Blockade of HIV-1 Infection of New World Monkey Cells Occurs Primarily at the Stage of Virus Entry

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Abstract

HIV-1 naturally infects chimpanzees and humans, but does not infect Old World monkeys because of replication blocks that occur after virus entry into the cell. To understand the species-specific restrictions operating on HIV-1 infection, the ability of HIV-1 to infect the cells of New World monkeys was examined. Primary cells derived from common marmosets and squirrel monkeys support every phase of HIV-1 replication with the exception of virus entry. Efficient HIV-1 entry typically requires binding of the viral envelope glycoproteins and host cell receptors, CD4 and either CCR5 or CXCR4 chemokine receptors. HIV-1 did not detectably bind or utilize squirrel monkey CD4 for entry, and marmoset CD4 was also very inefficient compared with human CD4. A marmoset CD4 variant, in which residues 48 and 59 were altered to the amino acids found in human CD4, supported HIV-1 entry efficiently. The CXCR4 molecules of both marmosets and squirrel monkeys supported HIV-1 infection, but the CCR5 proteins of both species were only marginally functional. These results demonstrate that the CD4 and CCR5 proteins of New World monkeys represent the major restriction against HIV-1 replication in these primates. Directed adaptation of the HIV-1 envelope glycoproteins to common marmoset receptors might allow the development of New World monkey models of HIV-1 infection.

Key words: human immunodeficiency virus • species restrictions • New World monkeys • receptors • virus entry

Introduction

The primate lentiviruses include the human immunodeficiency viruses (HIV-1 and HIV-2) and simian immunodeficiency viruses (SIVs).* In nature, HIV-1 and HIV-2 infect humans, HIV-1–related CPZ viruses infect chimpanzees, and SIV variants infect African monkeys (1–4). Humans infected by HIV-1 and HIV-2 and Asian macaques infected by certain SIV and HIV-2 strains often develop life-threatening immunodeficiency (AIDS) due to depletion of CD4-positive T lymphocytes (1, 2, 5–8). HIV-1 is the major cause of the global AIDS epidemic (9).

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**Abbreviations used in this paper:* CJ; *Callithrix jacchus*; CXCR, CXC chemokine receptor; ECL, extracellular loop; EGFP, enhanced green fluorescent protein; MM, *Macaca mulatta*; RT, reverse transcriptase; SHIV, simian-HIV; SIV, simian immunodeficiency virus; SS, *Saimiri sciureus*; VSV, vesicular stomatitis virus.

Infection by primate immunodeficiency viruses is dependent upon a number of host cell factors, some of which exhibit species-specific variation. An appreciation of the functional consequences of this variation for susceptibility to virus infection is important for understanding the origins and expansion of the HIV-1 epidemic. Furthermore, this knowledge can expedite the development of animal models of HIV-1 infection, which facilitate the design and improvement of therapeutic and prophylactic modalities and further our understanding of pathogenesis.

Currently available animal models of HIV-1 infection have limitations. HIV-1 infection of chimpanzees rarely results in immunodeficiency (10–15). Furthermore, the endangered status of these animals limits their availability and prohibits many experiments (16).

Asian rhesus macaques infected with certain strains of SIV and HIV-2 develop fatal AIDS-like illness, and this model has been useful in studying pathogenesis (17, 18). However, for therapeutic and vaccine studies, differences

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between SIV and HIV-1 often preclude extrapolation of results derived from this model to HIV-1–infected humans (19). For example, many antiviral drugs and immune responses directed against HIV-1 do not cross-react with SIV components. HIV-1 entry, which depends upon the binding of the viral gp120 envelope glycoprotein to host cell receptors (CD4 [20–22] and one of the chemokine receptors, CCR5 or CXC chemokine receptor [CXCR]4 [23–29]), is efficient in Old World monkey cells (30, 31). These cells, however, exhibit blocks to HIV-1 infection in an early stage of virus infection after virus entry (32). Chimeric simian–HIVs (SHIVs) encoding the HIV-1 envelope glycoproteins (and the HIV-1 Tat, Rev, and Vpu regulatory proteins) can infect and, in some cases, induce AIDS-like illness in Old World monkeys (30, 33). The consistency and rapidity of AIDS development in SHIV-infected macaques have made this model extremely useful in studies of viral pathogenesis and vaccine development. Nonetheless, because many antiviral agents and immune responses are directed against HIV-1 elements not included in the available SHIV chimerae, the development of new animal models involving infection with more complete HIV-1–like viruses is a worthy goal.

Mammalian species other than the primates have been examined as possible hosts for HIV-1. Rabbits were reported to be susceptible to HIV-1 infection, but the low level and reproducibility of virus replication in this species renders this model practically useless (34, 35). The poor ability of rabbits and rodent species to support HIV-1 infection has been investigated (36). The mouse and rabbit CD4 and CCR5 molecules do not support efficient HIV-1 entry into host cells (24, 37–40). Although rabbit CXCR4 is not permissive for HIV-1 entry (38), some HIV-1 strains can utilize murine CXCR4 (41, 42). Transgenic mice expressing human CD4 and coreceptor molecules fail to support HIV-1 replication even though virus entry occurs (43, 44). The identification of human cyclin T as a Tat cofactor allowed proviral transcriptional blocks to be overcome in mouse cells (45–48). Assembly defects exist for HIV-1 in murine cells that further complicate the development of small animal models of HIV-1 infection (49). Likewise, despite some detectable level of HIV-1 replication in cultured cells from CD4/CCR5 transgenic rabbits, in vivo replication of HIV-1 in this host was very limited (38, 50).

The existence of multiple blocks to HIV-1 infection in mammals evolutionarily distant from humans prompted us to investigate the nature of HIV-1 replication blocks in the New World primates, which are more closely related to the natural hosts of primate immunodeficiency viruses. Some early reports (51, 52) suggested that the PBMCs of some New World monkeys could support HIV-1 infection, although the validity of this claim is questionable; the same group reported that rhesus macaque PBMCs, which have been shown to exhibit stringent blocks to HIV-1 infection (32), also allowed HIV-1 replication (51, 52). Intraperitoneal inoculation of HIV-1 into two New World monkeys, cotton-top tamarins and common marmosets, did not result in seroconversion (53). The basis for the apparently minimal replication of HIV-1 in New World monkeys is unknown. Previous studies suggested that recombinant HIV-1 vectors pseudotyped with the vesicular stomatitis virus (VSV) G glycoprotein could infect and express a heterologous protein in New World, but not Old World, monkey cells (32). Thus, at least some of the early blocks to HIV-1 infection in Old World monkeys are not present in New World monkeys. The ability of HIV-1 to negotiate late stages of the retroviral life cycle in New World monkey cells is unknown. Here we investigate the basis for the restricted replication of HIV-1 in the cells of two New World monkey species.

Materials and Methods

Cell Lines and Primary Cell Cultures. 293T and Cf2Th (American Type Culture Collection) cells were grown in DMEM-10% FCS with antibiotics. Jurkat and CEMx174 human T cell lines were cultured in RPMI 1640–10% FCS with antibiotics. Squirrel monkey (*Saimiri sciureus* or SS), common marmoset (*Callithrix jacchus* or CJ), and rhesus macaque (*Macaca mulatta* or MM) PBMCs were isolated from fresh blood by Ficoll-Paque (Amersham Biosciences) density centrifugation. These animals were maintained in accordance with the guidelines of the Committee on Animals for the Harvard Medical School and the Guide for Care and Use of Laboratory Animals. All isolated primary cells were cultured (10^6 cells/ml) in RPMI 1640–10% FCS with antibiotics. Activation of primary cells was achieved by an initial 3-d stimulation with 1 μg/ml purified phytohemagglutinin (Murex Biotech) and subsequent culturing with 10 U/ml of recombinant human IL-2 (Collaborative Biomedical Products).

Virus Preparation. Replication-competent HIV-1 virus was generated by transfecting 10 μg pNL4–3 plasmid, which contains an infectious HIV-1 NL4–3 provirus (54), into 5×10^6 293T cells using the calcium phosphate transfection method. VSV G-pseudotyped NL4–3 virus was generated by cotransfecting 10 μg pNL4–3 and 2 μg of pHCMV-G (55, 56), a VSV G expression plasmid. A recombinant HIV-1 capable of a single round of infection and encoding the enhanced green fluorescent protein (EGFP) was generated by cotransfecting 293T cells with 10 μg pHIVec2.GFP (32), 10 μg pCMVΔP1ΔenvpA (55, 56), and 2 μg of plasmids expressing viral envelope glycoproteins. These included the pHCMV-G plasmid, which encodes the VSV G glycoprotein, and pSVIIIenv plasmids encoding the envelope glycoproteins derived from the HIV-1 strains HXBc2, 3.2, KB9, MN, MCGP, ADA, JR-FL, and YU2 (57–59). 1 μg of a Rev-expressing plasmid was cotransfected in the case where the pHCMV-G plasmid was used; this was not required when the pSVIIIenv plasmids, which encode functional HIV-1 Rev proteins, were used (60). 12 h after transfection, the cells were washed and cultured in fresh RPMI 1640–10% FCS with antibiotics. 24 h later, conditioned medium containing recombinant viruses was harvested and filtered (0.45-μm pore size). The level of recombinant virus in the medium was determined using a previously described reverse transcriptase (RT) assay (61).

