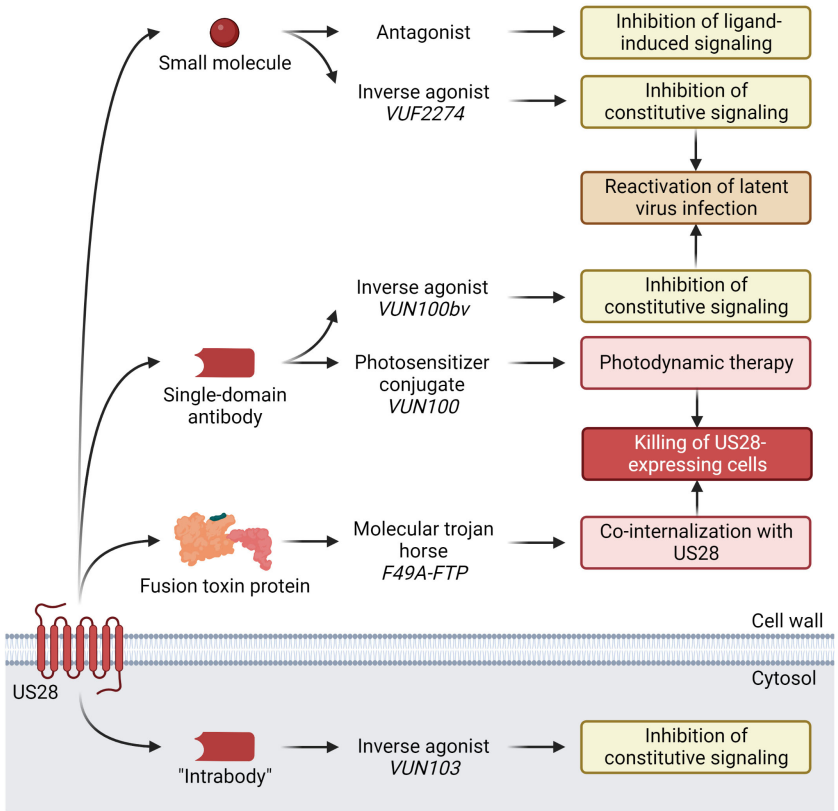

FIGURE 1
Current anti-HCMV US28-targeting modalities under development, discovered compounds, and their demonstrated effects. Small molecule VUF2274 and sdAb VUN100bv act as inverse agonists, i.e. inhibitors of US28 constitutive signaling. Attenuation of US28 signaling results in activation of the major immediate early promotor (MIEP), which leads to HCMV reactivation from latency. Another sdAb, VUN100, in conjugation with a photosensitizer binds to US28 on the surface of HCMV-infected cells. Upon stimulation with near-infrared light, the sdAb-photosensitizer conjugate is activated, producing reactive oxygen species that cause cell death. F49A-FTP consists of a US28-specific chemokine domain and a Pseudomonas exotoxin A (PE) domain that are fused. Upon binding to US28, it is co-internalized with the receptor. Inside the HCMV infected US28-expressing cell, the PE domain is released by furin cleavage. PE inactivates the eukaryotic elongation factor-2 (eEF-2), which halts host cell protein synthesis, resulting in apoptosis and cell death. The "intrabody" VUN103 is a sdAb that targets an intracellular epitope of US28. By displacing Gαq, it inhibits the constitutive signaling of US28 and exerts anti-proliferative effects on US28+ GBM tumor growth. Created with BioRender.com.

(FTPs). The inherent strengths, challenges, and potential clinical indications of these approaches will be discussed in this review.

2 Progress on drug targeting of US28

2.1 Small molecules targeting US28

A growing number of GPCR structures have facilitated the discovery of interacting small molecule compounds. For US28, the first compounds were discovered based on homology comparison to endogenous CKRs with known small molecule ligands as these were presented before the first US28 structure was solved. Several small molecule ligands acting as neutral antagonists or inverse agonists have displayed promising results in attenuating US28 signaling at micromolar concentrations (45). Among these, VUF2274 demonstrated the highest potency on US28 acting as an inverse agonist and interfered with CCL5 binding (45). The compound was observed to induce reactivation of latent HCMV infection potentially exposing it to the immune system (25), however, VUF2274 was originally discovered as a CCR1 antagonist (46) implying a selectivity issue if used as a drug. In search of potential drug candidates with limited cross-reactivity to endogenous receptors, two subsequent studies surveyed small molecule libraries based on VUF2274 (47, 48). Out of the latest study, several compounds emerged with agonistic or inverse agonistic profiles in Gαq-mediated signaling and capable of displacing CCL2 and CCL4, such as compound 56, 64 and 67 (48). These molecules were suggested as scaffolds for further development, but no advances on small molecules targeting US28 have been made since. Together, these studies demonstrate that US28, like other class A GPCRs, is highly targetable by small molecules though their clinical relevance as anti-HCMV therapeutics remains to be determined.

2.2 Single-domain antibodies to modulate US28 signaling

Apart from using small molecules to manipulate US28 activity, sdAbs are currently under investigation (49–52). Initially, a sdAb with sub-micromolar affinity to US28 was refined to create a bivalent sdAb with sub-nanomolar affinity. This compound partially inhibited ligand-dependent and constitutive US28 activity, leading to a reduction in US28 + GBM cell growth *in vitro* and *in vivo* (49), which shows therapeutic potential as US28 constitutive signaling can drive GBM proliferation (49). Subsequently, a US28-specific sdAb, VUN100, with nanomolar affinity for use in photodynamic therapy after conjugation with a photosensitizer was designed. Besides showing improved CX_3CL1 displacement compared to its predecessor, this compound displayed potent cytotoxicity *in vitro* on US28+ GBM cells (50). VUN100 was further refined into a bivalent version (VUN100bv) with improved affinity, acting as a partial inverse agonist inhibiting constitutive US28 signaling by 50% (51). This resulted in partial reactivation of HCMV in latently infected primary CD14+ monocytes, which lead to the hypothesis that VUN100bv could be used as a therapeutic in a "shock-and-kill" strategy where latent viral reservoirs are forced into lytic replication and subsequently killed by the host immune system. A fourth study described the generation of a sdAb (VUN103) targeting an intracellular epitope ("intrabody") that through displacement of G proteins completely inhibits constitutive US28 signaling and attenuates spheroid growth of U251 glioblastoma cells (52). Together, this set of studies underlines the possibility of US28 targeting and modulation through sdAbs. In the clinic, a sdAb-based strategy could potentially be used to treat HCMV diseases through attenuation of US28 signaling, leading to partial viral reactivation. This could expose the latent infection and improve the immune system's ability to combat the virus, potentially combined with existing anti-HCMV drugs.

2.3 US28-binding fusion toxin proteins kill infected cells

A different approach to novel therapeutics for HCMV disease has focused on the development of a US28-specific FTP (an immunotoxin strategy) (53–55). In this case, the drug is not intended to modulate US28 signaling but rather to kill US28-expressing cells. To generate a US28-targeting FTP, the preferentially US28-binding chemokine CX_3CL1 was fused to a modified version of the *Pseudomonas* exotoxin A (PE) lacking the cell entry moiety (53). Taking advantage of US28's constitutive internalization, the bound FTP is shuttled inside the cell where the toxin domain is released (53, 54). PE inactivates eukaryotic elongation factor-2 (eEF-2) by ADP-ribosylation which abolishes host cell protein synthesis, resulting in apoptosis and inevitable cell death (56). As CX_3CL1 also binds the endogenous receptor CX_3CR1, a mutated variant with high US28 selectivity (F49A-FTP) was generated. Exploiting the ubiquitous expression profile of US28 throughout both the lytic and latent cycle of HCMV infection, this FTP displayed potent and selective killing of infected cells in both stages (53, 55). The efficient elimination of HCMV-infected cells indicates a potential use in treatment of HCMV-associated diseases as demonstrated in patient-derived HCMV-infected CD34+ progenitor cells *in vitro*, forming the basis for a therapeutic strategy for eliminating latently infected cells before hematopoietic stem cell transplantation (55). Additionally, it showed efficacy on ganciclovir-resistant HCMV strains (53) thereby suggesting a use-case in clinical settings of treatment failure due to viral ganciclovir resistance. Following a successful trial-run (57), F49A-FTP was recently shown to reduce the load of latent HCMV by 80% in an *ex vivo* lung perfusion system (58), showcasing the potential for *ex vivo* elimination of HCMV in solid organ transplantations. These reports support a novel approach of eradicating latent virus reservoirs, which could prove particularly useful in organ transplantation settings provided improved clinical outcomes can be demonstrated.

3 Challenges of targeting US28 to treat HCMV diseases

3.1 Bridging the gap between bench and bedside

The reports on US28-targeting compounds are promising but crossing the gap between laboratory observations and *in human* effects is notoriously challenging for HCMV. The virus is highly adapted and species-specific after millions of years of co-evolution

with its host (59), making HCMV significantly different from other species' CMVs in its genetic content and immune modulation. The lack of a proper animal model for replicating *in vitro* effects *in vivo* is a persistent challenge in the field. Transgenic animal models have been applied with success, e.g. insertion of HCMV US28 into murine CMV (53, 60–62), however, findings from transferring HCMV-specific genes to another species' CMV are difficult to translate to humans and should always be considered with caution. Even though the distance between bench and bedside is increased by the lack of animal models for HCMV-associated diseases, US28 has the favorable position of a surface protein with basal, exploitable functionalities combined with homologies to endogenous class A GPCRs, which are inherently good drug targets (63).

Still, for transplantation-associated HCMV diseases, perhaps a better option is to utilize latently infected human organs unfit for clinical use in *ex vivo* systems (58). Here, the bigger hurdle is detecting and quantifying the latent HCMV load. Albeit not yet fully understood, latency is known to be established in a small fraction of CD34+ hematopoietic progenitor cells (HPCs) and CD14+ monocytes (64, 65), which are not abundant in most *ex vivo* organ settings. Additionally, gene transcription is minimal during latency (64). Reactivation assays have been described (58) but are time consuming, require steps of target cell extraction, viral reactivation, amplification using standardized cell cultures, and immunohistochemical staining of viral components, yielding more of an indirect measurement of HCMV activity. Modern techniques, such as RNA-seq, have shown promise in detecting latency transcripts (66) and may provide another approach to studying latency and *ex vivo* treatment effects. However, since reactivation is an inefficient process, genome- and transcript-based methods likely include abortive infections that will not reactivate. Alas, our current methods for detecting and quantifying the latent HCMV load and reactivation, and therefore evaluation treatment outcomes, are not ideal.

3.2 US28 genetic diversity

HCMV has a surprisingly diverse genome for a DNA virus displaying a high degree of sequence variability across many different genes including major immune modulators (67). For example, the chemokine-encoding UL146 gene is subject to extensive inter-strain diversity (68) that leads to structural and functional changes of the chemokine (69, 70). The US28 gene in contrast is quite conserved, strengthening its position as a therapeutic target (67). However, various genotypes have been observed (71–73), notably some with marked differences in the N-terminal (extracellular) tail of the receptor, which is important for chemokine binding. Indeed, molecular modeling has predicted changes in binding affinities of several endogenous chemokines to US28 variants (73). Variations of extracellular loops (ECLs) and the C-terminal (intracellular) tail have also been observed and, albeit less extensive, are not to be overlooked for their potential to alter US28 signaling. While the mechanism and biological significance are unclear, one study reported an increase in anti-CMV antibodies of renal transplant recipients carrying R267K-US28 (73). Additionally, antibody levels were reduced in HIV infected individuals carrying D170N-US28 and were accompanied by an increased HIV viral load and a reduction in sIFN-α/βR levels 12 months post initiation of anti-retroviral therapy (73). Functional differences between these naturally occurring variants remain unknown, but future research efforts exploring shifts in chemokine and drug binding along with signaling properties of the US28 variants will provide more knowledge. Furthermore, it is unclear to what extent these variants occur on a global scale as US28 genotyping studies and GenBank sequence deposits are limited. This combines to some uncertainties that should be addressed when progressing with US28-targeting drugs, as changes in drug affinities for US28 variants and downstream signaling can lead to altered drug effects. Additionally, treatment might induce US28 resistance mutations. These are risks that require clinical US28 sequencing before and during treatment to ensure and monitor the expected drug effects, however, clinically standardized tools to amplify and sequence US28 during latency is currently not available. Thus, these unknowns require attention when transitioning from lab to clinic.

3.3 What does it take to improve the clinical outcome?

The US28-targeting strategies discussed here rely on two distinct modes of action (MOA) (Figure 1 and Table 1). For small molecules and sdAbs, a "shock-and-kill" strategy has been proposed where HCMV infection is forced from latent to lytic phase. This is achieved by inhibition of constitutive US28 signaling with an inverse agonist that leads to activation of MIEP which initiates viral reactivation (74). Once reactivation is induced, this strategy relies on the host immune system or a combination treatment with a replication inhibitor to clear the infection (25). This will in theory allow clearance of HCMV infected cells before dissemination of infection. While elegant in its conception, this strategy rises some safety concerns if used in immunocompromised patients. Forcing HCMV reactivation requires a degree of control over the infection that currently is not always possible as seen in patient groups where infection can flare up despite administration of SOC prophylaxis (15), such as transplant recipients. This potentially limits the usefulness of the "shock-and-kill" strategy to patients with somewhat competent immune systems, which could be envisioned in an early treatment for US28+ GBM tumors, or to reduce the latent HCMV load in other cancer patients and R+ transplant recipients prior to immunosuppressive therapy.

The MOA of sdAb-photosensitizer conjugates and FTPs is cell toxicity, which does not rely on a competent immune system or a combination treatment to finish the job. However, this advantage requires a highly US28-specific ligand domain to limit adverse toxic effects emerging from off-target binding. Indeed, the promiscuous binding of US28 (26) might make it an easier target but also increase the risk of non-specific compound effects from off-target receptors. Promisingly, F49A-FTP did not induce acute lung injury or changes in cytokine levels in an *ex vivo* lung perfusion setting (58). As for other immunotoxins, potential compartmentalization of the FTP combined with release of the toxin moiety will need to be addressed to ensure its safety. While the sdAb-photosensitizer conjugate has the benefit of requiring site-directed light activation, peripheral effects of reactive oxygen species resulting from photodynamic therapy could affect its viability in some cancer and transplantation settings. Lastly, clearing latent infection from donor organs prior to transplantation (55, 58) raises

the question to what extent the viral reservoirs need to be reduced to influence the post-transplantation outcome. While early studies show that there is an association between the latent viral load and risk of recurrent infection (75, 76), it is unclear how the size of a latent HCMV reservoir in a donor correlates with the risk of reactivation and disease in the recipient, but it is not unthinkable that a small persistent HCMV pool can flare up to clinical significance in vulnerable patients. Overcoming these potential clinical challenges will be key to progression.

4 Concluding remarks

HCMV encodes three other genes with GPCR homology, US27, UL33, and UL78. These viral receptors are far less studied, and their functions and interaction partners remain largely unknown. UL33 and UL78 have been detected in some latency models *in vitro* whereas US27 is not expressed during latency (66) but has a role in viral dissemination in the lytic phase (77). Thus, US28 remains the prime target for novel therapeutics of HCMV-associated diseases.

Therapeutic targeting of CKRs has been a goal for more than 25 years. Despite the GPCR family being considered highly druggable, with nearly 500 successful drugs amounting to 34% of all FDA-approved drugs (63), strategies for developing treatments targeting CKRs have resulted in only three drugs approved for clinical use (Maraviroc, Plerixafor, and Mogamulizumab). Many more candidates have been tested and failed which shows that CKRs are not so straightforward targets as we initially had hoped. The reason is complex but can roughly be summed up to this—the chemokine system is highly promiscuous and redundant, vitally important for numerous biological processes, and disrupting it causes problems. Supporting this, the three approved drugs targeting CKRs are not designed to broadly alter inflammatory processes, but instead inhibits HIV-1 cell entry *via* CCR5 (78), promotes stem cell recruitment from the bone marrow *via* CXCR4 (79–81), and affects recruitment of a selected cell subset (regulatory T cells) to tumors *via* CCR4 (82).

On the quest for new CKR-targeting drugs, looking towards vCKRs could provide a solution for virus-associated diseases, but vCKR drug development is still in its youth. In this review, we have provided an update on HCMV US28 drug targeting and have discussed the major hurdles we currently face. Ongoing studies will reveal the further potential of the different US28-targeting strategies when progressing towards clinical adaptation.

Author contributions

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Conflict of interest

MMR is a co-founder and board member of Synklino A/S, Denmark, a biotech company focusing on HCMV treatment.

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Role of chemokine-like factor 1 as an inflammatory marker in diseases

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Immunoinflammatory mechanisms have been incrementally found to be involved in the pathogenesis of multiple diseases, with chemokines being the main drivers of immune cell infiltration in the inflammatory response. Chemokine-like factor 1 (CKLF1), a novel chemokine, is highly expressed in the human peripheral blood leukocytes and exerts broad-spectrum chemotactic and pro-proliferative effects by activating multiple downstream signaling pathways upon binding to its functional receptors. Furthermore, the relationship between CKLF1 overexpression and various systemic diseases has been demonstrated in both *in vivo* and *in vitro* experiments. In this context, it is promising that clarifying the downstream mechanism of CKLF1 and identifying its upstream regulatory sites can yield new strategies for targeted therapeutics of immunoinflammatory diseases.

KEYWORDS

chemokine-like factor 1, pathogenic mechanism, signaling pathways, immune-related disease, targeted therapy

1 Introduction

Chemokines were discovered in the early 1970s and late 1980s (1–3). They are a group of positive-charged cytokines with molecular weights of 8-10 kDA (4, 5) that regulate the infiltration of immune cells and the release of inflammatory mediators, making them an important component of the immune system. The involvement of chemokines in disease pathogenesis relies on their binding to receptors. The selectivity of different chemokines towards receptors is closely related to their ligand types (6). In 1999, chemokines were classified into four classic families using a systematic nomenclature based on different structures: CC, CXC, C, and CX3C (7). In 2001, Han et al. discovered and reported CKLF1, a novel chemokine with atypical structure, and its three variants (8). They also identified chemokine-like factor superfamily members (CKLFSF) by reverse transcription PCR techniques in the subsequent studies (9). Owing to the presence of a MARVEL (MAL and

Abbreviations: CKLFSF, Chemokine-like factor superfamily; MARVEL, MAL and related proteins for vesical trafficking and membrane link; CMTM, CKLF-like MARVEL transmembrane domain-containing; AS, Ankylosing spondylitis; ESR, Erythrocyte sedimentation rate; LN, Lupus nephritis; MAPK, Mitogen-activated protein kinase; MS, Multiple sclerosis; RA, Rheumatoid arthritis; VSMCs, Vascular smooth muscle cells.

related proteins for vesical trafficking and membrane link) domain, CKLFSF1-8 were renamed as CKLF-like MARVEL transmembrane domain-containing 1-8 (CMTM1-8) by the International Human Genetics Nomenclature Committee in 2005 (10). CMTM family (CMTMs), consisting of CKLF and CMTM1-8, is widely expressed in human tissues and plays multiple biological functions. CMTM1 negatively regulates the Ca^{2+} response in the ER and results in lymphoma cells apoptosis (11). CMTM2 is involved in the development and function of Leydig cells and modulates testicular testosterone production (12). CMTM3 mediates cell-cell adhesion and contributes to angiogenesis (13). CMTM4 plays an important role in tumors *via* regulating PD-L1 expression (14–16). CMTM5 exerts anti-atherosclerotic effects by suppressing migration and proliferation in the vessel wall (17). Deficiency of CMTM5 in oligodendrocytes leads to progressive axonopathy (18). Both CMTM5 and CMTM7 are biomarkers in human breast carcinoma (19). Targeting CMTM6 may improve the treatment of patients with clear cell renal cell carcinoma (ccRCC) (20). Over-expression of CMTM8 inhibits the invasion and metastasis of carcinoma cells, which provides a new potential target in the treatment of bladder cancer and other tumors (21, 22). CKLF1, as the most researched isoform, has potent broad-spectrum chemotactic and pro-proliferative capacity. Animal models and *in vitro* experiments have shown that CKLF1 acts in disorders affecting multiple systems by mediating different downstream signaling pathways. In this review, we outline the biology of CKLF1 and its receptors, discuss the links between CKLF1 and different diseases, and detail its downstream signaling pathways and therapies that target these mechanisms.

2 The structure of CKLF1 and its functions

CKLF1 is an intrinsically highly hydrophobic secretory protein obtained from pha-stimulated U937 cells. As a member of the CMTM family of proteins, CKLF1 has a contiguous CC structure similar to MDC (CCL22) and TARC (CCL17) but distinguishes from the CC family members by the absence of the C-terminal cysteine (8). There are differences in the expression levels of CKLF1 in adults and fetuses - higher levels of CKLF1 can be detected in peripheral leukocytes, spleen, lungs, and reproductive organs in adults. In contrast, higher expression was observed in fetal hearts, brains, and skeletal muscles (23). These findings suggest that CKLF1 may contribute to the physiological processes of human growth, and its abnormal expression could be a predictor of pathological states. Since CKLF1, MDC and TARC are structurally similar and are all situated on chromosome 16 (24), they may be evolutionarily conserved and have common biological activities. Furthermore, MDC and TARC bind specifically to CC chemokine receptor 4 (CCR4) and exert critical roles in allergic diseases (25). Therefore, CKLF1 may act as an immunoinflammatory factor and participate in pathogenesis through CCR4.

CCR4 is the most intensively studied CKLF1 receptor (26). It has seven typical transmembrane helices of G protein-coupled receptors (GPCRs). CCR4 is expressed in activated TH2 cells, Treg cells, activated natural killer cells, basophils, monocytes, platelets, and mature T-cell tumors (27). The C-terminal helix 8 of CCR4 facilitates the signaling and activation of chemokine receptors (28). CKLF1 has two stable secreted forms at the C-terminus – C19 peptide and C27 peptide. It can act *via* the Gi/o pathway upon binding to CCR4. C27/CCR4 exerts strong chemotactic properties, while the C19 peptide weakly activates CCR4 and may be a candidate antagonist of CKLF1 (29). As another GPCR receptor of CKLF1, CCR3 is mainly expressed in eosinophils and can also be detected on the surface of Th2 cells, basophils, and mast cells (30). Mouse model experiments have established that CCR3 plays a critical role in allergic airway inflammation (31). Moreover, the C19 peptide inhibits CCR3-mediated chemotaxis and has excellent therapeutic potential in managing allergic asthma (32). Meanwhile, CCR5 was initially identified as a receptor of chemokines, including CCL3, CCL4, and CCL5 (33). Its expression modestly reduces the risk of type 1 diabetes and celiac disease (34). CCR5+ leukocytes include Treg cells, CD4+ and CD8+ T cells, natural killer cells, dendritic cells, monocytes, and macrophages (35). A study by Chen et al. reported that CCR5 mediates neutrophil migration and participates in cerebral ischemia/reperfusion (I/R) injury by acting as a receptor for CKLF1 (36). Given the structural similarity of CKLF1 to members of the CC chemokine family, there are likely to be other typical or atypical CKLF1-binding GPCRs. Therefore, a further expansion of the CKLF1 receptor family is to be anticipated.

CKLF1 interacts with various cells, including neutrophils, lymphocytes, monocytes, and neuronal cells, playing an integral role in the transport of immune cells and the production of immune mediators. The activated GPCRs cause the G protein subunits to dissociate and the GDP to exchange with GTP following the binding of CKLF1 to GPCRs as CCR4, CCR3 and CCR5. The Gα and Gβ subunits further activate downstream signaling pathways and exert broad chemotactic activities. Dendritic cells (DCs) possess potent antigen-presenting functionality and are involved in Th1/Th2 polarization (37). *In vitro* experiments suggest CKLF1 may stimulate DC maturation through the NF-κB and MAPK (mitogen-activated protein kinase) pathway (38, 39). It has been found that peptides C19 and C27 stimulated the secretion of IL-12 in DCs and promoted the production of IFN-γ, which in turn affected the ability of DCs to activate Th1 cells while not affecting the activation of Th2 cells. The expression of CCR4 was detected on DCs, but whether the mentioned processes were mediated *via* CCR4 needs further investigation (40). In addition to participating in DC activation, CKLF1 may also regulate the activation of T lymphocytes. It has been shown that the mRNA levels and protein expression levels of CKLF1 were increased in activated CD4+ and CD8+ lymphocytes in a time-dependent manner (41). CD4+ T cells expressing CXCL5 and CD57 are localized explicitly to germinal centers and are called GC-Th cells. GC-Th cells are activated in a CD28 co-stimulatory signal-dependent manner, producing and secreting large amounts of CXCL13 as critical chemokines for B-cell entry into lymphoid follicles. Gene expression profiling revealed that GC-Th cells might induce the expression of CKLF1 and participate in the above process, but the exact regulatory mechanism is not yet clear (42). In addition, CKLF1 also mediates an important non-immune function, namely pro-proliferative capacity, targeting skeletal muscle cells, vascular smooth muscle cells, and bone marrow cells, providing therapeutic orientations for myasthenia gravis, vascular diseases, and hematological disorders (43–45).

3 CKLF1-related diseases

Various chemokines are involved in the development of many acute and chronic diseases by regulating the inflammatory environment. Chemokines do not correspond to diseases in a piecewise manner but are intertwined into networks. Many chemokine-receptor combinations may exert similar cellular functions in different diseases, while the same chemokine may bridge specific signaling pathways with different effects on disease progression. The role of CKLF1 in coordinating immune responses in different systemic diseases has been reported, and it would be meaningful to study the effects of CKLF1 targeting different cells in these diseases. In this section, we will present diseases associated with the undesirable effects of CKLF1, focusing on the underlying mechanisms of CKLF1 actions in different systemic diseases and the evidence supporting these effects of CKLF1. Here we list references containing reviews related to the diseases explored for readers to follow in more detail (Table 1).

3.1 CKLF1 and neurological diseases

3.1.1 Cerebral ischemia

Ischemic stroke is one of the leading causes of mortality and disability worldwide, resulting in 6 million deaths and 5 million permanent disabilities yearly (75). Although reperfusion may help to rescue the ischemic semidark zone, it can exacerbate neuroinflammation and worsen brain damage. Blood-brain barrier (BBB) disruption induced by neutrophil migration and release of pro-inflammatory factors participates in the pathophysiological process of cerebral ischemia-reperfusion (I/R) injury. Following the onset of stroke, activated microglia and astrocytes secrete pro-inflammatory mediators such as chemokines, with neutrophils migrating to the lesion site within a few hours. The infiltrating neutrophils release cytokines, including proteases, which activate glial cells *via* positive feedback, synergistically destroying the BBB and causing neuronal necrosis (76). The neutrophil/lymphocyte ratio is of great value in estimating the severity and prognosis of cerebral ischemic injury (46). Microglia are the primary cells governing the

TABLE 1 The association of CKLF1 with diseases.

CKLF1-mediated diseases	Main targeting cells	Effect on disease	Related Mechanisms	References
Cerebral ischemia	Neutrophils; Microglia	Early cerebral ischemia: promotes neutrophil migration, microglia M1 polarization, BBB destruction, and aggravates cerebral ischemic injury. Late cerebral ischemia: recruits nerve cells and promotes vascular regeneration for neurological recovery.	AKT/GSK-3β pathway; MAPK pathway; NF-κB pathway	(36, 46–51)
Brain development	SH-SY5Y cells and cortical neurons	Induces neuronal migration and promotes brain development	Non-extracellular calcium-dependent tyrosine kinase pathway	(52–54)
Bronchial asthma	Th2 lymphocytes and eosinophils	Recruits inflammatory cells to the bronchial mucosa, promotes the proliferation of bronchial smooth muscle cells and fibroblasts, and aggravates pulmonary fibrosis in bronchial asthma	NF-κB pathway	(55–59)
Allergic rhinitis	Th2 lymphocytes and eosinophils	Induces migration of inflammatory cells, promotes IgE production and the release of inflammatory factors and aggravates allergy symptoms	Not yet mentioned	(32)
Arthritis	Synovial lining cells Leukocytes	Causes proliferation of synovial lining cells, vascular proliferation and fibrosis, and diffuse inflammatory cell infiltration, which are involved in the mechanism of arthritis	MAPK pathway; NF-κB pathway	(60–63)
Psoriasis	Lymphocytes Endothelial cells	Induces lymphocyte migration to the skin, promotes microvascular dilation and endothelial cell proliferation and mediates the development of psoriasis	MAPK pathway	(64, 65)
Lupus Nephritis	Leukocytes	Promotes the accumulation of inflammatory cells to the site of injury where immune complexes are deposited and thereby aggravates the injury	Not yet mentioned	(66)
Antiphospholipid syndrome	Blood platelets	Affects platelet activity and function; is involved in hemostasis and thrombosis	Not yet mentioned	(67)
Inflammatory myopathy	Lymphocytes Muscle fibers	Attracts lymphocytes with regenerating muscle fibers to the site of inflammation and participates in the development of polymyositis (PM) and dermatomyositis (DM)	Not yet mentioned	(68)
Atherosclerosis and RS	Vascular smooth muscle cells (VSMCs) Mononuclear cells	Induces monocyte adhesion to vascular endothelium, promotes vascular smooth muscle cell migration, and accelerates thrombosis.	PI3K/AKT/NF-κB pathway	(69–71)
Abdominal Aortic Aneurysm	Macrophages Lymphocytes	Upregulates MMP-2 expression and accelerates (aortic wall structural protein) extracellular matrix degradation, leading to the development of AAA.	Not yet mentioned	(72)
Keloid scars	Not yet mentioned	Not yet mentioned	Not yet mentioned	(73)
Hepatocellular carcinoma	Tumor cells	Inhibits apoptosis, promotes malignant transformation, and induces HCC development and metastasis	IL6/STAT3 signaling pathway	(74)

intrinsic immune response of the brain. Microglia/macrophage polarization has been proven to regulate the development of various central nervous system disorders, including stroke, multiple sclerosis, and spinal cord injury (77, 78). Microglia are activated into two types in early cerebral ischemia: the pro-inflammatory M1 microglia, and the anti-inflammatory M2 microglia. The brain microenvironment favors M1 polarization, leading to brain injury progression and neurological deficits.

Preliminary experimental studies have confirmed that CKLF1 plays a central role in I/R injury. Research using a rat model of cerebral ischemic injury showed that CKLF1 expression was significantly elevated at the injury site after 3 hours and peaked after 48 hours. Knockdown of CKLF1 by HIF-1α-guided AAV in the ischemic area of the rat brain reduced the size and water content of the infarct area, confirming that CKLF1 exerts a pro-inflammatory effect in the early stage of cerebral ischemia to aggravate the injury. Immunohistochemical staining and an MPO activity assay showed that CKLF1/CCR5 mediated neutrophil migration through the AKT/GSK-3β pathway (75). In contrast, an earlier study reported that an anti-CKLF1 antibody inhibited neutrophil infiltration *via* the MAPK pathway (47). The different results may originate from the differences in CKLF1 distribution in the brain. The expression of CKLF1 is spatially specific in cerebral ischemic injury and primarily occurs in the cortex, thalamus, and hippocampus (46). Therefore, selective knockdown of CKLF1 at different sites may affect the experimental results differently. In addition, the selection of the time window may bring limitations to the experimental results - CKLF1 may mediate different signaling pathways or combine multiple signaling pathways to effect at different stages after the onset of ischemia-reperfusion injury. *In vitro* experiments have demonstrated that CKLF1 partially depends on CCR4 to regulate M1 polarization of BV2 microglia and induce oxygen-glucose deprivation/reperfusion (OGD/R) injury (48). Experiments using a mouse MCAO (middle cerebral artery occlusion) model showed that either exogenous or endogenous CKLF1 could promote M1 polarization in microglia during early cerebral ischemia, and the polarization process was associated with CKLF1/CCR4 axis-mediated activation of the NF-κB pathway (49). AQP4, MMP-9, and tight junction (TJ) proteins are markers that reflect BBB function. Overexpression of AQP4 is a significant cause of brain water imbalance (50). Inhibition of CKLF1 reduced AQP4 and MMP-9 levels, upregulated the expression of TJ proteins, including ZO-1 and Occludin, and attenuated brain edema in rats (51).

3.1.2 Brain development

The development of the cerebral cortex is accomplished through complex modulations on neurogenesis, neuronal migration, and neuronal connectivity. Disruptions in these processes can lead to neuropsychiatric disorders such as drug-resistant epilepsy, intellectual disability, and schizophrenia (79). Chemokines play a role in brain development by regulating synaptic transmission, cell migration, and other processes through autocrine or paracrine signaling (80). Not only is CXCR4/CXCL12 involved in inducing neurogenesis (81), but the CXCR4/CXCR7/CXCL12 axis may also guide cortical neuronal migration (82). *In vitro* experiments have shown that stromal cell-derived factor-1α (SDF-1α) acted on the migration of various neurons in the dentate gyrus, cerebral cortex, and brainstem nuclei. RANTES was found to have a stimulating effect on the migration of dorsal root ganglion cells (83). The expression level of CKLF1 mRNA in the adult brain was found to be well below that of the fetal brain (52). This discrepancy in expression may indicate that CKLF1 is involved in brain developmental processes. It was observed that CKLF1 induced the migration of neurons in the rat cerebral cortex in a dose-dependent manner at concentrations of 200 nM and 2000 nM (53), and this process was mediated through the non-extracellular Ca^{+}-dependent tyrosine kinase pathway (54).

3.2 CKLF1 and respiratory diseases

3.2.1 Bronchial asthma

Bronchial asthma is a heterogeneous chronic inflammatory disease with inflammation, hyperresponsiveness (AHR), and remodeling of the airways as the primary pathophysiological mechanisms characterized by Th2 lymphocyte and eosinophil infiltration. Airway epithelial cells and alveolar macrophages generate various chemokines in response to allergic or non-allergic stimuli, recruiting inflammatory cells toward the bronchial mucosa and participating in the onset and progression of asthma. The role of several chemokine receptors in asthma has been reported - CCR4 was found to be highly expressed in Th2 cells, and CCL17/CCR4, CCL22/CCR4 mediated the migration of Th2 cells and triggered allergic airway responses (84–86). CCR4-targeting inhibitors such as thymus and activation-regulated chemokine (TARC-PE38) have alleviated airway inflammation (87). Meanwhile, CCR3 is involved in eosinophil transport as a major receptor and acts with CCR4, CCR5, CCR6, CCR8, and CXCR4 to induce airway inflammation and AHR (88).

Mouse models provide excellent insights into the roles of specific chemokines in the respiratory system. Intramuscular injection of CKLF1 plasmid to BALB/c mice increased the total leukocyte count and eosinophil percentage in BALF, causing pathological lung modifications consistent with bronchial asthma (55). This process is facilitated by the CKLF1/CCR4-mediated NF-κB pathway (56). C19 peptide inhibited Th2 cell responses, eosinophilia, and AHR in a mouse allergic asthma model by acting on CCR4 and CCR3. In contrast, the C27 peptide caused a mild increase in total leukocytes in BALF of asthmatic mice, which was not statistically significant (57). It was found that inhibition of CKLF1 binding to CCR4 with antagonists alleviated airway symptoms in asthmatic mice and attenuated airway remodeling and lung fibrosis caused by epithelial cell and fibroblast proliferation (58). A clinical study confirmed that CKLF1 mRNA expression was significantly higher in the peripheral blood of asthmatic patients compared to controls and that CKLF1 level in the airways of these patients was much higher than in normal subjects (59).

3.2.2 Allergic rhinitis

Allergic rhinitis is an inflammatory disease of the nasal mucosa prominently marked by elevated IgE. It has pathogenesis similar to that of bronchial asthma featuring typical biphasic responses - with the early phase characterized by IgE activating basophils and mast cells to secrete cytokines, causing allergic symptoms including running nose and itching; then the pro-inflammatory factors further activate inflammatory cells, including eosinophils, initiating late-phase responses that lead to sustained symptoms (89). Th2 cells promote the synthesis and secretion of IgE, which is critical in the

above responses (90). Intranasal and intraperitoneal administration of C19 peptide to allergic rhinitis mice relieved symptoms and decreased serum IgE concentrations, with better efficacy of intranasal than intraperitoneal delivery (32). These findings substantiate that C19 peptide may be used as a therapeutic agent for allergic rhinitis by intranasal administration.

3.3 CKLF1 and rheumatic diseases

3.3.1 Arthritis

The main types of arthritis include rheumatoid arthritis (RA), osteoarthritis (OA), and ankylosing spondylitis (AS), with characteristic pathological changes being infiltration of the periarticular synovial tissue by mixed inflammatory cells. The successful development of TNF antibodies and soluble TNF receptor antagonists confirms the vital contribution of TNF in these diseases. TNF is known to induce the production of various chemokines, regulate inflammatory cell infiltration and vascular proliferation, and participate in the pathogenesis of arthritis. Several studies have identified receptors for chemokines, including CCR2, CXCR2, and CXCR3, on infiltrating cells from patients with arthritis (91–93). Among them, the presence of CCR5 is often associated with the deficiency of rheumatoid factor (94).

Given the scarcity of animal models of arthritis concerning CKLF1, most evidence was derived from clinical studies. A study (60) included 16 patients with OA, 15 with RA, and 10 with AS and measured levels of CKLF1, CCR4 mRNA, and plasma inflammatory markers. The results showed that CKLF1 levels were increased in synovial membranes of patients with RA, OA, or AS, and patients with RA presented with concomitant upregulation of CCR4 mRNA expression. The authors theorized that CKLF1 in RA acts through binding to CCR4, while in OA and AS, it may mediate disease progression through pathways other than the CKLF1/CCR4 axis. Several other clinical studies have found a positive correlation between CKLF1 levels and C-reactive protein (CRP)/sedimentation (ESR) in patients with RA, suggesting that CKLF1 may be a sensitive indicator for evaluating disease activity. However, in patients with OA and AS, findings contradict previous reports, which require verification through further studies (61–63).

3.3.2 Other rheumatic diseases

In addition to arthritis, CKLF1 also finds a role in other rheumatic diseases. CKLF1 and CCR4 levels were significantly elevated at the lesion sites in patients with psoriasis (64). The proposed mechanism is that CKLF1 induces lymphocyte migration to the skin and promotes microvascular dilation and endothelial cell proliferation, mediating the pathogenesis of psoriasis. The role of the C19 peptide in psoriasis is still controversial. It has been shown that the C19 peptide significantly reduced the number of neutrophils in patients with psoriasis and exerted a protective role in psoriasis through the MAPK signaling pathway (65). Despite being weaker than the C27 peptide, the C19 peptide has also been shown to promote the proliferation of vascular endothelial cells (64). The different biological effects induced by the C19 peptide may result from the variability of the local inflammatory environment (95). CKLF1 is expressed at low levels in normal kidneys and exerts a physiological chemotactic function. Overexpression of CKLF1 resulted in increased urinary protein in mice, which exhibited the pathological modifications of lupus nephritis (LN). CKLF1 levels in LN patients were positively correlated with the lupus activity index and could be used as a valid predictor of disease activity (66). Antiphospholipid syndrome (APS) manifests itself primarily by thrombosis and recurrent obstetric events. CKLF1 may compromise platelet activity and function by participating in the hemostatic and thrombotic processes, leading to the aggravation of APS (67). In inflammatory myopathies, intratubular thrombin induces the expression of CKLF1. CKLF1 may be a marker of myofiber regeneration in its ability to attract lymphocytes and regenerate myofibers (68).

3.4 CKLF1 and circulatory diseases

Atherosclerosis is known as an inflammatory mechanism-mediated disease. Studies on chemokines in atherosclerosis models are relatively well established. In response to inflammatory injury, the release of chemokines from activated endothelial cells and arterial smooth muscle cells induces monocyte adhesion to the vascular endothelium and contributes to forming foam cells. In addition, chemokines promote the migration of smooth muscle cells to the endothelium and their attachment to the plaque to form a thrombus (96). Monocyte chemotactic protein 1 (MCP-1), Fractalkine (CX3CL1), RANTES (CCL5), and eotaxin (CCL11) are involved in the development of atherosclerosis (97–100). Restenosis (RS) is highly prevalent at six months after percutaneous transluminal coronary angioplasty, with restenosis rates of up to 30-50% (101) in patients treated with balloon angioplasty or bare metal stents and still up to 12-20% with drug-eluting stents (102). The pathogenesis of RS is very similar to that of atherosclerosis, with extensive involvement of chemokines.

Many researchers have applied a balloon injury rat model to explore the effects of CKLF1. Early studies found that CKLF1 levels were significantly elevated in the neointima after injury and co-localized with vascular smooth muscle cells (VSMCs). C19 peptide potently inhibited VSMC migration and endothelial proliferation after CKLF1 transfection (69). A subsequent study showed that CKLF1 acted on G2/M-phase VSMCs to regulate the balance between proliferation and apoptosis and accelerate neoplastic endothelial formation. This process was regulated through the PI3K/AKT/NF-κB pathway (70). These results were confirmed in later clinical studies. It was found that the expression of CKLF1 and vascular adhesion molecule-1 (VCAM-1) was significantly increased in human carotid plaques compared to controls. Meanwhile, CKLF1 promoted the aggregation of human aortic smooth muscle cells (HASMCs) and monocyte adhesion through the NF-κB/VCAM-1 pathway (71).

