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DETAILED ANALYSIS OF BIOLOGICAL PROPERTIES OF CHRONIC HIV-1 ENVS IN HIV POSITIVE PATIENTS

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ABSTRACT

Human immunodeficiency virus (HIV) envelope glycoprotein (Env) is a major target for the development of vaccines and antiviral drugs because it is essential for viral entry into susceptible target cells. Over time, the viral envelope glycoprotein of human immunodeficiency virus type 1 (HIV-l) changes. Despite the availability of antiretroviral drugs, studies show that the (C2-V5) region diversifies at a rate of about 1% per year. Throughout an infection, many glycosylation sites are added to and enlarge the variable loops 1 and 2 (VI-V2) of the envelope glycoprotein. Errors in reverse transcription, a rapid viral replication cycle, and recombination all contribute to these envelope variations. However, the rate at which a mutation is fixed in a virus population depends on the selective advantage or disadvantage conferred by the mutation itself, as well as the level of viral replication capacity.

Keywords: Human immunodeficiency virus , glycoprotein , cellular tropism , anti-HIV therapy

1. INTRODUCTION

A study published in J. Virol 2009 by Sagar M et al. used a paternal virus to create replication-competent clones by inserting the VI-V5 region of subtype A envelopes isolated from acute and chronic HIV-1 infections. Clones containing VI-V5 envelope segments from chronically infected patients were found to have higher replication capacity in cells expressing low densities of CCR5, and the envelopes were also found to be more fiisogeriic than early phase envelopes. The authors of this study hypothesized that dwindling supplies of CCR5-expressing target cells might, in addition to host selection pressure, propel evolution in the HIV-1 envelope gene. In contrast, Doms KetalWW compared only two disease stages (transmitted/founder and chronic HIV-l envelops) phenotypically and immunologically. They observed no coreceptor tropism differences or CCR5 usage variations. This same team has examined subtype C and found very similar results. The relationship between viral tropism,

replication kinetics, fitness, and disease progression can be corroborated by gaining a deeper understanding of the dynamics of coreceptor usage of primary HIV-1 CCR5 using sfrains at various disease stages. There is a lack of understanding of the intricate interplay of factors that govern AIDS's emergence and development. Multiple studies (Bhattacharya et al., 2003; Dehghani et al., 2003; Gorry et al., 2002; Puffer et al., 2002; Ryzhova et al., 2002) have suggested that the degree to which HIV and SFV rely on CD4 is proportional to their affinity for CD4. Enhanced fropism in CD'C T-cell populations and efficient colonization in T-cell subsets that express low cell surface CD4 may result from the emergence of R5 variants that require less CD4. In the context of clade C infection, this could be a major route by which R5 sfrains destroys CD4+ T cells in the late stages of infection, leading to AIDS.

The correlation between viral tropism, replication kinetics, fitness, and disease progression rely on a firm grasp of the extent to which primary HIV-l strains utilize coreceptors at various stages of disease. Alterations in cytopathic ability, cellular tropism, coreceptor utilization, and immune evasion are just a few examples of how envelope protein evolution contributes to a great deal of virus diversity that plays a major role in modulating transmission (Berger et al., 1998; Cho et al., 1998; Cocchi et al., 1996; Wang e/a/. 1998; Yu et al., 1998).

This study analysed a large panel of envelopes spanning four disease stages, including the acute phase (median 18 days post-p24 antigen confirmation, range 2-85 days). A series of cell-based assays were used to distinguish between viral phenotypes in early (recently infected patients with history of infection within the last six to eight months), chronic (infected for more than three years with CD4 count over 500mm^3), and AIDS/late stage (CD4 count less than 250mm^3) viruses.

