Inhibition of HIV-1 Infection by Human α-Defensin-5, a Natural Antimicrobial Peptide Expressed in the Genital and Intestinal Mucosae

Lucinda Furci1, Monica Tolazzi1, Francesca Sironi1, Lia Vassena1, Paolo Lusso2

1 Unit of Human Virology, Department of Biological and Technological Research, San Raffaele Scientific Institute, Milan, Italy, 2 Department of Medical Sciences, University of Cagliari School of Medicine, Cagliari, Italy

Abstract

Background: α-defensin-5 (HD5) is a key effector of the innate immune system with broad anti-bacterial and anti-viral activities. Specialized epithelial cells secrete HD5 in the genital and gastrointestinal mucosae, two anatomical sites that are critically involved in HIV-1 transmission and pathogenesis. We previously found that human neutrophil defensins (HNP)-1 and -2 inhibit HIV-1 entry by specific bilateral interaction both with the viral envelope and with its primary cellular receptor, CD4. Despite low amino acid identity, human defensin-5 (HD5) shares with HNPs a high degree of structural homology.

Methodology/Principal Findings: Here, we demonstrate that HD5 inhibits HIV-1 infection of primary CD4+ T lymphocytes at low micromolar concentration under serum-free and low-ionic-strength conditions similar to those occurring in mucosal fluids. Blockade of HIV-1 infection was observed with both primary and laboratory-adapted strains and was independent of the viral coreceptor-usage phenotype. Similar to HNPs, HD5 inhibits HIV-1 entry into the target cell by interfering with the reciprocal interaction between the external envelope glycoprotein, gp120, and CD4. At high concentrations, HD5 was also found to downmodulate expression of the CXCR4 coreceptor, but not of CCR5. Consistent with its broad spectrum of activity, antibody competition studies showed that HD5 binds to a region overlapping with the CD4- and coreceptor-binding sites of gp120, but not to the V3 loop region, which contains the major determinants of coreceptor-usage specificity.

Conclusion/Significance: These findings provide new insights into the first line of immune defense against HIV-1 at the mucosal level and open new perspectives for the development of preventive and therapeutic strategies.

Introduction

With 2.6 million new infections in 2010, two thirds of which (65%) in sub-Saharan Africa, the HIV-1 pandemic remains one of the most important public health challenges worldwide [1]. The limited accessibility to expensive last-generation antiviral drugs and, most of all, the lack of a protective HIV-1 vaccine represent two formidable obstacles for the control of this infection [2]. Since more than 70% of the individuals living with HIV-1 are young women (aged 15–24 years) who acquired the infection through heterosexual contacts [1], effective prophylactic strategies, such as HIV microbicides, could be effective in preventing virus transmission at the mucosal level. The mucosal surface not only poses a physical barrier against pathogens but also hosts diverse defensive mechanisms of natural immunity. Thus, novel vaccination and prevention strategies might benefit from the elucidation of the innate defensive mechanisms that control the early events in HIV-1 invasion at mucosal sites [3].

Studies of vaginal transmission of simian immunodeficiency virus (SIV) demonstrated that between 100- and 1000-fold more virus is required to establish infection in macaques by vaginal application compared to intravenous inoculation [4]. Similar values were obtained from the study of a large cohort of 235 monogamous, HIV-discordant couples in Uganda [5], indicating that the genital mucosal tissue represents in itself a natural obstacle to infection [6]. This circumstantial evidence has been confirmed experimentally by the finding that vaginal fluids inhibit HIV-1 infection in cervicovaginal tissue models ex vivo [7]. The majority of such antiviral activity was identified in the cationic polypeptide fraction, which contains several natural antimicrobial proteins and peptides that act as effectors of the innate host defense and display to variable extent anti-HIV activity [8,9]. These include lysozyme, lactoferrin, secretory leukoprotease inhibitor (SLPI), and defensins.
Results

