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Targeting the CCL2–CCR2 axis for atheroprotection

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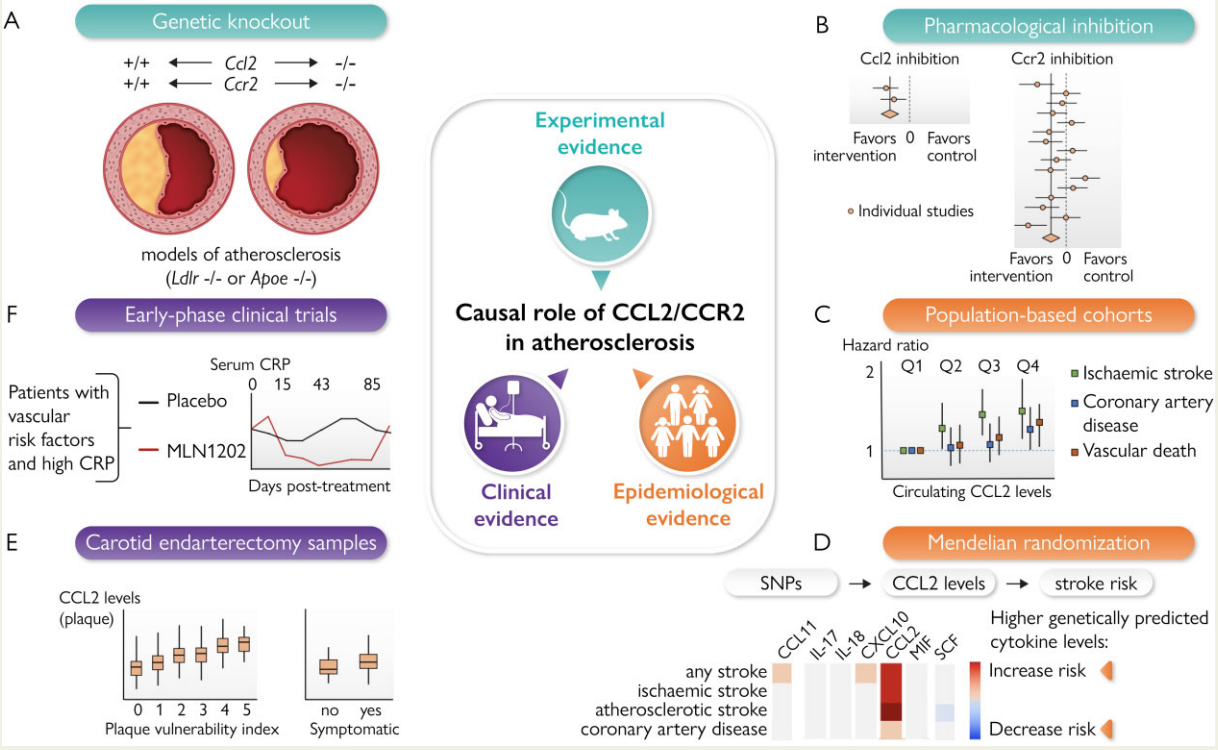

Graphical Abstract Triangulation of evidence supporting the translational potential of targeting the CCL2–CCR2 axis in atherosclerotic disease. (*A*) Studies in atheroprone mice (*Ldlr*$^{-/-}$ *or Apoe*$^{-/-}$) show that deletion of *Ccl2* or *Ccr2* is associated with smaller plaques in the aortic root and arch, as well as decreased monocyte infiltration.[66–69] (*B*) A meta-analysis of preclinical studies testing pharmacological inhibition of either Ccl2 or Ccr2 in atheroprone mice shows a beneficial effect of either approach on plaque size and plaque stability as depicted by the forest plots for aortic arch/root lesion size. The lines and the dots depict the individual study effects (standardized mean differences) and their 95% confidence

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intervals (CIs), whereas the diamonds the pooled effect sizes, as derived from random-effects meta-analyses (Hedge's *g*: −0.93, 95% CI: −1.46 to −0.40, *P*-value: 0.0006 for CCL2 inhibition and *g*: −0.73, 95% CI: −1.22 to −0.24, *P*-value: 0.003 for CCR2 inhibition). The vertical dotted line represents the reference (null effect) with studies on the left favouring interventions targeting Ccl2 or Ccr2 and studies on the right favouring the control group.[70] The detailed results along with the data on the individual studies and the inhibitors used by each study are presented in Supplementary material online, *Figure S1*. (*C*) Meta-analyses of prospective cohort studies show significant associations between higher midlife circulating CCL2 levels and higher risk of incident ischaemic stroke[71] or vascular death[72] in the general population over follow-up periods extending beyond 15 years (*P*-values for trend as derived from the Cox regression analyses adjusted for demographic and vascular risk factors: 0.009 for ischaemic stroke, 0.17 for coronary artery disease, and 0.004 for vascular death). The squares represent the hazard ratios for the second to fourth quartile of CCL2 levels (Q2–Q4), when compared with the first (Q1), and the lines correspond to 95% CIs, as derived from the Cox regression analyses adjusted for demographic and vascular risk factors. The horizontal dotted line at 1 represents the reference (null effect) with hazard ratios above it representing significant associations with higher risk of ischaemic stroke, coronary artery disease, or cardiovascular death. (*D*) Mendelian randomization analyses exploring multiple cytokines reveal that higher genetically predicted CCL2 levels are associated with a higher lifetime risk of atherosclerotic stroke and coronary artery disease.[73] The presented results are derived from fixed-effects inverse-variance weighted two-sample Mendelian randomization analyses. (*E*) In patients undergoing carotid endarterectomy, plaque CCL2 levels are associated with histopathological plaque vulnerability as revealed by an index of high macrophage content, low collagen deposition, high smooth muscle cell content, intraplaque haemorrhage, and large lipid core (range 0–5, left graph, *P*-value from ordinal regression analyses adjusted for demographic and vascular risk factors: 5.4×10^{-13}).[74] Moreover, CCL2 levels are higher in symptomatic vs. asymptomatic plaques (right graph, $P = 0.0001$ derived from the Mann–Whitney *U* test).[74] Shown are the median plaque CCL2 values (central line), the upper and lower quartiles (box limits), and the 1.5× interquartile range (whiskers). (*F*) Small early-phase II randomized trials have been conducted for CCL2 or CCR2 inhibition in the context of atherosclerosis; one of them found MLN1202 (a CCR2 antagonist) to decrease the levels of high-sensitivity C-reactive protein (CRP) among individuals with high CRP and vascular risk factors ($P < 0.05$ in the Mann–Whitney *U* test in all timepoints from 29 to 85 days post-treatment).[75]

Abstract

Decades of research have established atherosclerosis as an inflammatory disease. Only recently though, clinical trials provided proof-of-concept evidence for the efficacy of anti-inflammatory strategies with respect to cardiovascular events, thus offering a new paradigm for lowering residual vascular risk. Efforts to target the inflammasome–interleukin-1β–interleukin-6 pathway have been highly successful, but inter-individual variations in drug response, a lack of reduction in all-cause mortality, and a higher rate of infections also highlight the need for a second generation of anti-inflammatory agents targeting atherosclerosis-specific immune mechanisms while minimizing systemic side effects. CC-motif chemokine ligand 2/monocyte-chemoattractant protein-1 (CCL2/MCP-1) orchestrates inflammatory monocyte trafficking between the bone marrow, circulation, and atherosclerotic plaques by binding to its cognate receptor CCR2. Adding to a strong body of data from experimental atherosclerosis models, a coherent series of recent large-scale genetic and observational epidemiological studies along with data from human atherosclerotic plaques highlight the relevance and therapeutic potential of the CCL2–CCR2 axis in human atherosclerosis. Here, we summarize experimental and human data pinpointing the CCL2–CCR2 pathway as an emerging drug target in cardiovascular disease. Furthermore, we contextualize previous efforts to interfere with this pathway, scrutinize approaches of ligand targeting vs. receptor targeting, and discuss possible pathway-intrinsic opportunities and challenges related to pharmacological targeting of the CCL2–CCR2 axis in human atherosclerotic disease.