Phenotype comparison Five out of four clones at each developmental stage were eliminated. The assay reagents are expensive and in short supply, so this was done to ensure that the results would be accurate. Following are the criteria we used to choose participants for the experiments:

- Affecting Indian Patients
- Exclusively Clade C (Env)
- Uniquely dependent on CCR5

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2. CHRONIC ENVS WERE ASSESSED FOR INFECTIVITY

Pseudovirus Manufacturing

Pseudotyped viruses carrying patient Envelope were produced by co-transfection of env^{+}pSVIIIenv or env^{+}pcDNA 3.1/V5-His-TOPO with an env-defective HIV-1 backbone vector (pSG3ΔEnv) into 293T cells during log growth phase in 6-well tissue culture trays (Coming Inc) using calcium phosphate (Promega Inc) according to manufacturer's protocol. Cell supematants containing offspring pseudotyped viruses were extracted 48 hours post-transfection and kept at -152°C until use. The pseudovirus aliqoutes were RTactively tested.

Cavidi Tech RT-ELISA Kit For Determining Reverse Transcriptase Activity

A sample's RT activity can be measured with the use of the Lenti RT Activity kit. There are two stages to the process, first DNA synthesis and then DNA quantitation. Ploy The RNA template is attached to the base of a plate that is given. Besides the material to be analysed, a reaction mixture consisting of primer and a nucleotide (BrdUTP) is added to the plate. A DNA strand will be synthesized by the RT present in the sample. The double-stranded DNA/RNA is bound by an alkaline phosphatase (AP) conjugated a-BrdU antibody. The product is measured in volume by adding a colorimetric AP substrate (pNPP = para nitrophenyl phosphate disodium).

Day - 1

- The Pieces of a Kit Have Been Gather
- Mixed Together the Reagents for The Reaction
- The Polv a Plates Were Prepared
- HIV-1 Reduced-Intensity Rapid Testing Standard
- Samples Have Been Prepared
- Reaction On RT Has Begun

Day - 2

- Preparation Of Wash Fluid
- Ready To Use RT Product Tracer
- Put An End to RT Reaction
- Started Putting RT Product Tracer Together

- Alkaline Phosphatase Substrate Solution Was Made
- Extra Tracer Is Removed
- Activated Alkaline Phosphatase Reaction
- Consult The Food Label
- Data Processing

The average RT activity of the population was calculated. Any results from such dilutions should be disregarded.

The HlV-1 rRT Norm (Quantification of RT Activity)

Dilution Step	1	2	3	4	5	6	7	8	9	10	11	12
pg/well	93.5	41.6	18.5	8.21	3.65	1.62	0.721	0.32	0.142	0.0633	0.0281	0.0125
pg/	1870	831	369.4	164.2	73	32.4	14.4	6.41	2.85	1.27	0.562	25

After infecting TZM-bl cells with Env pscudotyped viruses having an equivalent amount of RT activity, we left them in a humidified CO_2 incubator at 37 degrees for two days. Relative luminescence units (RLU) were used, as stated elsewhere, to determine infectivity titers (Ringe el., 2010).