α-defensin-5 Inhibits HIV-1 Replication in Primary CD4+ T Lymphocytes

Since the mucosal surfaces are a virtually serum-free environment, and several proteins present in bovine serum are known to inactivate α-defensins [19,29,30] we first focused on optimizing the culture conditions for infection of primary human CD4+ T cells in serum-free medium. In agreement with previous observations [31], the lack of serum proteins in the assay significantly decreased the infectivity of HIV-1 resulting in a reduction in virus entry from 30 to 70% depending on the HIV-1 strain used (data not shown). Therefore, to increase virus uptake by target cells we used the spinoculation method, which was reported to significantly improve the efficiency of infection [32]. Indeed, this method yielded a substantially higher level of infection compared to conventional static protocols (data not shown). Thus, we tested the ability of increasing concentrations of HD5 to inhibit infection by a primary HIV-1 isolate (HIV-1J1005) in primary CD4+ T lymphocytes. As shown in Figure 1A, we found that HD5, in the absence of serum, exhibited a potent dose-dependent suppression of HIV-1 replication, with half-maximal inhibitory concentration (IC50) in the nanomolar range (400 nM). The broadly neutralizing mAbs 2G12 (gp120-specific) and Sim4 (CD4-specific) also inhibited infection, indicating that this infection protocol does not alter the physiological HIV-1 entry pathway mediated by envelope-receptor interaction by inducing non-specific membrane fusion events.

Next, we examined the effect of FBS on the antiviral activity of HD5. In agreement with previous observations [29], in the presence of serum HD5 failed to inhibit, and in some cases even slightly upregulated HIV-1 replication (Figure 1A). Since mucosal fluids typically have a low ionic strength [33] and the antimicrobial activities of HD5 are potentiated by low-salt media [34], we also explored the effect of ionic strength on HD5 activity. For this purpose, we infected CD4+ T lymphocytes with HIV-1J176 pre-incubated with HD5 in low-salt medium (10 mM phosphate buffer supplemented with 0.3% human AB serum, ITS supplement (Insulin, Transferrin, Sodium Selenite; Sigma) and 50 U/ml of rIL-2). Figure 1A shows that a low-salt environment decreased the amount of HD5 necessary to suppress viral replication (IC50 60 nM). MAbs 2G12 and Sim4 also potentily inhibited infection under low-salt conditions.

The breadth of anti-HIV activity of HD5 was investigated in activated CD4+ T lymphocytes using a panel of primary and laboratory-adapted HIV-1 isolates of different coreceptor-usage phenotype: three CCR5-specific (R5: HIV-1BaL, HIV-1J1005; HIV-1J176), two CXCR4-specific (X4; HIV-1IIIB, HIV-1J1005) and one dual-tropic (R5X4; HIV-1R5615). HD5, used at 1.4 μM, inhibited the replication of all the HIV-1 isolates tested (Figure 1B). These data indicate that HD5 inhibits different HIV-1 isolates irrespective of their coreceptor-usage phenotype. Most importantly, HD5 is effective against primary HIV-1 isolates, which are physiologically relevant and generally more resistant to antibody-mediated neutralization.