Keywords Vascular inflammation • Chemokines • Monocyte-chemoattractant protein-1 • Atherosclerosis • Coronary artery disease

Introduction

Cardiovascular disease (CVD) is the leading cause of morbidity and mortality worldwide.[1–3] Atherosclerosis is the prevailing pathology underlying CVD and may manifest with clinical sequelae in multiple vascular beds including coronary, cerebral, and peripheral arteries.[4,5] Given the role of LDLs as a principal driving force in the development of atherosclerosis, pharmaceutical interventions so far largely focused on LDL-lowering strategies.[6,7] Despite substantial progress in LDL-lowering and the control of other risk factors though, residual rates of CVD remain high calling for the identification of novel treatment paradigms for atherosclerosis.[1]

Over 20 years of preclinical research have provided overwhelming evidence for a causal role of inflammation in atherogenesis.[8,9] The observation that inflammatory biomarkers, in particular circulating C-reactive protein (CRP) and interleukin (IL)-6, associate with vascular risk in humans even at very low LDL levels,[10–12] has led to the concept of 'residual inflammatory risk' as a potential target for cardiovascular prevention.[3,13] Indeed, recent trials provided proof-of-concept for the inflammatory paradigm of atherosclerosis.[14–16] The Canakinumab Anti-inflammatory Thrombosis Outcome Study (CANTOS) demonstrated that a monoclonal antibody against IL-1β lowers the risk of recurrent vascular events among individuals with recent myocardial infarction.[14] The Colchicine Cardiovascular Outcomes Trial (COLCOT)[15] and the Low-Dose Colchicine-2 (LoDoCo2) trial[16] further showed that colchicine, an established drug with widespread inhibitory effects on inflammatory pathways,[17,18] lowers the risk of recurrent vascular events in patients with coronary artery disease (CAD).

Targeting residual inflammatory risk raises issues related to specificity and side effects. Treatment with low-dose methotrexate did not lower vascular risk in the Cardiovascular Inflammation Reduction Trial (CIRT),[19] thus emphasizing the importance of specifically targeting atherosclerosis-relevant inflammatory pathways.[14,19–21] Also, the CANTOS revealed considerable inter-individual variations in the efficacy of anti-IL-1β treatment, with benefits observed only among patients achieving low IL-6 and CRP levels.[22,23] Furthermore, neither the CANTOS[14] nor the colchicine trials[24] reduced mortality, and both canakinumab[14] and colchicine[15] were associated with adverse effects including fatal infections. Hence, there is interest in alternative anti-inflammatory approaches and drugs with improved properties.

Translational efforts have mostly focused on the inflammasome–IL-1β–IL-6 axis.[13] Yet, ample evidence from preclinical studies and early-phase clinical trials highlights the promise of alternative cytokines[9] for the development of a second generation of atherosclerosis-centred anti-inflammatory treatments.[8] CC-motif chemokine ligand 2 (CCL2) is a pivotal inflammatory chemokine regulating monocyte trafficking[25] that has been intensively studied as a potential target in atherosclerosis. While extensive preclinical data support a causal involvement of CCL2 and its receptor CCR2 in experimental atherosclerosis, it was not until recently that large-scale epidemiological studies highlighted the relevance of the CCL2–CCR2 pathway in human CVD, calling for clinical translation of strategies targeting this pathway.

Here, we outline the role of the CCL2–CCR2 axis in atherosclerosis and summarize data from preclinical studies, human genetics, population-based studies, and analyses of human atherosclerotic plaques pinpointing this pathway as a promising drug target in CVD. Furthermore, we discuss previous and ongoing efforts to interfere with the CCL2–CCR2 axis in preclinical atherosclerosis and in various human indications highlighting pathway-intrinsic opportunities and challenges pertaining to pharmacological targeting of this axis. We close by providing a roadmap towards clinical translation highlighting knowledge gaps that must be addressed before proceeding to trials targeting the CCL2–CCR2 axis in human atherosclerosis.

The CCL2–CCR2 axis

Chemokines are a family of small secreted proteins that regulate inflammatory cell trafficking between sites of haematopoiesis, secondary lymphoid organs, the circulation, and peripheral sites of inflammation,[25–28] and are pivotal mediators of atherosclerosis (*Figure 1*).[9,40,41] Originally discovered as the 'chemotactic inflammatory cytokine monocyte-chemoattractant protein-1', CCL2 is the most extensively studied chemokine.[25] As a 76-amino-acid-long polypeptide, it belongs to the CC-motif type chemokines featuring two adjoining amino-terminal cysteine residues.[42–44] The three-dimensional structures of CCL2 and CCR2 have been resolved[29,44,45] and provide important insight into ligand interactions and receptor activation (*Figure 1A*).

While CCL2 is expressed in most human tissues and secreted in response to inflammatory stimuli, CCR2 expression is largely limited to bone marrow, bloodborne cells, and secondary lymphoid organs in accordance with its primary expression in monocytes and to a lesser extent in highly activated T cells (*Figure 1B*).[46] CCL2 is a key regulator of monocyte trafficking, as it represents one of the strongest recruitment signals for monocytes to sites of inflammation.[47–49] CCL2 primarily acts on 'classical' monocytes, i.e. $CD14^{++}/CD16^{-}$ in humans and lymphocyte antigen 6 complex $(Ly6C)^{high}/CD43^{+}$ in mice, which strongly express its main receptor CCR2.[50,51] In humans, classical monocytes represent around 95% of the circulating monocyte pool.[52] Circulating CCL2 promotes the mobilization of classical monocytes from the bone marrow in a CCR2-dependent process.[53–56] Furthermore, CCL2 produced by endothelial cells, smooth muscle cells, and macrophages in atherosclerotic lesions gets anchored to the plasma membrane of endothelial cells by glycosaminoglycans and binds to CCR2 of circulating monocytes, promoting their adhesion to the endothelium and transmigration into the subendothelial space (*Figure 1D*).[9,41,57–59] Beyond strong chemotactic effects on monocytes, CCL2 has also been found under certain conditions to less strongly chemoattract T cells,[60] B cells,[61] natural killer cells,[62] basophils,[63] and neutrophils,[64] but the role of this action in atherosclerosis remains undetermined.

