ORIGINAL

Growth ability in various macaque cell lines of HIV-1 with simian cell-tropism

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Abstract : We have recently constructed a series of novel human immunodeficiency viruses (HIV-1s) that are tropic for a macaque cell line (mt ; macaque cell-tropic) to generate and establish a primate experimental system for HIV-1/AIDS study. In order to determine biological properties of these viruses effectively, several other macaque cell lines with distinct characteristics that can be routinely and easily used, instead of primary cells, for infection experiments are required. In this study, we have examined four macaque cell lines for their surface expression of virus receptor molecules and for their genotype of a major anti-viral capsid gene. Furthermore, we monitored the susceptibility of the cell lines to a standard simian immunodeficiency virus (SIV) clone and three representative basic mt HIV-1 clones. Results obtained here have clearly indicated that these cell lines are exquisitely useful to characterize various SIVs and more importantly, mt HIV-1s.
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Keywords **:** *HIV-1, macaque, Gag-CA, CypA, TRIM5α*

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) is tropic for humans and chimpanzees only, and the human is a sole animal species that develop AIDS. This singularly narrow host range, i.e., a property very much specialized to grow in humans, has been a major obstacle for basic studies on viral replication and pathogenesis *in vivo* (1). In an attempt to broaden the host range of HIV-1 to some primate species that are routinely used for various experiments, we have recently constructed several HIV-1 derivative viruses which are macaque cell-tropic (mt) (1-5 ; unpublished results). Mt viral genomes commonly contain a small portion of *gag* sequence encoding simian immunodeficiency virus from rhesus monkeys (SIVmac) capsid (CA) element, corresponding to the HIV-1 cyclophilin A (CypA)-binding loop, and the entire SIVmac *vif* gene (2). Representative basic clones were designated NL-DT5R (2), NL-DT5R6/7S (4) and NL-DT562 (5) (Fig. 1), and were CXCR4-positive lymphoid cell (X4)-tropic, X4 cell-tropic, and CCR5-positive lymphoid cell (R5)-tropic, respectively. The NL-DT5R6/7S construct carries an additional minimum sequence of SIVmac CA relative to NL-DT5R (Fig. 1) (4).

Simian cell-tropism of new viruses constructed in our laboratory was verified in HSC-F cells derived from a cynomolgus macaque (6). Although we have previously shown that the data obtained in HSC-F cells are parallel with those in primary lymphocytes (2, 7), viral properties may vary in the cells used. In particular, a species-specificity may exist in the sensitivity of the cell lines to HIV-1 derivative viruses. In this study, we therefore comparatively characterized four macaque cell lines of two monkey species (cynomolgus and rhesus macaques) available to us. Both monkey species are frequently

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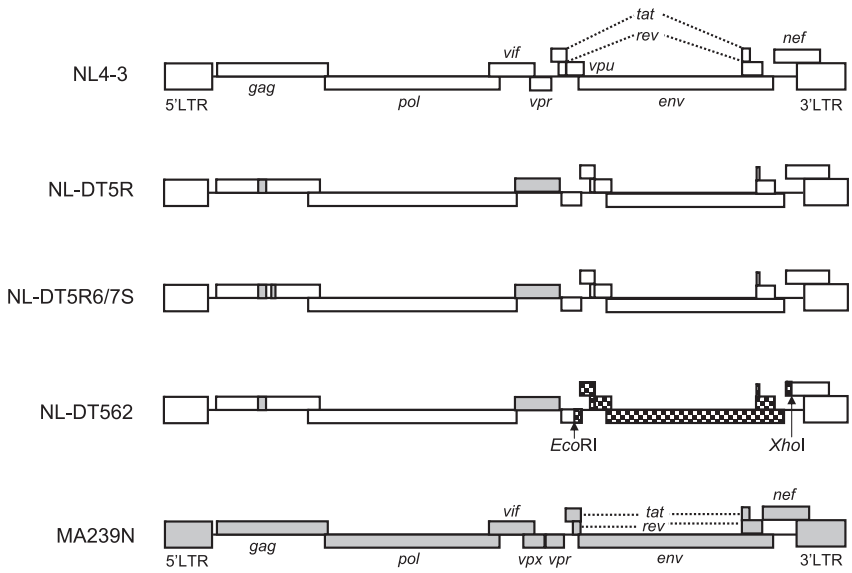