Human α-defensin-5 Inhibits HIV-1 Envelope-mediated Membrane Fusion

To understand if HD5, similarly to HNP1, blocks the early steps of the HIV-1 infectious cycle, we used a quantitative fusion assay based on vaccinia virus technology. PM1 cells expressing HIV-1 envelopes of two prototypic laboratory-adapted strains (IIB, BaL) and four primary isolates (two R5, J6366 and J2615, and two X4, J57 and J287) were cocultured with NIH-3T3 cells expressing recombinant CD4 and either CCR5 or CXCR4 in the presence or absence of increasing concentrations of HD5. HNP-1, which was previously shown to inhibit HIV-1 envelope-mediated fusion [19], was used as a positive control. Figure 2 shows that HD5 inhibited fusion mediated by all the HIV-1 envelopes tested in a dose-dependent fashion. The inhibitory activity was independent of the envelope coreceptor specificity, since both R5 and X4 HIV-1 were inhibited alike even though the IC50 values for the R5 viruses used in these assays were slightly higher than those for the X4 isolates.
(mean IC$_{50}$: 3.67±1.62 µM for R5 envelopes versus 2.77±0.38 µM for X4 envelopes). Consistent with the results obtained in cell-free HIV-1 infection assays, HD5 inhibited with comparable efficiency both laboratory-adapted and primary HIV-1 isolates and exhibited an inhibitory potency in the same range as that of HNP-1 (mean IC$_{50}$: 2.01±0.69 µM). Because envelope-mediated cell fusion closely mimics the molecular events leading to viral entry, these results demonstrate that HD5 blocks HIV-1 infection at its earliest stages, before the viral entry step.

The effect of FBS on HD5 activity was also tested in the fusion assay. As seen in cell-free infection assays, concentrations of FBS as low as 2.5% vol/vol, reduced the inhibitory activity of HD5 by more than one log (IC$_{50}$: 2.3 µM without FBS vs. 56 µM with FBS). An equivalent effect was observed in the presence of 2.5% normal human serum, whereas the levels of maximal fusion in absence of inhibitors were not influenced by the absence of serum in the assay medium (data not shown).

To gain insight into the mechanism(s) of inhibition of HIV-1 envelope-mediated fusion by HD5, we tested the effect of HD5 pre-treatment of either effector cells (expressing the HIV-1 envelope) or target cells (expressing CD4 and the coreceptor) followed by removal of the inhibitor during the fusion assay. Three different viral strains were used for this purpose: two X4 (HIV-1MN and HIV-1BaL) and one R5 HIV-1 isolates; solid bars X4 isolates; the striped bar a dualtropic isolate. The data represent mean values (±SD) of three separate experiments, each performed in triplicate. 

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Figure 1. Effect of HD5 on HIV-1 replication in purified CD4$^+$ T lymphocytes. (A) Effect of recombinant HD5 on infection by primary isolate HIV-1J176 in cell-free infection assays performed using the spinoculation method. HIV-1 virions were preincubated for 1 hour at 37°C with HD5 at the indicated concentrations in either RPMI-10 (10%FBS), RPMI-0.3-ITS (no serum) or 10 mM phosphate buffer 0.3-ITS (low salt). Gag p24 concentrations in the extracellular supernatant were measured on day 6 post-infection. Neutralizing mAbs directed to gp120 (2G12) or CD4 (Sim4) were used as positive controls to exclude non-specific infection. The data represent mean values (±SD) from 3 experiments each performed in triplicate. (B) Broad-spectrum inhibition of different HIV-1 strains by HD5. Purified CD4$^+$ T lymphocytes were infected with two laboratory-adapted strains (HIV-1MN and HIV-1BaL) or 4 primary clinical isolates (HIV-1J106, HIV-1MN, HIV-116195 and HIV-13170) in the presence of HD5 at 1.4 µM in RPMI-0.3-ITS. Gray bars indicate R5 HIV-1 isolates; solid bars X4 isolates; the striped bar a dualtropic isolate. The data represent mean values (±SD) of three separate experiments, each performed in triplicate.
throughout the fusion reaction period. As illustrated in Figure 3A, HD5 was able to inhibit fusion whether pre-incubated with the effector cells or with the target cells. For two of the HIV-1 envelopes tested, the inhibition was slightly more pronounced when HD5 was incubated with envelope-expressing cells. These data suggest that HD5 directly interacts with both the viral envelope and the cellular receptor molecules, in line with our previous observations with α-defensins-1 and -2 [19].

