

Preface

Although there have been many books on HIV and AIDS, surprisingly little has been published that focuses on the immunology of retroviral infections in general, and HIV in particular. *Retroviral Immunology: Immune Response and Restoration* is the first book of its kind to address the most important aspects of the immunology of retroviruses, including not only the virus-specific immune responses, but also genetic and virologic factors modulating these responses.

The book also deals directly with the emerging concept of immune restoration in retroviral infections, a particularly important subject to the thousands of clinicians who deal with this problem on a daily basis. With the advent of highly effective antiviral drug regimens to slow down the replication of HIV and the progression of AIDS, new challenges and opportunities are arising. Restoration of general immune function has brought with it not only complications of immune restoration-mediated disease, but also the realistic hope for meaningful restoration of the ability to control HIV replication with the immune system.

Leading scientists in the field have summarized the most current information regarding experimental and clinical aspects of retroviral infections. *Retroviral Immunology: Immune Response and Restoration* should prove an important point of reference for basic scientists and clinicians in this area of research. We are indebted to all of our authors for their excellent contributions.

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Role of Chemokines and Their Receptors in the Pathogenesis of HIV Infection

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INTRODUCTION

The chemokines comprise a rapidly expanding group of *chemotactic cytokines* now known to play a critical role in regulating leukocyte trafficking during development and in homeostasis, inflammation, and infection. In the brief period since the original description of platelet factor 4 (PF4) (1) and interferon-inducible protein of 10 kDa (IP-10) (2) this family has grown to more than 50 members. Numerous studies now link chemokines to the pathogenesis of a wide range of inflammatory processes including asthma, atherosclerosis, pneumonia, meningitis, psoriasis, rheumatoid arthritis, inflammatory bowel disease, and sarcoidosis, among others (3). Many also play roles in angiogenesis, hematopoiesis, and fetal development.

Chemokine and chemokine receptor genes have also been pirated by pathogens. Many herpes viruses encode chemokine-like molecules as well as functional chemokine receptors. In addition, several intracellular pathogens such as *Plasmodium vivax* (4), Poxviruses (5) and HIV-1 (6) utilize chemokine receptors to gain access to the cytoplasm of leukocytes. The recent discovery that HIV-1 uses chemokine receptors together with CD4 to enter cells has been a major advance in understanding the pathogenesis of HIV-1 infection and has revealed an entirely new class of therapeutic targets. In this chapter, we briefly review general aspects of chemokine biology (3,7,8), and then examine in detail the role of chemokines and their receptors in the pathogenesis of HIV-1 disease.

CHEMOKINES AND CHEMOKINE RECEPTORS

Structure

Chemokines are 8–10-kDa proteins sharing 20–95% amino acid homology (reviewed in 3,7,8). They are divided into families based on the arrangement of four conserved cysteine residues. In the α -chemokines, the first two cysteines are separated by a single amino acid (cysteine–X amino acid–cysteine, or CXC). In the β -family, these two cysteines are adjacent (cysteine–cysteine, or CC). Lymphotactin, a “C” chemokine, is homologous to CC members but lacks the first and third canonical

cysteines (9). Fractalkine contains a unique CX3C motif and may represent a new class in which the chemokine moiety sits atop a membrane-anchored mucin-like stalk (10). While a soluble cleavage product of the chemokine domain is chemotactic for T cells and monocytes, the membrane-bound form of fractalkine is expressed on activated endothelial cells and mediates leukocyte adhesion (11).

Several generalizations can be made with respect to the chemokine families. The CC or β -chemokines represent the largest group and generally attract monocytes, lymphocytes, basophils, and eosinophils but not neutrophils. The monocyte chemoattractant proteins (MCPs 1–5) and the eotaxin molecules (eotaxin 1–3) form a closely related subfamily within the β -chemokines (12). Members of the α -family containing the sequence glutamic acid–leucine–arginine amino (N)-terminal to the CXC motif (E-L-R-C-X-C) are chemotactic for neutrophils (e.g., interleukin-8 [IL-8], GRO), while those lacking this ELR sequence attract lymphocytes (e.g., interferon-induced protein 10 [IP-10], stromal cell-derived factor [SDF-1 α]). Many chemokines bind multiple receptor molecules, often overlapping with one another. Some, however, are presently known to bind only a single receptor (e.g., eotaxin and CCR3, MIP-1 β , and CCR5, and SDF-1 α and CXCR4). CC and CXC chemokines do not share common receptors.

The chemokine receptors belong to the seven-transmembrane G protein-coupled receptor (GPCR) group, which includes a diverse array of 1000–2000 signaling molecules constituting >1% of the vertebrate genome (13). While the families lack sequence homology, they share a common seven transmembrane core (TMI–VII) with three intracellular (i1–i3) and extracellular (e1–e3) loops. Two conserved cysteines in e1 and e2 are disulfide bonded. The TMI–VII domains are thought to form a circle in the cell membrane, with grouping of the corresponding ectodomains to generate chemokine-binding site(s). Ligand interaction changes the receptor core conformation, exposing G protein binding sites by which further signaling occurs. These models await confirmation by high-resolution structural analyses.