Virus Infections. Approximately 100,000 RT cpm of NL4–3 or VSV G-pseudotyped NL4–3 virus were added to 5×10^5 cells in a total volume of 500 μl in 24-well plates. The target cells were CEMx174 lymphocytes or primary MM, SS, or CJ PBMCs. 16 h after adding the virus to the cells, the plates were centrifuged at 2,000 rpm for 5 min. The medium was removed, and the cells were washed with PBS and resuspended in 1.5 ml

RPMI-10% FCS supplemented with IL-2 and antibiotics. At 24 h time points, 500-μl samples of the culture medium were collected from duplicate cultures and used to measure RT activity. Virus produced on day 2 after incubation of virus and cells was collected, and equivalent amounts (5,000 cpm) of RT activity were added to 5×10^5 CEMx174 cells in a total volume of 1 ml in 24-well plates. After incubation of the NL4–3 virus with primary MM, SS, or CJ PBMCs, no measurable virus was produced; in this case, 1 ml of culture supernatant was added to the CEMx174 cells. 1 d after infection the CEMx174 cells were washed with PBS and resuspended in RPMI-10% FCS with antibiotics. Subsequently, RT activity was measured in the CEMx174 cell supernatants every 24 h.

Cloning of New World Monkey CD4 and Chemokine Receptor cDNAs. Messenger RNA was extracted from primary SS or CJ PBMCs using the RNeasy Mini Kit (QIAGEN). After Dnase treatment using the RNase-Free Dnase Set (QIAGEN), first-strand cDNA was generated using the Reverse Transcription System (Promega). PCR was performed on 2 μl of the cDNA using the Expand High Fidelity PCR System (Roche Diagnostic Corp.). CXCR4 was amplified using primers CXCutr52: GCTCCAGTAGCCACCGCATCT and CXCutr31: AAAACTGTACAATATTGGTCAGTC. CCR5 was amplified using primers CC5utr53: GCTGAGACATCCGTTCCCCTAC and CC5utr31: GCCCAGGCTGTGTATGAAAACT. CD4 was amplified using primers CD4utr52: TCCCTCAGCAAGGCCACAATG and CD4utr31: GATCTCCCTGGCCTCGTGC. PCR products were sequenced using the same primers. Molecules were cloned into the mammalian expression plasmid pcDNA3.1(+) (Invitrogen). CD4 molecules were also cloned into pcDNA3.1(+) ZEO(+) (Invitrogen). Predicted amino acid sequence and alignments were generated using DNAstar software (DNAstar, Inc.). Quick-change point mutations were made using the following primers. For SS CD4 Y42S, 5′:AATATTCTGGGAGTTCAGAATTCCTTCGTGACTAGAGGTCAATCC and 3′:GGATTGACCTCTAGTCACGAAGGAATTCTGAACTCCCAGAATCTT. For SS CD4 Q48P, 5′:GCGATCGGTCAGCTTGGAGGGCCCTCTAGTCACGAAG and 3′:CTTCGTGACTAGAGGGCCCTCCAAGCTGACCGATCG. For SS CD4 R33Q, 5′:GTTCCACTGGAAAACCTCCGACCAGATAAAGATTCTGGGAGTTCAGAAC and 3′:GTTCTGAACTCCCAGAATCTTTATCTGGTCGGAGGTTTTCCAGTGGAAC.

For CJ CD4 Q59R, 5′:CCAAGCTGGCCAATCGCATTGACTGATCGTCTTGGGACCGAGGATCC and 3′:GGATCCTCGGTCCCAAGACGATCGCTTTGAGTCAATGCGATTGGCCAGCTTGG. For CJ CD4 Q48P, 5′:GCGATTGGCCAGCTTGGAGGGCCCTTTAGTCACGAAGGAG and 3′:CTCCTTCGTGACTAAAGGGCCCTCCAAGCTGGCCAATCGC.

Generation of Cell Lines Stably Expressing Receptors. Cf2Th cells were transfected with pcDNA 3.1 (+) plasmids expressing human, squirrel monkey or common marmoset CCR5 or CXCR4 proteins. Clones were selected in DMEM-10% FCS supplemented with 500 μg/ml G418 (GIBCO BRL). FACS® was used to enrich for cells expressing high levels of the receptors. These were then transfected with pcDNA3.1 ZEO plasmids expressing the CD4 molecule derived from the same species. Cells expressing the CD4 molecule and chemokine receptor were selected in medium containing 500 μg/ml G418 and 300 μg/ml Zeocin (Invitrogen) and FACS® was used to identify cells expressing high levels of both receptors.

Monoclonal Antibody Recognition of Receptor Molecules. Using the calcium phosphate transfection method, 5×10^6 Cf2Th cells were transfected with 10 μg of plasmid expressing the CD4 or chemokine receptor. After 48 h, the cells were detached with 5 mM EDTA in PBS, pelleted, washed, and resuspended in 95 μl PBS containing 2% FCS and 5 μl of monoclonal antibody (final antibody concentration was 10 nM). The monoclonal antibodies (and the target proteins) were: 12G5 (CXCR4), 3A9 (CCR5), 2D7 (CCR5), CD4v4 (CD4) (BD Biosciences), OKT4A (CD4; Ortho Diagnostic Systems), Q4120 (CD4; Sigma-Aldrich), and 44717.111 (CXCR4; R&D Systems). Samples were incubated for 20 min at 4°C, washed, and analyzed by flow cytometry (FACScan™; Becton Dickinson).

Virus Infection Assays. Using the calcium phosphate transfection method, 5×10^6 Cf2Th cells were transfected with 8 μg of plasmids encoding receptors. After 16 h, the cells were washed and plated at a density of 2.5×10^4 cells per well in 24-well plates. Untransfected cells were plated in parallel to provide negative controls. After 24 h, one well of cells from each experimental group was stained with antibodies to assess the level of receptor expression on the cell surface. To each of the duplicate remaining wells in the experimental group, 5×10^5 RT cpm of recombinant HIV-1 encoding EGFP was added in a total volume of 400 μl. Viruses contained various HIV-1 envelope glycoproteins or the VSV G glycoprotein, as discussed above. The cultures were spinoculated by centrifuging the 24-well plates at 1,200 rpm for 45 min at room temperature (62). 48 h later, the cells were harvested using 5 mM EDTA in PBS, fixed in 3.7% formaldehyde (Sigma-Aldrich), and analyzed by flow cytometry for EGFP expression.

Single-round infection assays with target cells stably expressing receptors were conducted similarly, except that 5×10^5 RT cpm of recombinant virus was spinoculated onto 4×10^4 cells per well in 24-well plates. EGFP-positive cells were photographed with a Nikon Eclipse TE 300 microscope and Roper Scientific CCD camera before cell harvesting.

HIV-1 gp120-CD4 Binding Assays. The HIV-1 gp120 glycoproteins from the R5 isolates ADA, YU2 and JR-FL were produced by pSVIIIenv plasmids in which stop codons had been introduced near the sequences encoding the natural gp120-gp41 junction. The plasmids were transfected into 293T cells using the Geneporter 2 transfection reagent (Gene Therapy Systems) and the medium harvested 48 h after transfection. The gp120 glycoproteins were purified by affinity chromatography using an F105 antibody column and quantified by silver staining and spectrophotometry.

To create target cells for the binding assays, 293T cells were transfected with plasmids encoding CD4 variants using Geneporter 2 reagent. The CD4 molecules used were human CD4, marmoset (CJ) CD4 proteins (wild-type and Q48P, Q59R, and Q48P/Q59R), and squirrel monkey (SS) CD4 proteins (wild-type and Q48P, Y42S, and Q48P/Y42S). Cells transiently expressing the CD4 variants were stained with the anti-CD4 antibody (Q4120; Sigma-Aldrich) to assess relative levels of surface CD4 expression. Parallel samples of transiently transfected cells were harvested with PBS/5 mM EDTA and resuspended in PBS/2% FCS at a density of 4×10^7 cells/ml. Then 2×10^6 cells were added to microcentrifuge tubes (Eppendorf) containing varying concentrations (0.01 nM to 3 μM) of the ADA, YU2, and JR-FL gp120 glycoproteins. After a 1-h incubation at 37°C, cells were washed in PBS/2% FCS and resuspended in 100 μl of PBS/2% FCS containing 1 μg of the C11 anti-gp120 antibody. After a 45-min incubation at 4°C, the cells were washed

once in PBS/2% FCS and resuspended in buffer containing 5 μl of anti–human IgG-PE (Jackson ImmunoResearch Laboratories). The cells were incubated for 20 min at 4°C, washed twice, and analyzed using a Becton Dickinson FACScan™ using CELLQuest™ software.