3.5 CKLF1 and tumors

The role of chemokines in tumors is not limited to the recruitment of leukocytes but may also interfere with cell proliferation and the generation of new blood vessels. Melanoma growth-stimulating activity (MGSA) and IL-8 may act as tumor

growth factors that directly contribute to the development of melanoma and non-small cell lung cancer (103, 104). It was revealed that CXC chemokines containing the Glu-Leu-Arg motif (ELR motif) promoted angiogenesis, while non-ELR CXC chemokines counteracted it (105). This may indicate that the bidirectional regulatory effects of chemokines in tumors are structure-related. As a novel chemokine, the structure of CKLF1 is highly characteristic. Exploring the role of CKLF1 in tumors may provide more possibilities for targeted therapy.

Abdominal aortic aneurysm (AAA) is closely correlated with structural protein degradation of the aortic wall mediated by inflammatory mechanisms. A rat AAA model study showed that CKLF1 levels were elevated in the AAA group compared to controls, with a significant positive correlation with MMP-2 levels, confirming that CKLF1 promotes AAA through upregulating the expression of MMP in the extracellular matrix (72). It was also found that basal CKLF1 levels were higher in keloid individuals than in those without keloids, and the expression of CKLF1 mRNA was higher in keloid tissues than in normal tissues, suggesting that CKLF1 could be instrumental in predicting keloids. However, the exact mechanism remains to be further elucidated (73). Most clinical studies on CKLF1 in malignancies have focused on hepatocellular carcinoma. One study reported that in patients with hepatocellular carcinoma, CKLF1 levels were significantly higher in cancer tissues compared to normal tissues, and the expression levels were higher in advanced cancer than in the early stages. Additionally, this research demonstrates that CKLF1 mediated its oncogenic effects through the IL-6/STAT3 signaling pathway and could assist in the staging and prognostic assessment of patients with hepatocellular carcinoma (74).

4 Mechanisms of CKLF1-mediated pathogenesis

The activation and crosstalk of diverse intracellular signaling pathways are involved in the mechanism of CKLF1-mediated pathologies, including the NF-κB pathway, MAPK pathway, JAK/STAT3 pathway, and PI3K/AKT pathway.

4.1 NF-κB signaling pathway

NF-κB is a crucial nuclear transcription factor widely present in animal tissues and participates in the regulation of processes, including immune inflammatory responses, cell proliferation and differentiation. The NF-κB transcription factor family consists of five proteins that share the Rel homologous structural domain: RelA (p65), RelB, cRel, NF-κB1 (p105/p50), and NF-κB2 (p100/p52). Without stimulatory signals, NF-κB is complexed with IκB in the cytoplasm and remains in dynamic homeostasis. In contrast, when the NF-κB pathway is activated by stimulating factors, it exerts effects through classical and non-classical pathways (alternative pathways) (106). In the classical pathway, PRRs (pattern recognition receptors), inflammatory cytokine receptors, TCRs (T cell receptors), and BCRs (B cell receptors) act as stimulatory signals to the IκB kinase (IKK) complex, causing IκB phosphorylation, which leads to the release of NF-κB dimers and their translocation to the nucleus, promoting the transcription of a variety of target genes (107). The NF-κB alternative pathway can be activated by members of the TNFR superfamily, with p100 processing being a key link in this pathway. An NF-κB-inducing kinase (NIK) and IKKα act as essential factors for p100/RelB processing, which leads to the activation of P52/RelB. This heterodimer may bind to different promoter regions and exert diverse biological effects upon entry into the nucleus (108).

Unlike other ligands of CCR4, such as TRAC and MDC, CKLF1 can activate the NF-κB classical signaling pathway involved in disease-causing mechanisms, which may have to do with its specific structure. A study on human amniotic mesenchymal stromal cells showed that CKLF1 mediates monocyte adhesion and smooth muscle cell (SMC) migration through the NF-κB/VCAM-1 pathway and that the use of PDTC, an NF-κB inhibitor, suppressed the CKLF1-induced elevation of VCAM-1 (71). A mouse MCAO model study showed that CKLF1 binding to CCR4 activated the NF-κB pathway and promoted microglia/macrophage polarization toward the M1 phenotype (49). CKLF1 also induces significant airway inflammation and pathological changes in mouse lungs by triggering NF-κB-regulated gene expression, which results in the release of multiple pro-inflammatory mediators, including IL-4, IL-13, and TNF-α (55). The chemotherapeutic drug cisplatin (CDDP) provokes elevated levels of CKLF1 and NF-κB in HK-2 renal tubular epithelial cells and mouse kidneys, inducing inflammatory injury and nephrotoxicity. In contrast, Kanglaite (KLT) and hydroxytyrosol (HT) could partially reverse the adverse effects of CDDP by acting on the IκB-NF-κB complex and inhibiting the CKLF1-mediated NF-κB pathway (109). There is still a lack of research on the effect of CKLF1 in the NF-κB alternative pathway. However, this does not mean that CKLF1 acts only through the classical pathway. Some non-TNFR receptors have been shown to serve as stimulatory signals in the NF-κB alternative pathway (108). With the exploration of CKLF1 receptors, further studies may bring more insights into its pathogenic mechanisms. (Figure 1)

4.2 MAPK signaling pathway

MAPK pathways are highly conserved in all eukaryotic cells that consist of typical three-tier core signaling modules: MAP3Ks, MAP2Ks, and MAPKs. Through hierarchical phosphorylation across these modules, MAPK pathways can mediate gene transcription and regulate biological processes, including cell proliferation, differentiation, and apoptosis. Mammalian cells share three well-defined MAPK signaling pathways: ERK pathway, JNK pathway, and p38 pathway. Rather than being independent of each other, the three may share some upstream regulators and downstream target genes. MAPK pathways can be activated by many stimuli, including cytokines, growth factors, and pathogen-associated molecular patterns (PAMPs) (110).

Recent studies indicate that CKLF1 acts as a stimulatory factor to regulate the MAPK pathway and that the chemotactic activity of CKLF1 is partially dependent on the MAPK pathway, particularly neutrophil infiltration. A study utilizing an I/R model found that the expression of CKLF1 was significantly upregulated. The use of CKLF1 antibody significantly reduced the phosphorylation levels of ERK, JNK, and p38, inhibited the production of cytokines (TNF-α, MIP-2,

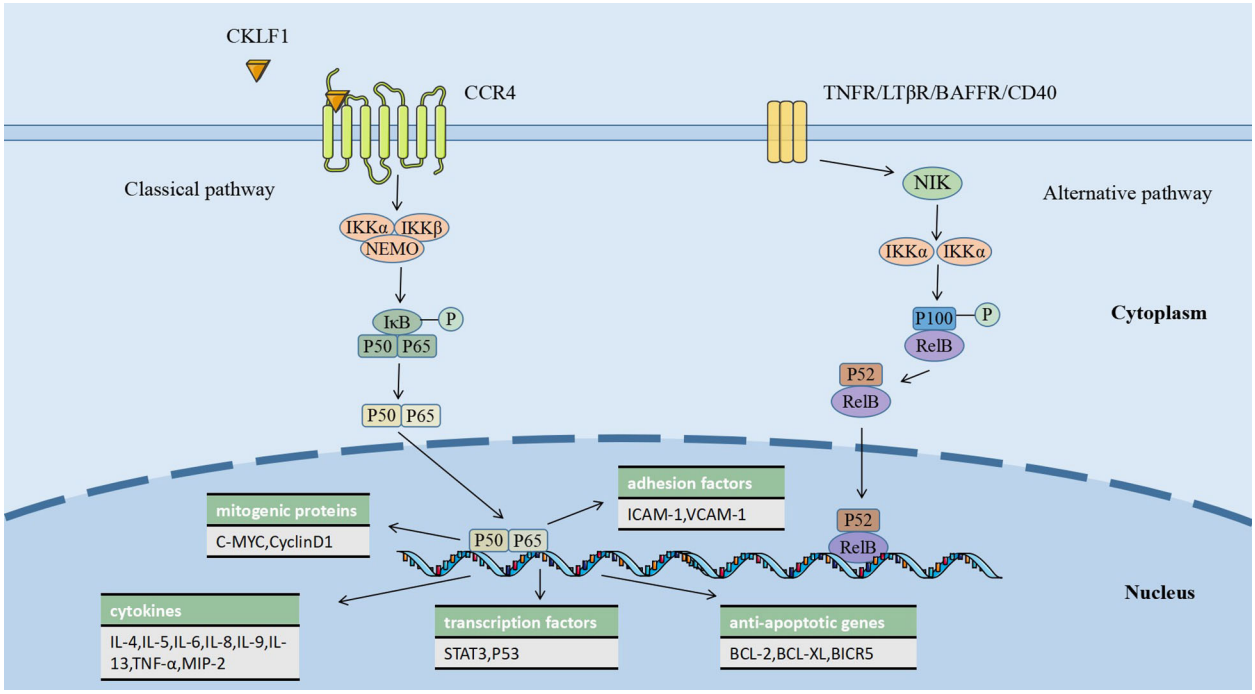

FIGURE 1
CKLF1 binds to CCR4 and activates the NF-κB classical pathway, causing a variety of biological processes, including the release of inflammatory factors in the nucleus, the transcription of anti-apoptotic genes, and the expression of mitogenic proteins, which are involved in the regulation of immune-inflammatory responses.

and IL-1β) and the expression of adhesion factors (ICAM-1 and VCAM-1), and reduced the recruitment of neutrophils to the ischemic area (111). Fang et al. further demonstrated that treatment with the C19 peptide reduced the phosphorylation level of MAPK, with more pronounced effects on JNK and p38 (112). *In vitro* experiments with human umbilical vein endothelial cells have shown that the C19 peptide antagonized ERK and p38 signaling pathways, suggesting that it might be beneficial in psoriasis by inhibiting inflammatory infiltration and microvascular proliferation (65). In addition, it was shown that phosphorylation of JNK and p38 was involved in microglia M1 polarization, and CKLF1 could bind to CCR4 receptors on microglia. However, whether CKLF1 activates MAPK pathways through CCR4 receptors to mediate microglia polarization remains to be further investigated (113) (Figure 2).

4.3 IL-6/JAK/STAT3 signaling pathway

The IL-6/JAK/STAT3 signaling pathway is involved in regulating cell growth and differentiation, making it potentially critical in tumorigenesis and metastasis. Activation of the IL-6/JAK/STAT3 pathway often indicates poor prognosis in cancer patients (114, 115). It was established that IL-6 was highly expressed in the inflammatory and tumor microenvironment and bound to IL-6R and gp130 to form a heterohexameric complex to initiate signaling pathways and induce activation of gp130-associated JAKs. Mammals harbor four JAKs: JAK1, JAK2, JAK3, and TYK2, all of which can be activated in this pathway, mediating the phosphorylation of tyrosine residues to form protein docking sites. The same study also illustrated that STAT3 acted as an important substrate for JAK and was phosphorylated at tyrosine 705 (Y705). The phosphorylated STAT3 is then dimerized and translocated to the nucleus to regulate gene transcription (116).

The involvement of CKLF1 in tumor development may be related to its role in mediating the IL-6/JAK/STAT3 pathway. STAT3 is an important regulator in the metabolism of tumor cells. Overexpressed CKLF1 increases pyruvate kinase activity and promotes lactate production, which is accomplished by regulating STAT3. A study based on a hepatocellular carcinoma model demonstrated that CKLF1 regulates the tumor microenvironment by binding to CCR4 to initiate the IL-6/JAK/STAT3 signaling pathway, upregulating STAT3-related cytokines such as TNF-α and IL-17A, and inducing the expression of cell cycle regulatory genes including BCL-XL and cyclinsD1. In addition, these authors found that CKLF1 was able to affect the cell cycle of adriamycin (DOX)-transfected cells through the IL-6/JAK/STAT3 pathway, promoting proliferation in the G2/M phase and inhibiting DOX-induced apoptosis (74) (Figure 3).

4.4 PI3K/AKT signaling pathway

PI3K (an oncogene) is one component of the PI3K/AKT pathway, which is widely involved in tumors and immune-inflammatory diseases. The activation of tyrosine kinase receptors (RTKs), cytokine receptors, and G protein-coupled receptors (GPCRs) may act as upstream stimulatory signals of the PI3K/AKT pathway to activate PI3K (117). Activated PI3K catalyzes the conversion of PtdIns(4,5)P2 phosphorylation to PtdIns(3,4,5)P3, which acts as a second messenger to recruit AKT to specific sites on the cytoplasmic membrane. AKT is activated by the combined action of PDPK1

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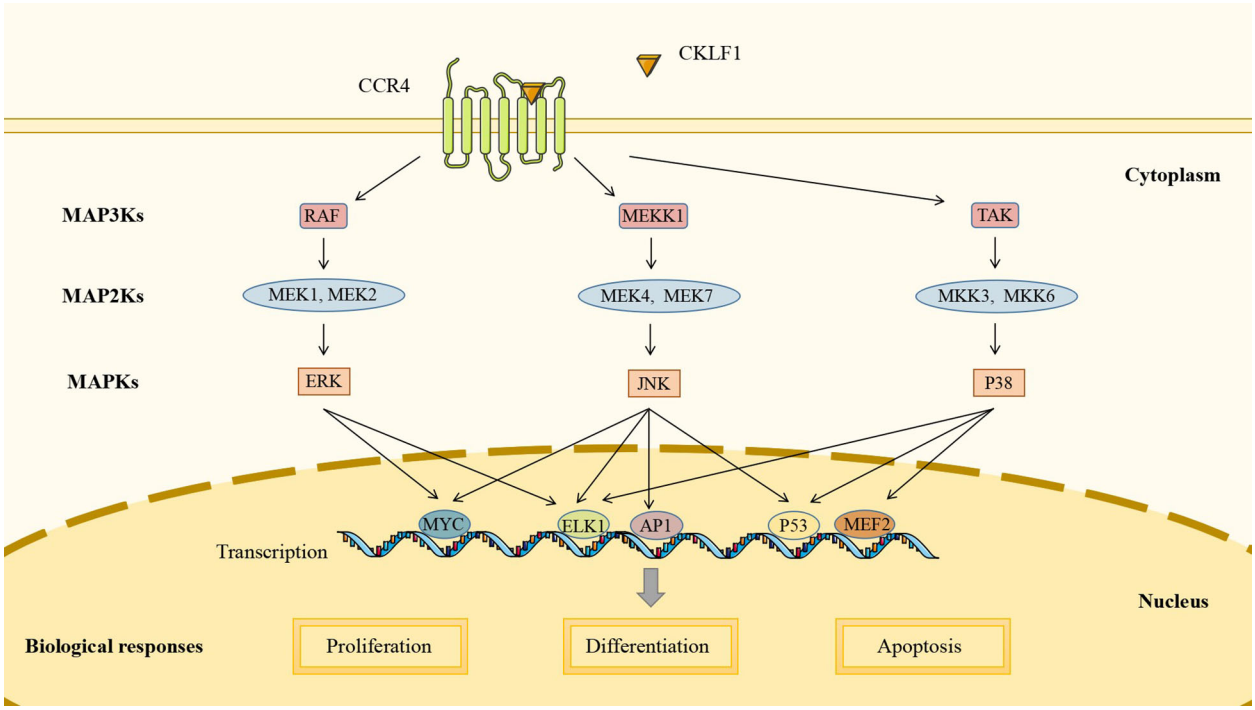

FIGURE 2
CKLF1 mediates cell proliferation, differentiation, and apoptosis by binding to CCR4 and activating the MAPK signaling pathway to induce intranuclear gene transcription.

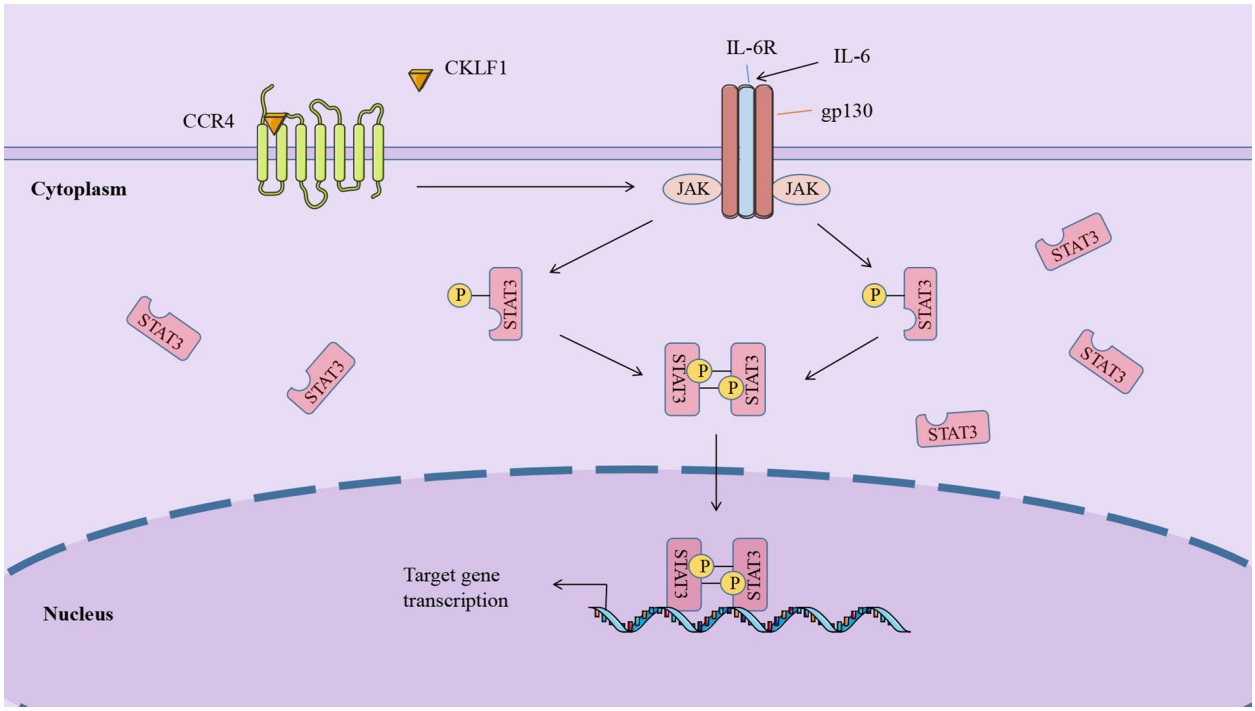

FIGURE 3
CKLF1 binds to CCR4 and induces the cell cycle regulatory gene expression through the IL-6/JAK/STAT3 signaling pathway, which is involved in tumor development.

(phosphatidylinositol-dependent protein kinase 1) and mTORC2 (mammalian target of rapamycin complex 2) protein kinase to act on diverse downstream substrates and carry out biological functions like regulating cellular metabolism (118, 119).

CKLF1 has been found to contribute to inflammatory injury through a PI3K/AKT-dependent mechanism. VSMCs at lesion sites after vascular injury secrete CKLF1, which binds to the G protein-coupled receptor CCR4 to activate the PI3K/AKT signaling pathway, which regulates target gene transcription decreases susceptibility to apoptosis of G2/M phase cells and accelerates VSMC accumulation (120). Studies have revealed that CCR5 expression was upregulated after nerve injury (121, 122). In parallel, the CKLF1/CCR5 axis can activate downstream GSK3 *via* the AKT pathway and mediate neutrophil migration (36) (Figure 4).

4.5 Other signaling pathways

In addition to the four kinds of classical inflammatory signaling pathways, CKLF1 also functions by mediating other signaling pathways. Protein tyrosine kinase 2 (PYK2) is commonly present in actin filaments and is involved in the actin skeleton reorganization process. CKLF1 regulates cell migration-associated actin backbone reorganization *via* the non-extracellular Ca^{2+}-dependent PYK2 pathway. A study found that CKLF1 in SH-SY5Y cells affected downstream phospholipase C-γ (PLC-γ) activity by binding to CCR4, triggering the hydrolysis of membrane phosphatidylinositides PIP2 and the production of second messengers DAG and IP3. IP3 then modulated the migration of SH-SY5Y cells by inducing the release of stored calcium ions and initiating autophosphorylation at the PYK2-tyr402 site (54) (Figure 5). NLRP3 inflammasomes are multimeric cytoplasmic protein complexes composed of the sensor protein NLRP3 linked to ASC and caspase-1, which are closely associated with oncological and metabolic diseases (123). CKLF1 was found to act on NLRP3-related signaling pathways and exert pro-inflammatory effects. CKLF1 expression was increased in the context of cerebral ischemic injury. Overexpressed CKLF1 bound to CCR4 and mediated the activation of downstream NLRP3 inflammasomes. The activation of caspase-1 then invoked the maturation and release of IL-1β, IL-18, and other pro-inflammatory cytokines, further exacerbating the inflammatory injury (124) (Figure 6).

4.6 Signaling crosstalk

The different signaling pathways mediated by CKLF1 are not independent of each other but present signaling crosstalk that is highly dependent on the cellular environment. CKLF1 can simultaneously activate multiple signaling pathways involved in the pathogenic process. IL-6 and STAT3 are key components of the IL-6/JAK/STAT3 pathway and are also important products downstream of NF-κB. STAT3 acts on the CKLF1-mediated NF-κB pathway through two routes: a. STAT3 inhibits IKK activity and attenuates NF-κB pathway-associated Th1 cell immunity; b. activated STAT3 inhibits nuclear NF-κB-IκB complex migration to the cytoplasm through P300-mediated acetylation. Nuclear retention of NF-κB-IκB facilitates prolonged activation of the NF-κB pathway (125). In addition to being a stimulatory signal for the JAK/STAT3 pathway, IL-6 activates PI3K/AKT signaling and induces the MAPK cascade *via* SHP2 (tyrosine phosphatase containing the SH2 domain) (126). Meanwhile, CKLF1 links inflammatory stimuli and cellular responses by mediating MAPK (127–130) and PI3K/AKT (65) pathways to activate NF-κB transcription factors. In addition, NF-κB may be involved in the activation process of NLRP3 inflammasomes by CKLF1, of which the exact mechanism needs further clarifi (131) (Figure 7).

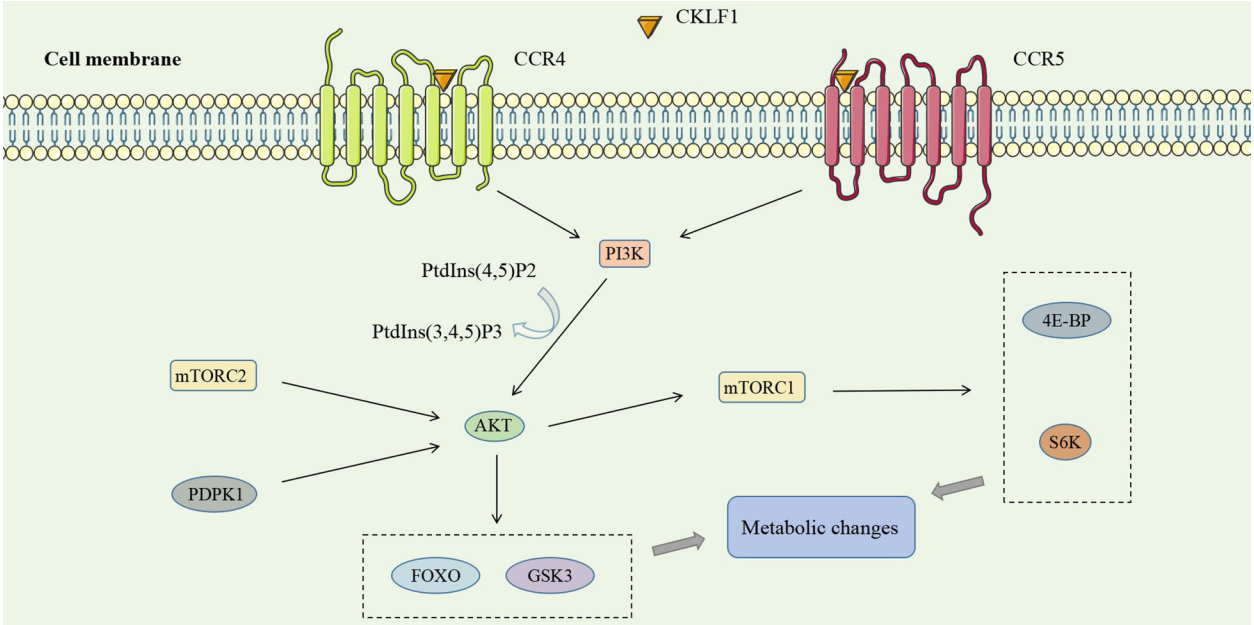

FIGURE 4
CKLF1 binds to both CCR4 and CCR5 and activates the PI3K/AKT signaling pathway, which plays a role in inflammatory injury through diverse downstream substrates of AKT and mTORC1.

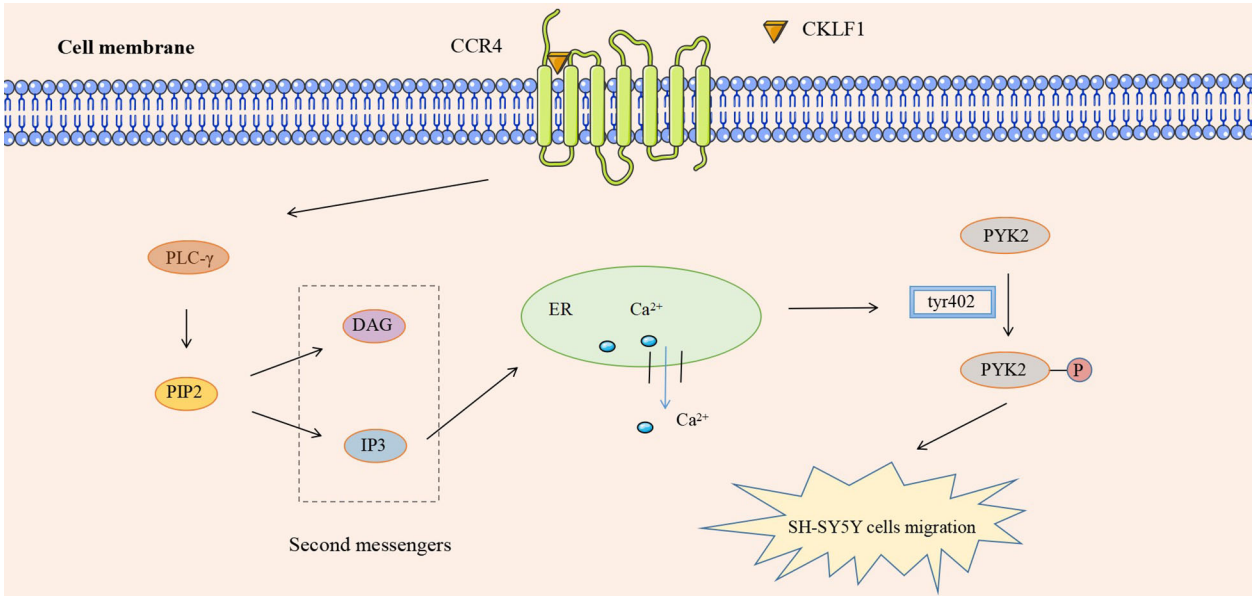

FIGURE 5
CKLF1 binds to CCR4 and induces the release of stored Ca2+ through the PYK2 pathway, activates autophosphorylation at the PYK2-tyr402 site, and regulates the migration of SH-SY5Y cells.

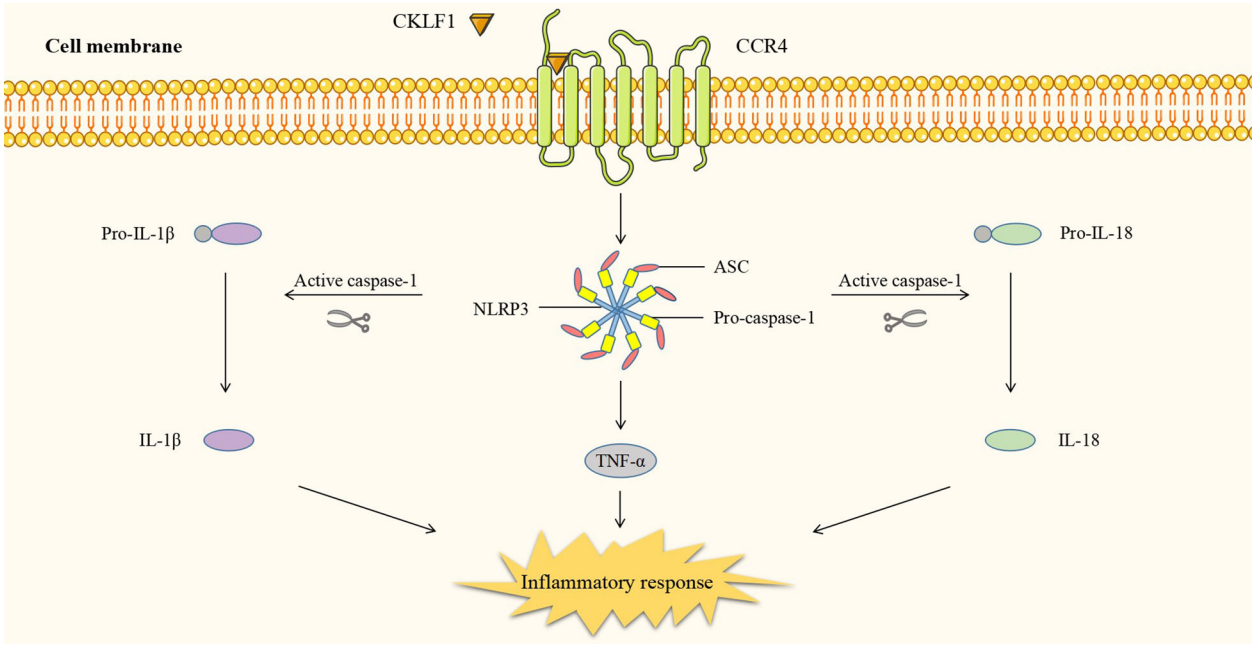

FIGURE 6
CKLF1 binds to CCR4 and mediates the activation of downstream NLRP3 inflammasomes. Activated caspase-1 induces maturation and release of pro-inflammatory cytokines and exacerbates inflammatory injury.

5 Treatments against CKLF1 mechanisms

Given the crucial role of CKLF1 in human diseases, treatments targeting CKLF1, CKLF1 receptors, and related pathways have become a hot research topic in the field of chemokines. (Table 2) Treatments targeting CKLF1 are highly specific and may be a preferred orientation for therapeutic options. A new 3-piperazinylcoumarin analogue (hereafter referred to as compound 41) has been identified as a potent antagonist of CKLF1. *In vitro* and mouse model experiments showed that compound 41 attenuated asthma pathological changes in CKLF1-transfected mice by inhibiting the binding of CKLF1 to CCR4 and affecting the activation of downstream NF-κB and other transduction pathways (56). By screening and optimization, investigators then obtained two derivatives of compound 41, IMMLG-5521 and IMM-H004. IMMLG-5521 inhibited CKLF1-induced eosinophil infiltration and TNF-α release and attenuated lung injury in rats (133). Subsequent

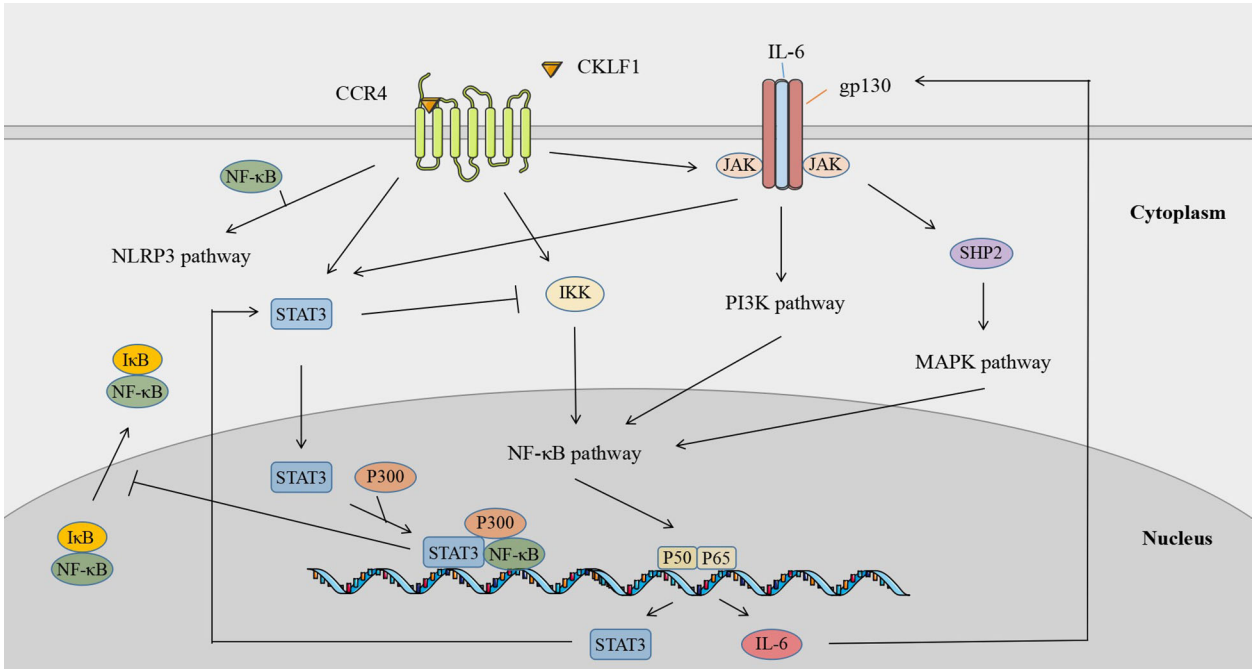

FIGURE 7
Signaling crosstalk across downstream pathways of CKLF1.

TABLE 2 Treatments against CKLF1 mechanisms.

Treatment strategy		Drugs	Limitations
Targeting CKLF1		Compound 41, IMMLG-5521,IMM-H004	Lack of clinical trial
Targeting CKLF1 receptors	CCR4	Compound 6b, compound 8a, 10E4, KW-0761	Species crossreactivity and pharmacokinetic properties remain to be solved
	CCR3	SB-328437 (132)	
	CCR5	Maraviroc (27)	
Targeting related pathways	NF-κB signaling pathway	WAY-169916, Bay-7082	Similar signaling processes in other cells are affected
	MAPK signaling pathway	SP600125, PD98059,SB203580	
	JAK/STAT3 signaling pathway	SOCS, CP-609550 , Stattic	
	PI3K/AKT signaling pathway	LY294002, wortmannin	

studies showed that IMMLG-5521 inhibited CKLF1-induced eosinophil infiltration and TNF-α release, attenuating lung injury in rats. At the same time, IMM-H004 decreased the activation of microglia *via* the CKLF1/CCR4 axis, and played a protective role in central nervous system diseases such as Alzheimer's disease and brain ischemia (48, 134, 135).

CCR4 is highly expressed in CKLF1-related diseases, and therapies targeting this receptor are being diligently developed. A study applied two identified CCR4 antagonists (hereafter referred to as compound 6b and compound 8a) to allergic rhinitis and asthmatic mice and observed that they somewhat alleviated symptoms (32). A CCR4-specific monoclonal antibody (referred to as 10E4 in this study) exhibits efficacy upon binding to the N-terminal end of CCR4 (136). KW-0761, a humanized monoclonal antibody targeting CCR4, has passed the phase I clinical trial and is intended to treat inflammatory diseases and tumors (132). In addition, the researches of antibodies targeting CCR3 and CCR5 have made some progress. Studies on applying the above antibodies in CKLF1-mediated disease are still lacking, but it could be hypothesized that they exert therapeutic effects by inhibiting the functional receptors of CKLF1. Classical inflammatory pathways dominate CKLF1 downstream signaling, and related inhibitors have been better characterized. For example, NF-κB transcription inhibitors WAY-169916 and Bay-7082, MAPK inhibitors SP600125, PD98059 and SB203580, JAK-STAT3 inhibitors SOCS, CP-609550, and Stattic, as well as PI3K pathway inhibitors LY294002 and Wortmannin (126, 137) can inhibit the biological function of CKLF1 by blocking signal transduction. Treatments targeting CKLF1 signaling pathways suffer from the drawback that similar signaling processes in other cells are affected. However, this drug category still provides a therapeutic option worthy of investigation.

6 Discussion

The discovery of CKLF1 opens up new insights into the immune-inflammatory mechanisms of diseases - excessive production of

CKLF1 disrupts the balance of the immune environment to exert harmful effects. Current studies have been directed at the downstream signaling pathways of CKLF1, whereas clarity on its upstream regulatory mechanisms is still lacking. Here, we explored the possible upstream regulatory mechanisms of CKLF1 based on the existing studies on the regulatory mechanisms of chemokine family members. Gene-level regulation is mainly reflected in single nucleotide polymorphisms (SNPs). The CCL2-2518-A/G polymorphism has been extensively studied and proven to be a risk factor for Alzheimer's disease (138, 139). SNPs of CXCL9-11 are closely associated with liver fibrosis (140). Apart from that, enhancers and promoters can activate transcription factors like NF-κB and AP-1 in response to inflammatory factors such as TNF-α and IL-6 and engage in the epigenetic regulation of chemokines (141). Post-transcriptional modifications, including DNA methylation and LncRNAs (long-stranded non-coding RNAs), were found to be associated with enhancer and promoter regions (142–144), which can exert a regulatory effect on chemokines by modulating inflammatory factor-responsive cis-acting elements. CHIP assay and luciferase assay showed that NF-κB could bind to the promoter region of the *CKLF1* (the core site of the *CKLF1* promoter is located at -238 to -249 bp) during ischemia and upregulate its expression (145). This finding confirms the epigenetic regulation mechanism of CKLF1. In recent years, cis-acting elements have emerged as favorable candidates for pharmacological interventions due to their broad activity and high level of target specificity against numerous pro-inflammatory genes. It is thus hypothesized that modulation of CKLF1 level by targeting the core site of the promoter of the CKLF1 gene may be beneficial for clinical treatments.

The main biological effects of CKLF1 are chemotactic activity and proliferation promotion. The good model of cerebral ischemia suggests that CKLF1 plays different roles in different stages of diseases, which may be a combination of the two biological effects. In the early stage of cerebral ischemia, CKLF1 recruits immune cells and aggravates brain injury; in the late stage of cerebral ischemia, CKLF1 promotes neurological recovery by promoting neuron and vascular regeneration (36). Current studies have focused on early stages of diseases, finding the time point at which the effect of CKLF1 changes is challenging. In addition, the role of CKLF1 on microglia polarization may be another reason for the difference. Microglia polarization has been found to be involved in the pathogenesis of a growing number of diseases. In immune-inflammatory diseases such as multiple sclerosis (146), M1 polarization in the early stage can induce the release of pro-inflammatory factors and exacerbate inflammatory response, while M2 polarization in the late stage is beneficial to inflammation regression and tissue repair. It has been confirmed that CKLF1 promotes M1 polarization in early cerebral ischemia, and future studies may further confirm the effect of CKLF1 on M2 polarization in the late stage of diseases. The diverse effects of CKLF1 in different stages of diseases suggest that the timing of using CKLF1 antagonists and agonists should be carefully selected.

Author contributions

JF conceived and revised this review. YL took charge of the original manuscript writing. HY drew the figure and table. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Prospects for targeting ACKR1 in cancer and other diseases

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The chemokine network is comprised of a family of signal proteins that encode messages for cells displaying chemokine G-protein coupled receptors (GPCRs). The diversity of effects on cellular functions, particularly directed migration of different cell types to sites of inflammation, is enabled by different combinations of chemokines activating signal transduction cascades on cells displaying a combination of receptors. These signals can contribute to autoimmune disease or be hijacked in cancer to stimulate cancer progression and metastatic migration. Thus far, three chemokine receptor-targeting drugs have been approved for clinical use: Maraviroc for HIV, Plerixafor for hematopoietic stem cell mobilization, and Mogalizumab for cutaneous T-cell lymphoma. Numerous compounds have been developed to inhibit specific chemokine GPCRs, but the complexity of the chemokine network has precluded more widespread clinical implementation, particularly as anti-neoplastic and anti-metastatic agents. Drugs that block a single signaling axis may be rendered ineffective or cause adverse reactions because each chemokine and receptor often have multiple context-specific functions. The chemokine network is tightly regulated at multiple levels, including by atypical chemokine receptors (ACKRs) that control chemokine gradients independently of G-proteins. ACKRs have numerous functions linked to chemokine immobilization, movement through and within cells, and recruitment of alternate effectors like β-arrestins. Atypical chemokine receptor 1 (ACKR1), previously known as the Duffy antigen receptor for chemokines (DARC), is a key regulator that binds chemokines involved in inflammatory responses and cancer proliferation, angiogenesis, and metastasis. Understanding more about ACKR1 in different diseases and populations may contribute to the development of therapeutic strategies targeting the chemokine network.

KEYWORDS

ACKR1, DARC, chemokine, cancer, inflammation

Introduction

Chemokine receptors (CKRs) are specialized seven-transmembrane domain surface receptors in the class A subfamily of the G-protein coupled receptor (GPCR) superfamily. Chemokine ligands are small, structurally-conserved proteins categorized by the configuration of a cysteine motif (CXC, CC, CX3C, C) in the N-terminus (1). The

classical function of chemokine GPCRs is to activate leukocyte migration along increasing chemokine concentration gradients towards their source, with different tissues producing distinct combinations of chemokines to attract specific cell types. Chemokine messages elicit complex, multicellular responses encoded in the combinatorial diversity of overlapping ligand-receptor specificities and dynamic membrane interactions. Receptor stimulation recruits β-arrestins, an intracellular effector that decreases activation of heterotrimeric G-proteins, scaffolds cytoskeletal adaptors that internalize surface receptors, and signals through distinct pathways (2). The chemokine network is tightly regulated with overlapping mechanisms to amplify, diversify, and resolve cellular signals (3). One arm of chemokine control is exerted through expression of atypical chemokine receptors (ACKRs), dedicated chemokine receptors uncoupled from G-protein cascades that regulate chemokine patterning and GPCR sensitivity (4). CKRs and ACKRs have complementary roles in exerting and modulating chemokine function. ACKRs have an independent role to bind, scavenge, and traffic chemokine ligands and maintain gradients so that cells are directed to their functional compartments (5). ACKRs can also directly regulate GPCR signaling through ligand depletion or resolution of activated intracellular cascades.