Evidence supporting the CCL2–CCR2 axis as a key driver of atherosclerosis

Owing to the key role of monocytes and macrophages in atherogenesis, the CCL2–CCR2 axis has received attention as a potential therapeutic target.[41] Adding to a strong body of experimental evidence supporting the CCL2–CCR2 axis as a key regulator of atherosclerosis, a coherent series of recent studies has created a new momentum for prioritizing this axis as a candidate target for cardiovascular prevention. Below, we discuss key findings from multiple settings providing triangulation of evidence[65] for the prioritization of the CCL2–CCR2 axis for further drug development (*Graphical Abstract*).

Preclinical studies

Following the discovery of CCL2 as a monocyte chemotactic molecule[43,76] and the demonstration that it is highly expressed in human atherosclerotic lesions,[47] investigators examined the consequences of deleting *Ccl2* or *Ccr2* primarily in hyperlipidaemic mice fed a Western-type diet. Compared with atherosclerosis-prone apolipoprotein E-deficient ($Apoe^{-/-}$) control mice, $Ccr2^{-/-}/Apoe^{-/-}$ mice showed a reduction of atherosclerotic lesions and decreased macrophage accumulation in plaques.[66,67] Mice heterozygous for *Ccr2* generally showed intermediate reductions in lesion size supporting a dose-dependent effect of *Ccr2* deletion, although this was not systematically explored.[66,67] Also, transplantation of $Ccr2^{-/-}$ bone marrow progenitor cells into atherosclerosis-prone apolipoprotein E3-Leiden transgenic mice resulted in suppression of atherosclerotic lesion formation.[77] Similar reductions were observed when deleting *Ccl2* in LDL receptor-deficient ($Ldlr^{-/-}$) mice,[68] transgenic mice overproducing human apolipoprotein B,[78] mice exposed to visceral fat transplantation,[79] and hyperlipidaemic $Apoe^{-/-}$ mice undergoing arterial injury for neointimal lesion formation.[80] Conversely, overexpression of *Ccl2* accelerated atherosclerosis in $Apoe^{-/-}$ mice.[69] Interestingly, combined deletion of *Ccl2* along with *Cxc3r1* and *Ccr5*, genes encoding other chemokine receptors traditionally considered to govern the recruitment of non-classical monocytes and neutrophils, respectively, led to additive reductions of up to 90% in atherosclerotic lesions.[81] Similarly, a combined deletion of *Ccr2* and *Cx3cl1* led to an

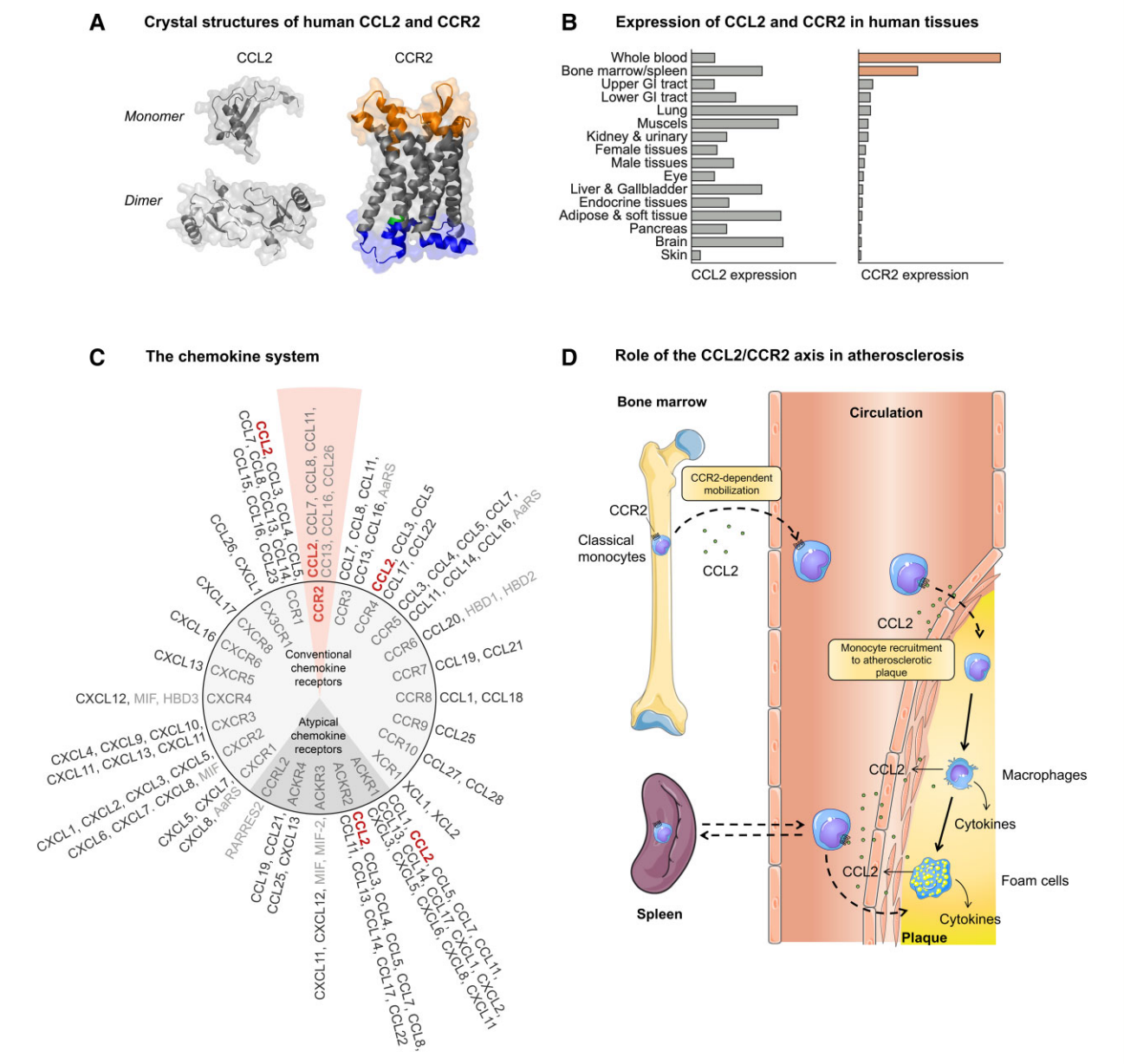

Figure 1 The CCL2–CCR2 axis and its role in atherosclerosis. (*A*) Shown are the crystal structures of CCL2 and CCR2. CCL2 is depicted both in its monomeric and dimeric forms. The individual structures of CCL2 and CCR2 were created with PyMOL version 2.4.1 and are based on the X-ray structures 1DOK and 5t1a, respectively.[29–32] The intracellular loops of CCR2 are coloured in blue and the G protein activation motif DRY is highlighted in green; extracellular loops are coloured in orange. (*B*) Expression of CCL2 and CCR2 in human tissues (data extracted from The Human Protein Atlas, https://www.proteinatlas.org). (*C*) Network of chemokines and their receptors. Based on differences in their N-terminal cysteine motif, CC-, CXC-, CX_3C-, and C-type motif chemokines are distinguished, with the CC- and CXC-chemokines representing the predominant classes.[25–28,33–36] With 49 human chemokines interacting with 18 classical chemokine receptors and 5 atypical chemokine receptors, the chemokine system constitutes a large network of ligands and G-protein-coupled receptors.[35–37] Different chemokines can induce distinct responses at the same receptor, while a single chemokine can interact with multiple receptors. This network is further complicated by atypical chemokines (in light grey) such as the human beta-defensins or macrophage migration inhibitory factor, and ligand hetero-oligomerization in an expanded chemokine interactome.[33,38,39] CCL2 and CCR2 are represented in bold red and the CCR2 network with its seven ligands is shadowed in orange. (*D*) Role of CCL2 and CCR2 in the mobilization of 'classical' monocytes from the bone marrow and their recruitment into atherosclerotic lesions.