Results

Susceptibility of New World Monkey PBMCs to HIV-1 Infection. To examine the susceptibility of New World monkey cells to HIV-1 infection, PBMCs from squirrel monkeys (SS) and common marmosets (CJ) were inoculated with the NL4–3 virus, a CXCR4-using HIV-1 isolate. Anticipating that the SS and CJ receptors might not optimally support HIV-1 entry, the PBMCs from these New World primates were also incubated with NL4–3 (VSV G), which is an NL4–3 virus transiently pseudotyped with the VSV G glycoprotein. The VSV G glycoprotein has previously been shown to allow HIV-1 to enter New World monkey cell lines, and is incorporated into the NL4–3 virions during their production from 293T cells transfected with an HIV-1 provirus. During the initial round of infection, the NL4–3 (VSV G) pseudotypes can bypass the typical requirement of HIV-1 for the CD4 and chemokine receptor proteins. The CD4-positive, CXCR4-positive human lymphocyte line CEMx174 was included as a positive control and rhesus macaque PBMCs were included as a negative control.

CEMx174 cells supported the production of the NL4–3 and NL4–3 (VSV G) viruses, which peaked on the second day after inoculation (Fig. 1 A, left). The viruses produced from the CEMx174 cells were able to initiate subsequent rounds of infection in CEMx174 cells (Fig. 1 A, right). In parallel experiments, the NL43 NL4–3 (VSV G) virus produced in 293T cells was incubated with the PBMCs from two rhesus macaques, an Old World monkey that restricts

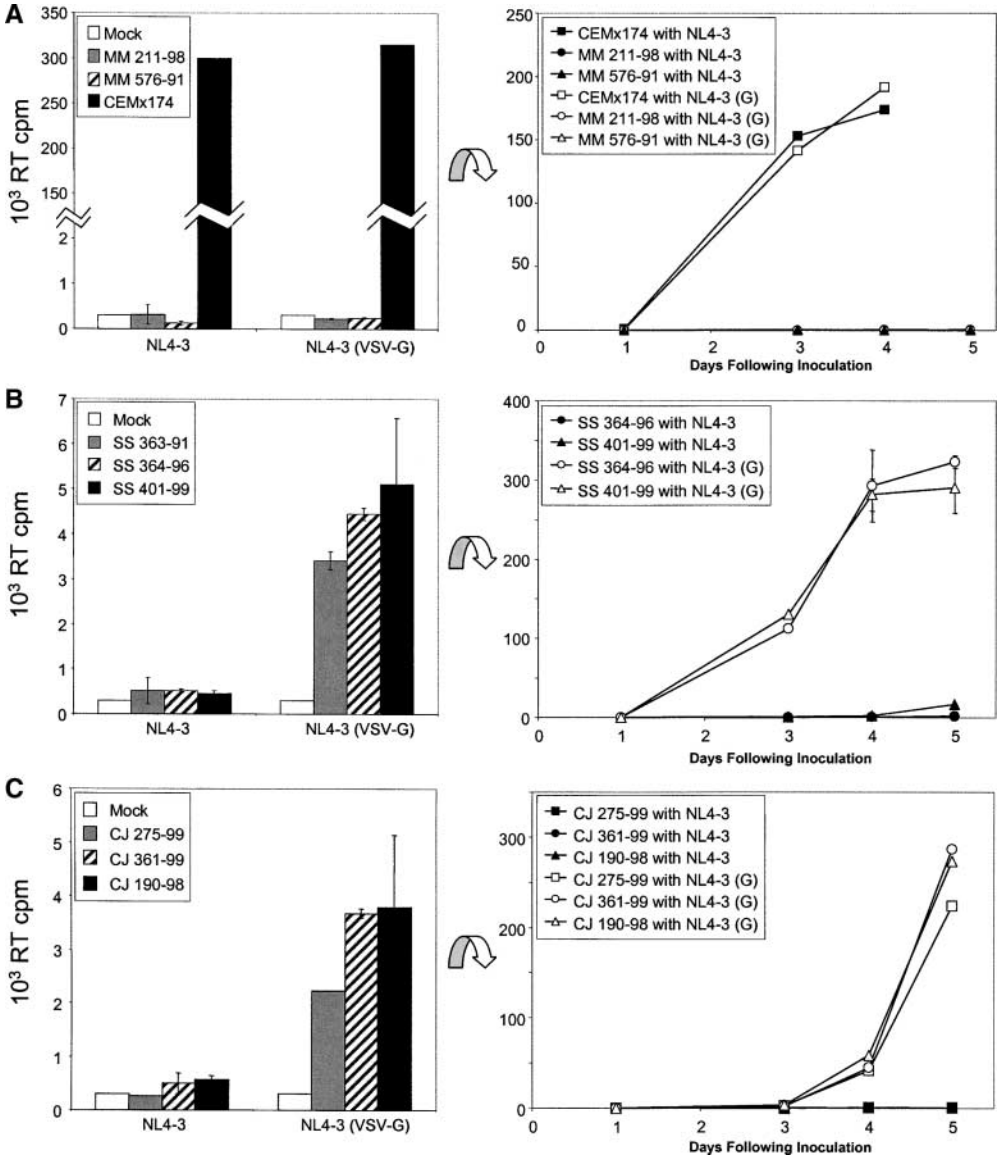


Figure 1. HIV-1 virus production from New World monkey PBMCs. Approximately 100,000 RT cpm of wild-type NL4–3 HIV-1 strain or the NL4–3 virus pseudotyped with the VSV G protein (NL4–3 (G)) were incubated with human CEMx174 cells as a positive control and rhesus macaque (MM) PBMCs as a negative control (A), PBMCs from three different squirrel monkey (SS) donors (B), or PBMCs from three different common marmoset (CJ) donors (C). Cells were washed at 16 h and RT units were measured in duplicate cultures at 24-h time points. Virus production on day 2, which for the primary monkey cells represents the peak value, is shown for each cell type in the bar graphs on the left. Equivalent RT units of virus produced from each culture were added to CEMx174 target cells to assay for their infectivity (line graphs on right). Where no detectable virus was produced after the round of initial infection (e.g., wild-type NL4–3 infection of New World monkey PBMCs), the entire supernatant was added. Target cells were washed at 16 h and RT units were measured at 24-h time points.

HIV-1 replication at a post-entry stage. Little or no virus was produced from the rhesus macaque PBMCs and supernatants from these Old World monkey cells were not able to initiate detectable infection of CEMx174 cells (Fig. 1 A).

The SS and CJ PBMCs from three independent donors did not produce virus after exposure to the NL4–3 virus, but did produce virus after incubation with the NL4–3 (VSV G) pseudotype (Fig. 1, B and C, left panels). Incubation of the latter group of PBMC supernatants with CEMx174 cells demonstrated that the virus produced by SS and CJ PBMCs was infectious for the CEMx174 cells (Fig. 1, B and C, right panels). Supernatants from the New World monkey PBMC that had been exposed to the NL4–3 virus did not initiate infections after incubation with CEMx174 cells.

Our results indicate that infection of New World monkey PBMCs by HIV-1 is inefficient. However, both the SS and CJ PBMCs were infected by HIV-1 that was pseudotyped by the VSV G glycoprotein and subsequently produced infectious HIV-1. Thus, although virus entry into New World monkey PBMCs appears to be inefficient, subsequent steps in the HIV-1 life cycle can be successfully negotiated in these cells. This is in contrast to Old World PBMCs, where blocks to HIV-1 replication after virus entry exist.

Squirrel Monkey and Common Marmoset Homologues to HIV-1 Receptors in Humans. To examine whether the inefficient entry of HIV-1 into New World monkey PBMCs resulted from species-specific properties of the viral receptors, the SS and CJ CD4, CXCR4, and CCR5 proteins were characterized. The polymerase chain reaction was used to amplify the cDNA from SS and CJ PBMCs, using primers specific for the 5′ and 3′ untranslated regions of the genes. Thus, the amplified products include the complete coding sequence of the genes, without the introduction of artifactual changes in these regions by the primers. Sequences were verified by cloning from three different CJ donors and two different SS donors.

The CXCR4 molecules of both New World monkeys (GenBank/EMBL/DDBJ accession nos. AF452612 and AF452613) exhibited a high degree of amino acid sequence identity with human CXCR4 (98.3 and 98.0% sequence identity for squirrel monkey and common marmoset CXCR4, respectively). Amino acid differences between the human and New World monkey CXCR4 molecules are found in the NH_2 terminus and the first and third extracellular loops (ECL1 and ECL3). Notably, neither of the New World monkey CXCR4 molecules exhibit the deletions in the NH_2 terminus or ECL2 that are found in mouse CXCR4 molecules.