Chemokine signals are crucial for immune cell recruitment, embryonic development, and retention of discrete cellular niches. Consequently, dysregulation of the chemokine network can contribute to a multitude of disease and CKRs are appealing therapeutic drug targets. GPCRs are the target of a third or more of all drugs, but chemokine GPCRs present unique challenges to drug design that prevent compounds from progressing to approved therapeutics (6, 7). Inhibitors of individual GPCRs can have deleterious side effects by perturbing the balance of these signal pathways and interfering in unrelated physiological functions that involve the target GPCR. A druggable chemokine network becomes more achievable when the interplay of signaling and regulatory components in the system is well-understood. Here we discuss the role of ACKR1/DARC in disease and potential therapeutic strategies.

The atypical chemokine receptor family

The four known atypical chemokine receptors, ACKR1-4, exhibit distinct expression patterns, chemokine-binding profiles, and cellular effects. The chemokine ligands of the atypical receptors are shown in Figure 1. ACKR1 is a promiscuous receptor for chemokines involved in diverse functions including angiogenesis, chemotaxis, and cellular retention signals. Expression is restricted to erythroid cells, cerebellar Purkinje neurons and the endothelial cell lining of capillary-draining venules, where ACKR1 binds and transports chemokines. ACKR2 binds the second-most chemokines and was thought to be restricted to binding CC-class chemokines until recent reports have described interactions with CXCL10 and CXCL14 (8–10). ACKR2 is primarily found in the lymphatic, not vascular, endothelium but it is also expressed in certain B-lymphocytes, myeloid immune cells, and developing trophoblasts (11–13). ACKR2 serves as a chemokine scavenger that constitutively recycles from membrane to endosome through a pathway involving β-arrestin (14). ACKR3 is a high affinity receptor for several proteins including endogenous opioid peptides and viral chemokine vCCL2/vMIP-II, but only binds two human chemokines, CXCL11 and CXCL12 (15, 16). ACKR3 expression has been described in a diversity of cell types with increasing evidence of ligand-specific, β-arrestin-mediated signaling pathways and multiple internalization mechanisms (17–19). ACKR4 binds CCL19, CCL20, CCL21, CCL22 and CCL25, a subset of chemokines associated with spatial organization of T-cells and dendritic cells (20). Knowledge of ACKR4 expression is incomplete, but it has been characterized as a component of endothelial barriers in tissues including the skin, spleen, and lymphatic vasculature and as a scavenger on fibroblasts in the dermis and intestinal submucosa (21–23). ACKR4 scavenging uses a similar internalization mechanism to ACKR2 involving β-arrestin recruitment, but without the downstream ERK1/2, Akt, or Src kinase activation attributed to ACKR3 (24). Candidate members of the ACKR family include CC chemokine receptor-like 2 (CCRL2/ACKR5) as a receptor for the chemotactic protein chemerin, and membrane-associated phosphatidylinositol transfer protein 3 (PITPNM3/ACKR6) as a receptor for CCL18 (25, 26). Overall, ACKRs bind the majority of CC and CXC chemokines and expression is spatially organized in tissues to maintain functional chemokine gradients and regulate GPCR signaling. ACKR1 has several advantages as a potential drug target because it is promiscuous and encompasses multiple important chemokine-induced pathways, while being uncoupled from direct signal transduction and exhibiting restricted tissue expression.

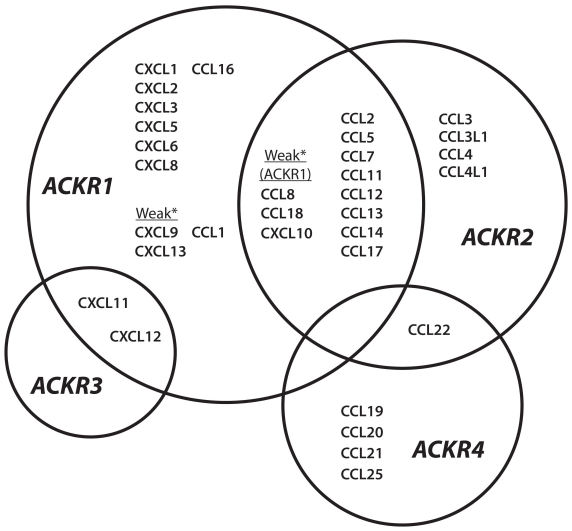

FIGURE 1
ACKR1-4 chemokine interaction network Chemokine ligands described for atypical chemokine receptors ACKR1, ACKR2, ACKR3, and ACKR4. *Chemokines are described as weak binders to ACKR1.

ACKR1 genetics

ACKR1 expression in humans was initially described as the "Duffy" or "Fy" blood group after a hemophiliac patient who developed hemolytic reactions from mismatched blood (27). The recognition sites of the "Fy-reactive" antibodies were mapped to distinct erythrocyte surface antigens, later revealed to correspond to regions of ACKR1. These include a conformational epitope (Fy3) capturing the extracellular loops, a linear pentapeptide sequence in the N-terminus (Fy6), and allelic N-terminal single nucleotide polymorphism (SNP) variants (FyA and FyB). Multiple ACKR1 phenotypes arise from SNPs in the upstream promoter and coding sequence of the *ACKR1* gene (28). The major isoform of ACKR1 is a 336 amino acid protein with two common alleles FyA (42Gly), FyB (42Asp), and the less common FyX, most associated with R89C (29).

A unique selective pressure from malaria parasites contributes to distinct population-specific and geographic patterns of ACKR1 expression (30). The N-terminus of ACKR1 is a recognition site for *Plasmodium vivax* and *P. knowlesi*, which invade erythrocytes during blood infection (31). Malarial resistance is conferred by the "Duffy-negative" or "erythrocyte silent" (Fy^{ES}) single nucleotide polymorphism (SNP), that alters the GATA1 transcription factor binding site in the *ACKR1* promoter, ceasing erythroid, but not endothelial, expression (32). The coevolutionary history of *Plasmodia* parasites and Fy^{ES} phenotype is complex, but the current evidence indicates that African *P. vivax* selected the "erythroid silent" polymorphism in the FyB allele in endemic regions. FyB^{ES} is now the prevalent phenotype of people in Africa, regions within the Arabian Peninsula, and with African ancestry (33, 34). The ancestral form of ACKR1 may have been FyB, which then adapted through the FyA variation (42G) conferring diminished susceptibility to *P. vivax* or the silencing polymorphism FyB^{ES} (rs2814778) (35, 36). The FyX variant is linked to both R89C and A100T mutations and decreases detection of ACKR1 expression (37). This effect may arise from a disruption in the first intracellular loop between the first and second transmembrane domains, and may interrupt trafficking to the membrane, impede protein folding, or cause formation of destabilizing inter/intra-molecular disulfide bonds (38, 39). The amino acid sequence of ACKR1 is depicted in Figure 2. Current understanding is that the primary drivers of differentiation of ACKR1 expression and the molecular basis of the Duffy blood group are the FyA/FyB alleles encoding Gly42 or Asp42 in the N-terminus and the Fy^{ES} SNP, which determines if ACKR1 is present on erythrocyte surfaces to display epitopes like Fy3 or Fy6. These genetic variations that alter ACKR1 expression and N-terminal sequence may have a significant impact on disease by changing the abundance and distribution of ACKR1 ligands (40, 41).

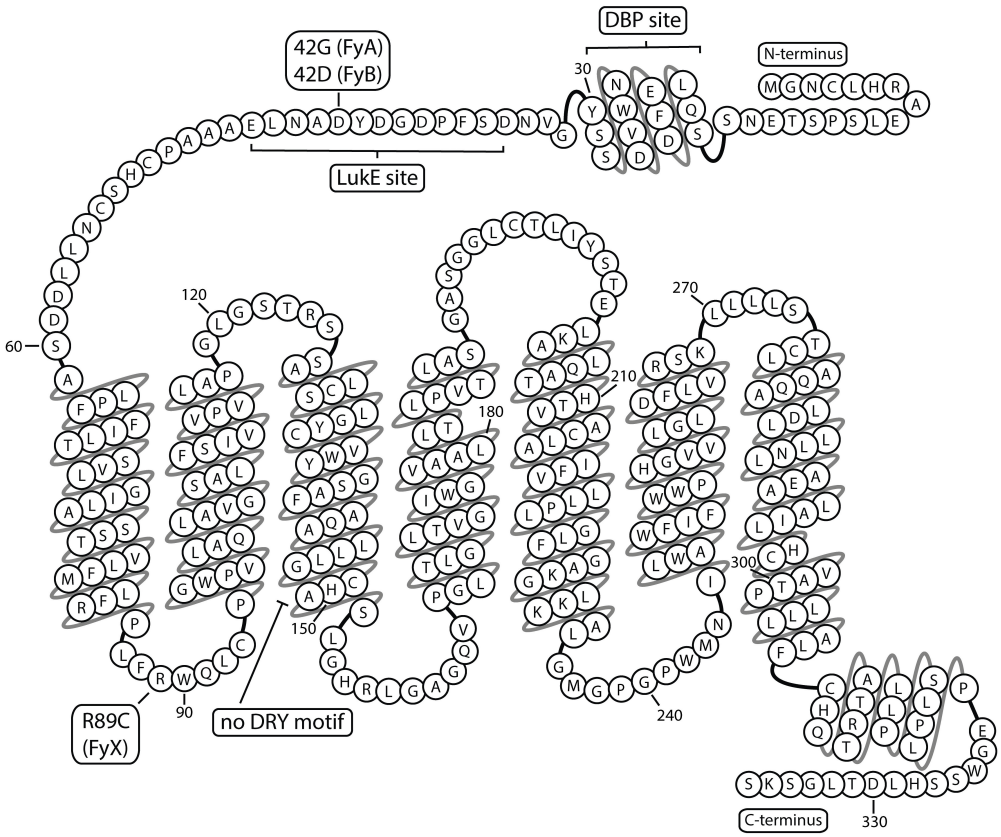

FIGURE 2
ACKR1 snake plot Atypical chemokine receptor 1 has seven transmembrane domains and multiple binding sites in the extracellular N-terminus. Residue 42 is depicted as aspartic acid corresponding to FyB variant. DBP, Duffy Binding Protein; LukE, Leukocidin E.

ACKR1 structure and function

Chemokine receptors are activated after binding ligands in a multi-step interaction using the receptor N-terminus that extends from the first α-helical transmembrane domain. The chemokine binding pocket is formed within the transmembrane helices and the extracellular connecting loop regions. Engagement of a typical chemokine receptor triggers conserved microswitches and conformational changes in the transmembrane helices followed by activation of intracellular secondary messengers (42). G-protein coupling occurs at a conserved "DRYLAIV" sequence motif found at the intracellular end of transmembrane helix 3. However, atypical receptors have sequence modifications at this position that prevent G-protein mediated signaling. While ACKR1 has no homologous motifs at this position, ACKR2 has DKYLEIV, ACKR3 has DRYLSIT, and ACKR4 DRYVAVT. Another common feature of GPCRs is a feedback inhibition mechanism wherein sustained receptor activation leads to phosphorylation of the C-terminus by G-protein coupled receptor kinases (GRKs). GRK activity supports association with β-arrestins, causing receptor internalization and alternative signaling. Both CKRs and ACKRs have serine and threonine-rich sequences in the intracellular C-terminal domain that are substrates for GRK-mediated phosphorylation. β-arrestin recruitment has been described for ACKR2-4, but while ACKR1 has analogous sites encoded in the C-terminus, investigation of GRK interactions has yet to be thoroughly explored (43). Thus, ACKR1 with the lowest sequence similarity to the other chemokine receptors, seems to have a distinct activation mechanism and network of intracellular interactions that is distinct from other ACKRs (44–46).

Solved structures of chemokine receptors are limited in the resolution of receptor N-terminal interactions, but studies support the importance of this domain for atypical chemokine receptor function (47). The ACKR2 N-terminus is selective for CC-type chemokines, and a protein derived from the critical domains has been proposed as an anti-inflammatory chemokine sink (48). The N-terminus of ACKR1 is among the longest of any chemokine receptors and contains extended regions of amino acids modeled to form electrostatic interactions with the basic and positively charged surfaces characteristic of chemokines (49). A distinguishing feature of ACKR1 is the capacity to bind multiple CXC and CC class chemokines, and the flexibility of this mostly disordered region allows for variable configurations to dock many different ligands (50). The binding interactions at the N-termini of ACKRs are shown in Table 1. Discrete ACKR1 N-terminal residues determine ligand affinity and different segments have been successfully engaged by antibodies or antibody-derived fragments to prohibit ligand binding (51, 52). A chimeric construct with the N-terminus of ACKR1 and the transmembrane domains and extracellular loops of CXCR2 retained the binding profile of full-length ACKR1, with high affinity for non-CXCR2 ligands CCL5 and N-terminally modified CXCL1 (53). The independence of the N-terminus for certain ligands also suggests utility of a soluble platform with the binding affinity of ACKR1, for example as a decoy for pathogens targeting erythrocytes. Additional detailed structural data describing interactions between the ACKR1 N-terminus and different chemokine ligands will contribute to understanding conserved and chemokine-specific binding mechanisms.

Initial surveys of ACKR1 functions suggested a binding preference for chemokines containing the sequence motif "ELR" in the N-terminus, a subgroup of CXC chemokines distinguished for its capacity for angiogenesis and inflammatory signaling through neutrophil receptors CXCR1 and CXCR2 (54, 55). One of the first reported angiogenic chemokines was CXCL8, and a model of neovascularization emerged with ELR$^+$ CXCR2 ligands stimulating endothelial migration and tube formation countered by ELR$^-$ CXCR3 ligands. Angiogenic effects have since been ascribed to non ELR$^+$ CXCL12 and other CC chemokines, particularly CCL2, suggesting a multifactorial system of CXC and CC chemokine receptors on endothelial cells and other immune cell types (56, 57). Evidence for the anti-angiogenic properties of ACKR1 was initially shown in a mouse by overexpressing ACKR1, decreasing CXCR2-mediated corneal angiogenesis in response to CXCL2 stimulation (58). Further investigation using radioligand displacement supported strong binding of ACKR1 to ELR$^+$ chemokines like CXCL5 and CXCL8 that signal through CXCR2, but highest binding affinities were calculated for CCL5, CCL7, and

TABLE 1 Ligands of atypical chemokine receptors 1-4.

	CC	CXC	non-CK
ACKR1	CCL2, CCL7, CCL11, CCL13, CCL14, CCL17 Weak*: CCL1, CCL8, CCL18	CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL8, CXCL11, CXCL12, Weak*: CXCL9, CXCL10, CXCL13	LukE, HlgA, PvDBP, PkDBP
ACKR2	CCL2, CCL3, CCL3L1, CCL4, CCL4L1, CCL5, CCL7, CCL8, CCL11, CCL12, CCL13, CCL14, CCL17, CCL22	CXCL10	HIV gp120, Staphopain A
ACKR3	vCCL2	CXCL11, CXCL12	Adrenomedullin, Adrenorphin, BAM18/22, Dynorphin A/B, MIF, Nociceptin NH2, Peptide E
ACKR4	CCL19, CCL20, CCL21, CCL22, CCL25	–	–

Atypical chemokine receptors bind chemokines of CC and CXC classes and have non-chemokine ligands. ACKR1 is targeted by *Plasmodium vivax* and *Plasmodium knowlesi Duffy* Binding Proteins (PvDBP and PkDBP) and by *Staphylococcus aureus* toxin proteins Leukocidin E (LukE) and γ-hemolysin A (HlgA). *Chemokines demonstrated weak binding affinity to ACKR1 in competition assays and their physiological relevance is uncertain. ACKR2 has been reported to bind HIV envelope glycoprotein gp120 and is a substrate for *S. aureus* cysteine protease Staphopain A. ACKR3 binds numerous peptides, the peptide hormone adrenomedullin, endogenous opioid peptides in the dynorphin, enkephalin, and nociceptin families, and macrophage migration inhibition factor (MIF).
-, none reported.

non-ELR$^+$ CXCL11 (59). The next functional categorization was regulation of "inflammatory" chemokines over "homeostatic" chemokines since chemokines CXCL12 and CCL21 showed weak ability to displace CXCL8 bound to ACKR1 (59). However, studies have since described many roles for both chemokines in inflammation and binding interactions have been reported between ACKR1 and CXCL12 (60, 61). ACKR1 binds most chemokines including the ELR$^+$ CXC subfamily, and chemokines CXCL10, CXCL13, and CCL1 that were reported as non-binders were found to have weak but sub-micromolar affinities for ACKR1 on human erythrocytes (59). ACKR1 does not bind every chemokine, for example CXCL4 and several lymphoid CC chemokines have been shown not to bind ACKR1-expressing cells (59, 62).

The binding profile of ACKR1 has been primarily surveyed using radioligand displacement assays with pre-bound, high-affinity ligands and erythrocyte ACKR1 that may underrepresent lower-affinity interactions with chemokines or the influence of other mediators on endothelial surfaces like glycosaminoglycans. This selectivity was reported to play a role in filtering chemokines at high endothelial venules (HEVs), where ACKR1 may restrict inflammatory chemokines from entering secondary lymphoid organs and interfering with chemokine sensitivity (62).

While ACKR1 is most readily detected on mature erythrocytes, ACKR1 expression is highest in the bone marrow on progenitor nucleated erythroid cells (NECs), where key cell contacts are made with hematopoietic stem cells (HSCs) (63). The erythroid silent variant (FyES), though providing malarial protection, loses this developmental cue, resulting in a neutrophil phenotype with altered surface markers and increased propensity to leave circulation (64, 65). The observed neutropenia, historically called "benign ethnic neutropenia" and now more accurately "Duffy-associated neutrophil count" (DANC), does not eliminate effective inflammatory immune responses and is hypothesized to be asymptomatic in otherwise-healthy patients (66–68).

Outside of the erythroid lineage, ACKR1 is expressed on endothelial cells of post-capillary venules, where affinity for certain chemokines results in immobilized gradients that direct cell migration (69–71). A hallmark of tissue inflammation is increased chemokine production, but chemokines must be concentrated and displayed in the vascular compartment with a coordinated gradient to effectively direct immune responses. Endothelial ACKR1 function involves a combination of chemokine retention, presentation to circulating leukocytes, and trafficking from tissues to the luminal surface (72). ACKR1 is distinguished from the other ACKRs by ligand-triggered chemokine transcytosis through venular endothelial cells. ACKR1 has been shown to transport chemokines from basolateral to luminal sides of endothelial cells and retain chemokines on the apical surface promoting signaling through GPCRs (73–76). One demonstration of this function is neutrophil diapedesis, where ACKR1 concentrated at endothelial junctions binds and exchanges CXCL1 and CXCL2 chemokines to direct neutrophils and prevent reverse migration (77). These functions at the endothelium have been shown to modulate neuroinflammation as well, by trafficking chemokines and immune cells across the blood-brain barrier (78, 79). ACKR expression is detected in the brain on cerebellar Purkinje cells, where it may regulate cellular excitation for smooth motor control (53, 80). Further studies of ACKR1 in different tissues, including neurons, and with non-chemokine ligands may reveal additional complexity and specialized functions.

ACKR1 and infectious disease

The extracellular domain of ACKR1 is a potential target to inhibit pathogenicity mechanisms of atypical malaria, *S. aureus*, and HIV. *Plasmodia* malarial parasites replicate and mature inside human reticulocytes and erythrocytes, and the "atypical" *P. vivax* and *P. knowlesi* parasites identify these targets by secreting Duffy Binding Protein (DBP), which binds to and oligomerizes around the N-terminal domain of ACKR1 (81). While *P. falciparum* secretes multiple soluble factors, atypical malaria invasion can be avoided with the erythroid silent polymorphism or by blocking the DBP-ACKR1 binding interface with inhibitory chemokines or antibodies (51, 82, 83). Crystal structures have been solved showing a dimer of PvDBP dimers binding a peptide corresponding to ACKR1 residues 14-43. The receptor peptide could be resolved between residues 19-30 as an amphipathic α-helix structure with Y30 oriented towards a positively charged pocket (84). An ACKR1 mimetic was designed from this N-terminal helix, with the DBP-binding residues grafted onto a stable scaffold (85). The engineered protein could successfully inhibit DBP dimerization and binding to erythrocytes. Non-*falciparum* malaria, particularly from *P. vivax*, is an increasingly widespread disease that can cause severe or fatal illness, and the dependence on ACKR1-mediated invasion provides a prime therapeutic target (86).

A role for ACKR1 has been proposed in HIV pathogenesis, however the potential mechanisms of interaction are unclear. HIV uses chemokine receptors CXCR4 or CCR5 as co-receptors for targeting leukocytes, and the CCR5 inhibitor Maraviroc can successfully prevent binding by viral glycoproteins (87). Some studies have proposed ACKR1 is involved in HIV interactions with erythrocytes that promote infection of other blood cells or maintain a viral reservoir (88–90). However, the FyES phenotype was not confirmed to alter HIV susceptibility or disease progression (91, 92).

ACKR1 is also a target for *Staphylococcus aureus* toxins LukED and HlgAB (93). *S. aureus* bacteremia is particularly dangerous because these pore-forming, bicomponent toxin systems cause hemolysis and vascular leakage when they engage ACKR1 on red blood cells and endothelial junctions (94, 95). A crystal structure of the LukE toxin protein and the ACKR1 N-terminus resolved residues 34-46 of the receptor with Y41 stabilized in a lysine and arginine-enriched viral pocket, similar to the mechanism of interaction observed in the crystal structure of PvDBP and ACKR1 (96). Further analysis using time-resolved mass spectrometry and resonance energy transfer from a C-terminal bioluminescent tag suggests toxin binding may modulate receptor conformation to form ACKR1 homodimers and even alter interactions with intracellular $G_{\alpha i}1$ subunits (97). Structure-guided strategies targeting ACKR1 could be useful to address pathogenicity mechanisms of significant infectious agents.

ACKR1 and pathoinflammation

Immune dysregulation involves an excess of chemokines and other soluble inflammatory mediators and can incur tissue damage from resultant immune cell infiltrates. Modulation of the chemokine network to treat autoimmune disease has yielded promising leads, but few have shown clinical effectiveness and safety (98, 99). Currently trials are ongoing for a CCR9 antagonist for Crohn's disease and a CCR1 antagonist for rheumatoid arthritis (100, 101). Reparixin, an allosteric CXCR1 and CXCR2 blocker, did not progress past a phase 3 trial as a drug adjuvant for pancreatic islet allotransplantation to treat type 1 diabetes, but it is still a candidate for ongoing trials for metastatic breast cancer and COVID-19 related acute lung injury (102–104). Alternatively, blocking chemokines may decrease autoinflammation, and an antibody drug bertilimumab targeting CCL11 was designed to prevent eosinophil-mediated autoimmune damage in bullous pemphigoid skin disorder and inflammatory bowel disease (105, 106). Administration of anti-CXCL10 antibody was a promising strategy to limit cytotoxic T-cell liver damage, but clinical utility was hindered by continuous CXCL10 secretion and retention on endothelial cells (107, 108).

Controlling chemokine concentrations *via* ACKR1 could contribute to the success of these drug strategies or offer new avenues for regulating immune responses. ACKR1 regulation may contribute to resolution of chemokine-driven inflammation. ACKR1 binds chemokines at the inflamed synovial endothelium, and diminished expression of ACKR1 may be associated with rheumatoid arthritis (109). People with the FyES phenotype that decreases erythrocyte ACKR1 were observed to have increased IgE in serum samples and higher susceptibility for asthma (110). Knocking out all ACKR1 expression in an endotoxin-induced mouse model of inflammation was shown to increase lung and liver damage from granulocytic infiltrates (111). These studies support a protective role for ACKR1 by decreasing circulating chemokine levels, particularly through expression on erythrocytes.

However, ACKR1 on the endothelial surface may have separate functions in chemokine retention and has been observed to increase leukocyte recruitment and activity (112). Endothelial ACKR1 expression may potentiate respiratory distress, as seen in patients with suppurative pneumonia, and require balance from erythrocyte ACKR1 to avoid acute lung injury (113, 114). This finding has been reinforced in mouse models of lung inflammation, where studies show that ACKR1 knockout mice are protected from neutrophil-mediated tissue damage (115, 116). ACKR1 receptors supporting chemokine-mediated leukocyte infiltration have also been reported to contribute to patient lesions of giant cell/temporal arteritis and nephrotoxicity in a mouse model of renal failure (117, 118).

ACKR1 can also facilitate neutrophil reverse transendothelial migration and indirectly cause systemic inflammation (119). Using aged mice subjected to IL-1 stimulation, ACKR1 was shown to concentrate mast cell derived CXCL1 at endothelial junctions, causing desensitization of CXCR2 on circulating neutrophils and dysregulated chemotaxis. Without tight regulation of chemokine patterns, the activated neutrophils migrated to the lung leading to vascular leakage, which could be a targetable mechanism for aging-related inflammation or acute lung injury such as COVID-19 pneumonia (120, 121). An increase in ACKR1 expression was also detected in humoral and cellular rejection of renal allografts, but it remains unclear if upregulation is induced by an inflammatory program, or which component of graft rejection would be influenced (122, 123).

Chemokines are also important mediators of chronic inflammatory damage in cardiovascular disease, including atherosclerosis, where chemokine concentrations, combinations, and oligomerization all contribute to initiation and progression of vascular lesions (124). ACKR1 involvement and targeting to treat atherosclerosis was initially proposed because endothelial dysfunction and chemokines like CXCL8 immobilized on erythrocyte membranes contribute to plaque formation and coronary artery disease (125, 126). In an atherosclerosis mouse model, knocking out ACKR1 led to diminished plaque formation, cellular infiltrate in the vessel walls, and activation of macrophages (127). As the chemokine network is further studied in the context of cardiovascular diseases, ACKR1 binding inflammatory chemokines may become a relevant drug target. More detailed investigation is required to discern the role of ACKR1 in acute and chronic phases of inflammation and what changes in cellular immune responses may be feasible by targeting ACKR1.

Cancer angiogenesis, metastasis, prognostics

Therapeutic cancer interventions include drugs to attack primary tumors or alter pro-metastatic signals and biomarkers for prognostic screening. Chemokine patterning and chemokine receptor signaling are integral to the proliferation and spread of tumor cells (128). A challenge to targeting CKRs in cancer is that the same chemokines that stimulate tumor growth and neovascularization can also activate and direct tumor-killing immune cells. For example, CCL5 signaling through CCR5 supports recruitment of anti-tumor natural killer cells and cytotoxic T cells, but also stimulates pro-tumor, tissue-resident myeloid cells and lymphocytes (129). Nevertheless, the chemokine receptor drugs that have demonstrated promising anti-cancer activity in clinical trials, particularly antagonizing CCR2, CCR4, CXCR2, and CXCR4, emphasizes the importance of studying chemokine regulation and receptor mechanisms (130).

Neovascularization of an emerging tumor is an essential process to tumor growth and vascular access that involves distorting the balance of pro and anti-angiogenic chemokines (131). Angiogenesis is difficult to target because it can be triggered by tumor cells through an increase in CXCR2 agonism, or by a change in the cellular tumor infiltrate that favor tumor-associated macrophages (132). The mechanism of ACKR1 regulating pro-cancer chemokine signaling involves interplay between endothelial cells and erythrocytes that influences the activation of GPCRs CXCR2 and CXCR3. ACKR1 and the ACKR subfamily may balance chemokine

abundance and patterning to benefit host immune cell recruitment that is lost in unregulated, aggressive cancer types (133, 134).

Studies show that when ACKR1 is expressed on malignant cells it is protective against tumor angiogenesis and subsequent metastasis. Proposed contributions of ACKR1 are shown in Figure 3. When transgenic ACKR1$^+$ non-small cell lung cancer cells were implanted in SCID mice, the resulting tumors had decreased vascularization, and metastatic potential (135). Immunoassay for chemokines secreted by ACKR1$^+$ tumor cells showed a decrease in CXCL5 and CXCL8, and chemokine detection suggested the chemokines were bound by ACKR1 and internalized or immobilized on the cell surface rather than removed from the tumor microenvironment. Another study injected mice with different cancer cell lines that expressed high or low levels of ACKR1 levels to show that cancer invasiveness was inversely related to ACKR1 activity (136). MDA-MB-231 breast adenocarcinoma cells were used to represent aggressive breast cancer with low endogenous ACKR1 expression, and MDA-MB-435 melanoma cells were used to model an ACKR1-expressing tumor (137, 138). Testing in either cell culture or the tumor xenografts showed that ACKR1 expression could prevent the spike of CCL2 and CXCL8 released into the growth media or tumor microenvironment. These findings were correlated with a breast cancer clinical cohort, where patients with higher levels of detectable ACKR1 had less invasive cancers and lower mortality rates. Altering the global ACKR1 expression also changes the tumor microenvironment. ACKR1 global knockout in a spontaneous murine prostate cancer model resulted in less dense, more necrotic tumors with increased intratumor concentrations of CXCL1 and CXCL2 (139). Overexpression of the endothelial ACKR1 in mice implanted with melanoma tumors demonstrated inhibition of tumor growth and vascularity and showed an increase in CD4$^+$ and CD8$^+$ T-cell and macrophage infiltration (140).

Angiogenesis is a continual process in healthy tissue that involves migration, proliferation, and differentiation and ACKR1 could influence feedback mechanisms triggered by CXCR2 signaling pathways. A study investigated how ACKR1 expression on non-malignant endothelial cells could decrease capillary formation and detected an upregulation of senescence biomarkers (141). In pancreatic cancer cells lines, co-expression of ACKR1 in

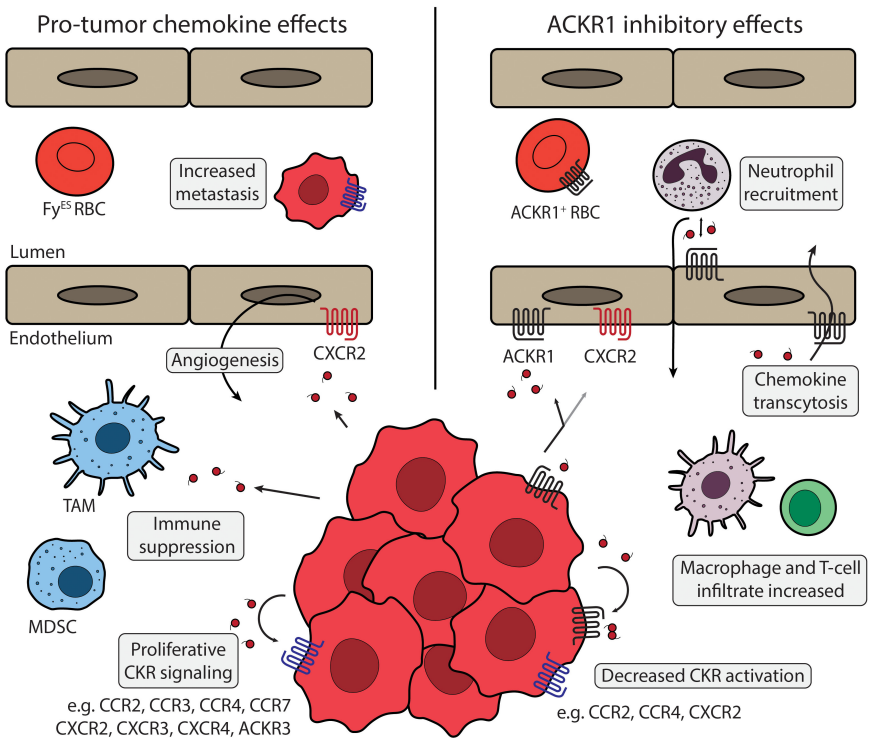

FIGURE 3
ACKR1 and tumor microenvironment Chemokine signaling in the tumor microenvironment is regulated by ACKR1 expression. Left panel describes chemokine effects that promote tumor phenotypes. ACKR1 (black) expression can be diminished on tumor cells or by the Fy^{ES} polymorphism. Angiogenesis can be triggered by chemokines secreted from TAMs, stromal cells, or by cancer cells themselves *via* activation of endothelial CXCR2 (red). Cancer cells release numerous chemokines, including CCL2, CCL5, CXCL8, and others that can act to suppress anti-tumor immunity. Various cancer types express a panel of CKRs (blue) including CCR1, CCR2, CXCR2, CXCR4, and others that support tumor proliferation and metastasis. Primary tumors can silence expression of chemokines like CXCL12 and increase expression of CKRs like CXCR4 to promote metastasis. Right panel shows proposed mechanisms of ACKR1 regulation. ACKR1 receptors on erythrocytes can act as a sink to buffer chemokine levels and may have interactions with ACKR1 expressed on endothelial cells. ACKR1 enrichment at endothelial junctions promotes neutrophil diapedesis *via* CXCL1 and CXCL2 exchange, and increased endothelial ACKR1 improves recruitment of macrophages, CD4+ and CD8+ T-cells. Expression of ACKR1 in cancer models or patient tumor samples has been shown to modulate CCL2 and CXCL8, ligands of CCR2, CCR4, and CXCR2. ACKR1 modulates many chemokines and regulation of multiple CKRs may contribute to the improved clinical outcomes observed. TAM, Tumor associated macrophage; MDSC, Myeloid-derived suppressor cell; Fy^{ES} RBC, "Erythroid-silent" erythrocyte; CKR, chemokine receptor.

CXCR2+ tumors was sufficient to inhibit CXCL8-triggered activation of STAT3 and mediators of epithelial-mesenchymal transition (142, 143). Blocking these oncogenic pathways is an important strategy to induce cellular senescence and restore the anti-tumor effects of immune defenses (144, 145). CXCR2 has a complex role in tumor progression, as receptor overstimulation and autocrine activation may also trigger and sustain a p53-mediated cellular senescence (146). Furthermore, it is possible ACKR1 could contribute cell cycle regulation through other interactions including the tumor suppressor CD82/KAI1, a multifunctional surface tetraspanin. A study found that $CD82^+$ cancer cells have increased adherence to $ACKR1^+$ vascular endothelial cells and suggested that a direct interaction leads to p21 cyclin-dependent kinase inhibition and prevention of metastatic escape (147). A follow-up study also detected p21 upregulation connected to CD82 and potentially ACKR1, and implied that CD82 opposes CXCL8 effects by downregulating secretion from tumors and displacing CXCL8 from endothelial ACKR1 (148). The data interpretation from these reports is limited without testing CXCR2 signaling or reliable antibody detection of ACKR1.

Another important target of anti-cancer therapeutics is metastasis, the major cause of cancer mortality (149). Blocking chemokine signaling is an appealing strategy because metastatic invasion of susceptible cellular niches is inefficient without chemokine-directed migration and often characterized by chemotactic GPCR overexpression (150). ACKR1 may play a role in fine-tuning the complex chemokine patterns that are hijacked by migrating cancer cells. Many of the studies that observed an inverse correlation between the proliferative potential of primary tumors and ACKR1 expression also reported a decrease in metastatic phenotype. Another possible mechanism is alteration of the chemokine oligomeric equilibrium. Chemokine dimers elicit distinct signaling from monomeric chemokines, potentially representing feedback inhibition that could be used as an antimetastatic cue (5, 151, 152). Multiple factors increase the propensity of chemokine dimerization, including GAGs and interactions with the N-termini of GPCRs (153, 154). ACKR1 also shows similar activity by binding preferentially to the dimeric form of CXCL12 (155). Improved quantitation of chemokine concentrations in different cellular compartments and the relation between dimerization and chemotaxis are needed to predict the effects of ACKR1 preferentially binding certain chemokines as dimers.

Testing ACKR1 genotype and expression in tumor biopsies may be a clinically useful cancer biomarker. Multiple studies have indicated that higher ACKR1 expression levels in breast cancer tumors improve relapse-free patient survival, while loss of ACKR1 expression, frequently in patients with African ancestry, is an indicator of increased tumor aggressiveness, metastatic propensity, and mortality (156–162). Detailed analysis is warranted for different cancer types, since comparing prostate cancer incidence within patient groups did not detect a strong correlation between the FyES polymorphism and increased cancer risk (163, 164). Additionally, blood typing to discern ACKR1 phenotype could be an effective, low-cost way to inform cancer treatment. ACKR1-mediated DANC neutropenia affects patient care by impeding administration of drugs like clozapine or azathioprine and leading to potentially unwarranted bone marrow biopsies (165–167). Patients with FyES phenotype are at increased risk of side effects from chemotherapy but using the same neutropenic cutoff values may unnecessarily delay initiation and prolong duration of cancer treatment (168–172). Adapting standard of care for patients with DANC could provide an opportunity to address disparate treatment outcomes with a precision medicine approach. Overall, a cancer-protective role for ACKR1 is supported by cell culture, mouse models, and genetic associations, and independent anti-angiogenic properties for endothelial, erythroid, and tumor ACKR1 expression can contribute to improved patient outcomes.

Discussion

ACKR1 exhibits favorable structural features, expression profile, and biological activity for development of therapeutic interventions. More investigation is needed to determine the extent of control over chemokine scaffolding by ACKR1 that can be attained by different classes of molecules. Antibodies binding to different ACKR1 epitopes do not uniformly inhibit chemokine binding, suggesting some capacity to alter ACKR1 specificity. Development of screening readouts for binding that can supplement competition assays will facilitate identification of small molecules. The independence of chemokine-binding and DBP recognition sites located in the extended N-terminus indicates that this domain could be isolated to provide an effective ACKR1 decoy, similar to a strategy proposed for the ACKR2 N-terminus. The positioning and functions of ACKR1 receptors in the hematopoietic compartment, on the surface of erythrocytes, and at the junctions of endothelial regions specialized for cell trafficking provide an opportunity to control immune cell migration into tissues. Additionally, further exploration of the impact of ACKR1 expressed at the blood-brain barrier and on different neuronal cell types may reveal a targetable role in regulating neuroinflammation. Still, the mechanisms of ACKR1 retaining or sequestering different chemokines have yet to be elucidated in detail, particularly in the context of the tumor microenvironment. Assigning ACKR1 expression to specific cell types within and around tumors of different origins will be needed to understand the correlation observed in experimental models between ACKR1 expression and decreased malignant phenotypes.

Targeting ACKR1 is an appealing approach for new compounds that modulate chemokine biology without interfering with the chemokine sensitivity and signaling functions of immune cell CKRs. ACKR1 in circulation is only reliably found in post-capillary venules and erythrocytes rather than myeloid or lymphoid cells, suggesting targeting ACKR1 would not directly impact immune effector function. While some studies report ACKR1 detection on other cells like bone marrow macrophages, these reports use a polyclonal antibody which has been shown to recognize non-ACKR1 surface markers (173, 174). Furthermore,

unlike the other ACKRs, ACKR1 functions seem independent of G-protein or β-arrestin signaling pathways (175). The restricted tissue and signaling capabilities suggest side effects of ACKR1 inhibition may be modest compared to the signaling GPCRs or other ACKRs. As ACKR1 biology and molecular pharmacology are examined in greater detail, development of new ligands to alter its function will be useful as research tools and may enable amelioration of specific disease pathologies.

Current opportunities for intervention should include shielding extracellular ACKR1 residues from virulence factors of important human pathogens. This approach may have multiple benefits, including preventing erythrocytic replication of *Plasmodia* and maintaining the integrity of endothelial junctions during *S. aureus* infections. Additionally, animal models, cancer cell experiments, ACKR1 biochemistry, and meta-analysis of clinical cohorts all indicate ACKR1 activity impedes cancer progression. This underscores the importance of elucidating ACKR1 chemokine-binding mechanisms and the impact on immune cell responses to tumors to take steps towards enhancement or reconstitution of ACKR1-mediated protection in cancer therapy. Until then, ACKR1 may be used as a prognostic indicator for the aggressiveness of different cancer types and may be inform treatment regimens for patients with different patterns of ACKR1 expression. The next steps include detailing the binding interactions of different chemokines to ACKR1 and the mechanisms that alter receptor expression and enable chemokine trafficking through cells. Future development and implementation of therapeutics that target the chemokine network should consider the role of ACKR1 in patient physiology and the possibility of targeting ACKR1 itself.

Author contributions

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Conflict of interest

Author BV has ownership interests in Protein Foundry, LLC and XLock Biosciences, LLC.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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The clinical efficacy of type 2 monoclonal antibodies in eosinophil-associated chronic airway diseases: a meta-analysis

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Background: Anti-type 2 inflammation therapy has been proposed as a treatment strategy for eosinophil-associated chronic airway disorders that could reduce exacerbations and improve lung function. We performed a meta-analysis of randomized controlled trials to assess the effectiveness of type 2 monoclonal antibodies (anti-T2s) for eosinophil-associated chronic airway disorders.

Methods: PubMed, Embase, Web of Science, and Cochrane Library were searched from their inception to 21 August 2022. Randomized clinical trials evaluating the effectiveness of anti-T2s versus placebo in the treatment of chronic airway diseases were selected. The outcomes were exacerbation rate and change in pre-bronchodilator forced expiratory volume in 1 s (FEV1) from baseline. The Cochrane Risk of Bias Assessment Tool 1.0 was used to evaluate the risk of bias, and the random-effects or fixed-effect model were used to pool the data.