Chemokine Signaling and Function

Chemokines mediate diverse biological effects including chemotaxis, activation of integrins and adhesion, coordinated lymphocyte and dendritic cell trafficking, and selective recruitment of polarized lymphocyte subsets (3,7,8,14). These functions play a central role in inflammatory and immune responses, as well as in pathologic states such as autoimmune disease, and are tightly regulated at numerous levels, for example, temporal–spatial control of ligand–receptor gene expression, chemokine binding by extracellular matrix, modulation of receptor signaling pathways, and interaction with other signaling pathways, for example stem cell factor/*kit* ligand (SCF/KL)/*c-kit* (15).

The intracellular events linking receptor activation to effector function are only beginning to be unraveled. Numerous biochemical phenomena have been described; however, the causal relationships among these remain to be fully defined. Chemokine receptors are coupled to the G_i subfamily of G proteins. Pertussis toxin (PTX), which ADP-ribosylates and irreversibly inactivates the $G\alpha$ subunit of the i class, inhibits the majority of chemokine-induced effects on leukocytes, including chemotaxis, calcium flux, and integrin activation (16). Other signaling events triggered by the majority of chemokine receptors include the activation of phospholipase C (PLC) leading to gen-

eration of inositol triphosphate, intracellular calcium release, and protein kinase C (PKC) activation. Phorbol myristate acetate (PMA) activation of integrins implicates PKC as a potential mediator by which chemokines activate integrins. Inhibition of chemokine-induced chemotaxis by wortmannin implicates phosphatidylinositol 3-kinase (PI3K) in chemokine receptor signal transduction. Chemokine signaling also leads to guanine nucleotide exchange on Rho, indicating the latter's activation (17). Rac and Rho are small GTP binding proteins involved in controlling cell locomotion via actin cytoskeletal rearrangement, membrane ruffling, and pseudopod formation.

Agonist-stimulated receptors also activate G protein receptor kinases (GRKs), which in turn phosphorylate serine and threonine residues in the tail of GPCRs (18). Receptor phosphorylation is followed by binding of arrestins and uncoupling from G proteins (desensitization). In addition, the arrestins function as adaptor molecules leading to clathrin-mediated endocytosis (internalization). These internalized receptors are either dephosphorylated by phosphatases and recycled to the cell surface or targeted for degradation. Desensitization and recycling of chemokine receptors may be an important mechanism by which leukocytes maintain their ability to sense a chemoattractant gradient during an inflammatory response, whereas degradation of chemokine receptors may lead to termination of migration. Thus, chemokine receptors activate multiple signaling pathways that regulate the intracellular machinery necessary to propel the cell in its chosen direction.

CHEMOKINE RECEPTORS ARE OBLIGATE CORECEPTORS AND DETERMINE HIV-1 TROPISM

Soon after the initial isolation of HIV-1 in the mid-1980s, its tropism for CD4-bearing cells rapidly led to the identification of CD4 as a viral entry receptor. Several early observations, however, suggested that the CD4 molecule by itself was insufficient for entry. For example, nonhuman cells transfected with CD4 still required a human-specific cofactor to support membrane fusion.

In addition, a single-receptor model could not explain the phenomenon of viral tropism. It was found that different isolates of human immunodeficiency virus type 1 (HIV-1) could be broadly classified into three phenotypic groups. Although all isolates could infect primary CD4⁺ lymphocytes, they differed in their ability to infect primary monocytes and immortalized laboratory T cell lines such as MT-2 cells. Viral strains could be phenotypically grouped as M tropic (able to infect primary monocytes), T tropic (able to infect laboratory T cell lines), or dual tropic (able to infect both), and this specificity was found to be determined by the V3 loop of the gp120 envelope molecule. These observations strongly suggested that there must be another receptor for HIV-1 in addition to the CD4 molecule.

In a landmark experiment, Berger and co-workers (19) used an expression cDNA cloning strategy to isolate "fusin," a seven-transmembrane G protein coupled receptor, which when coexpressed with CD4, permitted T-tropic (but not M-tropic) Env-mediated fusion and infection of nonhuman cells. At the time, fusin was an "orphan receptor" with homology to the receptor for CXC chemokine IL-8. These observations prompted speculation that the known inhibitory activity of RANTES on M-tropic HIV-1 (20) might occur via a CC chemokine receptor functioning as a fusion cofactor for M- but not T-tropic virus. In short order, five groups reported that CCR5—a receptor

for MIP-1 α , MIP-1 β , and RANTES—functioned as a coreceptor for M-tropic HIV-1 (21–25). “Fusin” was subsequently found to be a receptor for the CXC chemokine SDF-1 and renamed CXCR4. HIV-1 strains are now designated “R5” (CCR5), “X4” (CXCR4), or “R5X4” (CCR5/CXCR4 dual tropic) depending on coreceptor usage (26). In general, R5 strains are M tropic while X4 strains are T tropic.