The CCR5 molecules of the New World monkeys (GenBank/EMBL/DDBJ accession nos. AF452614 and AF452615) exhibited a lower overall level of sequence similarity to their human counterparts than was observed for the CXCR4 molecules. The SS and CJ CCR5 proteins exhibited 89.5 and 91.5% sequence identity, respectively, to human CCR5. Many of the amino acid differences between the New World monkey CCR5 molecules and human CCR5 involve the NH_2 terminus and extracellular loops. Both SS and CJ CCR5 proteins exhibit NH_2 termini in which glycine–proline replaces the tyrosine–threonine at residues 15 and 16. This difference may be important, as tyrosine 15 is sulfated and contributes to HIV-1 binding and entry (63–65). Both New World monkey CCR5 proteins have differences in ECL2 and ECL3 sequences from human CCR5, although the CJ sequences are more similar to the human sequences than the SS sequences are.

Of the three receptors, the New World monkey CD4 molecules diverge the most from their human counterpart, displaying 80.3 and 81% sequence identity to human CD4 for the SS and CJ molecules, respectively. An alignment of the SS and CJ CD4 sequence to the human and rhesus monkey proteins, which support HIV-1 infection, and to murine CD4, which does not support HIV-1 infection, is shown in Fig. 2 A. Of the four immunoglobulin-like extracellular domains of CD4, the NH_2-terminal domain is the most important for HIV-1 binding and entry. Phenylalanine 43 and arginine 59 of human CD4 make particularly important contacts with the HIV-1 gp120 glycoprotein (66–68). Both SS and CJ CD4 glycoproteins possess a phenylalanine residue at position 43, but exhibit numerous differences from human CD4 in the adjacent regions of the protein. For example, the CJ CD4 has a glutamine at residue 59.

Permissivity of New World Monkey Chemokine Receptors for HIV-1 Entry. The SS and CJ CXCR4 and CCR5 cDNAs were expressed transiently in Cf2Th canine thymocytes, which are permissive for HIV-1 or SIV infection only after complementation with appropriate CD4 and chemokine receptor proteins (69). Cf2Th cells transfected with the SS and CJ CXCR4-expressing plasmids stained with two conformation-dependent CXCR4-reactive monoclonal antibodies, 12G5 and 44717.111 (Table I). In transfected Cf2Th cells, human and New World monkey CCR5 proteins were recognized comparably by 3A9, an antibody that binds a linear, NH_2-terminal CCR5 epitope, and by 2D7, a conformation-dependent antibody directed against the CCR5 ECL 2 (Table I).

To determine the ability of the New World monkey chemokine receptors to support HIV-1 infection, Cf2Th canine thymocytes transiently expressing human CD4 and either human, SS or CJ chemokine receptors were exposed to recombinant HIV-1 bearing different HIV-1 envelope glycoproteins. The ability of the SS and CJ CXCR4 and CCR5 proteins to allow HIV-1 infection, relative to that of the human receptors, was assessed by monitoring the expression of EGFP, which is encoded by the recombinant virus, in the target cells. Fig. 3 A shows that the squirrel monkey and common marmoset CXCR4 molecules support the entry of viruses bearing several X4 or R5X4 HIV-1 envelope glycoproteins, with an efficiency roughly 50% of that seen for human CXCR4. The different infection efficiencies associated with cells expressing human

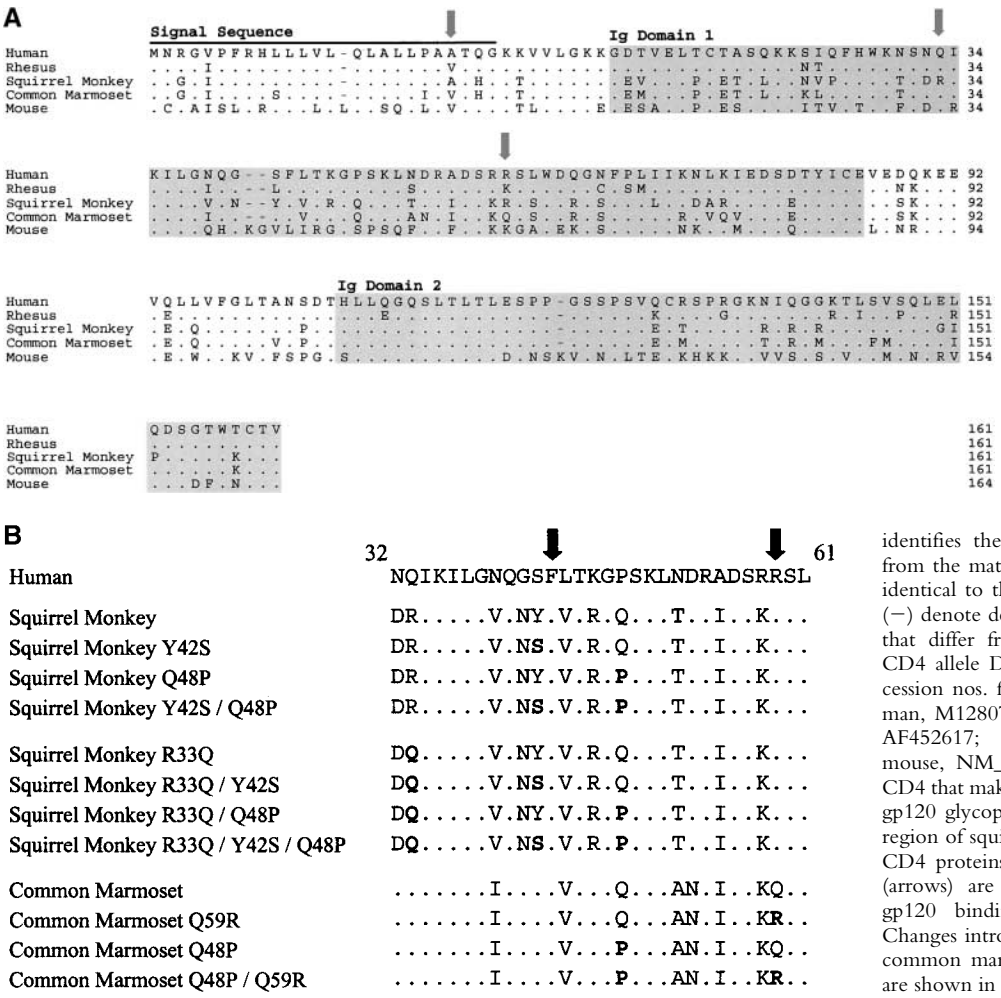


Figure 2. Predicted amino acid alignment of Ig domains 1 and 2 of New World monkey CD4 proteins. PCR primers specific for the 5′ and 3′ untranslated regions were used to amplify CD4 from cDNA generated from squirrel monkey or marmoset PBMCs. (A) The predicted amino acid sequences of these cloned CD4 molecules are aligned to the previously identified sequences of human, rhesus macaque, and murine CD4. The shaded boxes designate Ig domains 1 and 2. The solid line identifies the signal sequence, which is cleaved from the mature protein. Dots (.) denote residues identical to the human sequence and minus signs (−) denote deletions. Gray arrows denote residues that differ from the published squirrel monkey CD4 allele D86588. GenBank/EMBL/DDBJ accession nos. for the sequences are as follows: human, M12807; rhesus, D63347; squirrel monkey, AF452617; common marmoset, AF452616; mouse, NM_013488. (B) The region of human CD4 that makes important contacts with the HIV-1 gp120 glycoprotein is aligned with the analogous region of squirrel monkey and common marmoset CD4 proteins. Phenylalanine 43 and arginine 59 (arrows) are particularly important residues for gp120 binding (references 45, 77, and 79). Changes introduced into the squirrel monkey and common marmoset CD4 molecules in this study are shown in bold.

CXCR4 may relate to intrinsic differences in the efficiencies of the particular HIV-1 envelope glycoproteins and/or factors associated with individual preparations of each of the viral stocks.

Cf2Th cells expressing comparable levels of human CD4 and either human, SS, or CJ CCR5 were exposed to recombinant HIV-1 bearing the envelope glycoproteins of three primary R5 isolates (Fig. 3 B). Relative to the EGFP expression observed in target cells expressing human CD4 and CCR5, the levels of expression seen in cells expressing the SS or CJ CCR5 proteins were very low. Low but detectable EGFP expression was observed in the latter cells exposed to the viruses with the JR-FL or YU2 envelope glycoproteins. For both CXCR4 and CCR5 infection assays, Cf2Th cells expressing only human CD4 were used as negative controls and exhibited undetectable EGFP expression after exposure to the recombinant HIV-1 bearing the various HIV-1 envelope glycoproteins (data not shown). High levels of EGFP expression were observed in all target cells exposed to recombinant HIV-1 pseudotyped with the VSV G glycoprotein (data not shown).

