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The CCL2/CCR2 Axis in the Pathogenesis of HIV-1 Infection: A New Cellular Target for Therapy?

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Abstract: The identification of chemokine receptors as necessary co-receptors for HIV entry into target cells represented a breakthrough in the understanding of the pathogenesis of this viral infection. Since this initial discovery, it was unraveled that chemokines, in addition to their role in blocking viral entry by binding to co-receptors, have other functions in HIV pathogenesis. Indeed, chemokines can either inhibit or enhance HIV replication, and these effects may involve both entry and post-entry events of the viral life cycle. Depending on the balance of their negative *versus* positive effects on HIV replication and spreading, chemokines contribute to different outcomes of HIV pathogenesis. CCL2 is unique among the chemokines in that mostly enhancing effects on viral replication and pathogenesis have been reported. Either HIV infection itself or exposure to viral products can induce the expression of this chemokine and of its receptor CCR2, and high levels of CCL2/CCR2 are indeed found in HIV-infected subjects. The CCL2/CCR2 axis is tightly linked to the high level of immune activation and inflammation that is the hallmark of HIV infection even in patients undergoing antiretroviral therapy. In addition, more direct effects of CCL2 on HIV replication are becoming apparent. Thus, modulation of CCL2/CCR2-driven effects may have significant impact on HIV disease progression. In this review, we will discuss the complex interaction between CCL2/CCR2 and HIV and the emerging therapeutic approaches based on the inhibition of this axis.

Keywords: CCL2, CCR2, HIV, inflammation, pathogenesis, therapy.

INTRODUCTION

Since its discovery in the early 1980s, infection with human immunodeficiency virus (HIV) has reached epidemic proportions, particularly in developing countries. The World Health Organization estimated that approximately 35 million people were infected with HIV in 2013, of whom 2.1 were newly infected and 1.5 died. Highly active antiretroviral therapy (HAART) for the chronic suppression of HIV replication has been the main achievement in acquired immunodeficiency syndrome (AIDS) research. About 13 million people living with HIV were receiving HAART globally in 2013. Many of these patients are in the second decade of treatment, and have plasma HIV RNA levels below the limit of detection of clinical assay. However, ongoing viremia is detected at levels of 1 to 50 copies per milliliter in most of the patients on HAART. These virions may engage cellular receptors and activate pathways that could lead to chronic consequences such as cardiovascular and malignant diseases. Furthermore, many antiretrovirals show a suboptimal penetration into the central nervous system (CNS), and this could be responsible of low levels of viral replication and release from viral reservoirs, resulting in neuropathology. In addition, in low-income countries HIV contributes up to 40% of maternal mortality through tuberculosis and sepsis [1].

Since the introduction of HAART in the mid-1990s, HIV prevalence is increasing worldwide for the subsequent drastic reduction in mortality by HIV infection [2], with greater chance of developing AIDS-related diseases. In fact, infection with HIV causes depletion of T cells and reduced cell-mediated immunity, thus leading to a wide range of opportunistic infections and cancers. Furthermore, the virus directly damages many tissues, mainly brain, gut and lung, through infection and activation of mononuclear cells. In addition, it can be responsible for more subtle systemic organ damage, such as chronic CNS, cardiovascular, hepatic and pulmonary diseases through immune activation and effects on endothelia. HAART has enabled HIV-infected individuals to live with chronic infection, although with some side-effects and mortality, including reactions due to the immune reconstitution inflammatory syndrome (IRIS). As cohorts of infected people get older, age-related diseases will combine with chronic HIV infection leading to disabilities whose extent is yet unknown. Thus, there is a growing interest towards the outcomes of the interactions of HIV, antiretrovirals and ageing, and how to deal with the growing number of individuals with life-long HIV infection. Since non-AIDS clinical events represent the main causes of morbidity and mortality in people on HAART, the role of im-

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mune activation and inflammation in the pathogenesis of these conditions is receiving increased recognition.

Leukocyte movement during immune and inflammatory responses is a highly coordinated process that requires the integration of extracellular signals to ensure cell migration in response to physiological as well as pathological stimuli. Chemokines are a large family of small chemoattractant cytokines that play a central role in the induction of chemotaxis and in the regulation of cell trafficking. Based on the number and location of the highly conserved cysteine residues at the N-terminus, these molecules are structurally divided into four subgroups, namely CC, CXC, CX, and C chemokines. Several chemokines can dimerize in solution or upon their interaction with glycosaminoglycans (GAGs) [3].

Based on their function, chemokines are named as homeostatic and inflammatory. Homeostatic chemokines are constitutively produced by tissue cells and control basal leukocyte trafficking, for instance homing of leukocytes to secondary lymphoid organs and their recirculation through peripheral tissues. In contrast, inflammatory chemokines are induced in response to tissue injury, infections or stress factors. They control the recruitment of effector leukocytes to the site of inflammation. Some chemokines can have both inflammatory and homeostatic functions and are consequently called dual-function chemokines [3].

To perform their functions, chemokines interact with seven-transmembrane receptors coupled to heterotrimeric G-proteins [3]. There is extensive promiscuity between chemokines and their receptors, with a particular chemokine that can bind numerous different receptors, and a particular receptor that can interact with various different chemokines. This promiscuity within the system has generated the concept of chemokine redundancy [4]. However, redundancy may be mostly related to the potential ligand-receptor interactions and not to the biological functions *in vivo*, where chemokines are under temporal and spatial control. Moreover, several chemokine receptors can be expressed by a single cell either simultaneously or during different stages of its life, and the binding of different chemokines to a given receptor does not necessarily result in the same biological response. Overall, the diverse biological outcomes of receptor-ligand interaction seems to be determined by binding specificity and "how", "when" and "where" chemokines and their receptors encounter each other [5].

Although originally identified as regulators of leukocyte trafficking, chemokines can also control survival and effector functions of these cells. The expression of chemokines must be tightly regulated because alterations of their level or function might lead to a persistent inflammatory reaction, thus originating a pathogenetic event from the establishment of chronic inflammation. Furthermore, besides their roles in the immune system, chemokines control processes such as organogenesis, angiogenesis, hematopoiesis, fibrosis and tissue remodeling. Overall, many chemokines are involved in pathological processes including inflammatory and autoimmune diseases, transplant rejection, tumor growth and metastasis. Moreover, some pathogens can interfere with the host chemokines/chemokine receptors network promoting their own survival by either encoding chemokines/chemokine receptors or co-opting chemokine receptors for host cell entry [6].

CHEMOKINES IN HIV PATHOGENESIS

Discoveries over the past two decades have identified a close relationship between chemokines and HIV infection. The first connection was established by the work of Cocchi and colleagues showing that a subset of these factors, namely the CC chemokines macrophage inflammatory protein-1α (MIP-1α)/CC chemokine ligand 3 (CCL3), macrophage inflammatory protein-1β (MIP-1β)/CCL4 and regulated on activation, normal T expressed and secreted (RANTES)/CCL5, can function as inhibitors of HIV [7]. A breakthrough in the understanding of HIV pathogenesis came in 1996 with the discovery of a chemokine receptor like molecule, called LESTER, later designated as CXCR4, as an essential co-receptor for entry of T cell-tropic, syncytium inducing (X4 or SI) variants of HIV [8, 9]. In the absence of this second receptor, HIV can bind to the target cells through CD4, but the fusion process does not start. The recognition of the CC-chemokine receptor (CC) CKR-5 (afterward renamed CCR5) as the primary co-receptor for macrophage-tropic, non-syncytium inducing (R5 or NSI) strains of HIV was also reported in the same year by five independent research groups [10-14]. The two co-receptors play different roles during the natural course of HIV infection. In fact, CCR5 use is the general rule at the beginning of every infection in spite of the transmission route, whereas CXCR4 exploitation emerges later at the time of the immunological deficient phase of disease. Furthermore, CXCR4 use as co-receptor is associated with maintenance of CCR5 utilization in most cases [15]. Thus, the expression of the CCR5 binding chemokines CCL3, CCL4 and CCL5 during the early stage of infection can contribute to suppress virus replication and help the host to restrict virus dissemination. Indeed, a RANTES analogue, AOP-RANTES, is a potent suppressor of HIV infection *in vitro* and the CCR5 blocking drug maraviroc is approved for the treatment of HIV infection in humans. Immediately later on, it was shown that CCR5 polymorphisms, particularly a 32 bp gene deletion mutation leading to reduced or absent expression in heterozygotes or homozygotes, respectively, confer resistance towards HIV infection and disease progression [16, 17].

Since the initial discovery of the co-receptor function of chemokine receptors, a growing body of literature unraveled that, in addition to their role in blocking viral entry by binding to their receptors, chemokines have other roles in HIV pathogenesis. In particular, it soon appeared that chemokines can also enhance HIV replication. For example, CCR5-binding chemokines were also shown to up-regulate HIV-1 expression in monocyte-derived macrophages (MDMs) infected with R5 HIV-1 [18] and in dendritic cells (DCs) and peripheral blood mononuclear cells (PBMCs) infected with X4 viruses [19, 20]. Particularly in macrophages, the effect of CC chemokines on HIV-1 infection was highly dependent on the cell maturation stage and the time of chemokine addition with respect to virus infection. Moreover, alternative mechanisms of chemokine-mediated modulation of HIV replication involving post-entry steps of the virus life cycle have been reported. For instance, CCL5 increased HIV-1 infection in macrophages independently of CCR5 or any other known receptor, and of the normal route of virus entry. The CCL5-mediated enhancing effect of HIV-1 replication

was related to its tendency to form aggregates at high concentrations [21].

Another important aspect of the relationship between chemokines and HIV is represented by the observation that early interactions between cell surface and HIV external components and accessory proteins can stimulate the production of CC chemokines (i.e., CCL2, CCL3, CCL4, and CCL5) also in the absence of productive infection. Thus, chemokines produced by either infected or bystander uninfected cells triggered by viral products (i.e., gp120, Tat, Nef and p17), may regulate the course of HIV infection by both directly controlling the level of viral infection/replication and through the recruitment of immune cells. This may favor the infection of newly recruited immune cells, thus increasing viral spreading and contributing to AIDS pathogenesis. Overall, chemokines may contribute to different outcomes of HIV infection depending on the balance of their negative *versus* positive effects on viral replication and spreading. CCL2 appears to be unique among the chemokines thus far studied in the context of HIV infection, in that mostly enhancing effects on viral replication and pathogenesis have been reported.

CCL2

CCL2, also known as monocyte chemotactic protein-1 (MCP-1), is the first discovered member of the human CC chemokine family. CCL2 was originally isolated from mouse fibroblasts as identical to the *JE* gene, a platelet-derived growth factor (PDGF) inducible gene [22]. The human version of CCL2 (also called monocyte chemotactic and activating factor, MCAF) was cloned and purified in 1989 from human gliomas and myelomonocytic cells by two independent groups based on its monocyte chemoattractant properties [23, 24].

The human CCL2 gene is located on chromosome 17 and encodes for a 99 amino acid residue precursor protein with a hydrophobic signal peptide of 23 amino acids, whereas the mature peptide is composed of 76 amino acids and has a molecular weight of 13 kDa. The protein sequence displays a 60-70 % homology with other members of the CC family (i.e., CCL4, MCP-3/CCL7 and MCP-2/CCL8). Two regions in the primary structure of CCL2 were identified by mutagenesis as critical for biological activity, the first one from Thr-10 to Tyr-13 and the second one composed of Ser-34 and Lys-35. In addition, mutation of residues 28 and 30, but not of residue 30 alone, was shown to affect cell-type specificity of CCL2 [25].

CCL2, like other chemokines, can be post-translationally modified by specific enzymes [26, 27]. In particular, elimination of its N-terminal glutamate reduced CCL2 activity, while truncation of additional N-terminal residues generated a CCR2 antagonist [25]. Moreover, elimination of N-terminal residues 2–8 resulted in a dominant negative mutant that formed a heterodimer with full length mature CCL2 and abrogated its activity. In addition, different molecular mass forms of CCL2 with reduced chemotactic potency were identified which appear to be caused by O-glycosylation. An additional modification named nitration or nitrosylation was recently discovered on chemokines [28]. During nitration, tyrosine is converted into nitrotyrosine mediated by reactive nitrogen species. Nitrosylation is found in many pathological conditions such as neurodegenerative and cardiovascular diseases [26]. Nitrosylated CCL2 was detected in human prostate and colon carcinomas and hampered T-cell infiltration within the tumor tissues [28].

CCL2 is produced by many types of cells, including endothelial, epithelial, fibroblast, smooth muscle, monocytic, astrocytic, microglial and mesangial cells and DCs either constitutively or after induction by a variety of mediators, comprising growth factors and oxidative stress cytokines [i.e., PDGF, interleukin-1 (IL-1), IL-4, tumor necrosis factor-α (TNF-α), vascular endothelial growth factor (VEGF), lipopolysaccharides (LPS), interferon-γ (IFN-γ)], type I IFN and vitamin D [29-31]. However, monocytes/macrophages are the major source of CCL2 [25].

Many functions have been attributed to CCL2. It is a potent chemoattractor of monocytes, $CD4^+$ T cells and natural killer (NK) cells, thus controlling their recruitment to sites of infection and inflammation. In keeping with its role in leukocyte trafficking, CCL2 is involved in the pathogenesis of various chronic inflammatory conditions associated with monocyte/macrophage infiltration, such as multiple sclerosis (MS), rheumatoid arthritis (RA) and nephropathies [25, 29]. CCL2 is also involved in diabetes and diabetic nephropathy. In fact, a correlation between the level of this chemokine and high glucose concentration was demonstrated in these conditions. In addition, CCL2 is implicated in the disturbed differentiation of intestinal macrophages that occurs in the mucosa of inflammatory bowel disease (IBD) patients. It was also suggested that CCL2 might influence T cell immunity *versus* the development of polarized Th2 responses, thus being implicated in allergic conditions such as asthma, transplant rejections and AIDS [25, 29]. However, a direct role of this chemokine in the generation of Th2 responses was reported only in the mouse model [32]. CCL2 is one of the major inflammatory chemokines implicated in CNS inflammatory processes, increasing brain endothelial permeability and recruiting monocytes/macrophages and activated lymphocytes into the CNS. CCL2 is also involved in bone remodeling. This chemokine is not expressed in normal bone, but it is induced during osseous inflammation and mediates the recruitment of monocytes to areas of bone formation and resorption [29]. CCL2 also plays an important role in the cardiovascular system, both in physiological and pathological conditions. In fact, it was recognized as an angiogenic chemokine because it can induce the migration and sprouting of endothelial cells from aortal rings in the absence of an inflammatory response. Furthermore, CCL2 up-regulates sVEGF expression, increases vascular permeability and participates in VEGF-mediated angiogenesis. It is also implicated in atherosclerosis. In the development of this process, mononuclear phagocytes are primarily involved in the inflammatory processes. CCL2 stimulates the migration of these cells into the sub-endothelial space, where they commence phagocytosis of modified lipoproteins and become lipid-laden foam cells. Upon stimulation, macrophages produce significant amounts of CCL2 in atherosclerotic lesions. In addition, minimally oxidized low-density lipoproteins (LDLs) induce CCL2 production in vascular wall cells; hence this may represent a molecular link between oxidized lipoproteins and foam cell recruitment to the vessel wall [25, 29].

Owing to its involvement in a broad array of pathological processes, CCL2, like other chemokines, has important effects on cancer pathogenesis. Chemokines might work for or against cancer depending on the particular setting in which they are expressed. They can contribute to tumor growth and progression due to their pro-angiogenic properties, but they can also stimulate host antitumor responses due to their capacity to recruit and activate leukocytes. This is indeed the case of CCL2. Its expression in tumor cells significantly correlates with the amount of tumor-associated-macrophages (TAMs) and with the levels of VEGF in breast cancer cells [25]. In prostate cancer, CCL2 induced tumor cells to produce the pro-angiogenic factor VEGF-A which indirectly induced sprout formation in human bone marrow endothelial cells. *In vivo*, administration of neutralizing antibody (Ab) against CCL2 significantly reduced tumor blood vessel density and decreased the prostate cancer tumor burden [33]. However, CCL2 has also antitumor properties because it can increase the cytostatic activity against tumor cells by inducing FAS ligand expression in cultured endometrial stromal cells, thus driving cells to apoptosis [25].