additively lower atherosclerotic burden, pointing to the potential of simultaneously targeting multiple chemokines for atheroprotection.[82]

Complementing these observations, a meta-analysis of preclinical studies showed that pharmacological inhibition of the CCL2–CCR2 axis resulted in a substantial decrease in atherosclerotic lesion formation as well as reductions in macrophage accumulation and increases in smooth muscle cell content and collagen deposition in atherosclerosis-prone mice.[70] These changes in plaque morphology

support a stabilizing effect of CCL2–CCR2 inhibition on the plaque phenotype. The meta-analysis found heterogeneity in the efficacy of different interventions, which was, however, explained by differences in reductions in intralesional macrophages. The effects of Ccl2 or Ccr2 inhibition were significant across different vascular beds.[70] Interestingly, the effects on both atherosclerotic lesion size and plaque morphology were of the same magnitude when using pharmacological agents targeting either CCL2 or CCR2,[70] thus supporting both proteins as promising drug targets. It should be noted, however, that these results were mostly based on lesions developed in *Apoe*$^{-/-}$ or *Ldlr*$^{-/-}$ mice, which have a stronger inflammatory component than human atherosclerotic lesions.[83]

Observational and genetic epidemiological studies in humans

Despite abundant preclinical evidence, only recently did large-scale human studies demonstrate the relevance of the CCL2–CCR2 axis in atherosclerotic disease. In an analysis of human atherosclerotic plaques from 1199 individuals undergoing endarterectomy for the treatment of advanced carotid stenosis, CCL2 levels within plaques showed significant associations with markers of plaque instability.[74] Specifically, they were associated with a higher macrophage content, a larger lipid core, intraplaque haemorrhage, a lower smooth muscle and collagen content, and a pro-inflammatory plaque profile,[74] i.e. multiple characteristics that render plaques more vulnerable to rupture and subsequent complications such as stroke.[84,85] In accordance with these findings, CCL2 levels within plaque were higher in individuals with symptomatic stenosis (associated with an ipsilateral cerebrovascular event), when compared with those with asymptomatic disease.[74] These results expanded previous literature noting high CCL2 expression in human atherosclerotic plaques,[47] by demonstrating a connection between higher CCL2 levels and plaque instability.

While important, this study provides no proof for a causal role of CCL2 in human atherosclerosis.[74] Eventually, causality can only be demonstrated in an interventional trial. Yet, novel study designs exploiting human genetic data enable exploring such causal relationships.[86] A recent Mendelian randomization study (see Supplementary material online, *Text S1* for a description of the conceptual framework of Mendelian randomization) used data from 8293 individuals to identify genetic variants associated with the circulating levels of 41 cytokines[73] and tested whether genetically predicted circulating levels of these cytokines associate with stroke and CAD in case–control studies involving >60 000 cases.[73] As genetic predisposition to higher levels of a cytokine would indicate lifelong exposure to elevated levels of this cytokine independently of other vascular risk factors, associations with CVD could provide support for causal relationships.[87] Across all of the tested 41 cytokines genetically predicted levels of CCL2 showed the strongest associations with ischaemic stroke, and particularly large artery atherosclerotic stroke.[73] Higher genetically predicted CCL2 levels were further associated with CAD and myocardial infarction.[73] These results have since been replicated in other data sets with additional analyses further highlighting CCL2 levels as one of the links between obesity, a pro-inflammatory state, and CVD.[88]

Furthermore, these associations were validated with measured levels of circulating CCL2 in conventional epidemiological settings. A series of meta-analyses of seven population-based cohorts involving up to 21 401 middle-aged community-dwelling individuals free of CVD at baseline examined associations of circulating CCL2 levels with risk of ischaemic stroke, CAD, and cardiovascular mortality over a mean follow-up extending beyond 15 years.[71,72] Baseline CCL2 levels were associated with incident ischaemic stroke and[71] CAD, and cardiovascular mortality even after adjustments for conventional vascular risk factors.[72] These associations remained stable after adjustments for circulating IL-6 and CRP levels,[71,72] thus indicating underlying mechanisms partly independent of the IL-6 signalling cascade.

Previous smaller studies further found associations between circulating CCL2 levels and risk of recurrent vascular events, functional outcomes, or mortality after myocardial infarction.[89–93] While these results might be biased due to temporary increases in circulating CCL2 levels as a result of the inflammatory response induced by myocardial ischaemia, they also point to the role of circulating CCL2 as a potential prognostic biomarker for these patients. Evidence from preclinical studies supports that the CCL2–CCR2 axis governs monocyte infiltration to the infarcted myocardium leading to a deleterious inflammatory response that can increase infarct size and promote adverse cardiac remodelling.[94–98] In human myocardium, there are distinct resident macrophage populations with monocyte-derived CCR2+ macrophages representing an inflammatory population.[99] Following myocardial ischaemia in mice, the activation of cardiac Ccr2+ macrophages leads to the release of inflammatory cytokines that orchestrate monocyte and neutrophil recruitment resulting in worse outcome.[100] Thus, targeting the CCL2–CCR2 axis early after myocardial infarction could offer additional benefits beyond inhibition of monocyte recruitment to the atherosclerotic plaques.

Early-phase clinical trials

Taken together, these results triangulate evidence across different study designs and variable sources of animal and human data, thus supporting a key role of CCL2 in atherosclerosis. Data from interventional trials testing molecules targeting the CCL2–CCR2 axis in the context of CVD are scarce. At present, the only two agents that have been tested are the small-molecule drug bindarit and the monoclonal antibody MLN1202. Bindarit is an indazolic derivative that inhibits CCL2 production[101] and was tested in a Phase II trial for the prevention of restenosis in 148 patients submitted to coronary stenting using bare metal stents.[102] The study borderline failed on the primary efficacy endpoint (in-segment late loss on coronary angiograms) but found a significant reduction in in-stent late loss. Trials in other indications have shown that bindarit has a favourable safety profile and is well tolerated (see Supplementary material online, *Table S1*). Notably, bindarit was not designed as a CCL2 inhibitor and the specificity of the compound for targeting the CCL2–CCR2 axis is somewhat limited. The effect of bindarit on monocyte recruitment is likely due to a more general inhibitory effect on NFκB signalling with a reduction of other cytokines beyond CCL2.[101,103]

The second study was a Phase II trial that tested MLN1202, an anti-CCR2 monoclonal antibody, in 108 patients with risk factors for CVD. MLN1202 is a genetically engineered humanized neutralizing IgG1κ antibody that was created by inserting the complementarity-determining regions from an IgG2a mouse antibody into human IgG1.[104] Patients were required to have more than two risk factors for atherosclerotic CVD and circulating high-sensitivity CRP levels

>3 mg/L. Beginning at 4 weeks and continuing through 12 weeks after dosing, patients receiving MLN1202 exhibited significant decreases in high-sensitivity CRP levels as the primary outcome measure.[75] This decrease was independent of background therapy including lipid-lowering therapies. Lipid levels, glycaemic control, and IL-6 levels were unaffected and despite a temporary decrease in circulating monocytes the drug was well tolerated.[75] The study was not designed to test clinical endpoints, which would require a larger sample size and a longer follow-up. To our knowledge, MLN1202 has not been moved forward to a Phase-III trial.