These data suggest that the New World monkey CXCR4 molecules can act as functional receptors for HIV-1, in conjunction with human CD4. By contrast, the squirrel monkey and common marmoset CCR5 molecules are relatively inefficient receptors for R5 HIV-1 isolates in this setting.

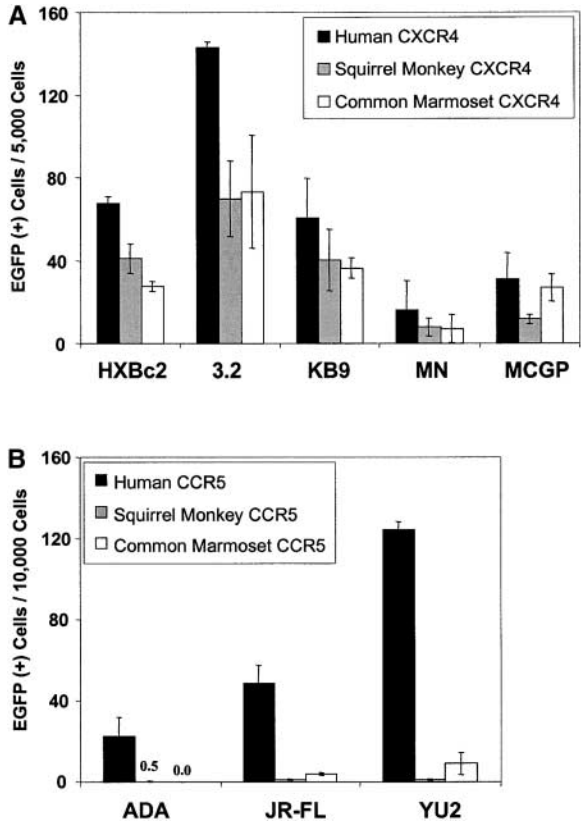
Permissivity of New World Monkey CD4 Molecules for HIV-1 Entry. Cf2Th canine thymocytes were transfected with plasmids expressing human, SS, or CJ CD4 proteins. The transfected cells were stained with three anti-CD4 monoclonal antibodies that recognize different CD4 regions. Human and New World monkey CD4 molecules were both efficiently recognized by the Q4120 and CD4v4 monoclonal antibodies, but the New World CD4 proteins were not recognized by the OKT4A antibody (Table I). OKT4A recognizes an epitope in domain 1 of CD4 that is near the HIV-1 gp120 binding site (70).

To compare the functionality of human and New World monkey CD4 molecules in supporting HIV-1 entry, Cf2Th cells expressing either human or New World monkey CD4 in combination with human or New World monkey CXCR4 were exposed to recombinant HIV-1 expressing EGFP, as described above. As was observed previously, cells expressing human CD4 and either SS or CJ CXCR4 proteins supported HIV-1 entry at roughly 50% the level seen for cells expressing human CD4 and human CXCR4 molecules (Fig. 4, A and B). Cells expressing ei-

Table I. *Antibody Recognition of HIV-1 Receptors from New World Monkeys*

	12G5	44717.111	3A9	2D7	Q4120	CD4v4	OKT4A
H CXCR4	+	+					
SS CXCR4	+	+					
CJ CXCR4	+	+					
H CCR5			+	+			
SS CCR5			+	+			
CJ CCR5			+	+			
H CD4					+	+	+
SS CD4					+	+	+
SS CD4 Y42S					+	+	−
SS CD4 Q48P					+	+	−
SS CD4 Y42S/Q48P					+	+	−
SS CD4 R33Q					+	+	−
SS CD4 R33Q/Y42S					+	+	−
SS CD4 R33Q/Q48P					+	+	−
SS CD4 R33Q/Y42S/Q48P					+	+	−
CJ CD4					+	+	−
CJ CD4 Q59R					+	+	−
CJ CD4 Q48P					+	+	−
CJ CD4 Q48P/Q59R					+	+	−

H, human; SS, squirrel monkey; CJ, common marmoset.



ther SS or CJ CD4 did not support efficient infection regardless of the coexpression of human or New World monkey CXCR4 molecules. A small percentage of EGFP-positive cells was observed after infection of the cells expressing both marmoset receptors exposed to the recombinant KB9 virus (Fig. 4 B). This was consistently higher than the background associated with cells transfected only with the human CD4-expressing plasmid. Recombinant HIV-1 pseudotyped with the VSV G glycoprotein infected all of the cells efficiently (data not shown). These results indicate that the New World monkey CD4 molecules are not efficient HIV-1 receptors.

Studies similar to those described above were performed with cells transiently expressing human or New World

Figure 3. Ability of New World monkey chemokine receptors to support HIV-1 entry. Cf2Th cells were cotransfected with (A) human CD4 and human, squirrel monkey, or common marmoset CXCR4, or (B) human CD4 and human, squirrel monkey, or common marmoset CCR5. Recombinant HIV-1 containing the indicated HIV-1 envelope glycoproteins and encoding EGFP was incubated with the cells. 48 h later, EGFP-positive cells were scored. To control for variation in transfection efficiency, the values shown were normalized based on CD4 expression levels determined by Q4120 antibody staining of cell cultures plated in parallel. The entry levels observed in cells expressing human CXCR4 (A) or CCR5 (B) were used as a baseline and all other entry values were multiplied by the percent that their associated CD4 expression level differed from the CD4 expression levels of the reference cells. In the cases where little or no infection was seen, the value is indicated numerically.

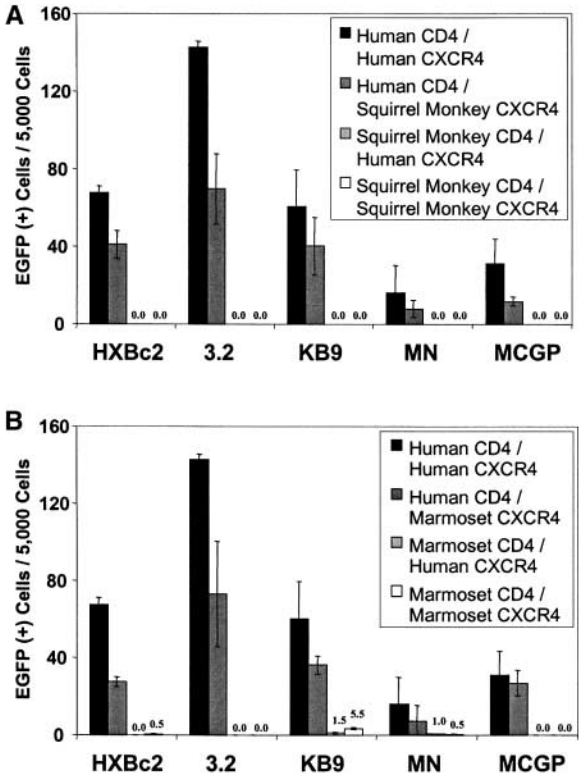


Figure 4. Ability of New World monkey CD4 to support entry of CXCR4-using HIV-1. Cf2Th cells were cotransfected with human CD4 and either human or New World monkey CXCR4, or with New World monkey CD4 and either human or New World monkey CXCR4. Recombinant, EGFP-expressing HIV-1 bearing the indicated CXCR4-using HIV-1 envelope glycoproteins was incubated with the cells, and EGFP-positive cells were scored as described in the Fig. 3 legend. Human receptors were compared with those of squirrel monkeys (A) or common marmosets (B).