Figure 1 : Proviral genome structure of various viruses used in this study. Clones NL4-3 (9), NL-DT5R (2), NL-DT5R6/7S (4), NL-DT562 (5), and MA239N (this study) are X4 HIV-1, X4 mt HIV-1, X4 mt HIV-1, R5 mt HIV-1, and SIVmac, respectively. SIVmac sequences are indicated by grey areas. To convert X4 virus into R5-tropic, NL-DT562 has the *Eco*RI-*Xho*I region from NF462 (5) as indicated.

and internationally used for infection experiments. For characterization of the cell lines in this study, we have focused on analyzing the viral receptors, anti-CA gene, and susceptibility to viral infection. We demonstrate here, as a result, that each monkey cell line is useful to examine properties of SIV and HIV-1 derivative viruses.

MATERIALS AND METHODS

Cells

A human monolayer cell line 293T (8) was maintained in Eagles's minimal essential medium containing 10% heat-inactivated fetal bovine serum. Human and simian lymphocyte cell lines used in this study were maintained in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum. Recombinant human IL-2 (AbD Serotec) was added to the medium (50 units/ml) for maintenance of rhesus lymphocyte cell lines. Original simian lymphocyte cell lines were generous gifts of Drs. Hirofumi Akari, Tomoyuki Miura, and Hidemi Takahashi.

Infection

To obtain input viruses for infection of lymphocyte cells, 293T cells were transfected with proviral clones (Fig. 1) by the calcium-phosphate coprecipitation method as previously reported (9). A newly constructed pMA239N carries a Glu codon (gaa) in place of an internal stop codon (taa) present in the *nef* gene of pMA239 (10). Target cells were infected with an equal amount of various viruses as determined by reverse transcriptase (RT) assays, and monitored for virus growth by RT assays as previously described (9). For infection of H9 cells, the spinoculation method (11) was used. All the infected cultures except for H9 cells were maintained in the presence of IL-2 as above.

RT assay

RT assay using ^{32}P-dTTP has been previously described (12).

Flow cytometry

Cells (10^6) were stained for cell surface viral receptors with mouse antibodies (anti-human CD4 antibody/FITC for CD4, anti-human CD184 antibody/PE for CXCR4 and anti-human CD195 antibody/PE for CCR5 (BD Biosciences)). Samples were then analyzed by FACS Calibur (BD Biosciences). Unstained cells were used as negative controls.

Cell sorting

Cells ($>10^7$) were stained with a mouse anti-human CD4 antibody conjugated with FITC (BD Biosciences). Samples were then sorted for surface CD4-positive cells by a cell sorter, JSAN (Bay bioscience). Cell sorting was repeated, and CD4-positive cells were pooled until desired cell numbers were obtained.

Genotyping of TRIM5 allele of macaque cell lines

PCR amplification of CypA insertion and of TRIM5 allele (exon eight) were performed according to the method described previously (13). To analyze CypA insertion, the amplification was done by Premix Taq (TaKaRa Ex Taq version) (Takara Bio) using genomic DNAs and primers reported previously (13). PCR products were then analyzed by agarose gel electrophoresis. For determination of TRIM5 allele, the exon eight was amplified with Prime STAR HS polymerase (Takara Bio) using genomic DNAs and primers reported previously (13) and cloned into pGEM-T easy vector (Promega). Exon eight heterozygosity was then examined by determining the sequences of multiple clones for polymorphic sites (14).

