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Mapping the determinants in owl monkey CD4 and CCR5 that
allow entry of early-stage HIV-1 Env variants

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Abstract

Mapping the determinants in owl monkey CD4 and CCR5 that
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Many physiological and immunological aspects of non-human primates make them ideal candidates for animal models of HIV-1 transmission and vaccine studies. However, HIV-1 does not infect most non-human primates because of species-specific host restriction factors and/or inability of the receptor for HIV-1, the CD4 proteins, to function efficiently as HIV-1 receptors for viral entry. This study aimed to determine if owl monkey species encode CD4 receptors and CCR5 coreceptors that support cellular entry by variants representing transmitted, circulating forms of HIV-1. The functionality of owl monkey CD4s as receptors and CCR5s as coreceptors was evaluated using infectivity assays with HIV-1 pseudotyped with envelope protein. Here, we have identified owl monkey CD4s that naturally function as receptors for HIV-1 entry and our data corroborate previous findings that N39 in the D1 domain of CD4 is important for usage as a receptor by transmitted HIV-1 envelope variants. Additionally, we have mapped residues in CCR5 necessary for transmitted HIV-1 envelope variant entry into cells. Two amino acid residues in CCR5, Y15 and T16, were found to be sufficient to support HIV-1 entry into cells for some, but not all, envelope variants tested. This research will assist in identifying new non-human primate models of HIV-1 infection that can support replication of transmitted forms of HIV-1 variants (and/or SHIVs) that are relevant for vaccine and therapeutic approaches.

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Introduction

Many physiological and immunological aspects of non-human primates make them ideal candidates for animal models of HIV-1 transmission and vaccine studies. However, chimpanzees and white-handed gibbons are the only mammals (besides humans) presently known to naturally support HIV-1 replication¹. Both of these primates are endangered and rarely progress to severe immunodeficiency¹. Macaques are currently the model organism of choice for the study of HIV-1 infection and preclinical assessment of HIV-1 vaccine candidates. However, naturally occurring host restriction factors hinder HIV replication in macaques⁴. To overcome this, HIV-1/simian immunodeficiency virus (SIV) chimeras called SHIVs are generated and used for transmission and infection studies^{2,3}. SHIVs usually encode antagonists of macaque restriction factors allowing them to circumvent restriction in macaques.

While the SHIV-macaque model has been a useful animal model for HIV-1, there are a number of limitations. SHIVS are typically designed to encode envelope surface glycoproteins (Envs) from circulating HIV-1 subtype B variants and rarely encode subtype A, C, or D which are the most prevalent circulating and transmitted HIV subtypes⁷. HIV-1 Env isolates used in SHIVs are usually isolated from chronic stages of infection rather than early stages making these SHIVs less applicable in transmission studies. Furthermore, many SHIVs must be lab-adapted before they can use macaque cell receptors (CD4s) to gain entry into macaque cells.

Because of the limitations of SHIVS, an HIV-1 animal model better representative of natural human HIV-1 transmission and infection is still desired and would ideally use SHIVs that can use host receptors and coreceptors without lab adaptation. HIV-1 requires interaction with a receptor and coreceptor to gain entry into cells, which is critical for viral replication. Envs of HIV-1 (gp160), specifically the gp120 subunit of gp160, interact directly with CD4 during

infection to allow the viruses entry into CD4+ T cells⁹. CD4 is a glycoprotein expressed on the surface of primate T cells that is necessary for antigen presentation and proper immune system function. Interaction of gp120 with CD4 causes a conformational change in gp120 that exposes the third variable (V3) loop region³². Once the V3 loop is exposed, it can interact with chemokine receptors CCR5 or CXCR4 expressed on the surface of the activated T cell³², which act as coreceptors to HIV entry. Binding of the V3 loop to a coreceptor induces a series of Env rearrangements that ultimately lead to the fusion of the viral and host cell membranes. Therefore, HIV entry into cells is necessarily dependent on the proper interaction of host CD4s and host coreceptors with gp120.

At least three sites in the D1 domain of mature CD4 in humans have been shown to influence the functionality of CD4s as mediators of HIV-1 entry^{5, 11}. A single nucleotide polymorphism between humans and rhesus macaques at mature CD4 D1 position 39 (Fig. 1A) has been shown account for the difference in CD4 functionality as a receptor for early-stage HIV-1 isolates⁵. Rhesus macaque CD4s encode an isoleucine at position 39 and do not mediate entry while human CD4 encode an asparagine at position 39 and do meditate entry (Fig. 1A)¹⁰.

The majority of circulating HIV-1 variants most relevant to transmission and vaccine studies cannot efficiently use the macaque CD4 receptor to gain entry into cells and therefore must be adapted before they are infectious to macaques^{5, 6}. The D1 domain of CD4 participates in the interaction of the T cell receptor (TCR) with MHC class II during antigen presentation as part of a typical human immune response⁸. Because of the importance of this interaction, it is expected that the D1 domain of CD4 would be conserved through evolutionary time. Contrary to this notion, it is known that the D1 domain of CD4 has many residues shown to be under

diversifying selection^{21, 22, 23}. This is hypothesized to be a result of selection pressure placed on CD4 by lentiviruses such as HIV, which required interaction with CD4 to gain entry into cells.

In an effort to identify monkey species for an improved HIV-1 model system, non-human primate genotyping studies were conducted in hopes of finding non-human primates naturally encoding CD4s which function as receptors for HIV-1 entry¹⁰. Genotyping studies have identified three species of owl monkey (Spix's - *Aotus vociferans*, Azara's - *Aotus azarae*, and Nancy-Ma's - *Aotus nancymaae*) naturally encoding asparagines at position 39 (N39) of some of their CD4 alleles¹⁰. Because these three owl monkey species possess alleles encoding asparagines at position 39, it was hypothesized that these owl monkey CD4s may function as receptors for HIV-1 entry. This study examines the functionality of these owl monkey CD4 alleles as receptors for HIV-1 entry and will contribute to understanding whether owl monkeys could be used as a practical animal model of HIV-1 infection.

For the purposes of this study, CD4 alleles have been numbered for each species as depicted in Fig. 1. Sequence analysis of CD4s in owl monkey (OM) species has determined that there are three Spix's OM CD4 alleles (called here, 2-4), one Azara's OM CD4 allele, and two NancyMa's OM alleles (called here, 1 and 2) each encoding an asparagine at position 39 while one Spix's OM CD4 allele (called here, 1) encodes an isoleucine (Fig. 1A)¹⁰. Indeed, it has been shown that Spix's OM CD4 allele-1 does not mediate human isolate HIV-1 entry while Spix's OM alleles 2-4 encoding an asparagine at position 39 (Fig 1A) do mediate entry of viruses expressing these Envs¹⁰ further supporting an important role for N39 in HIV-1 entry.

Given that some OM CD4 alleles appear to mediate HIV-1 entry, it is important to also know if OM coreceptor alleles mediate HIV entry. Transmitted HIV-1 variants have been shown to be exclusively CCR5 tropic (reviewed in Ronen et al. 2015³⁵) and are therefore the primary

focus of this study. CCR5 has an extracellular N-terminus followed by several transmembrane domains and then an intracellular C-terminus¹² (Fig. 1C). During typical HIV-1 infection, HIV-1 virions interact with the extracellular N-terminus of CCR5 via their V3 loop of their gp120 in order for viral entry to occur^{12, 32}. Several studies have shown that the N-terminus of CCR5 is essential for binding and infection of CCR5 tropic HIV-1 variants^{15, 16, 17, 18, 26, 30}. In particular, sulfated tyrosines in the N-terminus of CCR5 at amino acid positions 10, 14, and 15 have been shown to be most important for gp120 CCR5 interaction^{15, 16, 17, 18}. Furthermore, sulfated peptides encoding the human CCR5 N-terminus have been shown to competitively block CCR5 tropic HIV-1 infections of CCR5 expressing cells^{30, 31}. There is evidence that extracellular loop 2 (ECL-2, shown in Fig. 2A) of CCR5 may also be involved in CCR5 tropic HIV-1 viral entry with antibodies specific to ECL-2 (2D7) potently inhibiting viral entry^{19, 20}. Other studies indicate that the CCR5 N-terminus interaction with gp120 is most critical with ECL-2 possibly playing an additive or redundant role in gp120 binding and that as dependency on the N-terminus interaction is reduced (gp120 V3 loop mutations), the ECL-2 site can compensate for gp120 interaction²⁵. It is therefore important to consider residues in both the N-terminus of OM CCR5s as well as the ECL-2 sites when examining amino acid determinants of HIV-1 usage of OM CCR5s.