Results: Thirty-eight articles concerning forty-one randomized clinical trials with 17,115 patients were included. Compared with placebo, anti-T2s therapy yielded a significant reduction in exacerbation rate in COPD and asthma (Rate Ratio (RR) =0.89, 95%CI, 0.83-0.95, I^2 = 29.4%; RR= 0.59, 95%CI, 0.52-0.68, I^2 = 83.9%, respectively) and improvement in FEV1 in asthma (Standard Mean Difference (SMD)=0.09, 95%CI, 0.08-0.11, I^2 = 42.6%). Anti-T2s therapy had no effect on FEV1 improvement in COPD (SMD=0.05, 95%CI, -0.01-0.10, I^2 = 69.8%).

Conclusion: Despite inconsistent findings across trials, anti-T2s had a positive overall impact on patients' exacerbation rate in asthma and COPD and FEV1 in

asthma. Anti-T2s may be effective in treating chronic airway illnesses related to eosinophils.

Systematic Review Registration: https://www.crd.york.ac.uk/PROSPERO/, identifier CRD42022362280.

KEYWORDS

eosinophil-associated chronic airway diseases, efficacy, randomized controlled trials, meta-analysis, type 2 monoclonal antibodies

1 Introduction

Chronic airway diseases pose a serious public health risk, causing 3.91 million deaths in 2017, accounting for 7% of all death worldwide, which is mainly attributable to chronic obstructive pulmonary disease (COPD) and asthma (1).

Elevated blood eosinophils, sputum eosinophils, or exhaled breath nitric oxide fraction (FeNO) are common manifestations of eosinophilic airway inflammation, which are associated with increased risk of patient complications, recurrent acute exacerbations, pneumonia, prolonged hospitalization, and increased morbidity and mortality (2–9). Patients with persistent eosinophilic airway inflammation may benefit from inhaled glucocorticosteroids (ICS) (10–14). Nevertheless, long-term ICS therapy may result in several unfavorable adverse events, such as osteoporosis, diabetes, cataracts, and higher infection risk (15, 16). Additionally, ICS is not always well-tolerated by patients.

Several monoclonal antibodies targeting particular inflammatory pathways have been created to address the complications mentioned above. Pathogenic factor-induced cellular release of cytokines, including interleukin (IL)-4, IL-5, IL-9, IL-13, IL-25, IL-33, immunoglobulin E (IgE) and thymic stromal lymphopoietin (TSLP) are closely related to eosinophilic airway inflammation (17, 18). Except for blocking the downstream targets, activation of toll-like receptor 9 (TLR9) has been shown to balance the T helper (Th) 1/Th2 axis (19). Type 2 monoclonal antibodies (anti-T2s) are effective in decreasing FeNO and eosinophil levels (20–24). However, results from previous research, which investigated the effectiveness of anti-T2s in reducing exacerbation rate and improving lung function, have been controversial. Therefore, we performed a meta-analysis of randomized controlled trials (RCTs) to examine the efficacy of anti-T2s for chronic eosinophilic airway diseases, investigating the possibility of endotype-guided strategy in the management of chronic airway disorders.

2 Methods

2.1 Protocol

The study protocol was registered at the International Prospective Register of Systematic Reviews (number CRD42022362280).

2.2 Data sources and search strategies

PubMed, Embase, Web of Science, and Cochrane Library were searched from their inception to 21 August 2022. We used the following search strategy to find all studies evaluating anti-T2s, including IL-5, IL-4, IL-9, IL-13, IL-25, IL-33, IgE, TSLP, and TLR9 for patients with eosinophil-associated COPD and asthma: (mepolizumab OR reslizumab OR benralizumab OR tralokinumab OR lebrikizumab OR dupilumab OR anti-interleukin OR MEDI-528 OR GSK679586 OR omalizumab OR tezepelumab OR AZD1419 OR CYT003 OR itepekimab OR XKH001) AND (asthma OR chronic obstructive diseases). The detailed search strategy is shown in Supplementary Table 1. Languages had no restrictions. The pertinent review articles and their citations were also checked.

2.3 Study selection

Endnote X9 software was adopted to manage the eligible studies during the literature screening and automatically remove duplicate documents. The following particular inclusion criteria were met (1): Participants: individuals (6 years of age or older) with asthma or COPD who met one or more criteria for eosinophilic inflammation at study enrolment or within the previous year. (2) Interventions: with anti-IL-5, anti-IL-4, anti-IL-13, anti-IL-9, anti-IL-25, anti-IL-33, anti-TSLP, anti-IgE or TLR9 agonist therapy at any dose. (3) Randomized placebo-controlled trials. (4) Reporting the following outcomes: exacerbation rate and change in pre-bronchodilator forced expiratory volume in 1 s (FEV1) from baseline.

Excluded criteria were as follows: (1) Studies did not involve eosinophilic endotype. (2) Interventions were not related to type 2 inflammation. (3) Studies did not assess the exacerbation rate or FEV1. (4) Not RCTs or literature types were reviews, letters, second analysis, or conferences.

The source data, together with the rate ratio (RR) or mean difference (MD), are given or can be computed from the data. All references were independently reviewed by two authors (YW and MH) following the selection criteria. Any disagreements were resolved through conversation or by a third author (JL).

2.4 Data extraction and quality assessment

The preferred reporting items for systematic reviews and meta-analyses (PRISMA) statement was followed (25) (see Supplementary

Table 5). Two authors (YW and MH) independently retrieved data from eligible studies using Excel 2019 in a standardized data extraction form in a blinded manner based on the authors, publication year, research design, patient characteristics (age, gender, etc.), the type of anti-T2s used, the dosage, the length of the therapy, the definitions of the outcomes, the exacerbation rate, and the change in FEV1. A third author (JL) was consulted to settle disagreements. Furthermore, we evaluated the risk of bias using Cochrane Risk of Bias Assessment Tool 1.0, which included sufficient sequence generation, allocation concealment, blinding of participants and staff, inadequate outcome data, selective reporting, and additional bias (26). Two senior researchers (RY and YL) evaluated the reliability of the evidence using the GRADE-profiler software (V.3.6, The GRADE Working Group, 2010), items including the risk of bias, inconsistency, indirectness, imprecision, and publication bias, and the evidence was assessed as 4 levels: high quality, moderate quality, low quality, and very low quality.

2.5 Statistical analysis

We conducted a series of meta-analysis to compare the efficacy of anti-T2s with a placebo. For dichotomous data (exacerbation rate), intervention effects were reported using RR and 95% confidence intervals (CI), whereas standard mean differences (SMD) and 95%CI were used for continuous data (FEV1). Following the Cochrane Handbook, we aggregated two or three intervention groups into a single intervention group when research demonstrates more than two arms (26). The chi-squared test and the I^2 statistic were used to measure heterogeneity. Significant heterogeneity is indicated by an I^2 value of more than 50% (27). When there was significant heterogeneity, a random effect model was applied, and meta-regression analyses were performed to investigate the possible origins of heterogeneity. Planned considerations included ages, risk of bias, and demographics (exacerbation history, type 2 inflammatory criteria, and so forth). In comparisons involving at least 10 trials, publication bias was examined using a funnel plot and Eggers' test (28, 29). The influence of publication bias was estimated using the trim-and-fill method (30). Sensitivity analyses were carried out to assess the robustness of the overall effect sizes by removing one study at a time. Review Manager (V.5.4.1, The Cochrane Collaboration, 2020) and Stata (V.15.1) were used for all statistical analyses. A two-sided P value of 0.05 was considered statistically significant.

3 Results

3.1 Search results

A total of 7569 potentially pertinent articles were found. 3530 duplicate records from among all the potential studies were eliminated, leaving 4039 papers for screening. We found and obtained 131 papers in full text for review after examining the titles and abstracts. Ninety-three of these publications were excluded due to the following reasons: improper population (n = 39) (31–69), incorrect intervention (n = 4) (70–73), improper outcomes (n =29) (74–102), non-RCT (n = 13) (103–115), conferences (n = 3) (116–118), and second analysis (n = 5) (119–123). In the end, this meta-analysis included 38 articles with 41 studies (Figure 1).

3.2 Description of included trials

Thirty-eight articles, covering forty-one trials with 17,115 individuals, were included (Supplementary Table 2). The number of subjects in the studies ranges from 61 to 1545. Eleven of these studies employed benralizumab (124–133), three reslizumab (23, 134, 135), two dupilumab (21, 136), one lebrikizumab (137), eight mepolizumab (22, 138–143), seven omalizumab (111, 144–149), two tezepelumab (150, 151), one astegolimab (152), one itepekimab (153), one AZD1419 (154), one quilizumab (155), one CYT003 (156) and two tralokinumab (157). The duration of the treatment ranges from 12 to 56 weeks, and the follow-up was 12 to 84 weeks. Six trials administered the monoclonal antibody by intravenous infusion (IV), thirty-three studies by subcutaneous (SC), one by inhalation, and one study comprised both IV and SC arms. Thirty-four studies included patients with asthma, whereas seven researches included those with COPD.

All included patients with COPD who had an exacerbation history. In thirty-four studies with asthma patients, seven studies included severe asthma, five studies included moderate to severe asthma, one study included mild to moderate asthma, twelve studies included refractory, uncontrolled, or persistent asthma, and the remaining studies did not specify asthma severity; patients in eight studies required medium to high dose ICS plus long-acting β2-agonists (LABA), two studies required at least medium ICS, one study required 6-month maintenance treatment with systemic glucocorticoids, and one study required not receiving ICS; seventeen studies required exacerbation history.

The definition of 'type 2 inflammation' varied among studies. Four studies were defined by FeNO levels, three studies were defined by a sputum eosinophil count, twenty-two studies were defined by baseline blood eosinophil counts, one study was defined by eosinophil counts in blood or sputum, one study was defined by baseline blood eosinophil counts or IgE levels, five by IgE levels, and one study defines by combinatorial biomarkers, including FeNO levels, eosinophil counts in blood or sputum, whereas four studies did not specify the criteria.

3.3 Efficacy outcomes

In chronic airway illnesses associated with eosinophils, we contrasted anti-T2s with a placebo. The primary outcome was the exacerbation rate. A COPD or asthma exacerbation was defined as a clinical worsening for at least three days, a temporary increase in the ICS background dosage, the need for systemic corticosteroid treatment, the consumption of antibiotics, hospitalization, or mortality resulting from an airway disease. The secondary endpoint was the change in FEV1 from baseline measured by

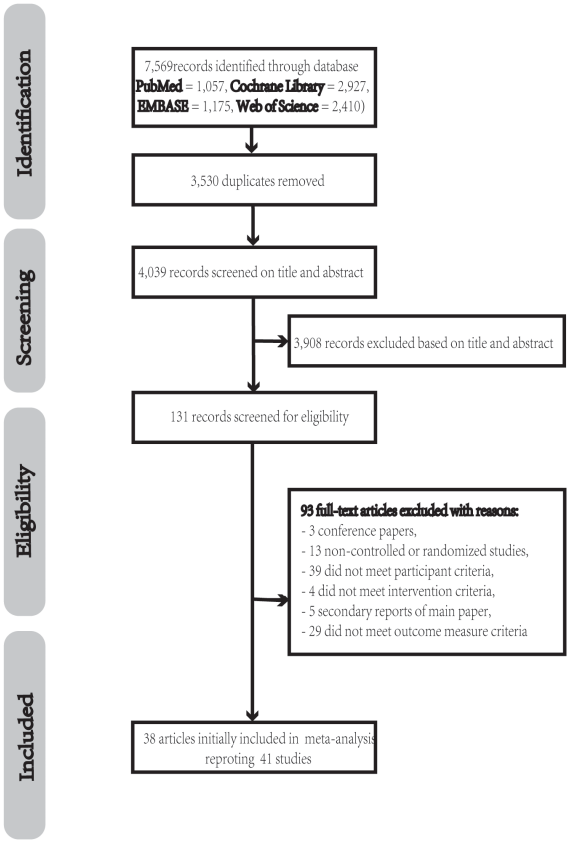

FIGURE 1
Flow chart of study identification, inclusion, and exclusion.

spirometry. Since the number of studies on asthma is much higher than studies on COPD, to eliminate the influence, the population was divided into the asthma group and COPD group for meta-analysis, respectively.

3.3.1 Exacerbation rate in COPD

There were seven studies included to analyse anti-T2s' efficacy in reducing the exacerbation rate of COPD. As a result, anti-T2s considerably reduced the exacerbation rate in COPD when compared to placebo (RR=0.89, 95%CI, 0.83-0.95, I^2 = 29.4%, Figure 2A).

3.3.2 FEV1 in COPD

There were four studies included to analyse anti-T2s' efficacy in improving FEV1 in COPD. As a result, anti-T2s improved pre-bronchodilator FEV1 in patients with COPD, whereas the difference was not statistically significant (SMD=0.05, 95%CI, -0.01-0.10, I^2 = 69.8%, Figure 2B). Considering the between-study heterogeneity, a subgroup analysis based on the sample size of studies was applied. In the subgroup analysis, studies with a sample size of less than 300 subjects exhibited anti-T2s' efficacy in improving FEV1 (SMD=0.14, 95%CI, 0.06-0.22, I^2 = 0%), while studies with sample size of more than 300 patients showed no effect on FEV1 improvement (SMD=0.05, 95%CI, -0.01-0.10, I^2 = 0%).

3.3.3 Exacerbation rate in asthma

There were twenty-five studies included. Anti-T2s considerably reduced the exacerbation rate in asthma when compared to placebo (RR=0.59, 95%CI, 0.52-0.68, I^2 = 83.9%, Figure 2C). Publication bias on Egger's test was present in this analysis (P=0.024, Figure 2E). But further investigation using the trim-and-fill test showed that this publishing bias did not affect the estimations (ie, no trimming was done because the data was unchanged).

Anti-IL-5 treatment was associated with a decreased incidence of asthma exacerbation compared to placebo in the subgroup analysis for different targets (RR=0.54, 95%CI, 0.48-0.61, I^2 = 52.1%). Similarly, anti-IgE therapy achieved a reduction in exacerbation of asthma (RR=0.69, 95%CI, 0.60-0.79, I^2 = 51.3%). The exacerbation rate was found decreasing with anti-IL-4/13, anti-TSLP, or TLR9 agonist medication when compared to placebo, although the difference was not statistically significant (RR=0.47, 95%CI, 0.22-1.02, I^2 = 77.1%; RR=0.65, 95%CI, 0.28-1.49, I^2 = 96.3%). Since the heterogeneity was partially decreased in subgroup analysis, different targets did not completely account for the between-study heterogeneity.

Univariable meta-regression using a random-effects model was performed and the results revealed that the criteria of 'type 2 inflammation', history of exacerbation, age, sample size, risk bias, severity, atopy, and different targets were not significantly associated with heterogeneity related to the exacerbation rate in asthma (Supplementary Table 3).

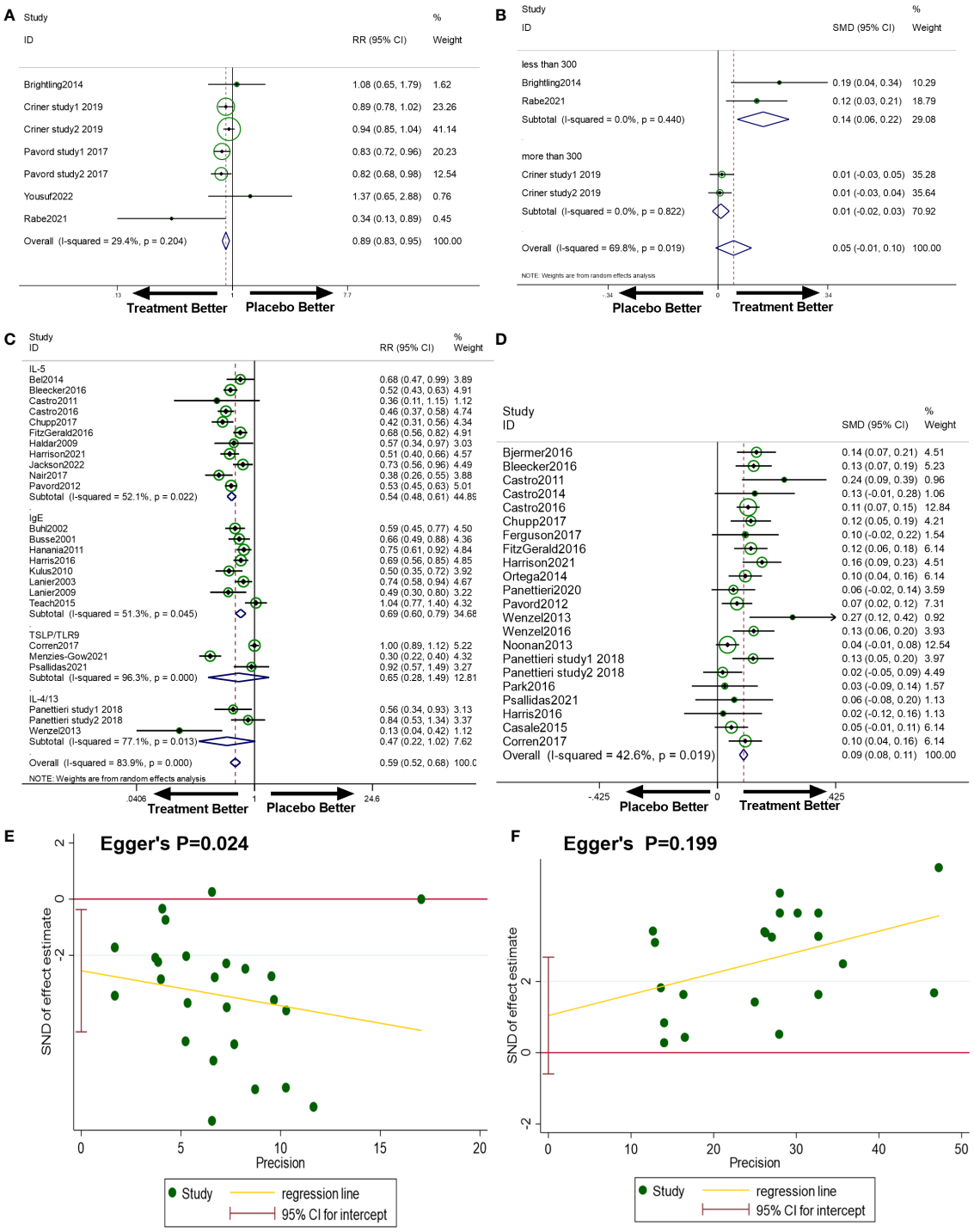

FIGURE 2
(A) The effect of anti-T2s versus placebo on exacerbation rate in COPD. Fixed-effect model. **(B)** The effect of anti-T2s versus placebo on FEV1 change from baseline in COPD. Random-effect model. **(C)** The effect of anti-T2s versus placebo on exacerbation rate in asthma. Random-effect model. **(D)** The effect of anti-T2s versus placebo on FEV1 change from baseline in asthma. Fixed-effect model. **(E)** Egger's test of exacerbation rate in asthma in the meta-analysis. **(F)** Egger's test of FEV1 in asthma in the meta-analysis. *CI*, confidence interval; SMD, standard mean difference; RR, rate ratio.

3.3.4 FEV1 in asthma

Data on pre-bronchodilator FEV1 were reported from twenty-two trials, of which seventeen reported a change in FEV1 from baseline, four reported a change in FEV1% from baseline, and one reported both.

Anti-T2s was associated with a substantial improvement in FEV1 change from baseline in a pooled analysis of twenty-two trials (SMD=0.09, 95%CI, 0.08-0.11, P<0.001, Figure 2D) with acceptable heterogeneity (I^2 = 42.6%, P=0.019). No publication bias existed (Egger's P=0.199, Figure 2F).

3.4 Risk of bias

A total of 25 researches (61.0%) adequately explained the randomization process. In 16 researches (39.0%), the random allocation was acknowledged, while in 25 studies (61.0%), allocation concealment was unclear. Research blinding was used in all of the investigations. 11 studies (26.8%) were at low risk for the outcome assessment's blinding. There was a low risk to the integrity of the outcome data in 23 trials (56.1%). There was a low risk of selection bias for 37 (90.2%). The data from each study was insufficient to determine if the risk of other biases was low or high (Figures 3A, B). In 32 studies included to assess the exacerbation rate, 20 studies in total (62.5%) provided a comprehensive explanation of the randomization procedure. In 11 investigations (34.4%), the random allocation was confirmed, while allocation concealment was uncertain in 21 studies (65.6%). In each study, research blinding was performed. 7 studies (21.9%) had a low risk of blinding in outcome assessment. In 19 trials (59.4%), there was a low risk in the integrity of the outcome data. For 28 studies (87.5%), the risk of selection bias was low. In 26 studies included to assess FEV1, 18 studies (69.2%) in total provided a thorough explanation of the randomization procedure. In 12 investigations (46.2%), the random allocation was acknowledged, and allocation concealment was ambiguous in 14 studies (53.8%). In each study, research blinding was applied. 9 studies (34.6%) had a low risk of blinding outcome assessment. In 11 trials (42.3%), there was a low risk of the integrity of the outcome data. 25 studies (96.2%) had a low risk of selective bias.

3.5 Sensitivity analysis

By removing one study at a time, sensitivity analyses were utilized to examine the impact of each study on the combined results. The outcome demonstrated that there had been no appreciable changes to the results' stability (Figures 3C–F), which supported the validity and dependability of our analysis.

3.6 Certainty of the evidence

Because of the considerable heterogeneity and inconsistent findings across trials, the evidence received a low-quality level in exacerbation rate in asthma and change in FEV1 from baseline in COPD, and a moderate-quality level in exacerbation rate in COPD and change in FEV1 from baseline in asthma (Supplementary table 4).

4 Discussion

This meta-analysis included 41 RCTs from 38 articles with 17,115 participants and investigated the effect of anti-T2s in patients with chronic eosinophilic airway diseases on exacerbation rate and FEV1. Our results showed that anti-T2s significantly reduces exacerbation rate in COPD and asthma, and improve FEV1 in asthma when compared to placebo, indicating that type 2 chronic airway disease patients can benefit from endotype-guided therapy as a treatment option.

Exacerbations are far more likely to cause morbidity and mortality (158, 159). One of the main objectives of chronic airway illness management is to reduce the exacerbation rate (22, 127). According to earlier investigations, increased blood and sputum eosinophilic counts are independent risk factors for exacerbations (160–162). Anti-T2s reduce the FeNO, eosinophil cationic protein, and eosinophil levels in airway inflammation (21–24), indicating that airway eosinophilia is a novel target, thus anti-T2s may be a potential approach to chronic eosinophilic airway disorders treatment. Our meta-analysis, which revealed a marked decline in the exacerbation rate in both COPD and asthma, validated the claim. Patients with COPD receiving anti-T2s medication in contrast to placebo experienced a reduced exacerbation. Although individuals with asthma receiving anti-T2s therapy had decreased exacerbations than those receiving a placebo, the heterogeneity was statistically significant. A subgroup analysis was conducted according to different targets, which revealed inconsistent results among subgroups. Anti-IL-5 and anti-IgE therapy both achieved a reduction in asthma exacerbation with acceptable heterogeneity, while anti-IL-4/13, anti-TSLP, and TLR9 agonist therapy had a decreased trend of exacerbations than placebo, and the difference was not statistically significant. The following factors may account for the inconsistent results among subgroups: (1) The inclusion criteria for each study within the current meta-analysis varied, which may have led to significant heterogeneity among study populations in terms of exacerbation risk, eosinophil count, and disease severity; (2) Different therapy regimens were varied; (3) Anti-IL-4/13 treatment has shown a less consistently positive impact on the exacerbation rate, as the previous study reported (163). Tralokinumab, an anti-IL-13 agent, did not affect the exacerbation rate in the study of Panettieri et al, but another cohort in the same article showed a statistically significant reduction in exacerbations (157). This may be an indication of the ambiguous impact of blocking the IL4/13 pathway on reducing the exacerbation rate in asthma (4). Anti-TSLP and TLR9 agonist therapy had fewer studies to evidence their efficacy, and the existing studies varied in ages of participants, disease severity, and so forth, resulting in obvious heterogeneity. However, the overall beneficial impact of anti-T2s in reducing asthma exacerbations is consistent across the meta-analysis, despite some lingering confounding factors.

The crucial identifying feature of chronic airway illnesses in the clinic and pathology is airflow limitation. The lung function test continues to be the gold standard in diagnosis and a crucial indicator of management, which is typically measured by the change in FEV1 (164). The results on FEV1 of asthma and COPD are inconsistent. Even though FEV1 in COPD patients improved, the difference was not statistically significant, which was consistent with previous studies (165–167). Overall, FEV1 considerably improved in asthma patients receiving anti-T2s. FEV1 alone may not be the optimum assessment for the management of chronic airway illnesses. The bias in results may

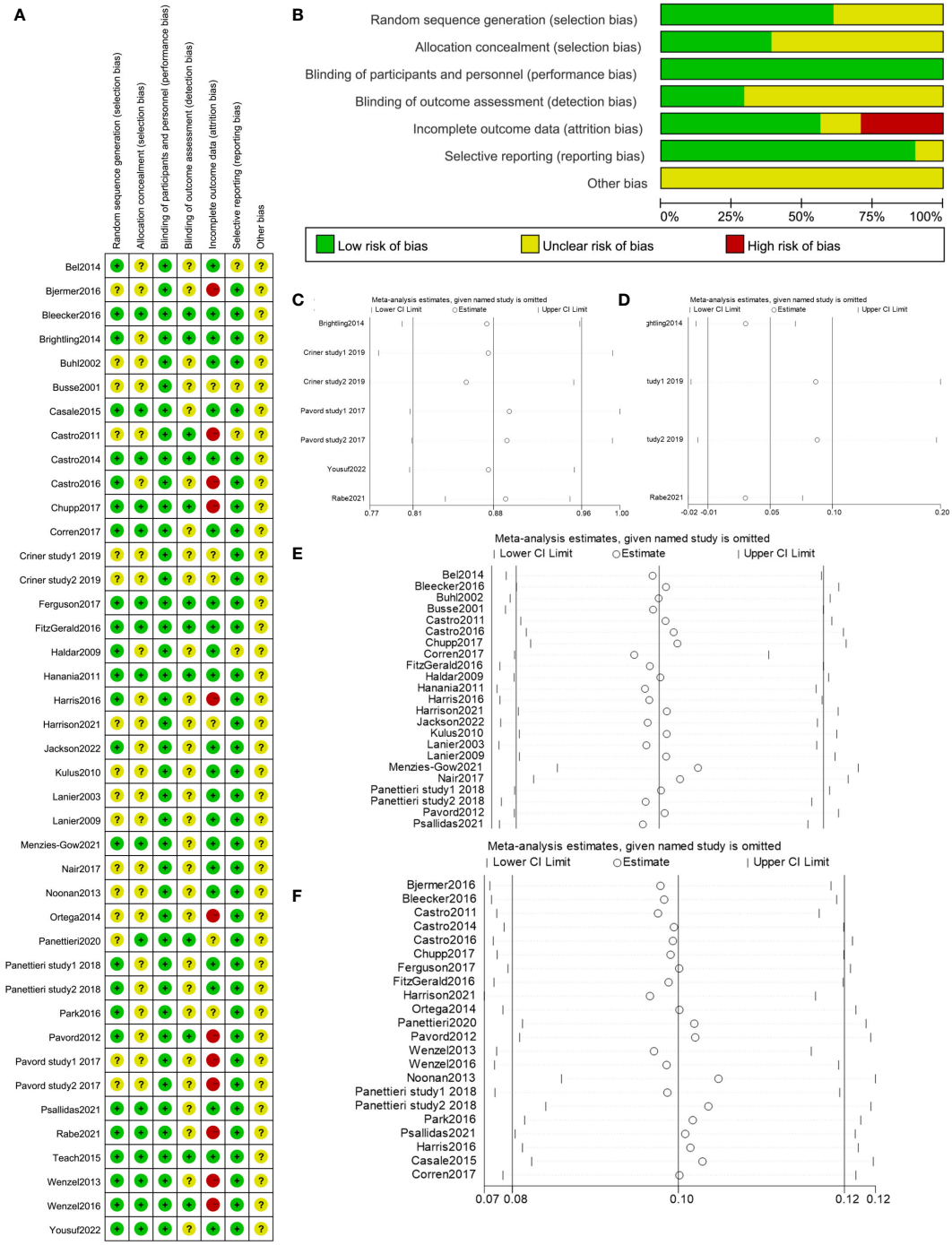

FIGURE 3
(A, B) Risk of bias summary of included studies. **(C)** Sensitivity analysis of exacerbation rate in COPD in the meta-analysis. **(D)** Sensitivity analysis of change in FEV1 from baseline in COPD in the meta-analysis. **(E)** Sensitivity analysis of exacerbation rate in asthma in the meta-analysis. **(F)** Sensitivity analysis of change in FEV1 from baseline in asthma in the meta-analysis.

be caused by variations in race, medication dosage, or even trial participants' status and severity. The conflicting results between asthma and COPD could be attributed to the varying baseline FeNO, blood, or sputum eosinophilia thresholds. In addition, it was observed that former smokers achieved more pronounced benefits than current smokers in a prespecified subgroup analysis, which might be explained by a broad pro-inflammatory effect of cigarette smoke, indicating that smoking status had an impact on the effectiveness of anti-T2s in treating COPD (50). Further research should be done to determine the COPD-specific threshold of type 2 inflammation and explore the effect of anti-T2s in COPD patients with different smoking status to address these deficiencies.

According to the Cochrane handbook, ambiguous allocation concealment might exaggerate the estimated effect in subjective

outcomes, while the bias in objective outcomes is not confirmed (26). In our study, the outcomes, including exacerbation rate and FEV1, are tending to be objective, and the impact of ambiguous allocation concealment remains unclear. Meanwhile, the GRADE system was applied to evaluate the reliability of our results, which had considered the bias judgments.

5 Limitations

Some potential restrictions must be taken into account. Firstly, it is difficult to determine the influence of the severity and initial therapy of included patients on the outcomes of the investigations. Secondly, a few of the research was conducted on a limited scale, which would limit their ability to investigate the true outcome. Thirdly, we failed to investigate the potential impact of disease severity, gender, and body mass index on outcomes given the limited data available. Fourthly, due to the finite number of specifically aimed at IL-4/13 pathway targeting, we were unable to further compare the effects of anti-IL-4 and anti-IL-4/13 treatment in subgroup analysis for exacerbation rate. Additionally, RCTs related to the anti-IL-9 agent were not included due to not meeting the inclusion criteria, RCTs related to anti-IL-25 therapy were in progress (NCT05128409), and RCTs of anti-T2s on COPD were under publication (NCT03615040, NCT03930732, NCT04456673). Finally, although using various intervention dosages and administration techniques, as recommended by the Cochrane handbook, we combined two or three intervention groups into a single arm, making it difficult to establish the ideal dosage. We should also be aware of the fact that different studies used various definitions of 'type 2 inflammation', and because no study included data on specific patients, we were unable to further examine the correlation between baseline levels of eosinophils or FeNO and treatment outcomes.

6 Conclusions

The current meta-analysis concluded that anti-T2s could considerably lessen exacerbations of chronic airway disorders. Therefore, anti-T2s may be effective in treating chronic airway illnesses associated with eosinophils. The findings highlight the effectiveness of endotype-guided treatment in chronic eosinophilic airway inflammation illnesses regardless of various background therapies and 'type 2 inflammation' criteria.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Author contributions

YW, JL, and JC conceived this meta-analysis. YW, MH, and JZ extracted the data and wrote the manuscript. YLu did statistical analyses and checked them. KG helps the methods. RY and YLi evaluated the reliability of the evidence. YLu, RY, YLi, JL, and JC revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1089710/full#supplementary-material

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CCL13 and human diseases

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CCL13/MCP-4 belongs to the CC chemokine family, which induces chemotaxis in many immune cells. Despite extensive research into its function in numerous disorders, a thorough analysis of CCL13 is not yet accessible. The role of CCL13 in human disorders and existing CCL13-focused therapies are outlined in this study. The function of CCL13 in rheumatic diseases, skin conditions, and cancer is comparatively well-established, and some studies also suggest that it may be involved in ocular disorders, orthopedic conditions, nasal polyps, and obesity. We also give an overview of research that found very little evidence of CCL13 in HIV, nephritis, and multiple sclerosis. Even though CCL13-mediated inflammation is frequently linked to disease pathogenesis, it's fascinating to note that in some conditions, like primary biliary cholangitis (PBC) and suicide, it might even act as a preventative measure.

KEYWORDS

CCL13, MCP-4, cytokines, human diseases, Th2, NF-κB, type 2 immunity

1 Introduction

Chemotactic cytokines are divided into four subfamilies based on how their amino-terminal (N-terminal) cysteines are arranged: CXC, CC, XC, and CX3C; their main function is to induce directional cell migration or the migration of cells drawn to chemotactic factors towards the source of the chemotactic factor along the signal of increased chemotactic concentration. CC chemotactic factor family member CCL13, also known as MCP-4 (monocyte chemoattractant protein 4) (1). CCL13 can bind to CCR1, CCR2, CCR3, CCR5, and CCR11, causing eosinophils, monocytes, T cells, and immature dendritic cells to migrate (2) (Figure 1). In addition to its chemotactic activity, CCL13 has been shown to induce eosinophil degranulation, basophil histamine release, adhesion molecule expression, and secretion of pro-inflammatory cytokines in epithelial, endothelial, and muscle cells. Besides which, research has revealed that CCL13 and its derived peptides have antibacterial activity against Gram-negative bacteria (3, 4). The antimicrobial activity of cytokines may be one of the body's defenses; therefore, elevated CCL13 in certain diseases may be associated with anti-infective properties.

Many organs, including the small intestine, thymus, colon, lung, trachea, stomach, and lymph nodes, express CCL13 at the transcription level. At the protein level, CCL13 has been reported to be present in knee chondrocytes, human proximal renal tubular epithelial cells, etc. (Table 1). mRNA expression of CCL13 is upregulated in various diseases, but

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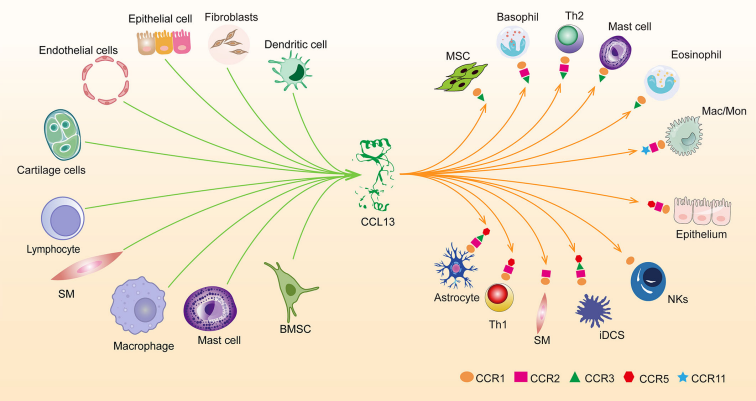

FIGURE 1
Overview of cells releasing CCL13 at the protein level and the expression of five receptors for CCL13 in cells. smooth muscle cells (SM), Macrophages (Mac), Monocytes (Mon), immature dendritic cells (iDCs), Natural killer (NKs), Helper T Lymphocyte (Th), Bone Marrow Stromal Cells (BMSC).

protein-level expression has rarely been validated. Studies have demonstrated that the proteomics of CCL13 exhibits inconsistency with mRNA expression. Therefore, CCL13 mRNA-based studies require validation by protein analysis to establish convincing conclusions (5).

This article seeks to present a thorough analysis of CCL13, a summary of the function that CCL13 plays in disease, and a discussion of its probable activation pathways (Figure 2). Additionally, we will discuss intervention strategies that can prevent CCL13 from functioning (Table 2).

2 CCL13 and respiratory diseases

CCL13 and other chemokines within its family have been extensively investigated in diseases such as asthma, COPD, allergic pneumonia, and upper and lower respiratory tract infections. These diseases are characterized by inflammatory cell infiltration, which is mediated by multiple chemokines. Inflammatory cell infiltration can be triggered by pathogens or non-pathogenic factors.

2.1 CCL13 and asthma

Asthma is a heterogeneous lung disease with different phenotypes and unique potential mechanisms. In the past decade, people have conducted extensive research on the cellular and molecular mechanisms of asthma. The aggregation of eosinophils, type 2 helper T cells (Th2), and monocytes in the airway leads to changes in lung structure, which then leads to the decline of respiratory function. The most important pathological process of

TABLE 1 Cells or tissues expressing CCL13 at the protein level in humans.

Locations	mRNA	Protein	Diseases	Species	References
Turbinate tissue	↑	-	CRSwNP	Human	(5)
Cartilage cells	↑	↑	Rheumatoid arthritis	Human	(6)
Blister fluid	not given	↑	AD	Human	(7)
HaCaT cells	↑	↑	AD	Human	(8)
Peripheral blood	not given	↑	Alopecia areata	Human	(9)
Proximal tubular epithelial cells	↑	↑	Glomerulonephritis	Human	(10)
Peritubular, periglomerular	not given	detectable	-	Human	(10)
Corneal stromal fibroblasts	↑	↑	Corneal injury	Human	(11)
Nasal mucosal epithelium	not given	↑	Rhinitis	Human	(12)
Plasma	not given	Late-pregnancy ↓ postnatal period ↑	Multiple sclerosis	Human	(13)
Plasma	not given	↑	Hodgkin lymphoma	Human	(14)
M2 TAM	not given	↑	OSCC	Human	(15)

AD, Atopic dermatitis; CRSwNP, Chronic rhinosinusitis with nasal polyps; and OSCC, oral squamous carcinoma; ↑: upregulation, ↓: downregulation, -: No obvious change.

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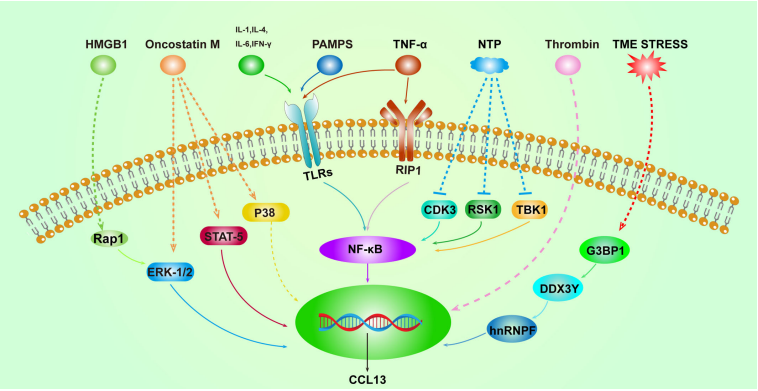

FIGURE 2
Overview of the activation pathways of CCL13. Solid lines represent well-defined relationships, and dashed lines represent intermediate molecular mechanisms unknown. High mobility group box 1 (HMGB1), Ras-Associated Protein 1 (Rap1), the tumor micro-environment (TME), and non-thermal plasma (NTP).

asthma is abnormal Th2 inflammation, which is mediated by Th2 cytokines, such as IL-4. About 50% of mild and moderate asthma and most severe asthma cases are induced by Th2-dependent inflammation. However, non-Th2 cytokines, including TNF-α, can also mediate Th2-low asthma (23). Airway epithelial cells can be activated by cytokines (IL-1β, IFN-γ,TNF-α, etc.) or PAMPS (Pathogen-related molecular patterns) *via* the TLRs-NF-κb pathway and releases CCL13, which recruits eosinophils and promotes the polarization of M2 macrophages to mediate the progression of asthma (2, 24, 25). When compared to patients with asthma who had their condition under control with glucocorticoids, people with uncontrolled asthma had greater CCL13 levels (26). Monocytes and eosinophils in sputum from children with asthma exhibited CCL13 and CCR3, but lymphocytes solely expressed CCL13; CCL13 is negatively correlated with peak expiratory flow and is downregulated in asthma remission (27). Elevated blood CCL13 concentrations have been observed in children with severe asthma, and it has been suggested that blood CCL13 levels can help in characterizing the severity of asthma in children (28). Mechanistically, CCL5, CCL7, CCL13, as well as CCL11 and CCL24, act synergistically to recruit eosinophils into the airways (29). Furthermore, Toll-like receptor 7 and 8 (TLR7/8) mediates the antiviral immune response by recognizing mainly viral RNA, and the increased response of the CCL13 gene to TLR7/8 agonists in the nasal mucosa of asthma patients may reflect the role of the virus in asthma progression (30). Interestingly, CDIP-2, which is a peptide derived from CCL13, has been shown to reduce chemokine-mediated function, decrease leukocyte recruitment, and reduce cytokine production by interacting with CCR1, CCR2, and CCR3 (31, 32). These results imply that CDIP-2 has the potential to alleviate airway inflammation and could be a promising therapeutic target for asthma. IL-13 is known to play a key role in the pathogenesis of asthma, and its upregulation has been associated with increased levels of CCL13 in the serum of asthma patients; Using Lebrikizumab, a monoclonal antibody that blocks the IL-13Rα1/IL-4Rα receptors, has had good results in lowering the serum concentrations of CCL17, CCL13, and total IgE in asthma patients (16). Yet it should be highlighted that, as shown in another trial, a single blockage of IL-13 was found to be insufficient to improve lung function in asthma patients who were not getting inhaled steroids (33). Bcl6 appears to be a promising inflammatory substance that inhibits histone

TABLE 2 Interventions to inhibit the function of CCL13 and their mechanisms.