Although CCR5 and CXCR4 are believed to be the primary cofactors for viral entry in vivo, numerous reports have shown that other chemokine receptors can function similarly. Thus, the dual-tropic primary HIV-1 isolate 89.6 uses CXCR4, CCR5, CCR3, and CCR2 (24)—an unexpectedly broad repertoire (while CCR5 shares 76% identity with CCR2, only 21% of its extracellular amino acids are homologous to CXCR4). Using an env-complementation assay, Choe et al. (25) found that CCR3 enhanced infection (9–32-fold) by viruses with ADA and YU2, but not BR20-4 or HXBc2 envelope glycoproteins. This effect was abolished by addition of CCR3 ligand eotaxin (60 nM). Other investigators have demonstrated coreceptor function for CCR8 (27), CX3CR1 (28,29), the orphan chemokine-like receptors STRL33/Bonzo (30,31), gpr1 (32,33), gpr15/BOB (31,34), ChemR23 (35), D6 (36), and the angiotensin receptor-related Apj (36,37). By contrast, the Duffy blood group antigen, a seven-transmembrane GPCR with 20% homology to CXCR4, does not support membrane fusion by HIV-1 89.6, IIIB, or JR-FL env proteins (24).

How are we to interpret these in vitro data? The clinical relevance of experiments using laboratory strains of HIV-1 or cell fusion assays is unclear. While in vivo genetic analysis has underscored the critical importance of CCR5 (see later), other coreceptors expressed in specific target tissues, such as CCR3 on microglia in the central nervous system, may play a critical role in the pathogenesis of HIV-1 infection. However, these hypotheses await definitive experimental validation.

STRUCTURE–FUNCTION STUDIES REVEAL CONFORMATIONALLY COMPLEX INTERACTIONS BETWEEN SPECIFIC HIV-1 STRAINS AND CORECEPTORS

As described in the preceding section, CD4 and chemokine coreceptors and viral Env protein are the main components involved in HIV entry into target cells. The HIV-1 Env protein complex is initially produced as a gp160 precursor, which is then proteolytically cleaved into two subunits: (1) The external gp120 subunit, which is heavily glycosylated and contains the CD4 and coreceptor binding sites; and (2) the membrane-spanning gp41 subunit, which is derived from the C-terminal portion of the precursor and contains an N-terminal, hydrophobic peptide directly involved in the fusion process. These two subunits remain noncovalently associated and oligomerize as trimers on the virion surface.

The mechanism of viral entry has been examined intensively, with the following overall mechanism now generally accepted (6,38): CD4 interaction with the gp120 subunit induces conformational changes, which presumably create, stabilize, or expose the coreceptor binding site on gp120. This is followed by binding of gp120 to coreceptor, conformational changes in the Env protein, and activation of gp41. Fusion of the viral membrane with the target cell membrane is mediated by exposing and extending the gp41 fusion peptide, allowing it to insert into the plasma membrane of the target cell. In support of this model, complexes of CD4, gp120 and coreceptor have been iso-

lated (39), and mutational, antigenic, and X-ray crystallographic studies (40–42) have confirmed conformational changes in gp120.

Chemokine function is mediated by coupling of their receptors to G proteins, initiating a complex array of intracellular signaling pathways. Although soluble Env protein can induce signaling via CCR5 and CXCR4 (43), it is now generally accepted, that Env-mediated fusion can occur independently from G protein coupled signaling (44–47).

The domains of the coreceptor involved in the interaction with gp120 have been extensively investigated. Mutational studies, receptor chimeras, antireceptor antibodies and other methods have yielded complex and sometimes contradictory results (6,48–51). The N-terminus and extracellular loops of the coreceptors seem to be important for gp120 binding and subsequent viral entry, whereas the intracellular loops and the so-called DRY motif required for G protein coupled signaling, do not seem to play an important role in coreceptor function. It should be noted that coreceptor modifications can have variable effects for different Env proteins.

gp120 contains 5 variable loops (V1–V5) interspersed with five relatively conserved regions (C1–C5). The V3 loop is critical for viral fusion and tropism, as well as coreceptor binding and specificity. X-ray crystallographic, mutational, and antigenic studies (40–42) suggest that the coreceptor interacts with the V3 loop, a “bridging sheet” of the V1/V2 stem, and an antiparallel, four-stranded structure including parts of the C4 domain.