Preliminary experiments have revealed that some CCR5s from old world monkeys (such as macaques) may function as a coreceptor for HIV-1 entry while CCR5s from new world monkeys (such as squirrel, marmoset, and owl monkeys) do not function as coreceptors (Sharma et al, unpublished) (Fig. 1A and 1B). We hypothesized that new world monkeys have non-synonymous polymorphisms in the N-terminus of CCR5 that may be responsible for their failure to function as an HIV-1 coreceptor. As depicted in Fig. 1B, CCR5s of humans and CCR5s of old

world monkeys that have been examined encode tyrosine (Y) at position 15 and threonine (T) at position 16. CCR5s of new world monkeys that have been examined encode an aspartic acid (D) at position 15 and an alanine (A) at position 16. It is known that during CCR5 tropic HIV-1 infection, the tyrosine at position 14 gets sulfated to contribute to Env binding and ultimately viral entry¹². Residues Y15 and T16 may contribute to rigidity of this interaction¹². If these residues are necessary for viral entry in humans, then mutating the new world monkey residues (D15, A16) to the human residues (Y15, T16) should allow viral entry (gain-of-function). Therefore, we hypothesized that mutagenesis of Spix's OM CCR5 allele-1 D15 to Y15 and A16 to T16 will allow new world monkey CCR5s to function as HIV-1 coreceptors.

This study has determined whether some OM encode functional receptors and coreceptors for HIV-1. Specifically, we examined whether OM (Spix's - *Aotus vociferans*, Azara's - *Aotus azarae*, and Nancy-Ma's - *Aotus nancymae*) CD4 alleles encoding an asparagine at position 39 are permissive to HIV-1 pseudotyped with early-stage HIV-1 Env clones. Furthermore, this study has identified CCR5 residues critical for transmitted early-stage HIV-1 Env variant entry into cells. Mutation of Spix's OM CCR5 allele-1 D15 and A16 to Y15 and T16 was able to restore functionality of this coreceptor for HIV-1 entry into cells when paired with a functional CD4 (encoding an asparagine at position 39). Future studies may be able to exploit the residues found in this study for specific inhibitory drug targets. Several Env variants were tested to explore differences in their ability to utilize OM CD4s and CCR5s for entry. Experiments reveal variation in efficiency of transmitted Env clone's ability to utilize various CD4/CCR5 pairs for HIV-1 entry into cells within and between subtypes. This research will assist in identifying new non-human primate models of HIV-1 infection that can support

replication of transmitted forms of HIV-1 variants (and/or SHIVs) that are relevant for vaccine and therapeutic approaches.

Methods

Pseudovirus production

HEK293T cells were seeded 16hrs prior to transfection at a density of 2×10^5 cells per ml in tissue culture plates in Dulbecco's modified Eagle medium (DMEM) (Invitrogen) with 10% heat-inactivated fetal bovine serum (FBS), 0.5% penicillin-streptomycin-fungizone, 0.5% 2mM L-glutamine (complete DMEM). Cells were then transfected using Roche FuGene6 transfection reagent and manufacturer's specifications with an *env*-deficient proviral plasmid (Q23 Δ EnvGFP or pBru Δ *env*-Luciferase) and *env* gene of interest (Table 1). 48hrs later, cell supernatants were harvested, centrifuged to remove cell debris (1,200g, room-temp, 5min) and concentrated using Amicon Ultra – 15 Centrifugal Filters (Ultracel – 100K) by centrifugation at 3,000g for 15min (room-temp) and then stored at -80°C.

The viral titer of each transfection supernatant was determined by infecting TZM-bl cells obtained from the National Institute of Health (NIH) AIDs Reagent Program. TZM-bl cells are derived from a HeLa cell line and stably express large amounts of CD4 and CCR5. These cells have a β -galactosidase gene integrated under the control of an HIV-1 long terminal repeat such that if these cells are infected, they express β -galactosidase. Forty-eight hours postinfection, cells were stained for β -galactosidase activity and appear blue after staining if β -galactosidase is present. The proportion of blue cells is determined and used to calculate viral titer as previously described¹³.

CD4 transient transfection infectivity assay

HEK293T cells were seeded 24hrs pre-transfection at 2.5×10^5 cells per well in 6 well tissue culture plates with 2ml complete DMEM. Cells were then co-transfected using Roche FuGene6 transfection reagent and manufacturer's specifications with 1 μ g human CCR5 plasmid

and 0.5µg CD4 plasmid of interest. Forty-eight hours later cells were harvested and re-seeded in 12 well plates in duplicate in 1ml complete DMEM at a density of 8×10^4 cells per well. In parallel, aliquots of cells were stained using Allophycocyanin (APC)-conjugated CD4 antibody (BD Biosciences, #551980) and Fluorescein Isothiocyanate (FITC)-conjugated CCR5 antibody (BD Biosciences, #561747) and examined via flow cytometry for CD4 and CCR5 expression levels. Six hours after plating, cells were infected by spinoculation (1,200g for 90 min.) with HIV-1 pseudoviruses at an MOI of 15 with 10ug/ml of DEAE-dextran. All pseudoviruses used were generated using a Q23ΔEnvGFP proviral clone with an *env* gene of interest (Fig. 2B). After 72hrs, the cells were washed once with 200µl of 1X phosphate-buffered saline (PBS), harvested using 200µl of 0.05% trypsin-EDTA (Gibco), and fixed in 200µl of 2% paraformaldehyde (PFA). The fixed cells were washed twice with 500µl of fluorescence-activated cell sorter (FACS) buffer (1x PBS containing 1% FBS and 1mM EDTA). The cells were resuspended in 400µl of FACS buffer, filtered through a 35um pore size nylon mesh cap (BD Falcon), and analyzed for GFP expression on BD FACSCanto II flow cytometer. The data from 10^4 cells were analyzed using FlowJo version 9.7.5.

CCR5 mutagenesis

A CCR5 mutant of the Spix's OM CCR5 allele-1 encoding 2 amino acid substitutions (D15Y, A16T) was generated using the QuickChange site directed mutagenesis kit (Stratagene) with the appropriate mutagenic primers. Sanger sequencing was used to ensure the correct mutations and that the rest of the sequence remained identical to the Spix's OM CCR5 allele-1.

Generation of stable cell lines

HEK293T were cultured in complete DMEM at 37°C and 5% CO₂. For generation of CD4 and CCR5 expressing cell lines, retroviral virus-like particles (VLPs) were generated in

HEK293T cells by cotransfecting pLPCX (retroviral vector encoding the CD4, Fig. 1A, or CCR5, Fig. 1B, of interest), pJK3 (MLV-based packaging plasmid)³³, and pMD.G (vesicular stomatitis virus glycoprotein [VSV-G] envelope plasmid)³⁴ 41 at a ratio of 1:1:0.5 using Fugene 6 (Roche) transfection reagent according to the manufacturer's protocol. 48hrs posttransfection, the supernatants containing VLPs were collected, filtered through 0.22-micrometer filters, and concentrated using Amicon Ultracel 100K filters (Millipore). The concentrated VLPs (200µl) were used immediately to transduce HEK293T cells that had been plated 24hrs prior at a density of 10^5 cells/well in a 6-well plate in 2 ml of drug-free complete DMEM. The cells were transduced in the presence of 10µg/ml of DEAE-dextran by spinoculation at 1,200g for 90 min. The following day, cells were split and transferred in new T75 flasks in 10 ml of drug-free complete medium and cultured for 48 h. The cells were then passaged and maintained in complete medium supplemented with 2µg/ml of puromycin (to select for CD4 and CCR5 expression). The transduced cells with high levels of CD4 and CCR5 expression were obtained by sorting the cells on a FACSAria II cell sorter using an APC-conjugated CD4 antibody (BD Biosciences, #551980) and FITC-conjugated CCR5 antibody (BD Biosciences, #561747) as described previously⁵. Cells line CD4 and CCR5 generated are listed in Fig. 3C.

