HIV-1 entry into CD4$^+$ cells is mediated by the chemokine receptor CC-CKR-5

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The β-chemokines MIP-1α, MIP-1β and RANTES inhibit infection of CD4$^+$ T cells by primary, non-syncytium-inducing (NSI) HIV-1 strains at the virus entry stage, and also block *env*-mediated cell–cell membrane fusion. CD4$^+$ T cells from some HIV-1-exposed uninfected individuals cannot fuse with NSI HIV-1 strains and secrete high levels of β-chemokines. Expression of the β-chemokine receptor CC-CKR-5 in CD4$^+$, non-permissive human and non-human cells renders them susceptible to infection by NSI strains, and allows *env*-mediated membrane fusion. CC-CKR-5 is a second receptor for NSI primary viruses.

THE replication of primary, non-syncytium-inducing (NSI) human immunodeficiency virus (HIV)-1 isolates in CD4$^+$ T cells is inhibited by the β-chemokines macrophage inflammatory protein-1α (MIP-1α), MIP-1β, and regulated-upon-activation, normal T expressed and secreted (RANTES)[1,2], but T-cell-line-adapted (TCLA) or syncytium-inducing (SI) primary strains are insensitive to these β-chemokines[2,3]. CD4$^+$ T cells from some HIV-1-exposed uninfected (EU) persons resist infection with NSI strains, but can be infected by TCLA and SI strains, and lymphocytes from some EU individuals secrete high concentrations of β-chemokines[3]. The β-chemokines are proteins of relative molecular mass 8,000 (M_r 8K). They are active on lymphocytes and monocytes by means of cell-surface receptors belonging to the family of G-protein-coupled seven-transmembrane-domain proteins[4–8]. One of these is the LESTR (also known as fusin) orphan receptor, the second receptor for TCLA HIV-1 strains[9], which is not a receptor for known β-chemokines[7–9].

β-Chemokines inhibit HIV-1 replication

To study how β-chemokines inhibit HIV-1 replication, we first used a virus entry assay based on single-cycle infection by an *env*-deficient virus, NL4/3Δ*env*, which also carries the luciferase reporter gene, complemented by envelope glycoproteins expressed *in trans*[10,11]. The use of PM1 cells, a variant of HUT-78 that supports replication of primary and TCLA HIV-1 strains, allowed comparison of *env* functions against a common cellular background[2,12]. The β-chemokines MIP-1α, MIP-1β and RANTES are most active against HIV-1 in combination[2,3], and strongly inhibited infection of PM1 cells by viruses complemented with envelopes from the NSI strains ADA and BaL (Table 1*a*). Individually, RANTES and MIP-1β were more strongly active than the other β-chemokines tested[13–15] (Table 1*a*). MIP-1α, MIP-1β and RANTES in combination did not inhibit infection of PM1 cells by the TCLA strains NL4/3 and HxB2 (Table 1*a*). Thus phenotypic characteristics of the HIV-1 envelope glycoproteins influence their sensitivity to β-chemokines in a virus entry assay.

EU CD4$^+$ T cells and NSI virus entry

The *env*-complementation assay was used to assess HIV-1 entry into CD4$^+$ T cells from two individuals, EU2 and EU3, which are exceptionally resistant to infection by NSI strains in conventional HIV-1 infection assays[3]. The cells of neither individual supported efficient entry of the NSI strain, JR-FL, but both allowed HxB2 entry; luciferase activity in cells from EU2 and EU3 after exposure to JR-FL was 300 and 200 c.p.m., respectively, compared with 5,440 and 29,560 c.p.m. from the same cells infected with HxB2. In contrast, JR-FL-infected CD4$^+$ T cells from control individuals LW4 and LW5 produced luciferase counts of 814,670 and 77,880 c.p.m. The cells of EU2 and EU3 were therefore capable of efficiently replicating the HIV-1 genome once virus entry had been achieved. MIP-1α, MIP-1β and RANTES strongly inhibited JR-FL infection of the CD4$^+$ T cells of LW4 and LW5, and weakly reduced HxB2 infection of both LW and EU cells (Table 1*c*).

To examine whether EU2 and EU3 had a genetic or acquired block to infection, we isolated CD4$^+$ T cell clones. All 21 clones from EU2 and the one clone isolated from EU3 produced high levels of β-chemokines (especially RANTES), irrespective of their Th phenotype. They were resistant to infection by the SF162 NSI strain, compared to 22 readily infectable CD4$^+$ clones from LW4 and LW5 (Table 2). The SI variant of SF162, R3H[3], was significantly more replication competent than SF162 in the EU clones, but the two strains replicated comparably in the LW clones. However, some EU clones resisted infection by both SF162 and R3H. That all the EU clones were essentially uninfectable by SF162 suggested that the mechanism of resistance had a genetic basis. One possibility was that constitutive overproduction of β-chemokines in the CD4$^+$ T cells of EU2 and EU3 rendered them resistant to infection. Anti-β-chemokine antibodies partly abolished resistance to HIV-1 SF162 infection of CD4$^+$ T cells from LW4 when they were co-cultured with cells from EU2 (and hence were exposed to β-chemokines secreted from the cells of EU2). However, CD4$^+$ cells from EU2 remained resistant to SF162 infection in the presence of these antibodies (Fig. 1*a*), suggesting that the resistance mechanism may be more complex than an overproduction of endogenous β-chemokines.

Inhibition early in HIV-1 infection

We determined when β-chemokines inhibited HIV-1 replication by showing that complete inhibition of the infection of PM1 cells required the continuous presence of β-chemokines for up to 5 h after the addition of BaL *env*-complemented HIV-1 (Fig. 1*b*). Pretreatment of the cells with β-chemokines for 2 or 24 h before infection had no inhibitory effect if the cells were subsequently washed before virus addition. Furthermore, adding β-chemokines

2 h after the virus only minimally affected virus entry (Fig. 1*b*). We next used an assay based on the polymerase chain reaction (PCR) to detect HIV-1 early-DNA reverse transcripts in PM1 cells after 10 h of infection. We found that reverse transcription of ADA, but not of NL4/3, was strongly inhibited in the presence of MIP-1β and RANTES (Fig. 1*c*). Thus inhibition by β-chemokines requires their presence during at least one of the early stages of HIV-1 replication: virus attachment, fusion and early reverse transcription.