The CD4-dependent interaction of gp120/CD4/coreceptor is undoubtedly the main mechanism for HIV-1 entry and fusion. However, certain Env proteins can interact with coreceptor in the absence of CD4 resulting in fusion and entry, as first described for HIV-2, and later for SIV and HIV-1 (52–58). However, this pathway is less efficient, and addition of CD4 in most cases enhances binding of gp120 and coreceptor. The biological relevance of this CD4-independent pathway is unclear. Nevertheless, the ability to select for CD4-independent strains of HIV-1 and simian immunodeficiency virus (SIV) *in vitro*, as well as the natural CD4-independence of feline immunodeficiency virus, suggest that chemokine receptors are probably the key receptors for viral entry.

CHEMOKINE RECEPTORS ARE MAJOR HOST GENETIC FACTOR IN INFECTION AND DISEASE PROGRESSION

Numerous host factors including determinants of innate and adaptive immune function (e.g., major histocompatibility alleles, chemokine/cytokine expression levels, mucosal immunity) as well as coreceptor mutations have been linked to disease susceptibility and progression. The complex interplay of these factors is only beginning to be unraveled (59,60). In the following sections, we focus on the recent findings regarding mutations/polymorphisms in chemokines and their receptors (Table 1).

CCR5 $\Delta 32$

Studies of individuals who remained uninfected despite multiple high-risk exposures to HIV-1 (“exposed-uninfected” or “EU” individuals) demonstrated that in some cases, their CD4⁺ T cells were resistant to infection *in vitro* (61). Analysis of two such individuals showed that (1) T cells were resistant to M-tropic but not T-tropic virus; (2) approx 10-fold higher levels of β -chemokines were secreted by T cells from one EU

Table 1
Inherited Polymorphisms in Chemokine and Chemokine Receptor Genes that Affect Susceptibility to HIV-1 Infection and Progression

Molecule	Polymorphism	Sites	Type	Freq	Genotypes		Mechanism
					-/-	+/-	
CCR5	CCR5 Δ 32	ORF	Del	10%	R	DP	Truncation
	m303	ORF	SNP	1%	R	ND	Truncation
	59029G/A	Prom	SNP	50%	DP	—	ND
CCR2	CCR2-64I	ORF	SNP	10%	ND	DP	ND
SDF-1	SDF-1 3' A	3' UTR	SNP	21%	DP	—	ND

Abbreviations: Freq, Allelic frequency; -/-, homozygous for the given allele; +/-, heterozygous for the given allele; ORF, open reading frame; SNP, single nucleotide polymorphism; Del, deletion; Prom, promoter; 3' UTR, 3' untranslated region of the mRNA; R, HIV-1 resistance; DP, delayed progression to AIDS relative to other genotypes; ND, not determined, NE, no effects.

Table (including allelic frequencies) adapted from Berger et al. (6).

compared to controls (62); and (3) both individuals were homozygous for a 32-bp deletion in the second extracellular loop of the CCR5 gene. This so-called Δ 32 mutation resulted in a frameshift and premature translation termination with consequent failure of the truncated receptor to reach the cell surface (63). Similar results were reported by other investigators (64–66). Of note, no obvious clinical effect of the Δ 32/ Δ 32 genotype was seen implying that CCR5 is nonessential, which has important therapeutic implications.

Heterozygosity for the Δ 32 deletion also appears to be beneficial, reducing cell surface coreceptor expression by gene dosage as well as heterodimerizing with wild-type CCR5 and trapping it in the endoplasmic reticulum (67). In vitro, using the M-tropic SF162 strain, Liu et al. (63) found that although EU CD4⁺ T cells (Δ 32/ Δ 32) did not support viral replication, parental (heterozygous) cells permitted an intermediate level of replication. Patients homozygous for the Δ 32 mutation were not found in an HIV-1-infected cohort of Caucasian subjects, and heterozygotes were 35% less frequent in this group compared to the general population (64). Analysis of 1955 patients from six acquired immune deficiency syndrome (AIDS) cohort studies showed that while heterozygosity did not appear to confer reduced susceptibility to infection, the Δ 32/+ genotype was associated with delayed progression to AIDS in infected subjects (65). Similarly, in studies of vertical transmission, heterozygosity in the child was not protective but may be associated with slower development of HIV-related disease (68–71). It should be noted that these effects were not consistently seen in all patient groups (72–74).