CCR5 stable cell line GFP infectivity assay

Stable cell lines were seeded in 12 well plates in duplicate in 1ml complete DMEM at a density of 8×10^4 cells per well. Sixteen hours after plating, cells were infected by spinoculation (1200g for 90 min.) with HIV-1 pseudoviruses at an MOI of 1 in the presence of 10ug/ml of DEAE-dextran per well. All pseudoviruses used were Q23ΔEnvGFP with *env* gene of interest. After 48hrs, the cells were washed once with 200µl of 1XPBS, harvested using 200µl of 0.05% trypsin-EDTA (Gibco), and fixed in 200µl of 2% PFA. The fixed cells were washed twice with

500µl FACS buffer. The cells were resuspended in 450µl of FACS buffer, filtered through a 35µm pore size nylon mesh cap (BD Falcon), and analyzed for GFP expression using BD FACSCanto II flow cytometer. The data from 10^4 cells were analyzed using FlowJo version 9.7.5.

CCR5 stable cell line luciferase infectivity assay

Stable cell lines were seeded in 96 well plates in 125µl of complete DMEM at a density of 10^4 cells per well. 200µl of sterile water was plated in outermost wells to prevent evaporation of media. 16 hours after plating, cells were infected by spinoculation (1200g for 90 min.) with HIV-1 pseudoviruses at an MOI of 5, 2.5, 1.25, 0.625, 0.3125, and 0.1563 in the presence of 10ug/ml of DEAE-dextran per well. All pseudoviruses used were pBru Δ env-Luciferase with *env* gene of interest. 4-6 hours after spinoculation, media was aspirated and replaced with 125µl complete DMEM. 48hrs after infection, 20µl of cell supernatants were collected into a separate 96 well plate, mixed with 50µl of Pierce™ *Gaussia* Luciferase Glow Assay Kit (Thermo Scientific, cat#: 16160) working stock (made according to kit recommendations), and then incubated for 10 min at room temperature. The samples were then immediately analyzed for luminosity using a luminometer. Data were gathered using Tecan© Magellan data analysis software and then plotted and analyzed further with Prism version 6.07 (GraphPad Software).

Results

Azara's and NancyMa's OM species naturally encode CD4 alleles that permit entry by transmitted variants of HIV-1.

Given that early-stage HIV-1 variants can use the Spix's OM CD4 allele-3 (Sp-3 CD4)¹⁰, we wanted to test whether CD4s from two other species, Azara's and NancyMa's OM species, were functional as receptors for early-stage HIV-1 Env variants. Azara's OM and NancyMa's OM each possess alleles encoding an asparagine at position 39 of CD4 (Fig. 1A)⁵. A CD4 allele from each of these two species (Fig. 1A) was cloned into an expression plasmid and transiently co-transfected it into 293T cells along with a plasmid encoding human CCR5 (Fig. 1B). Human CCR5 is known to be a functional coreceptor for CCR5 tropic Env variants and was paired with each CD4 to examine CD4 function independently¹⁰. Expression of both CD4 and CCR5 was measured using flow cytometry for each group of transiently transfected cells (Fig. 2A). Fig. 2A shows that each group of transiently transfected cells contains a majority of cells expressing both CD4 and CCR5 and that the proportion of cells expressing both CD4 and CCR5 is similar between each cell group (65.7%-75.1%). Transfected cells were then challenged with two pseudoviruses encoding transmitted subtype A HIV-1 Envs, Q23.17 and BG505.W6M.B1. These envelopes were isolated from viruses collected from an adult woman and an infant, respectively soon after they had become infected making them more relevant to studies of transmission (Table 1). Infection in cells expressing Azara's and NancyMa's CD4s was comparable to cells expressing human CD4 and was 6-7 fold greater than infection observed in cells expressing Rhesus CD4 (Fig. 2B).

CCR5 SpR5_Y15_T16 changes are sufficient to confer function as a coreceptor for entry.

Previous experiments using infectivity assays have shown that Spix's OM CCR5 allele-1 (Sp-1 CCR5) is not a functional coreceptor for transmitted HIV-1 Env variants when paired with human CD4 (Sharma et al. unpublished). Human CCR5 and Sp-1 CCR5 amino acid sequences were compared to determine amino acid residues that may be responsible for differences in coreceptor functionality. There is a 96.3% amino acid sequence similarity between human CCR5 and Sp-1 CCR5 with 2 residue differences at positions 15 and 16 hypothesized to be important for these CCR5's functioning as coreceptors for CCR5 tropic HIV-1 entry (Fig. 1B). To test this hypothesis, stable cell lines encoding either Spix's or human CD4s and CCR5s of interest (293T-CD4/R5) were infected with virus pseudotyped with early-stage HIV-1 envelopes (Table 1). Expression of CD4 (Fig. 3A) and CCR5 (Fig 3B) for each cell line was adequate for infection experiments and relatively equal between cell lines allowing us to compare infection between cell lines. Of note, all of the cell lines expressed receptors and coreceptors at levels comparable to the cell line expressing human receptors and coreceptors, which was used as a positive control (Fig. 3A, 3B, and 3C).

In order to test whether Spix's OM receptor/coreceptor combinations facilitate entry of early stage HIV-1 Env variants, cell lines expressing receptor/coreceptor combinations of interest were infected with a pseudovirus pseudotyped with a transmitted early-stage HIV-1 Env variant. Initial analysis (Fig. 3D) focused on infection by a single transmitted HIV-1 variant derived from an infant at the time of first HIV-1 infection (Table 1, BG505.W6M.B1). In agreement with prior studies¹⁰, the combination of either human CD4 or Sp-3 CD4 and the Sp-1 CCR5 does not permit efficient entry of this HIV-1 Env variant when compared to human CD4 and human CCR5¹⁰.

Next, we tested a mutant of Spix's OM CCR5 allele-1 (SpR5_Y15_T16) and observed that it functions as a coreceptor for pseudovirus pseudotyped with BG505.W6M.B1 Env when combined with a Sp-3 CD4 (Fig. 3D) and demonstrates a ~23 fold greater infection compared to the wild type Sp-1 CCR5 when paired with the same CD4. The CCR5 SpR5_Y15_T16 paired with human CD4 showed an ~11 fold increase in infection when compared to wild type Sp-1 CCR5 paired with human CD4. CCR5 SpR5_Y15_T16 paired with human CD4 showed infection levels comparable to the human CD4/human CCR5 expressing cell line. Therefore, the CCR5 SpR5_Y15_T16 mutant is sufficient to support infection by BG505.W6M.B1 (Fig 3D). To ensure the 293T-CD4/R5 stable cell lines were expressing functional CCR5 protein, a CD4-independent Env clone (QA255 CD4iA) was assayed in the same manner as the transmitted Env clones. QA255 CD4iA is a CD4-independent Env clone previously generated by passage of a chimeric virus expressing QA255.662M.C *env* in the context of a Q23.17 proviral clone through CD4-negative, CCR5-positive cells (BC7/R5 cells)³. This clone was assayed in our GFP infectivity assay in the same manner as the transmitted Env clones and infection was observed in all cell lines expressing human CCR5 regardless of CD4 pairing (Fig. 3E). Interestingly the infection of cells expressing Sp-MT CCR5 was low indicating that QA255 CD4iA Env is dependent on determinants other than CCR5 residues Y15 and T16.

CD4 and CCR5 pairs show different functionality as receptors and coreceptors for various transmitted HIV-1 Env variants.

To further examine whether there is infection variability between Env clones and elucidate similarities and differences in infection with these OM CD4 and CCR5 alleles, a variety of different Env variants were tested (Fig. 4). It has been shown that some subtype C Env variants utilize Sp-3 CD4 for entry while others do not¹⁰. This indicates that there may be

different infection phenotypes within various HIV-1 subtypes. None of the variants tested were able to efficiently infect cells expressing the Spix's OM CD4 allele-1 (Sp-1 CD4) regardless of pairing with CCR5 (Hu CCR5, Sp-1 CCR5, or CCR5 SpR5_Y15_T16, and data not shown) in agreement with Fig. 3D and data indicating that CD4s encoding an isoleucine at position 39 are non-permissive for HIV-1 entry⁵.

Infectivity experiments with a variety of transmitted Env clones from various subtypes indicate differential usage of receptor/coreceptor pairs within subtypes and between Env isolates (Fig. 4). For example, although human CD4 and Sp-3 CD4 both encode an asparagine at position 39, they differ at 21 other sites in the D1 domain, and diminished infection was observed for cell lines expressing Sp-3 CD4 regardless of CCR5 pairing for the two subtype C Envs tested. While BG505.W6M.B1, Q23.17, and MG505.W0.A2 are all subtype A Env isolates, differences in receptor/coreceptor usage were observed. Infection was observed in the cell line encoding human CD4 and Sp-1 CCR5 for Q23.17 Env whereas infection was low, near background levels for this cell line when infected with BG505.W6M.B1 and MG505.W0.A2 Envs.