Finally, CCL2 is involved in the pathogenesis of infections with either bacterial or viral pathogens, such as human rhinovirus (HRV), respiratory syncytial virus (RSV), human cytomegalovirus (HCMV), simian human immunodeficiency virus (SHIV), and HIV [34].

CCL2 is among the most studied members of inflammatory chemokines, and its blockade represents a potential point of intervention for the treatment of at least some of the diseases in which it has been implicated.

CCL2 RECEPTORS

The biological activity of CCL2 is mediated through the interaction with CCR2, one of the eleven CC-chemokine receptors characterized by seven trans-membrane domains and coupled to a guanosine triphosphate (GTP)-binding protein. In addition to CCL2, CCR2 can also bind CCL7, CCL8, MCP-5/CCL12 and MCP-4/CCL13 [35]. Unlike CCL2, CCR2 expression is relatively restricted to certain cell types, mainly monocytes, NK and T cells, although it can be induced in other cell types under inflammatory conditions. CCR2 can have either pro-inflammatory or anti-inflammatory roles depending on the cell type where it is expressed [antigen-presenting cells (APCs) and T cells *versus* regulatory T cells, respectively] [25].

Two alternatively spliced forms of CCR2 have been identified, namely CCR2A and CCR2B, which differ in their C-terminal tails. These isoforms are differentially expressed on different cell types. CCR2A is the main isoform expressed by mononuclear and vascular muscle cells, whereas the CCR2B isoform is predominantly expressed by monocytes and activated NK cells. The two isoforms may activate different signaling pathway thus exerting different actions [25].

Upon ligand binding to CCR2, a variety of cellular reactions trigger inositol triphosphate formation, intracellular calcium release, and protein kinase C (PKC) activation. CCL2 binding to CCR2 can also induce the activation of the mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinases 1/2 (ERK1 and ERK2), c-Jun N-terminal kinase 1 (JNK1) and p38, and of Janus kinase 2 (JAK2), phospholipase C (PLC) and the two isoforms of phosphatidylinositol-4, 5-bisphosphate 3-kinase (PI3-K) p85/p110 and C2α [29].

CCR2, as other chemokine receptors, can dimerize, giving rise to homodimers or heterodimers with other chemokine receptors. It represents the first receptor whereby chemokine receptor dimerization has been established. CCR2 homodimerization occurs in the absence of the ligand, but CCL2 dimers can favor CCR2 homodimerization, which may be necessary for chemotactic activity. CCR2 heterodimerization has important functional consequences. In particular, CCR2/CCR5 heterocomplexes activate calcium response and support cell adhesion rather than chemotaxis. CCR2/CXCR4 heterodimers have an allosteric trans-inhibitory effect on CCL2 binding to CCR2. Furthermore, this process is also important from a pharmacological point of view. In fact, the ability of CCR2 to dimerize may influence drug selectivity, because the specific antagonist of one receptor may cross-inhibit the other one [36].

Besides CCR2, CCL2 can bind some members of the family of atypical receptors, namely D6 and the Duffy antigen receptor for chemokines (DARC). These receptors are distinguished from "classical" chemokine receptors in that they are characterized by a greater promiscuity of ligand binding and by a lack of ability to signal following ligand binding. This last property is largely a consequence of a modified or missing canonical DRYLAIV sequence in the second intracellular loop which normally enables G protein coupling [37]. These receptors can act in several ways. In particular, upon binding to D6, the ligand is internalized and targeted for degradation, whereas the receptor is recycled to the plasma membrane. On the contrary, ligand binding to DARC, although resulting in internalization, does not end in degradation of the chemokine. It was shown that DARC can mediate the unidirectional trans-cellular transport of both inflammatory CC and CXC chemokines across the endothelial barrier [3, 37].

CCL2 can also bind viral receptors that act as chemokine receptor homologs, such as US28 from HCMV and UL12 and UL51 from human herpesvirus 6 (HHV-6) [36]. It was demonstrated that US28 accelerates inflammation in the presence of CCL2 [29].

THE CCL2/CCR2 AXIS IN THE PATHOGENESIS OF HIV INFECTION

CCL2, like other inflammatory chemokines, is rapidly expressed following infection to initiate leukocyte migration and activation. The recruitment of inflammatory cells to sites of infection to fight invading microbes is a major responsibility of the innate immune system. Fine tuning of chemokine expression, receptor interaction and subsequent cellular responses, is crucial to optimize protection and minimize deleterious effects of uncontrolled microbial growth and invasion. In animal models, the CCL2/CCR2 axis was shown to be an important mediator of the response to viruses such as murine cytomegalovirus (MCMV), West Nile virus (WNV) and influenza virus, due to its fundamental role in the recruitment of monocytes to sites of infection

[38]. However, in the context of HIV infection this chemokine seems to be more deleterious than protective. This is mostly due to its prominent inflammatory effects and to its role in recruiting leukocytes, thus contributing new targets for infection in a favorable environment for viral replication. In addition, CCL2 might also have direct effects on viral replication, at least in some types of cells.

In the forthcoming part of this review, we will summarize the complex interaction among CCL2/CCR2 and HIV, focusing on the modulation of CCL2 and CCR2 expression in HIV infection, on the mechanisms by which they affect viral replication, on the CCL2/CCR2 polymorphisms associated with the pathogenesis of HIV infection, and on the role this axis plays in at least some of the HIV-related disorders.

Modulation of CCL2 and CCR2 Expression by HIV and Their Effects on Viral Replication

A number of studies described the induction of the expression of CCL2 and CCR2 in the context of HIV infection. Different groups reported an up-regulation of CCL2 transcript levels in HIV-infected cells (Table **1**). In particular, an increase in CCL2 mRNA was described in MDMs and PBMCs infected *in vitro* with different strains of HIV-1 at various time points post-infection [39-42], as well as in astrocytes and human retinal pigment epithelial (HRPE) cells following viral exposure [43, 44]. The up-regulation of CCL2 transcripts was also found *in vivo*, as demonstrated by genome-wide assessments of monocytes and PBMCs obtained from HIV infected individuals [34, 45-49]. Interestingly, in some of these studies the up-regulation of CCL2 transcripts correlated with a high viral load and a low $CD4^+$ T cell count [34, 45, 47, 49]. In addition to CCL2 gene up-regulation, several *in vitro* studies revealed increased levels of the CCL2 protein in cells exposed to HIV (Table **1**). In particular, the effect of HIV-1 infection on CCL2 expression was investigated in different type of cells, mainly macrophages, PBMCs and mucosal tissues [39, 42, 50-53]. In some studies, an enhancement of CCL2 expression was linked to biological effects. Indeed, infection of MDM with HIV-1 resulted in CCL2 secretion increase at various time points post-infection, and the neutralization of this chemokine inhibited viral replication [50]. The same effect was also reported in ectocervical tissue explants [52].

Furthermore, an increased CCR2 expression in PBMCs was linked to leukocyte transmigration [54], whereas the induction of CCL2 release in HRPE cells was associated with the impairment of retinal pigment epithelial barrier function [44]. CCR2 up-regulation was also described in astrocytes [55], whereas modulation of CCL2 levels was reported in hepatic stellate cells (HSCs) and urethral tissues [53, 56].

In addition to infection itself, virus-derived proteins such as gp120 [42, 44, 57-63], Nef [64], matrix protein p17 [65] and transactivator protein Tat [39, 66-83] were shown to induce the expression and release of CCL2 in different cell types (Table **2**). The effect of gp120 exposure on CCL2 expression was mostly investigated in macrophages. In these cells, treatment with gp120 resulted in a strong increase of CCL2 secretion that was independent of viral glycoprotein interaction with the CD4 receptor [59]. Instead, this effect was mediated by co-receptor stimulation. Indeed, gp120-mediated triggering of CCR5 activated a phosphatidylcholine (PC)-specific and phosphoinositide (PI)-specific PLC dependent signaling pathway which resulted in MAPK ERK1/2 and NF-kB activation, ultimately leading to CCL2 expression [57, 58]. The effect of Tat has been mostly investigated in cells of the nervous system, particularly astrocytes, microglia and endothelial cells (Table **2**). Interestingly, enhanced CCL2 expression elicited by cell exposure to HIV-1 proteins correlated with transmigration of monocytes and microglia cells [63, 64, 70, 75, 77, 83] (Table **2**).

Furthermore, several *in vivo* studies revealed increased levels of the CCL2 protein in the cerebrospinal fluid (CSF) and blood of infected individuals [71, 84-99] (Table **1**), and some described the up-regulation of this chemokine in mucosal and renal tissues [100-103]. High levels of CCL2 in the CSF of patients were shown to correlate with a wide spectrum of HIV-related neurological complications [84-87, 89-92] (Table **1**), whereas increased amounts of this chemokine in the blood and cervical-vaginal lavages (CVL) were associated with high viral loads [94, 96, 99, 102].

Contrasting *in vivo* results were reported concerning the effect of antiretroviral regiments on CCL2 expression, with some reports showing reduced amounts of this chemokine following HAART [104], whereas others demonstrating any impact on its levels [89].

Enhanced expression of CCL2 and/or CCR2 in HIV infection may contribute to HIV-related complications in several ways, depending on the body district involved. The known mechanisms underlying the effects of CCL2/CCR2 in the co-morbidities associated with HIV are mostly related to their role in leukocyte recruitment and in sustaining the inflammatory status which represents a hallmark of HIV infection also in the post-HAART era. Some of these mechanisms will be discussed in the forthcoming paragraphs. However, in addition to its role in inflammation and cell migration, CCL2 was also shown to directly affect viral replication, as suggested by studies performed in PBMCs, T lymphocytes and macrophages [50, 105-107]. In particular, Vicenzi and colleagues investigated the effect of CCL2 addition on viral replication in cultures established from $CD8^+$ T cell-depleted PBMCs of HIV-infected individuals that were either co-cultivated with allogeneic T cell blasts (ATCB) of uninfected subjects or directly stimulated by mitogen plus IL-2. They reported that CCL2 stimulated HIV production in most patient cultures and co-cultures characterized by secreting relatively low levels (<20 ng/mL) of CCL2 [105]. They also found a positive correlation between CCL2 levels and HIV replication enhancement in co-cultures. Moreover, $CD14^+$ monocyte depletion from ATCB down-regulated virus replication during co-cultivation with CD8-depleted PBMC of infected subjects, and addition of CCL2 up-regulated HIV production in these CD14-depleted ATCB co-cultures. Overall these results suggest that CCL2 may represent a key factor enhancing HIV spreading, particularly in anatomical sites where infection of macrophages plays a prevalent role. The mechanisms underlying the effect of CCL2 on viral replication seem to be dependent on the cell type. In particular, Campbell and Spector reported that exposure of resting $CD4^+$ T cells to CCL2 resulted in a CCR2-dependent up-

Table 1. Modulation of CCL2/CCR2 expression in HIV-1 infection.

Type of Study	Cell/tissue Type	Virus	Effect	Biological Correlation	References
In vitro	**Macrophages**	HIV-1 BaL	↑ CCL2 release	↑ HIV-1 replication	Fantuzzi *et al.,* [50]
		HIV-1 BaL, primary isolates	↑ CCL2 mRNA and release		Mengozzi *et al.,* [39]
		HIV-1 BaL, BCF03, UG24	↑ CCL2 release		Schwartzkopff *et al.,* [51]
		HIV-1 BaL	↑ CCL2 mRNA		Woelk *et al.,* [40]
		HIV-1 BaL	↑ CCL2 mRNA		Vazquez *et al.,* [41]
	PBMCs	HIV-1 IIIB, JRFL, AdaM, SF162	↑ CCL2 mRNA and release		Wetzel *et al.,* [42]
		HIV-1 Ada, JR-CSF	↑ CCR2	Leukocyte transmigration	Eugenin *et al.,* [54]
	Astrocytes		↑ CCR2b		Cota *et al.,* [55]
		HIV-1 LAI	↑ CCL2 mRNA		Bethel-Brown *et al.,* [43]
	HSCs	HIV-1 IIIB	↑ CCL2 release		Tuyama *et al.,* [56]
	HRPE cells	HIV-1 NL4-3, 92TH014.12	↑ CCL2 mRNA	Impaired retinal pigment epithelial barrier function	Tan *et al.,* [44]
	Ectocervical tissues	HIV-1 CM235	↑ CCL2	↑ HIV-1 transcription	Rollenhagen *et al.,* [52]
	Urethral tissues	HIV-1 V29 infected PBMCs	bifasic modulation of CCL2 release		Ganor *et al.,* [53]
In vivo	**CSF brain tissues**		↑ CCL2		Conant *et al.,* [71]
			↑ CCL2	CMV encephalitis	Bernasconi *et al.,* [84]
			↑ CCL2	Encephalitis, high CSF HIV-1 RNA levels	Cinque *et al.,* [85]
			↑ CCL2	HAD, CSF viral load, dementia severity	Kelder *et al.,* [86]
			↑ CCL2	Cryptococcal meningitis	Christo *et al.,* [87]
			↑ CCL2	$CD4^+$ T cell counts <200 cells/μL	Spudich *et al.,* [88]
			↑ CCL2	Neurocognitive impairement	Yuan *et al.,* [89]
			↑ ratio CCL2/CXCL10	Cyptococcosis-associated IRIS	Chang *et al.,* [90]
			↑ CCL2	Neuropathology	Spitsin *et al.,* [91]
			↑ CCL2	PN	Wang *et al.,* [92]
	Plasma		↑ CCL2	Pneumococcal disease, survival	Carrol *et al.,* [93]

(Table 1) contd….

Type of Study	Cell/tissue Type	Virus	Effect	Biological Correlation	References
In vivo	**Plasma**		↑ CCL2	↑ viral load ↓ $CD4^+$ T cell count Association with higher thoracic aorta VWA and VWT	Floris-Moore *et al.,* [99]
			↑ CCL2	High viral load	Weiss *et al.,* [94]
			↑ CCL2	Correlation with CIMT values	Joven *et al.,* [95]
			↑ CCL2	High viral load	Miller *et al.,* [96]
			↑ CCL2	↓ HDL	Aragones *et al.,* [97]
			↑ CCL2		Chew *et al.,* [98]
	Jejunal biopsies		↑ CCL2		Wang *et al.,* [100]
	Duodenal tissues Blood		↑ CCL2 mucosal secretion ↑CCR2 on blood monocytes		Allers *et al.,* [101]
	CVLs		↑ CCL2	High GTVL	Mukura *et al.,* [102]
	Renal tissues		↑ CCL2		Kimmel *et al.,* [103]
	Monocytes		↑ CCL2 mRNA	High viral load	Pulliam *et al.,* [45]
			↑ CCL2 mRNA		Giri *et al.,* [46]
			↑ CCL2 mRNA	High viral load Low $CD4^+$ T cell count	Van den Bergh *et al.,* [47]
			↑ CCL2 mRNA		Gekonge *et al.,* [48]
	PBMCs		↑ CCL2 mRNA	High viral load	Duskova *et al.,* [49]
			↑ CCL2 mRNA ↑ CCL2 in serum	High viral load	Ansari *et al.,* [34]

The table reports the studies performed in humans or in cells of human origin. Abbreviations: PBMCs, peripheral blood mononuclear cells; HSCs, hepatic stellate cells; HRPE, human retinal pigment epithelial; CSF, cerebrospinal fluid; CMV, cytomegalovirus; HAD, HIV-associated dementia; IRIS, immune reconstitution inflammatory syndrome; PN, pheriperal neuropathy; VWA, Minimal, maximal and mean vessel wall thickness; VWT, vessel wall area; CIMT, carotid intima-media thickness; HDL, high-density lipoprotein; CVLs, cervical-vaginal lavages; GTVL, genital tract viral load.