Specific Binding of α-defensin-5 to HIV-1 gp120 and to Human CD4

Next, we evaluated whether HD5 was able to bind directly to the molecules involved in the first HIV-1 docking event on the target cell surface: gp120 and CD4. We previously demonstrated that α-defensins-1 and -2 compete with the binding of a gp120-specific monoclonal antibody, IgG1b12, to recombinant HIV-1 gp120 [19]. Thus, we tested the ability of increasing concentration of HD5 to compete with IgG1b12 in ELISA. As shown in Figure 3B, HD5 reduced in a dose-dependent fashion binding of IgG1b12 to gp120SF162, with an IC50 of 6.5 μM, demonstrating a direct interaction of HD5 with gp120. However, the competition was not complete even at the highest doses of HD5, suggesting an incomplete epitope overlap between the antibody- and the HD5-binding regions of gp120.

To investigate whether HD5 binds to CD4, the primary cellular receptor for HIV-1, increasing amounts of HD5 were used in ELISA to compete with the binding of a CD4-specific mAb, Leu3a, to a recombinant, truncated form of soluble CD4 (sCD4) immobilized on plastic. As shown in Figure 3C a dose-dependent inhibition of Leu3a binding was observed, with an IC50 of 1.8 μM, demonstrating that HD5 directly interacts with the CD4 molecule. Interestingly, the Leu3a-binding site maps to a region within the D1–D2 domain of CD4 that is directly involved in gp120 binding and inhibits gp120 binding and HIV-1 infection in vitro. In contrast, no inhibitory effect was seen when the same competition assays was repeated using mAb OKT4 (not shown), which recognizes an epitope within the D3 and D4 domains, both dispensable for gp120 binding. These results demonstrated that HD5 interacts with the D1–D2 domains of CD4, thus potentially hindering gp120 access to its binding site.

Mapping of the HD5-binding Region of gp120

To define the putative binding region of HD5 within the gp120 glycoprotein, we used a panel of gp120-specific mAbs of human or...
murine origin that recognize three major functional domains of gp120: the CD4-binding site, the coreceptor-binding site and the V3 loop. ELISA assays were performed using recombinant monomeric gp120 immobilized on plastic. A decreased binding activity was seen with mAbs directed against the CD4-binding region (F105, IgG1b12), or against CD4-induced epitopes that overlap with the coreceptor-binding site (17b, 48d and A1g8); by contrast, no significant interference was seen with the binding of mAbs 447-52D, B4a1 and 268-D, all directed against relatively conserved epitopes within the V3 domain (Figure 4). These data confirmed that HD5 directly interacts with HIV-1 gp120, binding to a region that overlaps, is contiguous to, or influences the conformation of the CD4- and coreceptor-binding domains. Similar results were obtained using a second recombinant gp120, derived from another R5 isolate, HIV-1SF162 (data not shown).