We discriminated between these sites of action by testing

TABLE 1 Inhibition of HIV-1 entry and membrane fusion

	Luciferase activity (%)					Fusion (%)	
	BaL	ADA	NL4/3	HxB2		HeLa-JR-FL	HeLa-BRU
(*a*) PM1 cells							
Control without virus	2	2	2	5			
Control with virus	100	100	100	100	No chemokines	100	100
+R/Mα/Mβ (50/50/50)	2	3	92	117	+R/Mα/Mβ (80/400/100)	1	95
+RANTES (100)	1	1	n.d.	n.d.	+RANTES (80)	8	100
+MIP-1α (100)	54	54	n.d.	n.d.	+MIP-1α (400)	39	100
+MIP-1β (100)	1	6	n.d.	n.d.	+MIP-1β (100)	13	93
+MCP-1 (100)	46	50	n.d.	n.d.	+MCP-1 (100)	99	98
+MCP-2 (100)	28	26	n.d.	n.d.	+MCP-2 (100)	72	93
+MCP-3 (100)	58	46	n.d.	n.d.	+MCP-3 (100)	98	99
(*b*) Macrophages							
Control without virus	1	1	n.a.p.	n.a.p.			
Control with virus	100	100	n.a.p.	n.a.p.	No chemokines	100	d.n.f.
+R/Mα/Mβ (50/50/50)	84	n.d.	n.a.p.	n.a.p.	+R/Mα/Mβ (80/400/100)	46	d.n.f.
+RANTES (100)	103	68	n.a.p.	n.a.p.	+RANTES (80)	80	d.n.f.
+MIP-1α (100)	93	90	n.a.p.	n.a.p.	+MIP-1α (400)	85	d.n.f.
+MIP-1β (100)	90	82	n.a.p.	n.a.p.	+MIP-1β (100)	63	d.n.f.
+MCP-1 (100)	107	90	n.a.p.	n.a.p.	+MCP-1 (100)	116	d.n.f.
+MCP-2 (100)	84	76	n.a.p.	n.a.p.	+MCP-2 (100)	72	d.n.f.
+MCP-3 (100)	88	96	n.a.p.	n.a.p.	+MCP-3 (100)	82	d.n.f.
(*c*) $CD4^+$ T cells	JR-FL	HxB2					
LW4					LW5		
Control without virus	1	1			No chemokines	100	100
Control with virus	100	100			+R/Mα/Mβ (106/533/133)	39	100
+R/Mα/Mβ (200/200/200)	14	68			+RANTES (106)	65	95
					+MIP-1α (533)	72	100
LW5					+MIP-1β (133)	44	92
Control without virus	1	1			+OKT4a (3 μg/ml)	0	0
Control with virus	100	100					
+R/Mα/Mβ (200/200/200)	15	73			EU2		
					No chemokines	d.n.f.	100
EU2					+R/Mα/Mβ (320/1600/400)	d.n.f.	100
Control without virus	n.a.p.	14			+RANTES (320)	d.n.f.	100
Control with virus	n.a.p.	100			+MIP-1α (1600)	d.n.f.	100
+R/Mα/Mβ (200/200/200)	n.a.p.	51			+MIP-1β (400)	d.n.f.	100
EU3							
Control without virus	n.a.p.	3					
Control with virus	n.a.p.	100					
+R/Mα/Mβ (200/200/200)	n.a.p.	n.d.					

Effect of β-chemokines on HIV-1 entry and *env*-mediated membrane fusion. Virus entry assay (left). PM1 cells were cultured as described[12]. Macrophages were isolated from peripheral blood by adherence to plastic in 96-well plates. The monolayer was washed extensively over 2 weeks to remove non-adherent cells and allow monocyte differentiation. Ficoll/hypaque-islated peripheral blood mononuclear cells (PBMCs) from laboratory workers (LW) or EU individuals were stimulated with PHA for 72 h before depletion of $CD8^+$ lymphocytes with anti-CD8 immunomagnetic beads (Dynal, Great Neck, NY). $CD4^+$ lymphocytes were maintained in culture medium containing interleukin-2 (IL-2) (100 U ml^{-1}; Hoffmann LaRoche, Nutley, NY), as described[3]. Target cells (10^5 to 2×10^5) were infected with supernatants (10–50 ng of HIV-1 p24) from 293 cells cotransfected with an NL4/3Δ*env*-luciferase vector and a HIV-1 *env*-expressing vector[10,11]. We then added β-chemokines (R & D Systems, Minneapolis) to the target cells simultaneously with virus, at the final concentrations (ng ml^{-1}) indicated in parentheses in the first column. The β-chemokine concentration range was selected based on previous studies[2,3]. After 2 h, the cells were washed twice with PBS, resuspended in β-chemokine-containing media, and maintained for 48–96 h. Luciferase activity in cell lysates was measured as described previously[10,11]. The values indicated represent luciferase activity (c.p.m. per ng p24 per mg protein), expressed relative to that in virus-control cultures lacking β-chemokines (100%), and are the means of duplicate or sexttuplicate determinations. Membrane fusion assay (right). $CD4^+$ target cells (mitogen-activated $CD4^+$ lymphocytes, PM1 cells or macrophages) were labelled with octadecyl rhodamine (Molecular Probes, Eugene, OR), and HeLa-JR-FL cells and HeLa-BRU cells (or control HeLa cells, not shown) were labelled with octadecyl fluorescein (Molecular Probes), overnight at 37 °C. Equal numbers of labelled target cells and *env*-expressing cells were mixed in 96-well plates and β-chemokines (or CD4 mAb OKT4a) were added at the final concentrations (ng ml^{-1}) indicated in parentheses in the first column. Fluorescence emission values were determined 4 h after cell mixing[16]. If cell fusion occurs, the dyes are closely associated in the conjoined membrane such that excitation of fluorescein at 450 nm results in RET and emission by rhodamine at 590 nm. Percentage fusion is defined as equal to $100 \times [(\text{exp RET} - \text{min RET})/(\text{max RET} - \text{min RET})]$, where max RET is the percentage RET obtained when HeLa-Env and $CD4^+$ cells are mixed, exp RET is the percentage RET obtained when HeLa-Env and $CD4^+$ cells are mixed in the presence of fusion-inhibitory compounds, and min RET is the percentage RET obtained when HeLa cells (lacking HIV-1 envelope glycoproteins) and $CD4^+$ cells are mixed. The percentage RET value has been defined[16], and each is the mean of triplicate determinations. These values were, for HeLa-JR-FL and HeLa-BRU cells respectively: PM1 cells, 11.5%, 10.5%; LW5 $CD4^+$ cells, 6.0%, 10.5%; EU $CD4^+$, cells, 0.1%, 4.1%; macrophages, 4.3%, 1.2%. Abbreviations: n.d., not done (inhibition by combinations of β-chemokines was so weak that individual β-chemokines were not tested); n.a.p., NL4/3 and HxB2 replicated so poorly in macrophages, and JR-FL replicated so poorly in EU2 and EU3 $CD4^+$ T cells, that no assessment of inhibition by β-chemokines was possible; R/Mα/Mβ, RANTES + MIP − 1α + MIP − 1β; d.n.f., EU2 $CD4^+$ cells did not fuse with HeLa-JR-FL cells, and macrophages did not fuse with HeLa-BRU cells.