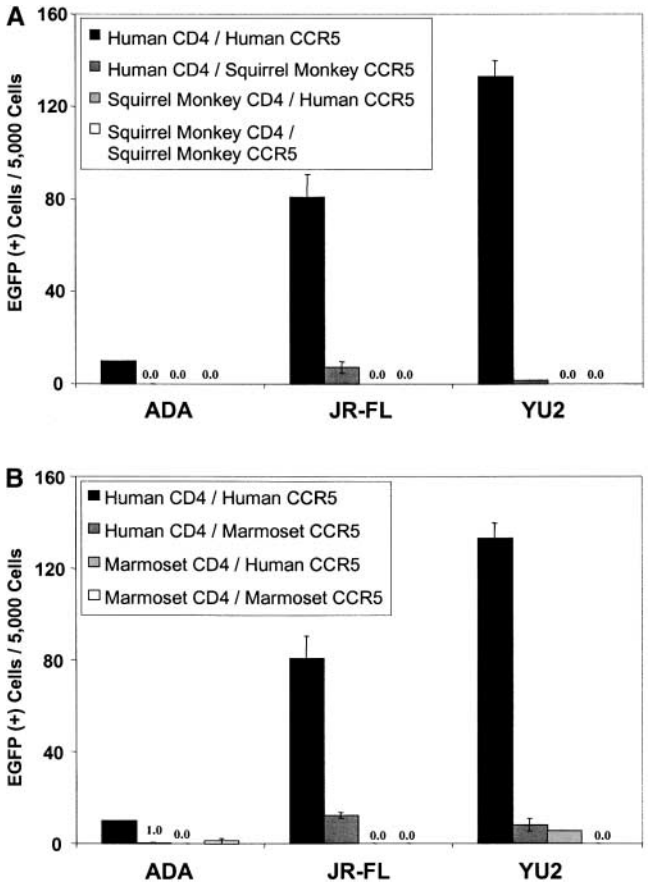


Figure 5. Ability of New World monkey CD4 to support entry of CCR5-using HIV-1. Cf2Th cells were cotransfected with human CD4 and either human or New World monkey CCR5, or with New World monkey CD4 and either human or New World monkey CCR5. Recombinant, EGFP-expressing HIV-1 bearing the indicated CCR5-using HIV-1 envelope glycoproteins was incubated with the cells, and EGFP-positive cells were scored as described in the Fig. 3 legend. Human receptors were compared with those of squirrel monkeys (A) or common marmosets (B).

monkey CD4 and CCR5 molecules. The results in Fig. 5 confirm that neither New World monkey CD4 nor CCR5 efficiently support the entry of viruses with R5 HIV-1 envelope glycoproteins.

In the studies described above, low but reproducible levels of HIV-1 entry occurred in cells expressing the CD4 and CXCR4 receptors from common marmosets. To verify this observation, Cf2Th lines stably expressing both CD4 and CXCR4 derived from humans, squirrel monkeys, and common marmosets were established. Fig. 6 shows that these cell lines stain with the CD4-specific antibody Q4120 and the CXCR4-specific antibody 12G5; staining with the CCR5-specific antibody 2D7 was equivalent to that seen with an irrelevant antibody (data not shown). Recombinant HIV-1 containing either CXCR4-using HIV-1 envelope glycoproteins or the VSV G glycoprotein were incubated with the Cf2Th cell lines expressing human, squirrel monkey, or common marmoset CD4 and CXCR4 glycoproteins. 48 h later, EGFP-positive cells were visualized (Fig. 7). Viruses with the HIV-1 envelope glycoproteins efficiently infected the cell line expressing the human receptors, and viruses with the VSV G glycoprotein infected all three cell lines efficiently. Cells expressing the squirrel monkey receptors were almost completely resistant to infection by viruses with the HIV-1 envelope glycoproteins. Some of these viruses, however, were able to infect the cells expressing common marmoset receptors at a low level. These results suggest that the CD4 and CXCR4 molecules of common marmosets can support the entry of HIV-1 with some X4 or R5X4 envelope glycoproteins, although these New World monkey receptors are much less efficient than their human homologues.

HIV-1 gp120 Binding to Wild-Type and Variant New World Monkey CD4 Molecules. The studies described above indicate that New World monkey CD4 molecules represent a major block to HIV-1 infection of these species. Based on the alignment of the human and New World monkey CD4 sequences and the known x-ray crystal structure of the HIV-1 gp120-human CD4 complex, amino acid residues in the monkey CD4 sequences that could potentially decrease gp120-CD4 binding affin-

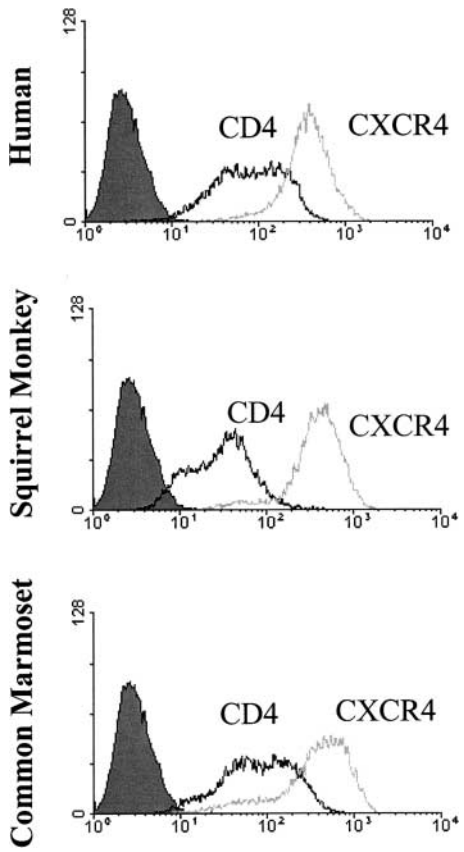


Figure 6. CD4 and CXCR4 expression levels in stable Cf2Th cell lines. Cf2Th cells stably expressing CD4 and CXCR4 of the indicated species were stained with Q4120 antibody (for CD4) and 12G5 antibody (for CXCR4). The 2D7 anti-CCR5 antibody was used as a negative control (shaded peak).

ity were identified. HIV-1 gp120 primarily contacts the CDR2-like loop of CD4, spanning CD4 residues 40–61, with CD4 residues phenylalanine 43 and arginine 59 playing critical roles in the interaction (66–68). Squirrel monkey CD4 retains phenylalanine 43 and arginine 59, but has a bulky tyrosine replacing the serine 42 found in human CD4. Moreover, a glutamine replaces the proline 48 in human CD4, a substitution that could alter the turn structure in the CD4 CDR2 loop (Fig. 2 B). Common marmoset CD4 also has a glutamine at residue 48, but in addition has a glutamine replacing the critical arginine 59 found in human CD4. To investigate the functional significance of these sequence differences, variants of the squirrel monkey and common marmoset CD4 molecules were created in which these identified residues were individually or in combination altered to the amino acid found in human CD4 (Fig. 2 B). The mutant CD4 proteins were efficiently expressed on the surface of transfected 293T cells, as judged by staining with the anti-CD4 monoclonal antibodies Q4120 and CD4v4 (Table I). The OKT4A antibody, which recognizes a CDR2 loop epitope in domain 1 of CD4, did not bind any of the mutant SS and CJ CD4 molecules.

The ability of the gp120 envelope glycoprotein from the ADA, JR-FL, and YU2 HIV-1 strains to bind 293T cells expressing the wild-type and variant New World monkey CD4 proteins was tested. The HIV-1 gp120 glycoproteins bound cells expressing human CD4 more efficiently than cells expressing common marmoset CD4 (Fig. 8). Approximate differences of 20-fold and 100-fold in affinity for the two CD4 molecules were observed for the ADA and JR-FL gp120 glycoproteins, respectively. A smaller difference in affinity was observed for the YU2 gp120 glycoprotein; the YU2 gp120 bound the CJ CD4 with an apparent K_d of $\sim$100 nM, a significantly higher affinity than was seen for the ADA or JR-FL gp120 glycoproteins. Thus, the gp120 glycoproteins from different HIV-1 strains apparently bind the CJ CD4 with a range of affinities. The CJ CD4 mutants Q48P and Q59R bound the gp120 glycoproteins of all three HIV-1 strains with an affinity that was better than that associated with the wild-type CJ CD4 protein. The double mutant CJ CD4 Q48P/Q59R bound the gp120 glycoproteins comparably to the human CD4 protein. Thus, the differences between the abilities of common marmoset and human CD4 to bind HIV-1 gp120 can be largely attributed to species-specific differences in CD4 residues 48 and 59.

In contrast to the results obtained with the CJ CD4, the squirrel monkey CD4 exhibited no detectable binding to any of the three gp120 glycoproteins (Fig. 8). The SS CD4 mutants also failed to bind the gp120 glycoproteins (data not shown). It appears that one or more amino acid differences between human and squirrel monkey CD4 other than those affecting residues 42 or 48 result in a major abrogation of gp120-binding ability.

Ability of Mutant New World Monkey CD4 Molecules to Support HIV-1 Infection. We wished to examine whether the alterations in glutamine 48 and glutamine 59 of common marmoset CD4 that restored gp120 binding would also allow more efficient function as an HIV-1 receptor. Cf2Th cells transiently expressing human CD4 or CJ CD4 variants along with human CCR5 were incubated with recombinant HIV-1 bearing the ADA, JR-FL, or YU2 HIV-1 envelope glycoproteins. Measurement of EGFP in the target cells revealed that, as expected, cells expressing human CD4 allowed more efficient infection than cells expressing the wild-type CJ CD4 molecule (Fig. 9, top panel). Compared with cells expressing the wild-type CJ CD4 protein, cells expressing the Q48P and Q59R variants supported more efficient virus infection. Cells expressing the double mutant (CJ CD4 Q48P/Q59R) were infected with efficiencies ranging from 50–80 percent of that seen for cells expressing human CD4.