Differential Effect of HD5 on HIV-1 Coreceptor Expression

After binding to CD4, gp120 engages a coreceptor, such as CXCR4 or CCR5, in order to promote viral entry into susceptible cells [35]. We questioned if the antiviral activity of HD5 could be in part mediated by interaction with the coreceptors. The effect of HD5 on coreceptor expression was studied by flow cytometry both on PM1 cells, which naturally express both CCR5 and CXCR4 [36], and on activated peripheral blood lymphocytes, which represent the most physiological ex vivo model. The activity of HD5 was compared with that of HNP1. Target cells were incubated with or without HD5 or HNP1 at 14.7 μM in RPMI-0 for 2 hours at 37°C and then stained with two anti-CXCR4 mAbs that inhibit infection by X4 HIV-1 strains [37]: 12G5, a conformation dependent mAb directed against a bridging epitope spanning the first and second extracellular loops of CXCR4 [38], and 44717.111, a conformation-dependent mAb that recognizes CXCR4 with higher efficiency that 12G5 on both PM1 and primary T lymphocytes [39]. Figure 5 shows that HD5 inhibited the binding of both 12G5 (mean fluorescence intensity [MFI] reduction 58.5 ± 2.9% on PM1; 70.04% ± 2.8% on primary T cells) and 44717.111 (MFI reduction 33.5 ± 5.5% on PM1; 56.9% ± 3.1% on primary T cells). Interestingly, in the PM1 cells model
upregulated HIV-1 replication in primary CD4+ T lymphocytes, but the physiological significance of the latter finding is questionable. HD5 is naturally secreted at mucosal surfaces [23], which constitute a virtually serum-free environment. We decided to address this issue by optimizing the culture conditions for testing HIV-1 infection and fusion in the presence of only trace amounts or in complete absence of serum-derived proteins. Using these assays, we were able to demonstrate that HD5 is a potent and broad-spectrum inhibitor of biologically diverse HIV-1 strains. Indeed, the inhibitory activity of HD5 was independent of the HIV-1 coreceptor-usage phenotype and was seen not only on laboratory-adapted strains but also on primary clinical isolates minimally passaged in vitro models versus in vivo models [33]. To mimic these conditions, we tested the effect of HD5 in low-ionic strength buffer characterized by low-salt concentrations [33]. To mimic these conditions, we tested the effect of HD5 in low-ionic strength buffer characterized by low-salt concentrations [33]. To mimic these conditions, we tested the effect of HD5 in low-ionic strength buffer characterized by low-salt concentrations [33]. To mimic these conditions, we tested the effect of HD5 in low-ionic strength buffer characterized by low-salt concentrations [33]. To mimic these conditions, we tested the effect of HD5 in low-ionic strength buffer characterized by low-salt concentrations [33]. To mimic these conditions, we tested the effect of HD5 in low-ionic strength buffer characterized by low-salt concentrations [33]. To mimic these conditions, we tested the effect of HD5 in low-ionic strength buffer characterized by low-salt concentrations [33]. To mimic these conditions, we tested the effect of HD5 in low-ionic strength buffer characterized by low-salt concentrations [33]. To mimic these conditions, we tested the effect of HD5 in low-ionic strength buffer characterized by low-salt concentrations [33]. To mimic these conditions, we tested the effect of HD5 in low-ionic strength buffer characterized by low-salt concentrations [33]. To mimic these conditions, we tested the effect of HD5 in low-ionic strength buffer characterized by low-salt concentrations [33]. To mimic these conditions, we tested the effect of HD5 in low-ionic strength buffer characterized by low-salt concentrations [33]. To mimic these conditions, we tested the effect of HD5 in low-ionic strength buffer characterized by low-salt concentrations [33]. To mimic these conditions, we tested the effect of HD5 in low-ionic strength buffer characterized by low-salt concentrations [33]. To mimic these conditions, we tested the effect of HD5 in low-ionic strength buffer characterized by low-salt concentrations [33]. To mimic these conditions, we tested the effect of HD5 in low-ionic strength buffer characterized by low-salt concentrations [33]. To mimic these conditions, we tested the effect of HD5 in low-ionic strength buffer characterized by low-salt concentrations [33]. To mimic these conditions, we tested the effect of HD5 in low-ionic strength buffer characterized by low-salt concentrations [33]. To mimic these conditions, we tested the effect of HD5 in low-ionic strength buffer characterized by low-salt concentrations [33]. To mimic these conditions, we tested the effect of HD5 in low-ionic strength buffer characterized by low-salt concentrations [33]. To mimic these conditions, we tested the effect of HD5 in low-ionic strength buffer characterized by low-salt concentrations [33]. To mimic these conditions, we tested the effect of HD5 in low-ionic strength buffer characterized by low-salt concentrations [33]. To mimic these conditions, we tested the effect of HD5 in low-ionic strength buffer characterized by low-salt concentrations [33]. To mimic these conditions, we tested the effect of HD5 in low-ionic strength buffer characterized by low-salt concentrations [33]. To mimic these conditions, we tested the effect of HD5 in low-ionic strength buffer characterized by low-salt concentrations [33]. To mimic these conditions, we tested the effect of HD5 in low-ionic strength buffer characterized by low-salt concentrations [33].
In the order of 90–450 genital mucosae although it could play a more important role in the principal target of HIV-1 infection. Decreased cell surface positioning of the highly conserved CD4-binding site in regions poorly accessible to neutralizing antibodies and the presence of multiple N-linked glycosylation sites accounting for over one-half of the molecular mass [57]. HD5 poses both a strong capacity to bind carbohydrates and glycoproteins including gp120hum (Ka = 24.5 nM) and gp120mul. (Keq106 nM) [47], and being a very small and tightly folded molecule it is likely to have access to hidden neutralization sites where full-length antibodies may not be able to reach.