MG505.W0.A2 is an Env clone isolated from a chronically infected mother who transmitted HIV-1 to her infant. This clone was isolated at the time of the infant's birth. BG505.W6M.B1 is the corresponding infant Env variant and was isolated from the infant born to this mother soon after transmission. These Env clones are therefore related with MG505.W0.A2 being representative of a chronic stage HIV-1 Env isolate and BG505.W6M.B1 representing a transmitted HIV-1 isolate. Interestingly, infection by MG505.W0.A2 was observed in cells expressing Sp-3 CD4 and human CCR5 but was lower in cells expressing Sp-3 CD4 with CCR5 SpR5_Y15_T16 (Sp-MT) contrary to the phenotype observed in this cell line for BG505.W6M.B1. This means that for MG505.W0.A2, the CCR5 SpR5_Y15_T16 mutant is not

sufficient to allow for rescued infection when paired with Sp-3 CD4 (Fig. 4) and that transmitted early-stage Env isolates such as BG505.W6M.B1 may be better able to use the CCR5 SpR5_Y15_T16 mutant.

Infection was observed for TRO.11 in all cell lines encoding a CD4 with an asparagine at position 39 regardless of CCR5 pairing (Fig. 4D). Infection was 3 fold less in cells expressing Sp-3 CD4 and Sp-1 CCR5 than cells expressing human CD4 and CCR5 yet 3 fold greater than the negative control (Rhesus macaque CD4 paired with human CCR5). Infection observed in cell lines expressing Sp-1 CCR5 encoding D15 and A16 when this coreceptor was paired with either human CD4 or Sp-3 CD4 implies that the Env variant TRO.11, without mutation at residues 15 and 16, can use Sp-1 CCR5 as a coreceptor in the context of these infectivity assays .

Examining the role of MOI on receptor and coreceptor usage.

Studies using transient transfection experiments have shown that infecting cells with a high MOI (MOI of 10) may lead to significant viral entry into cells even when the cells are expressing receptors that are not efficient for viral entry. Therefore, an experiment was performed infecting stable cell lines expressing various CD4/CCR5 pairs with a high MOI (MOI of 5) to determine whether MOI in this assay would allow more efficient entry in cells expressing the Sp-1 CCR5 coreceptor (Fig. 5). Fig. 5 shows infection as a percent of GFP expressing cells when stable cell lines expressing various CD4/CCR5 pairs were infected at an MOI of 5 with Q23ΔEnvGFP pseudotyped with BG505.W6M.B1 *env* (single replicate only). This experiment was identical to the experiments performed in Fig. 3D but with an MOI of 5.

Under these conditions, a several-fold increase in infection occurred (Fig. 5) in each cell line, especially those that were non-permissive to HIV-1 entry in GFP infectivity experiments with an MOI of 1 (Fig. 3D). While a several fold increase in infection was observed in each cell

line, some trends seen when infecting with an MOI of 1 remained. Infection levels observed in cell lines expressing Sp-1 CD4 regardless of CCR5 pairing and cell lines expressing Sp-3 CD4 paired with Sp-1 CCR5 were low and comparable to the negative control cell line expressing Rhesus macaque CD4 and human CCR5. The stable cell line expressing human CD4 and Sp-1 CCR5 showed a ~20 fold increase in infection and the stable cell line expressing Sp-3 CD4 and Sp-1 CCR5 showed a ~24 fold increase in infection. The cell line expressing human CD4 and CCR5 however, only showed an apparent ~2 fold increase in infection. This indicates that the use of a high MOI in these experiments can exaggerate receptor and coreceptor apparent usage for HIV-1 entry and that an MOI of 1 is sufficient for detecting differences in the efficiency of receptor/coreceptor usage for HIV-1 entry.

To further examine the effect of MOI on receptor/coreceptor usage for HIV-1 entry and the efficiency of receptor/coreceptor pairs for HIV-1 entry, luciferase reporter pseudoviruses pseudotyped with a subset of Env clones assayed in the GFP infectivity experiments were generated. The ability of CD4/CCR5 pairs to function as mediators of pseudovirus entry were evaluated via a luminometer 48hrs after infection by measure of luminosity of cell line supernatants as a function of luciferase expression (Fig. 6). These luciferase infectivity experiments corroborate GFP infectivity data (Fig. 5) and more effectively show differences in the efficiencies of the ability of CD4/CCR5 pairs to function as mediators of pseudovirus entry at various MOIs. Furthermore, luciferase infectivity experiments confirm that the CCR5 mutant SpR5_Y15_T16 functions as an HIV-1 coreceptor when combined with a Spix's OM CD4 encoding an asparagine at position 39 (Sp-3 CD4) for certain transmitted HIV-1 Env clones such as BG505.W6M.B1, Q23.17, and TRO.11 (Fig 6). Interestingly, this assay (Fig. 6) which has a relatively large dynamic range, suggests there is low level infection of some transmitted HIV-1

Env clones of cells expressing Sp-1 CCR5 paired with a CD4 functional for HIV-1 entry¹⁰ (human CD4 and Sp-3 CD4 both encoding an asparagine at position 39, Fig. 1A).

When assayed with an MOI of 5 with BG505.W6M.B1 (GFP infectivity assay) the cell line expressing human CD4 and CCR5 showed an apparent ~2 fold increase in infection while cell lines expressing CD4/CCR5 combinations that showed little infection at an MOI of 1 showed much greater fold increase in infection. The discrepancy in fold increase in infection is clarified by the luciferase infectivity data. Luciferase infectivity data for BG505.W6M.B1 show infection in the cell line expressing human CD4 paired with Sp-1 CCR5 although much less infection than the humanized cell line at all MOI's. This suggests that for BG505.W6M.B1 Env, low levels of infection occur in cells expressing Sp-1 CCR5 and human CD4.

Discussion

In this study, we examined CD4 and CCR5 determinants of HIV-1 infection and explored the ability of transmitted HIV-1 Env variants to use owl monkey CD4s and CCR5s as receptors and coreceptors for viral entry. Sulfated tyrosines Y10 and Y14 of human CCR5 that have been shown to interact with gp120 during HIV-1 entry and are critical for successful CCR5 usage as a coreceptor^{15, 16, 17, 18}. However, these sulfated tyrosines are conserved between CCR5 alleles (Fig. 1B) that function and do not function as coreceptors for HIV-1 entry. It is believed that Y15 and T16 contribute to the rigidity of the sulfated tyrosine (Y10, Y14) interactions with gp120 and are therefore important for successful CCR5 usage as a coreceptor for entry. Indeed, we observed that the mutant of Spix's OM CCR5 allele-1 (SpR5_Y15_T16) functions as an HIV-1 coreceptor when combined with a functional Spix's OM CD4 and demonstrates a ~23 fold greater infection compared to the wild type Sp-1 CCR5 when paired with the same CD4 for BG505W6.B1 Env. Similar trends were observed for Q23.17, MG505.W0.A2, and TRO.11 Envs. These observations suggest residues at positions 15 and 16 are important for CCR5 functionality as a coreceptor for CCR5 tropic HIV-1 as mutating 2 CCR5 amino acid residues is sufficient to restore HIV-1 entry for otherwise non-permissive CCR5 OM alleles. Studies examining primate populations for potential animal model candidates may be able to use this information to target primate species encoding a Y15 and T16 with this study showing that CCR5s encoding these residues may be more likely to function as coreceptors for CCR5 tropic HIV-1 entry.

This study also found that three species of OM (Spix's - *Aotus vociferans*, Azara's - *Aotus azarae*, and Nancy-Ma's - *Aotus nancymae*) naturally encode CD4 alleles functional as a receptor for HIV-1 entry. Population studies genotyped an additional 21 Azara's and 35 NancyMa's OMs and found that all monkeys examined were homozygous for an asparagine

encoded at amino acid position 39 of CD4¹⁰. This supports the notion that all Azara's and NancyMa's OM and ~75% of the Spix's OM may have CD4 alleles that are compatible with early stage HIV-1 isolates.