Table 2. Induction of CCL2 expression by HIV-1 proteins.

Stimulus	Cell Type	Signaling Pathway Involved	Biological Effect	References
gp120	**Macrophages**	CCR5/PC- and PI-PLCs/ERK1/2		Spadaro *et al.,* [57]
		CCR5/PC-PLC/NF-kB		Fantuzzi *et al.,* [58]
		CD4 independent		Fantuzzi *et al.,* [59]
		MAPK p38		Del Cornò *et al.,* [60]
	PDCs			Del Cornò *et al.,* [61]
	PBMCs			Wetzel *et al.,* [42]
	HSCs	CCR5/MAPK p38/NF-kB		Bruno *et al.,* [62]
	HRPE cells		Impaired retinal pigment epithelial barrier function	Tan *et al.,* [44]
	Renal proximal tubular cells		Monocyte migration	Kapasi *et al.,* [63]

(Table 2) contd….

Stimulus	Cell Type	Signaling Pathway Involved	Biological Effect	References
Nef	**Astrocytes**	Myristoylation-/ calmodulin-dependent	Monocyte chemotaxis	Lehmann *et al.,* [64]
p17	**Monocytes**	AP-1		Marini *et al.,* [65]
Tat	**Astrocytes**	P2X7R/ERK1/2	Neuronal death	Tewari *et al.,* [66]
		CDK9		Khiati *et al.,* [67]
		C/EBPbeta		Abraham *et al.,* [68]
		MEK1/2		Kutsch *et al.,* [69]
			Monocyte transmigration	Weiss *et al.,* [70]
				Conant *et al.,* [71]
	Brain endothelial cells	Inhibition by PPARα/ PPARγ		Huang *et al.,* [72]
	Astrocytic/monocytoid cells			Boven *et al.,* [73]
	Monocytes	Subtype B specific, TNF-α dependent		Campbell *et al.,* [74]
	Astrocytic cells	MH2 domain of Smad3	PBMC transmigration	Eldeen *et al.,* [75]
		SP1/AP1/NF-kB		Lim *et al et al.,* [76]
	Microglia		Microglia migration	Eugenin *et al.,* [77]
		PI3K/ERK1/2		D'Aversa *et al.,* [78]
				McManus *et al.,* [79]
	Glial cells	Smad-3/Smad-4		Abraham *et al.,* [80]
	Macrophages			Mengozzi *et al.,* [39]
	KS cells			Kelly *et al.,* [81]
	Endothelial cells			Lee *et al.,* [82]
		PKC/PI3K	Monocyte transmigration	Park *et al.,* [83]

The table reports the studies performed in cells of human origin. Abbreviations: PDCs, plasmacytoid dendritic cells; PBMCs, peripheral blood mononuclear cells; HSCs, hepatic stellate cells; HRPE, human retinal pigment epithelial; KS, Kaposi sarcoma; PC-PLC, phosphatidylcholine-specific-phospholipases C; PI-PLC, phosphoinositide-specific- phospholipases C; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; P2X7R, P2X purinoceptor 7; CDK9, Cyclin-dependent kinase 9; C/EBPbeta, CCAAT-enhancer-binding protein beta; MEK, MAPK/ERK kinase; PPAR, peroxisome proliferator-activated receptor; Smad, small mother against decapentaplegic; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; PKC, protein kinase C.

regulation of CXCR4 expression which increased the ability of these cells to be chemoattracted by gp120 and rendered them more permissive to X4 HIV-1 infection [106]. These results suggest that CCL2 has the capacity to render a large population of lymphocytes more susceptible to HIV-1 in the course of infection, and this effect might be particularly relevant in late stages of the disease when both X4 viruses and high levels of CCL2 are present. In addition, our group investigated the effect of blocking the high levels of CCL2 endogenously expressed by macrophages, either constitutively or following infection with HIV-1. We reported that infection of MDMs with HIV-1 in the presence of anti-CCL2 Ab resulted in a potent inhibition of p24 Gag release with respect to control cells, in the intracellular accumulation of this viral antigen and in marked changes in cell size and morphology [50]. These results suggest that CCL2 might represent an autocrine factor critical for enhancing virion production possibly by affecting the macrophage cytoskeleton. Furthermore, in a very recent study we showed that additional, early post-entry steps of the HIV-1 life cycle are also impaired upon CCL2 blocking in macrophages [107]. In fact, we found that neutralization of this chemokine potently reduced the proportion of p24 Gag^{+} cells, without affecting HIV-1 entry and reverse transcriptase activity. Conversely, we found that CCL2 blocking potently limited the accumulation of viral DNA. Furthermore, we showed that CCL2 neutralization resulted in the up-modulation of several genes involved in the defense response to viruses, among which apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3 A (APOBEC3A). Thus, endogenous CCL2 may represent an autocrine factor acting as a negative regulator of the expression of innate intracellular viral antagonists and its targeting may represent a novel therapeutic strategy for enhancing cellular defenses to thwart HIV-1 infection. Finally,

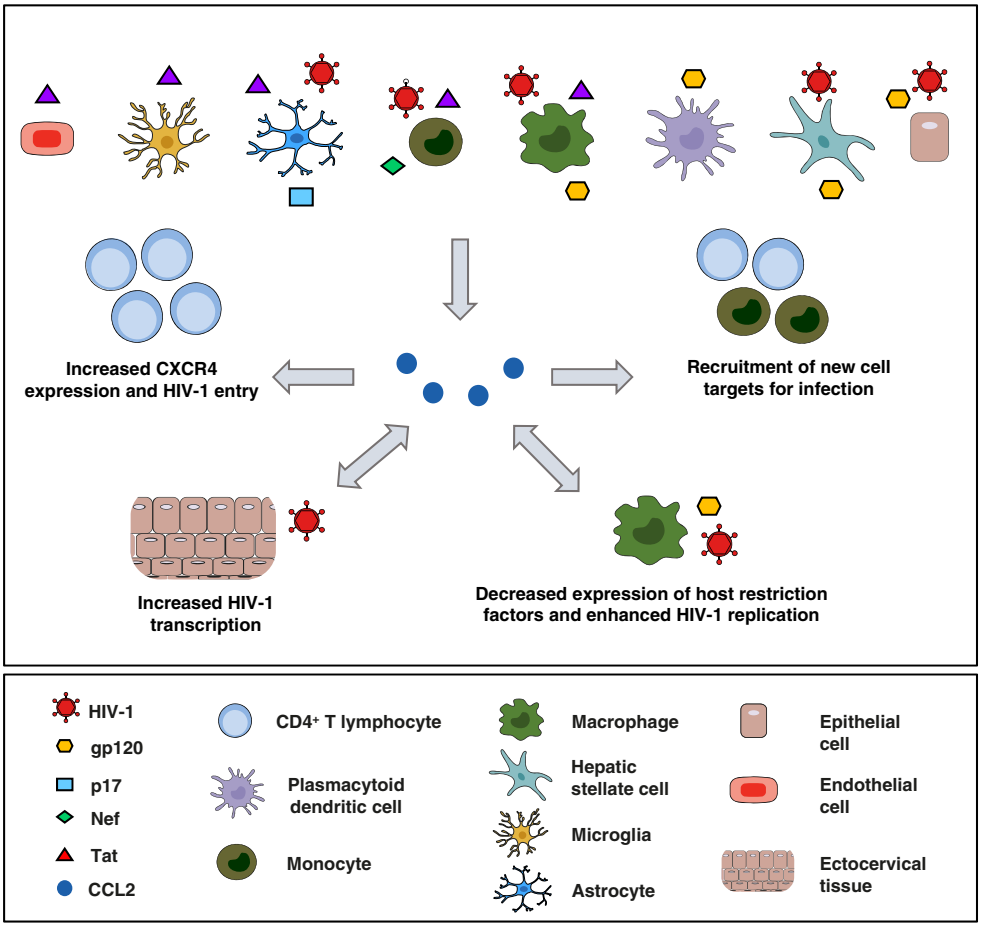

Fig. (1). Model for CCL2-driven mechanisms of enhanced HIV-1 replication. Exposure to HIV-1 or viral proteins upregulate CCL2 expression in several types of cells, particularly monocytes/macrophages, astrocytes and epithelial cells. The resulting high levels of CCL2 affect HIV-1 replication in different ways. It recruits new cell targets for infection and increases CXCR4 expression on resting $CD4^+$ T lymphocytes rendering them susceptible to infection with X4 viruses. In macrophages, CCL2 acts as an autocrine factor negatively regulating host restriction factor expression thus enhancing HIV-1 replication. In ectocervical tissue cells CCL2 increases HIV-1 transcription. Overall, these effects enhance viral replication and may contribute to increasing viral load *in vivo* in HIV-1 infected individuals.

neutralization of CCL2 in HIV-1 infected ectocervical tissues from post-menopausal women resulted in decreased viral transcription, suggesting that this chemokine may contribute to enhance HIV-1 replication in mucosal tissues at least in these conditions [52]. The mechanisms by which CCL2 may directly affect HIV replication are summarized in the model depicted in (Fig. **1**).

CCL2/CCR2 Polymorphisms in the Pathogenesis of HIV Infection

A growing body of literature suggests that CCL2/CCR2 allelic polymorphisms affecting their expression or function may impact on the course of a wide spectrum of inflammatory diseases [108].

CCL2 Polymorphisms

Several single nucleotide polymorphisms (SNPs) were described in the CCL2 gene and showed to delineate a number of common haplotypes. Some of these SNPs, namely -2136T, -2518G, -2835A and -764G, were associated with increased CCL2 production at both mRNA and protein levels [109].

CCL2 genetic polymorphisms were shown to affect individual susceptibility to HIV infection, disease progression, and even the presence of HIV-associated manifestations [110], though the mechanisms are not yet completely known and many works are conflicting (Table **3**).

The CCL2 polymorphism -2518 A/G **(**alternatively designed -2578**)** was extensively investigated in HIV infected patients. This genetic variant was associated with faster progression to AIDS and neuropsychological impairment/HIV-associated dementia (HAD) in HIV seropositive subjects in some studies [95, 111-119], but not in others [118, 120-125] (Table **3**). In particular, Gonzalez and colleagues reported that, in adults homozygous for the CCL2-2578G allele, this polymorphism was associated with a 50% reduction of the risk of acquiring HIV. They also showed that once HIV infection is established, the CCL2-2578G allele was associated with accelerated disease progression and a 4.5-fold increased

Table 3. Effects of CCL2 polymorphisms in HIV infection.

Polymorphism	Cohort	Findings	References
2518 A/G	240 HIV+ (78 with CMV end-organ disease). Controls: CMV+ without CMV end-organ disease. From USA	Association with-lower nadir $CD4^+$ T cell counts	Affandi *et al.,* [111]
	183 HIV+ ±atherosclerosis. Controls: 348 unrelated subjects	Increased risk for atherosclerosis	Alonso-Villaverde *et al.,* [112]
	129 HIV+ (43 with LD)	Association with carotid atherosclerosis	Coll *et al.,* [113]
	164 HIV+ (Caucasian)	Association with higher mean $CD4^+$ T cell count; higher response to therapy in CCL2/CCR2 double mutant patients	Coll *et al.,* [119]
	1,115 HIV+ adults, 592 children perinatally exposed to HIV (322 HIV+, 270 HIV-), 1,035 control blood donors	Homozygosity associated with reduced risk of infection; association with disease progression and increased risk of HAD in established infection	Gonzalez *et al.,* [118]
	226 HIV+, 384 HC (Caucasian)	Absence of the G allele more frequent in patients with serum HIV RNA <200 copies/mL	Joven *et al.,* [95]
	98 USA HIV+	CSF CCL2 levels highest in G/G, intermediate in G/A, lowest in A/A	Letendre *et al.,* [114]
	Repository CSF specimens: 27 perinatally HIV+ children with HAE, 13 HIV- controls	Association with high HIV DNA levels and high CCL2 concentration in CSF	Shiramizu *et al.,* [115]
	263 HIV+ patients (112 with TB). Controls: 155 HIV- PTB patients and 206 HC	No differences in genotype frequencies	Alagarasu *et al.,* [122]
	143 HIV+ patients (National NeuroAIDS Tissue Consortium) 26 with HAD	No contribution to risk for HAD	Levine *et al.,* [121]
	181 HIV+; Controls: 568 HIV- high risk population	No significant survival benefit to seroconversion and disease progression	Roy and Chakrabarti, [123]
	1059 USA HIV-1+ children	No association with disease progression or CNS impairments	Singh *et al.,* [125]
	121 HIV-1+ adults	No association with disease progression and neuropsychological impairment	Singh *et al.,* [124]
	318 individuals (73 LTNP, 109 HIV+ UP, 36 EU, 100 HC), from Europe (white)	No differences in genotype frequencies between groups; higher prevalence of GG genotype in HIV-1+ compared to EU	Viladés *et al.,* [116]
	183 HIV- and 227 HIV+ adolescents from the REACH project; 173 HIV+ adults from the HERO study. From USA	No association with $CD4^+$ T counts and plasma viral load	Wang *et al.,* [120]

(Table 3) contd….

Polymorphism	Cohort	Findings	References
2578 A/G **2835 C/A** **2136 A/T** **1811 A/G** **927 G/C** **3726 T/C**	287 HIV+ cases, 796 HC	No differences in genotype frequencies; no association with plasma CCL2 levels in HC; higher plasma CCL2 levels in HIV+ 2835 AA, 2578 GG, 1811 AG (H1, H2, H5 haplotypes)	Alonso-Villaverde *et al.,* [117]
2136 A/T **767 C/G**	2012 European-Americans (EA: 534 seronegatives, 878 seroprevalents, 600 seroconverters) and 1052 African-Americans (AA: 249 seronegatives, 579 seroprevalents, 224 seroconverters), from 5 USA cohorts	High frequencies of mutants and H7 haplotype in uninfected EA repeatedly exposed to HIV-1; linkage disequilibrium with a SNP in eotaxin	Modi *et al.,* [127]
rs7210316 **rs8068314** **rs4795893** **rs1860190** **rs1860189** **rs2857654** **rs3917884** **rs1024610** **rs3917886** **rs11575010** **rs2857656** **rs4586** **rs13900** **rs3917890**	567 HIV serodiscordant couples, from Africa	No influence on early events of HIV-1 infection	Hu *et al.,* [126]

Abbreviations: CMV, Cytomegalovirus; LD, lipodystrophy; CSF, cerebrospinal fluid; HAD, HIV-associated dementia; HC, healthy controls; HAE, HIV-1-associated encephalopathy; TB, tuberculosis; PTB, pulmonary tuberculosis; CNS, central nervous system; LTNP, long-term non-progressors; UP, usual progressors; EU, exposed uninfected, 2518 is alternatively designed as 2578.

risk of HAD. Thus, they suggest a dual role for CCL2 expression in HIV infection: it ensured partial protection from viral infection, while once the infection occurred, its pro-inflammatory properties and ability to stimulate HIV replication may contribute to accelerate disease progression and increase the risk of HAD [118]. The variant homozygous CCL2-2518 GG genotype was over represented in HIV-infected subjects from a white Spaniards cohort [116], and the CCL2-2518G allele conferred an increased risk for atherosclerosis to HIV-infected patients [112, 113]. In patients with serum HIV RNA below 200 copies/mL, the absence of the CCL2-2518G allele was significantly more frequent than its presence [95], and Caucasian patients carrying CCL2-2518G showed significantly lower nadir $CD4^+$ T cell counts [111]. Furthermore, in HIV seropositive perinatal children with HIV-associated encephalopathy (HAE) the CCL2-2578G allele was associated with high HIV DNA amounts and high CCL2 levels in CSF compared to the wild type allele [115]. The CCL2-2578 variant was associated with elevated CCL2 levels in the CSF of HIV seropositive adults [114]. Furthermore, plasma CCL2 concentrations were significantly higher in carriers of the CCL2-2835 AA and CCL2-1811 AG genotypes in HIV positive subjects, but not in the control population [117].

