CCR6/CCL20 chemokine axis in human immunodeficiency virus immunity and pathogenesis

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Abstract
Recent studies in human immunodeficiency virus (HIV) have garnered interest for the role of CC chemokine receptor 6 (CCR6) and its known ligands, CC chemokine ligand 20 (CCL20) and human β-defensins, in viral entry, dissemination and antiviral immunity. Several studies have suggested that CCR6 may also act as a weak co-receptor of HIV entry, in addition to the canonical CXC chemokine receptor 4 (CXCR4) and CCR5. However, the pathogenic significance has yet to be demonstrated as the observations for preferential infection of CD4⁺CCR6⁺ over CD4⁺CCR5⁺ T cells appear to be independent of CCR6 expression. This indicates means for preferential infection other than CCR6 co-receptor use. Attention has also turned to the inadvertent role of the CCR6/CCL20 axis in attracting key immune cells, including T₁₁7 cells and dendritic cells, to sites of infection and propagating the virus to other sites of the body. This review article will summarize the latest evidence that the CCR6/CCL20 chemokine axis is playing an important role in HIV pathogenesis and immunity. Further work with in vivo studies is needed to establish the biological and, hence, therapeutic significance of these findings.

CCR6/CCL20 AXIS BIOLOGY
It is well recognized that the human immunodeficiency virus-1 (HIV-1) uses chemokine receptors, CC chemokine receptor 5 (CCR5) and CXC chemokine receptor 4 (CXCR4), to gain entry into CD4⁺ immune cells. Recent times, however, have focused on the virus’ uses of an expanded repertoire of chemokine receptors for this purpose [1, 2]. One such receptor is CCR6 – a seven-transmembrane domain G-protein-coupled receptor whose gene is found on chromosome 6q27 [3]. Unlike most members of the chemokine superfamily, CCR6 has only one confirmed chemokine ligand, CCL20 [3]. CCL20 is constitutively expressed in lymphoid tissue, lungs and gut and is upregulated in inflammation [4]. This observation suggests that the chemokine plays both homeostatic and inflammatory roles. CCL20 mRNA expression has also been found in keratinocytes, neutrophils and T₁₁7 cells [3].

CCR6 was discovered as a functional chemokine receptor in 1997 after the then-orphan receptor, GPR-CY4 (CCR6), was transfected into cell lines and CCL20 established as the only chemokine that induced calcium flux and mobilization on binding [5]. As a chemokine receptor, CCR6 (CD196) is chiefly found on dendritic cells (DCs), memory T cells and selected B cell subtypes, and is involved in the chemotaxis and correct positioning of such cells in effector organs [3, 4]. T₁₁7 cells, a subtype of effector memory T cells, are emerging as a key subset of T cells involved in the pathogenesis of chronic inflammation and autoimmunity. Characteristically, they are phenotyped as expressors of CCR6 and the master transcription factor, RORγt [6]. CCR6 has also been found expressed on NK cell subsets [7] as well as γδ T cells [8].

Mice deficient in CCR6 show structural and functional deficits of the mucosal immune system, suggesting a role for CCR6 and/or its ligand in this domain [9, 10]. Of particular interest, CCR6⁻/⁻ mice had smaller Peyer’s patches and a reduced number of M cells in the follicle-associated epithelia [11]. Recent research has also pointed to a putative role for CCR6 in optimal germinal centre (GC) kinetics and B memory cell function, with CCR6⁻/⁻ mice showing accelerated but dysfunctional humoral immune responses [12–14].

CCR6 has been noted to bind, besides its predominant ligand CCL20, to antimicrobial peptides known as β-defensins, which is thought to be due to the similar
molecular structure between β-defensins and CCL20 [15]. Interestingly, β-defensins may activate and initiate chemotaxis to cells bearing CCR6 [16]; yet this observation has been disputed in recent times because β-defensins do not consistently chemoattract CCR6-positive cells but also CCR6-negative cell lines [15]. These contradictions could be explained by the individual cell lines used, level of expressed CCR6, concentration of ligands used and utilization of other receptors apart from CCR6 [15].