The Δ 32 allele is found almost exclusively among Caucasians, and exhibits a north-to-south geographic cline across Eurasia (75–77). In a screen of 3342 individuals, the frequency of this deletion was 20.93% (Ashkenazi), 14.71 (Iceland), 11.13 (Britain), 9.78 (Russia: Udmurtia), 8.62 (Spain), 2.94 (Pakistan), 2.07 (Saudi Arabia), 0.50 (Thailand), 0.45 (Nigeria) (77). The near absence of the mutation in native African, American Indian and East Asian ethnicities suggests a recent and unique origin, which has been estimated at ~1200 AD by haplotype analysis (76). The current high allele frequency of 5–15% as well as the recent finding that other naturally occurring CCR5

mutations are predominantly codon altering (nonsynonymous; 78) suggest a positive selective force for these alleles. Given the estimated origin of $\Delta 32$ approx 700 yr ago, some speculate that this allele might have conferred a protective effect during the Black Death (1346–1352 AD) or against other widespread pathogens targeting macrophage (e.g., *Shigella*, *Salmonella*, *Mycobacterium tuberculosis*) (76).

Homozygosity for the $\Delta 32$ deletion provides nearly complete protection against the M-tropic isolates studied thus far, implying that CCR5 is in fact the critical coreceptor in HIV-1 infection. The identification of rare HIV-1+ $\Delta 32/\Delta 32$ individuals, however, indicates that other routes of viral entry in vivo exist. In one such patient with hemophilia A, a homogenous SI, T-tropic quasispecies was found. This individual's viral isolates used CXCR4 exclusively as coreceptor during the period of observation, which began 4 yr postinfection (79,80). Consistent with the increased virulence of X4 virus, relatively rapid CD4 loss was reported (see also 81,82). These observations together with a Danish study showing decreased survival after diagnosis of AIDS in $\Delta 32$ carriers (83) have raised concern that CCR5 antagonists may be detrimental.

Other Disease-Modifying Receptor Mutation/Polymorphisms

The CCR5 $\Delta 32$ allele is present in only a fraction of EU individuals. Moreover, $\Delta 32/+$ heterozygous long-term nonprogressors do not appear to differ significantly from wild-type nonprogressors with respect to immunologic and virologic parameters (84). These observations imply the existence of additional genetic or epigenetic factors that influence susceptibility to infection and disease course. The complexity of such interacting factors has made direct comparison of cohorts problematic and reduced the likelihood of identifying single-gene effects. Nevertheless, a number of additional disease-modifying mutations/polymorphisms have been discovered (see 6):

- *CCR5 m303*: One EU heterozygous for the $\Delta 32$ allele was found to have a T→A mutation at position 303 of CCR5 on the other allele (i.e., genotype CCR5 m303/CCR5 $\Delta 32$) (85). The m303 mutation results in premature translation termination and lack of functional receptor expression. Its precise frequency in the general population is not known.
- *CCR5 59029 A/G* promoter polymorphism: McDermott et al. (86) identified an A/G polymorphism at position 59029 in the CCR5 promoter. In an in vitro CAT assay system, the 59029-G promoter was 45% less active than 59029-A, although this site is not part of a known transcription factor binding element. Patients with genotype 59029-G/G progressed to AIDS significantly more slowly than those homozygous for 59029-A. CCR5 transcription is complex—consisting of at least two promoters, multiple start sites, complex alternative splicing, and polymorphisms in regulatory as well as other 5' noncoding sequences (87). Consistent with this, other possible disease-modifying promoter polymorphisms are beginning to be reported (88,89).
- *CCR2-V64I*: A conservative valine → isoleucine change in the first transmembrane domain of CCR2 delayed disease progression in some (90–93), but not all (94,95) cohorts studied. These results were unexpected, as few HIV-1 strains use CCR2 as coreceptor. In vitro studies of CCR2b, the major CCR2 isoform, showed that the V64I mutation is expressed comparably to wild-type protein and retains coreceptor function. In addition, this polymorphism did not affect MCP-1 induced calcium mobilization, or expression/coreceptor activity of CCR5 or CXCR4 in cotransfected cell lines (96). The V64I polymorphism was associated with a small decrease in CXCR4 levels in unstimulated peripheral blood mononuclear cells (PBMCs). Recently, it has been reported that CCR2-V64I is in complete linkage disequilibrium with a CCR5 promoter mutation (position 59653) (92,97) but not associated with decreased CCR5 expression.

Table 2
Chemokine Receptors Known to Support HIV-1 Entry and Blocking Ligands

Receptor	Ligands	Virus
CCR2	MCP-1, MCP-2, MCP-3, MCP-4	R5X4
CCR3	RANTES, MCP-2, MCP-3, MCP-4, Eotaxin-1, Eotaxin-2, Eotaxin-3	R5X4
CCR5	RANTES, MIP-1 α , MIP-1 β , MCP-2	R5, R5X4, HIV-2
CCR8	I-309	R5X4, HIV-2
CXCR4	SDF-1 α , SDF-1 β	X4, R5X4
CX3CR1	Fractalkine	R5X4, HIV-2
APJ	(Unknown)	R5X4
ChemR23	(Unknown)	
GPR15/Bob	(Unknown)	R5X4
STRL33/Bonzo	(Unknown)	R5X4
D6	(Unknown)	
US28 (cytomegalovirus)	RANTES, MIP-1 α , MIP-1 β	R5X4

The preceding discussion attests to the complexity of identifying disease-modifying genetic factors in heterogenous populations. While the dominant role played by CCR5 in disease pathogenesis is clear, analyses of other polymorphisms/mutations have not been completely concordant. This may reflect numerous factors including tight linkage of CCR1, CCR3, CCR4, CCR8, and CXCR1 (98), sampling bias (e.g., use of seroprevalent versus seroincident cohorts), statistical power, and epigenetic phenomena, among other possibilities.