Pharmacological agents targeting CCL2 or CCR2

The involvement of the CCL2–CCR2 axis in multiple conditions including autoimmune disorders, viral infections, cancer, and CVD has sparked the development of drugs targeting this pathway. *Figure 2* provides an overview of agents that have entered clinical trials or have been tested in experimental models of atherosclerosis. The majority of studies tested small molecules and monoclonal antibodies mostly targeting CCR2. Aside from CVD, drugs targeting the CCL2–CCR2 axis have been tested in a wide range of indications with some trials still ongoing. A detailed overview of the trials is provided in Supplementary material online, *Tables S1* and *S2*. The pharmacological properties of different drug classes are discussed in Supplementary material online, *Text S2*.

Challenges and opportunities pertaining to pharmacological targeting of the CCL2–CCR2 axis in atherosclerosis

Despite extensive efforts to pharmacologically target chemokine signalling, only three agents have made it to the market: maraviroc, a CCR5 allosteric antagonist for HIV;[105,106] plerixafor, a CXCR4 antagonist, for mobilizing haematopoietic stem cells for autologous transplantation in non-Hodgkin's lymphoma and multiple myeloma;[107,108] and mogamulizumab, an anti-CCR4 monoclonal antibody for cutaneous T-cell lymphoma.[109,110] While pharmacological targeting of the CCL2–CCR2 axis in preclinical models of atherosclerosis has been remarkably successful, translation into clinical trials for molecules targeting this axis has been lagging behind. This may be rooted in challenges related to the promiscuity of the CCL2–CCR2 axis, difficulties in choosing between CCL2 and CCR2 as the optimal target, the complex molecular structures of CCL2 and CCR2 and their presumed binding interface, the physiological diurnal variation in CCL2–CCR2 axis activity, and issues related to drug delivery to the desired tissues. These challenges, along with opportunities to overcome them, will be discussed below.

Promiscuity of the CCL2–CCR2 axis

The CCL2–CCR2 axis is a good example of the complexity and redundancy inherent to the chemokine ligand–receptor network (*Figure 3A*). CCR2 has been reported to bind to seven ligands with a range of affinities and potencies. Aside from CCL2, these include CCL7, CCL8, CCL11, CCL13, CCL16, and CCL26. While monocyte mobilization is chiefly promoted by the CCL2–CCR2 axis, it is also supported by the CCL7–CCR2 axis,[54,116,117] but CCL7 has a broader role in leucocyte homeostasis, as it also regulates the recruitment of eosinophils, neutrophils, dendritic cells, and T cells. The functional relevance of pathways involving alternate CCR2 ligands beyond CCL2 and CCL7 remains largely unknown. Yet, some of these may likewise impact atherosclerosis.[117,118] CCL2 in turn also interacts with the CC-chemokine receptors CCR1 and CCR4 and it can engage in CC-type heterodimer formation with CCL5, although the functional consequence of this interaction is incompletely understood.[38] It is important to obtain differential structural information on the binding interface for the various ligands and receptors, when considering tailored drug approaches for selective targeting of CCL2, as, for example, obtained for binding of CCL2 and CCL7 to CCR2 by mutational analysis and ligand chimera.[30] The implications of the redundancy of CCL2 interactions with other CCRs are insufficiently understood,[119,120] but studies implying CCL2 as a non-canonical ligand of CCR4 and the role of this axis in promoting the recruitment of inflammation-dampening regulatory T cells highlight the need for an improved structural understanding of the CCL2–CCR4 interface.[121,122] CCL2 also binds to the atypical chemokine receptors ACKR1 and ACKR2. While traditionally considered decoy receptors for circulating chemokines, it is now evident that chemokine binding to ACKRs might also promote downstream signalling through the β-arrestin pathway and regulate the activity of co-expressed conventional chemokine receptors.[123,124] The functional relevance of these interactions remains to be explored. Despite the promiscuity, targeting-specific signalling cascades may be possible. For example, the development of CCR2-biased or probe-dependent antagonists that selectively block a certain chemokine or signalling pathway, sparing interactions with alternate chemokine ligands,[125–127] might be a promising approach. However, whether going for a selective vs. a non-selective strategy requires a deeper understanding of the alternative pathways beyond the main ligand and receptor and their roles in atherosclerosis.

CCL2 targeting vs. CCR2 targeting

While effective strategies have been developed that target either CCL2 or CCR2, it is debated whether receptor-targeting would be superior to ligand targeting in the context of CVD. As illustrated, CCR2 pathways elicited by alternate ligands beyond CCL2 also influence the recruitment of other leucocyte populations that may be relevant for atherosclerotic disease,[117,118] which would argue for broadly blocking CCR2 signalling elicited by all of its ligands. After all, CCR2 is a prototypical G-protein-coupled receptor (GPCR) and is considered to be druggable.[128] Moreover, the spectrum of substance classes targeting CCR2 is wide. Small-molecule antagonists encompass orthosteric antagonists that directly interfere with ligand binding and/or ligand-induced receptor activation as well as allosteric antagonists. Such studies were instigated by the CCR2 crystal structure and molecular dynamics simulations indicating a potential drug-binding pocket at an intracellular allosteric site.[29,129,130] CCR2-targeting approaches further include organometallics and dual CCR2/CCR5 antagonists. Potential additional receptor-targeting modalities include CCR2-targeting monoclonal antibodies, truncated CCL2 variants