**CCR6 AS AN HIV CO-RECEPTOR**

The receptor CCR6, together with its ligand CCL20, is emerging as an important molecular pair in the pathogenesis and immunity of HIV-AIDS. HIV enters host cells by the binding of its envelope protein gp120 to CD4 and associated host cell co-receptors. This attachment subsequently triggers viral fusion with the cell’s membrane [17]. HIV tropism for selected CD4* host cells is thought to be, in part, mediated by chemokine co-receptor utilization [18]. This is a dynamic process and it is not unusual for HIV to undergo co-receptor switching during the course of the infection [18].

Early studies performed by Nedellec et al. [2] established that NP-2/CD4/CCR6 constructed cells permitted a low entry of HIV-1 pseudotyped with two simian immunodeficiency virus (SIV) envelope glycoproteins, gp160 and gp140, compared with other chemokine co-receptor constructs. Conversely, HIV envelope-pseudotyped viruses (subtypes A–D) taken from 15 patients did not permit entry via CCR6 above background.

A later study co-cultured HIV-1 (subtype B), HIV-2 and SIV isolates with CCR6-transfected human glioma NP-2 cell lines [19]. The percentage of subtype-infected cells was determined by indirect immunofluorescence assays. Compared with CCR5- and CXCR4/NP-2-transfected cell lines, CCR6/NP-2 was not permissible to HIV-1 after 8 weeks of co-culturing. CCR6/NP-2 permitted HIV-2 viral entry at a delayed rate compared with CCR5/NP-2 and CXCR4/NP-2. In comparison, NP-2/CCR5 was highly permissible to SIV, whereas NP-2/CCR6 permitted infection after about 8 weeks and NP-2/CXCR4 did not allow infection at all. The conclusion from these observations indicates that CCR6 has the ability to facilitate viral entry and replication as a weaker co-receptor compared with CCR5 and CXCR4. However, the artificial nature of these in vitro studies and the relative weakness of CCR6 as a co-receptor raise some questions about the biological significance of these findings.

The precise molecular mechanisms for CCR6 co-receptor use have not been elucidated yet. In the previously mentioned investigation [19], it was established that mutations in the C2 region of HIV-2 as well as V1 and V2 of SIV were associated with a CCR6 co-receptor usage. Subsequently, a naturally occurring isoform of CCR6 (CKR-L3), which is truncated at the N-termminus domain by five amino acids, and its ability to permit entry of HIV were studied [20]. Compared with CCR6, CKR-L3 showed a superior co-receptor ability for HIV-2 and SIV entry in NP-2/CD4 cell lines, allowing an infection of a greater number of cells at an earlier time point. Only one out of five HIV-1 primary isolates (and one out of six laboratory isolates) managed to gain entry via both receptors, although to a lesser extent when compared with HIV-2 and SIV. The efficacy for chemotaxis was not explored in this study. However, compared with the CCR5 control, HIV-2 and SIV only demonstrated evidence of entry 1–6 weeks later. These findings suggest that the first five amino acids at the N-termminus are dispensable for co-receptor use and impact negatively on this function in CCR6. The CKR-L3 isoform and its potential differential expression on immune cells have not yet been characterized in the literature. Consequently, whether or not this predisposes an individual to a greater viral burden is not yet known.

From the above study, it is plausible that the N-termminus, hence, plays an important role in mediating viral entry. This could be seen in its closely related co-receptor, CCR5, whose N-termminus domain was established to be important – but not essential – for viral entry [21]. Chemokine receptor intracellular signalling is thought not to be essential for HIV binding and entry [22]; instead, physical interactions with the N-termminus and extracellular domains (ECDs) appear to be important for chemokine and viral ligand binding and engagement of the receptor [23, 24], as studied using the prototypic HIV co-receptor, CCR5.