CHEMOKINES ARE POTENT INHIBITORS OF HIV-1 ENTRY

The existence of cytokine inhibitors of HIV-1 replication was first proposed in 1986, when it was found that CD8⁺ T lymphocytes could mediate viral suppression in vitro at least in part through released soluble factors (99,100). The first such factors to be isolated from CD8⁺ T cells were MIP-1 α , MIP-1 β , and RANTES (20). These chemokines could inhibit M-tropic but not T-tropic strains of HIV-1 in vitro, suggesting that they might bind a common receptor used by M-tropic HIV-1 strains. As noted above, this indeed proved to be the case. Soon thereafter, the orphan chemokine receptor CXCR4 was identified as the specific “coreceptor” for T-tropic strains of virus. The discovery of MIP-1 α , MIP-1 β , and RANTES as ligands of CCR5 that block R5 strains of HIV-1 in vitro, and the identification of SDF-1 as a CXCR4 ligand that blocks entry of X4 but not R5 virus confirmed the hypothesis that chemokine receptor ligands are specific antagonists of viral entry (101,102).

Although all strains of HIV-1 are classifiable as R5, X4, or R5X4 a growing list of other chemokine receptors aside from CCR5 and CXCR4 have been found to serve as entry receptors for HIV-1 in vitro (Table 1). Their specific chemokine ligands generally block viral entry. Because of the epidemiological evidence regarding the critical importance of CCR5 in disease transmission and the fact that CCR5 and/or CXCR4 are found on monocytes, dendritic cells, and CD4⁺ T lymphocytes, the predominant

primary cell types known to be infected *in vivo*, it is unclear what role these other receptors and their ligands might play in the pathogenesis of infection.

A possible exception to the specific mechanism of chemokine blockade of specific chemokine receptors involved in viral entry is the chemokine MDC. This chemokine, which is a ligand for CCR4 and not CCR5 or CXCR4, has been reported to block entry of both R5 and X4 strains of HIV-1 through an undetermined mechanism (103). This activity remains controversial, however, as several other studies have failed to validate these findings (104).

MECHANISM OF CHEMOKINE-MEDIATED INHIBITION OF HIV-1

Chemokines have been postulated to inhibit HIV-1 entry into cells by (1) direct competition with HIV-1 envelope for chemokine receptor binding and (2) induction of chemokine receptor internalization. Experimental evidence suggests that both can contribute to suppression of viral entry, and clearly these mechanisms are not mutually exclusive. Because chemokine action involves binding to receptor followed by receptor activation, phosphorylation and internalization, chemokines may potentially suppress viral entry through both pathways.

Support for simple competitive inhibition of HIV-1 entry comes from experiments using peptide (e.g., T22 105, ALX40-4C 106) and small molecule (e.g., AMD3100 ([107,108]) antagonists of CXCR4. Several such compounds are capable of binding CXCR4 without signaling through the receptor (and presumably not downregulating it), and have been found to inhibit entry of X4 strains of HIV-1 into cells. This suggests that binding of a receptor by a ligand alone is enough to compete with viral entry. Furthermore, a study of varying N-terminal truncated forms of SDF-1 indicated that CXCR4 binding affinity was inversely correlated with ability to antagonize viral entry, again suggesting that receptor binding can compete with viral entry (109).

Other data indicate an important role for receptor downregulation as the mechanism of chemokine-mediated inhibition of HIV-1 entry. Studies of N-terminal modified RANTES and SDF-1 have revealed that downregulation of chemokine receptors has a potent effect on viral entry (110–112). Modifications of these chemokines which enhance receptor downregulation (presumably through more efficient internalization and/or impaired receptor recycling to the cell surface) markedly increase viral blocking activity. This increase in activity can be observed even in the absence of Gi-mediated signaling, as noted for aminooxypentane–RANTES, or decreased receptor affinity, as in the case of methionine–SDF, indicating that downregulation of the chemokine receptor is the key factor in the enhanced antiviral activity of these compounds. In addition, a small molecule inhibitor of HIV-1 cell fusion (NSC 651016) was shown to downregulate CCR5 and CXCR4 (113). Furthermore, carboxy (C)-terminal non-signaling truncation mutants of CXCR4 while able to support viral entry were not inhibitable by the addition of SDF-1, its natural ligand (114,115). Thus, although chemokine receptor signaling is not required for viral entry, it is required for chemokine-induced inhibition of HIV-1 entry.