whether β-chemokines inhibited binding of JR-FL or BRU gp120 to soluble CD4, or of tetrameric CD4–IgG2 binding to HeLa cells expressing oligomeric JR-FL envelope glycoproteins[16]. No inhibition by any of the β-chemokines was found in either assay, whereas the CD4 monoclonal antibody OKT4a was strongly inhibitory (not shown). Thus β-chemokines inhibit a step after CD4 binding, when conformational changes in the envelope glycoproteins lead to fusion of viral and cellular membranes[17]. Cell–cell membrane fusion is also induced by the interaction between gp120 and CD4, and can be monitored directly by resonance energy transfer (RET) between fluorescent dyes incorporated in cell membranes[16]. OKT4a completely inhibits membrane fusion of PM1 cells with HeLa cells expressing the envelope glycoproteins of either JR-FL (HeLa-JR-FL) or BRU (HeLa-BRU), confirming the specificity of the RET assay[16]. RANTES and MIP-1β (and, to a lesser extent, MIP-1α) strongly inhibited membrane fusion of HeLa-JR-FL cells with PM1 cells, whereas fusion between PM1 cells and HeLa-BRU cells was insensitive to these β-chemokines (Table 1*a*).

Similar results were obtained with primary CD4$^+$ T cells from LW5 (Table 1*c*), although higher concentrations of β-chemokines were required to inhibit membrane fusion in the primary cells than in PM1 cells. In marked contrast to the cells of LW5, the CD4$^+$ T cells of EU2 did not fuse with HeLa-JR-FL cells, although they clearly fused with HeLa-BRU cells in a β-chemokine-resistant manner (Table 1*c*). The RET assay demonstrates that β-chemokines interfere with *env*-mediated membrane fusion, and establishes that envelope glycoproteins from a primary, NSI strain cannot fuse with CD4$^+$ T cells from an EU individual, giving an indication of how these cells resist HIV-1 infection.

Cell-specific variables

Specific characteristics of envelope glycoproteins from different strains influence the susceptibility of HIV-1 to β-chemokines. There are, however, cell-type-specific variables to consider. For example, fusion of HeLa-JR-FL with human macrophages (normal donor) was only weakly inhibited by MIP-1α, MIP-1β, RANTES, MCP-2 and MCP-3, and not at all by MCP-1 (Table 1*b*). Similarly, infection of macrophages by BaL or ADA in the *env*-complementation assay was only minimally sensitive to very high concentrations (500 ng ml^{-1}) of MIP-1α, MIP-1β and RANTES (Table 1*b*, and data not shown). Hence, infection by the same virus (BaL or ADA) is either inhibited (T cells) or not inhibited (macrophages) by β-chemokines (Table 1); given the isogenic nature of the NL4/3Δ*env* reporter virus[10,11], the target cell must be an important variable. Mitogen-activated primary CD4$^+$ T cells are intermediate between macrophages and PM1 cells in their β-chemokine sensitivity, and there is also inter-donor variability. Thus BaL *env*-mediated entry was at 18, 36 and 101% of control levels in peripheral blood lymphocytes from three individuals in the presence of 50 ng ml^{-1} each of MIP-1α, MIP-1β and RANTES.