RESULTS AND DISCUSSION

Expression level of cell surface receptors for HIV-1 in human and macaque cell lines

First and primary barrier of viral productive infection is the receptor molecule(s) on cell surface. As an initial step to characterize various cell lines of simian origin, the expression level of receptors for HIV-1 (CD4, CXCR4, and CCR5) was examined by flow cytometry using HIV-1 susceptible human H9 (15) and M8166 (10) cells as controls. Simian cell samples analyzed here included those of HSC-F (derived from a cynomolgus macaque), HSR1.4F (rhesus macaque), HSR5.4 (rhesus macaque), MT-IL2I (rhesus macaque), and their derivative lines. The HSC-F, HSR1.4F, HSR5.4, and MT-IL2I cell lines were similarly generated through immortalization by *herpesvirus saimiri* (6). Figure 2 shows the expression profiles of viral receptors in H9, M8166, and HSC-F cells. As is clear, human H9 and M8166 cells were positive for CD4 and CXCR4 but not CCR5, consistent with a previous observation that these cells support the growth of X4 HIV-1 but not R5 virus (16). In contrast, simian HSC-F cells, which have been used in numerous studies on SIVs, SIV/HIV-1 chimeric viruses (SHIVs) and mt HIV-1s, expressed all the viral receptors (CD4, CXCR4 and CCR5). As shown in Figs. 3 and 4, although the rhesus macaque cell lines, HSR5.4, HSR1.4F and

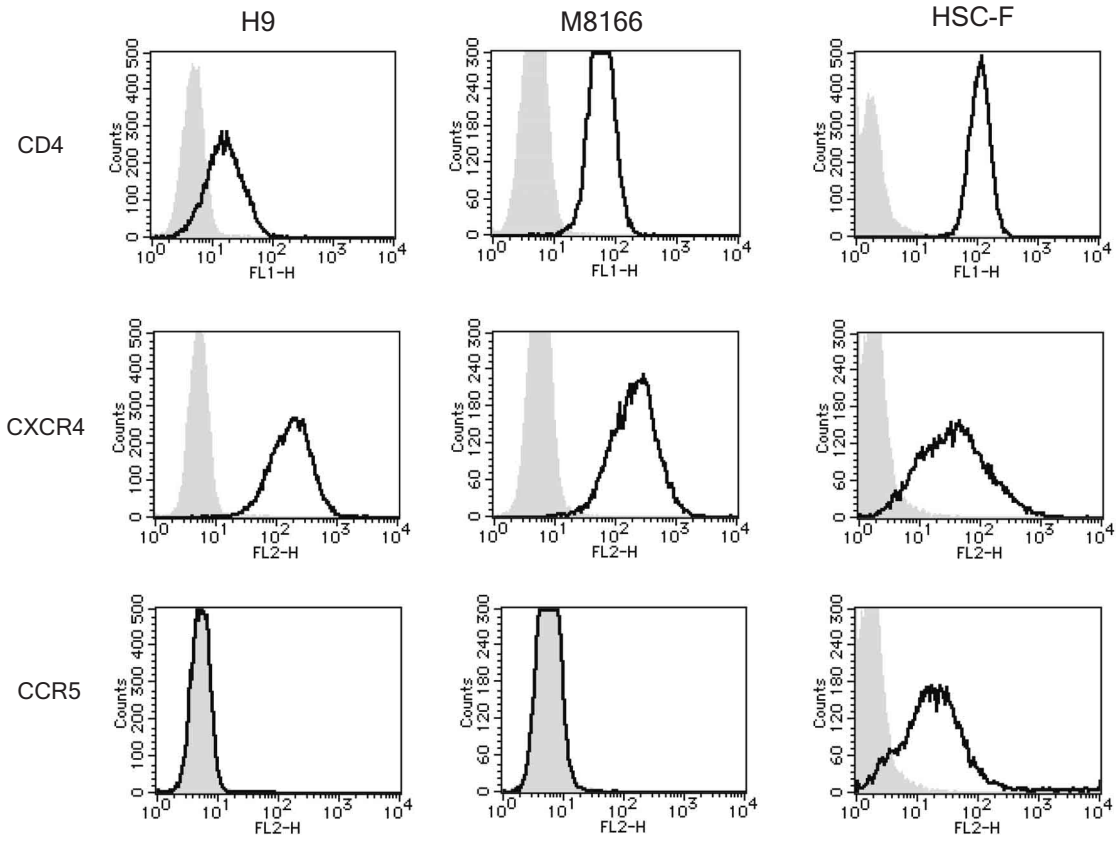


Figure 2 : Flow cytometry profiles of human H9, M8166 and cynomolgus macaque HSC-F cells for virus receptors. Cells were stained with mouse anti-CD4, -CXCR4 or -CCR5 antibody as described in MATERIALS AND METHODS, and analyzed by FACS Calibur.