Intervention means	Mechanism	Disease	Species	References
Lebrikizumab	IL-13 signaling was blocked by IL-13Rα1/IL-4Rα receptors	Asthma	Human	(16)
PD98059	Inhibition of MEK1	RA	Human	(17)
Non-thermal plasma	Blocking NF-κB activation in an IκB independent manner	AD	Human	(8)
ASN002	Inhibition of CCL13 chemotaxis through dual JAK/SYK blocking	AD	Human	(18)
Imiquimod	Direct inhibition of Th2-associated cytokine expression.	Hypertrophic scar	Rabbit	(19)
Immunoglobulins	Regulating peripheral T cell chemokines	MS	Human	(20)
Tesamorelin	Enhancing the GH axis to improve immune activation	AIDs	Human	(21)
Tasquinimod	Blocking the S100A9-TLR4 interaction deactivates NF-κB	CTCL	Human	(22)

RA, Rheumatoid arthritis; AD, Atopic dermatitis; MS, Multiple sclerosis; AIDS, Acquired Immune Deficiency Syndrome; and CTCL, Cutaneous T-cell lymphoma.

acetylation of the chromatin of the gene cluster in lung epithelial cells by binding to a specific site of the CCL13 gene, leading to the downregulation of CCL13 (34).

The role of the CCL13-Th2 axis in promoting the entry of M2 macrophages and eosinophils into the airways and triggering airway inflammation in asthma is well established. While targeting type 2 cytokines to downregulate CCL13 has shown effectiveness, blocking upstream mediators of CCL13 alone has not been proven to be sufficient in achieving favorable outcomes.

2.2 CCL13 and COPD

Chronic Obstructive Pulmonary Disease (COPD) is a serious chronic respiratory condition which is currently the third greatest cause of death worldwide (35). Airway inflammation and damaging emphysema characterize COPD. In terms of pathology, it entails infiltration of inflammatory cells, modification of the airways, and irreversible damage of the lungs, among these, chronic inflammation is essential for the emergence of COPD (36). CCL13 has been linked to the development of COPD and has been shown to significantly increase when human lung tissue is stimulated by respirable smoke extracts from cooking (37). Contrarily, analysis of macrophages from bronchoalveolar lavage fluid (BALF) of COPD patients revealed primarily non-polarized macrophages with reduced gene expression of CD163, CD40, and CCL13, which are involved in pathogen recognition and processing; non polarized macrophages are primarily to blame for the decreased phagocytic capacity and the reduced ability to recognize and handle pathogens in lung macrophages (38). Moreover, a study by Ghebre et al. (39) revealed that the microbiome spectrum in sputum from patients with asthma and COPD seems to reflect different lineages of inflammatory mediators, and the upregulation of CCL13 and other type 2 mediators suggests the possibility of the infection of the bacterial phylum Bacteroidetes; type 1 mediators such as CXCL10 are more closely related to the phyla Actinobacteria and Firmicutes; pro-inflammatory mediators such as IL-1β are increased following the bacterial phylum Proteobacteria infection; these inflammatory lineages imply heterogeneity in bacterial related exacerbations of asthma and COPD, and suggest that endotype may be more important than the diagnosis of the disease itself. However, Proteobacteria, Actinobacteria, and Firmicutes are associated with other types of media. At last, bronchoalveolar lavage and biopsy samples' content of CCL13 and other MCP family members may not be specific in identifying asthma, tuberculosis, nodular disease, and chronic bronchitis (40).

The different stages of the illness may play a role in the contradictory findings of CCL13 in the progression of COPD. CCL13 may add to chronic inflammation and tissue damage in the early phases of COPD. The detection and removal of pathogens by macrophages, as well as tissue healing and regeneration, may be promoted by CCL13 in later phases, when the lung tissue is already damaged. It's also conceivable that CCL13's dual function is context-dependent and affected by a variety of elements, including the pathogens involved, the host immune system, and additional environmental variables. Clarification of CCL13's function in the development of COPD and its possible medicinal uses requires more study.

2.3 Acute eosinophilic pneumonia and hypersensitivity pneumonitis

Acute eosinophilic pneumonia (AEP) is a condition characterized by a significant infiltration of eosinophils in the lungs. Interestingly, eosinophil migration in endothelial cells is only induced by AEP-BALF, but not BALF from patients with hypersensitivity pneumonitis. The accumulation of eosinophils in AEP is mediated by CCR3 ligands, such as CCL13 and, CCL24 (41). The CCR3 antagonist YM-355179 has been shown to have potential in the treatment of eosinophil-associated allergic inflammatory disorders by blocking chemokine-mediated intracellular Ca^{2+} influx; this mechanism prevents the accumulation of eosinophils in affected tissues, thereby reducing the severity of the associated inflammation (42).

Although CCR1 and CCR3 are receptors for CCL13-induced eosinophil-triggered allergies, it is crucial to remember that CCR1 also has a function in CCL13-induced allergic pneumonia. Further investigation into the role of CCR1 in this mechanism may shed light on the etiology of allergy illnesses linked to eosinophils and possibly reveal novel targets for therapy.

2.4 Acute upper and lower respiratory tract infections

Acute lower respiratory infection (ALRI) is a prevalent infectious disease that affects individuals of all ages. ALRI in young children are known to be most frequently brought on by the respiratory syncytial virus (RSV) (43). According to a study, levels of CCL13 were found to be higher in patients with ALRI who were hospitalized from the start to day 6 after admission, but it wasn't correlated with the severity of their symptoms; in fact, it was even downregulated in severe cases of patients with a CURB-65 score of >3 (suggestive of severe pneumonia) (44). Based on the available evidence, the relationship between CCL13 and ALRI remains unclear. While some studies suggest a potential role for CCL13 in ALRI, there is currently no definitive evidence linking the expression level of CCL13 to the severity of ALRI symptoms, further research is needed to better understand the potential role of CCL13 in the pathogenesis and clinical course of ALRI. Finally, In upper respiratory tract infections, CCL13 and CCL7 have been suggested to play a role in the recruitment of macrophages in children with confirmed viral infections of the upper respiratory tract (45).

3 CCL13 and rheumatism

3.1 CCL13 and RA

Rheumatoid arthritis (RA) is a chronic inflammatory disease that is one of the most prevalent autoimmune disorders. The disease

primarily affects the joints, resulting in inflammation of the synovial membrane, which can cause joint swelling, stiffness, and pain (46). Serum CCL13 levels and expression in synovial and cartilage tissues are elevated in RA patients. The joint is the main site of inflammation in RA, and the cells known to produce CCL13 in the joint are synovial fibroblasts and chondrocytes (6, 47). Synovial fibroblasts promote chondrocyte catabolism and the development of synovial bone fragmentation, and they also have an aggressive inflammatory, stromal regulatory, and invasive character. These elements work together to cause joint destruction (48). Oncostatin M (OSM) is necessary for IL-6 and TNF- to positively regulate CCL13 expression in synovial fibroblasts. STAT-5, ERK-1/2, and p38 are involved in the production of CCL13 in response to OSM, and the increased CCL13 inhibits H_2O_2-induced apoptosis in synovial fibroblasts, enhances intra-synovial macrophage infiltration and angiogenesis, and aids in the course of the disease (47, 49). Studies have also demonstrated that the MAPK pathway mediates CCL13-induced proliferation of synovial fibroblasts and that these effects can be completely inhibited by PD98059 (17). In rheumatoid arthritis, estrogen has been found to decrease synovial fibroblast death and increase CCL13 expression, and this process may explain why women are more likely than males to get RA (50). In cartilage tissue, CCL13 is substantially expressed at both the mRNA and protein levels. CCL13, which is released by chondrocytes and aids in the deterioration of joints in RA, encourages the formation of rheumatoid synovial cells (6). Hintzen et al. (47, 49) reported that whereas all types of examined fibroblasts showed evidence of efficient OSM-induced signaling, only synovial fibroblasts were found to release CCL13. Although synovial fibroblasts are the primary source of CCL13 in the context of RA, several studies have demonstrated that CCL13 can be produced in response to different stimuli by other types of fibroblasts, such as dermal fibroblasts, colonic subepithelial myofibroblasts, and nasal polyp fibroblasts (51–53).

3.2 CCL13 and OA

Inflammation is one of the key mechanisms in the complicated joint condition known as osteoarthritis(OA), and imaging is a crucial way to diagnose OA (54). Inflammatory molecules known as the danger-associated molecular pattern (DAMP) contribute to the inflammatory process in OA joints. Alkaline calcium phosphate crystals, which are OA-associated DAMPs, can downregulate the expression of CCL13 and MRC1 (M2 markers) in macrophages (55); however, clinical studies have shown that CCL13 levels in serum and synovial fluid (SF) correlate with the severity of OA as determined by imaging (56). Patients with KL4 knee OA have significantly higher levels of CCL13 in serum and SF than those with KL2 and KL3, and patients with KL3 knee OA have significantly higher levels of CCL13 in SF than those with KL2 (The KL grading system is the most commonly used to assess the severity of joint disease in patients with OA, with grade 0 being the normal knee and grade 4 being the most severely affected) (56). It is evident that CCL13 exhibits distinct expression patterns in OA, and this pattern of expression in macrophages merits exploratory research.

3.3 CCL13 and other rheumatic diseases

Sjogren's syndrome (SS) is an autoimmune disease, antibodies associated with SS can upregulate the expression of CC chemokines (CCL2, CCL13, and CCL20) in human salivary gland epithelial cells *via* the TACE/TNF-α/NF-κB signaling pathway (57). Nevertheless, in a cross-sectional study comparing several illnesses, serum CCL13 levels were measured in patients with systemic sclerosis (SSc), dermatomyositis, systemic lupus erythematosus (SLE), and healthy individuals, and it was discovered that only SSc patients had elevated levels of serum CCL13 compared to the control group (58, 59). In conclusion, while CCL13 cannot be used to diagnose rheumatism, it may be coupled with other disease-specific markers to increase the accuracy of the diagnosis.

4 CCL13 and skin diseases

Atopic dermatitis (AD) and alopecia areata are the two primary dermatological disorders linked to CCL13 that have been described in the literature, with the former being more clearly linked.

4.1 CCL13 and AD

Atopic dermatitis (AD) is a prevalent chronic inflammatory skin disease. The development of AD is influenced by various factors, including genetic predisposition, skin barrier dysfunction, and innate and adaptive immune dysregulation (60). CCL13 has been extensively studied as a Th2-associated marker in various tissues, including human PBMCs, dendritic cells, macrophages, and lesional skin tissue from patients with AD. However, the findings have not always been consistent. In lesional skin tissue from AD patients of all ages, CCL13 mRNA expression is significantly increased and has been shown to contribute to IgE synthesis (61–63). Furthermore, in older AD patients, CCL13 is considered a risk factor for atherosclerosis, indicating that these people might gain from screening for and treatment of cardiovascular disease (64). Proteomic analysis of blister fluid in AD patients has shown that CCL13 is one of the most upregulated proteins (7). However, the basal mRNA expression of CCL13 is downregulated in the PBMCs of AD patients, and CCL13 secretion into PBMCs is significantly increased upon stimulation with TLR2 ligands (65). It has been suggested that age-specific therapies may be beneficial for AD because CCL13 levels decrease significantly in diseased tissue and blood With increasing age (64). Targeting CCL13 for AD treatment is in its infancy, and an *in vitro* experiment (8) has pointed out that TNF-α promotes CCL13 gene expression in both IκB dependent and non-dependent pathways by binding to RIP1(receptor interacting protein 1); Non-thermal plasma (NTP) inhibited NF-κB pathway in a non-IκB-dependent manner and downregulated CCL13 expression in AD mice, and the combination of NTP and 1% hydrocortisone cream was found to be more effective. In 2018, the FDA authorized the oral medication ASN002, a dual JAK/SYK inhibitor, for the treatment of moderate to severe atopic dermatitis; ASN002 significantly reverses the transcriptome of lesional skin to a

nonlesional phenotype and inhibits key inflammatory pathways involved in the pathogenesis of AD, including CCL13-mediated Th2-associated inflammation (18).

Indeed, age is a significant factor in determining the function of CCL13 in AD, and more investigation is required to understand why CCL13 expression in AD-lesioned tissues and PBMC differs.

4.2 CCL13 and alopecia areata

Alopecia areata is a common autoimmune disorder characterized by the immune-mediated destruction of hair follicles. One of the main events in the etiology of alopecia areata is the loss of hair follicle immune privilege, although the underlying processes are complicated and may involve both local and systemic immune dysregulation (66). Alopecia areata has traditionally been thought to be associated with Th1 activation, but recently there is ample evidence that Th2 may also mediate the development of alopecia areata. GWAS studies suggest a role for Th2-associated genes in the pathogenesis of alopecia areata (67). Elevated Th2 markers in the blood and skin of patients with alopecia areata may be involved in systemic inflammation and could be a potential indicator of the severity of the disease (9, 68). Upilumab, an IL-4R inhibitor, blocks the Th2 axis in patients with alopecia areata, and these patients showed downregulation of Th2-related markers (CCL13, CCL18, CCL26, CCL24) starting at week 12 of treatment with dupilumab, significant upregulation of the hair keratin gene-set at week 24, and clinical improvement in patients with alopecia areata at week 48 (69). This demonstrates how Th2-related cytokines can suppress the expression of hair keratins and be harmful in alopecia areata. To ascertain the function of particular Th2 inhibition in the treatment of alopecia areata, larger size and longer clinical trials are still necessary before the Th2 axis may be targeted for the treatment of alopecia areata.

5 CCL13 and digestive system disease

5.1 CCL13 and hepatic disease

According to Townsend et al.'s research, CCL13 expression is reduced in hepatitis D patients; this change in chemokine levels may contribute to the faster disease development in hepatitis D patients (70). On the other hand, secondary bacterial infections in cirrhotic individuals may be linked to the increase of CCL13 transcript levels in their serum and duodenal mucosa (71). Although little is known about CCL13's role in the development of cirrhosis, recent research suggests that it may slow the advancement of the disease through type 2 immunity. However, viruses can counteract this effect in a number of ways. Elevated CCL13 may be a protective factor and needs to be further investigated, given that Gram-negative bacteria are more frequently seen in bacterial infections associated with cirrhosis.

Primary biliary cholangitis (PBC), is a chronic autoimmune liver disease characterized by cholestasis and the presence of anti-mitochondrial antibodies in the bloodstream (72). It was shown that Th2 chemokines were downregulated in early PBC, where serum CCL13 was elevated in early and decreased in late of PBC, and negatively correlated with PBC staging ($r = -0.373$), preventing early disease progression (73). Given the marked eosinophil infiltration in the portal vein in patients with early PBC, the increased CCL13 in the serum of patients with early PBC might be associated with this, and the downregulation of CCL13 in the late stages seems to indicate that it is not involved in the subsequent progression of PBC.

5.2 CCL13 and IBD

Inflammatory bowel disease (IBD) is an idiopathic inflammatory disease of the intestinal tract that primarily includes ulcerative colitis (UC) and Crohn's disease (CD). Although its pathogenesis is unclear, chronic lesions of CD show an excessive Th1 response and a Th2 pattern is present in the mucosa of lesions in early CD and UC (74). Studies have shown that the effects of pharmacological interventions alone on chemokines in patients with IBD appear to exhibit targeting. Only CXCL10 was significantly downregulated in the blood of patients with CD treated with atorvastatin, while the other eight chemokines, including CCL13, were not significantly changed (75). Interestingly, in a clinical study that included UC and CD, after treatment with vedolizumab, although serum CCL13 levels increased in the treatment group as a whole; when patients were subdivided into groups with and without responders, CCL28 was down-regulated in responders, whereas CCL13 was up-regulated in non-responders, and they concluded that CCL13 levels at the initiation of treatment may predict the potential prognostic value of vedolizumab (76). The mechanism of CCL13 in IBD may be related to its induction of adhesion molecule expression in epithelial cells, and increased expression of adhesion molecules allows more cells of the innate immune system (such as monocytes or neutrophils) to enter the site of inflammation. The greater CCL13 levels in non-responders following therapy may be partially explained by this.

6 CCL13 and renal diseases

Studies on individuals who experienced acute renal allograft rejection and vasculitis glomerulonephritis revealed that CCL13 was primarily expressed in peritubular, periglomerular, and perivascular sites and was linked to the infiltration of $CD3^+$ lymphocytes and $CD68^+$ monocytes/macrophages. Proximal tubular epithelial cells also displayed low levels of CCL13 protein expression, which could be seen by protein blotting. The upregulation of CCL13 expression is a result of pro-inflammatory cytokines in response and plays an important role in monocyte/macrophage recruitment and retention in renal inflammation (10). In addition, kidney donor quality significantly affects renal transplantation outcomes, such as recipients of kidney donors from the elderly who exhibit low post-transplant renal function and short graft lifespan; a single-center, retrospective, observational study showed that compared to

standard criteria donors and living donors, elderly and expanded criteria donors have upregulated CCL13 at the transcriptional level and may be involved in post-transplant renal inflammation and renal function impairment (77).

7 CCL13 and ocular disorders

Ocular uveitis can be a symptom of a variety of connective tissue disorders, including seronegative spondyloarthritis (SpA), nodular disease, pseudoarthrosis, recurrent polychondritis, and granulomatous polyangiitis. Yet uveitis that manifests as SpA has its own distinctive features, notably with a strong link to the presence of HLA-B27 (78). For this reason, uveitis presenting with spondyloarthritis is collectively referred to as B27-associated uveitis, which typically develops in young adults but can also affect children and adolescents. Studies have shown that people with HLA-B27-associated uveitis have 255-fold higher levels of CCL13 in their aqueous humor than healthy individuals. Additionally, compared to granulomatous uveitis, non-granulomatous uveitis exhibits a considerably higher level of CCL13 (79). This suggests that CCL13 is involved in the pathogenesis of non-granulomatous uveitis and that the associated immune responses may be more effective in this type of uveitis, particularly in HLA-B27-associated uveitis.

Patients with ischemic retinal vein occlusion (RVO) had modestly elevated levels of vitreous CCL13, which correlated with vitreous hemorrhage and may have indicated how severe the retinal inflammation was (80). In patients with primary rheumatogenic retinal detachment (RD), CCL13 levels are higher in vitreous fluid (VF) than in macular holes (MH), and several cytokines seem to be involved in the immune initiation and profibrotic processes after RD, and these cytokines may be related to the increased intraocular fluid volume and higher mobility of the fluid around the refractive apparatus of the anterior chamber (81). Furthermore, *in vitro* experiments have demonstrated that treatment of corneal stromal fibroblasts with thrombin resulted in a significant increase in CCL13 mRNA expression (up 2-fold) and protein production (from 0 to 14 pg/ml) and that CCL13 played a crucial role in mediating the thrombin-triggered immune response (11).

8 CCL13 and nasal polyps and rhinitis

Nasal polyposis (NP) is an inflammatory disease with Th2 skewing, and it is widely believed that certain chemotactic agents and Th2 factors play a crucial role in its pathogenesis. One study showed that fibroblasts may be a major source of Th2 chemokines and that TLR2, 3, 4, and 5 ligands can synergistically induce the production of CCL13 in nasal polyp fibroblasts when combined with IL-4, while TLR7/8 or 9 ligands do not induce its production (53). The expression of Th2 markers, including CCL13, was not found to be altered in nasal polyposis (NP) patients receiving glucocorticoid (GC) therapy; this lack of change in expression levels may suggest that T-cell-driven NP inflammatory mediators are resistant to the effects of GC treatment (82).

Chronic rhinosinusitis with nasal polyps (CRSwNP) is a condition characterized by a type 2 immune response, with overproduction of IgE and infiltration of eosinophils (83). Much of the research on the immunopathology of CRSwNP has relied on transcriptomics. However, Workman et al. (5) noted that proteomic analysis can reveal inconsistencies with mRNA expression, with tissue proteomic and transcriptomic analyses of CRSwNP polyp tissue found significantly elevated CCL13 mRNA levels, but no change in protein expression, and this implies that research based on mRNA of CCL13 should be supported by protein.

Allergic rhinitis is also an inflammatory disease characterized by excessive production of local type 2 cytokines and increased eosinophils in tissues. In patients with seasonal allergic rhinitis, under nasal allergen stimulation, the level of CCL13 in nasal secretions increases 3.7-fold, while IL-10 and IL-4 significantly decrease; CCL13 may worsen, while IL-10 may alleviate nasal mucosa allergy (12). In addition, similar to asthma, increased responsiveness of CCL13 to TLR7/8 agonists has also been observed in allergic rhinitis. The response to nasal administration of Resiquimod (a specific TLR7/8 agonist) can be used to simply assess nasal mucosal responsiveness (30).

9 CCL13 and obesity and its complications

In recent years, much interest has been generated in the fundamental mechanisms causing the global rise in obesity. Many health issues related to obesity, including coronary atherosclerosis, have been linked in studies to the emergence of persistent low-grade inflammation in the body (84). A positive energy balance and an increased anabolic state, especially in adipocytes, may be the initial stimulus in the case of obesity. This results in the release of chemokines that activate an adaptive inflammatory response, which encourages the healthy expansion of adipocytes while lowering energy stores (85).

It has been demonstrated that adipocytes from obese individuals exhibit higher expression of CCL13 compared to those from lean individuals (86), and elevated levels of CCL13 are positively associated with BMI (87, 88). According to *in vitro* studies, CCL13 expression in preadipocytes is low at baseline but steadily rises following differentiation (89).

Integrated analysis of miRNA and genome-wide data from epicardial adipose tissue (EAT) in patients with coronary artery disease (CAD) suggests that altered metabolic and inflammatory regulation is a hallmark of EAT in CAD. Furthermore, miR-103-3p/CCL13 appears to be a novel candidate that plays a role in EAT function and CAD (90). Additionally, CCL13/CCR2 has been shown to promote the formation of carotid plaques and may act as a link between the activation of platelets and monocytes (91). Coincidentally, data from mouse models confirm that when fed a high-fat diet, mice lacking CCR2 eat less and are less likely to become obese, and CCR2 antagonist therapy lowers inflammatory aspects of obesity, such as macrophage infiltration in adipose tissue (92). Earlier on, a study of adipokines in obesity and related comorbidities suggests that CCL13, a brand-new biomarker for

extreme obesity, may exacerbate subclinical atherosclerosis in persons with obesity by affecting circulating levels of major atherosclerotic markers. After bariatric surgery, CCL13 was significantly reduced, indicating that reduced levels of CCL13 may be one of the mechanisms leading to cardiovascular risk reduction in obese patients following bariatric surgery (93).

Together with CAD, obesity has become a significant risk factor for periodontitis. Notably, levels of CCL13 and high-sensitivity C-reactive protein (hs-CRP) were found to be significantly elevated in both gingival crevicular fluid and serum, and a positive correlation between CCL13 and periodontal parameters (94). When the relationship between obesity, chronic periodontitis, and serum CCL13 concentrations was examined, Pradeep et al. (95) discovered that obese patients with chronic periodontitis had significantly higher serum CCL13 concentrations than the non-obese group. All these discoveries implies that CCL13 and hs-CRP may be markers of chronic inflammation in obesity and periodontal disease.

10 CCL13 and tissue repair

Wound healing is a complex process that occurs in response to skin damage, and involves a series of reparative events that aim to restore the protective skin barrier (96). When the wound is inflamed, the probability of forming a hypertrophic scar will greatly increase (97, 98). CCL13 is barely expressed in normal scar formation but is expressed for a longer period in hyperplastic scar formation (99). An animal study showed that Imiquimod significantly inhibited the expression of CCL13 mRNA and upregulated Th1-associated chemokines in rabbits at 21 days to 63 days after surgery to reduce collagen deposition and the extent of fibrosis and thus inhibit scar hyperplasia in a manner that regulated the Th1 and Th2 axes (19).

The process of bone healing is largely affected by the stability of the fixation (biomechanics) as well as the blood supply to the healing site. This repair process is composed of several phases, including inflammation, repair, and remodeling (100). High mobility group box 1 (HMGB1), a ubiquitous inflammatory factor in fractures, promotes the secretion of CCL4 and CCL13 from mesenchymal stem cells (MSCs) by activating the Ras-associated protein-1 (Rap1) signaling pathway, and these chemokines mediate the migration of MSCs, which in turn promotes fracture healing (13, 101, 102).

11 CCL13 and neurological disorders

Multiple sclerosis (MS) is an immune-related central nervous system disease (103). In MS, upregulation of CCL13 levels in the brain tissue and cerebrospinal fluid can induce monocyte chemotaxis and the secretion of inflammatory cytokines in lymphocytes, ultimately, causing oligodendrocyte activation and myelin destruction (20, 104). The downregulation of CCL13 protein expression in the plasma of MS patients in the third trimester of pregnancy and its increase after delivery may be related to the immune tolerance established during pregnancy, this expression pattern of CCL13 may reflect the disease activity of MS during pregnancy (14). Indeed, the haplotype gene in CCL13 is associated with susceptibility to MS in both rat and human genomes (104). In addition, the administration of intravenous immunoglobulin to patients with MS downregulates CCL13 expression in peripheral T cells, which in turn may inhibit T cell proliferation (20, 104).

12 CCL13 and mental illness

Mental illness refers to a range of clinical manifestations arising from brain dysfunction that is influenced by various biological, psychological, and social environmental factors, resulting in varying degrees of impairment in cognitive, emotional, volitional, and behavioral mental activities (105). In recent years, despite significant advances in molecular mechanisms in neuroscience, few biomarkers have made their way into clinical psychiatric practice (106). CCL13 has been shown to drive chemotaxis of pro-inflammatory cells to the inflamed or injured central nervous system (CNS), therefore, chemokines could potentially serve as novel diagnostic and therapeutic targets in psychiatric disorders (107).

In individuals with post-traumatic stress disorder (PTSD), there is an increase in serum levels of CCL13, CCL20, and CXCL6, which may indicate a higher risk for developing PTSD. In contrast, CX3CL1 may serve as a marker for recovery; more than any of these, CCL13 shows a positive correlation with scores on the PTSD Checklist (PCL), suggesting that CCL13 levels may be associated with the severity of PTSD symptoms (108). Another study revealed that plasma levels of CCL13/CCL2 ratios were approximately twofold higher in individuals with PTSD, without significant changes observed in cerebrospinal fluid levels, these ratios remained constant over circadian time, regardless of gender, body mass index, or age at the time of trauma, and may be potential circadian biomarkers for chronic PTSD (109). All of these suggest that the diagnosis of PTSD based on a single marker is difficult, and a combination of multiple substances may be more promising. However, unlike in PTSD, brain tissue levels of CCL13 were significantly lower in the dorsolateral prefrontal cortex of individuals who completed suicide, revealing that chemokine alterations may be suicide-specific immunological mechanisms (110). Interestingly, in a 12-year follow-up study, individuals who attempted suicide had lower levels of CCL13, CCL11, CCL4, CCL2, and CCL17 in their cerebrospinal fluid and plasma compared to those with psychosis who had never experienced suicidal thoughts (111). This long-term study strongly demonstrates that chemokines such as CCL13 are of great research value, as they are likely to play a role in the maintenance of immune homeostasis in the organism, which is critical for mood stabilization and regulation.

13 CCL13 and AIDS

The pathogenesis of Acquired Immune Deficiency Syndrome (AIDS) is primarily a result of cellular immunodeficiency caused by

the direct and indirect actions of HIV (112). The primary targets of HIV have activated $CD4^+$ T lymphocytes (113); and in untreated HIV-infected patients, the macrophage phenotype has been reported to shift from M1 in the early stages of infection to M2 in the later stages and can inhibit viral replication (114).

According to a multicenter AIDS cohort study, CCL13 detectability was found to be greater than 80%, and elevated plasma levels of CCL13 may be associated with more rapid disease progression in HIV-infected individuals (115, 116). In a multicenter AIDS cohort study (MACS) conducted from 1984 to 2009, CCL13 was found to be higher in the group of SUP (exposed to HARRT with HIV RNA suppressed to less than 50 copies/ml plasma) compared to the HAART-naive (NAI) group, on the timeline, CCL13 increased significantly in the first year of viral suppression, followed by a uniformly flat trajectory, HIV suppression appeared to increase the levels of the M2-associated chemokines (117). In addition, the growth hormone-releasing hormone (GHRH) analog tesamorelin significantly reduced CCL13 expression in HIV populations with metabolic dysregulation, and systemic and end-organ inflammation, suggesting that enhancement of the GH axis may improve immune activation in this population (21).

14 CCL13 and cancer

Cancer growth and response to therapy are both significantly influenced by inflammation, with chronic inflammation increasing tumor progression and therapeutic resistance. Acute inflammatory responses, on the other hand, frequently promote dendritic cell (DC) maturation and antigen presentation, which can drive anti-tumor immune responses. Multiple chemokines, such as those in the CCL and CXCL families, have been identified as key regulators of inflammation initiation and regression (118). The majority of tumor-associated macrophages (TAMs) in most solid tumors are M2-type TAMs (M2 TAMs), which are essential for controlling the immunosuppressive tumor microenvironment, encouraging tumor angiogenesis, and promoting tumor spread (15). CCL13 is a crucial chemokine for M2 TAMs and may be involved in its function of it (Table 3).

High blood CCL13 levels were independently a marker for predicting distant metastasis in colorectal cancer, according to logistic regression analysis, and they were substantially linked with advanced age, advanced T-stage, distant metastasis, and UICC stage in colorectal cancer (119). In cases of gastric cancer, the levels of CCL13 reflect the distinct response patterns of fibroblasts in specific tumor sites toward cancer cell invasion. Elevated levels of CCL13 can be indicative of submucosal invasion by tumor cells (120). In M2 TAMs, stress granule (SG) formation was stimulated by tumor micro-environment (TME) stress, and SG increased DDX3Y/hnRNPF mediated mRNA stability of CCL13, which in turn enhanced CCL13 expression and promoted the metastasis of oral squamous cell carcinoma metastasis, however, the above molecular expression and phenotype are reversed upon knockdown of the G3BP1 (15). Intriguingly, tissues from individuals with metastatic salivary adenoid cystic carcinoma(SACC) showed higher expression of CCL13 in the absence of tumor recurrence or perineural invasion than in the presence of tumor recurrence (121). Moreover, CCL13 is linked to 2-microglobulin (2-MG) levels in multiple myeloma, poor prognosis in prostate adenocarcinoma, and cell proliferation in breast cancer (122–124). Likewise, hepatocellular carcinoma (HCC) tumor tissues contain considerably more CCL13 mRNA than normal tissue, however this is unrelated to the clinical prognosis (125). In ovarian cancer, CCL13 triggers epithelial-mesenchymal transition *via* the p38 MAPK pathway (128). Chronic hypoxia does not seem to affect the expression of CCL13, according to *in vitro* experiments using breast cancer, hepatocellular carcinoma, and lung adenocarcinoma cells (129).

Further to that, it has been discovered that CCL13 protein expression is augmented in the plasma of pediatric Hodgkin's lymphoma patients and is related to a sluggish early response; and for Hodgkin's lymphoma patients, biologically-based risk stratification algorithms that incorporate CCL13 could be taken into consideration to enhance treatment results and reduce toxicity (126, 127). In cutaneous T-cell lymphoma

TABLE 3 Effect of CCL13 on phenotype in tumors or its clinical significance.

Cancer species	Significance of elevated CCL13	References
Oral squamous cell carcinoma	Promotion of tumor cell metastasis	(15)
Colorectal cancer	Markers of distant metastasis	(119)
Gastric cancer	Suggests tumor invasion into the submucosa	(120)
Salivary adenoid cystic carcinoma	Means a lower risk of recurrence	(121)
Multiple myeloma	Associated with β2-MG production	(122)
Prostate adenocarcinoma	Poor prognosis	(123)
Breast cancer	Promotes cancer cell proliferation	(124)
Hepatocellular carcinoma	Unclear	(125)
Pediatric Hodgkin's lymphoma	Associated with slower response to early treatment	(126, 127)
Cutaneous T-cell lymphoma	Mediating immunosuppression	(22)

(CTCL), CCL13$^+$ monocytes/macrophages play a role in mediating immunosuppression by interacting with malignant T cells, and blocking the S100A9-TLR4 interaction with tasquinimod has been shown to inactivate the NF-κB pathway, leading to inhibition of CTCL tumor cell growth and induction of apoptosis (22).

15 Discussion

In summary, the main pathogenic mechanism of inflammation involves the recruitment and activation of immune cells, specifically Th2 and M2 macrophages, to inflamed tissues through the chemokine CCL13. Despite the potential of blocking the chemokine system as an important area of anti-inflammatory drug development, only a few drugs, such as ASN002 (a dual-action inhibitor of JAK/SYK), lebrikizumab (IL-13Rα1/IL-4Rα receptor blockade), maraviroc (a CCR5 antagonist), and plerixafor (a CXCR4 antagonist), have received FDA approval (16, 18, 33, 130, 131). However, a single blockade of CCL13 upstream or downstream does not appear to be effective due to high structural overlap and functional crossover between chemokines (33). Additionally, many other immune agents that can modulate chemokines are still not well studied and the molecular mechanisms are still unclear. For another, it is worth noting that CCL13 has been reported to have a positive effect on human diseases such as PBC and suicide, and further exploration of its mechanism is warranted. It should be noted that elevated CCL13 may be linked to antimicrobial resistance, and it ought to take this into account given the potential of CO infections like COPD and cirrhosis (39, 71).

It is crucial to keep in mind that while examining the connection between CCL13 and illness, age is a component that cannot be disregarded. Much research on CCL13 on both youngsters and the elderly has yielded conflicting findings (61, 64). Likewise, as some research suggests that the two may exhibit divergent expression patterns, simultaneous monitoring of lesion locations and peripheral blood chemokines is required (37, 38, 65). Furthermore, insufficient research has been done on the locations where the CCL13 protein is expressed. Several research (5, 132) indicate that CCL13 is not consistently expressed at the levels of transcription and translation, and rather than focusing exclusively on identifying changes in expression at the transcriptional level, future research should validate results through protein analysis.

Author contributions

LL wrote the main manuscript text and prepared the figures and tables. LW made suggestions on the framework of the review. YS reviewed and revised the first draft. LM gave instructions on the drawing. YR and FY helped search literature. FD designed the study, revised and gave final approval of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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New pairings and deorphanization among the atypical chemokine receptor family — physiological and clinical relevance

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Atypical chemokine receptors (ACKRs) form a small subfamily of receptors (ACKR1–4) unable to trigger G protein-dependent signaling in response to their ligands. They do, however, play a crucial regulatory role in chemokine biology by capturing, scavenging or transporting chemokines, thereby regulating their availability and signaling through classical chemokine receptors. ACKRs add thus another layer of complexity to the intricate chemokine–receptor interaction network. Recently, targeted approaches and screening programs aiming at reassessing chemokine activity towards ACKRs identified several new pairings such as the dimeric CXCL12 with ACKR1, CXCL2, CXCL10 and CCL26 with ACKR2, the viral broad-spectrum chemokine vCCL2/vMIP-II, a range of opioid peptides and PAMP-12 with ACKR3 as well as CCL20 and CCL22 with ACKR4. Moreover, GPR182 (ACKR5) has been lately proposed as a new promiscuous atypical chemokine receptor with scavenging activity notably towards CXCL9, CXCL10, CXCL12 and CXCL13. Altogether, these findings reveal new degrees of complexity of the chemokine network and expand the panel of ACKR ligands and regulatory functions. In this minireview, we present and discuss these new pairings, their physiological and clinical relevance as well as the opportunities they open for targeting ACKRs in innovative therapeutic strategies.

KEYWORDS

ACKR1, ACKR2, ACKR3, ACKR4, ACKR5, D6, CXCR7, GPR182

1 Introduction

Chemokines (or chemotactic cytokines) are small soluble proteins (8–14 kDa) that guide cell migration and orchestrate several vital processes, including leukocyte recruitment during immunosurveillance. They are also involved in numerous inflammatory diseases and the development and spread of many cancers (1). They act through classical chemokine receptors (CKRs) that belong to the seven-transmembrane domain G protein-coupled receptor (GPCR) superfamily. Functionally, chemokines can be categorized as homeostatic or inflammatory according to their properties. Structurally, based on specific cysteine motifs in their N termini they are classified as CC, CXC, XC and CX_3C chemokines and their receptors are consequently named CCR, CXCR, XCR and CX_3CR (2).

Over the past years, an important subfamily of chemokine receptors has emerged as key regulators of chemokine functions. Formerly named chemokine-binding proteins, decoys, scavengers or interceptors, the standard nomenclature for this membrane protein family is now atypical chemokine receptors (ACKRs) (3, 4) (Figure 1). ACKRs are generally expressed on lymphatic and vascular endothelium, the epithelium of barrier organs and to a lesser extent on circulating leukocytes, in contrast to the classical chemokine receptors that are mainly found on hematopoietic and immune cells (5, 6). Although ACKRs form a rather diverse group and do not cluster phylogenetically, they do share several characteristics. Among their main common features is the inability to trigger the canonical G protein-mediated signaling or to directly induce cell migration in response to chemokines. Despite this atypicality, ACKRs fulfill essential regulatory functions in the chemokine–receptor network. Their well-established role is the tight regulation of chemokine concentration, for instance in inflammatory processes, and the formation of chemokine gradients for the signaling chemokine receptors, which is accomplished by the capture, transport or internalization of chemokines into degradative compartments or their presentation on cells (4, 7, 8). Other distinctive properties of ACKRs are their unconventional cellular localization, trafficking and expression profile. Indeed, most ACKRs are predominantly found in endosomal vesicles and several can cycle constitutively between the plasma membrane and the intracellular compartments, efficiently scavenging the bound chemokines (3, 7, 9–11). Although these functions were previously considered to mainly rely on β-arrestins, recent reports showed that they are not indispensable (12–17). Dimerization with canonical receptors and consequent alteration of expression and signaling properties is another characteristic of ACKRs that allows modulation of the chemokine network (8, 18, 19).

To date, out of the 23 chemokine receptors recognized by the International Union of Basic and Clinical Pharmacology (IUPHAR), four are members of the ACKR family (ACKR1–4) (20). This group of atypical receptors will presumably increase in the near future, both in terms of number and relevance. Indeed, for each of the ACKRs, recent pairings with chemokines or, as in the case of ACKR3 non-chemokine ligands, have been reported, and it is expected that new members, such as the recently deorphanized promiscuous chemokine scavenger GPR182 (ACKR5), will further enlarge this family.

In this minireview, we present and discuss these new pairings, their physiological and clinical relevance but also the growing number of properties that unify this somewhat heterogeneous receptor subfamily.

2 Pairing of dimeric CXCL12 with ACKR1

ACKR1 (formerly DARC for Duffy Antigen Receptor for Chemokines) is the oldest known chemokine receptor. It is barely

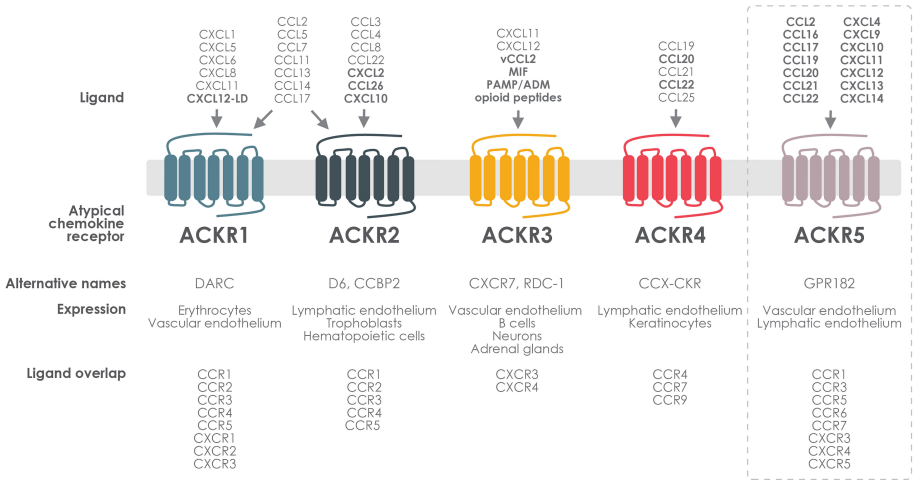

FIGURE 1
ACKR expression, ligand selectivity and crosstalk with classical chemokine receptors. Atypical chemokine receptors are expressed on different types of endothelial or immune cells. ACKR1 and ACKR2 bind a broad spectrum of inflammatory chemokines that they share with CXCR1–3 and CCR1–5. ACKR3 binds the homeostatic chemokine CXCL12, which it shares with CXCR4, and the inflammatory CXCL11, shared with CXCR3. ACKR3 also binds MIF and small non-chemokine peptides such as the proadrenomedullin-derived peptides, ADM and PAMP, as well as several opioid peptides. ACKR4 interacts with a limited number of mainly homeostatic chemokines that it shares with CCR4, CCR7 and CCR9. ACKR5 binds a wide range of both CC and CXC chemokines shared with CCR1, CCR3, CCR5–7 and CXCR3–5 and is still awaiting official IUPHAR recognition as an atypical chemokine receptor (dashed rectangle). Newly identified pairings are indicated in bold. CXCL12–LD: CXCL12 locked dimer.

recognizable as one from its primary amino acid sequence and its phylogenetic association (21, 22) and was initially described as blood group antigen and as a receptor for the Duffy Binding Proteins (DBP) from *Plasmodium knowlesi* and *Plasmodium vivax* malaria parasites (23–25). ACKR1 is prominently expressed on erythrocytes and venular endothelial cells, but not on capillaries or arteries (26–28). ACKR1 owes its distinctive regulatory function to its ability to internalize chemokines in polarized cells, mediating their transcytosis and increasing their bioavailability by presenting bound chemokines to other chemokine receptors in a spatiotemporally well-defined manner (29). Although ACKR1 is unable to promote the degradation of its ligands, it can compete with classical receptors for chemokine binding or reduce their availability in defined regions via internalization. By this mechanism, ACKR1 was proposed to play a role in impairing chemokine-induced angiogenesis (30, 31). On erythrocytes, ACKR1 binds circulating inflammatory chemokines with high affinity and can act as a "sink" or as a "buffer". Indeed, a number of studies showed that ACKR1 modulates inflammatory responses by depleting its ligands (32, 33).