The chemokines are basic proteins and bind avidly to negatively charged heparin and heparan sulfate (116–119). Heparan sulfate proteoglycans serve to capture chemokines in the extracellular matrix and on the surface of cells, which may serve to establish a local concentration gradient from the point source of chemokine secretion (116). In

addition, proteoglycans can influence the biological activity of chemokines, and several studies have demonstrated that they play an important role in chemokine-induced inhibition of HIV-1 entry (120–122). Because chemokines are secreted from HIV-1-specific CTL bound to proteoglycans this interaction is likely to be relevant *in vivo*.

CHEMOKINE LIGANDS AS HOST GENETIC FACTOR IN INFECTION AND DISEASE PROGRESSION

Despite evidence that numerous chemokine ligands can inhibit HIV-1 entry via their receptors *in vitro*, the *in vivo* relevance of this phenomenon remains unclear. Whether chemokines serve a directly antiviral role *in vivo* is debatable, in contrast to the well-established clinical impact of CCR5 mutations on susceptibility to infection and disease progression. Clinical correlations of chemokine levels in HIV-1 infection have provided circumstantial evidence that chemokines may have roles both in protection from infection as well as affecting the rate of disease progression (reviewed in 123).

Although some heavily HIV-1-exposed yet uninfected individuals are resistant to infection by virtue of CCR5 $\Delta 32$ mutation homozygosity, the vast majority are not of this genotype. One proposed immunologic mechanism is protection by MIP-1 α , MIP-1 β , and RANTES. This is based upon the observation of higher production of these CC chemokines by mitogen-activated PBMCs from highly exposed yet uninfected homosexuals (124) and hemophiliacs (125), as compared to other seronegative controls. Similarly, CD4⁺ lymphocytes from highly EU individuals with wild-type receptors for CCR5 were found to be more sensitive to the HIV inhibitory effects of β -chemokines (124). Long-term nonprogressing infected individuals appear to have increased production of MIP-1 α , MIP-1 β , and RANTES as well, indicating that these chemokines may affect the rate of disease progression (125a). Although these results correlate apparent protection from infection and disease progression with increased chemokine production by PBMCs, it is not clear whether protection is mediated by the chemokines themselves or an associated factor such as the activated T cells that produce them.

Further indirect evidence for an impact of chemokines on the course of disease includes a correlation of a polymorphism in the 3' untranslated region of SDF-1. This is a G to A transition at bp 809 of the 3' untranslated region of the mRNA of SDF-1 β , also designated SDF-1 3'A (this is found only with SDF-1 β , and not in SDF-1 α , which is a splice variant of the same gene). An initial report suggested a statistically significant correlation of homozygosity for this polymorphism with delayed progression to AIDS, particularly in patients infected for longer periods (126). The postulated mechanism was an effect of this polymorphism on posttranscriptional modulation of SDF-1 levels; however, this has never been demonstrated. A second study failed to confirm the association. In fact, in this second cohort, 3' A homozygosity was associated with more rapid progression to death (97). The reasons for this discrepancy are unclear, and the precise molecular effect(s) of the 3'UTR mutation remains to be determined.

THERAPEUTIC IMPLICATIONS

The discovery that HIV requires a chemokine coreceptor to invade host cells and that chemokines can inhibit HIV-1 entry into cells has prompted many investigations into therapeutic strategies that target these receptors in an attempt to block HIV entry. The concept that blocking HIV-1 entry into cells is a useful drug strategy was sup-

Table 3
Anti-HIV Therapeutic Strategies Based on Chemokine Receptors

Strategy	Therapy	Target
Immune restoration	Downregulation of CCR5 on CD4 ⁺ T cells	CCR5
Gene therapy	Antisense	CCR5, CXCR4
	Ribozymes	CCR5, CXCR4
	Single chain mAbs	CCR5, CXCR4
	Intrakines	CCR5, CXCR4
Immunotherapy	mAbs	CCR5, CXCR4
Chemokines	MIP-1 α , MIP-1 β , RANTES	CCR5
	SDF-1	CXCR4
Chemokine analogs	Met-RANTES	CCR5
	AOP-RANTES	CCR5
	Met-SDF-1	CXCR4
Peptide antagonists	T22	CXCR4
	ALX40-4C	CXCR4
Small molecule antagonists	AMD3100	CXCR4
	NSC 651016	CCR5, CXCR4

Adapted from Cairns et al. (128).

ported by studies using a peptide T-20 that inhibits the interaction of gp41 with the cell membrane (127). This inhibitor was able to significantly reduce viral load in HIV infected patients and revealed that blocking HIV entry could complement existing anti-retroviral drug therapy (127). We briefly summarize some approaches being developed (Table 3), which have also been reviewed by Cairns et al. (128).