To examine the ability of the mutant CJ CD4 proteins to serve as receptor for CXCR4-using HIV-1, Cf2Th cells transiently expressing human CD4 or CJ CD4 variants along with human CXCR4 were incubated with recombinant HIV-1 bearing the envelope glycoproteins of the HXBc2, 3.2, KB9, MN, or MCGP HIV-1 strains. The observed EGFP levels in the target cells corroborated the results presented in Fig. 9 (data not shown). For each recom-

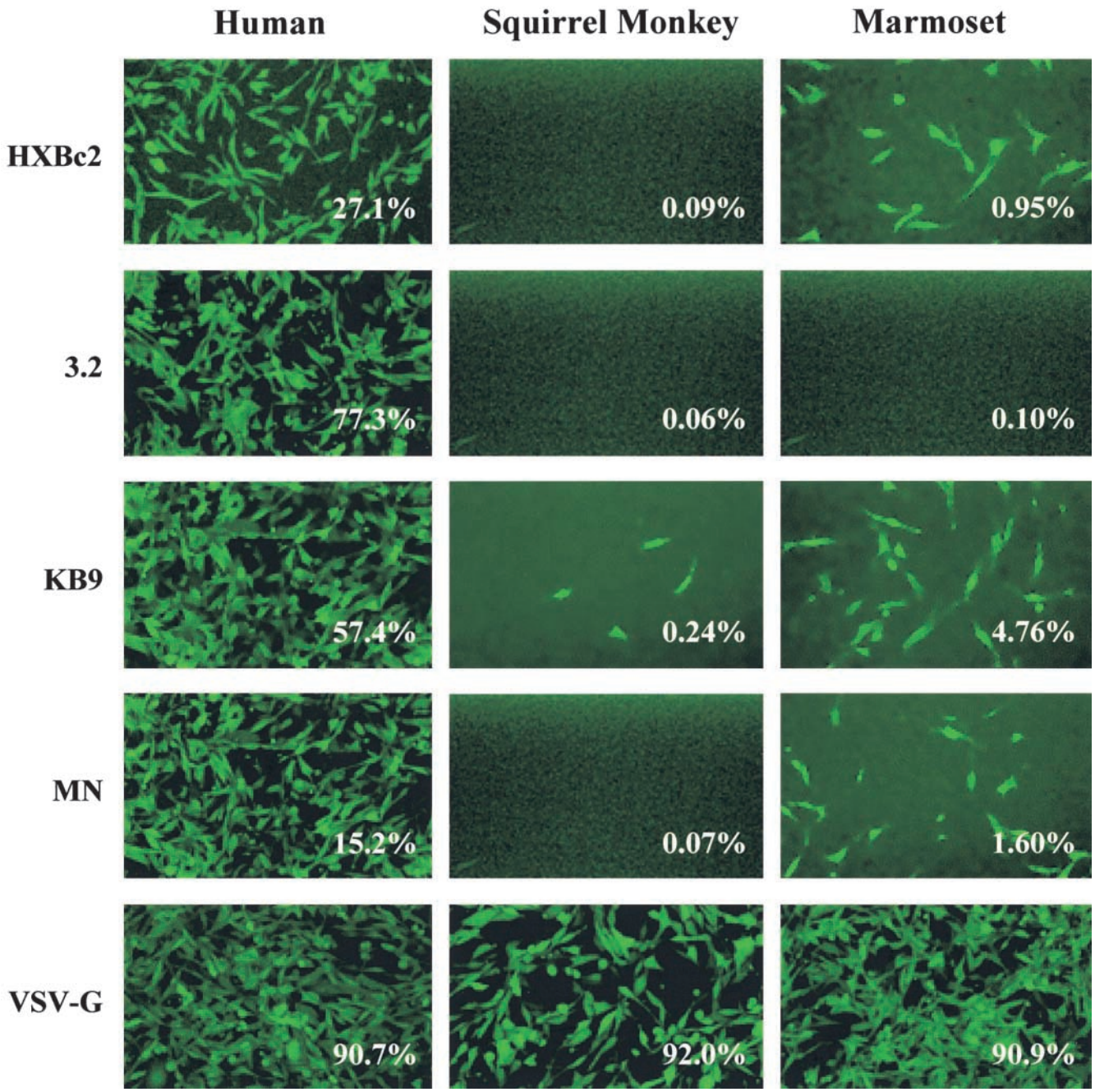


Figure 7. Infection of cells stably expressing CD4 and CXCR4 of different species. Recombinant, EGFP-expressing HIV-1 bearing the indicated envelope glycoproteins were incubated with Cf2Th cells stably expressing CD4 and CXCR4 molecules of the indicated species. EGFP-positive cells are shown and were quantified by flow cytometry, with the percentage of EGFP-positive cells indicated in the corner of each panel.

binant virus, the level of infection observed was lowest in cells expressing wild-type CJ CD4 and progressively higher in the cells expressing CJ CD4 Q48P, CJ CD4 Q59R, CJ CD4 Q48P/Q59R, and human CD4.

In assays similar to those described above, the wild-type squirrel monkey CD4 and its mutant derivatives were co-expressed with human CCR5 in Cf2Th cells. The cells were incubated with recombinant HIV-1 containing the envelope glycoproteins of CCR5-using HIV-1 isolates. No EGFP expression was detected in these cells (Fig. 9, middle panel), indicating that the squirrel monkey CD4 and mutant derivatives were unable to support HIV-1 entry. Thus, as was deduced from the gp120 binding assays, differences between human, and squirrel monkey CD4 other than those at residues 42 or 48 account for the poor function of the latter molecule as an HIV-1 receptor. Fig. 2 A shows that the squirrel monkey CD4 allele that we cloned differs from a published squirrel monkey allele in three amino ac-

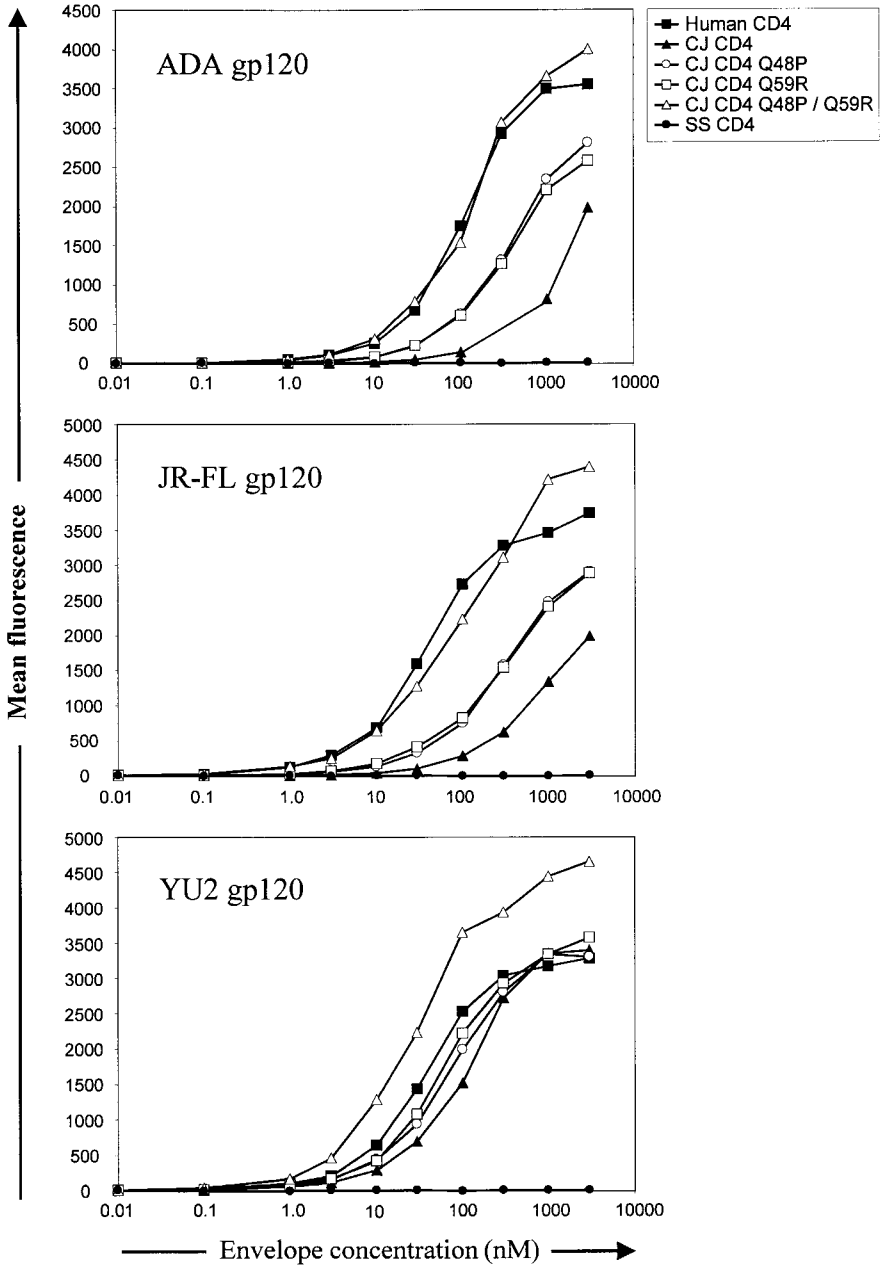


Figure 8. Binding of soluble gp120 glycoprotein to New World monkey CD4 molecules. 293T cells were transfected with plasmids encoding human, marmoset (CJ), or mutant marmoset CD4 molecules and assayed for binding to soluble purified gp120 by flow cytometry. The concentration of the gp120 envelope glycoprotein used in the assay is shown on the x-axis, and the mean fluorescence associated with detection of bound gp120 is on the y-axis. SS CD4 showed no binding to gp120 molecules at any concentration and was used as a negative control.