CC-CKR-5 is a second receptor

The simplest explanation of our results is that the binding of certain β-chemokines to their receptor(s) prevents, directly or

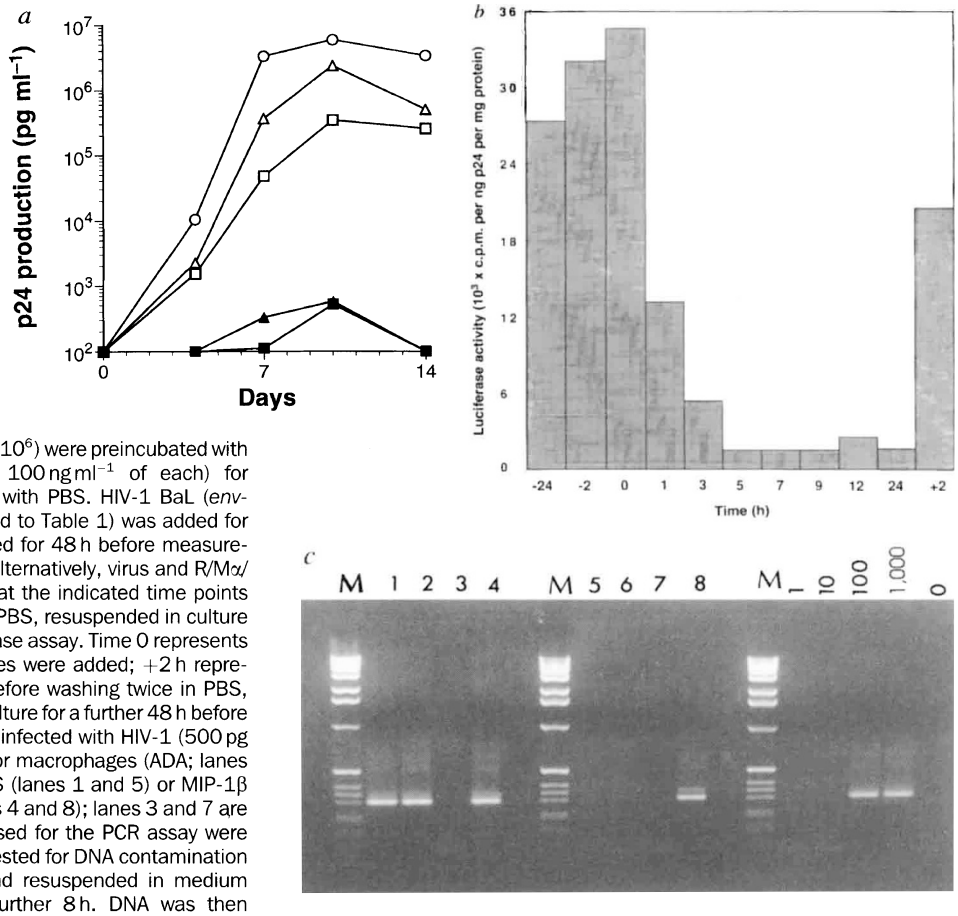


FIG. 1 Specificity, time course and stage of β-chemokine inhibition of HIV-1 replication. *a*, Mitogen-activated CD4$^+$ lymphocytes from LW4 (open circles), and equal numbers of CD4$^+$ lymphocytes from LW4 and EU2 in the absence (open squares) or presence (open triangles) or polyclonal neutralizing antibodies to RANTES, MIP-1α and MIP-1β, or CD4$^+$ lymphocytes from EU2 in the absence (filled squares) or presence (filled triangles) of these neutralizing antibodies, were inoculated with 600 $TCID_{50}$ of HIV-1 SF162. Virus replication was monitored by p24 antigen production[3]. Antibodies to RANTES, MIP-1α and MIP-1β (R&D Systems) were added at 200, 50 and 100 μg ml^{-1} respectively, and were replenished on day 7 of the culture. *b*, PM1 cells (1×10^6) were preincubated with RANTES + MIP − 1α + MIP − 1β (R/Mα/Mβ; 100 ng ml^{-1} of each) for 24 h (−24) or 2 h (−2), then washed twice with PBS. HIV-1 BaL (*env*-complemented virus, 50 ng of p24; see legend to Table 1) was added for 2 h, then the cells were washed and incubated for 48 h before measurement of luciferase activity in cell lysates[10,11]. Alternatively, virus and R/Mα/Mβ were added simultaneously to cells, and at the indicated time points (1 h, 3 h, etc.) the cells were washed twice in PBS, resuspended in culture medium, and incubated for 48 h before luciferase assay. Time 0 represents the positive control, to which no β-chomokines were added; +2 h represents the mixture of virus with cells for 2 h before washing twice in PBS, addition of R/Mα/Mβ and continuation of the culture for a further 48 h before luciferase assay. *c*, PM1 cells (1×10^6) were infected with HIV-1 (500 pg p24) grown in CEM cells (NL4/3; lanes 1–4) or macrophages (ADA; lanes 5–8), in the presence of 500 ng ml^{-1} RANTES (lanes 1 and 5) or MIP-1β (lanes 2 and 6), or with no β-chemokine (lanes 4 and 8); lanes 3 and 7 are negative controls (no virus). All viral stocks used for the PCR assay were treated with DNAse for 30 min at 37 °C, and tested for DNA contamination before use. After 2 h, cells were washed and resuspended in medium containing the same β-chemokines for a further 8 h. DNA was then extracted from infected cells using a DNA/RNA isolation kit (US Biochemicals). First-round nested PCR was performed with primers: U3+, 5′-CAAGGCTACTTCCCTGATTGGCAGAACTACACACCAGG-3′; preGag, 5′-AGCAAGCCGAGTCCTGCGTCGAGAG-3′; and the second round with primers; LTR-test, 5′-GGGACTTTCCGCTGGGGACTTTC-3′; LRC2, 5′-CCTGTTCGGGCGCCACTGCTAGAGATTTTCCAC-3′; in a Perkin Elmer 2400 cycler with the following amplification cycles: 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, 72 °C for 7 min. M indicates 1 kb DNA ladder; 1, 10, 100 and 1,000 indicate the number of reference plasmid (pAD8) copies. The assay can detect 100 copies of reverse transcripts.

otherwise, the fusion of HIV-1 with CD4+ T cells. HIV-1 requires a second receptor for entry into CD4+ cells[18–20]. This function is supplied, for TCLA strains, by LESTR[9], so we considered that a related β-chemokine receptor might act as a second receptor for primary, NSI strains on CD4+ T cells. Several receptors for MIP-1α, MIP-1β and RANTES have been identified[6,7], and β-chemokines exhibit considerable cross-reactivity in receptor usage[4–8]. However, we identified CC-CKR-1 and, especially, CC-CKR-5 as the most likely candidates, based on their tissue expression patterns and ligand-binding properties[4,7,8,15,21]. CC-CKR-1, CC-CKR-5 and LESTR are each expressed at the mRNA level in PM1 cells (data not shown). We therefore amplified by PCR, cloned and expressed these and other β-chemokine receptors.