On the contrary, Coll and colleagues showed that patients on antiretroviral therapy carrying the mutated CCL2-2518G allele had a higher mean $CD4^+$ T cell count throughout the follow-up period with respect to those with the common allele. Also, patients with both the CCL2-2518G and CCR2-64I mutated alleles most likely maintained an undetectable viral load [119]. Moreover, the CCL2-2578G and CCL2-2136T alleles were not associated with HIV-related virological/immunological outcomes [120], and CCL2-2578G was not differently expressed between healthy subjects and HIV-infected patients with or without tuberculosis [122]. Finally, the analysis of fourteen CCL2 SNPs in a large prospective cohort of HIV serodiscordant couples showed no association with both HIV acquisition and viral load in the high-risk study population [126].

The frequencies of the CCL2-2136T, CCL2-767G (alternative name of 764) and CCL2-1385A variants were significantly elevated in exposed uninfected (EU) European-American subjects repeatedly exposed to HIV through high-risk sexual behavior or contaminated blood products. Interestingly, frequencies of the CCL2-2136T and CCL2-767G variants, together with the CCL11-1385A mutation, were clustered and significantly elevated in EU individuals [127].

Several transcription factors, including interferon regulatory factor 1 (IRF-1), poly [ADP-ribose] polymerase 1 (PARP-1), signal transducers and activators of transcription 1 (STAT-1) and Pre-B cell leukemia transcription factor (Pbx)-regulating protein-1 (Prep1)/Pbx complexes, were

shown to bind differentially to various CCL2 allele [118, 128-130]. Interestingly, polymorphisms in Prep1 were recently associated with HAD development [131]. Since Prep1 preferentially binds the CCL2-2518G allele, these results support previous findings suggesting a key role of CCL2 in the onset of HIV-associated neurocognitive disorders (HANDs).

CCR2 Polymorphisms

Several biallelic SNPs in the CCR2 gene were studied in relationship with various disease susceptibility or severity [108]. The most studied CCR2 mutation is a dGT to dAT polymorphism in the coding region at nucleotide position 190 which leads to expression of an isoleucine instead of a valine at amino acid position 64 (designated as CCR2-V64I). Such allele was shown to be polymorphic in almost all populations examined, possibly because this mutation is ancient, occurring before the out of-Africa migrations of modern humans [132]. The CCR2-V64I polymorphism occurs at an allele frequency of up to 36%, depending on the ethnic group of the studied population [133].

The CCR2-V64I genetic variant was associated with a protective role against HIV infection and a slower disease progression in some cross sectional and prospective studies [119, 133-147] but not in others [16, 116, 122-124, 148-160] (Table **4**). In particular, in a HIV-exposed Zimbabwean pediatric population the frequency of this allele was significantly higher in uninfected compared to infected children, thus suggesting a possible protective role for this genotype against HIV infection [147]. Fang and colleagues found an intriguing association between CCR2-V64I and HLA B58 in a cohort of long-term survivors (LTSs) HIV-infected sex worker women with high viremia in Nairobi [143]. HLA B58 is normally expressed in 1% of Caucasians, while it is highly represented in Africans [161] and it was associated with significantly slower HIV disease progression, lower mortality rate and cytotoxic T lymphocyte (CTL)-driven attenuation of HIV [162]. The close association between the CCR2 mutation and HLA B58, significant in HIV-infected but not in HIV-seronegative women, may suggest that the V64I allele could affect HIV infection almost in part through the HLA B58 haplotype. To our knowledge, this is the only association between CCR2-V64I and HLA B58 haplotype. Furthermore, patients carrying both the CCL2-2518 and CCR2-V64I alleles had a better response to a protease inhibitor (PI)-based regimen [119]. The CCR2-V64I allele was also associated with lower levels of viremia in early chronic HIV infection [145], which is a strong determinant of prognosis [163]. In a wider meta analysis the same authors showed that the CCR2-V64I variant was associated with a decreased risk for progression to AIDS and death, and lower HIV RNA levels after seroconversion. However, this allele was not associated with a clear protective effect on the risk for death after development of AIDS [144]. A significant favorable association between CCR2-V64I homozygosity and $CD4^+$ T cell count was also found in HIV-infected subjects in Thailand [142].

Conversely, the presence of the CCR2-V64I mutation was reported to represent a risk factor for heterosexual transmission of HIV in women in Scotland [154]. This allele was also found to affect neuropsychological impairment in HIV-infected adults. However, this effect was not associated with increases in plasma or CSF viral load, or $CD4^+$ T cell count, suggesting that it may be linked to immune/inflammatory responses against the virus within the CNS rather than impacting on viral entry or replication directly [124]. Conversely, no association of the CCR2-V64I genotype with either HIV-related neuropsychological impairment or disease progression was found in children with symptomatic HIV infection [157, 159]. Moreover, the children homozygous for the CCR2B-V64I allele and the heterozygous children were equally distributed in the infected and uninfected populations [159]. Although the reasons for the discrepancies in the involvement of this CCR2 polymorphism in CNS impairment between adults and children are unknown, they may reflect different pathogenic mechanisms of HIV-related neurological disease in adults compared to children. In addition, a number of studies reported a lack of association between the CCR2-V64I allele and host resistance to HIV infection [16, 152, 153, 158, 160]. No significant survival benefit regarding seroconversion rates, disease progression or death was also associated to mutant genotypes of CCR2 and CCL2 in Indian patients [123]. Moreover, no significant difference in the expression of CCR2-V64I was found between healthy subjects and HIV-infected patients with or without tuberculosis in an Indian cohort [122]. Finally, the CCR2-V64I allele was expressed at similar frequencies in both progressors and long-term non progressors (LTNPs) patients [156].

The mechanisms by which the CCR2-V64I polymorphism affects the course of HIV infection are not clear and are difficult to elucidate. Since relatively few HIV-1 strains can use CCR2 as co-receptor *in vitro* [164], the mechanisms underlying the reported effects are probably unrelated to viral entry. In keeping with this, the V64I residue is located in a trans-membrane region not associated with any known protein binding site, both allele are expressed at similar levels on human PBMCs, they function equally well as HIV-1 co-receptors in *in vitro* studies, and CCR2-V64I PBMCs are permissive for HIV-1 infection regardless of viral tropism *in vitro* [164]. Since it was suggested that chemokine-receptor dimerization may help to prevent HIV-1 infection [165], the possible protective effect of CCR2-V64I could be due to its ability to heterodimerize with the CCR5 and/or CXCR4 receptors, thus reducing their cellular expression. In this regard, it was reported that CXCR4 can dimerize with the CCR2-V64I mutant, but not with wild-type CCR2, thus indicating that Val 64 may be critical for dimer stabilization [164, 166]. Furthermore, it was suggested that the CCR2-V64I mutation tracks, through linkage disequilibrium, with other mutations, particularly in the regulatory or promoter region of CCR5, and that population-specific variants/haplotypes in the genes encoding CCR2 and CCR5 show patterns that may contribute to explain disparities in infection or disease progression [146, 155, 167].

Taken together these CCL2/CCR2 genotype/phenotype association studies result in confusing and conflicting data. These inconsistencies can be attributed at least in part to methodological issues and cohort features. For example, it has been proposed that the protective effect of the CCR2-

Table 4. Effects of CCR2V64I mutation in *in vivo* studies.

Finding	Cohort	Correlates	References
Protective effect	132 HIV+, from Europe (white)	$CD4^+$ T cell counts	Easterbrook *et al.,* [138]
	221 HIV+, from Asia	$CD4^+$ T cell counts	Ammaranond *et al.,* [142]
	6 HIV+ LTS (non-subtype B), from Africa	Association with HLA-B58	Fang *et al.,* [143]
	19 prospective cohort studies and case-control studies from USA, Europe, and Australia	Disease progression, viral load. No protective effect on the risk for death after developing AIDS	Ioannidis *et al.,* [144]
	666 HIV+, 287 HIV-1-, 35 EU, from USA	Disease progression	Kostrikis *et al.,* [135]
	22 HIV discordant couples, 156 HIV-1-NBD, from Asia	Transmission of infection	Louisirirotchanakul *et al.,* [139]
	567 HIV discordant couples, from Africa	Viral load	Malhotra *et al.,* [146]
	34 HIV+ and 36 EU children, 36 HIV-, from Africa	Genotype frequencies	Mhandire *et al.,* [147]
	10 cohorts of HIV-1 seroconverters of European (n=1,635) or African (n=215) ancestry, from USA, Europe, and Australia	Disease progression	Mulherin *et al.,* [134]
	32 HIV+, 147 HIV-, from Africa	Resistance/susceptibility to infection	Nkenfou *et al.,* [141]
	3,003 individuals (5 prospective AIDS cohorts)	Disease progression. No influence on the incidence of infection	Smith *et al.,* [136]
	364 HIV+, from Europe (Caucasian)	Syncytium-inducing variants	van Rij *et al.,* [137]
	57 HIV+, 70 EU, 112 HC, from USA	Resistance/susceptibility to infection	Zapata *et al.,* [140]
	3034 HIV+ (10 adult/adolescent cohorts)	Disease progression, viral load	Ioannidis *et al.,* [145]
No effect	161 HIV+, 99 HIV-, from Africa	Viral load, immune activation markers, risk of infection	Adjé *et al.,* [149]
	263 HIV+ TB±, 155 HIV- TB+, 206 HC, from Asia	Resistance/susceptibility to infection ± TB	Alagarasu *et al.*, [122]
	164 HIV+, from Europe	$CD4^+$ T cell counts, viral load. Association with probability to maintain undetectable viral load following PI-treatment in patients bearing both CCL2-2518G and CCR2-64I	Coll *et al.,* [119]
	215 HIV- (EU and NBD), from Europe	Disease progression, overall survival, $CD4^+$ T cell counts. No differences in genotype frequencies	Eugen-Olsen *et al.,* [150]
	180 HIV+, 221 HC, from Asia	Susceptibility to infection	Kaur *et al.,* [152]
	287 HIV+, 388 HC, 49 IDU HIV EU, from Asia	Resistance to infection	Li *et al.,* [153]
	316 HIV+, 94 EU, 425 HIV- from USA	Resistance to infection	Liu *et al.,* [16]
	91 HIV+, 91 HC, from Asia	Rate of infection, viral load	Lu *et al.,* [155]
	395 HIV+ (255 AIDS progressors, 140 non progressors, 20 LTNP), 16 high risk HIV- (San Francisco men's Health Study)	Transmission of infection, disease progression	Michael *et al.,* [151]
	70 LTNP (French ALT), 83 progressors (IMMUNOCO), from Europe	Genotype frequencies	Magierowska *et al.,* [156]

(Table 4) contd….

Finding	Cohort	Correlates	References
	108 IDU seroconverters, from Europe	Disease progression, $CD4^+$ T cell counts, virus load	Schinkel *et al.,* [148]
	181 HIV+, 568 HIV-, from Asia	Seroconversion rates, disease progression	Roy and Chakrabarti, [123]
	1049 HIV+ children, from USA	Disease progression	Singh *et al.,* [157]
	121 HIV+ adults	Disease progression, neuropsychological impairment	Singh *et al.,* [124]
	35 EU partners of HIV+ individuals, 75 HC and 50 HIV+ controls, from Asia	Susceptibility to infection	Suresh *et al.,* [158]
	376 HIV+ and 369 HIV- infants born to HIV-1+ mothers (220 from Africa)	Genotype frequencies, disease progression	Teglas *et al.,* [159]
	73 LTNP, 109 HIV+ UP, 36 EU, 100 HC, from Europe (white)	Genotype frequencies	Viladés *et al.,* [116]
	330 HIV+, 474 HIV- (215 IDU, 259 with sexually transmitted diseases), 3,165 HC, from Asia	Transmission of infection	Wang *et al.,* [160]
Risk factor	144 (HIV+ and EU) and 57 HIV+ index partners, 50 controls with polycystic kidney disease, from Europe	Susceptibility to infection	Lockett *et al.,* [154]

Abbreviations: LTS, long-term survivors; ; EU, exposed uninfected; NBD, normal blood donors; HC, healthy controls; TB, tuberculosis; PI, protease inhibitor; LTNP, long-term non-progressors; IDU, intravenous drug users; UP, usual progressors.

V64I mutation could be masked if seroincident, rather than seroprevalent, cohorts are studied [168]. Moreover, at least in some studies, any beneficial effect of CCL2/CCR2 mutations could be masked by a high immune activation status observed in subjects included in the cohort [149], as it may increase the number of activated $CD4^+$ T cells susceptible to HIV infection and then plasma HIV viral load [169]. In addition, the impact of CCL2/CCR2 polymorphisms on HIV disease may be different in CNS and non-CNS sites, with mechanisms affecting the course of viral infection in a very complex and context-dependent manner.

CCL2/CCR2 and HIV-associated Neurologic Disorders

HIV can cause a wide range of neurocognitive complications grouped under the name of HANDs. Depending on the degree of disability, three categories have been defined, namely (i) asymptomatic neurocognitive impairment (ANI), (ii) HIV-associated mild neurocognitive disorder (MND) and (iii) HAD, which represents the most severe form. HANDs can occur when HIV enters the nervous system and impairs the activity of nerves involved in cognitive functions. Symptoms of HANDs include confusion and forgetfulness, behavioral changes, pain due to nerve damage. HANDs remain a mounting problem, despite successful HAART, as seropositive individuals live longer. Furthermore, while current therapy reduces peripheral virus, it does not limit the low level, chronic neuroinflammation that is ongoing during the neuropathogenesis of HIV infection. Thus, several HIV-infected individuals develop HAND that continues to be a major public health issue [170].

HIV-encephalitis (HIVE) represents the main HAND substrate. It is a metabolic encephalopathy induced by viral infection and fueled by activation of brain mononuclear phagocytes, mainly perivascular and parenchymal macrophages and microglia. These cells serve as reservoirs for persistent infection and sources of soluble neurotoxins. The role of CCL2 in the encephalopathy caused by HIV is well documented [171, 172]. This encephalopathy results from intense infiltration of mononuclear cells, productive replication of the virus in monocyte-derived macrophages/microglia, abortive replication in astrocytes and activation of macrophages/microglia and astrocytes leading to neuronal degeneration in the brain of HIV infected persons. HIV-1 clade-specific differences in the induction of neuropathogenesis were described in the severe combined immune deficiency (SCID) mouse HIV encephalitis model with intracranial injection of macrophages infected with representative clade B (ADA) or clade C (Indie-C1) HIV-1 isolates. In fact, differences were found in a key characteristic of HIV-1 that influences HAD, namely increased monocyte infiltration. In particular, macrophages infected with HIV-1(Indie-C1) recruited monocytes poorly compared to those infected with HIV-1(ADA). Interestingly, monocyte recruitment was shown to be CCL2 dependent [173].