The other structures which may be important include the ECDs of the chemokine receptor. Using CCR6–CCR5 chimeras in which the ECDs of CCR5 were substituted for CCR6 ECDs in various combinations, one group found that replacement of one or more ECDs prevents the efficient binding of CCR6 to its CCL20 ligand [25]. Because HIV uses selected ECDs of CCR5 to gain cell entry [26, 27], it is possible that CCR6 may permit entry in a similar manner. It is likely not dependent solely on static molecular structures, but requires a complex and subtle conformation of multiple aspects of the three-dimensional receptor structure.

**ANTI-HIV ACTIVITY OF HUMAN β-DEFENSINS VIA CCR6**

Human β-defensins (hBDs) have been described to possess direct anti-HIV properties [28, 29], and CCR6 may also play an indirect role in modulating HIV activity by acting as a receptor for these peptides. Although the mechanisms of anti-HIV activity are poorly understood, one possible mechanism is through forming post-entry complexes with HIV, thus reducing its virulence, as demonstrated by one recent study of human oral epithelium [30].

By binding to and activating CCR6, hBDs were able to promote HIV antigen-specific T cell proliferation in the spleen and at mucosal sites, perhaps in part by stimulating local inflammatory activity [31]. CCR6 activation has also a direct role to play in post-entry anti-HIV immunity via the
induction of apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like 3G (APOBEC3G), which interferes with HIV reverse transcription [32]. Anti-HIV activity was abrogated when CCR6 was eliminated via pertussis toxin or by utilizing a cell line without CCR6 expression, thereby confirming the specificity of this finding [32]. This could provide a mechanism into the anti-HIV activity often seen with hBD-2.

Interestingly, further work has found that higher copy number variations of hBDs are correlated with an increased viral load and impaired CD4+ T cell immune reconstitution, likely because through CCR6, hBDs recruit T_{H17} cells, which can become infected [33].

**INVolVEMENT OF CCR6 IN CELLULAR HIV PATHOGENESIS**

**T_{H17} cells**

The archetypal and best-studied CCR6+ T cells affected by HIV are the T_{H17} cells. T_{H17} cells are specialized CD4+ T cells characterized by the master transcription factor RORγt/RORC2 and the chemokine receptor CCR6 [34]. Because of their CCR6/CCL20 profile, T_{H17} cells are attracted to key immunological sites in HIV infection, such as the gut and lymph nodes (LNs) [35].

T_{H17} cells appear to be important in the control of opportunistic infections in HIV subjects and appear to be the T cell subset predominately infected by HIV [36]. In HIV-infected individuals, CCR6+ T cells harboured more viral DNA than CCR6− T cells [35]. The X4 HIV strain infected mainly T_{H1}T_{H17}, T_{H17} and, to a lesser degree, T_{H2} cells. Because a similar surface expression of CXCR4 was observed on all four cell types, this suggests that there are additional post-entry mechanisms that permit viral replication [35]. Similarly, CCR5 expression on T cell subsets correlated poorly with levels of integration of the R5 HIV strain. Although T_{H1}T_{H17} cells expressed the highest levels of surface CCR5, T_{H17} cells, with moderate CCR5 expression, permitted the highest R5 HIV integration. Nevertheless, these T_{H1}T_{H17} cells tend to be enriched in HIV DNA and demonstrate superior HIV latency compared with other central memory CD4+ T cells [37]. Part of the reason for this phenomenon may lie in the tendency for these cells to migrate to areas where HIV inoculation is high (e.g. the gastrointestinal tract), owing to high CCR6 expression.

Although it may be tempting to think that CCR6 plays a significant role in infection, the effect may be, in part, due to the presence of other classical HIV co-receptors. Alvarez et al. [38] found that although CCR5 expression remained constant between T_{H17} and non-T_{H17} (CD4+CCR6−) cells, there was higher expression of CXCR4 in the former, suggesting that HIV may target T_{H17} cells through this receptor. However, there was also increased CD4 and α4β7 (an integrin that is involved in HIV binding) expression, which could confound these findings. In addition, T_{H17} cells do not express CCR5 ligands, in contrast to T_{H1} cells, which was a factor associated with HIV permissiveness [38].