The expression of CC-CKR-5 in HeLa-CD4 (human), COS-CD4 (simian) and 3T3-CD4 (murine) cells rendered each of them readily infectable by ADA and BaL in the *env*-complementation assay of HIV-1 entry (Table 3). LESTR, CC-CKR-2a, CKR-3 and CKR-4 could not substitute for CC-CKR-5 in this assay, and CC-CKR-1 expression permitted only very limited entry of ADA and BaL into HeLa-CD4 cells, but not into COS-CD4 or 3T3-CD4 cells (Table 3). However using a PCR-based assay, we have consistently observed a low level of NL4/3 and ADA entry into each of these CC-CKR-1-expressing cell lines (data not shown). The expression of LESTR in COS-CD4 and 3T3-CD4 cells allowed HxB2 entry in the *env*-complementation assay, and HxB2 readily entered untransfected (or control plasmid-transfected) HeLa-CD4

TABLE 2 Production of β-chemokines and infectability of CD4+ T-cell clones

		p24 (pg ml^{-1})		Chemokines (pg ml^{-1})		
Clone	Th	SF162	R3H	RANTES	MIP-1α	MIP-1β
EU2.1	1	0	0	3,427	0	227
EU2.2	2	0	1,123	3,009	526	1,153
EU2.3	1	0	5,341	1,880	0	34
EU2.4	1	0	1,661	1,998	23	42
EU2.5	1	83	0	15,951	156	1,253
EU2.6	1	8,090	442	4,630	156	618
EU2.7	1	0	53,478	3,055	0	156
EU2.8	1	0	44,864	2,168	0	118
EU2.9	1	0	1,940	1,368	0	68
EU2.10	1	0	0	2,939	83	202
EU2.11	1	0	84	1,602	0	98
EU2.12	1	0	725	3,590	361	1,988
EU2.13	1	0	46,894	1,483	0	34
EU2.14	1	0	42,326	2,055	17	88
EU2.15	1	0	113	3,872	78	164
EU2.16	0	0	780	28,701	7,778	5,344
EU2.17	1	207	39,030	2,726	0	96
EU2.18	1	0	797	2,505	28	251
EU2.19	0	0	0	1,435	127	997
EU2.20	0	237	0	4,454	195	1,483
EU2.21	2	0	0	1,071	237	126
EU3.1	2	2,063	0	8,341	445	1,855
LW4.1	1	105,730	70,222	185	48	436
LW4.2	0	127,286	44,936	642	170	490
LW4.3	0	65,018	84,528	699	36	412
LW4.4	2	28,922	42,326	148	22	65
LW4.5	1	150,940	95,488	246	0	0
LW4.6	0	61,594	99,834	246	29	81
LW4.7	1	114,188	154,024	47	16	25
LW4.8	0	11,871	1,077	782	130	201
LW4.9	2	39,974	4,704	43	30	30
LW4.10	n.p.	22,794	39,030	53	36	51
LW4.11	2	3,439	1,202	<156	90	116
LW4.12	2	49,722	40,550	273	27	337
LW4.13	n.p.	555,548	363,020	169	0	0
LW5.1	2	66,160	93,432	227	22	25
LW5.2	n.p.	104,912	159,208	280	0	0
LW5.3	2	46,836	169,850	336	0	23
LW5.4	0	72,222	44,726	829	124	229
LW5.5	n.p.	129,638	284,338	127	0	0
LW5.6	1	163,028	120,736	401	0	0
LW5.7	2	114,844	109,892	0	32	62
LW5.8	n.p.	91,732	251,338	151	0	0
LW5.9	1	134,924	280,600	<156	124	14

CD4+ lymphocytes were purified (>96% purity) from peripheral blood, then maintained in culture as described in the legend to Table 1. After one week, lymphocytes were labelled with anti-CD8 antibody and negatively sorted on a FACS Vantage cell sorter (Becton Dickinson, San Jose, CA) to further deplete CD8+ T cells. The purified cells were cloned at 10, 2.5 and 0.5 cells per well in the presence of allogeneic feeder cells, an anti-CD3 antibody and IL-2, and were periodically restimulated. The expression of both CD3 and CD4 on the clonally expanded cells was confirmed by FACS analysis, and some cells were tested for clonality by determining Vβ T-cell receptor usage using sequence-specific primers. The concentrations of interferon-γ (IFN-γ), IL-4, RANTES, MIP-1α and MIP-1β were determined in the supernatants from 1×10^6 cloned cells using commercial ELISA kits (R&D Systems). Clones secreting IFN-γ but not IL-4 were defined as Th1, those secreting IL-4 but not IFN-γ were defined as Th2 clones, and those secreting both cytokines were defined as Th0 clones. Cells (2×10^5) from each clone were inoculated with 200 $TCID_{50}$ of the NSI HIV-1 strain SF162 or of its SI variant SF162R3H[3]. HIV-1 replication was assessed by p24 antigen production on day 14, using a commercial ELISA (Abbott Labs, Abbott Park, IL)[3]. Abbreviations: n.p., clones that produced neither IFN-γ nor IL-4, for which no Th phenotype could be assigned.

cells (Table 3). Entry of BAL and ADA into all three CC-CKR-5-expressing cell lines was almost completely inhibited by the combination of MIP-1α, MIP-1β and RANTES, whereas HxB2 entry into LESTR-expressing cells was insensitive to β-chemokines (Table 3, and data not shown). These results show that CC-CKR-5 functions as a second receptor for some primary, NSI HIV-1 strains in a manner broadly analogous to that established for LESTR and TCLA strains[9].