Several studies reported specific up-regulation of CCL2 in CSF and brain of patients with HAD [71, 85, 86], as well as in a murine model of HIVE [174] and in macaques with Simian Immunodeficiency Virus (SIV) encephalitis (SIVE) [175], but not in the brain tissue of patients without cognitive deficits [71]. On the contrary, Kamat and colleagues described elevated CCL2 levels in patients on suppressive

HAART even in the absence of clinical manifestations [176]. Interestingly, a close temporal relationship was found between the elevation of CCL2 and the rebound of viral load in the CSF of patients following interruption of antiretroviral therapy, suggesting that these parameters are co-regulated or that one is a stimulus for the other [177]. Higher CCL2 levels in the CSF were also found in HIV patients with $CD4^+$ cell counts below 500 cells/mm^3 with respect to those with $CD4^+$ cell counts above 500 cells/mm^3 [178], while median CSF CCL2 levels were comparable in "elite" viral controllers with respect to that in uninfected subjects and HIV-infected patients on HAART [179]. In a cohort with advanced HIV infection, CCL2 amounts in CSF tended to be associated with time to HAD [180], while plasma CCL2 levels tended to be associated with time to death [181]. High CCL2 levels in the CSF appeared mostly due to infiltration of monocytes across the blood brain barrier (BBB) [45] and, in turn, CCL2 was suggested to be responsible for triggering the CSF pleocytosis occurring in patients who experienced large increases in viral load after interrupting antiretroviral therapy [182]. Indeed, HIV-1 infection of leukocytes results in their increased transmigration across the BBB in response to CCL2, as well as in BBB disruption. In particular, it was reported that CCL2 affected macrophage migratory movement through regulation of voltage gated K^+ channels [183]. Exposure to CCL2 or Tat, which triggers CCL2 expression, also induced the actin rearrangement and the formation of microglial processes, thus facilitating microglia migration [77]. In an *in vitro* model of BBB (co-culture of endothelial cells and astrocytes), Tat-mediated release of CCL2 by astrocytes induced the transmigration of peripheral blood monocytes and lymphocytes [184]. This CCL2-dependent transmigration involved only HIV-infected cells and was associated with BBB disruption, as demonstrated by its greater permeability, the reduced number of tight junctions and the production of metalloproteases. These results suggest that CCL2 is essential for virus entry into the CNS. Interestingly, in the absence of CCL2, but not of other chemokines (i.e. CXCL10, CCL3 or CCL5), HIV-infected leukocytes did not transmigrate *in vitro*, nor did this condition alter BBB integrity and permeability. The enhanced capacity of HIV-infected leukocytes to transmigrate in response to CCL2 correlated with their augmented expression of CCR2 in an *in vitro* tissue culture model of human BBB [54]. Interestingly, CCR2 was significantly increased on $CD14^+CD16^+$ monocytes in individuals with HANDs compared to infected people with normal cognition, and CCR2 expression on this monocyte subset was proposed as a novel peripheral blood biomarker of HANDs [185].

Perivascular astrocytes were identified as the main CCL2 producers in the CNS in a variety of neuroinflammatory conditions, and CCR2 was reported to be widely expressed by resident immune cells, such as microglia [186-188]. An up-regulation of CCL2 expression was observed in the vascular endothelium of mouse brain injected with Tat *in vivo* [189], while *in vitro*, Tat significantly increased CCL2 expression and release in human fetal astrocytes [71], microglia [78] and microvascular endothelial cells [83]. Gene array analysis revealed a remarkable increase in CCL2 gene transcription in astroglial cells isolated from HIV-1-infected-U937/astrocytes co-cultures compared with those from uninfected co-cultures [190]. Furthermore, an *ex vivo* study in slices of rat hippocampus, a structure damaged in HAD, showed that Tat induced CCL2 production in a time- and dose-dependent manner and that this effect was associated with an increase in TNF-α levels [191].

A role for the CCL2/CCR2 axis was proposed in HAD worsening following abuse of cocaine, which is often assumed by HIV-infected patients. Dhillon and colleague in fact showed that THP-1 cells infected *in vitro* with X4 HIV-1 and exposed to cocaine expressed higher CCR2 mRNA levels and secreted higher amounts of CCL2 respect to infected untreated control cells [192]. Cocaine also enhanced CCL2 secretion in *in vitro* models of human monocytes [193] and rodent microglia [194].

CCL2 could also have beneficial effects in HAD. In fact, some *in vitro* studies showed that CCL2 can protect human fetal astrocytes and neurons from Tat-induced apoptosis, thus suggesting a dual role for CCL2, favoring neurodegeneration in the setting of excessive chronic up-regulation and conferring neuroprotection in optimal regulation conditions. The neuroprotective effect of CCL2 against Tat-induced cytotoxicity in neurons was mediated by CCR2-dependent triggering of the PLC/phosphatidylinositol 3 (IP3) pathway, leading to the activation of transient receptor potential cation (TRPC) channels and resulting in [Ca2+]i increase and subsequent stimulation of the ERK/ cAMP response element-binding protein (CREB) pathway [195].

In addition to the CNS, HIV affects also the peripheral nervous system (PNS). Peripheral neuropathy (PN) is a recurrent neurological complication in HIV-infected patients, and a distal sensory polyneurophaty (DSP) is the most frequent type of PN in these patients. Although the exact pathogenesis of HIV-DSP is unknown, pro-inflammatory cytokines and macrophage activation are implicated in this process. Furthermore, patients are more prone to become symptomatic after starting nucleoside reverse transcriptase inhibitor (NRTI) treatment. Wang and colleagues recently performed a cross-sectional neurological study of HAART-naive subjects during the first year of HIV infection. They reported that patients with signs of PN had elevated levels of CCL2 in the CSF compared to subjects without PN [92]. On the contrary, plasma levels of CCL2 in HIV-infected subjects were not associated with neuropathy [98], and other authors did not found association between CSF levels of CCL2 and DSP [196]. Data obtained in a rodent model of neuropathy induced by perineural treatment with gp120/hCD4 and NRTI strongly support a role for the CCL2/CCR2 axis in DSP [197, 198].

Taken together these data indicate that a deregulated CCL2 production plays a key role in HIV-infected leukocyte infiltration into the CNS and the subsequent pathology characteristic of HANDs (Fig. **2**). Thus, therapeutic strategies aimed at reducing CCL2/CCR2-mediated cellular transmigration across the BBB and brain tissue damages may help in preventing the neuropathogenesis of HIV infection.

CCL2/CCR2 and HIV-associated Cardiovascular and Metabolic Disorders

HIV infection itself and undesirable secondary effects of antiretroviral drugs are associated with metabolic complica-

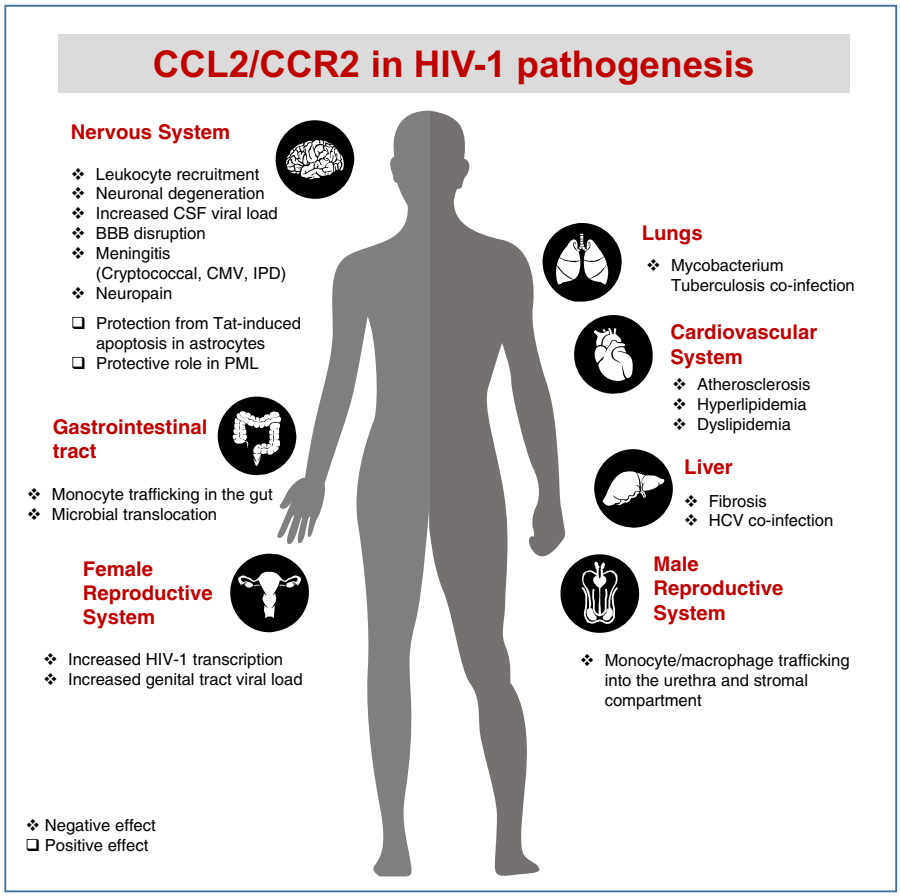

Fig. (2). Schematic representation of the CCL2/CCR2 involvement in HIV infection related disorders. The figure summarizes the described CCL2/CCR2 driven effects in the diseases associated to HIV infection in different organs/tissues.

tions, including dyslipidemia, insulin resistance, altered body fat distribution (lipoatrophy and lipohypertrophy) and hypertension. Cardiovascular diseases (CVDs) are also particularly worrying due to the high prevalence of cardiovascular risk factors in HIV-infected subjects, the HIV-sustained inflammation which promotes atherosclerosis and the above mentioned metabolic changes [199, 200]. Inflammation is of paramount importance in the development of atherosclerosis and HIV, together with associated opportunistic agents, may contribute inflammatory stimuli that could initiate or exacerbate atherogenesis [201]. CCL2, among several pro-inflammatory cytokines and chemokines altered in HIV infection, plays a crucial role in the pathogenesis of atherosclerosis and vascular dysfunctions in infected individuals.

A number of studies investigated the link between CCL2 and atherosclerosis in HIV-infected patients. In particular, higher CCL2 plasma levels in HIV-infected individuals correlated positively with viral load and negatively with $CD4^+$ T cell counts and were associated with higher minimal, maximal and mean vessel wall thickness (VWT) and vessel wall area (VWA) in the thoracic aorta, highlighting the correlation between CCL2 and atherosclerotic burden in this site [99]. Furthermore, increased plasma levels of CCL2, together with TNF-α, were associated with the presence of coronary artery calcium (CAC), a validated surrogate marker of arterial injury, and this association was independent of traditional CVD risk factors [202]. This study supports the hypothesis that CCL2-driven inflammation contributes to the development of atherosclerosis in the HIV-infected population. In addition, Parra and colleagues found that CCL2 and oxLDL plasma levels were significantly higher in HIV-infected subjects with atherosclerosis as compared to those without atherosclerosis, as assessed using carotid intima media thickness (CIMT) [203]. Therefore, the increased CVD risk was linked, at least in part, to the chronic oxidative stress and inflammatory status present in these patients. Thus, the mesurement of serum CCL2 and oxLDL concentrations, together with that of CIMT, may be a useful additional tool to better evalute the CVD risk in these patient population, and may facilitate therapeutic decisions concerning CVD prevention, particularly in the clinical management of HIV-infected subjects in whom treatment is complex.

HIV-infected individuals have more subclinical coronary atherosclerosis and endothelial dysfunction than HIV-uninfected subjects. McKibben and coworkers investigated the association among subclinical coronary atherosclerosis, CCL2 levels, and the serologic markers of monocytes activation sCD163 and sCD14. The levels of these factors were higher among HIV-infected men in spite of viral suppression by HAART, and were associated with lower $CD4^+$ T cell counts. In addition, increases in each of these biomarkers were significantly associated with coronary artery stenosis, even after adjustment for traditional CVD risk factors. In particular, elevated levels of CCL2, but not of sCD14 or

sCD163, were associated with greater levels of non-calcified plaque [204]. This is of particular interest, since a greater amount of non-calcified plaque was found in HIV-infected compared to uninfected men [205].

Several studies focused on the role of CCL2 polymorphisms in atherosclerosis. In this regard, mutations in the promoter region of the CCL2 gene were shown to have an atherosclerosis-promoting effect. In particular, HIV-uninfected patients diagnosed as having ischemic heart disease exhibited a higher prevalence of the CCL2-2518G/G genotype [206]. Furthermore, this polymorphism was associated with an increased risk for atherosclerosis [112]. In addition, the CCR2-V64I polymorphism was associated with reduced coronary artery calcification, and its homozygosity had a protective effect with respect to the development of coronary artery disease [206, 207]. Interestingly, these CCL2 and CCR2 polymorphisms are also associated with faster and lower disease progression to AIDS, respectively (see Table **3** and **4**). Besides, Coll and colleagues reported that HIV-infected patients with clinically manifest lipodystrophy (LD) had higher CCL2 plasma concentration which positively correlated with carotid intima-media thickness (IMT), and increased risk for sub-clinical atherosclerosis, compared to those without LD. These observations further support the relationship between CCL2-driven inflammation and atherosclerotic disease [113]. They also analyzed CCL2 genotypes and found that the CCL2-2518G allele and the presence of LD were associated with the development of carotid atherosclerosis. Furthermore, the association of plasma CCL2 levels with a series of major cardiovascular disease risk factors was studied in a healthy population and compared to a group of HIV-infected individuals. CCL2 concentrations were higher in HIV-infected patients, whereas an increase in circulation levels of CCL2 in healthy subjects was associated with aging, smoking and serum lipid alterations. This association, however, was lost in the group of HIV-infected individuals, indicating the prevalent influence of inflammation and/or cytokine imbalance in this population. The inheritance of the CCL2-2518G allele predisposed to higher levels of the chemokine in both groups, but this association was significant only in the healthy population [95]. The same group conducted an analysis of CCL2 genetic variants and its relation to plasma levels, metabolic trait and subclinical atherosclerosis in HIV-infected patients. They found that individuals with the H1, H2 and H5 haplotypes had higher CCL2 levels than those carring the H3, H4 and H6 haplotypes. Nevertheless, only carriers of the H1 and H5 haplotypes had higher insulin resistance and sub-clinical atherosclerosis. On the contrary, carriers of the H2 haplotype, which also showed high plasma CCL2 levels, presented less deleterious metabolic disorders [117].

Besides atherosclerosis, other metabolic abnormalities, such as lipoatrophy (subcutaneous adipose tissue wasting) and lipohypertrophy (central adipose tissue accumulation), contribute to CVD in the HIV-infected population [200, 208]. In this regard, the expression of CCL2 and other inflammatory markers was significantly elevated in lipoatrophic subcutaneous adipose tissue of HAART-treated lipodystrophic compared to non lipodystrophic patients, and positively correlated with liver fat content [209]. Moreover, CCL2 and other biomarkers linked to vascular inflammatory pathways were higher in HIV-infected compared to HIV-exposed, uninfected children and were independently associated with HIV viral load and/or hyperlipidemia [96].

Another complication typical of HIV infection is dyslipidemia, which is characterized by hypertriglyceridemia, low high-density lipoproteins (HDL) cholesterol and high LDL cholesterol with a prevalence of small LDL particles [200, 208]. In particular, altered HDL cholesterol levels are common in HIV-infected subjects and are usually ascribed to the effects of either antiretroviral drugs or infection itself. In this regard, the association between plasma concentrations of HDL and inflammatory response was evaluated in HIV-infected patients treated with abacavir/lamivudine (ABC-3TC) or tenofovir/emtricitabine (TDF-FTC) [97]. In this study, HIV-infected patients showed significantly increased baseline plasma concentrations of CCL2, C reactive protein (CRP), tissue plasminogen activator (tPA) and IL-6 then control subjects, and these markers negatively correlated with the plasma concentration of HDL. After two years of follow up, the decrease in plasma inflammatory biomarker levels was significant only for CCL2, tPA, and IL-6. However, plasma CCL2 levels decreased only in patients who did not receive TDF. The decrease of CCL2 levels was not significant among those individuals in which atherosclerotic lesions progressed. Surprisingly, the levels of CCL2 and CCR2 gene expression were lower in subjects with the highest cholesterol concentrations. Moreover, a lower CCL2 gene expression was found in patients with the highest plasma levels of LDL-cholesterol and plasma triglycerides. They also observed a negative correlation with total cholesterol concentrations in patients receiving ABC-3TC. In contrast, plasma HDL cholesterol concentrations positively correlated with CCL2 levels in patients receiving TDF-FTC. These results strengthen the idea that HIV infection modulates CCL2-driven inflammatory pathways and highlight the complexity of phenomena associated with antiretroviral therapy initiation, suggesting the need for a closer monitoring with respect to CVD risk during the initial phases of treatment.