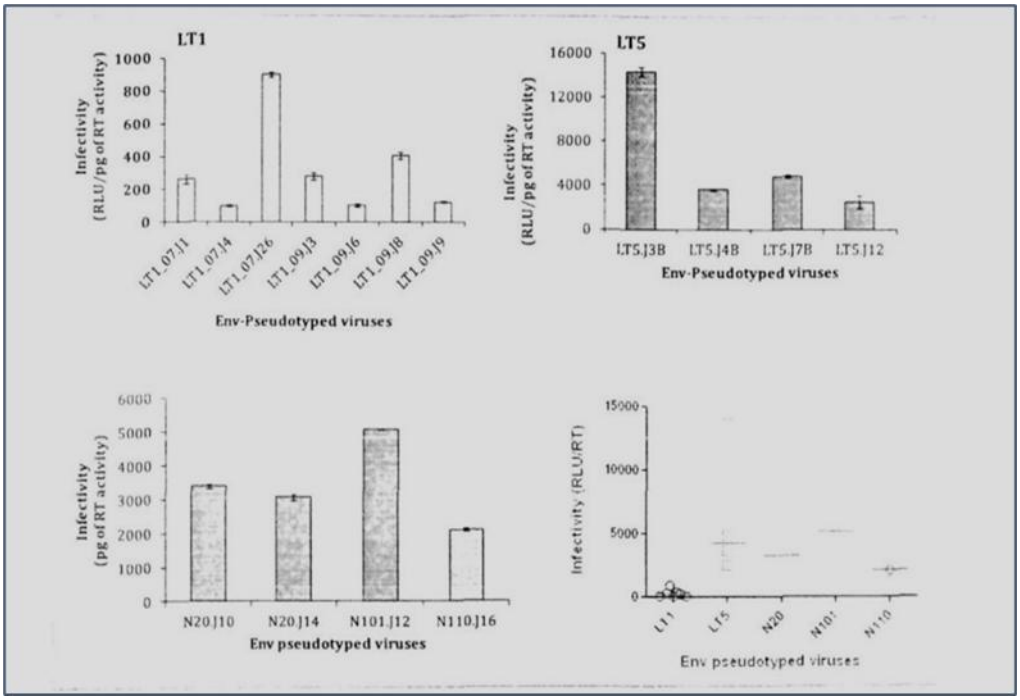

Figure 1: RT Activity of Env-pscudotyped Viruses Was Standardized to Reflect Equivalent Viral Particles. Infection of TZM-bl Cells Followed.

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The LT-1-J26, LT-53B, and NISA101-J12 Clones Had Higher Infection Potential, According to The Findings.

TZM-bl cells were infected with Env-pseudotyped viruses (equal quantities of p24 activity) and incubated for 2 days in a CO_2 incubator at 37°C so that the infectivity potential of the envelops could be compared. Below is a detailed account of how we determined p24. As was previously described, infectivity titers were determined by counting the number of times a sample fluoresced in a dark room (Ringe et al., 2010).

Mode of Examination

- The Microelisa Plate for the test, 25µl of Disruption Buffer was applied to each well.
- The linear range of detection for this assay is 3.1–100 pg/ml. Complete Tissue Culture Medium is used to dilute the p24 Standard as follows:

p24 Standard (pg/ml)	p24 Standard Volume		Media Volume
100	50 µl of 1 ng/ml	+	450 µl
50	250 µl of 100 pg/ml	+	250 µl
25	250 µl of 50 pg/ml	+	250 µl
12.5	250 µl of 25 pg/ml	+	250 µl
6.25	250 µl of 12.5 pg/ml	+	250 µl
3.1	250 µl of 6.25 pg/ml	+	250 µl

- Both replicates of 100 µl of diluted p24 Standard were introduced to microelisa wells containing Disruption Buffer.
- Negative controls were made by substituting 100 µl of Complete Tissue Culture Media for the Disruption Buffer in four separate wells.
- To microelisa wells containing Disruption Buffer, 100 µl of the prepared Test Samples were added. A light tap on the plate's side brought everything together.
- At 37 0.5°C for 60 2 minutes, the Microelisa plate was sealed with a Plate Sealer and incubated.

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3. CCR5 DEPENDENCY STUDY GOALS

Efficacy in CCR5 use can be approximated by administering CCR5 antagonists. We employed TAK779 and Maraviroc to see if envs that represent different disease stages have different sensitivities, which would indicate different efficiencies in using CCR5.

The viral envelope protein gp l60 interacts with the receptor/coreceptors on the surface of the host cell, allowing the human immunodeficiency virus (HIV) to infect the cell. Although CCR5 is used by the vast majority of main HIV-1 strains, other viruses are able to use the chemokine receptor CXCR4 as a coreceptor (termed X4 viruses) or use both CCR5 and CXCR4 as coreceptors (termed X4 and X2 viruses) (termed R5X4 virus). CCR5 has become a very appealing target for anti-HIV therapy due to its status as the most common coreceptor for clinical HIV isolates and its role in maintaining normal physiology within the human genetic knockout population. Several small chemical CCR5 antagonists with strong antiviral activity in cell culture and human clinical studies have been discovered (Kondru R et al, 2007).