CC-CKR-5 allows membrane fusion

We confirmed that CC-CKR-5 functions as a second receptor in assays of *env*-mediated membrane fusion. Transient expression of CC-CKR-5 in COS-CD4 and HeLa-CD4 cells allowed both cell lines to fuse strongly with HeLa-JR-FL cells (Fig. 2*Ac*, *d*), whereas no fusion occurred when control plasmids were used (Fig. 2*Aa*, *b*). Expression of LESTR instead of CC-CKR-5 did not allow either COS-CD4 or HeLa-CD4 cells to fuse with HeLa-JR-FL cells, but did allow fusion between COS-CD4 cells and HeLa-BRU cells (data not shown), consistent with previous results[9]. A quantitative end-point for cell–cell fusion was also obtained, β-galactosidase is expressed in the fused cells and stains them blue in the presence of the X-gal substrate[22]. The numbers of blue syncytia per well of Fig. 2*A* were: *a*, 7; *b*, 0; *c*, 3,340; *d*, 5,100.

The β-chemokine receptors were also tested in the RET fusion

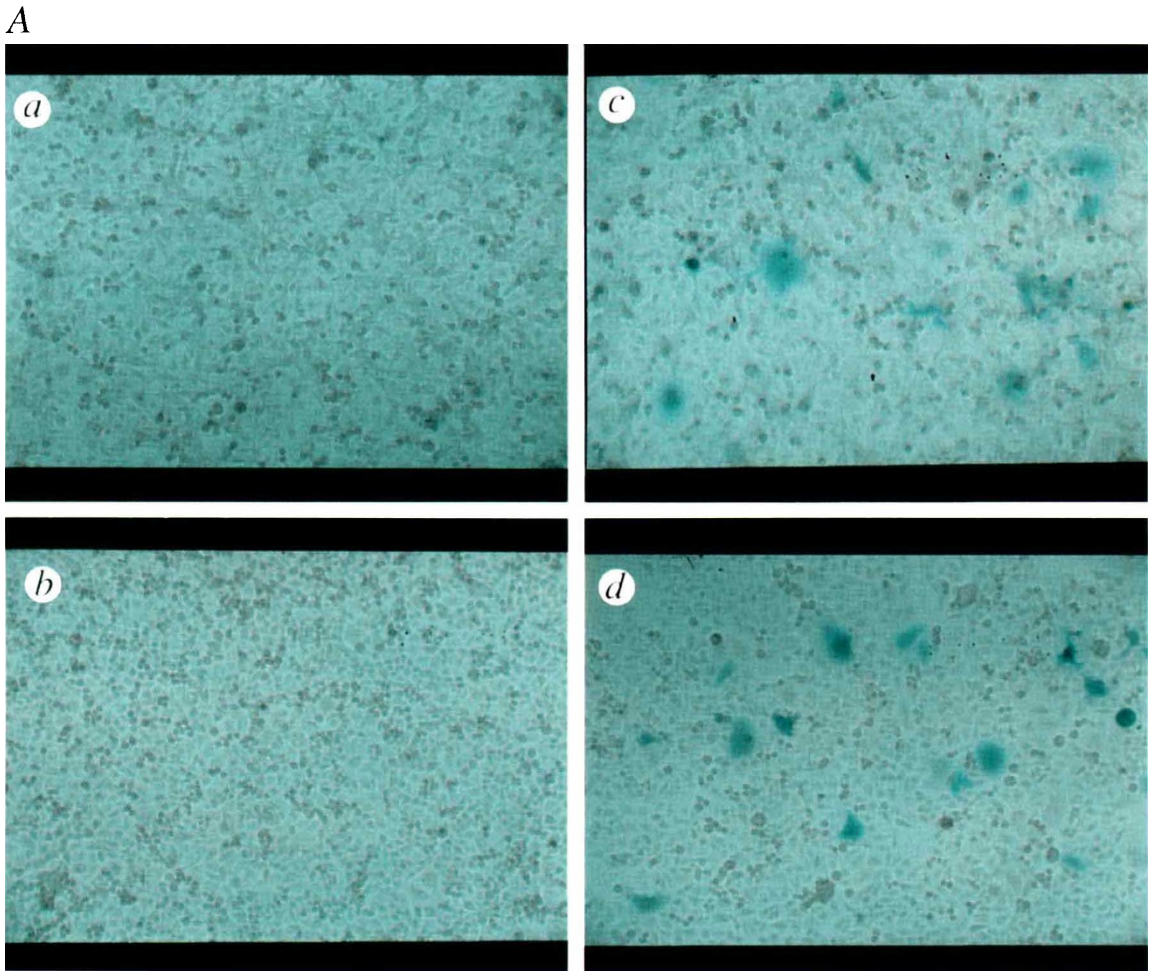
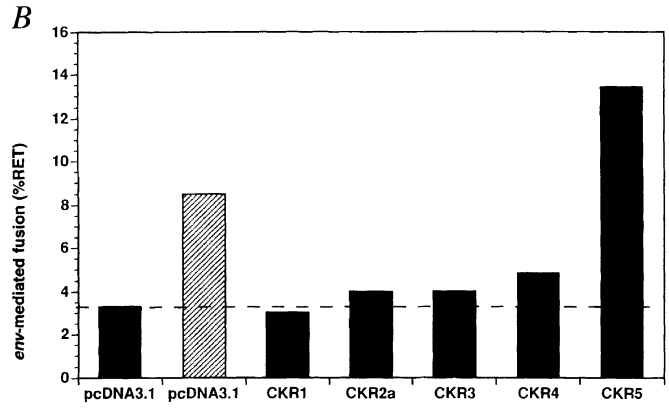