ACKR1 is the most promiscuous chemokine receptor with over ten chemokine ligands from the CC and CXC chemokine families (34–36). Studies carried out in the 1990s identified several chemokine ligands for ACKR1, which included CXCL1, CXCL4, CXCL7, CXCL8, CCL5, and CCL2 (34, 37). Since then, many more have been discovered with a broad range of affinities. Among the additional chemokines, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL11, CCL7, CCL11, CCL13, CCL14 and CCL17 exhibit strong binding to ACKR1 (36). Most of ACKR1 ligands are classified as inflammatory chemokines, with the receptor exhibiting no preference for either CC or CXC chemokines (36). In contrast, the majority of homeostatic and angiostatic ELR-chemokines show weak or no binding (36, 38, 39).

Recently, using biophysical analysis and immunofluorescence microscopy, ACKR1 was shown to bind with the dimeric form of CXCL12 (40). CXCL12 plays an important part in tissue development, vascular integrity, hematopoiesis, and immunity. Its effects through the interaction with the classical receptor CXCR4 and the atypical receptor ACKR3 have been studied extensively (41–43). It has now been suggested that ACKR1 promotes CXCL12 dimerization, which could potentially interfere with its monomeric signaling (44). The interaction between the CXCL12 dimer and ACKR1 suggests a potential new function for ACKR1 to modify the chemokine's monomer–dimer equilibrium, further deepening the complexity of the functional regulation of CXCL12 (40).

3 Pairing of CXC and CC chemokines with the promiscuous CC chemokine scavenger ACKR2

ACKR2 (formerly D6 or CCBP2), identified in 1997, was until recently reported to exclusively bind inflammatory CC chemokines (45). The main ACKR2 ligands include CCL2–8, CCL11–14, CCL17 and CCL22, which are shared with the classical inflammatory receptors CCR1–5 (46–49). By scavenging these chemokines, ACKR2 is proposed to drive the resolution phase of inflammation and prevent exacerbated immune responses (50–55).

The pairing of ACKR2 with CC chemokines dates from when many chemokines, especially from the CXC class, were not yet known or readily available (45, 46, 49). A recent effort to systematically evaluate the activity of a full array of human and viral chemokines on ACKR2, by examining their ability to induce β-arrestin recruitment, revealed at least one more CC, CCL26, and two CXC chemokines, namely CXCL2 and CXCL10 as ligands of ACKR2 (56) with different potencies and efficacies (Supplementary Figure 1).

CCL26 was identified as a low-potency partial agonist of ACKR2, able to compete with other partial agonists for the binding and uptake by the receptor. CCL26 was previously demonstrated to bind and activate CCR3, although it has also been proposed as a ligand of CX_3CR1 (57, 58). Though the functional relevance of the interaction between ACKR2 and CCL26 remains largely unknown, this chemokine–receptor pair may play a major role in a range of immune-mediated diseases. For instance, in persistent asthma, CCL26 was shown as the most effective inducer of eosinophil migration (59), while ACKR2, which is constitutively expressed in the lung, was shown to reduce airway reactivity by scavenging chemokines (60). Furthermore, considering ACKR2 was described to prevent spread of psoriasiform inflammation (61) and high serum levels of CCL26 were correlated with atopic dermatitis severity (62), it is possible that this new pairing will shed light on mechanisms of autoimmune inflammation. CXCL10, previously known to bind exclusively to CXCR3, is the strongest CXC chemokine identified activating ACKR2. CXCL10 was shown to act as a partial agonist of ACKR2 with potency in the low nanomolar range, inducing approximately half of the maximal response measured with its known full agonist CCL5. This partial agonist behavior was reminiscent of the activity towards its long-established signaling receptor CXCR3 relative to the full agonist CXCL11 (63, 64). Moreover, the potency of CXCL10 towards ACKR2 was approximately three times stronger than towards CXCR3. The rapid mobilization of ACKR2 to the plasma membrane induced by CXCL10 was similar to that observed in the presence of CC chemokines (65, 66), while imaging flow cytometry revealed specific and efficient uptake of labelled CXCL10 by ACKR2-expressing cells. Importantly, the ACKR2-driven intracellular accumulation of CXCL10 was also associated with a reduction of its availability in the extracellular space, pointing towards a regulatory role of ACKR2 for this CXC chemokine. Of note, CXCL10 is a pivotal inflammatory CXC chemokine in many physiological and pathological processes, including angiogenesis, chronic inflammation, immune dysfunction, tumor development and dissemination (67, 68), in which ACKR2 was also shown to be involved (6).

Noteworthy, CXCL2 also showed activity towards ACKR2, although it was weak in comparison to CXCL10 or to the activity it displays towards its classical receptor, CXCR2 (69–72). CXCL2

has no scavenger reported and is an important inflammatory chemokine and a powerful neutrophil chemoattractant. Interestingly, it has recently been reported that ACKR2-deficient mice show increased neutrophil infiltration in different tissues (73) and a higher anti-metastatic activity of neutrophils than normal mice (74). It remains to be investigated whether the enhancement of these neutrophil-related processes results from the suppression of CXCL2 regulation by ACKR2.

4 Pairing of a CC chemokine and non-chemokine endogenous peptides with ACKR3

ACKR3 (CXCR7 or RDC-1) is the second to last deorphanized chemokine receptor. It was initially shown to bind and be activated only by CXC chemokines, namely CXCL12 and CXCL11, which are also ligands for CXCR4 and CXCR3, respectively (41, 75). ACKR3 is expressed by endothelial cells, mesenchymal cells, B cells (76–78), in diverse regions of the central nervous system and in the adrenal glands (79–81). ACKR3-deficient mice die perinatally due to semilunar heart valve malformation and ventricular septal defects and show disrupted lymphangiogenesis and cardiomyocyte hyperplasia, despite no alterations in hematopoiesis (82, 83). Similarly to other scavenging receptors, ACKR3 is generally present intracellularly, and cycles continuously between the plasma membrane and the endosomal compartments (84–86). The scavenging function of ACKR3 was convincingly illustrated in studies using zebrafish embryos, where it shapes CXCL12 gradient during development (42, 87).

In 2018, a study demonstrated that the broad-spectrum antagonist CC chemokine vMIP-II/vCCL2 encoded by the sarcoma-associated herpesvirus (HHV-8) can bind and activate ACKR3 with potency somewhat lower than the endogenous CXC chemokines (88). ACKR3 scavenging of vCCL2 was proposed to impact the life cycle and immune escape of HHV-8 by controlling the availability of this important chemokine and its activity on both viral and host receptors. The identification of vCCL2 as a third chemokine ligand for ACKR3 and the first CC chemokine was also particularly valuable in the understanding of the activation mechanism and function of this atypical receptor (70).

ACKR3 was also shown to be the receptor for the pseudo-chemokine macrophage migration-inhibitory factor (MIF) (89). MIF is an inflammatory cytokine that functions as a chemoattractant and participates in innate and adaptive immune responses by promoting macrophage activation and B-cell survival (90–92). MIF is also a mediator in numerous inflammatory conditions and cancers (91, 93). MIF binding to ACKR3 was shown to promote receptor internalization and to contribute to cell signaling and B-cell chemotaxis (89). Moreover, MIF-induced ACKR3 signaling in platelets was described to modulate cell survival and thrombus formation (94).

Besides chemokines and pseudo-chemokines, ACKR3 was shown to bind several small peptide ligands. ACKR3 was proposed as a scavenger receptor for the two pro-angiogenic peptides adrenomedullin (ADM) and proadrenomedullin N-terminal 20 peptide (PAMP) (95) both encoded by the *Adm* gene, regulating their activity for the cognate receptors CLR/RAMPs and MgRX2, respectively (96, 97). These findings were in line with the observation that *Ackr3* knockout recapitulates the *Adm* overexpression phenotype and that silencing *Adm* expression counterweighs lymphatic and cardiac aberrations observed in *Ackr3* knockout mice (96). Nevertheless, the respective contribution of the two *Adm*-encoded peptides in the phenotype observed requires further investigation as ADM binds to ACKR3 at high micromolar concentrations, whereas processed forms of PAMP have potencies in the nanomolar range (95). ACKR3 was also shown to be a high-affinity scavenger for a broad spectrum of opioid peptides, especially enkephalins and dynorphins, binding and internalizing them. ACKR3 was thus proposed to reduce the availability of these peptides in important opioid centers in the central nervous system, where it is co-expressed with the classical opioid receptors. Modulation of the negative regulatory function of ACKR3 by molecules such as LIH383 or conolidine, an analgesic alkaloid used in traditional Chinese medicine, was shown to potentiate the activity of endogenous opioid peptides towards classical receptors, possibly opening alternative therapeutic avenues for opioid-related disorders (13, 98–101).

5 Pairing of the CC chemokines CCL20 and CCL22 with ACKR4

ACKR4 was deorphanized in 2000 (102). It was proposed to bind CCL19, CCL21, CCL25 and CXCL13, which are the ligands for CCR7, CCR9 and CXCR5, respectively (12, 102, 103). By scavenging these chemokines, ACKR4 was shown to regulate the trafficking and positioning of T cells and dendritic cells (104, 105). ACKR4 is best known for its role in shaping the gradient of CCL19 and CCL21 for CCR7-expressing dendritic cells in the subcapsular sinuses of the lymph nodes in the initial phase of the adaptive immune response (106, 107).

In a recent study, CCL20, previously known to bind exclusively CCR6, was identified as a novel ligand for ACKR4 (108). The authors predicted this chemokine–receptor pairing based on CCL20 sequence and expression similarities with CCL19 and CCL21. They demonstrated that CCL20 triggers β-arrestin recruitment to ACKR4, and is efficiently scavenged by ACKR4-expressing cells, both *in vitro* and *in vivo*. They proposed that by scavenging CCL20, ACKR4 regulates its availability for the classical receptor CCR6 and thereby plays a role in the positioning of CCR6-positive leukocytes within secondary lymphoid tissues for effective humoral and memory immune responses (108).

A parallel systematic pairing analysis using β-arrestin recruitment as readout confirmed CCL20 as a new full agonist

ligand for ACKR4 with nanomolar potency (109). This study also found that CCL22 acts as a potent partial agonist of ACKR4. CCL22, which is a key player in both homeostasis and resolution of inflammatory responses was until then known for its ability to interact with CCR4 and ACKR2. Interestingly, in line with a previous report (110) this study also disproved the agonist activity of CXCL13 towards ACKR4 (109).

6 Deorphanization of GPR182/ACKR5 as a promiscuous scavenger receptor for both CC and CXC chemokines

Until very recently the G protein-coupled receptor 182 (GPR182, formerly known as ADMR) was classified as a class A orphan GPCR. Phylogenetically, it clusters within the chemokine receptor family owing to its 40% sequence similarity to ACKR3 (111). GPR182 was previously suggested as a receptor for adrenomedullin (112), which was later not confirmed (113). It was initially described to be present in several organs (80, 111), further studies identified its prevalent expression in endothelial cells in mouse and zebrafish (114), where it was proposed as a regulator of hematopoiesis.

In 2021, GPR182 was deorphanized and proposed as a new atypical chemokine receptor for CXCL10, CXCL12 and CXCL13 (115). The study confirmed the GPR182 expression in the endothelial compartment by using a transgenic mouse model expressing mCherry fluorescent protein under the control of mouse *Gpr182* promoter. GPR182 was detected in vascular endothelium of lungs, bone marrow, lymph nodes, Peyer's Patches, liver and spleen but not in the vascular endothelium of conductive arterial vessel. It was also detected in lymphatic vessels from skin, intestine and lymph nodes. As its closest paralogue ACKR3, GPR182 was shown to bind CXCL12 with nanomolar affinity. CXCL10 was also a strong ligand for GPR182 and several other binders could be identified from a large set of human chemokines screened in binding competition studies with fluorescently labelled CXCL10, including CXCL13, CCL19 and CCL16.

More recently, a study highlighted GPR182 expression in lymphatic endothelial cells in human melanoma (116). In accordance with the first report, GPR182 was suggested as a novel atypical chemokine receptor for an extended spectrum of chemokines of different families and was tentatively named ACKR5. The authors primarily identified the CXCR3 ligand CXCL9 as able to bind GPR182. Competition binding studies with a set of 35 chemokines revealed the ability of GPR182 to interact also with the other CXCR3 ligands, CXCL10 and CXCL11 as well as promiscuous binding for chemokines belonging to the four different classes (CCL, CXCL, CX_3CL and XCL). The authors suggested that GPR182 might be able to recognize GAG-binding motif, which is critical region for chemokines to adhere to the endothelium. Different GAG-biding peptides were able to disrupt CXCL9–GPR182 interaction, which led the authors to consider the GAG-binding motif as determinant for chemokine interaction.

Interestingly, both studies demonstrated the absence of Gprotein signaling in response to chemokine binding to GPR182 (115, 116), which is a common feature in the atypical chemokine receptor family (3, 4). Of note, a strong constitutive interaction with β-arrestin-2 was observed but no ligand-induced β-arrestin recruitment could be detected (115, 116). However, β-arrestins were suggested to be responsible for the rapid and spontaneous receptor internalization (115). An important scavenging ability was highlighted by rapid uptake of labelled chemokine in GPR182-expressing cells and the increased plasma levels of CXCL10, CXCL12 and CXCL13 in both full- and endothelial compartment GPR182 knockout mice (115). These mice also showed alteration in hematopoiesis, which is consistent with GPR182 scavenging of CXCL12 (115), a chemokine notably involved in this process (115, 117). Absence of GPR182 also determined increased intratumoral concentration of different chemokines (CCL2, CCL22, CXCL1, CXCL9 and CXCL10) (116), which was suggested to contribute to an increased recruitment of tumor infiltrating lymphocytes and, therefore, hypothesized as potential target for improved immunotherapy (116).

Further studies are needed to validate GPR182 ligand specificity, as this aspect is not entirely consistent between the two studies. Both studies do however propose GPR182 as a broad-spectrum atypical chemokine receptor. This is particularly interesting as it would represent the only scavenger receptors identified so far for chemokines like CXCL9, CXCL13, CCL16 and CCL28. In the absence of detectable ligand-induced GPR182 signaling, it is challenging to determine precisely the receptor selectivity as well as its molecular characterization. It renders the official inclusion of GPR182 in the atypical chemokine receptor family by the IUPHAR particularly complex.

7 Discussion

Significant progress has been made over the last decade towards a better comprehension of the functional and molecular aspects underlying the activity of ACKRs in health and disease. They have been gaining continuous consideration and are presently regarded as one of the most important receptor family standing at the forefront of the chemokine research and holding great therapeutic potential (6, 118–120).

The unifying characteristic of ACKRs and unique integration criteria is so far their inability to trigger G protein signaling in response to chemokine binding. However, ACKRs often share other properties, such as the predominant intracellular localization or the

ability to constitutively cycle between the plasma membrane and the intracellular compartments. Furthermore, early and more recent pairings suggest that ACKRs are commonly responsive to chemokines from different families. Indeed, the ability to bind and respond to both CC and CXC chemokines was historically described for ACKR1 (121) and — although it was subsequently challenged (109, 110) — for ACKR4 (102). This cross-family selectivity has now been extended to ACKR2 (56), ACKR3 (88) and ACKR5 (115, 116) and therefore appears to represent an additional functional characteristic of ACKRs (3) that is not observed among the classical chemokine receptors.

Despite the many similarities, each ACKR presents its own distinct particularities in terms of expression pattern, ligand selectivity, function and mode of action. For instance, while most ACKRs interact with β-arrestins, ACKR1 seems to be an exception. ACKR3 also stands out in its atypicality as it is highly prone to activation (70) and can act as a receptor also for non-chemokine small peptide ligands (13, 95, 98). Whether these two properties are linked and exclusive to ACKR3 or shared with other ACKRs remains to be investigated. Finally, GPR182 (ACKR5) seems to be a highly promiscuous receptor continuously scavenging chemokines with high basal β-arrestin association (115, 116).

While it may seem surprising that several chemokine–ACKR pairings have been identified only recently, it was made possible thanks to different technological and scientific advances. For the long-established ACKRs, the better understanding of their function, mode of action and the commercial availability of chemokines as recombinant proteins have facilitated the recent pairings. Most importantly, the development of various sensitive assays allowing to accurately detect the activity of chemokines on the receptors independently of G protein signaling, e.g. via the induction of β-arrestin recruitment or the modification of the receptor trafficking or localization, have been instrumental to identifying new ligand–receptor interactions (122). In case of GPR182, which shows high level of basal cycling activity and β-arrestin interactions, a combination of experimental approaches allowed for its deorphanization. Receptor sequence comparison, precise determination of the expression profile and the use of binding competition studies confirmed by increased chemokine plasma concentration in knockout mice, were required to circumvent the problems related to the absence of direct chemokines-induced effects on the receptor (115, 116). For this receptor, additional independent investigations are now needed to precisely define the panel of chemokines it can scavenge and obtain an official inclusion by the IUPHAR in the ACKR family as ACKR5.

The chemokine–receptor network is well recognized for its highly intricate interactions where a chemokine may interact with several receptors, while a chemokine receptor has usually multiple ligands (Figure 2). On the other hand, some chemokines may be exclusive of a single classical receptor. However, the recent pairings described above identified at least one ACKR for a number of these chemokines, such as CCL20 (CCR6), CCL25 (CCR9), CXCL2 (CXCR2), CXCL9 and CXCL10 (CXCR3), CXCL13 (CXCR5) expanding the panel of ACKR ligands and functions. To date, out of the 45 human chemokines, several of them binding to XCR1 (XCL1 and XCL2), CCR8 (CCL1 and CCL18), CCR10 (CCL27 and CCL28), CCR3 (CCL15 and CCL24), CCR1 (CCL23), CXCR1 (CXCL6), CXCR6 (CXCL16), CX_3CR1 (CX_3CR1) and the orphan chemokine CXCL17 have not been paired with an ACKR (Figure 2).

The recent new pairings suggest that a systematic reassessment of chemokine–receptor interactions for ACKRs but also long-established classical chemokine receptors may still be necessary. Indeed, owing to the functional selectivity and biased signaling reported for some chemokines and receptors, the attempts to uncover new pairings should not be limited to monitoring G protein signaling or β-arrestin recruitment. Other approaches such as measuring fluorescent ligand uptake, receptor trafficking or chemokine degradation in both agonist and antagonist modes should also be considered, as important crosstalks may remain unexplored.

The novel pairings among ACKRs add an unforeseen level of complexity to their functions and regulatory roles for chemokines and non-chemokine ligands, while they also open interesting therapeutic opportunities, notably for cancer and chronic pain. For instance, the identification of ACKR2 and GPR182 as scavenger receptors for CXCL10 and/or CXCL9, in addition to their well-established inflammatory CC chemokine ligands such as CCL2, CCL4 and CCL5, may be exploited in approaches seeking to turn cold tumors to hot tumors to improve the effectiveness of immunotherapies. Indeed, these newly identified chemokines for ACKR2 and GPR182 are key players in driving NK cells and CD8+ T cells into the tumor bed (123–126). Therefore targeting their receptors may consequently increase the chemokine levels in the tumor microenvironment and subsequently sensitize them to immunotherapy (56, 118). On the other hand, targeting ACKR3 and blocking its proposed opioid peptide scavenging function was proposed as a new avenue to develop safer drugs with less side effects, which is critically needed to treat chronic pain (100, 101).

However, considering the importance and multiplicity of their functions, the constantly growing number of ligands identified, the complexity of their biology and the interconnectivity with multiple systems, the targeting of ACKRs remains a great challenge. So far, only small molecules, peptides, modified chemokines and antibody fragments targeting ACKR3 have been reported, partly owing to the long-established importance of the CXCR4–CXCL12 axis in cancer, autoimmune and cardiovascular diseases (13, 70, 119, 120, 127–131). Nevertheless, the increasing number of studies showing implication of other ACKRs, including ACKR5, in cancer development, progression but also protection together with the increasing availability of screening assays specific for each ACKR will likely favor in the new future the development of modulators for other members of the family (100, 122).

In the coming years, the ACKR family may be further enlarged (132). Indeed, CXCR3B, the extended isoform of CXCR3, was

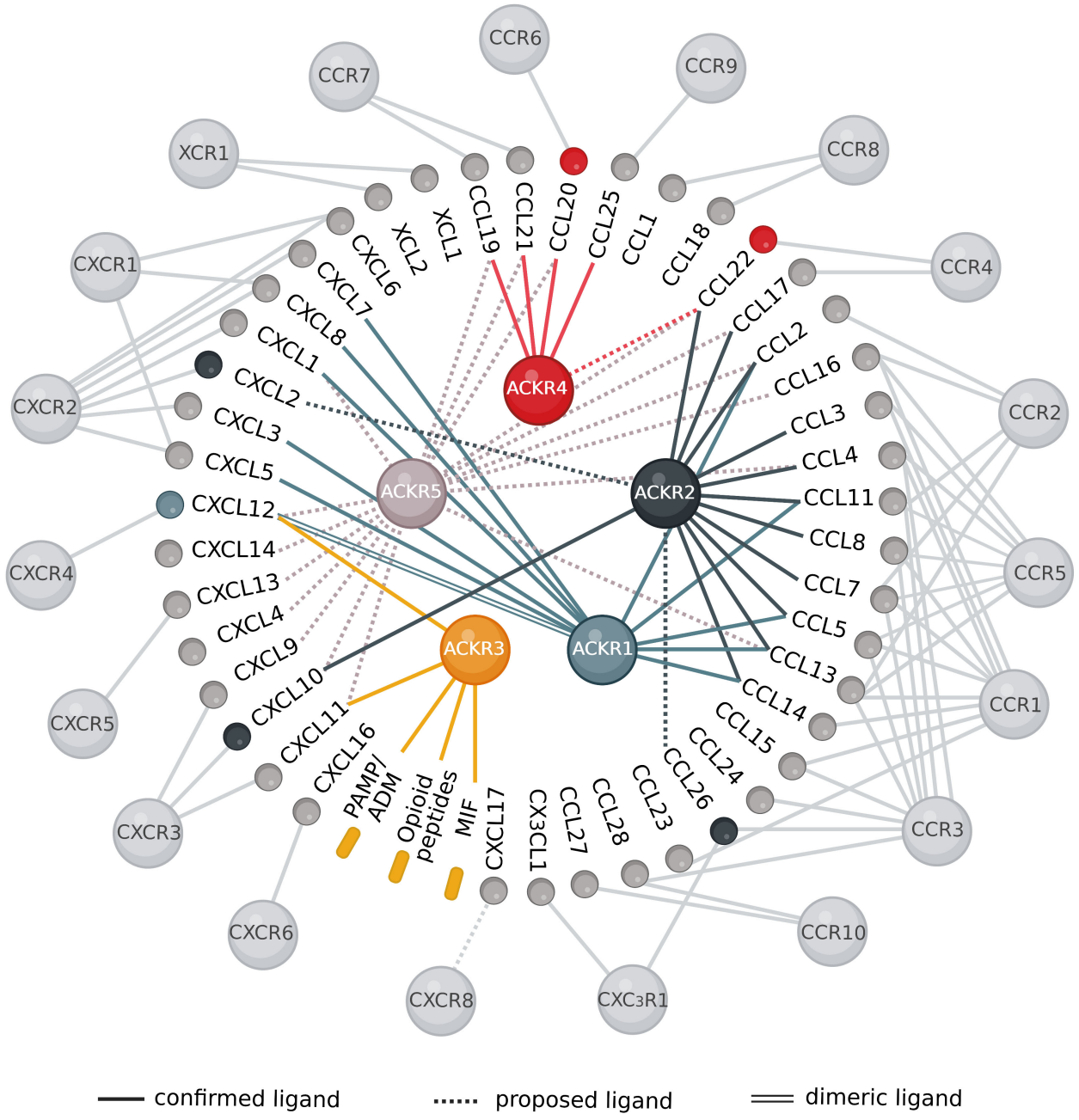

FIGURE 2
Overview of the chemokine interaction network with classical and atypical receptors. The interactions between different chemokines and their signaling and regulatory receptors are highly promiscuous. Most chemokines can bind several receptors and the majority of the receptors have multiple ligands. Receptors and chemokines are represented as spheres, while non-chemokine ligands are represented as rounded rectangles. There are 45 chemokines, 19 classical chemokine receptors (light grey) and 5 atypical chemokine receptors: ACKR1 (light blue), ACKR2 (dark blue), ACKR3 (yellow), ACKR4 (red) and the newly proposed ACKR5/GPR182 (light grey). Colored chemokines and non-chemokine ligands represent recently identified pairings, dashed lines indicate proposed ligands and double lines designate the binding of the dimeric ligand to the receptor. Created with BioRender.com.

recently proposed to display attributes of ACKRs (133), while CCRL2 and PITPNM3 await validation with regard to chemokine binding and direct regulatory functions (134–136). Additional studies will reveal whether the latter two share common functional properties with the established and newly deorphanized atypical chemokine receptors.

In summary, investigations on ACKR are still in a highly dynamic phase and the recent identification of new pairings for established members of the family and of GPR182 as new member will certainly reinforce the interest of the community for this fascinating class of receptors. A better understanding of their functional complexity and heterogeneity is still needed in light of

the extended panel of ligands they regulate and the therapeutic potential they seem to hold.

Author contributions

MS and AC designed the manuscript. MS, GD'U, RL, AAB, and AC wrote the manuscript. MS, GD'U, RL, MT, DL, and AC revised the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1133394/full#supplementary-material

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Emerging roles of a chemoattractant receptor GPR15 and ligands in pathophysiology

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Chemokine receptors play a central role in the maintenance of immune homeostasis and development of inflammation by directing leukocyte migration to tissues. GPR15 is a G protein-coupled receptor (GPCR) that was initially known as a co-receptor for human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV), with structural similarity to other members of the chemoattractant receptor family. Since the discovery of its novel function as a colon-homing receptor of T cells in mice a decade ago, GPR15 has been rapidly gaining attention for its involvement in a variety of inflammatory and immune disorders. The recent identification of its natural ligand C10orf99, a chemokine-like polypeptide strongly expressed in gastrointestinal tissues, has established that GPR15-C10orf99 is a novel signaling axis that controls intestinal homeostasis and inflammation through the migration of immune cells. In addition, it has been demonstrated that C10orf99-independent functions of GPR15 and GPR15-independent activities of C10orf99 also play significant roles in the pathophysiology. Therefore, GPR15 and its ligands are potential therapeutic targets. To provide a basis for the future development of GPR15- or GPR15 ligand-targeted therapeutics, we have summarized the latest advances in the role of GPR15 and its ligands in human diseases as well as the molecular mechanisms that regulate GPR15 expression and functions.

KEYWORDS

GPR15, C10orf99, GPCR, chemokine, homing, inflammation

Introduction

The major task of the intestinal immune system is to tolerate innocuous food antigens and commensal microbes while fighting ingested pathogens. Failure to balance tolerogenic and inflammatory reactions can result in diseases, such as inflammatory bowel disease and gastrointestinal infections. Multiple immune mechanisms, including the balanced activities of regulatory T (Treg) cells and effector T (Teff) cells, contribute to the maintenance of intestinal immune homeostasis. Trafficking of these immune cells to the intestines is tightly controlled by a wide array of chemokines, chemokine receptors, and adhesion molecules expressed by leukocytes, the vascular endothelium, and the epithelium (1). For instance, the

expression of CCR5, CCR6, CCR9, and CCR10 together with α4β7 integrin has been shown to target lymphocytes in the small intestine and colon (2–5). One of the recent advances in this field is the identification of G-protein coupled receptor 15 (GPR15) as a new chemoattractant receptor that mediates the homing of T cells to the colon in response to the natural ligand C10orf99 produced in the colon (6–8). A growing number of studies have suggested important roles of GPR15 in immune homeostasis and pathology of gastrointestinal (GI) tissues (6, 9–11). Similarly important is the discovery of GPR15-independent activity of C10orf99, as well as additional GPR15 ligands, that appear to be involved in the pathology of a broader range of tissues. Collectively, these findings indicate that GPR15 and its ligands are promising new targets for intervention. However, currently there is no approved drug that can specifically modulate GPR15 or GPR15 ligand activities. This review aimed to provide a basis for the development of GPR15- or GPR15 ligand-targeted interventions by summarizing recent research advances in the roles of GPR15 and its ligands in human pathophysiology, as well as the regulatory mechanisms of GPR15 expression and functions.

GPR15

GPR15 is a member of the Class A GPCR family that was cloned in 1996 (12) and identified in 1997 as a co-receptor for SIV, macrophage-tropic, non-syncytium-inducing HIV type 1 (M-tropic HIV-1), and HIV-2 (13, 14). Although this receptor was found to mediate T cell trafficking to the colon (6) and later "deorphanized" when a chemokine-like protein C10orf99 was identified as a functional endogenous ligand (7, 8), GPR15 is an "orphan" in terms of relatively low sequence similarity to its paralogues; it resembles a probable orphan receptor GPR25 with highest similarity of 36%, which is marginally higher than that to angiotensin II receptors, apelin receptor, and other chemokine receptor members (https://www.ensembl.org). GPR15 is also unique in that it lacks cysteines in the NH2-terminal region and the third extracellular loop, which are thought to form a disulfide bond and are required for optimal ligand binding and/or receptor activation in many GPCRs (15). Nevertheless, the NH2-terminal region of GPR15 carries several Tyr and acidic residues, a feature shared by multiple chemokine receptors (16). Sulfation of Tyr residues is known to promote the receptor binding of HIV/SIV (16, 17) as well as chemokine ligands (18–20), and the sulfated Tyr residues in the GPR15 NH2 terminus were recently shown to be required for optimal binding to C10orf99 (21). In humans, *Gpr15* mRNA is highly expressed in the colon and lymphoid tissues, including peripheral blood lymphocytes and the spleen (13), while different studies have reported GPR15 protein expression in the colonic and small bowel mucosa, lymphoid cells, testis, liver, prostate, vascular endothelium, and skin (7, 22, 23). In peripheral blood, GPR15 is expressed in T cells (primarily $CD4^+$) and at lower levels in B cells, monocytes, and neutrophils (11, 24, 25). It is of note that GPR15 expression was not induced by retinoic acid (6), that is known to regulate lymphocyte migration to the small intestine by enhancing the expression of CCR9 and integrin α4β7 (26, 27). This may contribute to the colon-specificity of $GPR15^+$ cell homing, combined with the abundant expression of C10orf99 in the colon compared with the small intestine (7, 8).

Role of GPR15 in disease pathology

GPR15 expression in mouse T cell subsets and colitis

A novel function of GPR15 as a colon-homing receptor was discovered in 2013 by Kim et al. using *Gpr15*-deficient mice (6). Knock-in mice with *Gpr15* gene replaced with the GFP sequence showed preferential expression of GFP in $Foxp3^+$ Tregs in the large intestine lamina propria (LILP); approximately 60–70% of LILP $CD4^+Foxp3^+$ cells expressed GPR15, compared to 7–20% of $CD4^+Foxp3^-$ cells. The results of a cell transfer assay confirmed the efficient homing of $GPR15^+$ cells to LILP in an α4β7-dependent manner. The *Gpr15* gene knockout (KO) reduced Treg numbers in LILP and exacerbated colitis induced by *Citrobacter rodentium* infection. In addition, in a non-infectious colitis model in $Rag2^{-/-}$ mice, where CD40 stimulation induced innate immune cell-mediated colitis (28–30), adoptive transfer of Tregs from wild-type mice reduced colitis severity and tissue damage, but Tregs from *Gpr15* KO mice failed to do so (6). These observations suggest that GPR15 is required to dampen the immune response in the large intestine by directing homing of Tregs in mice.

However, a subsequent study by Nguyen et al. using *Gpr15* KO mice demonstrated that GPR15 is also important for the colon migration of pathogenic Teff cells that cause inflammation (11). In a T cell-mediated colitis model in which adoptive transfer of $CD45RB^{hi}$ T cells (naïve CD4 T cells depleted of Tregs) to immunodeficient recipient mice resulted in the generation and intestinal trafficking of Teff cells to cause colitis (31–33), $Rag2^{-/-}$ mice that received naïve *Gpr15*-KO T cells were protected from developing colitis. In addition, GPR15 was found to be induced in *in vitro*–generated mouse Th17 effector cells under conventional polarizing conditions (11). Moreover, a more recent study by Xiong et al. clearly showed substantial GPR15 expression in all Th1, Th2, and Th17 subsets isolated from mouse LILP, although at a lower frequency compared with that of Treg cells (34). Hence, GPR15 is capable of directing the colon homing of both Treg and Teff $CD4^+$ T cells in mice, and the impact of this receptor in colitis pathology will depend on the experimental settings regarding the relative requirement of Treg and Teff subsets for the development of colitis.

GPR15 expression in human T cell subsets and colitis

How are these mouse studies translatable to humans? The original study by Kim et al., who discovered the colon homing function of GPR15 in mouse Tregs, observed an increased amount of *Gpr15* mRNA in the human $CD25^-CD4^+$ T cell population than in Treg-enriched $CD25^+CD4^+$ T cells from colon tissues of patients

with colorectal cancer (6), implying a difference between mice and humans. The following study by Nguyen et al. found that in human colon tissues, GPR15 expression was highly enriched in the IL-5$^+$ or IL-13$^+$ Th2 subset, particularly in patients with ulcerative colitis (UC), and there was little or no GPR15 expression in Treg cells (11). *In vitro* polarization of human peripheral blood mononuclear cells (PBMCs) and mouse spleen cells by cytokines also revealed disparate GPR15 expression patterns in T cell subsets between the two species; GPR15 was expressed primarily in Th2 cells in humans, whereas GPR15 was expressed in Treg and Th17 subsets in mice (11). Further analyses of *Gpr15* gene and master T cell transcription factors for Th2 (GATA3) and Treg cells (Foxp3) led to the conclusion that the following mechanism underlies preferential expression of GPR15 in Th2 cells in humans: (i) GATA3 promotes *Gpr15* gene expression in human Th2 cells by binding to the 3′ enhancer of *Gpr15*, while (ii) Foxp3 binding to the enhancer suppresses *Gpr15* expression in human Treg cells, and (iii) this GATA3 binding does not occur in mouse Th2 cells and Foxp3 binding is much weaker in mouse Treg cells because of the sequence difference in the *Gpr15* enhancer. These authors concluded that GPR15 is preferentially expressed in Teff cells rather than in Tregs in humans and this is reflected in the inflamed colon of patients with UC.

In another study, Fischer et al. utilized a humanized mouse model to comparatively determine the role of GPR15 and α4β7 integrin, in which human peripheral blood T cells from patients with UC were transferred into mice and examined for migration to the colon in dextran sulfate sodium (DSS)-induced colitis (35). Expression of both GPR15 and α4β7 integrin was elevated in Tregs but not in Teff cells in the colon of patients with UC compared with those in healthy controls and patients with CD. Pre-treatment of these cells with siRNA for GPR15 only affected Teff cell homing but did not affect Treg homing, whereas treatment with an α4β7 antibody (vedolizumab) suppressed both Teff and Treg homing, suggesting that GPR15 is important for Teff homing but not Treg homing in humans (35).

Despite all these findings suggesting species differences, a simplified interpretation such as "GPR15 is expressed by Teff cells in humans, while it is expressed by Treg cells in mice," requires caution. Indeed, multiple studies from different groups, including later studies from the same group of Nguyen et al., have shown that GPR15 is expressed by human Tregs in the peripheral blood at similar or even higher levels than that in Teff cells (6, 7, 35–37), which is not consistent with the generalized model that GPR15 expression is promoted in Th2 cells and suppressed in Tregs in humans. Furthermore, Adamczyk et al. reported that GPR15 was expressed at an even lower frequency in Teff cells than in Treg cells in colon tissues of either healthy controls or patients with UC (36). Interestingly, they observed significantly increased expression of GPR15 in both Treg and Teff cells from the uninflamed region, but not from the inflamed region, in patients with UC compared with that in tissues from healthy controls. This is also consistent with a more recent study by Xiong et al. that observed substantial expression of GPR15 (nearly 40% on average) in Tregs from uninflamed regions of UC patient colons and a positive correlation between GPR15 and Foxp3 expression in human colonic Tregs (34). These findings are in contrast to the predominant GPR15 expression by Th2 cells observed in UC colon by Nguyen et al. (11). The reason for this discrepancy is not clear, and further human studies are necessary; however, the differential expression of GPR15 in Tregs in the inflamed and uninflamed regions of the UC colon could provide some insights. The fact that GPR15 expression was not increased in Tregs from the inflamed region of the UC colon implies that GPR15 is not an exclusive master regulator for the migration of Tregs into the inflamed colon. On the other hand, the increased GPR15 expression on Tregs in the non-inflamed region also raises the possibility that GPR15 drives Tregs to the colon of patients with UC, but its expression is downregulated in the inflamed environment. For instance, increased local production of the GPR15 ligand might enhance receptor internalization and subsequent degradation. In addition, since dysbiosis in UC is characterized by reduced levels of bacterial metabolites, including short chain fatty acids (SCFAs) (38) and GPR15 expression was found to be upregulated by SCFAs (36, 39, 40), the diminished local production of SCFAs could result in the reduced expression of GPR15 on Tregs in the inflamed colons of patients with UC. Interestingly, the aforementioned study by Adamczyk et al. (36) found that the majority of Teffs and Tregs in the peripheral blood did not co-express GPR15 and α4β7 integrin, and this lack of co-expression was also detectable in colonic biopsies of healthy individuals and patients with UC. This indicates the phenotypic heterogeneity of T cells, especially Tregs (41, 42) and highlights the need for careful analysis of the expression of multiple molecular factors potentially involved in the colonic migration of T cells.

Colorectal cancer

In addition to colitis, GPR15 has also been implicated in the pathogenesis of CRC. Tregs infiltrating the tumor sites create an immunosuppressive tumor microenvironment that prevents the development of effective anti-tumor immune responses (42, 43). Adamczyk et al. compared gene expression in Treg cells from tumors and healthy colonic tissues of colitis-associated CRC mice induced by azoxymethane (AOM)/DSS treatment (10). They identified a specific set of genes that are preferentially expressed in tumor-associated Tregs, including GPR15. Similar to mice, the frequency of GPR15$^+$ Tregs, but not that of GPR15$^+$ Teffs, in the tumor sites of patients with CRC was significantly higher than that in non-tumor sites, suggesting a distinct role for GPR15 in Treg delivery to CRC sites in humans (10). Importantly, genetic deletion of *Gpr15* in mice significantly decreased the infiltration of tumor-associated Tregs, reduced the Treg/CD8$^+$ T cell ratio, and diminished tumor development (10), suggesting that GPR15 is responsible for directing the colon migration of Treg cells that support the growth of CRC. Thus, GPR15 represents a promising novel target for modifying T cell-mediated anti-tumor immunity in CRC.

Eosinophilic esophagitis

EoE is an allergic disease characterized by chronic esophageal inflammation with prominent recruitment of eosinophils (44). Inflammation in EoE critically involves Th2 cells that produce cytokines such as IL-5 and IL-13, which promote eosinophil recruitment and activation and exacerbate epithelial barrier dysfunction, respectively (44, 45). In a recent study, Morgan et al. (9) conducted single-cell RNA analysis of T cells in tissues from patients with EoE. They found that *Gpr15* expression was increased in highly polarized pathogenic effector Th2 (peTh2) clonotypes detected in both esophageal tissue and peripheral blood of patients with EoE, and *Gpr15* was the most significantly upregulated transcript in these cells in the esophagus compared with peripheral blood. While the genes encoding integrin α4β7 were broadly expressed by T cells in both the esophagus and duodenum, GPR15 was only expressed in esophageal T cells, and CCR9, a chemokine receptor known for gut homing (46), was only expressed in duodenal T cells. In addition, the authors detected the expression of C10orf99 and CCR9 ligand CCL25 only in the esophageal epithelium and duodenal epithelium, respectively (9). This is consistent with the fact that the esophagus is one of the tissues that most strongly expresses C10orf99 (https://gtexportal.org). These findings collectively support the model that GPR15 expression promotes esophageal homing of peTh2 cells and exacerbates inflammation during EoE, and in addition, GPR15 may serve as a marker for esophagus-migrating peTh2 cells in the peripheral blood of patients.

Rheumatoid arthritis

An earlier study on RA reported that GPR15 is expressed by macrophages in synovial tissue and monocytes and neutrophils in peripheral blood, and its expression is upregulated in patients with RA compared to non-RA controls (24). A more recent study examined GPR15 expression on T cells from patients with RA and found that the frequency of $CD4^+/CD8^+$ $GPR15^+$ T lymphocytes was higher in patients with RA than in healthy subjects (47). In addition, the frequency of $CD4^+/CD8^+$ $GPR15^+$ T lymphocytes was higher in the synovial fluid of patients with RA than in that of patients with osteoarthritis. Immunostaining results of synovial tissue sections demonstrated that GPR15 and GPR15L are present in the synovial tissues of patients with RA (47). These findings implicate the GPR15-GPR15L axis in RA pathogenesis, which involves both innate and adaptive immune cells.