While we have demonstrated functionality for viral entry with Azara's and NancyMa's CD4s, their CCR5s have not yet been tested. Azara's and NancyMa's CCR5s are extremely similar in amino acid sequence to Sp-1 CCR5 with Azara's CCR5 allele-1 encoding only a single amino acid substitution (position 200) and NancyMa's CCR5 amino acid sequence being identical. Because both of these CCR5 alleles are nearly identical and completely identical in the N-terminus region which interacts with Env during viral entry, it is hypothesized that Azara's and NancyMa's CCR5 would behave similarly to Sp-1 CCR5 in the infectivity assays described here. If these CCR5 alleles are found to function similarly to the Sp-1 CCR5, with certain Env variants such as TRO.11 demonstrating infection in cell lines expressing these CCR5's, Azara's and NancyMa's OM species may be candidates for development of better animal models of HIV-1 infection.

Importantly, the HIV-1 Env variants used in this study are isolates from circulating and transmitted forms of HIV-1 and represent major circulating HIV-1 subtypes (Table 1). GFP and luciferase infectivity assays both showed differential receptor/coreceptor pair usage for different Env variants within and between HIV-1 subtypes (Fig. 4 and 6). This differential usage of receptor/coreceptor pairs emphasizes the importance of studying a variety of HIV-1 isolates. Indeed, within a single subtype, differences in receptor/coreceptor usage were observed indicating the importance of studying a variety of HIV-1 Env isolates even within a single subtype is important for fully understanding receptor/coreceptor usage of circulating and prominent subtypes. Alignments of Env sequences used in this study may reveal patterns and

Env residues important for understanding the differences observed in receptor/coreceptor usage and will be examined in future studies.

Data from both GFP and luciferase infectivity assays suggest that receptor and coreceptor usage for viral entry may not depend only on individual receptors/coreceptors expressed, but also on the pairing of receptors and coreceptors. For example, for Q23.17 Env, infection was observed in cell lines expressing human CD4 and Sp-1 CCR5 yet infection observed in cell lines expressing the same CCR5 (Sp-1 CCR5) paired with Sp-3 CD4 was ~50 fold lower. However, infection observed in cell lines expressing Sp-3 CD4 paired with human CCR5 was comparable to infection observed in cell lines expressing human CD4 and human CCR5. This implies that Q23.17 Env can use Sp-1 CCR5 as a coreceptor only when paired with human CD4 but not when paired with Sp-3 CD4 even though Q23.17 can use Sp-3 CD4 when paired with human CCR5. This finding suggests that when investigating receptor and coreceptor functionality, it is important to consider receptor and receptor pairing.

There are some HIV-1 Env variants that appear to use the Spix's CD4 and CCR5 (Sp-3 CD4 and Sp-1 CCR5) receptor combination with higher efficiency, suggesting that some viruses may be able to infect Spix's OM T cells. Sp-1 CCR5 functions as a coreceptor for entry at levels within 2-3 fold of human CCR5 levels for some HIV-1 Env variants (TRO.11) when paired with a CD4 encoding an asparagine at position 39 including the Sp-3 CD4 (Fig 6). The indication that some HIV-1 Env variants are able to use Spix's OM receptors and coreceptors suggest OMs could be developed as a model organism for SHIVs expressing envelope isolated from circulating HIV-1.

Future studies are needed to test whether SHIVs encoding envelope from transmitted HIV-1 isolates can replicate in Spix's OM peripheral blood mononuclear cells (PBMCs).

Infectivity experiments in this study show that virions pseudotyped with TRO.11 Env are able to infect cell lines encoding wild type Spix's OM CD4s and CCR5s, although less efficiently than cells expressing human CD4 and CCR5. Importantly, TRO.11 is a transmitted Env variant and therefore relevant to transmission and vaccine studies. Because TRO.11 is representative of a transmitted Env variant and our data suggest it may be able to naturally infect Spix's OM cells expressing some Spix's CD4 and CCR5 alleles, TRO.11 may be an excellent Env candidate for development of SHIVs for use in OM animal models.

In future experiments, a SHIV encoding TRO.11 Env could be passaged in OM PBMCs to create more infectious SHIVs as shown previously with other SHIVs encoding transmitted envelopes. For SHIVs to replicate in OMs, it may be important to alter the virus to circumvent naturally occurring restriction factors such as TRIM5 α and APOBEC3 in these species. However, Hoffman et al. 1999¹⁴ has shown PBMCs isolated from many species of New World monkeys are successfully infected by VSV-G-pseudotyped HIV-1 vectors. This suggests that post-entry and before genome integration, New World monkeys may not possess restriction factors to HIV-1 and that restriction to viral replication most likely occurs at the point of viral entry. The implication that New World monkeys may not possess restriction factors makes them an even more ideal candidate for an animal model.

Several studies have shown that virion envelope content and stoichiometry between virion envelope and cell receptor/coreceptor can significantly influence infectivity assays (reviewed in Brandenberg et al. 2015²⁸). Therefore, additional experiments, such as quantitative western blots of pseudovirus envelope, will be needed to show that pseudovirus envelope content has not influenced the phenotypes observed in this study.

In conclusion, the data presented here suggest that OM encode CD4 alleles that naturally function as receptors for early-stage HIV-1 entry. Additionally, our data indicate that virions pseudotyped with some early-stage Env variants may be able to infect cell lines encoding wild type CD4s and CCR5s. This presents the possibility that OM may function as a new animal model of HIV-1 infection more relevant to transmission studies than current models. Our data corroborates the finding that N39 in the D1 domain of CD4 is important for usage as a receptor by transmitted HIV-1 Env variants. We have also shown that CCR5 residues 15 and 16 are important for usage of CCR5 as a coreceptor for several early-stage Env variants. These residues may represent targets for genotyping studies seeking new candidates for animal models of HIV-1 infection.

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A: CD4

Name	Functionality	1	10	20	30	40
Human	Permissive	KKVVLGKKGDTVELTCTASQKKS	IQFHWKNSNQIKILGN	QGSFLTKGP		
Rhesus Macaque†	non-Permissive	KKVVLGKKGDTVELTCNASQKKN	TQFHWKNSNQIKILGI	QGSFLTKGP		
Spix OM-1	non-Permissive	KTVVLGEKGETVELPCETSLKKN	VQFHWKTS	SDQIKILGI	QGSFLTRGQ	
Spix OM-2	Permissive	KTVVLGEKGETVELPCETSLKKN	VQFHWKTS	SDQIKILGN	QGSFLTRGQ	
Spix OM-3	Permissive	KTVVLGEKGETVELPCETSLKKN	VQFHWKTS	SDQIKILGN	QGSFLTRGQ	
Spix OM-4	Permissive	KTVVLGEKGETVELPCETSLKKN	VQFHWKTS	SNQIKILGN	QGSFLTRGQ	
Nancy Ma's OM-1	Tested in thesis	KTVVLGEKGETVELPCETSLKKN	VQFHWKTS	SDQIKILGN	QGSFLTRGQ	
Nancy Ma's OM-2	Tested in thesis	KTVVLGEKGETVELPCETSLKKN	VQFHWKTS	SDQIKILGN	QGSFLTRGQ	
Azara's OM	Tested in thesis	KTVVLGEKGETVELPCETSLKKN	VQFHWKTS	SDQIKILGN	QGSFLTRGQ	

*.*****:*.***** * :* **. ***** :*:***** *****:*

B: CCR5

Name	Functionality	1	10	20	30	40
Human	Permissive	MDYQVSSPI	YDIN	Y	YTSEPCQKINVKQIAARLLPPLYSLVFI	FGFVGNMLV
Rhesus Mac†	Permissive	MDYQVSSPT	YDID	Y	YTSEPCQKINVKQIAARLLPPLYSLVFI	FGFVGNILV
Pig-Tail†	Permissive	MDYQVSSPT	YDID	Y	YTSEPCQKINVKQIAARLLPPLYSLVFI	FGFVGNILV
Squirrel	non-Permissive	MDYQVSSPI	YDID	Y	GFSEPCRKIDVKQMGQALLPPLYSLVFI	FGFVGNMLV
Marmoset	non-Permissive	MDYQVSSPI	YDID	Y	GFSEPCRKIDVKQMGHALLPPLYSMVFI	FGFVGNMLV
SpixCCR5-1	Tested in thesis	MDYQVSSPI	YDID	Y	DASEPCQKIDVKQMGHALLPPLYSMVFI	FGFVGNMLV
SpixCCR5-2	Unknown	MDYQVSSPI	YDID	Y	DASEPCQKIDVKQMGHALLPPLYSMVFI	FGFVGNMLV
AzaraCCR5-1	Unknown	MDYQVSSPI	YDID	Y	DASEPCQKIDVKQMGHALLPPLYSMVFI	FGFVGNMLV
AzaraCCR5-2	Unknown	MDYQVSSPI	YDID	Y	DASEPCQKIDVKQMGHALLPPLYSMVFI	FGFVGNMLV
NancyMaCCR5	Unknown	MDYQVSSPI	YDID	Y	DASEPCQKIDVKQMGHALLPPLYSMVFI	FGFVGNMLV