FIG. 2 HIV-1 *env*-mediated membrane fusion of cells transiently expressing C–C CKR-5. *A*, Membrane fusion mediated by C–C CKR-5 expression in HeLa and COS cells was demonstrated by transfecting control pcDNA3.1 into COS-CD4 (Z28T1) cells (*a*) or HeLa-CD4 (P42) cells (*b*), or transfecting pcDNA3.1-CKR5 into COS-CD4 cells (*c*) or HeLa-CD4 cells (*d*), then coculturing the transfected cells for 24 h with HeLa-JR-FL cells at a 1:1 ratio. The Z28T1 and P42 cells carry the inducible LTR-lacZ construct, and HeLa-JR-FL cells express HIV-1 tat. The β-gal staining was performed as described previously[22]. *B*, Membrane fusion mediated by β-chemokine receptors expressed in HeLa cells was demonstrated. Cells were transfected with control plasmid pcDNA3.1 or plasmid pcDNA3.1-CKR constructs using lipofectin (Gibco BRL). The pcDNA3.1 plasmid carries a T7-polymerase promoter, and transient expression of β-chemokine receptors was boosted by infecting cells with 1×10^7 plaque-forming units of vaccinia encoding the T7-polymerase (vFT7.3) 4 h post-lipofection[9]. Cells were then cultured overnight in R18-containing media, and tested for their ability to fuse with HeLa-JR-FL cells (black columns) or HeLa-BRU cells (hatched column) in the RET assay. Background fusion (pcDNA3.1 plasmid) is indicated by the broken line. The background level of fusion with control HeLa cells in this assay is typically between 3% and 4% RET, irrespective of the transfected plasmid, so the percentage RET seen with C–C CKR-1, CKR-2a, CKR-3 and CKR-4 is not considered significant.



assay. Expression of CC-CKR-5, but not of CC-CKR-1, CKR-2a, CKR-3 or CKR-4, allowed fusion between HeLa-CD4 cells and HeLa-JR-FL cells to an extent greater than the constitutive level of fusion between HeLa-BRU cells and HeLa-CD4 cells (Fig. 2*B*). Fusion between HeLa-JR-FL cells and CC-CKR-5-expressing HeLa-CD4 cells was inhibited when RANTES, MIP-1α and MIP-1β (500 ng ml^{-1} of each) were added at the same time as the fusion process was initiated by coculturing the cells. However, the extent of fusion inhibition by the β-chemokines depended on how long the HeLa-CD4 cells had been infected with the T7-polymerase-expressing vaccinia virus before they were co-cultured with the HeLa-JR-FL cells to initiate membrane fusion. Thus inhibition was 71, 46, 28 and 0% at 5, 7, 9 and 21 h, respectively, after infection by the vaccinia virus. This is probably because sustained overexpression of CC-CKR-5 overcomes the blocking effect of the CC-CKR-5 ligands.

Implications of second-receptor function

Taken together, our results establish that MIP-1α, MIP-1β and RANTES inhibit HIV-1 infection by interfering with the virus–cell fusion reaction subsequent to CD4 binding. Furthermore, we show that CC-CKR-5 can serve as a second receptor for entry of some primary NSI strains of HIV-1 into CD4$^+$ T cells, and that CC-CKR-5 coexpression with CD4 allows membrane fusion mediated by the *env*-gene from NSI primary viruses. These findings are linked by the observation that the interaction of β-chemokines with CC-CKR-5 inhibits membrane fusion and HIV-1 entry. Overexpression of CC-CKR-5 in the membrane-fusion assay reduces the ability of β-chemokines to block fusion, which is suggestive of a competitive mechanism of inhibition. Further studies will be necessary to prove this, however, as other mechanisms, such as receptor downregulation, could also account for inhibition of CC-CKR-5-mediated fusion by β-chemokines.

We do not know if CC-CKR-5 is the only second receptor for NSI strains that can function on primary CD4$^+$ T cells. Inter-donor variation in the β-chemokine sensitivity of HIV-1 infection suggests there may be some overlap in second receptor usage on activated CD4$^+$ T cells by different NSI strains. Furthermore, the identity of the second receptor on macrophages is not yet known. Entry (and fusion) of NSI HIV-1 strains into macrophages was relatively insensitive to all β-chemokines tested, which contrasts with the observations from CD4$^+$ T cells. Although another G-protein-coupled seven transmembrane domain protein may, perhaps, serve as a second receptor in macrophages, we do not rule out the possibility that CC-CKR-5 also fulfils this function on these cells. SI and TCLA strains do not enter or fuse with macrophages. Although it is not yet clear whether LESTR is expressed on macrophages, it was first cloned from macrophage/monocytes[4,23]. There may be as-yet unexplained subtleties in second-receptor function and regulation in macrophages, and conceivably in other non-lymphoid cells. We note that the Duffy blood-group antigen on human erythrocytes is a promiscuous β-chemokine receptor[24,25]. This might account for the observation that a heat- and protease-resistant factor present in human erythrocyte membranes can confer HIV-1 fusion competence to murine cells expressing human CD4, when heterokaryons of the two cell types are made[26,27]. The Duffy antigen may not be a physiologically relevant second receptor, but might function *in extremis*.