Although our results indicate that HD5 inhibits the earliest stages of HIV-1 infection, preventing viral entry into the target cell, we cannot exclude an effect on downstream steps in the virus life cycle, as previously shown for HNP-1 [20,58]. Interestingly, it was demonstrated that HD5 and HNP5 inhibit herpes simplex virus infection by acting both at the entry and post-entry levels [41]. A growing body of evidence [17,20,21] is compatible with the notion that multiple mechanisms cooperate toward inhibition of HIV-1 infection by human defensins, and further studies will be required to define these complex mechanisms of viral inhibition.

HD5 is expressed and stored in secretory granules of the Paneth cells located at the base of the crypts in the small intestine. The massive HIV-1 replication that takes place in the gastrointestinal tract early in the course of the infection not only plays a critical role in the massive depletion of CD4+ T cells within the lamina propria, but also results in a seemingly irreversible damage to the architecture of the intestinal mucosa leading to crypt hyperplasia, decreased number of Paneth cells and decreased levels of luminal defensins [59,60]. These observations, together with our data on the antiviral effect of HD5 suggest a possible role of HD5 in the pathogenesis of HIV-1 infection, which needs to be addressed in further studies.

Our data also show that HD5 inhibits HIV-1 infection in primary CD4+ T lymphocytes at concentrations similar to those measured in the female and male lower genital tracts [23,43], suggesting that HD5 can function as a natural immune barrier against HIV-1 sexual transmission. Moreover, HD5 may share the mechanism of action of cyanovirin-N, a lectin-like protein that binds to mannose moieties in gp120 and of mAb IgG1b12, with which it competes for the same binding site on CD4 receptor. Both of these virus-targeting entry-inhibitors have been reported to protect macaques from vaginal SHIV-challenge [61].

A potential exploitation of the antiviral properties of HD5 could be the exogenous application of synthetic defensin peptides as part of a preventive microbicide strategy. So far, it has been difficult to establish an association between levels of mucosal antimicrobial peptides and decreased HIV-transmission [62] since higher genital levels of anti-HIV immune factors are invariably associated with bacterial vaginosis and sexually transmitted infections, which are known to increase HIV susceptibility [63]. However, the prospect that HD5 might function as a safe, nonimmunogenic and noninflammatory topical microbicide is supported by the fact that this defensin is normally found at antiviral concentrations in the female genital tract [23,43]. Moreover, the documented antimicrobial effect of HD5 against herpes simplex viruses, papillomavirus and pathogenic bacteria like Neisseria gonorrhoeae and Chlamydia trachomatis, all of which cause genital infections that act as cofactors in HIV-1 transmission, suggests that this defensin has the potential to function as a broad-spectrum topical microbicide [64]. Pre-clinical studies in suitable animal models will be important to assess the potential efficacy of HD5 in the prevention and control of HIV-1 infection.
Methods