This first small chemical CCR5 antagonist, TAK-779, is a quaternary ammonium anilide (Baba et al., 1999). Because of its low oral bioavailability, this chemical was taken off the market. Both TAK-220 and TAK-652, which are structurally dissimilar, are currently undergoing clinical testing (Imamura et al., 2006; Seto et al., 2006). All GPCRs, it is theorized, have a similar general structure and all feature a pocket formed by the TM helices. As with the retinal binding of Rhodopsin, small molecule drugs can occupy the pocket and act as either an agonist or an antagonist. CCR5 antagonists may also bind to this conserved pocket, as shown by previously published results. TAK-779 is projected to interact with the Tyr108 and Trp86 aromatic side chains in multiple ways. T-shaped stacking between Tyr108 and the phenyl group of TAK-779's bicyclic ring is believed to be an interaction site. Over all, TAK-779 was found to interact strongly with Trp86 on TM2 and Tyr108 on TM3, and weakly with Glu283. Thrl95 on TM5, Ile198 on TM5, Phel09 on TM3, and Trp248 and Tyr251 on TM6 also have weak hydrophobic contacts.

Maraviroc (MVC) is a chemical based on atriazolotropane; it is a small molecule CCR5 antagonist, and it is now on the market for the treatment of HFV. MVC has shown to have very effective pharmacological characteristics and antiviral activity (Kondru R et al, 2007).

All of these small-molecule CCR5 inhibitors work by disrupting the interaction between gpl20 and CCR5. This prevents HIV-1 from entering its target cells (Dragic et ai, 2000; Tsamis et al., 2003). Data suggest that antagonists impede viral entrance via allosteric effects (Watson et al., 2005), albeit the molecular mechanism of this activity is not well understood. While HIV gpl20 binds to the exterior of CCR5, primarily through contact with the N terminus and the second extracellular loop (ECL) of CCRS (Dragic et al., 2000; Tsamis et al., 2003; Nishikawa et al., 2005; Mskeda et al., 2006; Seibert et al., 2006), small-molecule CCR5 antagonists sit in the pocket formed by the (Kondru R et al, 2007).

Suppression of TZM-bl Cell Proliferation by The CCR5 Inhibitor MARAVIROC

The methodology below details the use of the CCR5 inhibitor Maraviroc in 96 well trays containing TZM-bl cells to perform entry inhibition experiments.

Guidelines:

- In 96 well plates, TZM-bl cells were seeded at a density of 1 x 10^4 cells per well. In a humid environment containing 5% carbon dioxide, the plates were incubated at 37^0C for one whole day. The next day, the plates had been incubated, and then they were examined using an inverted microscope. The experiment was performed on a monolayer of HeLa cells that was between 60 and 70% confluent.
- Interim stock of 200 µM Maraviroc for inhibition assay. A further working concentration of 5000 nM was generated from an interim supply of 200 µM.
- On a freshly emptied 96-well plate, add 110 µl of the inhibitor working dilution (5000 nM) generated in accordance with Procedure 2. Rows B through H received an additional 10% DMEM.
- In order to transfer the 10 µl ratio from row A to B, the ingredients in row A must be well combined. Similarly, moved 10 µl from Row B to Row C, and so on, all the way up to Row G. Pipette tips were swapped out between rows wherever possible.
- After removing the TZM-bl cell plate from the incubator, the medium was discarded and the cells were covered with inhibitor dilutions (100 µl in each well) produced as indicated above.
- We treated the TZM-bl cells to a 1-hour incubation at 37°C and 5% carbon dioxide. During incubating, Maraviroc travels to the CCR5 receptor and blocks its activity.

- The infectious unit content (200 $TCID_{50}$) of envelope pseudo typed viruses was normalized after 1 hour of incubation with TZM-bl (JC53-bl cell line) infections. Hence, TZM-bl cell-containing wells were inoculated with two replicates of each pseudotype virus (200 $TCID_{50}$ in a total volume of 100 µl). An additional 200 µl of fluid was added to the well's total. Substituting 10% DMEM for the pseudovirus in the confrol wells.