In women living with HIV, further factors, in addition to HIV itself and impact of HAART, predispose to metabolic complications as osteoporosis, lipid and glucose disturbances and cardiovascular risk. Among these factors, the loss of the protective effects of estrogen in menopausal womens seems to play a prominent role [199]. Although there is not a direct correlation between CCL2, estradiol and HIV-induced atherosclerosis, growing evidence suggests a possible link among them. In particular, estradiol inhibited the expression of CCL2 in human coronary artery smooth muscle cells [210] and in injured arteries of ovariectomized rats [211]. Furthermore, trans-dermal estrogen therapy significantly decreased levels of CCL2 [212], whereas a hypo-estrogenic state in women treated with gonadotropin-releasing hormone (GnRH) agonist increased circulating levels of this chemokine. In addition, Tani and coworkers studied the changes in circulating levels of cytokines and chemokines in women during menopausal transition. They found that CCL2 levels were higher in late menopausal transition and postmenopause, and positively correlated with follicle-stimulating hormone (FSH) [213]. Thus, CCL2 may be susceptible to hormonal change and its increase in menopausal transition may be involved in the development of CVD.

Overall, these findings strongly support the relationship between CCL2 and cardiovascular and metabolic disorders in AIDS patients, and underline the need for a deeper analysis not only of traditional cardiovascular risk, but also of immunologic factors, particularly CCL2, as potential contributors to these morbidities (Fig. **2**).

CCL2/CCR2 and HIV Infection in Mucosal Tissues

Most HIV infections results from mucosal transmission. In fact, vaginal and rectal transmission is responsible for the majority of infections in adults, whereas oral ingestion of maternal fluids usually accounts for pediatric HIV infections [214]. The involvement of CCL2 in mucosal tissues infection has been poorly investigated. Below, we will draw the attention on the evidence in the literature of such an involvement.

Female Reproductive Tract Mucosal Tissues

The female reproductive tract (FRT) mucosal surface represents the primary site of transmission for several sexually transmitted infections, and accounts for around 40% of all HIV-1 transmissions [215-217]. Nevertheless, the immunological correlates of protection against HIV infection in the FRT are poorly known. Furthermore, the immune system of the FRT is regulated by the cyclical variations of the sex hormones estradiol and progesterone during the menstrual cycle. These hormones act either directly or indirectly on epithelial and immune cells in the FRT modulating immune functions in a site specific way throughout the reproductive tract [215]. A number of studies described the effects of changes in hormone levels in the FRT on the establishment of HIV-1 infection [215]. In this regard, lower estradiol levels in post-menopausal women may enhance HIV-1 transmission by modulating early innate immune responses. Indeed, low levels of estradiol induce the expression of soluble inflammatory mediators, thus leading to an inflammatory status that sustain HIV-1 replication. On the contrary, higher levels of estradiol during pre-menopause may reduce the expression of pro-inflammatory cytokines and immune cell activation, both of which reduce viral infection [218]. Rollenhagen and colleagues compared HIV-1 infection in *ex vivo* explants of ectocervical tissue from post- and pre-menopausal women, showing an enhanced release of HIV-1 p24 Gag in the former. This effect was not due to a difference in HIV-1 integration, whereas it was linked to higher levels of viral transcription in post- compared to pre-menopausal tissues. Increased HIV-1 transcription was associated with greater levels of CCL2 and other inflammatory cytokines/chemokines in post-menopausal tissues. Interestingly, blocking of CCL2 determined a decrease of HIV-1 trasncription levels [52]. These results suggest that post-menopause is associated with increased secretion of CCL2 and other pro-inflammatory mediators that foster HIV-1 replication in the FRT. Mukura and coworkers evaluated the levels of expression of inflammatory mediators in CVLs from HIV positive women and their correlation with the genital tract viral load (GTVL). They found higher concentrations of CCL2 and other soluble factors in CVL of women with detectable GTVL with respect to those with undetectable GTVL [102]. These results indicate that GTVL is associated with local inflammatory mediators, among which CCL2, that play an important role in HIV infection in women.

Male Genital Tract Mucosal Tissues

Besides the FRT, also the male genital tract can transmit infection. The limited accessibility of the male genital tract tissues has led to a lack of information concerning the precise mechanism of HIV transmission in this district. HIV infection in men has gained extensive attention in recent times due to the identification of the protective effect of male circumcision in HIV transmission, which suggested an essential role of the male foreskin as HIV entry site [216]. Thus far, the protective effect of circumcision is far from being complete, thus highlighting the existence of other penile epithelia as possible HIV entry sites. The penile urethra is another potential HIV entry site, since it is lined only by a pseudo-stratified non-keratinized columnar epithelium. In this regard, a recent study showed that the exposure of urethral explants to HIV-1 was associated with high levels of CCL2 release, which decreased urethral macrophage exit from the epithelial compartment. The high levels of CCL2 may be responsible for the continuous recruitment into the urethra of circulating blood monocytes, which will then differentiate locally into resident urethral macrophages. Furthermore, the decreased secretion of this chemokine as a consequence of urethral exposure to cell-associated HIV-1 might favor urethral macrophage exit from the epithelial to stromal compartment [53].

Gastrointestinal Tract Mucosal Tissues

Another common site of initial infection is the gastrointestinal tract (GIT). In fact, the GIT mucosa has a critic role as entry portal during mother-to-child and sexual transmission of HIV infection [219]. Gut disease is characterized by several GIT clinically relevant complications following HIV infection, such as severe diarrhea, body-mass wasting and various colorectal infections [220]. The GIT is a primary site for HIV infection, replication, and dissemination and the principal cells implicated may differ in relation to the tract of the gut implicated and the route of transmission. In particular, the gut-associated lymphoid tissue (GALT) harbors the majority of the body lymphocytes [221, 222], thus a cell-to-cell spread of HIV can readily occur by means of immunological synapses, thousand fold more efficient in comparison to infection with cell-free virions [223]. Moreover, resident HIV-infected macrophages are able to survive for long periods of time and represent a major long-lived viral reservoir due to their abundance at mucosal sites [224]. In addition, along the GIT, drug efflux transporters reduce the level of antiretroviral drugs available for gut cells [223], thus allowing unrestricted HIV replication [225]. Indeed, the GALT is the best characterized anatomical reservoir for HIV [226]. All together these features of HIV infection in the gut allow the virus to escape eradication by the immune system and by the anti-retroviral drug suppressive effect.

Another key aspect of HIV infection in the gut is represented by microbial translocation, which plays a crucial role in immune activation and disease progression during chronic HIV infection. In fact, microbial translocation across the damaged intestinal mucosa leads to increased levels of circulating LPS and other bacterial products, which contribute to

aberrant immune activation in HIV infected patients, and have been proposed as the main cause of the chronic immune activation associated with disease progression [227].

Concerning the involvement of CCL2 in the pathological processes associated with HIV infection in the gut, higher CCL2 protein levels were detected in jejunal biopsies from AIDS patients with or without active cryptosporidiosis respect to normal subjects [100]. Furthermore, Allers and coworkers recently reported that duodenal mucosa of untreated HIV-infected individuals was enriched in macrophages, and this effect was associated with a decrease of blood monocytes and an increase of the gut-homing molecule integrin β7 on these cells. They also found an increase of both CCR2 expression on integrin $\beta7^+$ monocytes and mucosal secretion of CCL2. These results suggest that the CCL2/CCR2 axis may be involved in monocyte trafficking in the gut during the course of HIV infection, thus contributing to the accumulation of activated macrophages that may promote microbial translocation through the mucosal barrier due to local inflammation and tissue injury [101].

Overall, these results suggest an involvement of the CCL2/CCR2 axis in the inflammatory processes associated with HIV infection and in the control of viral replication in mucosal tissues (Fig. **2**). Thus, strategies aimed at reducing the CCL2-driven pathological leukocyte migration in these tissues might help to control viral load and microbial translocation, thus reducing the detrimental effects on overall immunity in HIV infected individuals.

CCL2/CCR2 and HIV-associated Co-infections

The immunodeficiency caused by chronic HIV infection determines an increase of the risk of co-infections with pathogens that in normal conditions are controlled by the immune system. Moreover, treatment with antiretroviral drugs in the setting of HIV co-infections does not always restore the pathogen-specific immune response to normal levels. The differences in the presentation of the major HIV-associated opportunistic diseases in HIV-immunosuppressed persons with respect to immunocompetent individuals are that these are more frequent, present with atypical clinical and histopathological features, have higher infection loads, are more difficult to treat and often recur after treatment [228]. In the following paragraphs we will focus on some of the major co-infections for which a role of CCL2 was suggested (Fig. **2**).

Mycobacterium Tuberculosis

Individuals with HIV/AIDS are at high risk of Mycobacterium tuberculosis (Mtb) infection and active tuberculosis is one of the major causes of death among HIV seropositive subjects. CCL2 is a critical factor in HIV/Mtb co-infection and this topic was recently reviewed by Ansari and coworkers [229]. TB is associated with high pleural fluid levels of CCL2 and TNF-α [230], which are both implicated in transcriptional activation of HIV-1. Neutralization of CCL2, but not of TNF-α, reduced Mtb-induced HIV-1 gag/pol mRNA in pleural fluid mononuclear cells [231]. CCL2 secreted by Mtb-infected alveolar macrophages [232-234] recruits $CCR2^+$ leukocytes, allowing the virus to infect and replicate in these permissive cells [230, 235, 236]. In turn, the persistent high HIV viremia leads to macrophage dysfunction and reduced killing of Mtb [230, 237]. Furthermore, high CCL2 levels have been detected in the bronchoalveolar lavage (BAL) fluid of HIV-1/Mtb patients [232], and might drive the generation of a Th2 dominant environment that may suppresses Mtb-specific IFN-γ-mediated Th1 immunity. Activation of HIV-1 long-terminal repeats (LTR) induced by CCL2 in infected macrophages and $CD4^+$ T cells [238] also determines the induction of pro-inflammatory genes such as CCL2, TNF-α and IL-6 [237, 239]. A persistent activation of signaling pathways and consequent secretion of inflammatory mediators, including CCL2, may drive a chronic inflammatoty status that may be lethal to HIV/Mtb patients. Thus, besides anti-retroviral and anti-TB therapy regimens, approaches to decrease CCL2 expression could restrain severity of HIV/Mtb co-infection [229].

Hepatitis C Virus

Hepatitis C virus (HCV) is frequent among people at risk for or infected with HIV since it is transmitted in some of the same ways of HIV, namely through drug injection and condom-less sex. Viral hepatitis progresses more rapidly and causes more liver-related health problems in people with HIV than in those without HIV. Thus, HCV-related liver disease represents one of the principal causes of non-AIDS-related death among HIV-positive individuals. Despite successful implementation of HAART, low $CD4^+$ T cell counts persist in HIV/HCV co-infected with respect to HIV mono-infected subjects. In turn, HIV negatively affects the natural history of HCV infection by inducing rapid virus replication, accelerated fibrosis and poor response to antiretroviral therapy [240].

The complex cellular environment of the human liver comprises HSCs, macrophages (Kupffer cells), endothelial cells, and circulating immune cells. Continuous hepatic inflammation is a key factor in the progression of chronic liver diseases, including hepatitis C. Liver inflammation is regulated by chemokines, which modulate the migration and activity of hepatic cells [241]. In particular, CCL2 is one of the best characterized chemokines during hepatic fibrogenesis, involved both in the initiation of liver inflammation and in keeping chronic injury, perpetuating inflammatory responses [242, 243]. Its contribution to liver disease in the setting of HIV/HCV co-infection was recently reviewed by Ansari and colleagues [244].

A faster progression of liver fibrosis to cirrhosis was reported in HCV/HIV co-infected patients. HSCs represent a key cell type in the pathogenesis of fibrosis. In this regard, Bruno and colleagues reported that HIV-1 gp120 modulates several aspects of HSC biology, including expression of pro-inflammatory cytokines, mainly CCL2, and directional cell movement [62]. These results identify a direct pathway possibly linking liver fibrogenesis and HIV infection through viral envelope proteins and CCL2. Furthermore, Tuyama and coworkers demonstrated that HIV-1 can infect HSC and promote CCL2 and collagen I expression [56]. These findings further support direct profibrogenic and CCL2-driven pro-inflammatory effects of HIV on HSCs. Interestingly, the CCL2-2518 polymorphism, associated with faster disease progression to AIDS (see Table **3**), was also associated with significantly higher hepatic appearence and severity of HCV-related liver disease [245].

Overall, CCL2 appears to be a relevant mediator of hepatic fibrosis and offers a critic contribution to HIV/HCV co-infection. Thus, this chemokine may be a possible marker of disease control and an interesting target of anti-fibrotic intervention in HIV/HCV co-infected patients.

CNS Opportunistic Infections

Advanced HIV infection is characterized by a symptomatic disease with severe immune deficiency as a result of persistent viral replication, chronic immune activation, and progressive deterioration of immune functions. This generate a favorable milieu for opportunistic infections in the CNS. Several of the opportunistic infections that affect the CNS in HIV-infected individuals are AIDS defining conditions and include cryptococcal meningitis (CM), CNS cytomegalovirus and toxoplasmic encephalitis, progressive multifocal leukoencephalopathy (PML), and CNS tuberculosis. All these conditions are associated with high mortality. Most CNS opportunistic infections are a consequence of the reactivation of latent pathogens. In patients undergoing antiretroviral therapy, IRIS might reveal previously unsuspected CNS opportunistic infections. Most of them are treatable, but several challenges remain in their management [246]. Despite treatment of CNS opportunistic infections in conjunction with HAART resulted in improved survival, CNS opportunistic infections still represent a serious burden worldwide. A number of studies evaluated the role of CCL2 in some CNS opportunistic infections.

Cryptococcus infection occurs *via* the lungs, and is disseminated from this site to other body districts, predominantly the CNS. CM represents the most common clinical manifestation of cryptoccocal infection in HIV-infected individuals [228]. Christo and coworkers found that CSF levels of CCL2, among other chemokines, were increased in HIV-infected patients with CM, when compared to patients with toxoplasmic encephalitis or without opportunistic infections [87]. Furthermore, Chang and coworkers found that in HIV-1 infected subjects, CM is characterized by elevated CSF levels of CCL2, CCL3 and CXCL10 compared to blood. In addition, at HAART initiation, a higher ratio of CCL2/CXCL10 and CCL3/CXCL10 was found in the CSF of patients who developed Cryptococcosis associated immune reconstitution inflammatory syndrome (C-IRIS). The relative abundance of chemokines in patients who developed C-IRIS may be responsible of monocyte and neutrophil infiltration into the CSF [90].

Streptococcus pneuomoniae represents the main cause of pneumonia and meningitis in children in the developing world and is the principal cause of community-acquired bacteremia in children in sub-Saharan Africa. The burden of invasive pneumococcal disease (IPD) has augmented in areas with a high prevalence of HIV infection. Carrol and colleagues evaluated the relation between chemokines in children presenting IPD and the influence of HIV infection. They found that HIV-infected children had higher plasma CCL2 and CXCL8 levels than HIV-uninfected children, and that chemokines levels correlated with pneumococcal bacterial loads. Furthermore, plasma levels of CCL2 and CXCL8 were significantly higher in non-survivors than in survivors among HIV-infected but not HIV-uninfected children. In addition, multivariate analysis of the survivors/non-survivors revealed that lower plasma CCL2 levels were associated with an increased chance of survival [93]. Thus, in IPD CCL2 is associated with survival, pneumococcal bacterial loads, disease presentation, and outcome.

Another widespread opportunistic infection is CMV encephalitis. Bernasconi and coworkers investigated the levels of CCL2 in the CSF and serum of HIV infected subjects with CNS opportunistic disorders. They found that serum CCL2 levels were not significantly altered in patients with CNS opportunistic disorders. Instead, they found a dramatic elevation of CCL2 levels, but not of other chemokines, in CSF of individuals with CMV encephalitis and a minor CCL2 increases in the CSF of subjects with PML, CM, toxoplasmic encephalitis and primary CNS lymphoma. In addition, they also examined some cases at autopsy and found that macrophages represented the most abundant type of inflammatory cells in CMV-related necrotizing lesions. Thus, CCL2 may be responsible of the recruitment of monocytes in the CNS of these patients [84]. The correlation between CCL2 and CMV encephalitis in HIV-infected patients was also evaluated by Cinque and colleagues. They found a strong association between HIV encephalitis and high CSF CCL2 levels, which also correlated with high CSF HIV RNA amounts but not with plasma viremia [85].