Proteins and Antibodies

Synthetic human HD5 was purchased from Peptides International (Louisville, KY). Recombinant HIV-1 gp120 proteins from HIV-1 isolates SF162 and BaL, recombinant human soluble CD4 (sCD4), human anti-HIV-1 gp120 mAb directed to the V3 loop (447-52D, B4a1 and 268-D), to the CD4-binding site (F105 and IgG1b12) and to CD4-induced epitopes adjacent to the coreceptor-binding site (17b, 49d and Alg1) and anti-CXCR4 mAb D12G were obtained from the NIH AIDS Research and Reference Reagent Program (ARRRP, Rockville, MD). Murine anti-V3 loop mAb D19 was obtained by standard hybridoma technology as previously described [65]. Additional mAbs used were anti-CD4, Leu3a (Becton Dickinson, San Jose, CA) and OKT4 (Ortho Diagnostics, Raritan, NJ), anti-CCR5 clone 2D7 (BD Pharmingen, La Jolla, CA), anti-CXCR4 mAb clone 4417.111 (R&D Systems, Minneapolis, MN), anti-CD8, -CD14, -CD19 and -CD56 (ImmunoTools, Friesoythe, Germany).

Viruses and Persistently Infected Cell Lines

Laboratory-passaged HIV-1 isolates IIIB, MN and BaL were obtained through the NIH ARRRP. All the primary HIV-1 isolates, kindly provided by Dr. Gabriella Scarlatti (DIBIT-HSR, Milan), were obtained by cocultivation of patient peripheral blood mononuclear cells (PBMC) with activated PBMC from healthy blood donors and minimally passaged in vitro exclusively in primary cells, as previously reported [66]. Infectious viral supernatants were used to prepare chronically infected PM1 and SupT1 as previously described [65]. Serum-free virus stocks were prepared by ultracentrifugation of the viral stocks, originally produced in the presence of 10% FBS, on a 20% sucrose cushion as described [67], with slight modifications. Briefly, cell debris was initially removed by centrifugation at 470g for 5 min and subsequent filtration through a 0.22-μm pore-size filter. The virus stocks were layered on top of a 20% (wt/vol) sucrose solution in TNE buffer (20 mM Tris [pH 8.0], 150 mM NaCl, and 2 mM EDTA). The virus was pelleted at 19,000 × g for 2 h at 4°C and resuspended in serum-free RPMI supplemented with glutamine and antibiotics (RPMI-0). Viral stocks were titrated on activated human CD4+ T cells. Aliquots were frozen at −80°C. HeLa and SupT1 cell lines were obtained from the American Type Culture Collection. The PM1 cell clone and NIH3T3 mouse fibroblast cells stably expressing human CD4 and either CCR5 or CXCR4 were provided by the NIH ARRRP. The study protocol was approved by the institutional review board at the San Raffaele Research Institute (Milan, Italy). Written consent was obtained from all study participants.

Acute HIV-1 Infection Assay

PBMCs were isolated from healthy blood donors by standard Ficoll-HyPaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation from concentrated leukocytes of healthy blood donors and stimulated with 5 μg/ml PHA (Sigma, St. Louis, MO) in complete RPMI medium supplemented with 10% FBS and 50 U/ml recombinant IL-2 (Proluken; Chiron Corporation, Emeryville, CA) (RPMI-10). After 3 days in culture, CD4+ T cell population was enriched by negative immunomagnetic selection using Dynabeads M-450 (Invitrogen Carlsbad, CA) coated with monoclonal antibodies to CD8, CD14, CD19 and CD56 (Sigma, St. Louis, MO) according to the manufacturer's protocol. Infection was carried out by spinoculation for 1.5 hours as previously described [32] with minor modifications. Briefly, CD4+ T cells were washed twice in RPMI-0 and plated (10^5/well) in 96-wells U-bottom microtiter plates with 50 50% tissue culture infective dose (TCID50) of serum-free HIV-1 virions, preincubated for 1 hour at 37°C with HD5 at the indicated concentrations. The plates were centrifuged at 1,000g for 1.5 hrs and washed twice with RPMI-0 to remove unbound virus, after which HD5 was added back to the cells. Residual virus associated to cells after washes was below 100pg of p24/ml (day 0 time point). Infected cells were cultured in 200 μl of RPMI-1640 supplemented with 0.3% human AB serum, ITS supplement (Insulin, Transferrin, Sodium Selenite; Sigma) and 50 U/ml of IL-2 (RPMI-0.3-ITS) in the presence or absence of inhibitors. Culture in ITS serum-free media supplement and 0.3% human AB serum to RPMI-0 culture medium did not significantly alter the viability of purified CD4+ lymphocytes or PM1 and SupT1 cell lines, as analyzed using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega Madison, WI), nor the expression of CD4 and the CCR5 and CXCR4 coreceptors, as analyzed by flow cytometry as previously described [19] (data not shown).