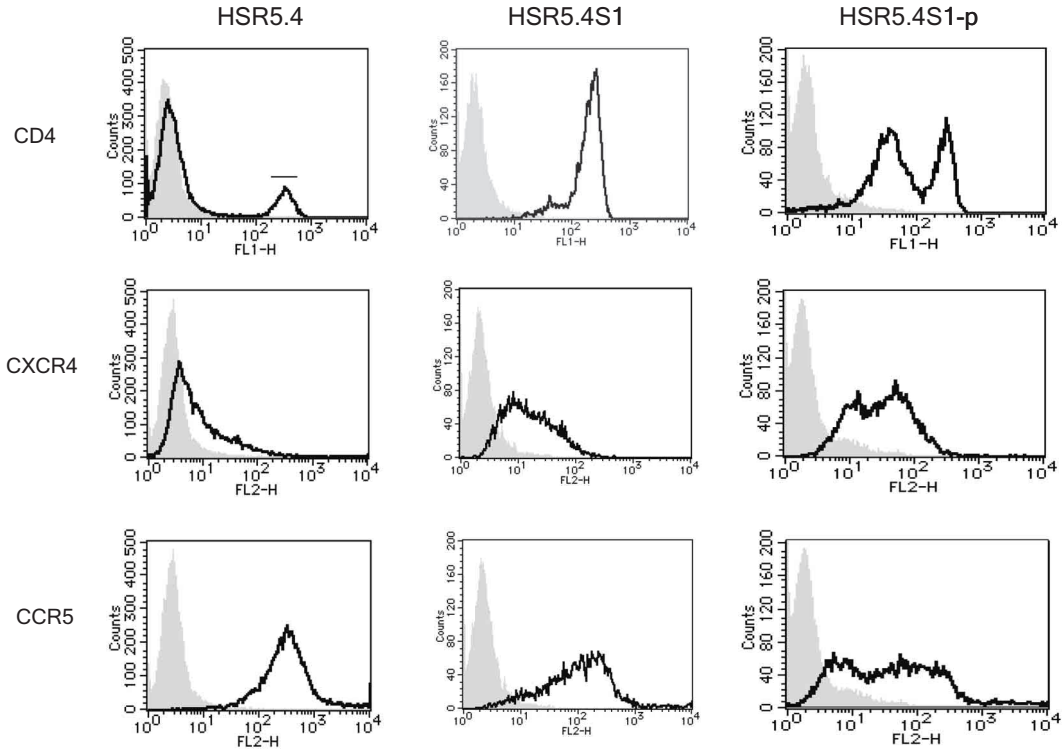


Figure 3 : Flow cytometry profiles of rhesus macaque HSR5.4, HSR5.4S1 and HSR5.4S1-p cells for virus receptors. Cells were stained and analyzed as described in the legend to Figure 2. HSR5.4S1 and HSR5.4S1-p indicate the HSR5.4 cells sorted for CD4 and their prolonged culture (2 months), respectively.