Novel strategies are being developed to sequester or prevent the expression of chemokine receptors in order to make cells resistant to HIV. These include inducing CCR5 downregulation or gene therapy approaches to prevent chemokine receptor expression. Activation of CD4⁺ T cells with monoclonal antibodies (mAbs) to the cell surface receptors CD3 and CD28 results in downmodulation of CCR5 and the production of soluble factors that inhibit R5 as well as X4 virus replication (129,130). These CD4⁺ T cells are resistant to R5 viruses but are still susceptible to X4 viruses. Resistance of stimulated cells to HIV-1 infection is short lived in vitro, with reacquisition of HIV infectability occurring within one week after the stimuli are removed. However, these findings illustrate that CCR5 levels can be experimentally manipulated and support studies to explore this concept further.

Gene therapy approaches are also being explored and are based on the premise that insertion of anti-HIV genes into target cells will render them resistant to HIV infection and/or replication. In current gene therapy strategies, cells are taken from the donor and transduced with a vector capable of expressing genes that interfere with chemokine receptor function. Several different types of gene therapy vectors can be developed that affect levels of a specific gene in unique ways. For example, these vectors can encode

antisense RNA to arrest translation of chemokine receptors, ribozymes (enzymatic RNA molecules) designed to specifically recognize and cleave other RNAs, intrakinins (modified chemokines) designed to be retained intracellularly and trap chemokine receptors in the endoplasmic reticulum, or single chain mAbs designed to bind and prevent expression and function of chemokine receptors.

Other anti-HIV strategies are designed to more directly interfere with the chemokine receptor HIV gp120 interaction. These approaches include blocking mAbs, natural chemokine receptor ligands, peptide antagonists and small molecule inhibitors. In addition to its potential benefit for chronically infected individuals, passive immunization with anti-CCR5 mAbs may also be useful as post-exposure prophylaxis and for the prevention of maternal–fetal transmission.

Because of their ability to inhibit HIV-1 entry into cells, chemokines are being explored as therapeutic agents. In addition, modified chemokines with more potent HIV inhibitory activities, such as AOP–RANTES and Met–SDF-1 are promising compounds particularly since some of these (e.g., AOP–RANTES) lack inflammatory activity. Two peptides that specifically block CXCR4–HIV interaction are T22 (130) and ALX40–4C (106). T22 is an 18 amino acid peptide isolated from the horseshoe crab and ALX40–4C is a cationic peptide containing nine arginines. These peptides block X4 envelope as well as SDF-1 interactions with CXCR4.

Because of issues of bioavailability and expense, much effort has been focused on the discovery of small molecule chemokine receptor inhibitors. The biopharmaceutical industry has significant experience in developing small molecule antagonists for seven transmembrane spanning G protein coupled receptors and the industry is actively trying to develop such molecules for the chemokine receptors. Recent reports (107,108,131) describe the feasibility of using a small molecule, AMD3100, to block HIV entry. AMD3100 is a member of the heterocyclic family of compounds called bicyclins. AMD3100 binds to CXCR4 and inhibits the interaction between X4 envelope and CXCR4 as well as the interaction of SDF-1 and CXCR4. In vivo studies of AMD3100 in the severe combined immunodeficiency (SCID)–human mouse model have shown that it is nontoxic, and efficacious steady state levels can be maintained by subcutaneous injections or implantable minipump administration. However, its poor oral bioavailability may limit clinical development. Small molecule antagonists of CCR5 have also been developed by several companies and are now registered in the patent database. The identification of such agents capable of inhibiting the interaction of HIV-1 with CXCR4 and CCR5 establishes “proof of principle” for this approach, and provides a basis for rational drug design. If such inhibitors can be developed with adequate oral bioavailability and acceptable toxicity, they could prove to be invaluable additions to our current pharmacologic armamentarium.

SUMMARY

The discovery that chemokine receptors are obligate coreceptors for HIV-1 entry into cells, hailed by the journal *Science* in 1998 as the discovery of the year, has without a doubt been a major advance in our understanding of the pathogenesis of HIV-1 infection. Intense investigation in this area has revealed that chemokine receptors determine viral cell tropism, and that CCR5 is a major genetic determinant of disease susceptibility and progression. In addition, we have learned much about the structural

requirements for HIV-1 entry into cells and have shown that chemokine and chemokine analogs are potent inhibitors of viral entry. All of this has underscored the exciting possibility that chemokine receptor antagonists or molecules that interfere with the HIV-1 gp120 chemokine receptor interaction will prove to be novel and welcome additions to our armamentarium of antiretroviral agents.

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