***** ***: * .*****:*.*****:*.*****:*.*****:*

C

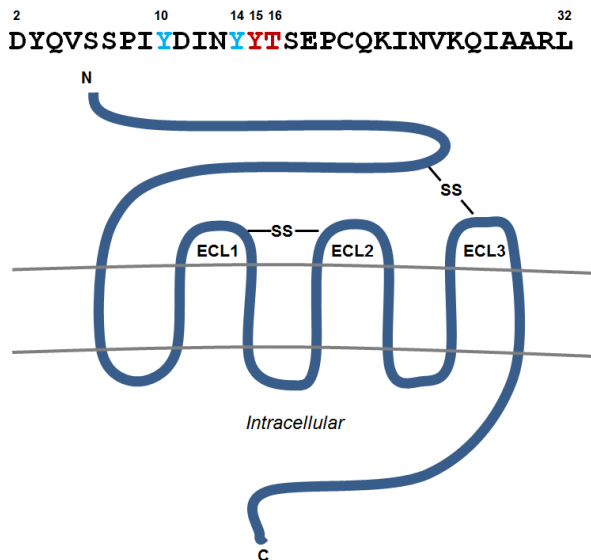


Fig. 1 – Amino acid sequences of a portion of (A) mature primate CD4s of interest. Sites examined in this study are highlighted in yellow. Amino acids differing from human CD4 are highlighted in grey. OM indicates owl monkey species. Numbers after species identification indicate different alleles identified in these primates. Functionality column indicates whether the receptor is permissive to human isolate HIV-1 entry when paired with a permissive CCR5^{10, 11} (Sharma et al, unpublished). (B) Primate CCR5s of interest with sites of interest highlighted in yellow. Amino acids differing from human CCR5 are highlighted in grey. Sulfated tyrosines positions 10 and 14 shown to be most important for gp120-CCR5 interaction^{a, b, c, d} are highlighted in blue. Numbers after species identification indicate different alleles found to exist in these primates. Functionality column indicates whether the receptor is permissive to human isolate

HIV-1 entry when paired with a permissive CD4^{10, 11} (Sharma et al, unpublished). (C) Figure adapted from Huang et al. 2007. Schematic representation of human CCR5. Sequence corresponds to the extracellular N-terminus of CCR5²⁻³². Residues of interest are depicted in red. Sulfotyrosines (Tys) critical for interaction with HIV-1 are depicted in blue¹². Extracellular loops and disulfide linkages (-SS-) are labeled.

† - Indicates old world monkey species. Other species listed (besides human) are new world monkey species.

Env clone	Subtype	Mode of transmission*	Source†	GenBank accession no.	Time postinfection (wks)
Q23.ENV.17	A	M-F	PBMC	AF004885	11
BG505.W6M.B1	A	M-C	PBMC	DQ208457	6
MG505.W0.A2	A	M-F	PBMC	DQ208449.1	-
Q255 CD4iA	A	M-F	Lab adapted‡	-	-
TRO.11	B	M-M	ccPBMC	AY835445	4
Du172.17	C	M-F	ccPBMC	DQ411853	12
Du422.1	C	M-F	ccPBMC	DQ411854	8

Table 1 – Adapted from Meyerson et al. 2015. Description of Env clones used in this study.

*M-F, male to female. M-M, male to male. M-C, mother to child.

†PBMC - Env was cloned from uncultured PBMCs isolated directly from patient

‡ccPBMC - patient PBMCs (or virus from these PBMCs) underwent short-term coculture with PBMCs from HIV-1-negative donors in order to amplify virus before cloning.

‡ - Adapted to be CD4 independent

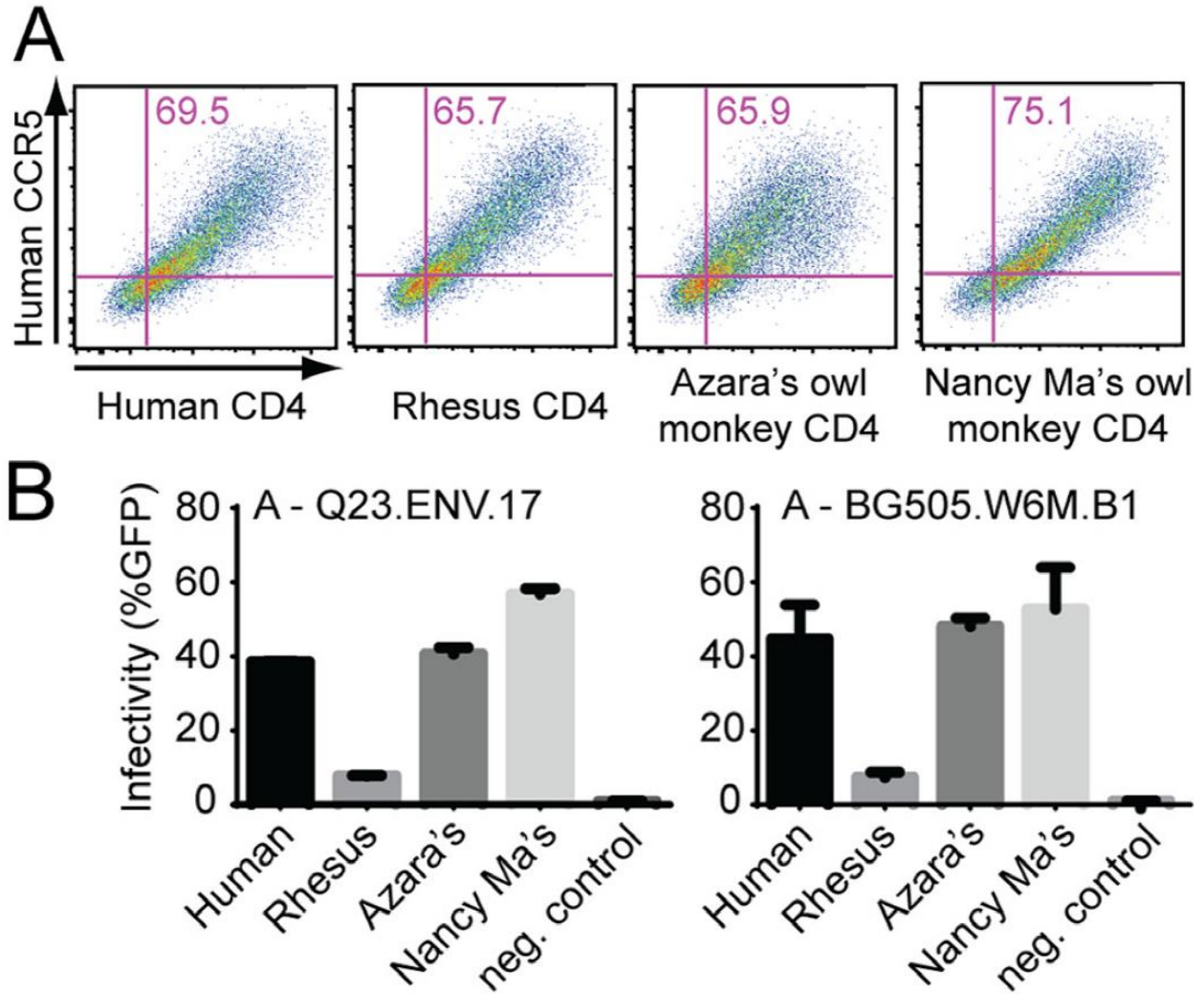


Fig. 2 - (A) Flow cytometry scatter plots exhibiting expression of CD4s of various primate alleles (indicated along the *x* axis) and human CCR5 expression (indicated along the *y* axis) in 293T cells. Numbers in each plot indicate the proportion of cells expressing both CD4 and CCR5. (B) 293T cells from panel A were infected with the indicated Env-pseudotyped virions. Infection was measured by flow cytometry as percent GFP-positive cells 72hrs postinfection. All pseudotyped virions were generated using a Q23ΔEnvGFP backbone. Error bars represent the standard deviations between replicate wells. The results are representative of three independent experiments.