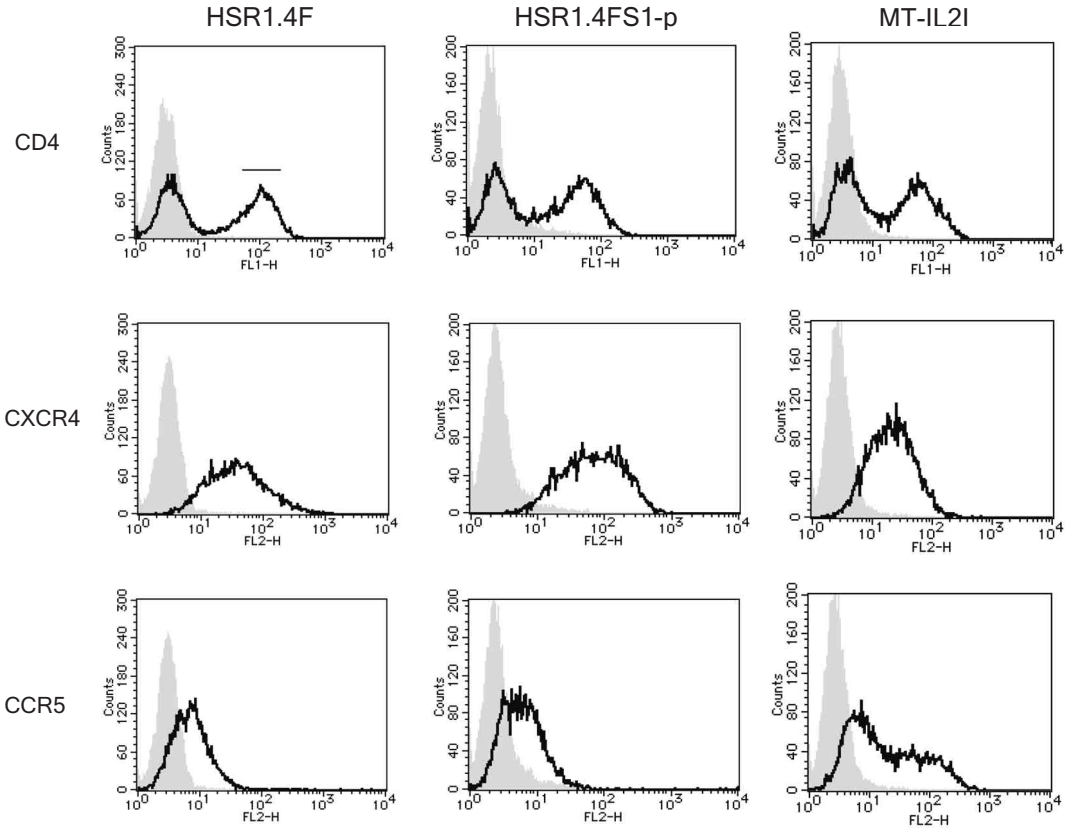


Figure 4 : Flow cytometry profiles of rhesus macaque HSR1.4F, HSR1.4FS1-p and MT-IL2I cells for virus receptors. Cells were stained and analyzed as described in the legend to Figure 2. HSR1.4FS1-p indicates the HSR1.4F cells sorted for CD4 and cultured for 2 months.

MT-IL2I, also expressed three receptors, we noticed that there were two major cell populations (CD4-positive and -negative). The HSR5.4 and HSR1.4F cell lines, therefore, were sorted for CD4-positive cells with an anti-CD4 antibody and a population that would be mostly composed of CD4-positive cells from each cell line was obtained. However, the sorting for CD4-positive cells appeared to give only a transient effect, and CD4-positive and -negative cell populations were observed after a while (HSR5.4S1-p in Fig. 3 and HSR1.4FS1-p in Fig. 4). A similar result was also obtained for MT-IL2I (data not shown). Extensive cell cloning might be required to obtain rhesus macaque cell lines that are stable for the CD4 expression on cell surface.