Skin inflammation

The mouse skin epithelium contains a specialized population of γδ T cell receptor $(TCR)^+$ cells called dendritic epidermal T cells (DETCs), which exclusively express the monoclonal Vγ3Vδ1 TCR and are implicated in protection of skin homeostasis, host defense, and wound healing (48). DETCs mature in the fetal thymus and migrate to the skin during late embryogenesis, after which they are maintained through self-renewal. An earlier study by Lahl et al. found that GPR15 is highly expressed on fetal thymic DETC precursors and that *Gpr15* KO substantially reduces the frequency of epidermal DETCs in neonatal (Day 1) mice compared with *Gpr15* $^{-/+}$ heterozygote neonates (23). This suggested that GPR15 is essential for migration of embryonic DETC to the skin, which is also consistent with the high expression of *C10orf99* in keratinocytes of embryonic (day 16) and neonatal skin (7). In a more recent study, Sezin et al. conducted a profiling of T cell populations in the skin of adult (8-16 weeks) *Gpr15* KO mice and found that the DETCs were reduced by approximately 60% compared to wild-type littermates (49). In addition, the niche of DETCs in the epidermis was populated by αβ TCR^+ cells; approximately 40% of all $CD3^+$ cells in *Gpr15* KO mice were αβ TCR^+ compared to only 10% in wild-type mice (49). Furthermore, these changes were also associated with shifts in the composition of skin microbiome in *Gpr*15 KO mice (49). These studies collectively highlighted a pivotal role of GPR15 in the skin homing of DETCs in mice, which appears to impact the composition of T cell populations and microbiome even in the adulthood.

On the other hand, the role of GPR15 in the skin disease settings remain somewhat elusive. Deficiency in *Gpr15* did not alter the course of disease neither in the imiquimod-induced psoriasiform dermatitis nor in the IL-23-induced dermatitis model, despite the increased expression of C10orf99 in the inflamed skin (50). However, in the antibody transfer mouse model of bullous pemphigoid-like epidermolysis bullosa acquisita (BP-like EBA), an autoimmune subepithelial and mucocutaneous blistering disease, the *Gpr15* KO was found to markedly aggravate the skin pathology (51). Importantly, this was associated with an increased accumulation of γδ TCR^+ cells in the dermis (51), suggesting a possibility that GPR15 may counteract antibody-mediated skin inflammation through direct and/or indirect mechanisms that limit the recruitment of γδ TCR^+ cells into the dermis.

GPR15 ligands and their roles in pathophysiology

C10orf99 in GPR15 signaling

C10orf99 was reported as a natural GPR15 agonist in 2017 (7, 8). Mature human C10orf99 is a short, 57-amino acid basic protein (pI = 11.28) with two pairs of Cys residues that form intramolecular disulfide bridges, implying that this protein is related to a CC chemokine. The tissues expressing C10orf99 include the digestive tract (particularly the colon and esophagus), skin, tonsils, cervix, and bladder. In the mouse colon, C10orf99 expression did not appear to be regulated by colonic inflammation or the presence of commensal bacteria (7). C10orf99 has several unique features that differ from those of the canonical chemokines. Secondary structures typically found in chemokine family proteins, such as loops, β-strands, and helices, were not identified in C10orf99 by structure prediction programs (8). In contrast to most chemokines that

require their N-termini for receptor binding and activation (52), N-terminal deletion of C10orf99 by up to 10 amino acids showed no marked change in GPR15-dependent calcium signaling (8). Instead, the hydrophobic C-terminal region of C10orf99, which is highly conserved among species, is critically required for receptor binding and signaling (8, 21, 53). In addition, unlike canonical chemokines and chemokine receptors, C10orf99 and GPR15 show highly specific interactions; C10orf99 does not cross-activate any of the known 22 chemokine receptors, and GPR15 does not respond to any of the 27 known chemokines (8). Functionally, C10orf99 interaction with GPR15 leads to inhibition of cAMP production, which can be reversed by pertussis toxin, indicating that GPR15 is a Gαi/o-coupled receptor, similar to most chemokine receptors. C10orf99 activates extracellular signal-regulated kinase (ERK)1/2, induces calcium release, promotes β-arrestin recruitment and receptor endocytosis, and induces chemotaxis in GPR15-expressing immune cells (7, 8, 21, 53, 54) (Figure 1A).

C10orf99 in psoriasis

C10orf99 was implicated in immune regulation prior to the discovery of its GPR15 agonistic activity. Its first implication in human diseases came from global analyses of psoriasis-associated genes in 2009 which reported significant upregulation of *C10orf99* in psoriatic skin (55, 56). A large-scale gene knockout study in 2010 showed that disruption of *C10orf99* gene leads to an increased ratio of $CD4^+/CD8^+$ cells and a decreased serum IgM level in mice (57). Multiple studies have demonstrated the regulated expression of *C10orf99* in the epidermis of the skin and its association with psoriasis. Consistent with the epidermal migration of fetal thymic DETCs that express GPR15 (23), *C10orf99* mRNA is highly expressed in the fetal and neonatal epidermis of mice, but is low or nearly absent in the uninflamed adult epidermis both in mouse and human (7, 51). However, *C10orf99* expression was highly upregulated in wounded skin (7), antibody-mediated model of BP-like EBA (51), and imiquimod-induced models of psoriasis in mice (8, 51, 58), and in patients with psoriasis (8, 58–60) or atopic dermatitis (59, 61). *C10orf99* has also been indicated as a reliable marker gene for the classification of psoriasis (60).

A major question is whether C10orf99 plays a determining role in disease progression or whether it merely indicates loss of epithelial integrity. It appears that C10orf99 has GPR15-independent functions. Yang et al. reported that C10orf99 acts as an antimicrobial peptide that exhibits broad-spectrum antimicrobial activity (62) (Figure 1A), as has been reported for some chemokines (63). In addition, local sustained delivery of nanoparticle-encapsulated C10orf99 peptide promoted granulation tissue formation and wound healing in a full-thickness dermal defect rat model (64). Similarly, overexpression of *C10orf99* gene in transgenic mouse was reported to reduce skin inflammation and remodeling after an imiquimod challenge in a published patent application (65). These findings implicate a protective role for C10orf99 in the inflammatory skin.

However, a later study using human keratinocyte HaCaT cells under inflammatory conditions showed that *C10orf99* knockdown decreased cell proliferation, whereas overexpression of C10orf99 promoted their proliferation (58) (Figure 1A). A more recent study also showed that *C10orf99* transfection into normal human epidermal keratinocytes induced the expression of inflammatory mediators and reduced the expression of barrier-related genes such as filaggrin and loricrin (59). The addition of synthetic C10orf99 peptide reduced the expression levels of barrier-related genes in human keratinocyte 3D cultures, suggesting that C10orf99 binding induces specific signaling in keratinocytes (59). Furthermore, local depletion of *C10orf99* by lentiviral shRNA vectors or systemic knockout of *C10orf99* in mice effectively ameliorated imiquimod-induced psoriatic dermatitis, supporting the proinflammatory role of C10orf99 (58, 59). Collectively, these results indicate that C10orf99 is a primary inducible regulator that reduces barrier formation and induces the inflammatory response of keratinocytes under psoriatic conditions (Figure 1A). As already mentioned, deficiency in the *Gpr15* gene did not alter the course of disease in imiquimod-induced psoriasiform dermatitis or the IL-23-induced dermatitis model, suggesting that C10orf99 modulates psoriasiform dermatitis via GPR15-independent pathways (50).

The notion of GPR15-independent activity of C10orf99 is further supported by a recent study by Tseng and Hoon (61) who discovered that C10orf99 can act as an endogenous pruritogen during inflammation that activates Mas-related G protein-coupled receptors (MRGPRs). These authors found that C10orf99 selectively stimulates mouse dorsal root ganglion neurons that express Mrgpra3 and evokes intense itch responses and vasodilation. C10orf99 also caused mast cell degranulation through the stimulation of MRGPRX2 and Mrgprb2, and genetic disruption of *C10orf99* expression attenuated scratch responses in an imiquimod-induced psoriasis model (61). Together, these studies suggest that elevated expression of C10orf99 during psoriasis can aggravate the disease by promoting the proliferation and inflammatory response of keratinocytes, reducing barrier formation, and inducing itch responses and vasodilation by acting on neurons and mast cells (Figure 1A).

C10orf99 in cancer cell growth

The above-mentioned studies indicate that C10orf99 has multiple receptors and cellular substrates that are involved in physiologically different reactions. An earlier study by Pang et al. reported another function of C10orf99: the growth inhibition of cancer cells (66). C10orf99 was found to interact with the transmembrane protein Sushi Domain Containing 2 (SUSD2), hence termed a colon-derived SUSD2 binding factor (CSBF) (66). The authors showed that the C-terminally IgG-Fc-tagged recombinant C10orf99/CSFB protein binds to SUSD2 expressed in CRC cell lines and inhibits cell growth through G1 cell cycle arrest (66) (Figure 1A). However, this model requires further investigation because the inhibitory effect of C10orf99 on CRC cells could not be reproduced in a follow-up study by a different group (62) who used the untagged C10orf99 protein and observed its cytotoxic effect only on a specific B-cell lymphoma line.

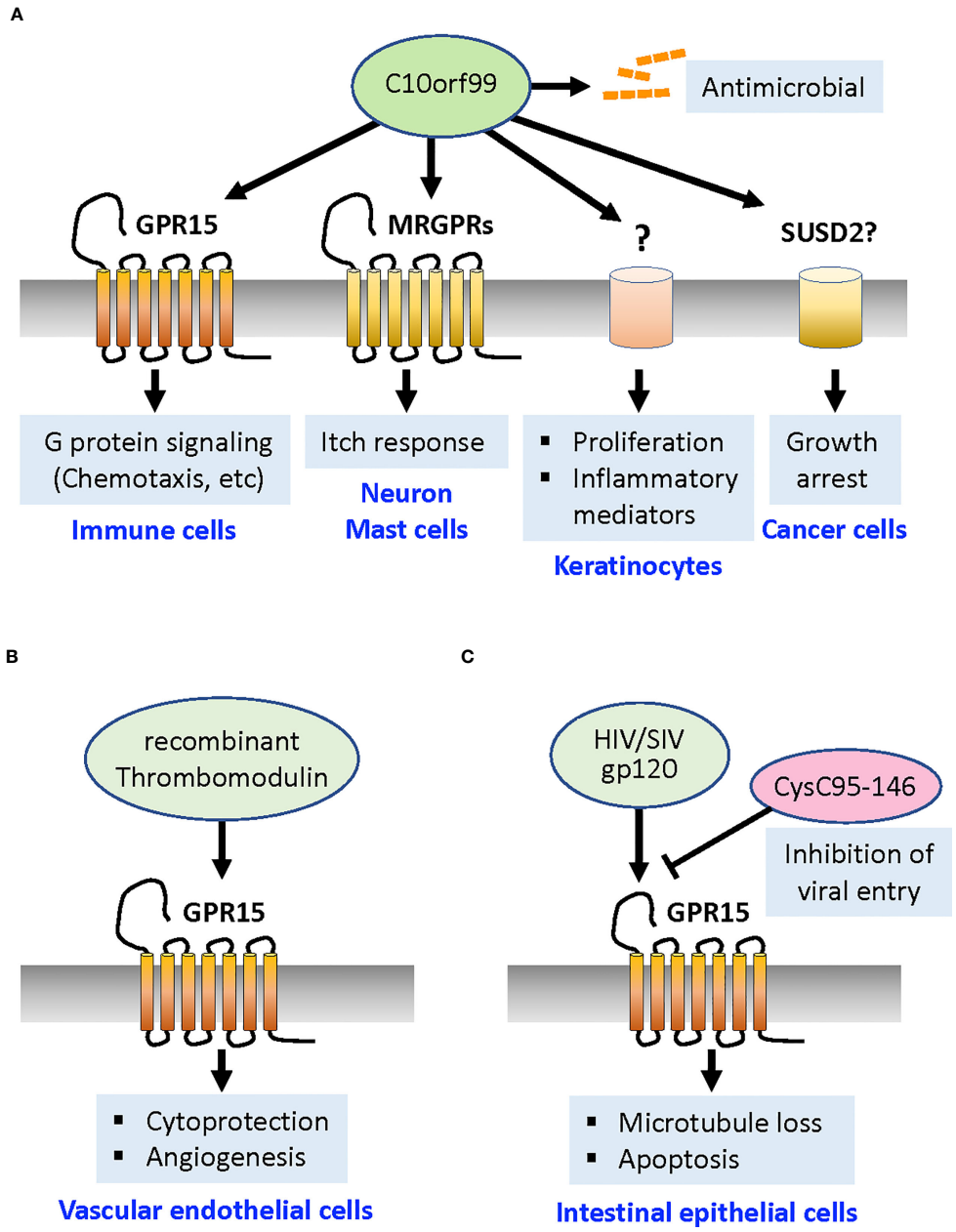

FIGURE 1
GPR15 ligands and their functions. **(A)** A natural GPR15 ligand C10orf99 activates the Gαi-mediated signaling pathways that induce chemotaxis of GPR15$^+$ T cells and other immune cells toward this ligand. C10orf99 also functions independently of GPR15 to exert antimicrobial activities. In the psoriatic skin, C10orf99 evokes itch response by activation of MRGPRs on sensory neurons and mast cells, and promote proliferation and production of inflammatory mediators of keratinocytes through unknown receptor/mechanism. C10orf99 also induces growth arrest of cancer cells by activating SUSD2 receptor (although controversial). **(B)** Recombinant thrombomodulin acts on GPR15 expressed by vascular endothelial cells via its fifth region of EGF-like domain to confer cytoprotection from apoptosis signal and promote angiogenesis. **(C)** HIV protein gp120 induces loss of microtubule and apoptosis in the intestinal epithelial cells. The C-terminal fragment of cystatin C (CysC95-146) competes for GPR15 and inhibits the entry of HIV and SIV. *MRGPRs*, Mas-related G protein-coupled receptors; *SUSD2*, Sushi Domain Containing 2 receptor; *HIV*, human immunodeficiency virus; SIV, simian immunodeficiency virus.

Thrombomodulin as a ligand to GPR15 in vascular endothelial cells

A novel function of GPR15 in ECs, mediated by a ligand that is completely distinct from C10orf99 in structure, was demonstrated in 2017 (67) (see more comprehensive review (68)). Pan et al. found that the recombinant soluble protein TME5, which encodes the fifth region of the epidermal growth factor (EGF)-like domain of TM, binds to the GPR15 expressed by human vascular ECs *in vitro* (Figure 1B). TM is a type I transmembrane protein constitutively expressed by ECs and acts as an anticoagulant by binding to thrombin and activated protein C (69). The blood level of soluble TM is known to be elevated under various pathological conditions involving endothelial damage, such as sepsis, COVID-19 infection,

progressive systemic sclerosis, and diabetes (70–73). Recombinant human soluble TM (rTM, ART-123), consisting of the extracellular domain of TM, has been used to treat disseminated intravascular coagulation (DIC) (74, 75). Pan et al. demonstrated that recombinant TME5 rescued growth inhibition and apoptosis caused by the calcineurin inhibitor FK506 in vascular ECs isolated from wild-type but not from FK506-treated *Gpr15* KO mice (67). This cytoprotective effect was mediated by the activation of ERK1/2 and increased level of anti-apoptotic proteins (67). In addition, TME5 enhanced the migratory activity of ECs and increased their production of nitrogen oxide. Moreover, *in vivo* Matrigel plug angiogenesis assay revealed that TME5 stimulates angiogenesis in wild-type mice but not in *Gpr15* KO mice (67) (Figure 1B). TME5 also ameliorates inflammation in a murine sepsis model in a GPR15-dependent manner through suppression of NF-kB activity and release of pro-inflammatory cytokines in macrophages (76). GPR15 in T cells also appears to mediate TME5-induced anti-inflammatory effects in a murine model of acute graft-versus-host disease (GVHD) caused by allogeneic hematopoietic stem cell transplantation (77).

Interestingly, in a more recent study testing the potential anti-tumor effect of rTM, it was suggested that GPR15 mediates rTM-induced growth inhibition of pancreatic ductal adenocarcinoma (PDAC) (78). The anti-tumor effect of rTM was only observed in PDACs with high GPR15 expression, and rTM suppressed NF-kB and ERK1/2 activation in a GPR15-dependent manner. This inhibition of ERK1/2 activity by rTM is not consistent with its cytoprotective effect in ECs that involves activation of ERK1/2 (67), suggesting that the consequence of rTM-GPR15 interaction is dependent on the cell context. Further investigations in different cell types are necessary to delineate TM-induced GPR15 signaling pathways.

HIV gp120 in enteropathy

Chemokine receptors are expressed in numerous non-hematopoietic cells and play important roles beyond chemotaxis of leukocytes, such as development, angiogenesis, and apoptosis (79–81). GPR15 is abundantly expressed on the basolateral surface of the intestinal epithelium, unlike CXCR4 and CCR5, which are present mainly at or near the luminal surface (22). It was previously reported that HIV-1 surface protein gp120 induces calcium signaling, microtubule loss, and physiological changes, including increased paracellular permeability in the intestinal cell line; these changes resemble HIV enteropathy (82, 83). Fantini et al. found that these gp120-induced effects were inhibited by anti-GPR15 neutralizing antibody or selective G protein inhibitor pertussis toxin (22, 84). They also found that GPR15 mediates viral strain-specific gp120-induced calcium signaling at low, physiologically reasonable gp120 concentrations, which are up to 10,000-fold lower gp120 concentrations than those of the principal HIV co-receptors. These findings suggest that gp120 is involved in HIV enteropathy via its interaction with the GPR15. GPR15 has also been implicated in the apoptosis of intestinal epithelial cells in an SIV infection model (85) (Figure 1C).

Regulation of GPR15 expression

The molecular mechanisms that have been reported to regulate *Gpr15* gene expression are depicted in Figure 2.

Regulation of GPR15 expression by aryl hydrocarbon receptor

The AhR is a ligand-activated transcription factor that has been studied for many decades as a sensor for environmental contaminants, such as dioxins. Research in the past 15 years has led to the emergence of AhR as a key physiological regulator of immune responses, affecting both innate and adaptive systems by sensing a variety of endogenous, dietary, microbial (e.g., tryptophan metabolites), and environmental ligands (86). AhR is known to regulate the differentiation, homing, and immunosuppressive functions of Tregs (87, 88) and numerous studies have shown that AhR ligand activation can reduce inflammation and ameliorate disease (89–92). AhR is also downregulated in the intestinal tissue of patients with IBD (93). Using genetic mouse models, Zhou et al. found in 2017 that GPR15 expression is significantly reduced in AhR-deficient colonic Treg cells (94). A more recent study by the same group (34) demonstrated that the AhR-dependency of GPR15 expression was not confined to Tregs, but was observed for all murine $CD4^+$ T cell subsets in the colon. In addition, when wild-type mice were fed with an AhR-ligand-deficient diet, the addition of the dietary AhR ligand indole-3-carbinol (I3C) (95) significantly enhanced GPR15 expression in intestinal $CD4^+$ T cell subsets, while this effect was abrogated in Tregs (but not in other $CD4^+$ subsets) in engineered mice in which *Ahr* was specifically ablated in Tregs (34). Moreover, in a short-term homing assay, in which *in vitro* differentiated Tregs (iTregs) were transferred to $Rag1^{-/-}$ mice, the number of $Ahr^{-/-}$ Tregs migrating to the colon was approximately 3-fold lower than that of $Ahr^{+/+}$ Tregs. Importantly, forced expression of GPR15 in $Ahr^{-/-}$ Tregs significantly enhanced their homing to the colon but not to other organs, suggesting that AhR promotes colon-specific homing of Tregs by enhancing GPR15 expression in mice (34). Notably, GPR15 expression in iTregs generated from human PBMCs was enhanced or reduced by the AhR agonist or antagonist, respectively, indicating that AhR regulates GPR15 in humans as well (34). This is supported by another study by McAleer et al. (96) who found regulation of GPR15 expression by an AhR agonist and antagonist in human $CD4^+$ T cells. In a separate recent study, Swaminathan et al. reported the regulation of GPR15 expression by AhR in activated human PBMCs, sorted effector/memory $CD4^+$ T cells, and *in vitro* polarized human Th2 and Treg cells (37). Collectively, these studies have uncovered a novel role of AhR in controlling colon homing of $CD4^+$ T cells by positively regulating GPR15 expression in mice and humans. It is interesting to note that, retrospectively, there was already an indication of AhR regulation of GPR15 in 2007 when *Gpr15* was identified as a novel dioxin-inducible gene (97).

A recent study demonstrated that the AhR intrinsically promotes differentiation and function of resident memory $CD8^+$

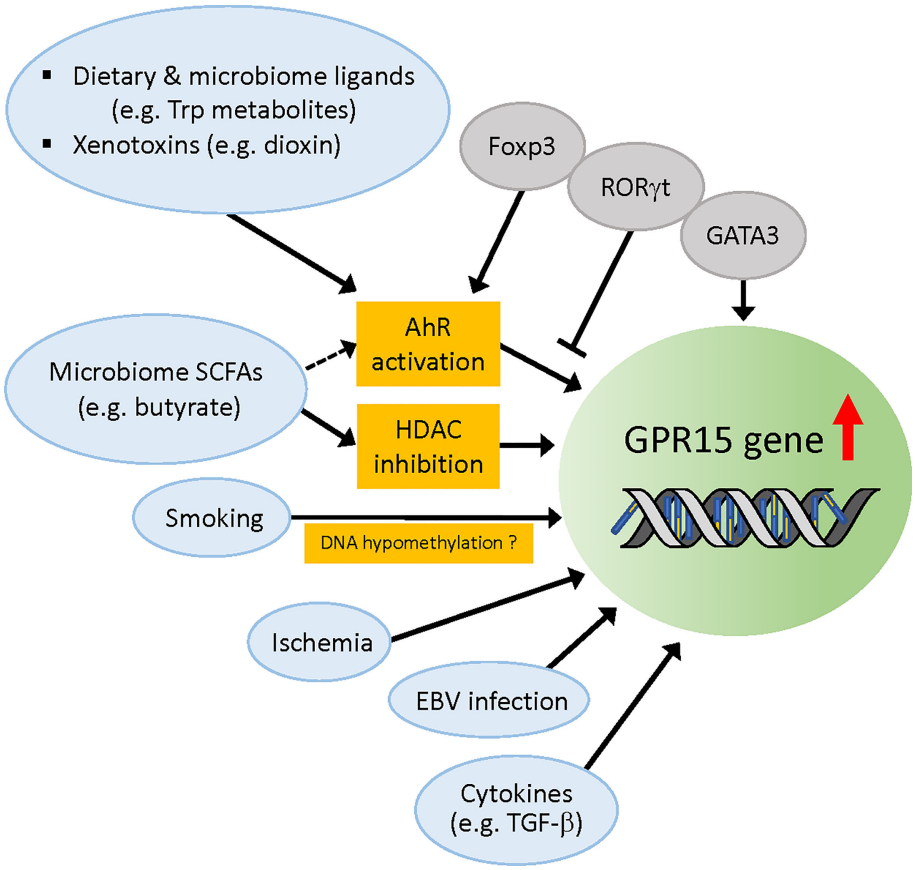

FIGURE 2
Regulation of *Gpr15* gene expression. Molecular factors that have been reported to upregulate *Gpr15* expression are depicted. Dietary and microbiome ligands such as tryptophan metabolites activate AhR that directly acts on *Gpr15* gene. Foxp3 cooperates with AhR (although contradictory data exist) while RORγt competes with AhR, and GATA3 enhances *Gpr15* expression independently of AhR. Foxp3 and GATA3 are reported to differentially bind *Gpr15* enhancer and regulate *Gpr15* expression in the T cells of human and mouse (not depicted in this figure). SCFAs from microbiota such as butyrate upregulate Gpr15 expression by inhibition of HDAC but might also by activation of AhR. Smoking habit is strongly associated with DNA hypomethylation of *Gpr15* and increased mRNA expression of *Gpr15* in blood leukocytes, but the evidence for the direct effect of cigarette smoke extract on *Gpr15* DNA methylation is lacking. Instead, the increased frequency of peripheral $CD3^+GPR15^+$ T cell population appears to account for the apparent hypomethylation of *Gpr15* DNA in blood samples from smokers. Infarction-related ischemia in mice and ischemic condition in cell culture induces *Gpr15* expression in cardiomyocytes. EBV infection in B cells enhances *Gpr15* expression by an unidentified mechanism. TGF-β induces GPR15 expression *in vivo*. *AhR*, aryl hydrocarbon receptor; *SCFAs*, short chain fatty acids; HDAC, histone deacetylase; *EBV*, Epstein-Barr virus.

T cells, including those in the intestinal epithelium (98). Nevertheless, genetic deletion or activation of AhR did not affect GPR15 expression in $CD8^+$ T cells (34), suggesting an AhR-independent mechanism for GPR15 expression in $CD8^+$ T cells. The crucial role of AhR in regulating GPR15 expression in $CD4^+$ but not in $CD8^+$ T cells suggests a potential therapeutic target in intestinal disorders, e.g., colorectal cancer where disruption of *Gpr15* gene reduced infiltration of $CD4^+$ Tregs but not $CD8^+$ T cells into tumor sites and inhibited tumor growth (10).

Direct activation of *Gpr15* expression by AhR and regulation by other transcription factors

How does AhR regulate GPR15 expression? Chromatin immunoprecipitation sequencing (ChIP-seq) of AhR using iTregs and Th17 cells identified *Gpr15* as one of the top genome-wide targets of AhR in both cell types (34). In addition, mutations in the DNA-binding region abolished the ability of AhR to promote *Gpr15* expression in iTregs, suggesting that AhR regulates transcription by directly binding to the *Gpr15* locus. Consistent with this notion, both aforementioned studies (34, 37) identified AhR binding sites/xenobiotic response elements (XREs, 5′-GCGTG-3′) within the *Gpr15* 3′ enhancer sequence that are conserved across mammalian species. As expected, the *Gpr15* promoter-driven luciferase reporters attached to these XRE-containing enhancer regions exhibited AhR agonist-dependent activity that was inhibited by an AhR antagonist (34, 37). Further analyses by Xiong et al. (34) revealed that Foxp3 cooperates with AhR, potentially via interactions with AhR at the *Gpr15* locus, to enhance GPR15 expression in Tregs. In contrast, RORγt, which is frequently expressed in gut Tregs and Th17 cells, negatively regulates GPR15 expression, at least in part, by competing with

AhR for binding to the *Gpr15* locus (34). In addition, Swaminathan et al. showed that GATA3 and AhR independently and synergistically promoted GPR15 expression (37). Intriguingly, however, these authors also found a significant decrease in GPR15 expression by Foxp3 in the presence of either AhR or GATA3 in the luciferase assay, contrary to the findings of Xiong et al. (34). Although further investigation is necessary to elucidate the crosstalk between AhR and other regulatory factors, the discovery of AhR-mediated regulation of GPR15 expression and colon homing of T cells has significantly advanced our understanding of the roles of GPR15 in the intricate mechanisms of T cell migration to target tissues.

GPR15 expression in T cells can be induced by SCFAs such as butyrate, propionate, and acetate (36, 39, 40), which are the main metabolites produced by the microbiota in colon. These SCFAs synergistically enhance basal and ligand-induced expression of AhR-responsive genes in a gene- and cell context-dependent manner, likely through the inhibition of histone deacetylase (HDAC) (99). Interestingly, a recent study by Marinelli et al. provided evidence suggesting that butyrate acts as an AhR ligand to enhance transcription of AhR ligand-dependent genes, independent of its HDAC inhibitor activity, in intestinal epithelial cell (IEC)-AhR reporter cell lines (100). Although this observation needs to be confirmed in other cell types, the study raises an interesting possibility that SCFA butyrate can upregulate GPR15 expression through activation of AhR in colon-migrating T cells and possibly also in colon epithelial cells.

Regulation of GPR15 expression by pathophysiological factors

Smoking and DNA methylation

A number of genomic analyses of blood cells, mostly methylation analysis, have been performed to identify the molecular targets responsible for smoking-induced reprogramming. Blood T cells and B cells expressing GPR15 have approximately 50% methylation at cg19859270, a CpG site within the single exon of *Gpr15*, while non-GPR15-expressing cells are nearly 100% methylated at this site (101). Many studies have shown that smoking is associated with decreased methylation of cg19859270 (102–108) (also see review (109)) and increased *Gpr15* mRNA expression (105, 107, 108, 110, 111) in blood leukocytes. Smoking was also shown to increase the frequency of peripheral $GPR15^+CD3^+$ T cells (107, 112). The effect of smoking is slowly reversible after cessation (102, 110, 113). These data suggest a potential mechanism by which smoking decreases *Gpr15* DNA methylation, which leads to an increase in *Gpr15* mRNA expression, resulting in increased GPR15-positive T cells in the blood. However, although it is conceivable that hypomethylation of *Gpr15* DNA *per se* contributes to the increased expression of *Gpr15* mRNA, the direct causal effect of smoking on *Gpr15* DNA methylation is questionable. By analyzing GPR15 protein expression in leukocyte subtypes, Bauer et al. (107) found that the increased proportion of $CD3^+GPR15^+$ T cells in the blood of smokers was responsible for the apparent smoking-induced hypomethylation of the *GPR15* gene, since cg19859270 hypomethylation was specifically found in GPR15-expressing cells. In addition, treatment of PBMC cultures with aqueous cigarette smoke extract (CSE) did not induce a higher proportion of this T cell subtype, suggesting that DNA hypomethylation at the cg19859270 site observed in smokers did not arise from the direct effect of tobacco smoking compounds on DNA methylation but rather from the enrichment of a smoking-induced $GPR15^+$ T cell population in the peripheral blood (107). This study also indicates that the frequency of $GPR15^+$ T cells in the blood can be effectively used as a highly reliable biomarker for tobacco smoking. It remains to be elucidated how smoking leads to an increased population of $GPR15^+$ T cells independent of CSE, and whether or how the increased $GPR15^+$ T cells in the blood impact immune homeostasis and smoke-related disease pathology.

Ischemia

In a recent study by Haase et al. (114), transcriptome analysis showed upregulated *Gpr15* mRNA expression and downregulated *Gpr15* DNA methylation in PBMCs from early onset myocardial infarction (MI) individuals compared to controls. The MI risk prediction analysis indicated that the effect of smoking on MI was fully mediated by *Gpr15* mRNA expression; however, the associations between *Gpr15* mRNA expression and *Gpr15* DNA methylation with MI were found to be independent of smoking status. In addition, cardiac *Gpr15* expression was significantly upregulated in a mouse model of infarction-related ischemia (>6-fold increase at five days after MI) as well as in an ischemic cardiomyocyte culture model (4-fold after 24 h induction of ischemic stress) (114). These data imply that GPR15 might play a role, independent of smoking, in the pathogenesis of acute MI and conditions of ischemia, such as artery narrowing by plaques. Interestingly, *Gpr15* KO mice had reduced survival compared to wild-type mice after MI induction (114), which raises the possibility that MI-induced cardiac GPR15 expression represents a protective response to oxidative stress. Further investigation is warranted to elucidate the pathophysiological role of GPR15 in MI. It will be particularly interesting to determine whether and how GPR15 in cardiomyocytes is activated by known or novel ligands to induce signaling.

Epstein–Barr virus

The RNAseq data in the GTEx portal (www.gtexportal.org) showed the strongest expression of *Gpr15* in EBV-transformed lymphocytes (median transcripts per million (TPM) was 252.9, while median TPM of whole blood was 1.0) among diverse human tissues. The mechanism of EBV-induced GPR15 expression is yet to be understood. EBV infects the oropharynx but frequently induces B-cell proliferation, which causes tumors in the gut of immunosuppressed individuals, such as transplant recipients treated with immunosuppressive drugs, namely post-transplantation lymphoproliferative disorders (PTLDs) (115, 116). Delecluse et al. found that EBV infection induces integrin $\alpha4\beta7$ expression in tonsillar B cells (117). Since $\alpha4\beta7$ is the key for homing of B cells to the gastrointestinal tract (118), this study

suggests that the induction of α4β7 is one of the mechanisms through which EBV-infected cells enter the gastrointestinal mucosa-associated lymphoid tissue. It will be interesting to determine whether elevated GPR15 expression in EBV-transformed B cells may contribute to their specific homing to the colon by synergizing with α4β7 to enhance the pathogenesis of PTLDs.

Cytokines

GPR15 expression can be induced *in vitro* by a combination of TGF-β1 and either IL-6 or IL-21 preferentially in mouse Tregs (6). However, *Il21r*$^{-/-}$*Il6*$^{-/-}$ mice crossed with *Gpr15*$^{gfp/+}$ mice had a similar level of GFP expression as that of control mice, suggesting that TGF-β1 is a key regulator of GPR15 expression *in vivo* (6). An earlier study has shown that both TGF-β1 and TGF-β2 can induce *de novo* Foxp3 expression in CD4^{+}CD25^{-} cells (119). This raises a possibility that the effect of TGF-β on GPR15 expression may involve the increase of Foxp3 expression that would promote *Gpr15* transcription through enhancement of the AhR activity (34).

Regulation of GPR15 by posttranslational modifications

Regulation of cell surface expression of GPR15 by phosphorylation

Cell surface expression levels and ligand responsiveness of chemokine receptors are tightly regulated by various post-translational modifications (PTMs). The trafficking of *de novo* synthesized GPR15 to the plasma membrane is dependent on the phosphorylation of the penultimate Ser359 residue in the cytoplasmic C-terminal tail (120). Phosphorylation of Ser359, which can be mediated by AKT (121), induces binding of 14-3-3 proteins to the receptor (120). The 14-3-3 binding sterically masks the upstream di-Arg (RxR) motif composed of Arg352 and Arg354, which is the binding motif of COPI, a coatomer protein complex that mediates the retrieval of cargo protein-loaded membrane vesicles from the Golgi to the endoplasmic reticulum (ER) (122). Thus, Ser359 phosphorylation promotes the expression of GPR15 at the cell surface (Figure 3A).

In addition to receptor insertion into the plasma membrane, the cell surface expression of GPR15 is also controlled by endocytosis. GPR15 is constitutively internalized in the absence of exogenous ligand (C10orf99) stimulation (123). This constitutive endocytosis requires the phosphorylation of Ser357, which can be mediated by protein kinase A (PKA) or PKC, but does not require the upstream Ser/Thr cluster in the C-terminal tail, which is commonly phosphorylated by G protein-coupled receptor kinases (GRKs) and is critical for recruiting β-arrestin (124) (Figure 3B). Constitutively endocytosed receptors have been successfully utilized for the delivery of therapeutic agents to target cells; for example, drugs conjugated with specific antibodies to receptors (125, 126). Therefore, ligand-independent endocytosis of GPR15 provides a basis for developing interventions targeting this receptor in human inflammatory/immune diseases, where GPR15-expressing cells may play pathogenic roles (9–11, 47).

Regulation of GPR15-C10orf99 interaction by Tyr sulfation and O-glycosylation

PTMs of GPR15 also regulate receptor-ligand interactions. Sulfation of Tyr residues in the extracellular N-terminus of chemokine receptors positively regulates the binding of chemokine ligands through electrostatic interactions (127, 128). GPR15 has also been recently found to be sulfated on Tyr residue(s), which enhances the binding of the endogenous ligand C10orf99 (21). It is interesting to note that latent membrane protein 1 (LMP1), which is encoded by EBV, induces tyrosine sulfation of CXCR4 through upregulation of tyrosylprotein sulfotransferase-1 (TPST-1) and promotes metastasis of cancer cells (129), a mechanism that could also be applied to GPR15.

GPR15-C10orf99 interaction is also regulated by O-linked glycosylation at the receptor N-terminus (21, 120). In contrast to Tyr sulfation, the O-glycans on the N-terminal Ser/Thr cluster negatively regulate ligand binding, which is at least in part due to the α2,3-linked sialic acid that caps O-glycans. This is similar to CCR7, in which sialylated O-glycans add steric hindrance to the receptor N-terminus to limit ligand binding (130). Consistent with their effects on ligand binding, Tyr sulfation and O-glycosylation of GPR15 differentially regulate the magnitude of receptor signaling (21). Thus, GPR15 represents a unique chemoattractant receptor in which two different PTMs on the N-terminus, Tyr sulfation and O-glycosylation, play contrasting roles in ligand binding and consequent signaling (Figure 3C), which is distinct from the reportedly cooperative roles of these two PTMs in the case of CCR5 (18) and CCR8 (20). The highly regulated glycosylation and sialylation during the differentiation and activation of T cells (131, 132) underpins the notion that the strength of the GPR15–GPR15L interaction is dynamically regulated by PTMs in both physiological and pathological conditions.

Inhibitors of GPR15

There is currently no publication reporting small-molecule compounds that inhibit C10orf99-induced GPR15 signaling. Wang et al. (133) predicted the 3D structure of human GPR15 and applied structure-based virtual screening approaches to discover potential antagonists that could bind to the predicted active sites. By screening a chemical database consisting of 62,500 small molecules, they isolated a set of compounds that satisfied the threshold of a high docking score. However, their antagonistic effects on GPR15 signaling have not yet been demonstrated. Hayn et al. screened peptide libraries generated from human hemofiltrate, which essentially represents the entire blood peptidome, to identify novel endogenous ligands of GPR15 (134). Using primate lentiviruses, these authors discovered a C-terminal fragment of cystatin C (CysC95-146) that specifically inhibits GPR15-dependent entry of HIV-1, HIV-2, and SIV, but does not inhibit

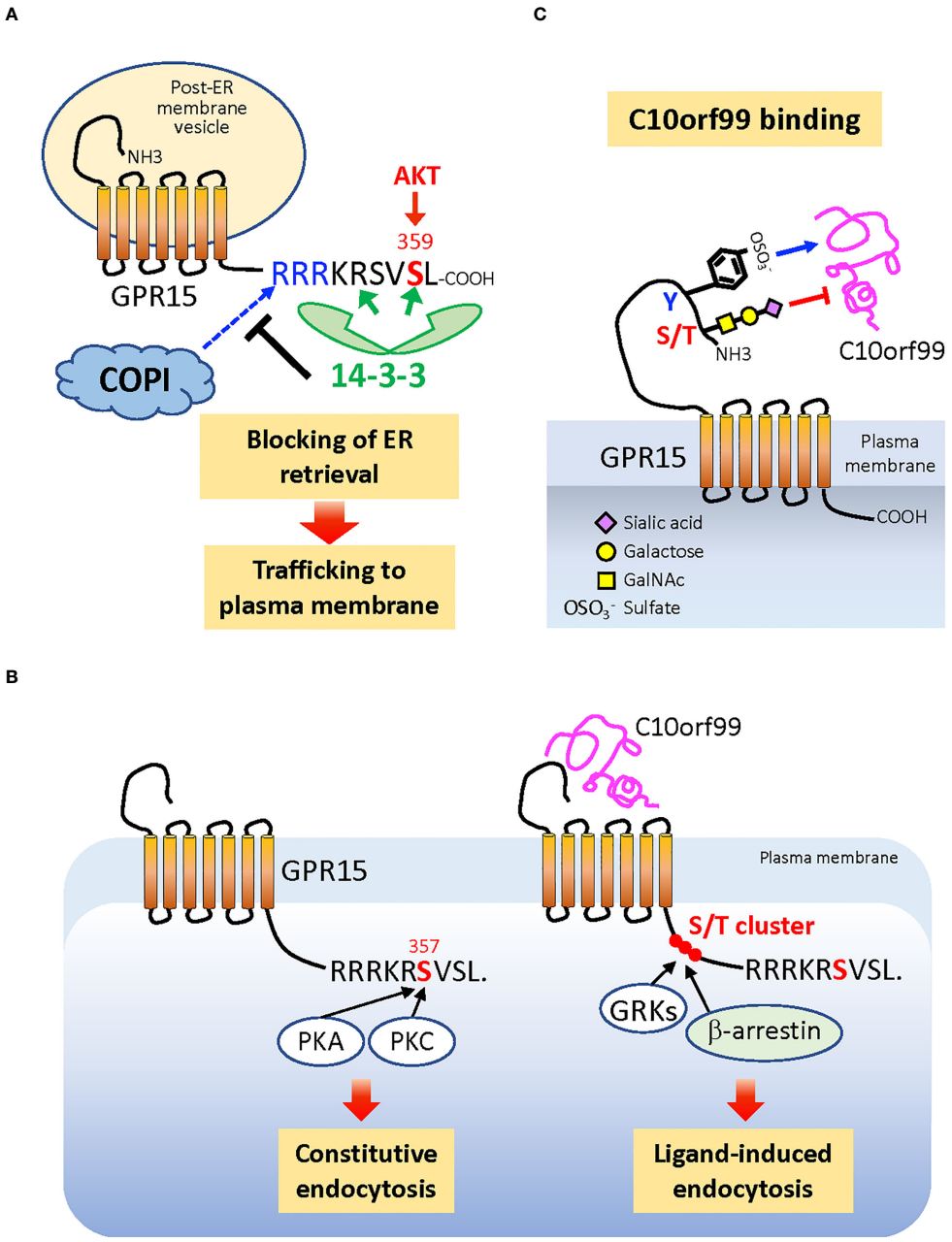

FIGURE 3
Posttranslational modifications that regulate cell surface expression and ligand binding of GPR15. **(A)** Phosphorylation of C-terminal penultimate Ser359 of the nascent GPR15 protein, which can be mediated by AKT, recruits dimeric 14-3-3 proteins to the C-terminus, which in turn blocks the binding of COPI to the adjacent di-Arg (RxR) motif. This prevents GPR15-loaded membrane vesicles from being retrieved back from the Golgi to the ER and allows GPR15 trafficking to the plasma membrane. **(B)** The cell surface level of GPR15 is also regulated by the constitutive (ligand-independent) endocytosis that requires phosphorylation of Ser357, which is the target of PKA and PKC. The more upstream Ser/Thr cluster that is commonly phosphorylated by GRKs and responsible for recruiting β-arrestin to many GPCRs is not required for the constitutive endocytosis of GPR15, while most likely required for the C10orf99-induced endocytosis. **(C)** The Tyr residues in the N-terminal extracellular region of GPR15 are sulfated ($OSO3^-$) while the Ser and/or Thr residues are O-glycosylated and capped with α2,3-sialic acids. Sulfated Tyr residues promote, while sialylated O-glycans inhibit, the binding of C10orf99 to the receptor. *ER*, endoplasmic reticulum; *PKA*, protein kinase A; *PKC*, protein kinase C; *AKT*, protein kinase B; *GRKs*, GPCR kinases; *COPI*, coatomer protein complex I.