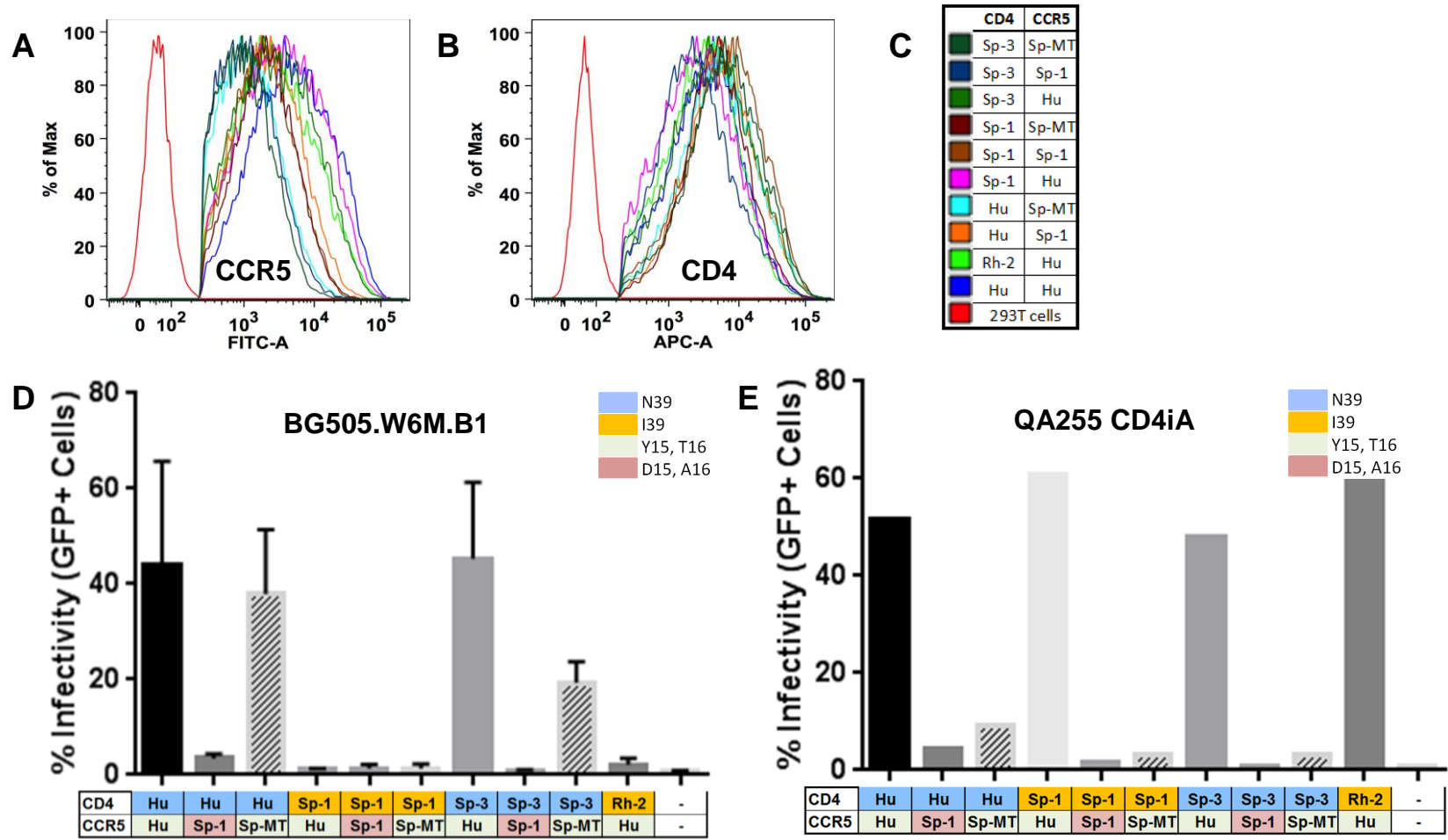


Fig. 3 - (A) Flow cytometry fluorescence intensity plots exhibiting expression of CCR5 on 293T-CD4/R5s. (B) Flow cytometry fluorescence intensity plots exhibiting expression of CD4 on 293T-CD4/R5s. (C) List of 293T-CD4/R5s. Color corresponds to panels A and B. (D, E) 293T-CD4/R5 cell lines (x-axis) from panel C were infected with indicated Env-pseudotyped virions. Infection was measured by flow cytometry as percent GFP-positive cells 48hrs postinfection. All pseudotyped virions were generated using a Q23ΔEnvGFP proviral clone. Colors correspond to residues of interest encoded in the CD4 or CCR5 allele. Error bars represent the standard deviations between replicate wells. “-” indicates 293T cells that do not express CD4 or CCR5 and were infected as a negative control. Hu=Human, Rh=Rhesus macaque, Sp=Spix owl monkey, MT=SpR5_Y15_T16, #’s=allele as depicted in Fig 1. (D) Cells infected with BG505.W6M.B1 Env-pseudotyped virions. Results are representative of four independent experiments. (E) Cells infected with QA255 CD4iA Env-pseudotyped virions. Results are representative of a single replicate only.