Others have found that increased CCR5 surface expression could be correlated with the susceptibility of early differentiated IL-17+CD4+ T cells, leading the authors to postulate that this may be a possible mechanism for viral permissiveness [39]. One earlier study [40] used all-trans-retinoic acid (ATRA) (a compound that upregulates α4β7 integrin expression) to study the mechanisms of the preference of the R5-HIV strain to infect CCR6+ memory T cells over CCR6− cells. Indeed, pseudotyped HIV that enter host cells independently of chemokine co-receptors showed a preference for infection of ATRA-treated CCR6+ cells (over untreated CCR6− cells, and compared with treated and untreated CCR6−/− cells), suggesting that the mechanism by which HIV is selective towards ATRA-treated CCR6+ cells is likely through post-entry mechanisms such as integration and transcription [40]. Subtle molecular differences in T_{H17} cells may also be responsible for their increased permissiveness to HIV infection with genome-wide transcriptional profiles of T_{H17} versus T_{H1} cells (as controls) revealing an increased expression of molecules responsible for promoting TCR signalling that are essential for HIV transmission (e.g. ZAP-70 and permissiveness [41]).

A consequence of T_{H17} cell infection is the tendency for this subtype to be depleted in the gut during HIV infection, as compared with healthy controls [42, 43]. In treatment-naive patients, some researchers established that peripheral T_{H17} cells, contrary to the gastrointestinal tract, are not reduced [35, 42, 44], whereas others confirm a reduction in these cells [45–47]. Antiretroviral therapy (ART)-treated patients have lower peripheral blood T_{H17} cells than untreated patients [35, 44, 48]. This may reflect chronicity of the infection or a compensatory rise in uninfected T_{H17} cells in early infection patients. Possible mechanisms for T_{H17} and other CCR6+ T cell depletion are the sequestration and subsequent apoptosis of cells in the spleen [46], loss of T_{H17} precursor cells, or loss of CD4+IL-21+ cells which promote T_{H17} polarization [48, 49].

**T_{H17}-like cells and precursors**

Recent analysis of CD4+ T cell subtypes has recognized a genotypically distinct group of memory T cells, designated stem cell memory T cells [50]. Most of these cells were, in fact, CCR6+ precursors of T_{H17} and T_{H17}-regulatory cells [50, 51]. In HIV-infected individuals on ART, T_{H17}-regulatory cells (IL-17+Treg cells) were found to be preferentially reduced compared with Treg cells [51]. These observations suggest that CCR6 is a marker of this precursor population and that they may be preferentially infected in HIV infections, leading to failure of full T_{H17} polarization and, therefore, decreased T_{H17} cells in HIV patients [48].

Novel transient T_{H17}-like subsets, CCR6+CXCR3−CCR4− and CCR6+CXCR3+CXCR4− cells, have been identified that represent distinct differentiation phases of T_{H17} cells. Like T_{H17} cells, they are permissive to HIV infection, but only CCR6+CXCR3+CCR4− cells are reduced in chronically infected HIV patients on ART. Indeed, ART appeared to...
In addition, low myeloid DCs (CCR6+ cells) play a significant role in the establishment of infection [60]. The initial innate immune response, including DCs, plays a critical role in defending against SIV infection, providing some credence to the notion that innate immunity, has been successful in preventing acute pre- and post-SIV exposure, a compound known to inhibit DCs. Administration of intravaginal glycerol monolaurate CCL20 at the cervical cells of macaques after SIV challenge by Li et al. [59], remains yet to be fully characterized. A paper than equivalent cells in uninfected individuals [53]. Furthermore, early differentiated CD4+ T cells with a Th1 phenotype are more susceptible to HIV infection and are reduced in chronically infected individuals [39]. Like Th17 cells, CCR6+ GC-resident Th17 cells showed preferential depletion in macaques infected with SIV; however, a related subset, CXCR5+ GC Th17, demonstrating a Th1-like phenotype and able to provide B cell help, was markedly increased in SIV infection [54]. HIV also appears to inhibit the migration of CCR6+ B cells to CCL20 via desensitization of CCR6 [55].