ids. However, in our cloned allele, two of these residues, alanine 22 in the signal sequence and arginine 59, are identical to those found in human CD4. At the third residue (residue 33), our SS CD4 clone differed from all known CD4 sequences by virtue of an arginine in place of glutamine. To test if this substitution accounted for the poor functionality of SS CD4 as an HIV-1 receptor, arginine 33 was converted to glutamine in the context of the wild-type and mutant SS CD4 proteins (Fig. 2 B). All of these molecules were expressed efficiently on the cell surface and were recognized by the Q4120 and CD4v4 antibodies, but were not recognized by OKT4A (Table I). The SS CD4 variants were coexpressed with human CCR5 in Cf2Th cells, which were incubated with recombinant HIV-1 EGFP-reporter virus containing CCR5-using envelope glycoproteins. The conversion of arginine 33 to glutamine, in either wild-type or mutant SS CD4 contexts, did not restore functional HIV-1 receptor activity (Fig. 9, bottom panel).

Discussion

Our results indicate that the major restriction limiting HIV-1 infection of New World monkey cells operates at the level of virus entry. The inefficiency with which squirrel monkey and common marmoset CD4 molecules support HIV-1 binding and entry creates a significant hurdle for all strains of HIV-1 to replicate in cells of these species. In addition, New World monkey CCR5 proteins are inefficient receptors for HIV-1. As most naturally transmitted

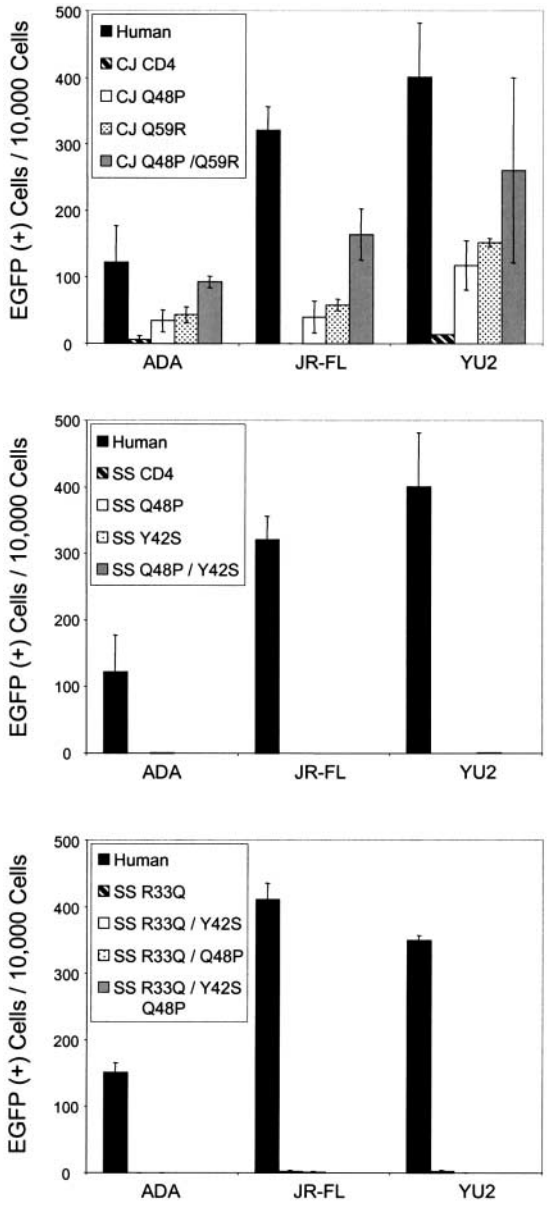


Figure 9. Effect of amino acid changes on the receptor activity of common marmoset (CJ) CD4 or squirrel monkey (SS) CD4. Cf2Th cells were transfected with plasmids encoding human CCR5 and the wild-type common marmoset (CJ) or squirrel monkey (SS) CD4 proteins, or the indicated mutant CD4 proteins. The cells were then incubated with recombinant, EGFP-expressing HIV-1 bearing the ADA, JR-FL, or YU2 envelope glycoproteins. After 48 h, cells were assayed for EGFP expression, as described in the Fig. 3 legend.

primate lentiviruses utilize CD4 and CCR5 as receptors, the species-specific polymorphisms in these molecules would be sufficient to limit natural infection of New World monkeys, even if they were exposed to these agents. Of the HIV-1 receptors, New World monkey CD4 exhibits the greatest degree of sequence divergence from human CD4. Two differences involving CD4 residues 48 and 59 explain, to a large extent, the inability of the CD4 glycoprotein of common marmosets to support HIV-1 binding and entry. In human CD4, proline 48 contributes to a β-turn that results in the antiparallel orientation of the C′′ and D strands of the protein, each of which makes important contacts with HIV-1 gp120 (66, 68). The C′′ strand includes phenylalanine 43, which fills a receptor-binding pocket on gp120 and the D strand includes arginine 59, which forms a salt bridge with gp120 aspartic acid 368 (66). The glutamine at position 59 of common marmoset CD4 would be unable to form such a favorable bond. The resulting decreases in the affinity of the HIV-1 gp120-CD4 interaction apparently explain the poor receptor function of the marmoset protein.

The squirrel monkey CD4 did not detectably bind or support infection of HIV-1, although it was efficiently expressed on the surface of target cells. The basis for this poor activity as an HIV-1 receptor requires further investigation. Our studies indicate that sequences present in squirrel monkey CD4 other than arginine 33, tyrosine 42, and proline 48 prevent this molecule from acting as a functional receptor molecule for HIV-1. Future studies changing other nonhomologous amino acid residues to their human counterparts should be able to identify the determinants of SS CD4 that restrict its usage as an HIV-1 receptor.

The New World monkey chemokine receptors exhibit less sequence divergence from their human counterparts than does CD4. The CXCR4 proteins of these monkeys are most conserved among the three molecules, and also exhibit the highest levels of HIV-1 receptor activity. This is consistent with the reported ability of some R5X4 and X4 HIV-1 strains to utilize CXCR4 molecules from evolutionarily distant mammalian species, including rodents. New World monkey CCR5 exhibited very poor coreceptor function for HIV-1 infection. Differences in the NH_2 terminus, including a glycine replacing a critical sulfated tyrosine at residue 15 of human CCR5, probably contribute to the functional coreceptor differences between New World monkey and human CCR5 proteins.

Our results indicate that HIV-1 can effectively negotiate all the steps in the viral life cycle following virus entry in New World monkey cells. The early, post-entry block to HIV-1 infection seen in Old World monkeys does not appear to be operative in New World monkeys. Cellular cofactors in New World monkey cells must be able to interact functionally with the HIV-1 Tat, Rev, and Vif proteins to allow successful completion of the late phase of virus replication (71–74). Likewise, New World monkey HP68 and cyclophilin A are apparently compatible with HIV-1 Gag components to allow capsid formation and the attainment of virion infectivity, respectively (75–78).

The discrete nature of the replication blocks to HIV-1 in New World monkeys raise the possibility that these hurdles might be bypassed by directed adaptation of the virus. Our results suggest that such attempts might be more successful with common marmosets than squirrel monkeys. Common marmosets would be attractive hosts in an animal model of HIV-1 infection, as they are small, relatively inexpensive, and easy to breed (79). Common marmosets exhibit species-wide homogenization of their major histocompatibility loci and a high incidence of multiple births, facilitating

transplants and adoptive transfer experiments between siblings (80). The peripheral blood lymphocytes of common marmosets can be readily transformed by Herpes saimiri or ateles and are easily propagated in recombinant human IL-2 (81). These features make common marmosets ideal for studying interactions of the virus with the immune system. Future studies will explore the possibilities of establishing such a model.

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