Table 1: Envs Susceptibility to Anti-CCR5 Drugs

CLONE ID	nM MARAVIROC	nM TAK779
16936	0.5	0.9514447
25711	5.41	1.8775503
H16055	1.28	0.8716466
00836	0.22	0.001
26191	3.31	1.2974495
7.J20	0.96	1.1433284
4-2.J41	1.31	0.9145391
5-4.J16	1.11	0.8921888
2-3.J7	1.88	0.8063513
4.J22	7.31	1.6297937
LT1.J1	1.3	1.2129484
LT1.J4	0.61	0.09
LT1.J6	0.43	0.5
LT-1-J9	1.59	0.001
LT1.J3	8.05	0.001
LT1.J8	1.54	0.001
LT1.J17	1	8.1967213
LT1.-J26	1	0.1086957
LT5.J3b	6.72	0.0001
LT5.J7b	7.8	0.0001
NISA-101.J12	1.56	0.6434892
NISA-20.J10	0.05	0.001
NISA-110.J16	6.28	30
NISA-20.J14	1.55	0.001
VB100.J38	18.27	1600
VB106.J38	8.52	0.001
VB95.J22	11.36	0.16
VB27.J2	18.52	1300
JRFL	2.6	60
JR-CSF	0.75	1
SF162	NOT DONE	10
YU-2	0.5	1

GraphPad Prism was used to examine IC_{50} values in order to determine if there were any significant changes in sensitivity. At a confidence level of 99%, we calculated median IC_{50} values using the Wilcoxon signed-rank test. The P values (2-tailed) and the corresponding confidence intervals (99%) for the non-parametric correlation coefficients were calculated in Graphpad Prism.

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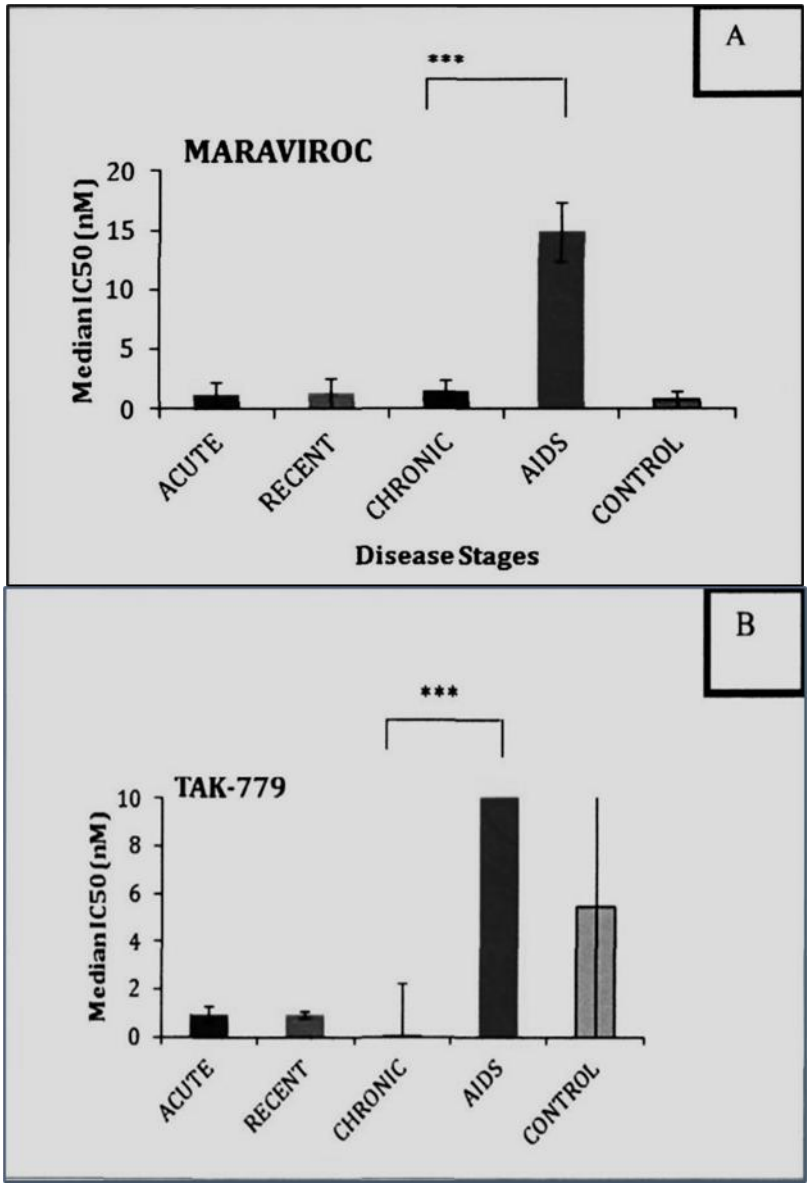

Figure 2: Comparison of The Median IC_{50} of Env Pseudotyped Viruses Against CCR5 Antagonists Marviroc (A) and TAK779 (B).