The involvement of CCL2 was also investigated in HIV-infected patients with PML, a rare but severe CNS opportunistic infection caused by the reactivation of latent John Cunningham (JC) virus. In this regard, Marzocchetti and colleagues found that CCL2 levels, but not those of TNF-α and CCL5, were significantly higher in PML patients than in control and that the higher concentration of CCL2 correlated with lower JC viral load. Although CCL2 levels did not differ in PML patients previously treated with antiretroviral drugs when compared to those who were not, in nine of them CCL2 showed a trend towards a positive correlation with the time of therapy. In addition, they found that higher levels of CCL2 in the CSF were associated with longer survival in subjects with $CD4^+$ T cell counts below 100 cells/ul. They speculate that high CCL2 levels might be associated with a JC virus-specific immune response [247]. Thus, at least in these patients, the CCL2-mediated inflammatory reaction should be considered as a favorable event in the course of AIDS-associated PML. However, since a potent inflammatory reaction may cause irreversible brain damage and death, this process needs to be strictly regulated.

THE CCL2/CCR2 AXIS AS A THERAPEUTIC TARGET IN HUMAN DISEASES

As a consequence of their involvement in a variety of human pathologies, chemokines and their receptors represents extremely attractive therapeutic targets for pharmaceutical and biotechnology companies. Indeed, industrial development pipelines are full of new chemokine-targeting drugs for the treatment of an array of inflammatory diseases and malignancies. The first example of the druggability of the chemokine system derived from the function of chemokine receptors in HIV infection. Indeed, selective inhibition of CCR5 and CXCR4 with small molecules demonstrated the clinical relevance of targeting chemokine receptors to control

viral infection [248, 249]. Maraviroc, an anti-CCR5 compound, represented the first FDA-approved chemokine receptor-targeting drug and is currently used in the clinic for the treatment of HIV infection.

CCL2 is one of the most important chemokines which regulate migration and infiltration of monocytes/macrophages. Either CCL2 or its receptor CCR2 have been demonstrated to be induced and involved in several inflammatory diseases. CCL2 and CCR2, possibly more than any other chemokine system, have been shown to play key pathological functions *in vivo* in several murine models of disease, including atherosclerosis, experimental autoimmune encephalomyelitis, obesity-induced diabetes and cancer [250]. Thus, inhibiting the CCL2/CCR2 axis has been regarded as a promising novel approach for the treatment of inflammatory diseases and malignancy. As a consequence, targeting CCL2 and CCR2 is a very active area of drug development with potential application for a large number of important acute and chronic human diseases.

In general, chemokine activity can be blocked by chemokine receptor antagonists, chemokine or chemokine receptor specific neutralizing Abs, or by inhibiting intracellular signaling pathways activated by chemokine interaction with their receptors [251]. In this review we will concentrate our attention on the first two classes of drugs and on a new original approach employing aptamers.

Pharmaceutical Development of Small Molecule Inhibitors

CCR2 Antagonists

One key point of intervention is the inhibition of the ligand/receptor interaction, and the preferred solution is orally available small molecule inhibitors. Many companies have reported programs to identify CCR2 antagonists. General challenges in the development of CCR2 agents have included reduced activity in the mouse model and selectivity for either other chemokine receptors or ion channels, which can lead to undesirable cardiovascular side-effects. A number of reviews described the CCR2 antagonists reported in publications and patents in recent years [36, 252, 253]. The field remains extremely active with many pharmaceutical companies continuing to register patents, publish articles and present at meetings. In this review we will update to the present focusing on the compounds that have already entered in clinical trials.

(Table **5**) summarizes active and past CCR2 small molecule antagonist clinical trials in patients with different conditions. Although the majority of these studies have been completed, clinical data have been issued only for few of them. The majority of these drugs showed a favorable safety profile. However, most of them failed from a therapeutic point of view, at least for the indications for which they were tested.

Chemocentryx has two candidate molecules targeting CCR2 that have progressed to clinical trials. The lead drug candidate, CCX140-B, is highly selective for CCR2 relative to other chemokine receptors such as CCR5, even at high doses, and it was shown pre-clinically to be free of the cardiovascular danger signals associated with other CCR2 antagonists. Preclinical toxicology studies showed that this compound is suitable for evaluation in humans in diabetic nephropathy (DN) [254, 255]. Indeed, CCX140-B is being developed as an orally delivered therapy for the treatment of such condition [256]. CCX140-B appeared to be well tolerated and no safety issues were observed that would prevent its further clinical development in DN. CCX872-B is a second generation CCR2 inhibitor which completed phase I clinical development in 2014 [257]. A phase Ib clinical trial in patients with pancreatic cancer is ongoing.

Merck developed MK-018, a potent CCR2 antagonist that showed good pharmacokinetic (PK) profiles in preclinical studies and demonstrated efficacy in animal models. This drug entered in phase II clinical trials for MS as well as RA, but no significant improvement compared with placebo was reported for any of the end points studied [4, 258].

Johnson & Johnson developed two independent CCR2 antagonists. JNJ-41443532 is an orally available, selective, reversible antagonist of CCR2. Its potential for the treatment of type II diabetes mellitus (T2DM) was explored in a phase II proof-of-concept clinical trial. In this study, administration of JNJ-41443532 was generally well tolerated in patients, but resulted in modest improvement in glycaemic parameters compared with placebo [259]. JNJ-17166864 is highly selective for CCR2, but it has poor oral bioavailability. Thus, it entered human clinical trials in the form of a nasal spray for the treatment of allergic rhinitis (AR). No clinical data from this trial were found in the literature.

Bristol–Myers Squibb was very active in the field of CCR2 antagonists. The compound BMS-741672 was evaluated in two phase II clinical studies in patients with type II diabetes. Although both trials were completed, no clinical data were revealed.

AstraZeneca developed AZD2423, a selective, potent and reversible antagonist of CCR2. Data from phase I studies in healthy volunteers showed adequate safety, tolerability and PK of AZD2423. This drug was evaluated in phase II clinical trials in patients affected by post-traumatic neuralgia (PTN) and painful diabetic neuropathy (PDN). In these studies the drug failed to show efficacy despite good evidence of target engagement [260, 261]. The compound was also evaluated in phase II trials in patients with Chronic Obstructive Pulmonary Disease (COPD), but no reports were issued concerning the results of these studies.

Pfizer developed PF-04136309, which was evaluated in patients with osteoarthritic pain of the knee. No clinical data from this trial were found in the literature. Based on the results obtained in an orthotopic model of murine pancreatic cancer [262], this drug is currently being evaluated, in combination with standard chemotherapy, in pancreatic cancer patients with locally advanced non-metastatic disease.

Antagonists With Dual-Receptor Specificity

The promiscuity of chemokines for multiple receptors represents one of the principal characteristics of the chemokine receptor family. Not surprisingly therefore, one of the main challenges in the development of chemokine receptor antagonists has been the selectivity between members of the CC chemokine receptor family. In particular,

Table 5. Summary of clinical trials of drug candidates targeting CCR2.

Type of Compound	Company	Compound Name	Trial	Condition	Status	References
Small molecule antagonist	Chemocentryx	CCX140-B	NCT01440257 (phase II)	T2DM	active	
			NCT01447147 (phase II)	DN	active	
			NCT01028963 (phase II)	T2DM	completed	Pirow *et al.,* [256]
		CCX872-B	NCT02345408 (phase I)	Pancreatic cancer	active	
	Merck	MK-0812	NCT00239655 (phase II)	RRMS	completed	
			NCT00542022 (phase II)	RA	completed	Beaulieu *et al.,* [258]
	Johnson & Johnson	JNJ-41443532	NCT01106469 (phase I)	Healthy	completed	
			NCT01105780 (phase I)	Healthy, PK study	completed	
			NCT01230749 (phase II)	T2DM	completed	DiProspero *et al.*, [259]
		JNJ-17166864	NCT00604123 (phase II)	AR	completed	
	Bristol-Myers Squibb	BMS-741672	NCT00683423 (phase II)	NP	completed	
			NCT00699790 (phase II)	T2DM	completed	
	AstraZeneca	AZD2423	NCT00977626 (phase I)	Healthy	completed	
			NCT01215279 (phase II)	COPD	completed	
			NCT00970775 (phase I)	Healthy	completed	
			NCT01153321 (phase II)	COPD	completed	
			NCT00940212 (phase I)	Healthy	completed	
			NCT01200524 (phase II)	Nerve pain	completed	Kalliomaki *et al.*, [260]
			NCT01223014 (phase I)	Healthy	completed	
			NCT01201317 (phase II)	NP	completed	Kalliomaki *et al.*, [261]
			NCT01233830 (phase I)	Healthy	completed	
	Pfizer	PF-04136309	NCT01413022 (phase I/II)	Pancreatic Neoplasms	active	
			NCT01226797 (phase II)	Chronic Hepatitis C	terminated[a]	
			NCT00689273 (phase II)	Knee Osteoarthritis	completed	

(Table 5) contd….

Type of Compound	Company	Compound Name	Trial	Condition	Status	References
Antibody	Millennium	MLN1202	NCT01015560 (phase II)	Bone Metastases	completed	
			NCT01199640 (phase II)	MS	completed	Sharrack B, [288]
			NCT00715169 (phase II)	Atherosclerosis	completed,	Gilbert *et al.*, [289]

The table reports the clinical trials found in http://www.clinicaltrialssearch.org and https://clinicaltrials.gov

[a] the study has been terminated due to difficulty in enrolling the targeted number of patients

Abbreviations: T2DM, Type 2 Diabetes Mellitus; DN, Diabetic Nephropathy; RRMS, Relapsing-Remitting Multiple Sclerosis; RA, Rheumatoid Arthritis; PK, pharmacokinetic; AR, Allergic Rhinitis; NP, Neuropathic Pain; COPD, Chronic Obstructive Pulmonary Disease; MS, Multiple Sclerosis.

CCR2 and CCR5 genes, which share significant sequence homology (73%), are closely located on the same chromosome, apparently arising from an event of gene duplication. One of the early CCR5 antagonist developed, TAK779, was found to be a potent antagonist of both CCR5 and CCR2 and cross-reactivity with CCR5 is a common property to several CCR2 antagonists. Although drug receptor promiscuity is not usually desirable because of off target effects, in some cases it may be therapeutically advantageous. The use of promiscuous drugs has been an important step forward in the treatment of complex diseases such as schizophrenia, mood disorders and cancer, and has demonstrated that small molecule drugs can bind to multiple GPCRs [4].

At least some of the clinical failures in several chemokine receptor antagonist based therapeutic programs are due at least in part to targeting complex diseases with an antagonist to a single receptor. Thus, the problem of receptor redundancy may be overcome by alternative approaches in which more than one chemokine receptor is inhibited through the use of promiscuous non peptide antagonists. This type of approach might be more successful than receptor specific antagonists in the treatment of complex diseases such as AIDS. Based on the hypothesis that inhibition of both CCR2 and CCR5 will overcome any potential degeneracy in the system that might limit efficacy, some companies have capitalized on the tendency for antagonists to show excellent affinity for more than one receptor to develop compounds that inhibit both receptors. As shown in (Table **6**), Tobira Therapeutics, Pfizer and Bristol-Mayers Squibb have active programs involving dual CCR2/CCR5 antagonists. While the candidate compounds from the last two companies are being tested in diabetic patients, the drug candidate being developed by Tobira Therapeutics is of particular interest for the purpose of this review because its first indication is HIV infection.

Cenicriviroc (CVC; also known as TBR-652 and previously TAK-652 from Takeda) is a first-in-class dual CCR2/CCR5 antagonist in phase III and II of development for the treatment of HIV infection and non-alcoholic steatohepatitis (NASH), respectively. By blocking CCR5 it inhibits HIV entry into host cells, but has also significant potential for anti-inflammatory effect in patients due to CCR2 inhibition. Thus, unlike maraviroc, in addition to the direct anti-HIV effects CVC could be effective in treating the chronic immune activation associated with suppressed HIV infection. Hence, CVC might play a differentiated role in the management of HIV-infected patients. Due to its long half-life (35-40 hours), CVC remains in the blood for prolonged periods, and can thus be taken just once daily. Another advantage of CVC is that it generally does not interfere with enzymes in the liver that are used to process many other drugs.

Up to date, CVC safety and tolerability profile has been evaluated in about 580 subjects in phase I and II trials, including a safety study in subjects with liver cirrhosis and 115 HIV-1 infected patients treated for 48 weeks. These studies confirmed CVC mechanism of action and supported its longer term safety and efficacy. In particular, a phase IIa randomized, placebo-controlled, double-blind, dose-escalating study assessing the antiviral activity, PK, pharmacodynamic (PD), safety and tolerability of oral once-daily CVC mono-therapy for 10 days in HIV-1–infected, antiretroviral treatment–experienced, CCR5 antagonist–naive subjects, was conducted in 2010 (study 201). The results of this trial demonstrated that HIV-infected subjects treated with CVC had a significant reduction in viral load, which persisted for up to two weeks after treatment discontinuation [263, 264]. As a consequence of these positive results, a phase IIb randomized, double-blind, double-dummy, dose-finding study of CVC in comparison to efavirenz (EFV), both in combination with FTC/TDF, in treatment-naïve HIV-1-infected subjects was conducted (study 202). Data from this trial showed rates of viral suppression of 76% for patients taking 100 mg CVC, 73% with 200 mg CVC, and 71% with EFV. Although non-response rates were higher with CVC, this was largely due to greater drop-out of patients [265, 266]. Adherence may be improved by lowering pill burden with a new tablet formulation. The analysis of immune and inflammatory biomarkers revealed a decrease of sCD14 levels and an increase of CCL2 amounts, indicative of CCR2 engagement, in the CVC arms. The available efficacy and safety data from these clinical trials supported its further evaluation as a backbone for new multi-drug combination therapy for HIV in phase III registration studies as part of a fixed dose combination of CVC and the antiretroviral drug 3TC. Tobira plans to proceed in clinical development of CVC/3TC in treatment-naive HIV-1 infected patients by developing a single-tablet, fixed-dose combination.

Table 6. Clinical trials of small molecule antagonists targeting CCR2/CCR5.

Compound Name	Company	Trial	Condition	Status	References
Cenicriviroc	Tobira Therapeutics	NCT02128828 (phase II)	HIV Neurocognitive Impairment	recruiting	
		NCT01092104 (phase I/II)	HIV-1 infection	completed	Lalezari *et al.*, [263] Marier *et al.*, [264]
		NCT02120547 (phase I)	Liver Insufficiency	completed	
		NCT02217475 (phase II)	Nonalcoholic Steatohepatitis	recruiting	
		NCT01827540 (phase I)	HIV Infection/AIDS	completed	
		NCT02330549 (phase II)	Prediabetic State, Nonalcoholic Fatty Liver Disease	enrolling by invitation	
		NCT01338883 (phase II)	HIV-1 infection	completed	Gathe *et al.*, [265] Feinberg *et al.*, [266]
		NCT01474954 (N.A.)	HIV Infection	terminated	
PF-04634817	Pfizer	NCT01712061 (phase II)	Diabetic Nephropathy	recruiting	
		NCT01140672 (phase I)	Healthy	completed	
		NCT01247883 (phase I)	Healthy	completed	
		NCT01791855 (phase I)	Renal Insufficiency	recruiting	
		NCT01098877 (phase I)	Healthy	completed	
		NCT01994291 (phase II)	Diabetic Macular Edema	recruiting	
BMS-813160	Bristol-Myers Squibb	NCT01049165 (phase I)	Healthy	completed	
		NCT01752985 (phase II)	Diabetic Kidney Disease	recruiting	

The table reports the clinical trials found in http://www.clinicaltrialssearch.org and https://clinicaltrials.gov

CCL2 Synthesis Inhibitors

Bindarit is an Angelini original indazolic derivative initially developed as anti-inflammatory agent testing its potential in rheumatic diseases [267]. The discovery that its anti-inflammatory effects *in vitro* and *in vivo* are related to inhibition of CCL2 synthesis leads to a new clinical development plan on selected diseases. The drug is best known for its transcriptional inhibition of the monocyte chemoattractant subfamily of CC chemokines including CCL2 [268]. Even though CCL2 is the best characterized target of bindarit, the drug also inhibits other inflammatory mediators, such as CCL7, CCL8, and IL-12 [269]. Recently, a modulatory effect of bindarit on the classical NF-kB pathway has been identified. In particular, bindarit was shown to specifically inhibits p65 and p65/p50-mediated activation of the CCL2 promoter [269].