Sequence changes in the *env* gene that occur during adaptation of HIV-1 to growth in cell lines, and conceivably those involved in the phenotypic switch[28,29] from NSI to SI, might be the result of alterations in envelope glycoprotein configuration that allow the use of LESTR as well as, or instead of, CC-CKR-5 or a related receptor. It will be important to determine whether envelope glycoproteins bind directly to second receptors, and how this

TABLE 3 Infection of CD4-expressing cells by primary, NSI HIV-1 strains

		pcDNA3.1	LESTR	CKR-1	CKR-2a	CKR-3	CKR-4	CKR-5	R/Mα/Mβ CKR-5
COS-CD4	ADA	798	456	600	816	516	534	153,000	3,210
	BaL	660	378	600	636	516	618	58,800	756
	HxB2	5,800	96,700	5,240	5,070	5,470	5,620	4,850	5,000
HeLa-CD4	ADA	678	558	4,500	912	558	600	310,000	6,336
	BaL	630	738	1,800	654	516	636	104,000	750
	HxB2	337,000	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	356,000
3T3-CD4	ADA	468	558	450	618	534	606	28,400	1,220
	BaL	606	738	660	738	534	558	11,700	756
	HxB2	456	24,800	618	672	732	606	618	606

The β-chemokine receptor genes *C–C CKR-1*, *C–C CKR-2a*, *C–C CKR-3*, *C–C CKR-4* and *C–C CKR-5* have no introns[4–8,15,21]. They were isolated by PCR performed directly on a human genomic DNA pool derived from the PBMCs of 7 healthy donors. Oligonucleotides overalapping the ATG and the Stop codons, and containing *Bam*HI and *Xho*I restriction sites for directional cloning into the pcDNA3.1 expression vector (Invitrogen Inc.), were used. LESTR (also known as fusin or HUMSTSR)[4,9,23] was cloned by PCR performed directly on cDNA derived from PM1 cells, using sequences derived from the NIH database. All oligonucleotide sequences are listed below. PCR was performed in a Perkin Elmer 2400 cycler with the following amplification cycles: 94 °C for 5 min, 30 cycles of {94 °C for 1 min, 60 °C for 1 min, 72 °C for 2 min}, 72 °C for 10 min. The human CD4-expressing cell lines HeLa-CD4 (P42), 3T3-CD4 (sc6) and COS-CD4 (Z28T1) (ref. 22) were transfected with the different pcDNA3.1-CKR constructs by the calcium-phosphate method, then infected 48 h later with different reporter viruses (200 ng HIV-1 p24 per 10^6 cells) in the presence or absence of β-chemokines (400 ng ml^{-1} each of RANTES, MIP-1α and MIP-1β). Luciferase activity in cell lysates was measured 48 h later[10,11]. The β-chemokine blocking data are only shown for C–C CKR-5, as infection mediated by the other C–C CKR genes was too weak for inhibition to be quantifiable. We have not yet formally demonstrated that the clones C–C CKR genes are functional as β-chemokine receptors, so the inability of C–C CKR-1, CKR-2a, CKR-3, and CKR-4 to function as HIV-1 second receptors could be due to defective expression at the protein level. Listed below are the 5′ and 3′ primer pairs used in first (5-1 and 3-1) and second (5-2 and 3-2) round PCR amplification of the CKR genes directly from human genomic DNA, and of LESTR from PM1 cDNA. Only a single set of primers was used to amplify CKR-5. LESTR: L/5=AAG CTT GGA GAA CCA GCG GTT ACC ATG GAG GGG ATC; L/5-2=GTC TGA GTC TGA GTC AAG CTT GGA GAA CCA; L/3-1=CTC GAG CAT CTG TGT TAG CTG GAG TGA AAA CTT GAA GAC TC; L/3-2=GTC TGA GTC TGA GTC CTC GAG CAT CTG TGT. CKR-1: C1/5-1=AAG CTT CAG AGA GAA GCC GGG ATG GAA ACT CC; C1/5-2=GTC TGA GTC TGA GTC AAG CTT CAG AGA GAA; C1/3-1=CTC GAG CTG AGT CAG AAC CCA GCA GAG AGT TC; C1/3-2=GTC TGA GTC TGA GTC CTC GAG CTG AGT CAG. CKR-2a: C2/5-1=AAG CTT CAG TAC ATC CAC AAC ATG CTG TCC AC; C2/5-2=GTC TGA GTC TGA GTC AAG CTT CAG TAC ATC; C2/3-1=CTC GAG CCT CGT TTT ATA AAC CAG CCG AGA C; C2/3-2=GTC TGA GTC TGA GTC CTC GAG CCT CGT TTT. CKR-3: C3/5-1=AAG CTT CAG GGA GAA GTG AAA TGA CAA CC; C3/5-2=GTC TGA GTC TGA GTC AAG CTT CAG GGA GAA; C3/3-1=CTC GAG CAG ACC TAA AAC ACA ATA GAG AGT TCC; C3/3-2=GTC TGA GTC TGA GTC CTC GAG CAG ACC TAA. CKR-4: C4/5-1=AAG CTT CTG TAG AGT TAA AAA ATG AAC CCC ACG G; C4/5-2=GTC TGA GTC TGA GTC AAG CTT CTG TAG AGT; C4/3-1=CTC GAG CCA TTT CAT TTT TCT ACA GGA CAG CAT C; C4/3-2=GTC TGA GTC TGA GTC CTC GAG CCA TTT CAT. CKR-5: C5/5-12=GTC TGA GTC TGA GTC AAG CTT AAC AAG ATG GAT TAT CAA; C5/3-12=GTC TGA GTC TGA GTC CTC GAG TCC GTG TCA CAA GCC CAC.

might be achieved. The pattern of second receptor useage by HIV-1 strains of different genetic subtypes remains to be explored. However, the replication in peripheral blood lymphocytes of primary NSI strains from several genetic subtypes is inhibited by β-chemokines (A. Trkola and J.P.M., unpublished data), implying that viral phenotype is more important than genotype in determining β-chemokine sensitivity. Any binding site for the β-chemokine receptors on the envelope glycoproteins must, therefore, use highly conserved residues, or must have a strongly conserved structural feature, such as charge, that is independent of major variations in primary sequence.

Finally, at least some EU individuals have $CD4^+$ T cells that are relatively incompetent at fusing with NSI strains of HIV-1, probably because of a defect in second-receptor usage. Whether this is a result of autocrine ligation of CC-CKR-5 by overexpressed β-chemokines, or whether CC-CKR5 is dysfunctional for HIV-1 entry into these cells, has yet to be determined. □

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