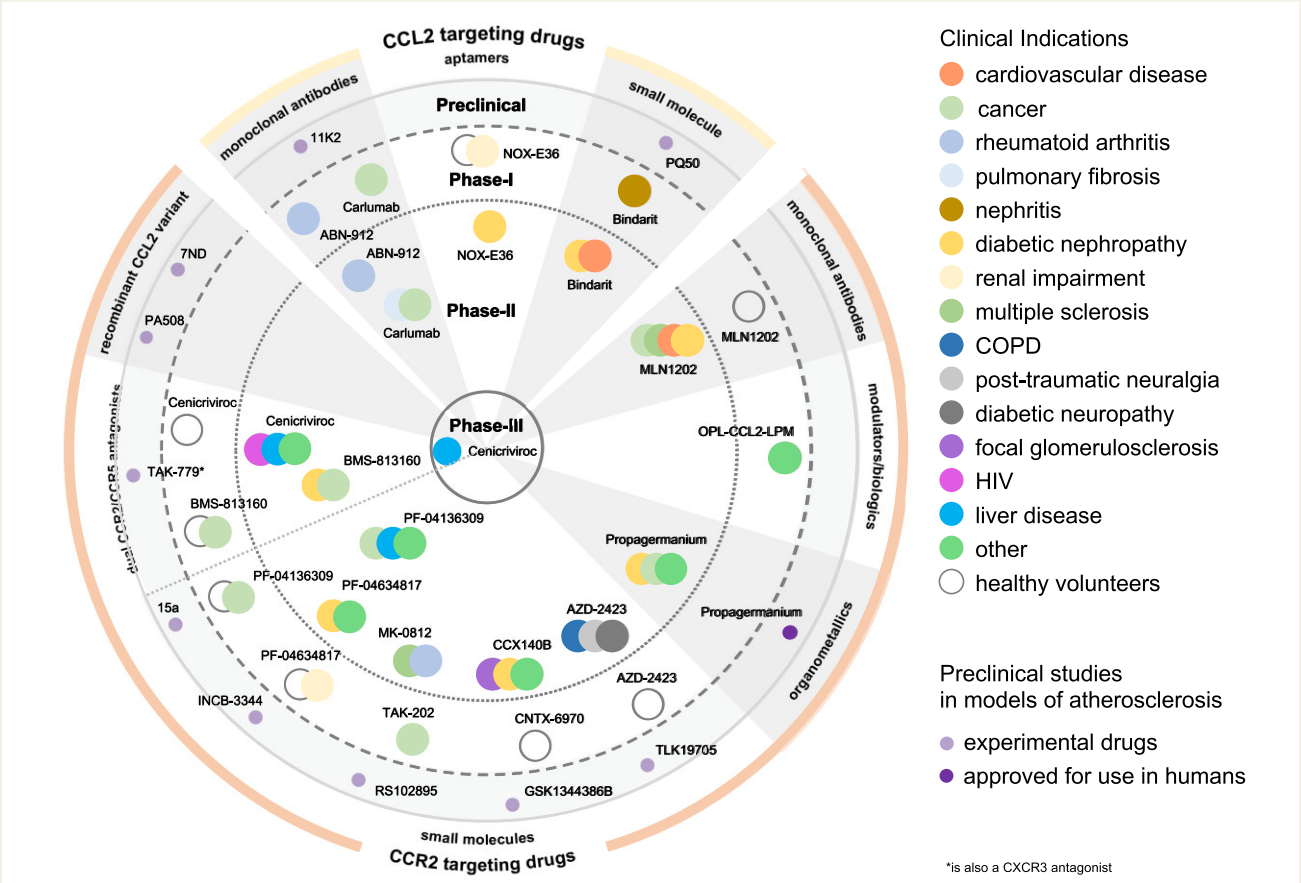

Figure 2 Available pharmacological agents targeting the CCL2–CCR2 axis. Shown are the pharmacological agents that are targeting CCL2 or CCR2 and have been tested in preclinical models of atherosclerosis (outer circle) and in Phase I–III clinical trials for different clinical indications (inner circles). Agents are classified by target protein and drug type. The different clinical indications are depicted by different colours. Information on clinical trials was retrieved from the Therapeutic Drug Database (http://db.idrblab.net/ttd/) and ClinicalTrials.gov.

functioning as receptor antagonists, and cell-toxic CCL2 fusion proteins that serve as modulators (*Figures 2* and *3*). Still, the ligand bias paradigm including potential homeostatic activities by some agonists may argue against a broad anti-CCR2 strategy. In this light, targeting CCL2 might be advantageous as it would result in less pronounced promiscuity compared with targeting CCR2. Among the drug classes that have been studied are monoclonal antibodies, aptamers, and two types of small-molecule compounds: a glutaminyl cyclase inhibitor and an inhibitor of protein biosynthesis, which indirectly target CCL2 by reducing its half-life and expression rate, respectively.[102,131] Alternative ligand-directed strategies include a chemokine receptor mimicry approach, as recently shown for the MIF/CXCR4 pathway[132] or virus-derived chemokine-binding proteins such as the myxoma pox virus protein T1, which binds CC-chemokines with high affinity.[133,134] Despite the pharmacological challenges pertaining to targeting either of the two proteins, a meta-analysis of preclinical studies found no difference in the efficacy of CCL2-based strategies compared with CCR2-based strategies in lowering plaque burden and vascular inflammation.[70] As such, there is equipoise in targeting the ligand or receptor of the CCL2–CCR2 axis.

Complexity of the molecular structure of CCL2 and CCR2

While a co-crystal structure of CCR2 with CCL2 has yet to be resolved, recent studies[29–32] have provided valuable structural information for a putative binding model of the CCL2–CCR2 complex. Applying the lipidic cubic phase method, previously established for other chemokine receptors,[135–137] Handel and Domaille[45] crystallized CCR2 as a ternary complex together with the orthosteric antagonist BMS-681 and the allosteric antagonist CCR2-RA-[R]. CCR2 showed a canonical Class A GPCR structure with a flexible N-terminal region and seven transmembrane helices linked by three extracellular and three intracellular loops. Together with the CCL2 structures showing the typical structural features of a CC-chemokine and mutational analyses of CCL2,[30–32,45] this enables to predict a model for the CCL2–CCR2 complex with an extensive ligand/receptor interface[29,30] (*Figure 3B*).

The interaction between a chemokine and its receptor is that of a protein with another protein thus differing from interactions between many GPCRs and their small-molecule ligands. While smaller than most cytokines, chemokines represent rather large GPCR ligands compared with small molecule or peptidic ligands. Thus, the