Viral sensitivity ($p<0.05$) indicated that viruses in the acute, recent, and chronic stages all depend on CCR5 in a comparable way. Median IC_{50} values for late-stage viruses against both inhibitors were much higher than those for chronic envs, indicating that late-stage viruses can mediate infection with very low levels of CCR5 on the target cell.

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4. HOW ENVS FROM DIFFERENT STAGES OF A DISEASE JOIN CELLS

A virus replicates, it produces infectious particles called virions, which diffuse through the fluid between cells and tissues and enter cells by binding to certain glycoproteins on the surface of the virus. There is mounting evidence, however, that other viruses, HIV-1 included, can hijack these established physiological communication pathways as well. in order to promote direct cell-cell viral dissemination; nevertheless, the proportional importance of the cell-cell transmission route in spontaneous infection has not yet been resolved. Several researchers, led by Nicola M. (et al.2009).

Cell-associated HIV can spread to cells that haven't been infected yet in many different ways. This can involve both cells that are actively infected (cis-infection) and cells that caught the virus but didn't get infected (trans-infection). Transmission events can be different on a molecular level depending on the cell type of the counter partners, their relative frequencies, and the rate of infection. (Irene A.A. et al., 2012) This was said to depend on a variety of extracellular interaction structures, such as T-T cell viral synapse, DC-T-cell viral synapse, Macrophage-T-cell, polysynapses nanotubes, and filopodia.

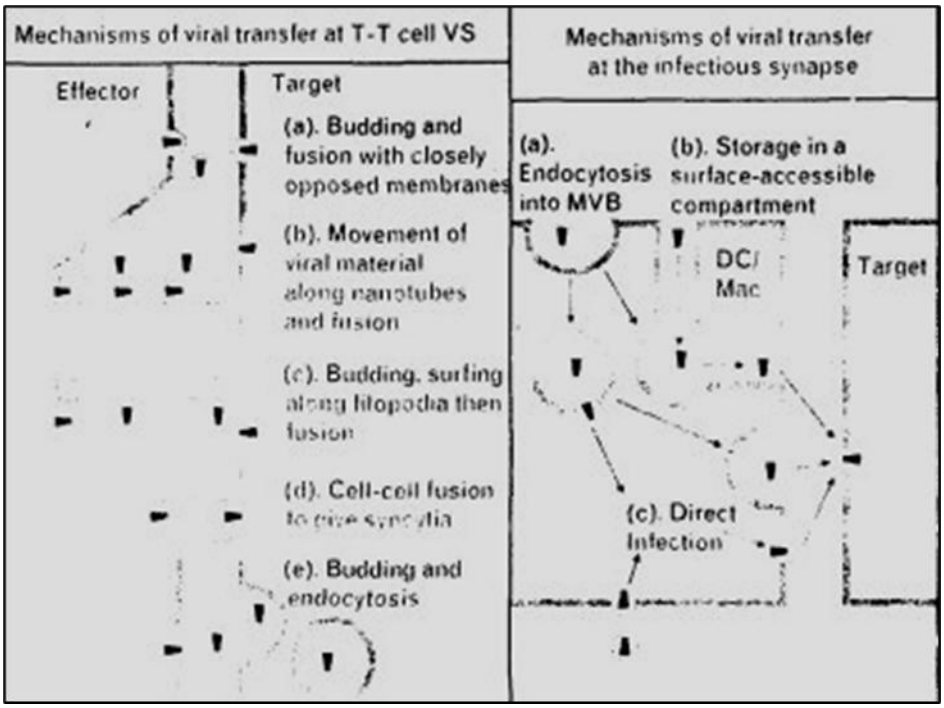

Figure 3: Mechanisms of How Viruses Move from One T Cell to Another at The Virological Synapse