CD8+CCR6+ mucosal-associated invariant T cells in infected HIV patients tend to have lower surface CCR6 expression compared with healthy control mucosal-associated invariant T cells [56, 57], possibly leading to their failure to migrate to key immunological sites.

Other lymphocytes

A range of other CCR6+ T cells appear to be targeted by HIV, which has implications for subsequent migration and spread of infection and immune system dysregulation. These include CXCR5+ peripheral follicular helper T (Tfh) cells, which are reduced in the blood of HIV patients and displayed impaired B cell help and secreted less cytokines than equivalent cells in uninfected individuals [53]. Furthermore, early differentiated CD4+ T cells with a Th1 phenotype are more susceptible to HIV infection and are reduced in chronically infected individuals [39]. Like Th17 cells, CCR6+ GC-resident Tfh cells showed preferential depletion in macaques infected with SIV; however, a related subset, CXCR5+ GC Th17, demonstrating a Th1-like phenotype and able to provide B cell help, was markedly increased in SIV infection [54]. HIV also appears to inhibit the migration of CCR6+ B cells to CCL20 via desensitization of CCR6 [55].

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DCs

At sites of infection, DCs become infected in rare cases or commonly, bind or sequester HIV to cross-infect CD4+ T cells in LNs [58]. Whether CCL20 plays a role in CD4+ cell-derived DC trafficking, which is CCR6+ and susceptible to CCL20 [59], remains yet to be fully characterized. A paper by Li et al. [60] demonstrated that the early induction of CCL20 at the cervical cells of macaques after SIV challenge was paralleled with an increase in vaginal recruitment of DCs. Administration of intravaginal glycerol monolaurate pre- and post-SIV exposure, a compound known to inhibit innate immunity, has been successful in preventing acute SIV infection, providing some credence to the notion that the initial innate immune response, including DCs, plays a significant role in the establishment of infection [60].

In addition, low myeloid DCs (CCR6+) and plasmacytoid DCs are noted in the blood of HIV patients. This is negatively correlated with the concentration of plasma CCL20 [61] and HIV viral load [62]. This has led to some authors to speculate that the CCR6/CCL20 axis plays a role in recruitment of DCs to key immunological sites [61].

A detailed study of the role of DCs in SIV infection found that both CCR6 and CCL20 mRNA are expressed at the subcapsular region of LNs at an early stage of infection. However, as the infection progresses, expression of both decline significantly. Assuming this plays a role in DC trafficking, the authors speculated that this axis may assist the early recruitment of DCs to LNs, but as the infection ensues, other chemokine axes (e.g. CCR7–CCL21) take over in their movement [63]. Indeed, this is the premise of one early paper by Dieu et al. [64], which showed that as DCs mature in the periphery, they lose their CCR6 and the corresponding CCL20 responsiveness and upregulate CCR7 for CCL19 chemotaxis. The findings of low CCR6/CCL20 may hence reflect the decreased recruitment of cells and/or chemokine receptor switching.

CCL20 in Viral Pathogenesis and Immunity

Few studies have focused solely on the CCL20 ligand in the role of HIV transmission. One in vitro study [65] found that HIV-positive semen – but not saliva – stimulated HEC-1A (human endocervical epithelial) cells to produce more CCL20 protein, and that there was a trend for increased viral load associated with more CCL20 being produced. Semen lactoferrin is one possible molecule that drives CCL20 production as its concentration was proportional to the amount of CCL20 produced by HEC-1A cells. The relevance of these findings is that HIV-infected semen may therefore promote viral dissemination via the CCL20-mediated recruitment of DCs and lymphocytes at the cervix. Lending some support to this theory, an earlier study by the same group [66] found that semen-induced secretion of CCL20 from a vaginal epithelial cell line (SiHa) was able to attract Langerhans cells via CCR6. The study, however, did not demonstrate significantly more CCL20 secretion via HIV-positive semen [66]. This observation is in keeping with the induction of DCs in the cervix after CCL20 is upregulated at cervical cells following macaques' SIV inoculation [60].