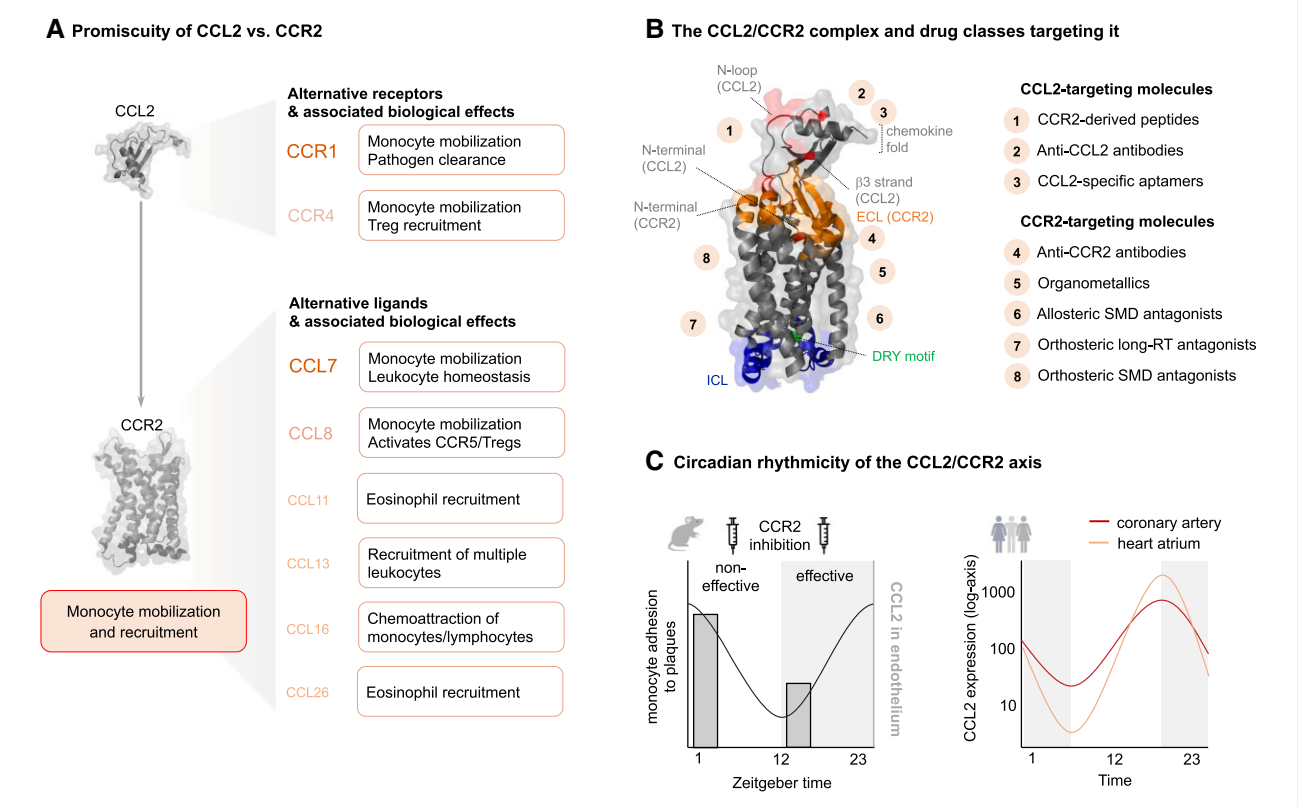

Figure 3 Challenges related to pharmacological targeting of the CCL2–CCR2 axis. (*A*) Promiscuity of the CCL2–CCR2 axis at the level of the ligand and the receptor, associated biological effects, and relation to atherosclerosis. Tregs, regulatory T cells. Information on alternative receptors and ligands of CCR2 and CCL2, respectively, is derived from the IUPHAR/BPS database: https://www.guidetopharmacology.org/. (*B*) Predicted 3D molecular structural model of the CCL2–CCR2 complex. This hypothetical model of the complex considers the unifying two-site model of chemokine/receptor binding and activation,[111–113] and the structural information provided by the extensive structure–activity studies, the X-ray and NMR structures of CCL2, and the recently elucidated X-ray structure of CCR2 together with small-molecule inhibitors.[29–32] According to the two-site model of chemokine–receptor binding and activation the chemokine N-loop and the adjacent β3-region form Site I together with the N-terminal region of the receptor, an interaction suggested to be critical for the ligand–receptor binding step. Site II is formed by the chemokine N-terminus interacting with certain receptor transmembrane helices and/or their connecting extracellular loops and has been suggested to mainly govern receptor activation.[111–113] The N-loop, N-terminal, chemokine-fold, and β3-strand of CCL2, as well as the N-terminal, the extracellular loops, intracellular loops, and G protein-binding DRY motif of CCR2, are indicated. The model was created in the PyMOL using the structures of CCL2 (PDB 1DOK) and CCR2 (PDB 5t1a). Furthermore, potential drug approaches/classes targeting CCL2 or CCR2 are depicted taking into account the structural considerations of CCL2 and CCR2. (*C*) Experimental (left) and human (right) data supporting a circadian rhythmicity of the CCL2–CCR2 axis. In an atherogenic mouse model, classical monocyte recruitment to atherosclerotic lesions, as well as CCL2 endothelial deposition, were shown to follow a diurnal pattern with a peak in the transition from the active (grey) to the resting (white) phase. Chrono-pharmacological inhibition of CCR2 before this peak (Zeitgeber time 17) showed a reduction in atherosclerotic lesion size and lesional macrophage accumulation in the aortic root, as opposed to no effect when the inhibition was applied after the peak (Zeitgeber time 1).[114] Human data[115] also support a diurnal pattern in CCL2 expression in the coronary artery and heart atrial tissues with peaks in the transition from the active (white) to the resting (grey) phase (data modified from the online repository CircaDB: http://circadb.hogeneschlab.org/about).

orthosteric binding pockets of chemokine receptors are relatively large, feature polar residues, and contribute to an extensive interaction.[29,138–140] While it has been argued that it is difficult to block such interaction surfaces with a small-molecule-based approach, the structure of the ternary CCR2 complex with BMS-681 and CCR2-RA-[R] highlights previously unanticipated binding modes of small molecules directed against a chemokine receptor.[29] BMS-681, for example, not only interacts with polar residues in the open binding site of CCR2, but also engages in hydrophobic interactions with helices. Moreover, similar to the long target residence time of Compound 15a, allosteric antagonists can result in insurmountable antagonism leading to receptor blockade even when high or enduring concentrations of the chemokine ligand are present.[141] This feature may be desired in diseases such as atherosclerosis, but no preclinical or clinical data are available yet for such allosteric antagonists targeting CCR2.

Together, targeting chemokine receptors with small-molecule inhibitors is a promising approach, while this has proven to be almost impossible for larger cytokines.[142–145] Anti-CCR2 or anti-CCL2 antibodies, aptamers, CCL2 variants, or peptide-based receptor mimics (*Figure 3B*) may offer additional favourable properties in targeting the large and partly flexible CCL2–CCR2

interface. However, the conformational constraints of such inhibitors may limit interactions with the hydrophobic transmembrane moieties of the receptor.

Circadian rhythmicity of the CCL2–CCR2 axis

An additional aspect to consider in future studies is chronopharmacologic targeting. Monocyte activity is under tight circadian control and their recruitment to atherosclerotic lesions has been shown to oscillate in mice (*Figure 3C*).[114] This diurnal phenotype seems to be regulated by rhythmic release of CCL2 with circulating CCL2 levels and CCL2 immobilization along the wall of large arteries peaking at transition from the activity to the resting phase. Indeed, injections of RS102895, a small-molecule CCR2 antagonist, during the active (but not resting) phase in rodents reduced atherosclerotic lesion formation and macrophage accumulation.[114] A circadian pattern of rhythmicity has also been shown for CCR2 surface expression on Ly6C^{high} monocytes in mice subjected to myocardial infarction with higher expression rates at the beginning of the active phase.[146] These findings highlight potential advantages of optimizing drug efficiency based on timed interference strategies although their relevance for human biology remains to be determined.