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The possible ways that a virus could move from a T cell that has been infected and is making copies of itself to another T cell are shown. These are: (a) Budding and fusion with closely-opposed membranes. This shows the close interaction between the two cells through a virological synapse, with intact virions budding into the cleft and then fusing at the plasma membrane of the target cell in a CD4 and coreceptor-dependent manner. (b) Budding and fusion at the end of a nanotube. This is similar to (a), but the cell bodies are not close to each other. Instead, they are far apart, and the cells talk to each other through a nanotube that runs between them. (c) Budding, surfing along filopodia, and then fusion: The target cell sends out filopodia, which attach to the infected cell and allow intact virions to travel along them until they reach the cell body of the target cell, where they fuse. (d) Cell-to-cell fission to form syncytia: The membranes of the effector cell and the target cell fuse to form a multinucleated syncytium. (e) Endocytosis: The virus buds off of the effector cell and is endocytosis, a CD4-dependent but coreceptor-independent process. It's not clear what will happen to the virus inside the endosome, but the virion could join with the endocytic membrane and cause an infection. On the right: How viruses move from one cell to another at the infectious synapse. The possible ways that a virus can move from an APC to a target T cell. Like in the left panel, this section talks about possible ways for a virus to get in. (a) Endocytosis into MVB: The virus is taken into the cell by the APC, possibly through DC-SIGN interactions, and stored intact in a late endosome or MVB. When the virus comes in contact with a target cell, it is moved to the cell's surface and released in its whole form to infect the target cell. A virus could also join with the endocytic membrane and infect the APC this way. b) Virus that can get to the surface: The virus gets into deep membrane holes that can still be reached from the outside. From the endosomal pathway, a virus can also be moved to such a place. When the virus comes in contact with a target cell, it moves out of the depression and infects the target cell. c) Infection from a virus Figure from "Nicola M. et al. 2009 Current Opinion in HIV and AIDS 4:143-149."

Even though ART Therapy is being used, HIV-1 can still spread from cell to cell (Sigal A. et al., 2011). Trkola A et al. showed in 2012 that CD4bs reactive agents had lost all of their ability to stop fusion, while the MPER-specific antibodies 2F5,4E10 and the gp41-directed fusion inhibitor T-20 still had a lot of ability to stop fusion. [IC_{50} is only 3 times higher than for a virus that is not in a cell.] In this study, we tested the ability of the assembled panel of patient-derived envs to fuse in a way that changed over time. As viruses that can spread more

quickly will spread more quickly both between cells and between cells, the disease will get worse.

For this experiment, equal amounts of envelope plasmid and env defective backbone plasmid DNA were put into 293T cells at the same time. This was done with all of the available envelope clones and each envelope clone went through a total of six transfections. As a control, only a backbone plasmid with a broken env gene was also transfected. After being incubated for a full day, an equal number of TZM-bl cells were put on top of the 293T cells. After the TZM-bl cells were put in, luciferase readings were taken at 4, 8 and 18 hours.