C10orf99-induced signaling. This indicates that CysC95-146 is an endogenous inhibitor of GPR15-mediated HIV and SIV infections that does not interfere with the physiological function of this GPCR. In another study by Guo et al. (135), *Gpr15* was found to be upregulated in CRC tissues, and silencing of *Gpr15* by siRNA inhibited the growth, migration, and invasion of CRC cells. These authors found that the expression of GPR15 is post-transcriptionally regulated by microRNA-1225 (miR-1225), the expression of which is significantly downregulated in CRC cells. Overexpression of miR-1225 caused suppression of GPR15 and inhibited the proliferation of CRC cells, suggesting its therapeutic potential (135).

Conclusion and open questions

Over the past decade, a growing number of studies have indicated the involvement of GPR15 and its ligands in a variety of human immune disorders, making them promising therapeutic targets. In the past two decades, 45 drugs targeting chemokine receptors have been tested in clinical trials and only three have been approved by the Food and Drug Administration (FDA) (136). One of the major reasons for the poor success of chemokine receptor inhibitors may be the redundancy of chemokine-chemokine receptor interactions *in vivo*. In this regard, the unusual lack of cross-reactivity of GPR15 and C10orf99 with known chemokines and chemokine receptors, respectively, may offer an advantage for developing specific and clinically effective inhibitors. GPR15 is expressed in multiple cell types and plays pro-inflammatory or regulatory roles in both humans and mice. Further investigations to determine the relative contribution of specific cellular subsets in individual disease settings are necessary to enable the implementation of GPR15-targeted therapy. In this regard, development of conditional GPR15 KO mice likely facilitates elucidation of cell-intrinsic roles of GPR15. In addition, further discovery and characterization of new GPR15 ligands and regulatory mechanisms of GPR15 expression will expand our understanding of GPR15 biology in broader pathophysiology.

The questions that remain open include:

(1) GPR15 is strongly expressed by colon epithelial cells; however, the implication of epithelial GPR15 in disease is limited to HIV enteropathy and the growth of CRC cells. Studies in cell culture systems have demonstrated that chemokine/chemokine receptor signaling, such as CXCL8/CXCR1 and CXCL12/CXCR4, contributes to the maintenance of the epithelial barrier by stimulating the migratory repair process, termed restitution, of wounded epithelium (137, 138). It remains to be determined whether autocrine activation of GPR15 by C10orf99 in colon epithelial cells plays a role in barrier integrity through restitution. Also interesting will be whether GPR15 expression in colon epithelium is regulated by AhR stimulation by dietary and/or commensal metabolites.

(2) It is interesting that C10orf99 functions as an inducible "inflammatory" chemoattractant in psoriatic skin while this protein appears be a "homeostatic" chemoattractant in the colon where its expression was not altered by inflammation or the presence of commensal bacteria (7). It is still unclear what causes the increase in C10orf99 expression at the onset and/or during psoriasis. This is important since such regulatory mechanisms could also be applied to other C10orf99-expressing tissues including colonic and esophageal epithelia. Additionally, it is unknown whether the direct effects of C10orf99 on keratinocyte proliferation and inflammatory response are mediated by MRGPRs or other receptors.

(3) It remains open whether/how the different ligands such as C10orf99 and TME5 activate different signaling pathways (e.g., G-proteins) through GPR15. This may be cell context-dependent. It would also be interesting to investigate whether GPR15 undergoes different PTMs when expressed in different types of cells (e.g., lymphocytes, epithelial cells, and endothelial cells), which could potentially enable preferential interaction with particular ligands.

(4) Many studies indicate a correlation between smoking habit, hypomethylation of GPR15 DNA, and the increase of peripheral blood GPR15$^+$ T cells; however, it is not clear how the smoking leads to the increase of GPR15 expression and whether the increase is specific to any T cell subsets (e.g., Tregs or Teffs). Those questions will be relevant for understanding the potential role of GPR15 in the smoking-related diseases.

Author contributions

YO and SS wrote the manuscript and made figures. Both authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Development of tolerance to chemokine receptor antagonists: current paradigms and the need for further investigation

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Chemokine G-protein coupled receptors are validated drug targets for many diseases, including cancer, neurological, and inflammatory disorders. Despite much time and effort spent on therapeutic development, very few chemokine receptor antagonists are approved for clinical use. Among potential reasons for the slow progress in developing chemokine receptor inhibitors, antagonist tolerance, a progressive reduction in drug efficacy after repeated administration, is likely to play a key role. The mechanisms leading to antagonist tolerance remain poorly understood. In many cases, antagonist tolerance is accompanied by increased receptor concentration on the cell surface after prolonged exposure to chemokine receptor antagonists. This points to a possible role of altered receptor internalization and presentation on the cell surface, as has been shown for agonist (primarily opioid) tolerance. In addition, examples of antagonist tolerance in the context of other G-protein coupled receptors suggest the involvement of noncanonical signal transduction in opposing the effects of the antagonists. In this review, we summarize the available progress and challenges in therapeutic development of chemokine receptor antagonists, describe the available knowledge about antagonist tolerance, and propose new avenues for future investigation of this important phenomenon. Furthermore, we highlight the modern methodologies that have the potential to reveal novel mechanisms leading to antagonist tolerance and to propel the field forward by advancing the development of potent "tolerance-free" antagonists of chemokine receptors.

KEYWORDS

chemokine receptors, antagonist, antagonist tolerance, clustering, oligomerization, signaling

Abbreviations: GPCR, G-protein coupled receptor; ACKR3, Atypical Chemokine Receptor 3; CCR, C-C Chemokine Receptor; CXCR, C-X-C Chemokine Receptor.

Chemokine receptors: functions and pharmacology

Chemokine receptors belong to a subfamily of rhodopsin-like class A G-protein coupled receptors. Their primary function is to orchestrate directional migration of cells (chemotaxis) in response to stimulation by small extracellular proteins (chemokines). Chemokines are secreted at the sites of infection or inflammation and create a concentration gradient that is sensed by the chemokine receptors on the surface of immune cells. Activation of chemokine receptors promotes engagement of immune cells in the inflammatory response. Recognition of chemokine receptors by chemokines is frequently promiscuous with several different chemokines being able to bind and activate the same receptor. However, some chemokine receptors have monogamous cognate ligand binding partners rather than being promiscuous.

While obtaining insight into activation of chemokine receptors from X-ray structures has been challenging due to difficulties associated with crystallization of chemokine-bound receptors, mechanistic details are emerging with the advent of cryo-electron microscopy and advanced crystallography studies. The structures of agonist-occupied CCR2, CCR5, viral chemokine receptor US28, and CXCR2 (1–5) are consistent with the previously proposed two-step, two-site mechanism (6, 7). This mechanism involves the initial recognition of the chemokine by the flexible N-terminus of the receptor. The encounter complex matures after the chemokine inserts its N-terminal region into the helical bundle of the receptor, shifting its conformational ensemble towards the active state characterized by repositioning of helix 6. While the general features of activation are preserved among chemokine receptors, for which structural information is available, the interactions of chemokines and their receptors differ in detail. For example, the N-terminus of CXCL8 does not penetrate deep into the helical bundle of CXCR2, but, instead, interacts with a shallow pocket, primarily establishing contacts with electrostatic residues in TMs 5 and 6 (5). Differences in chemokine:receptor interaction modes suggest the existence of several distinct activation mechanisms. Conventional chemokine receptors in the active state accelerate the exchange of GDP for GTP in the coupled Gα (primarily Pertussis toxin sensitive Gαi), while the atypical chemokine receptors ACKRs do not. Instead, the ACKRs tend to act as chemokine scavengers but can also modulate the function of conventional chemokine receptors through heterodimerization (8). Interestingly, the structures of agonist-bound ACKR3 resemble those of active conventional chemokine receptors (9). However, distinct conformations and dynamics of ACKR3's intracellular loops and the more compact cytoplasmic pocket may explain its bias against G-protein activation.

GTP-loaded Gαi disengages from the receptor and from the Gβ/γ heterodimer and interacts with adenylyl cyclase to inhibit production of cAMP. At the same time, Gβ/γ activates MAPK, PI3K, phospholipase C, Ca2+ flux, and promotes remodeling of the cytoskeleton necessary for the formation of cellular protrusions. Together, the signaling of Gα and Gβ/γ initiates chemotaxis of cells towards increased concentrations of the chemokines. After G-protein signaling is initiated, chemokine receptors recruit GRK, PKC, and Pim1 kinases to their intracellular portions and undergo phosphorylation. The phosphorylation sites attract β-arrestins 1/2 that interact with membrane phosphoinositides and assemble the clathrin machinery, promoting receptor endocytosis and desensitization (10, 11). Additionally, β-arrestins participate in signal transduction, primarily through the MAPK pathway (12) (Figure 1).

The concept of homologous (limited to the stimulated receptor) (13–15) and heterologous (includes the stimulated receptor and other receptors) (16, 17) desensitization is well studied. Chemokine receptor desensitization is the result of prolonged contacts with the agonist, leading to reduced cell surface receptor levels and activation. Mechanistically, desensitization is linked to rapid receptor internalization followed by degradation or by slow repopulation of the cell surface by the recycled or newly synthesized receptors. This process can be influenced by the extracellular environment, receptor type, and the exact effectors and downstream signaling involved in receptor function (13). Desensitization was found to be ligand type dependent for chemokine receptors that have multiple ligands. One of the best examples of this phenomenon is CCR4 that can be activated by either CCL22 or CCL17 (18, 19). CCL22 stimulates CCR4 desensitization at a much higher level compared to CCL17 (20). This sensitivity to chemokine types might be required for the sequential functions of CCL17 and CCL22, allowing for precise recruitment of Th2 lymphocytes into tissues (20).. Heterologous desensitization of chemokine receptors can be exemplified by the cross-talk between opioid and chemokine receptors (21). For instance, pretreatment of cells with RANTES, a chemokine for CCR5 or with CXCL12, a CXCR4 specific chemokine, reduces the subsequent efficacy of DAMGO, μ-opioid receptor's agonist. Remarkably, simultaneous treatment with chemokine and μ-opioid receptor agonists produced a significantly greater effect, indicating a rapid desensitization of the opioid receptor. This reaction also works in the opposite direction, where the activation of the μ-opioid receptor desensitizes the chemokine receptors, but to a lesser degree (22).

Selective activation of either G-proteins or receptor internalization can be achieved through biased agonism, a phenomenon that has received significant attention in the context of chemokine receptors (23–25). Biased agonists have been identified for multiple chemokine receptors, such as CXCR4 (26), CXCR3 (27, 28), CCR2 and CCR5 (29). There are additionally a few instances of a natural ligand or receptor modifications that stimulate biased signaling. Specific examples can be found in the N-terminal modifications of CCL15, a CCR1 ligand (30), and TM helical modifications of CCR5 (31). Structural characterization of two CCR1-bound truncation variants of CCL15 (one balanced agonist and one biased agonist) revealed differences in their binding modes and resulting receptor conformations (32). The residue Y291 in helix 7 of CCR1 acts as a "toggle switch" for biased signaling. Binding of the balanced agonist causes Y291 to form hydrogen bonds with Y113 in helix 3 and Y255 in helix 6, promoting the conformation of the receptor favorable for recruitment of β-arrestin. However, upon binding of the biased agonist, Y291 exists in two alternative conformations, one

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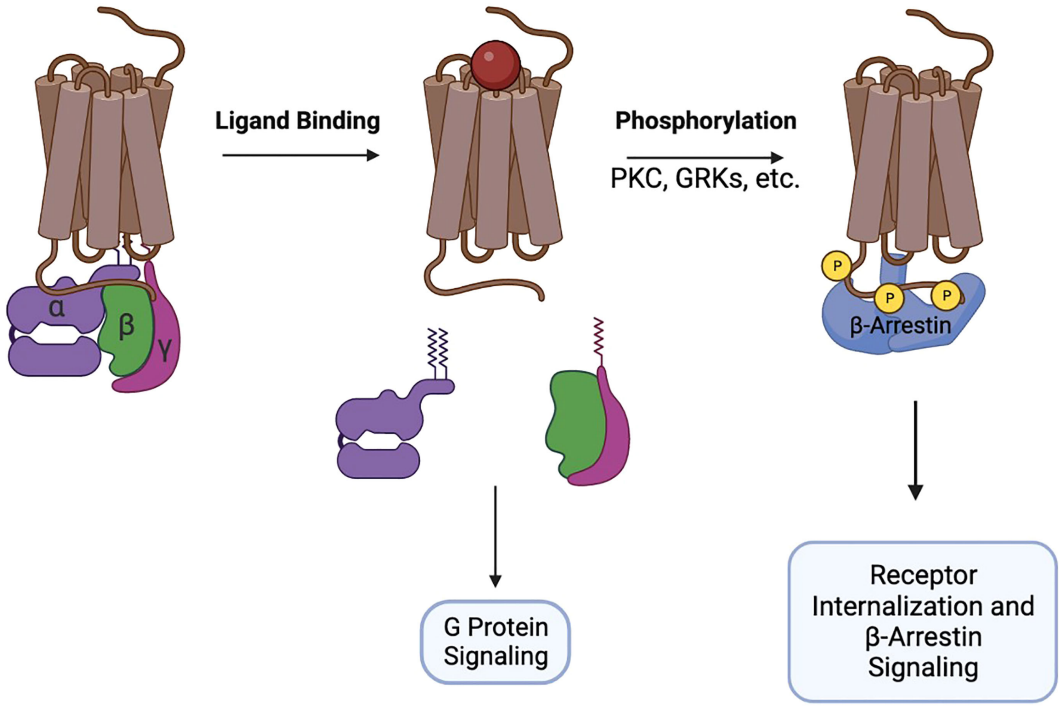

FIGURE 1
Brief schematic of GPCR pharmacology.

resembling the balanced agonist bound state and one resembling the apo-state of the receptor. Thus, CCR1 bound to the biased agonist displays reduced recruitment of β-arrestin (32).

Partial agonists that induce incomplete response in the stimulated receptor have been described for multiple chemokine receptors (29, 33). In some cases, these are promiscuous chemokines that induce more robust responses in the context of other receptors (34, 35) or chemokines with altered N-termini (36–40). The presence of subdued functional outcomes in chemokine receptors stimulated with partial agonist chemokines suggests several possible receptor binding modes that engage alternative mechanisms of activation. Sometimes, the partial agonistic activity of chemokines is related to their ability to form homo-dimers (41–43), while small molecule and peptide partial agonists likely employ allosteric mechanisms (44, 45).

Inverse agonists of chemokine receptors that inhibit their basal activity by stabilizing the inactive conformations can be successful therapeutics. For example, maraviroc, an inverse agonist of the HIV cellular entry portal CCR5, is approved by the FDA for treatment of AIDS (46, 47). Motixafortide, an inverse agonist of CXCR4, has been successfully tested in clinical trials and might gain FDA approval as a hematopoietic stem cell mobilizing agent (48). Other inverse agonists of chemokine receptors have been identified and some of these might also lead to the development of promising therapeutics (49) or used as tools to study the functions of chemokine receptors (50–52). Inverse agonists of chemokine receptors utilize allosteric mechanisms of action (53–55). The mechanistic structural data describing the inhibitory activity of maraviroc has been particularly insightful (3, 56, 57). Maraviroc's phenyl group interacts with hydrophobic residues in helices 3 and 6 of CCR5 and prevents the downward movement of helix 6 that is necessary for receptor activation (57). The phenyl group also blocks the activation-promoting signal transmitted through M287 in helix 7 (56, 58).

The ability of chemokine receptors to stimulate chemotaxis of cells explains their role in multiple diseases (59). In acute and chronic inflammatory disorders, chemokine receptors drive excessive chemotaxis of leukocytes to the sites of inflammation. These disorders include asthma, acute respiratory distress syndrome, autoimmune diseases, such as multiple sclerosis and rheumatoid arthritis, and others (60–65). The critical importance of proper management of these conditions is highlighted by the devastating COVID-19 pandemic where the most common comorbidities were linked to the proinflammatory state (66). Chemokine receptors participate in the pathogenesis of cardiovascular disease by recruiting leukocytes to the areas of arterial damage and by promoting smooth muscle migration into the intima and thrombus formation over atherosclerotic plaques (67). Chemokine receptors play an important role in viral and bacterial infections. Chemokine receptors facilitate HIV cellular entry (68), and mediate exacerbation of immune response in coronavirus (69) and Ebola infections (70). Normal chemokine receptor signaling is subverted by poxviruses and herpesviruses that induce production of viral chemokines and chemokine receptors (71). Chemokine receptors balance pathogenic and protective immune responses in Mycobacterium tuberculosis infections (72). Chemokine receptors are also key mediators of cancer-related inflammation and can promote angiogenesis, tumor growth, and

metastasis (73). Thus, it is not surprising that chemokine receptors are important therapeutic targets in many diseases. However, the development of chemokine receptor antagonists has been challenging primarily due to toxicity and low efficacy of these molecules in clinical trials (74). While toxicity of chemokine receptor antagonists is likely related to their promiscuous binding to secondary targets and associated off-target effects, the issue of low efficacy is harder to explain. One potential reason for the low efficacy in clinical trials is divergent functions of chemokine receptors in animals used for the preclinical development and in humans. In addition, we previously observed that after prolonged treatment with antagonists of chemokine receptors CXCR4 and CCR3, cells eventually became tolerant to the antagonists and were able to mount a robust chemotactic response even in the presence of the inhibitors (75, 76). Antagonist tolerance can also explain multiple failures in clinical trials with chemokine receptor antagonists, particularly those that reported low efficacy of the molecules under investigation. Although antagonist tolerance has been reported for several GPCRs (77–80), this phenomenon remains understudied and the underlying mechanisms are not delineated. Here, we summarize the available knowledge on antagonist tolerance in the context of therapeutic targeting of chemokine receptors and encourage deeper investigation of this problem.

Optimism in the community remains high as there are multiple groups currently studying chemokine receptors. Seven clinical trials testing potential therapeutics targeting chemokine receptors are ongoing at the time of this publication, and there have been over 30 since 2005, twelve of which specifically focused on chemokine receptor antagonists. Despite much effort and significant financial investment, only three drugs have ever been approved for clinical use. These three (plerixafor, maraviroc and mogamulizumab) have proven therapeutic efficacy for their approved purpose despite the development of tolerance.

Plerixafor, a CXCR4 antagonist, releases hematopoietic progenitors from the bone marrow by blocking binding of the chemokine CXCL12 to the receptor and inhibiting downstream signaling. Mobilizing these cells allows their collection and purification from peripheral blood for transplantation. Plerixafor, also known as AMD3100 or Mozobil, is additionally used to treat non-Hodgkin's lymphoma or multiple myeloma (81). Plerixafor is the only clinically approved CXCR4 antagonist, which inhibits downstream activation of G-proteins and β-arrestin. Apheresis occurs 11 hours after administration of the drug. Plerixafor displaces stroma-attached progenitor cells by blocking CXCL12 from homing cells to the bone marrow. This prevents progenitor cells from returning to the bone marrow, where they develop chemoresistance. The short treatment time for Plerixafor may aid in overcoming tolerance related issues, however, in cases of longer treatment times tolerance development remains a concern. Drugs similar to AMD3100, like AMD11070 and Filgrastim, which suffer from prolonged apheresis fall victim to tolerance (82, 83). Mogamulizumab is another anti-chemokine/anti-CCR4 antibody that targets lymphomas as well, and has been clinically approved for Cutaneous T-cell lymphomas and Adult T-cell leukemia/lymphoma (84). This drug increases antibody-dependent cellular cytotoxicity (ADCC) (73). Mogamulizumab increases ADCC by high-affinity binding with the Fc receptor on effector cells (85). Mogamulizumab inherently deals with the common problem of tolerance by labeling the tumor cells expressing CCR4 for destruction through the mechanism of ADCC.

The third and final chemokine receptor drug approved by the FDA through clinical trials is maraviroc. This drug targets the cellular entry of HIV by interfering with the coreceptor CCR5 (86–88). Other indicators of success are a high nadir CD4 cell count, detectable viral load, protease inhibitor exposures and young age. Maraviroc is always used in combination with other HIV drugs and, therefore, has its own challenges in determining efficacy and tolerance. The combination of drugs may create unintended or exacerbate existing issues potentially speeding up tolerance development on top of other negative effects.

Addressing chemokine receptor tolerance should be essential when considering efficacy of antagonists but is often overlooked. Low efficacy is a surprisingly frequent problem during chemokine antagonist clinical trials because the preclinical phase of development is expected to be highly selective for efficacious drugs. This being said, there are a number of possible reasons for the low efficacy, tolerance being one of these. Tolerance, manifested by cell surface receptor accumulation is cited as a reason for the low efficacy of AMD3100 in prolonged treatments (75), but this idea of tolerance is hardly mentioned elsewhere. In many completed and terminated chemokine antagonist clinical trials, efficacy is investigated but not the specific factors leading to inefficacy are not mentioned or remain overlooked. For example, the CXCR2 antagonist AZD5069 was studied in its role in controlling severe exacerbations in patients with asthma in a clinical trial by AstraZeneca (89). It was determined that CXCR2 antagonist did not reduce the frequency of severe exacerbations, but no connection was made to specific mechanisms. Another case of inefficacy is demonstrated by chemokine CCR2 receptor antagonists. Although the preclinical phase of development should select highly efficacious and selective drugs, efficacy became a major issue in NCT00992186, which studied a biased antagonist (90). PF-04634817, designed to treat Diabetic Macular Edema was said to be well tolerated with a high level of CCR2 antagonism, inefficacy was a major issue, with no reasons concluded. Carlumab, another CCR2 antagonist also had problems with inefficacy, with 0 complete or partial responses in reducing tumors. The list goes on with similar outcomes, inefficacy was observed but no conclusion was made regarding the contributing factors leading to this result.

Investigation into lack of efficacy is oftentimes overlooked leading to a loss of usable information for future studies where the same factors, tolerance included, could be having a significant impact. Another issue plaguing clinical trials is that efficacy and tolerance are often disguised by other problems, such as species-dependent biology or disease pathogenesis, resulting in unsuccessful clinical trials. In a clinical trial, NCT01160224, for an oral CCR3 antagonist, GW76694, 68% of subjects reported adverse events. The issue of efficacy is grouped into broader categories of why the drug is ineffective without further exploration into the mechanistic effects of the antagonist itself. These broader categories, involving questions regarding the role of CCR3 in the pathogenesis of asthma, potentially overshadow the molecular mechanisms at play, such as tolerance. This is just another example of a lack of inquiry into the biochemical basis for the failure of clinical

drugs. To more coherently investigate chemokine antagonists, reasons for inefficacy must be thoroughly investigated. Through investigations such as those done by Hitchinson *et* al (75) tolerance will likely have an important impact. By considering lack of efficacy as contingent of tolerance, the current paradigms of chemokine receptor antagonism may be challenged. The mechanisms at play by antagonists within GPCR pharmacology is much less understood compared to the abundant agonist information. This is likely a major obstacle to the successful development of chemokine receptor antagonists.

Antagonist tolerance

Although antagonist tolerance in the context of chemokine receptors is not well documented, there are multiple reported cases regarding the development of tolerance to antagonists of other GPCRs. A large portion of these studies focuses on tolerance to antipsychotics, specifically those targeting Dopamine receptors. These studies tend to only examine clinical occurrence and symptoms as a result of tolerance but do not focus on the mechanisms of antagonist tolerance (91–93). The few studies beyond the scope of antipsychotics explore tolerance as a secondary aim, usually in cases where the use of antagonists is preceded by inverse agonist treatments.

An important GPCR target of investigation of antagonist tolerance has been the Histamine $H_{1,2,3}$ receptor. Cases exploring histamine receptor antagonists acting as inverse agonists oftentimes connect this activity to the development of tolerance (94–96). This view considers tolerance as part of a larger mechanistic process where the mode of the interaction of pharmacological agents with their targets depends on the cellular environment, previous treatments, and changes over time. These molecules tend to be evaluated for potential use against various diseases (97–99) without addressing the reasons for failure to produce new and effective antagonists that can succeed in clinical trials. Even among studies exploring the difficulties in developing antagonists and ways to overcome these challenges (100, 101), investigation of antagonist tolerance is often omitted. A good launch point is applying FDA-approved antagonists of chemokine receptors to situations that require prolonged use and asking questions about tolerance and its mechanisms (75). However, many more studies need to be done. Given the lack of information specifically regarding tolerance to antagonists of chemokine receptors, in this review we will explore how cellular responses to antagonists of other GPCRS may be mechanistically informative or related to the development of tolerance to antagonists of chemokine receptors.

Mechanistic insights into antagonist tolerance

Similarities between antagonist tolerance and agonist tolerance

Given significant gaps in knowledge of antagonist tolerance, it is helpful to compare it to a better studied phenomenon of agonist tolerance. There is an excellent in-depth review by Raehal et al. (102) that discusses the development of agonist tolerance to opioids. The primary concepts that are relevant to both agonist and antagonist tolerance are regulation of receptor levels on the cell surface and complexity of the signaling cascades related to both events. Raehal et al. discuss how different drugs for the same opioid receptor lead varying levels of tolerance development. Typically, agonist tolerance is associated with a reduction in receptor levels on the cell surface, while in antagonist tolerance receptor levels on the cell surface rise. The conformational state induced by the agonist, signaling pathway stimulated and secondary or off-target effects all play key roles in determining the timeline and the severity of tolerance. As a brief example; DAMGO, a drug that induces a significant phosphorylation of the μ-opioid receptor utilizing GRK2 has been shown to produce lessened tolerance as compared to morphine, which has been shown to promote phosphorylation of the μ-opioid receptor through GRK and PKC (103). The idea that signaling, cellular environment and conformational state influence the presence and severity of tolerance is one we believe applies to antagonist tolerance as well.

Timeline for tolerance

The time needed for the development of antagonist tolerance is likely dependent on many factors, including the antagonist type, the dosing regimen, and the lack for target selectivity. For example, the tolerance to Biperiden, a selective antagonist of the muscarinic M1 receptor, begins to develop clinically around the 3rd night of drug administration (104). Clozapine, an antipsychotic, which targets the dopamine D2 receptor begins to exhibit reduced effectiveness within 2 days and its potency gradually decreases further (105, 106). However, other antipsychotics targeting the same receptor, such as Thioridazine, show a slight reduction in efficacy beginning at 4 days with a very slow development of tolerance afterwards (106). Two of the most frequently prescribed antipsychotics, Haloperidol and Risperidone, are heralded for their ability to avoid tolerance in patients. However, the progressive nature of the side-effects, associated with reduced on-target efficacy, suggests the potential involvement of antagonist tolerance (107–109). Tolerance development for both typical and atypical antipsychotics has been partially associated with the dosing method (intermittent vs continuous) as well as the non-specific binding events with the serotonin 5-HT receptor, ideas that we will discuss further in this review (108, 110). The phenomenon of tolerance is a crucial variable when considering the treatment of psychosis and other mental disorders and this concept should be applied to other GPCRs, especially chemokine receptors. When it comes to chemokine receptor antagonist tolerance, CXCR4-expressing Jurkat cells develop tolerance to Plerixafor (AMD3100) after 72 hours of treatment (75).

Cell surface density increases in antagonist tolerance

In all cases of reported tolerance to antagonists targeting GPCRs, including chemokine receptors, an increase in receptor

density on the cell surface is invariably observed. Although, there may be several different mechanisms leading to increased receptor expression in antagonist tolerance, it is likely related to inhibition of receptor turnover by antagonists that can inhibit recruitment of β-arrestin to the receptor. Internalization is a key regulatory mechanism for transmembrane receptors (111). Endocytosis maintains an appropriate concentration of receptor at the cell surface to prevent overpopulation, which can impact receptor function and signaling (112). GPCRs and especially chemokine receptors are fairly prone to hetero- and homo-oligomerization at high receptor density; both processes can significantly affect receptor signaling and regulation (113, 114). It is not surprising that directly affecting the cell surface concentration and receptor trafficking mechanisms may have effects on receptor oligomerization and structure. A prime example of antagonist-induced receptor density increases comes from observations by O'Dowd et al. who explored Dopamine D1, D5, and 2 serotonin receptors antagonist-induced cell surface density changes (115). They found that antagonist's dose-dependently increases receptor density up to 15-fold. Even at lower antagonist concentrations, up to 8.5-fold density increases were seen. Hess et al. observed the effects of a dopamine D1 and D2 antagonist, cis-flupentixol on receptor upregulation and cataleptic effects of the drug (116). Researchers found that there was a significant upregulation of D2 receptors following treatment with cis-flupentixol. Moreover, the time to cataleptic effects in rats was increased, leading to the conclusion that upregulation of the receptor was associated with antagonist tolerance. Hitchinson et al. came to a very similar conclusion, suggesting that prolonged treatment of cells with Plerixafor (AMD3100) increased CXCR4 on the cell surface and led to the development of antagonist tolerance. Thus, there is significant evidence linking tolerance to increased levels of receptors on the cell surface after prolonged administration of the antagonists.

Potential oligomerization of receptors in antagonist tolerance

The mechanistic importance of GPCR oligomerization has been a long-studied topic and yet its functional significance is not firmly established. Recent studies have suggested that oligomerization of GPCRs plays key roles in signaling and regulation (117, 118) and this sentiment has been echoed as especially important for chemokine receptors (114, 119). It is also emphasized that the formation of oligomers may impact drug discovery, as targeting a receptor dimer or even trimer may require adaptations for ligands, which only target monomeric receptors (120, 121). As we described previously, changes in receptor cell surface density have been observed after treatment with antagonists and this has been correlated to tolerance. This increase in receptor density likely elevates receptor oligomerization, which may be the underlying cause of the observed antagonist tolerance. This receptor oligomerization can be in the form of homo- or hetero-oligomers, both with a significant potential to alter receptor structure and function. Examining available structures of oligomerized CXCR4 reveals structural changes caused by oligomerization (122). These changes in structure can remodel the drug binding sites and reduce drug binding, leading to tolerance. In addition to forming homo-oligomers, evidence suggests GPCRs can readily hetero-oligomerize at the cell surface, such as the dopamine D_2/Adenosine A_{2a}, CXCR4/ACKR3 (atypical chemokine receptor 3), Serotonin 5_{HT}/mGlu2, and many others. At high receptor cell surface density, hetero-oligomers can co-exist with homo-oligomers, further altering receptor signaling and responses to treatment with antagonists. To explore the potential effects of this increased heteromerization we will take a look at two systems, the Adenosine A_{2a} and Dopamine receptor interactions and CXCR4 interactions with multiple other GPCRs.

In the case of heteromeric A_{2a}/D_2, the A_{2a} receptors directly interact with and antagonize dopamine D_2 receptors (123–125). Further exploration has found that targeting of A_{2a} with agonists can decrease D_2 signaling and treatments with adenosine antagonists can increase D_2 signaling, such interactions can also be seen in A_1/D_1 heteromers (123). This interaction not only impacts the signaling and function of the complexed receptors but also extends to ligand binding. There have been a few unique changes in ligand binding events observed from the interaction between A_{2a} and D_2, namely, an allosteric network, which had 2 distinct effects: changed A_{2a} antagonists to have agonist activity and the appearance of modulated D_2 agonist and antagonist affinity and efficacy (125).

Similarly, the concept of heteromerization can be applied in the context of a chemokine receptor and antagonist pair. CXCR4 has many heteromerization partners, including but not limited to the atypical chemokine receptor ACKR3, alpha- and beta-adrenergic receptors and CXCR3 (126–128). Early studies of Plerixafor asserted that it was selective to CXCR4 (129, 130), however, other studies found that it also acts as an ACKR3 agonist (131). Given that Plerixafor can target ACKR3 and CXCR4, which can form a heteromeric complex, there is a possibility for additional ligand effects and signal dampening. ACKR3 can also act as an allosteric modulator of CXCR4 (126). ACKR3 alters CXCR4 signaling in cancer by changing ligand binding, internalization, and signal propagation (132, 133). Remarkably, co-transfection of CXCR4 and ACKR3 caused degradation of CXCR4 and internalization of ACKR3 (134) and increased front cell velocity (135). Hetero-oligomerization of chemokine receptors, much like homo-oligomerization, can have significant impacts on the ligand binding and signaling pathways. When considering drug discovery, any changes to the receptor density and availability of heteromeric partners can influence the efficacy of drugs and lead to the development of unintended consequences, such as tolerance.

Addressing antagonist tolerance

Chemokine receptor antagonists tend to be unable to maintain their therapeutic efficacy when prolonged (three days

or longer) administration is necessary. Equipotent inhibition of chemotaxis and receptor endocytosis by unbiased antagonists may be the root for this progressive reduction in efficacy over time. Unlike unbiased antagonists, biased antagonists selectively inhibit G-protein signaling, while leaving β-arrestin recruitment and receptor internalization intact. Thus, biased antagonists do not lead to receptor accumulation on the cell surface over time and can avoid the development of tolerance. For example, the peptide R321 targeting the eosinophil chemokine receptor CCR3 acts as a biased antagonist by blocking G-protein signaling and eosinophil recruitment into the lungs. This biased antagonist then still allows β-arrestin recruitment and receptor endocytosis (76). Currently, there are no clinically approved CCR3 antagonists: antagonist tolerance may have been the cause of failure of the previously tested CCR3 antagonist in clinical trials against asthma. However, biased antagonists of CCR3 may be a promising alternative. Like R321, the CXCR4 biased antagonist X4-2-6 permits β-arrestin recruitment and receptor internalization but inhibits the G protein signaling. The G-protein signaling is blocked by the simultaneous interaction of X4-2-6 with CXCR4 and CXCL12, leading to a partial expulsion of CXCL12's N-terminus from the helical bundle of the receptor. The remaining contacts between CXCL12 and the receptor allow activation of β-arrestin recruitment. The biased antagonists have the potential to address antagonist tolerance in the context of chemokine receptors by preventing signaling but permitting endocytosis, which prevents receptor accumulation on the cell surface. This mechanism of tolerance avoidance by X4-2-6 and R321 was tested by comparing potency between the novel peptides and unbiased antagonists before and after prolonged treatments. For example, the ability of X4-2-6 and Plerixafor to inhibit CXCR4-mediated chemotaxis of Jurkat T lymphocytic leukemia cells before and after the 72-hour treatment was determined. While the 72 hour exposure to Plerixafor led to a significant increase in the drug's IC50 value for the inhibition of CXCL12-induced chemotaxis (75), cells treated with the biased antagonist X4- 2-6, had similar IC50 with and without pretreatment with the peptide. A very similar outcome was obtained with the CCR3 biased antagonist R321 in AML14.3D10-CCR3 cells. Both R321 and X4-2-6 are derived from the second transmembrane helix of their respective chemokine receptors and also contain sequences derived from the extracellular loop 1 of CXCR4 and CCR3 (75, 76). The loop sequences likely facilitate binding of the chemokines, while the sequences corresponding to the transmembrane regions can interact with the receptors. Chemokine binding even in the absence of the receptors might contribute to the inhibitory activity of the peptides through the potential chemokine sequestration mechanism, complicating delineation of contribution of biased antagonism to successful blocking of chemokine receptor signaling. Thus, further studies are needed with simpler peptides and/or small molecule biased antagonists to understand how the biased mechanism of chemokine receptor inhibition prevents antagonist tolerance.

Although biased antagonism is an effective approach to avoid tolerance, there may be additional ways to accomplish similar outcomes. Homo- and hetero- oligomerization of chemokine receptors might be directly related to the development of antagonist tolerance. A potential methodology to overcome tolerance would be inhibiting the formation of receptor oligomers. Drug discovery related to disruption of oligomers is already underway and has produced reliable data suggesting the potential druggability of oligomerization interfaces (136, 137). The logical idea of disrupting oligomer formation is utilizing peptides synthesized from the transmembrane helices of GPCRs. A review by Gallo et al (138) does an excellent job in discussing the current progress in peptide development and how researchers are slowly addressing the pitfalls of using peptides as drugs. The review discusses multiple successful attempts at using TM synthetic peptides to disrupt the formation of oligomers, such as $A_{2A}R$ homodimers and the cannabinoid receptor 2/5-hydroxytryptamine:2A heterodimer (139). The use of TM synthetic peptides as drugs is still a growing idea but the preliminary data gives an optimistic outlook on the future.

Concluding remarks

Tolerance to chemokine receptor antagonists remains unaddressed and this likely impedes successful development of chemokine receptor inhibitors for clinical use. To date, there is no comprehensive mechanistic understanding of antagonist tolerance. However, some clues can be derived from a handful of studies on chemokine receptors and other GPCRs (Figure 2). Antagonist tolerance tends to be accompanied by receptor accumulation on the cell surface possibly due to inhibition of receptor turnover by the antagonists. Thus, we propose that high receptor density plays a key role in the mechanism of tolerance. This is different from the low receptor levels at the cell surface commonly observed in agonist (primarily opioid) tolerance, which has different mechanisms. It seems likely that accumulation of chemokine receptors in the plasma membrane leads to their clustering and oligomerization. Under these conditions, rising numbers of receptor oligomers can far exceed the numbers of receptor oligomeric assemblies commonly seen in untreated cells and start to alter signaling pathways in response to drugs. As only three chemokine receptor antagonists have been approved for clinical use, different approaches to inhibition of chemokine signaling are sorely needed. Biased antagonists that block G-protein signaling but allow receptor internalization have been reported to avoid antagonist tolerance. There are likely other approaches to either avoid or alleviate tolerance. It will be interesting to test if inhibitors of receptor oligomerization or blockers of signaling associated with tolerance can be successful

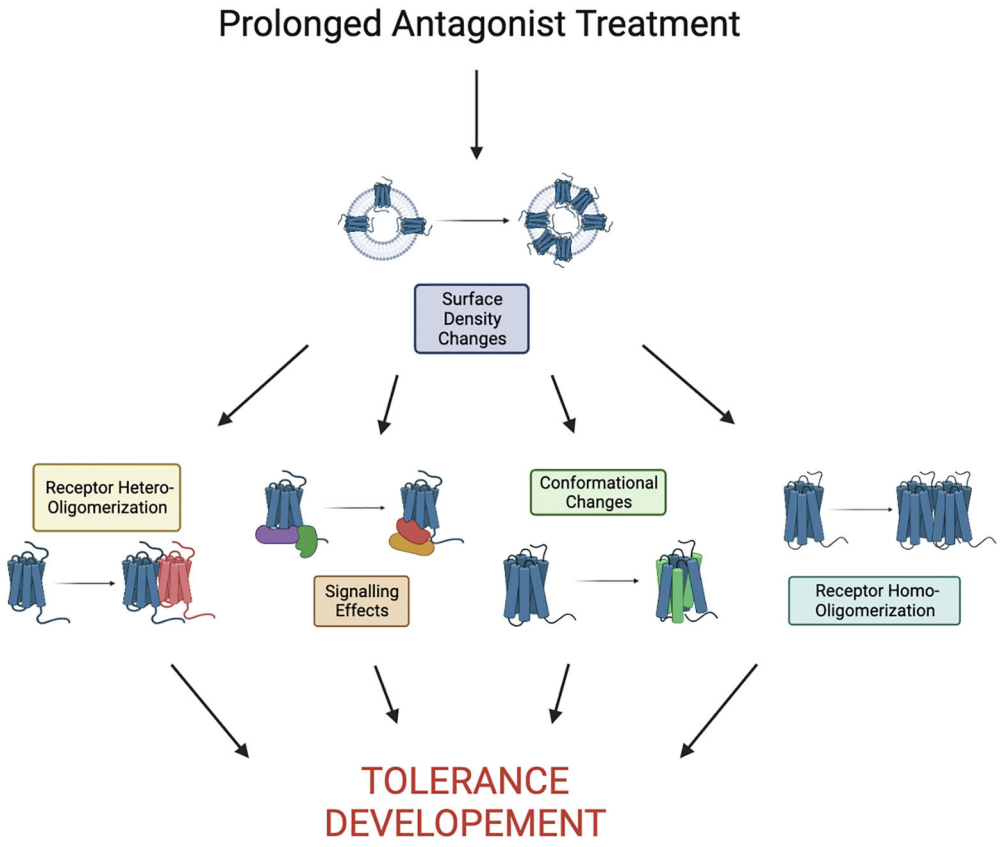

FIGURE 2
Proposed pathway to tolerance as a result of prolonged antagonist treatment. Here we define prolonged as the time it takes to see development of tolerance, it is largely receptor/drug dependent. Development of tolerance may be a result of any combination or all of the shown effects.

alternatives to conventional chemokine receptor antagonists. Given the wide array of diseases and conditions associated with chemokine receptors, more study into the mechanisms of tolerance will propel the development of therapeutics targeting chemokine receptors.

Author contributions

VG conceived the idea for the article, wrote the introduction, and edited the manuscript. PG wrote 75% of the manuscript, generated a summary figure, and included references. HN wrote 25% of the manuscript. ME provided clinically relevant comments on the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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