Systemic side effects of CCL2–CCR2-targeting molecules: optimizing delivery

Given the central role of the CCL2–CCR2 axis in monocyte mobilization for fighting infections,[53] targeting this axis might come at the cost of an impaired host defence. One solution might be an optimized delivery of molecules addressing the CCL2–CCR2 axis with nanoparticle formulations that allow directing therapeutics to the desired site of action with high accuracy.[147] Therapeutic silencing of *Ccr2* mRNA with a short interfering-RNA delivered through lipid nanoparticles led to reductions in atherosclerotic lesion size and lesional macrophages in *Apoe*$^{-/-}$ mice.[148] Nanoparticles encapsulating siRNA sequences targeting *Ccl2* have been directed to bone marrow endothelial cells in an effort to inhibit the release of monocytes and improve healing in mouse models of myocardial infarction with promising effects.[98] Studies focusing on other pathways have achieved nanoparticle delivery to the fibrous cap, lesional macrophages, and endothelial cells in preclinical *in vivo* models of atherosclerosis.[149–152] Thus, nanoparticles encapsulating CCL2–CCR2-targeting molecules and directed towards atherosclerotic lesions or the circulating monocyte pool seem an attractive tool for optimizing delivery and limiting potential side effects. However, the potential of such approaches for use in human atherosclerosis remains to be explored.

Considerations for clinical translation

The success of previous treatment paradigms targeting inflammation in CVD highlights the importance of careful trial design before testing interventions targeting the CCL2–CCR2 axis. While two Phase II trials have already been successfully undertaken, they were not designed to demonstrate clinical efficacy in patients with established atherosclerosis.[75,102] Several aspects should be considered before moving to a clinical endpoint trial.

First, there is uncertainty regarding the optimal readout for a Phase II trial targeting the CCL2–CCR2 axis. Given the efforts and costs of clinical endpoint trials, such a proof-of-concept study would be needed before moving to a Phase-III trial. High-sensitivity CRP has been used in previous trials testing anti-inflammatory treatments in CVD[75,153,154] and circulating levels of CCL2 correlate with CRP levels,[71] but whether CRP represents a meaningful readout for CCL2–CCR2-targeting therapies remains to be determined. Serial changes in carotid intima-media thickness have been proposed as a surrogate marker of atheroprogression,[155] but are characterized by relatively low intra- and inter-rater reliability, are non-specific for vascular inflammation, and the slow progression would require long follow-up to capture meaningful changes. Markers of plaque composition as assessed by vascular imaging represent a promising readout. Meta-regression analyses from preclinical studies suggest that the beneficial effect of CCL2–CCR2 inhibition on atherosclerotic lesion size relates to a reduction in macrophage accumulation within plaques.[70] Quantification of activated macrophage content within plaques is possible with advanced imaging modalities, such as positron emission tomography (PET) involving specific tracers[156–160] or ultrasmall superparamagnetic iron-oxide-enhanced magnetic resonance imaging.[161,162] An increasing number of studies show that increased uptake of ^{18}F-FDG in carotid PET imaging is associated with higher risk of future strokes.[156,163,164] Recently developed radiotracers may increase specificity.[165] Indeed, a radiotracer developed to specifically capture CCR2 expression (^{64}Cu-DOTA-ECL1i) shows high promise as a potentially specific biomarker for response to treatments interfering with the CCL2/CCR2 axis. *In vivo* imaging of abdominal aortic aneurysm in rats and *ex vivo* imaging of surgically removed human aortic tissues revealed high specificity for CCR2+ macrophages.[166] An *in vivo* study evaluating this method in patients with established carotid and femoral atherosclerosis is currently ongoing.[167] Alternative circulating biomarkers of CCL2–CCR2 activity and monocyte trafficking, such as monocyte count or markers of macrophage activity[168] would require further investigation. Combinations of circulating inflammatory biomarker panels with imaging assessment of plaque inflammation might represent suitable readouts for testing approaches targeting the CCL2–CCR2 axis in Phase II trials.

Second, there is a need to define the optimal trial population. As the absolute benefits of prevention are typically larger in patients with established CVD, early-phase trials targeting anti-inflammatory mechanisms are typically conducted as secondary prevention trials and focus on patients with myocardial infarction.[14–16,19] Yet, studies targeting the CCL2–CCR2 axis might require a slightly different population. Specifically, such trials might benefit from an enrichment with atherosclerotic stroke patients as recent epidemiological and experimental studies support a more prominent role of CCL2 in carotid atherosclerosis and ischaemic stroke,[70–73] when compared with CAD. It also remains uncertain whether patients should be selected on the basis of evidence of increased activity of the CCL2/CCR2 pathway as determined either by circulating biomarkers or by evidence of local inflammation in established lesions. Plaque imaging might again represent a useful tool for selecting the patients with a highest probability to benefit.

Third, choosing the right pharmacological agent will be critical. While the field of pharmacological targeting of CCL2 or CCR2 has

rapidly advanced studies in humans mostly focused on indications other than CVD. Data from preclinical studies could guide the selection of atheroprotective agents suitable for trials in humans, taking into account the known differences in lesion morphology between humans and experimental atherosclerosis.[83] However, there is considerable heterogeneity in the efficacy of different agents.[70] As such, agents leading to larger reductions in macrophage content along with efficacy in lowering atherosclerotic lesion burden, such as 11K2,[48] 15a,[169] TLK19705,[170] and propagermanium,[171] should be prioritized for studies in humans. In the light of experimental evidence supporting additive effects of concurrently blocking more than one chemokine axis,[81,82] and the availability of dual inhibitors for CCR2 and CCR5, it should be further clarified, whether such approaches would offer benefits beyond single inhibition of CCL2 or CCR2. Dual inhibitors have not, however, been tested in experimental atherosclerosis. Additional parameters to be considered include pharmacodynamic and pharmacokinetic properties allowing delivery to the inflamed subendothelial tissue, mode of administration (e.g. oral vs. injection), potential interactions with current standard treatments, and the binding affinity and specificity of the agent to inhibit CCL2 or CCR2. The generation of new compounds with improved properties might offer additional options for future trials.

Fourth, the side effects of approaches targeting the CCL2–CCR2 axis remain to be determined. While there is no alarm from available trials (see Supplementary material online, *Tables S1* and *S2*) in terms of serious side effects, early-phase trials are commonly underpowered to detect safety signals. Preclinical studies support a role of CCL2–CCR2 in monocyte emigration from the bone marrow, which could be crucial during bacterial infections.[53] Also, there is evidence for a role CCL2 in thrombus resolution, which could be important, if envisioned in patients at high risk of thrombotic events.[172,173] The inhibition of the CCL2–CCR2 axis may further lead to a blockade of the recruitment of anti-inflammatory monocytes promoting atheroregression in advanced unstable plaques.[174] Finally, potential rebound effects after stopping treatment should be considered and have not yet been explored in experimental studies.

Conclusions

Recent large-scale clinical trials provided proof-of-concept for the efficacy of anti-inflammatory treatments in CVD, but also highlighted the importance of carefully selecting suitable pathways and drug candidates. A large body of experimental, genetic, and epidemiological evidence supports a causal role of the CCL2–CCR2 axis in atherosclerotic disease. At the same time, major advances in the pharmacological targeting of these proteins raise hope for a stepwise transition towards clinical testing. This will require addressing questions related to study design. Meanwhile, the undisputed biological role of monocyte recruitment to atherosclerotic lesions, along with the unique body of data supporting its causal role in human atherosclerosis, earmark the CCL2–CCR2 axis as a strong target for the next generation of anti-inflammatory approaches in CVD.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

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