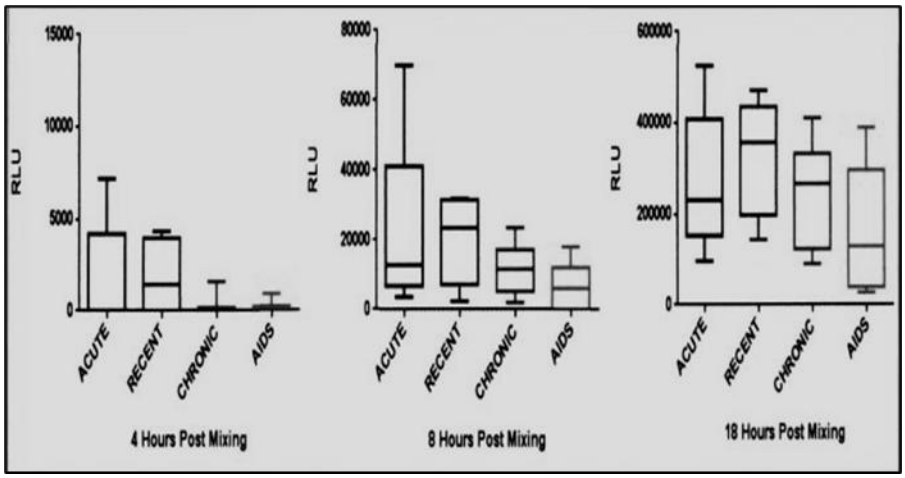

Figure 4: Comparing the Ability of Envs from Different Disease Stages to Cause Infection in CD4+/CCR5+ (TZM-bl) Target Cells

5. CONCLUSION

HIV-1 is spread by direct contact with mucous membranes or through a needle stick. Because of the genetic bottleneck that occurs during mucosal transmission, only a single form of human immunodeficiency virus type 1 (HIV-1) is established in the recipient in approximately 80% of cases of sexual transmission. Although R5 tropic viral transmission has been reported to be more efficient than X4 or dualtropic virus transmission, it is clear that a large degree of selection occurs in this scenario. Many research focusing on acute and primary infections have investigated the immunologic phenotypic signatures associated with HFV-l strains of transmitted strains. We have collected five clones of acute HIV-l infection from recent heterosexually transmitted infections in Pune and five clones of HIV-l positive individuals with a history of exposure within the last six to eight months, all of which have been previously characterized. In this study, five individuals who had been chronically

infected for at least three years without any antiretroviral therapy (ART) and showed broad to moderate broadly neutralizing antibody activity were used to create a stable of fourteen functional chronic Envs.

These variants differ genetically, allowing them to exploit CD4 and CCR5 in novel ways and possibly giving rise to a subclass of CCR5 variants that directly eliminate T cell populations. It has been hypothesized that the emergence of R5 variants requiring less CD4 will allow for robust colonization in T-cell subsets that express low cell surface CD4 and confer expanded tropism within $CD4^+$ T-cell populations (Bhattacharya et al, 2003; Dehghani et al, 2003; Gorry et al, 2002; Puffer et al, 2002). stage of infection and the development of AIDS, and this is especially true for clade C infection. In India, where sexual transmission is a key contributor to the spread of HIV/AIDS, clade C HIV is the most common subtype. Cell surface expression of CD4 and either CCR5 or CXCR4 are required for virus entry (Berger, 1997; Clapham and McKnight, 2002), however the relative utilization of CCR5 or CXCR4 is recognized to play a critical role in preferred transmission of HIV-l invivo. Some members of the chemokine receptor repertoire do contribute, albeit rarely, in preferential transmission, especially during the late and/or symptomatic phase of HIV infection (Berger, 1998; Berger, Murphy, and Farber, 1999; Coetzer et al, 2008; Nedellec et al, 2009; Shimizu et al, 2008; Shimizu et al, 2009).

The majority of new HIV cases are due to transmission between heterosexual partners. The investigations reveal that the single variant out of a complicated swarm of quasipecies is often the cause of mucosal transmission. The mucous membrane has been hypothesized to operate as a bottleneck in the population, suggesting that the strains that are successfully transmitted must possess unique characteristics. In addition, viruses that use CCR5 as a coreceptor are more likely to be transmitted through the mucosal route. Patients who had recently contracted HIV through heterosexual transmission were used to isolate the acute envelops for this study, with a mean isolation time of 18 days following p24 antigen confirmation (range: 2-85 days). However, I was unable to detect evidence of a substantial distinction between the median IC50 values for acute early and chronic envs. However, the IC50 values for both inhibitors were noticeably greater for the late-stage viruses. This data suggests that subtype C late-stage viruses require much lower levels of CCR5 for infection. In line with earlier publications, we found no discernible difference in CCR5 usage between acute early and chronic envs, demonstrating that an increased capacity to utilize CCRS is not

necessary for efficient transmission and development of new infections. Envelope infectivity was found to be positively correlated with sensitivity to the CCR5 antagonists Maraviroc and TAK-779, and with higher infectivity being linked with lower sensitivity.

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