Bindarit showed preclinical efficacy in several animal models of human diseases through potent suppression of CCL2 transcription [270-276], and positive results were obtained in clinical trials in patients with DN [277], lupus nephritis (LN) [278] and coronary restenosis [279] (Table **7**). Such efficacy was associated with the ability of this compound to reduce CCL2 production. Bindarit completed phase II clinical development and is available for licensing. The Angelini pipeline also comprises the identification and development of new MCPs inhibitors with therapeutic potential in oncology, pain and inflammation, and gastroenterology.

Clinical Therapeutic Antibodies Against CCL2/CCR2

General Considerations

The development of therapeutic Abs represents an alternative approach with respect to small compounds for targeting the chemokine system. Knowledge on how to efficiently generate monoclonal Abs (mAbs) with high therapeutic potential has greatly evolved in the past century. Consequently, these pharmaceuticals have gained ground over traditional

Table 7. Summary of clinical trials of drug candidates targeting CCL2.

Type of Compound	Compound Name	Company	Trial	Condition	Status	References
Small molecule synthesis inhibitor	Bindarit	Angelini	N.A. (phase II)	Lupus Nephropathy	completed	Ble *et al.*, [278]
			NCT01109212 (phase II)	DN	completed	Ruggenenti *et al.*, [277]
			NCT01269242 (phase II)	Coronary Restenosis	completed	Colombo *et al.*, [279]
Antibody	Carlumab	Centocor	NCT00786201 (phase II)	IPF	completed	Martinez *et al.*, [284]
			NCT01204996 (phase I)	Cancer	completed	Brana *et al.*, [281] Sandhu *et al.*, [282]
			NCT00992186 (phase II)	Prostate Cancer	completed	Pienta *et al.*, [283]
Spiegelmer	NOX-E36	Noxxon Pharma	NCT01085292 (phase I/II)	T2DM	completed	
			NCT01547897 (phase II)	T2DM, Albuminuria	completed	Vater *et al.*, [291]
			NCT01372124 (phase I)	Renal Impairment	completed	
			NCT00976729 (phase I)	Chronic Inflammatory Diseases, T2DM, SLE	completed	

The table report the clinical trials found in http://www.clinicaltrialssearch.org and https://clinicaltrials.gov

Abbreviations: DN, Diabetic Nephropathy; IPF, Idiopathic Pulmonary Fibrosis; T2DM, Type 2 Diabetes Mellitus; SLE, Systemic Lupus Erythematosus.

small-molecule drugs because of several advantages. In fact, Ab-derived therapeutics can inhibit the function of the target antigen in a very selectively way, thus limiting off-target effects. Moreover, they can also elicit additional strong immune responses through two different and powerful mechanisms. Indeed, the Fc portion of Ab heavy chains can mediate complement-dependent cytotoxicity (CDC) or Ab-dependent cellular cytotoxicity (ADCC) by binding specific complement factors or Fc receptors expressed on monocytes/macrophages, respectively. These effects further improve *in vivo* Ab efficacy. On the other hand, small molecule drugs are favored for their small size, hence their quick clearance, and their capacity to enter the cell, which allow them, unlike Abs, to target intracellular molecules. One of the major limitation of Abs is that they are recognized by the patient as being of foreign origin, thus originating an anti-globulin response. Recombinant DNA technology allowed to convert the rodent Abs into a more human form. Different degrees of humanization can be obtained ranging from chimeric Abs with a combination of human constant regions with rodent variable regions to fully humanized Abs where also the variable regions are of human origin. Thus, therapeutic mAbs are progressively more going in the clinic for the treatment of various diseases [280].

Clinical Trials of Ab-derived Therapeutics Against CCL2

Carlumab/CNTO-888 is a fully human anti-CCL2 Ab developed by Centocor. Phase I and II clinical trials were conducted with this compound in patients with solid tumors (Table **7**). In these studies carlumab was demonstrated to have an acceptable safety profile, but no long-term suppression of serum CCL2 or significant tumor responses were observed [281-283]. Thus, the drug is not being developed further in oncology. Carlumab was also tested in a phase II study to evaluate its efficacy and safety in patients with idiopathic pulmonary fibrosis (IPF) (Table **7**). Also in this trial the drug did not show measurable benefit in patients [284].

The clinical utility of carlumab was limited by the finding of increased CCL2 levels in serum in response to escalating doses of the drug [282, 283, 285]. Although the reason for this is unknown, the weak binding affinity of the Ab, resulting in a large amount of free CCL2 being dissociated from the carlumab–CCL2 complex, was proposed as a potential explanation for the lack of clinical efficacy. The same effect was observed with the anti-CCL2 mAb ABN912 developed by Novartis and tested in patients with RA [286]. In this study the total serum levels of CCL2 increased dose-dependently in patients treated with ABN912. This effect might be explained by rapid binding of intravascular CCL2 by ABN912 and subsequent mobilization of the chemokine from extra-vascular compartments, e.g. from GAGs. Consistent with the increase in circulating CCL2, no clinical improvement was seen in patients treated with ABN912.

In conclusion, the results of these studies do question the strategy of targeting CCL2 with neutralizing Abs, because subsequent increases in serum levels might be a general phenomenon for CCL2 and all other chemokines that bind to

GAGs, and are therefore stored in large quantities in the extracellular matrix. In general, this observation suggests that strategies targeting CCR2 might be more potent than inhibition of CCL2.

Clinical Trials of Ab-derived Therapeutics Against CCR2

MLN1202 is a humanized anti-CCR2 mAb developed by Millennium. This drug was tested in patients with RA, but the treatment did not result in amelioration of synovial inflammation [287] (Table **5**). This result was in agreement with those of other clinical trials that were aimed at interfering with the CCL2/CCR2 ligand/receptor pair in RA patients, thus suggesting that targeted CCL2/CCR2 blockade is not an effective approach for such condition. MLN1202 was also tested in MS patients (Table **5**). Although Millennium reported that it was effective in reducing the number of lesions in the brain, the compound was no longer developed for such indication, suggesting that the activity may be insufficient to compete with current therapies [288]. Finally, MLN1202 was evaluated in patients at risk for atherosclerotic CVD. In this study, patients who received MLN1202 exhibited a significant decrease in CRP levels. Furthermore, patients with the -2518G allele in the CCL2 gene had significantly greater reduction in CRP levels than patients with the wild-type genotype [289] (Table **5**).

CCL2 Inhibition With Spiegelmers

Spiegelmers represent a class of next generation aptamers discovered and developed by NOXXON Pharma. An aptamer is a nucleic acid structure that binds to a target molecule in a manner similar to an Ab recognizing an antigen. The term Spiegelmer, derived from the german word Spiegel (mirror), refers to the substance class of structured mirror-image oligonucleotides that are designed to bind and inhibit pharmacologically relevant target molecules. Spiegelmers are biostable aptamers which possess a structure that prevents enzymatic degradation. In fact, they are entirely made up of unnatural (mirror-image) L-nucleotides which render them nuclease resistant and exceptionally biostable. Indeed, the mirror-image configuration confers plasma stability and immunological passivity. Furthermore, Spiegelmers modified with a higher molar mass, such as PEGylated L-RNA aptamers, show a prolonged plasma half-life. These properties make them interesting substances for drug development [290]. These compounds combine the benefits of small molecule drugs and biopharmaceuticals.

Emapticap pegol (NOX-E36) is a Spiegelmer that binds and neutralizes human CCL2, being developed for the treatment of DN. This drug underwent regulatory safety studies, demonstrated good safety profiles in healthy volunteers and was taken into phase IIa studies in patients (Table **7**). In all clinical studies NOX-E36 was safe and well tolerated, and a dose-dependent reduction of peripheral blood $CCR2^+$ monocytes was observed. This demonstrates that the drug effectively antagonizes the CCL2/CCR2 axis in humans. Proof-of-concept for emapticap pegol has recently been demonstrated in a double-blind, randomized, placebo-controlled phase IIa exploratory study in DN patients [291].

Lessons Learned From Clinical Trials of CCL2/CCR2 Targeting Drugs

Chemokines and their receptors, mostly due to their pivotal role in controlling leukocyte moving and inflammation, play a key role in the pathogenesis of a vast array of human pathologies. However, with the exception of selective CCR5 antagonists for HIV infection inhibition and CXCR4 inhibitors for hematopoietic stem cell mobilization, the promise of obtaining new therapeutics targeting chemokine functions has not yet been realized. Also for CCL2 and CCR2, the hope of developing effective therapeutics targeting this ligand-receptor pair has not been achieved till now. Ab or small molecule chemical blockade of CCL2/CCR2 signaling is one strategy for therapeutic intervention that showed promise in animal models of inflammatory diseases. However, this approach has not yet translated to effective human therapies. Instead, there have been several failed phase II clinical studies, which have tempered the initial enthusiasm for these proteins as drug targets. In fact, the majority of the drug developed till now did not meet the developing company's criteria for moving into phase III.

Although it is often difficult to ascertain definitive reasons for the suspension of clinical development, since little primary research is published in this area and negative results of clinical trials are often not issued, several perspectives and reviews have suggested that these decisions could be attributed to a lack of efficacy. The reasons that could account for why a drug can fail in clinical trials include inappropriate target selection for a given disease, ineffective dosing level of the drug or timing of its administration, and redundancy of the therapeutic target. These developmental hurdles are intimately linked to the lack of reliable animal models of at least some of the targeted diseases, mostly due to differences between the immune system of humans versus animals (relevance of the target to the human disease and species differences in chemokine receptors for antagonists), and the lack of a good PD assay to accurately measure levels of active drug in the plasma [4, 5]. Concerning the redundancy of the target, it may reflect the fact that multiple receptors can be involved in driving the pathophysiology of the disease. A potential solution of this problem could be to develop drugs that target more than one receptor, known as polypharmacology, which could be a novel way to generate effective therapeutics. As discussed above, some companies are actively involved in the clinical development of dual CCR2/CCR5 antagonists. These drugs, together with the CCR2 antagonists and the CCL2 targeting compounds depicted in (Fig. **3**), are present in the pipelines of the companies with still active CCL2/CCR2 programs. Major indications for such compounds are DN, restenosis, NASH and HIV infection.

CONCLUSIONS

The CCL2/CCR2 axis is crucially involved in immune activation and inflammation which are hallmarks of HIV infection, thus contributing to several HIV-associated disorders in infected patients. In addition, a growing body of evidence suggests that CCL2 is exploited by HIV to boost its

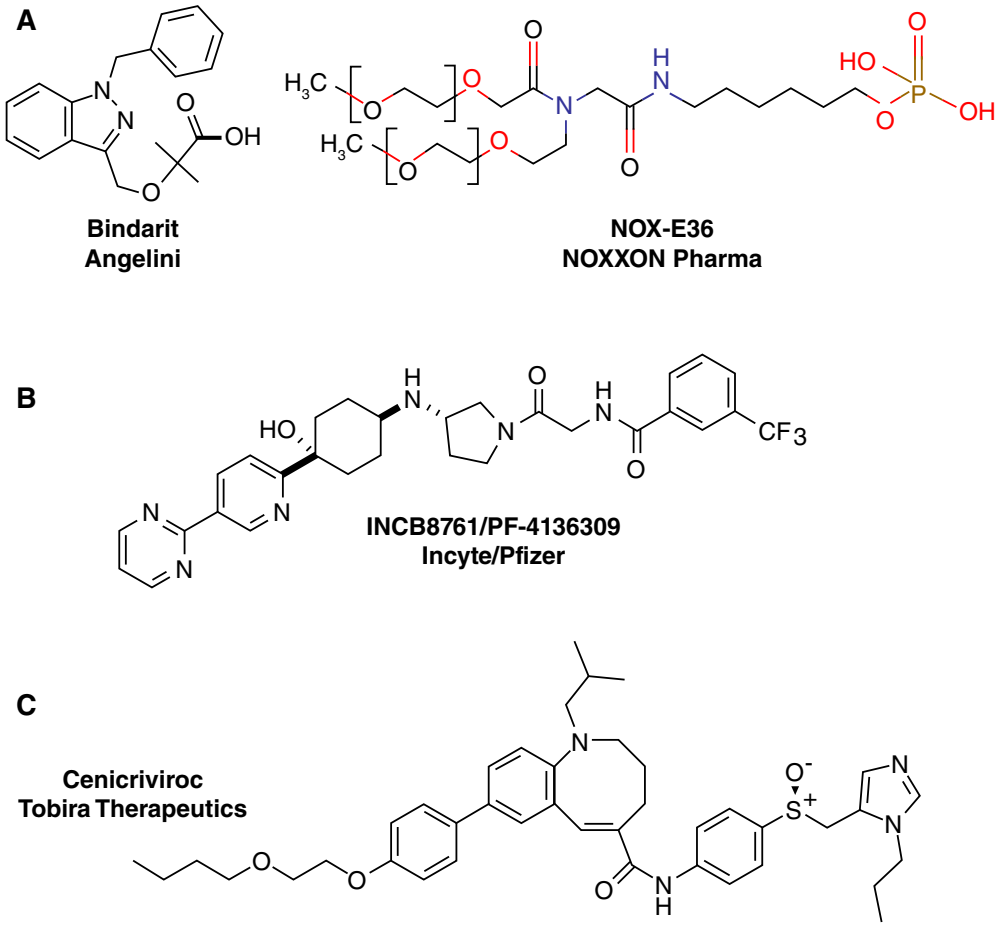

Fig. (3). CCL2/CCR2 targeting compounds with active programs. The figure shows the molecular structures of the drugs targeting CCL2 (**A**), CCR2 (**B**) and CCR2/CCR5 (**C**) that are currently in clinical development and for which the chemical structures have been disclosed. Other compounds are present in active CCR2 (CCX140-B and CCX872-B from Chemocentryx) and CCR2/CCR5 (BMS-813160 and PF-04634817 from Bristol-Myers Squibb and Pfizer, respectively) targeting programs, but their structures are undisclosed.

replication by recruiting target cells and rendering them more susceptible to viral replication, either by enhancing co-receptor expression on T lymphocytes or by inhibiting the expression of innate viral antagonists in macrophages. Further investigation exploring the molecular mechanisms by which CCL2/CCR2 affect HIV replication and pathogenesis may help to define the precise contribution of this chemokine/chemokine receptor pair in these processes, thus opening the way for the investigational treatment of HIV-associated co-morbidities with drugs interfering with CCL2/CCR2. Targeting this chemokine/chemokine receptor pair represents a very active area for drug development with potential application for a variety of important acute and chronic human diseases. Indeed, several compounds have been developed and tested in clinical trials. Although most of these drugs failed from a therapeutic point of view in the indication tested, the majority resulted to be safe and well tolerated. Hence, at least some of these drugs may represent interesting therapeutic options that could be exploited in HIV infected subjects to control viral replication as well as the associated immune activation and inflammation, thus hopefully resulting in further improving quality of care and life expectancy of HIV-infected patients in the post-HAART era.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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