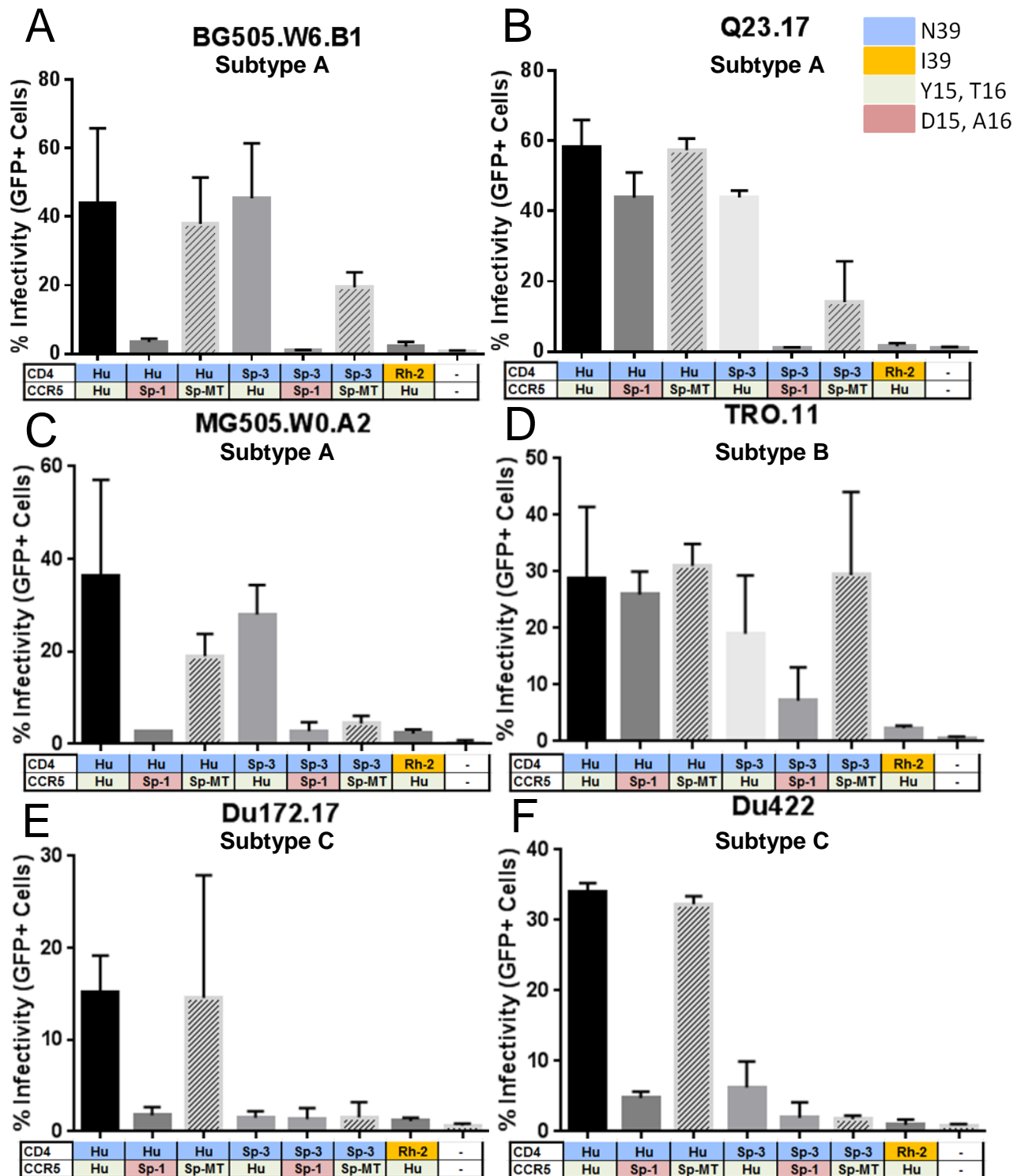


Fig 4. - 293T-CD4/R5 cell lines (x-axis) were infected with BG505.W6M.B1 (A), Q23.17 (B), MG505.W0.A2 (C), TRO.11 (D), Du172.17 (E), and Du422.11(F), Env-pseudotyped virions. Infection was measured by flow cytometry as percent GFP-positive cells 48hrs postinfection. All pseudotyped virions were generated using a Q23ΔEnvGFP backbone. Error bars represent the standard deviations between replicate wells. The results are representative of two independent experiments each. “-” indicates 293T cells that do not express CD4 or CCR5 and were infected as a negative control. Hu=Human, Rh=Rhesus macaque, Sp=Spix’s owl monkey, MT=SpR5_Y15_T16, #’s=allele as depicted in Fig 1.

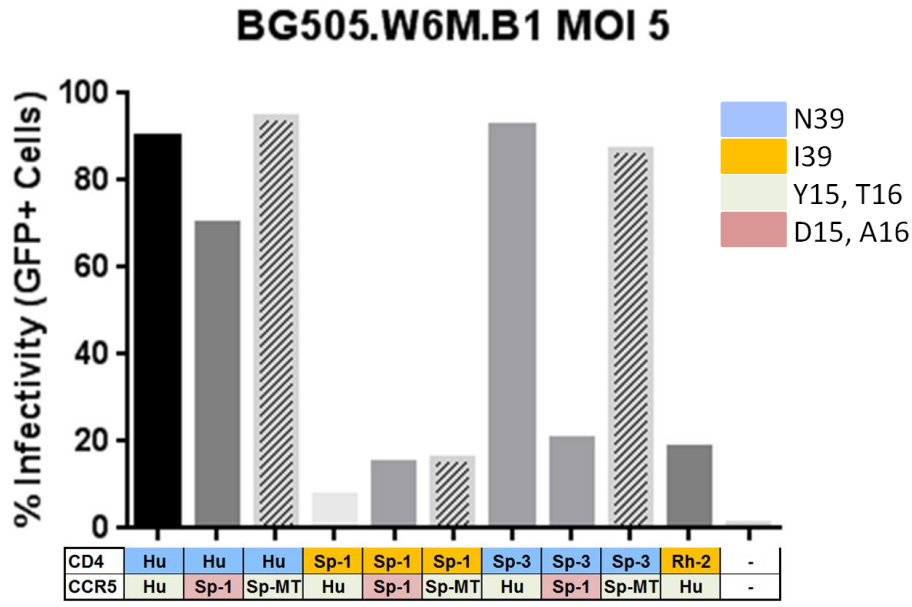


Fig 5. - 293T-CD4/R5 cell lines (x-axis) were infected with BG505.W6M.B1 Env-pseudotyped virions (MOI 5). Infection was measured by flow cytometry as percent GFP-positive cells 48hrs postinfection. All pseudotyped virions were generated using a Q23ΔEnvGFP backbone. Error bars represent the standard deviations between replicate wells. “-” indicates 293T cells that do not express CD4 or CCR5 and were infected as a negative control. Hu=Human, Rh=Rhesus macaque, Sp=Spix’s owl monkey, MT=SpR5_Y15_T16, #’s=allele as depicted in Fig 1.

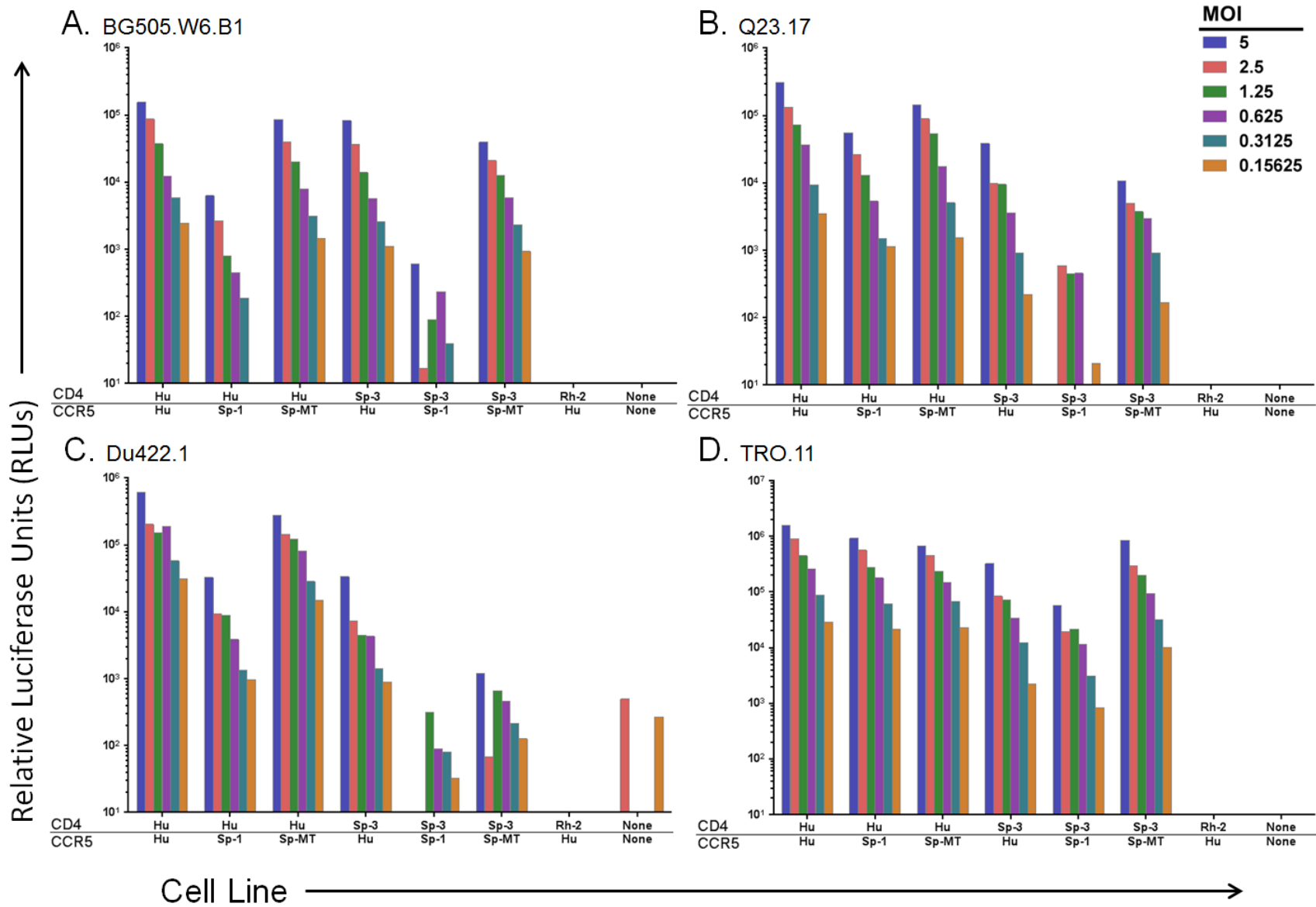


Fig. 6 – 293T-CD4/R5 cell lines (x-axis) were infected with BG505.W6M.B1 (A), Q23.17 (B), Du.422.1 (C), TRO.11 (D) Env-pseudotyped virions. Infection was measured by luminosity of cell supernatants as a function of luciferase expression 48hrs postinfection. All pseudotyped virions were generated using a pBruΔEnvLuciferase backbone. The results are representative of one independent experiment each. Color is indicative of MOI. “None” indicates 293T cells that do not express CD4 or CCR5 and were infected as a negative control. Hu=Human, Rh=Rhesus macaque, Sp=Spix’s owl monkey, MT=SpR5_Y15_T16, #'s=allele as depicted in Fig 1.