The HIV-1 p24 Ag concentrations in the culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA) at days 4 and 6 post-infection as previously described [68]. Briefly, p24 antigen from a detergent lysate of virions was captured by an immobilized anti-p24 polyclonal antibody (D7290; Aalto Bio Reagents, Dublin, Ireland). Bound p24 Ag was then detected using an alkaline phosphatase-conjugated anti-p24 monoclonal antibody (BC 1071-AP; Aalto Bio Reagents) and the AMPAK ELISA amplification system (DAKO A/S, Glostrup, Denmark).

HIV-1 Envelope-mediated Cell Fusion Assay

The HIV-1 Env-mediated fusion assay was performed as described [65] using a modification of the test originally developed by Berger and coworkers [69]. In the modified assay, HIV-1 Env effector cells were T cell lines (PM1 or SupT1) chronically infected with laboratory strains or primary HIV-1 isolates, (coinfected with recombinant vaccinia virus VCB21R encoding the E Coli lacZ reporter gene linked to the T7 promoter). Target cells were NIH 3T3 mouse fibroblast cells stably expressing human CCR5 or CXCR4 and human CD4 (infected with vaccinia recombinant vT7 encoding bacteriophage T7 RNA polymerase, vT117). Effector and target cells were mixed in 96-well plates (10^5 each cell type per well) and incubated at 37°C in RPMI-0 with or without inhibitors; after 2 h cells were lysed with a nonionic detergent, and β-galactosidase activity was quantitated by spectrophotometry.

ELISA Assays

Flat-bottom, 96-well Maxisorp plates (NUNC, Roskilde, Denmark) were coated with recombinant sCD4 (5 μg/well) or HIV-1 gp120 (2 μg/well) in phosphate-buffered saline (PBS) for 18 hrs at 4°C. Bovine serum albumin (BSA) 3% was used for blocking the plates. Antibodies were added in 100 μl PBS+0.05%BSA and incubated for 1 hr at 37°C, after which the plate was washed and incubated for an additional hour at room temperature with the secondary antibody. Peroxidase-conjugated, affinity-purified goat-anti-mouse IgG or goat-anti-human IgG (Dako, Glostrup, Denmark) were used as secondary antibodies. The reaction was revealed by using an appropriate substrate. The specific signal was calculated by subtracting background values obtained from replicate wells containing all the reagents except the specific ligand for each assay. Binding of mAbs 17b and 48d, directed to CD4-induced epitopes, was performed after preincubation of immobilized gp120 with sCD4 (2 μg/well) in PBS for 20 min at 37°C [63].
Flow Cytometry

PM1 or PHA-activated peripheral blood lymphocytes were incubated with or without α-defensins in RPMI without FBS and then stained with the mAbs for 20 minutes at room temperature. Cells were then washed with PBS supplemented with 1% FBS and then stained with the mAbs for 20 minutes at room temperature. Incubated with or without staining.

In some experiments, the cells were fixed with 2% paraformaldehyde before treatment with α-defensins and mAb staining.

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Author Contributions

Conceived and designed the experiments: LF PL. Performed the experiments: MT FS LV. Analyzed the data: LF FS LV PL. Contributed reagents/materials/analysis tools: LF MT FS LV. Wrote the paper: LF PL.
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