The CCR6/CCL20 axis may be involved in HIV pathogenesis and immunity by more than just chemotaxis. One study established that CCL20, along with other chemokines, was able to facilitate efficient HIV-1 integration in resting CD4+ T cells by binding to CCR6 displayed on these cells. This occurred independently of cell activation and was likely achieved by the rearrangement of the cytoskeleton that facilitated effective transportation of the virus into the nucleus [67]. Because most CD4+CCR6+ cells are Th17 like cells, this is a possible mechanism by which these cells become targeted by the virus and latency established. It also suggests that in areas of high CCR6 expression, such as in the gut mucosa, latency and a viral reservoir can be induced. Of course, other factors need to be taken into account, including the fact that other chemokine ligands have been shown similarly to induce latency as well [68].

On the other hand, in line with direct antimicrobial properties of CCL20 [69], CCL20 demonstrated anti-HIV activity when directly incubated with cells, but not just prior to or post-infection, suggesting a direct interaction with the virus and not pre- or post-entry mechanisms [70]. As CCL20 is found at numerous epithelial and mucosal sites, the chemokine may therefore contribute to the innate defense against HIV entry.
DISCUSSION AND CONCLUSION

Although CCR5 and CXCR4 remain the two primary HIV co-receptors for viral entry, an expanded repertoire of chemokine receptors, including CCR6, is emerging in the HIV paradigm. Although many CCR6+ cells show preferential infection, it is unlikely that this is all due to CCR6 co-receptor usage. However, only limited experiments have been performed in vitro, and to what extent the virus uses CCR6 in vivo is yet to be explored. At present, evidence supports the dominant in vivo usage of CCR5 and CXCR4 [71], and data on a significant biological impact of CCR6 co-receptor utilization are absent. It is certainly possible that CCR6 could be used transiently or evolves as part of natural viral genetic recombination [72]. This remains to be explored in future work.

Where CCR6 may be more relevant in HIV biology is in acting as a receptor for hBDs and CCL20. A major role of the latter appears to be the inadvertent provision of a pathway for HIV dissemination via the recruitment and subsequent migration of cells during the course of anti-HIV immunity. As a result, blockade of chemokine pathways, including CCR6/CCL20, may be a possible avenue for therapeutic intervention to prevent dissemination of the virus to key immunological sites. Conversely, CCR6 directly functions as an inhibitory receptor for HIV by interfering with viral reverse transcription [32] and direct antiviral activity through hBDs (see Anti-HIV activity of human β-defensins via CCR6).

Thus, CCR6 appears to be a double-edged sword in HIV infection, which contributes to immunity and spread of HIV. An appropriate model that summarizes its relative contributions can be seen in Fig. 1. The relative contribution of CCR6 as a co-receptor appears relatively minor; but additional clarification is needed in in vivo studies to further characterize this aspect. Treatment of latency is a major barrier in HIV medicine, and further understanding of the signalling pathways involved would be vital in developing strategies to prevent this. The CCR6/CCL20 axis is, hence, an emerging molecular pair in our understanding of the growing HIV-AIDS pandemic.

Fig. 1. Putative model for the involvement of CCR6/CCL20 in HIV pathogenesis and immunity. (1) HIV breaches epithelial surface and damaged cells release CCL20 chemokines, which have anti-HIV properties. (2) CCR6+ DCs are attracted to the site and are infected by HIV or bind/sequester the virus. (3) DCs migrate to draining LNs, and CD4+ T cells are also infected. (4) Naive CD4+ T cells differentiate into T_h 17 cells, and as they are CCR6+, they migrate down the chemokine gradient to the site of infection. (5) T_h 17 cells become infected by HIV and are gradually depleted.

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