Analysis of TRIM5 allele in macaque cell lines

We next examined HSC-F, HSR5.4S1, HSR1.4F and MT-IL2I cells for polymorphism of TRIM5 (Fig. 5), a major anti-retroviral CA genetic locus (17), on the basis of the following observations. The restriction factor TRIM5α has been demonstrated to work as a mediator of innate immunity and to inhibit retroviral replication in a species-specific manner (18-20). It has been shown that TRIM5α is polymorphic especially within the exon eight, which encodes SPRY (B30.4) domain important for a species-specific restriction (Fig. 5A) (13, 14). At least seven TRIM5 alleles have been reported and named *Macaca mulatta* TRIM5 allele 1 to 7 (*Mamu1* to 7) (Fig. 5A). The ability to restrict replication of various retroviruses varies among *Mamu* genes (13, 14). *Mamu7* has a frame-shift in the exon eight, generating a fusion gene with a downstream CypA-coding region, and eventually encodes a TRIMCyp fusion protein composed of TRIM5 and CypA (13). It has been shown that a cynomolgus macaque also expresses this TRIMCyp, and that rhesus and cynomolgus TRIMCyp proteins do not restrict the replication of HIV-1 and SIVmac (13, 21). In addition, a recent study has shown that *Mamu1* to *3* more efficiently inhibit SIVmac replication than *Mamu4* to *6*, indicating that the polymorphism of TRIM5α significantly influences SIVmac replication (22).

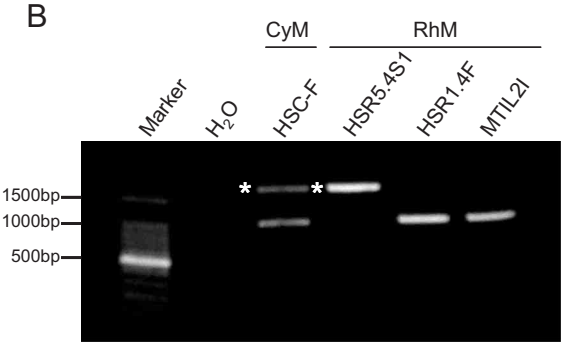
To determine whether each macaque cell line has the TRIMCyp gene (*Mamu7* for rhesus macaques in Fig. 5A), the CypA insertion was monitored by the PCR amplification followed by agarose gel electrophoresis. As shown in Fig. 5B, whereas

A

Polymorphic sites in the exon eight of rhesus macaque TRIM5 allele

Amino acid	307	327	333	339-340	339 341	405 407	414 416	420 422	488 490	
Mamu1	T	P	A	TF	P	G	V	S	M	TRIM5α
Mamu2	T	P	A	TF	P	G	A	S	M	TRIM5α
Mamu3	P	P	A	TF	P	G	V	S	M	TRIM5α
Mamu4	P	P	S	ΔΔ	Q	G	V	L	M	TRIM5α
Mamu5	P	T	S	ΔΔ	Q	G	V	L	M	TRIM5α
Mamu6	P	T	S	ΔΔ	Q	G	V	L	T	TRIM5α
Mamu7	P	P	T	ΔΔ	Q	G	V	S	M	TRIMCyp

B



C

Amino acid	307	327	333	339-340	339 341	405 407	414 416	420 422	488 490	
HSC-F										TRIM5α TRIMCyp
HSR5.4S1	P	P	T	ΔΔ	Q	G	V	S	M	Mamu7
	P	P	T	ΔΔ	Q	G	V	S	M	Mamu7
HSR1.4F	P	P	A	TF	P	G	V	S	M	Mamu3
	P	P	S	ΔΔ	Q	G	V	L	M	Mamu4
MTIL2I	T	P	A	TF	P	G	V	S	M	Mamu1
	P	P	A	TF	P	G	V	S	M	Mamu3

Figure 5 : TRIM5 genotype of macaque cell lines. (A) Reported polymorphism in the exon eight of TRIM5 (14). (Δ) represents the lack of the amino acid indicated. (B) Determination of the CypA insertion. PCR products were run by agarose gel electrophoresis, and visualized by ethidium bromide staining. H_2O (water) is a negative control. (*) indicates the insertion. (C) Genotyping of TRIM5 alleles. Sequences of the exon eight of TRIM5 gene in the cell lines were determined, and classified as shown. No TRIM5 polymorphism has been reported for cynomolgus macaques. (Δ) represents the lack of the amino acid indicated.

cynomolgus HSC-F cells were clearly heterozygous for TRIMCyp and TRIM5α, rhesus HSR5.4S1 cells are homozygous for TRIMCyp. In contrast to theses cells, HSR1.4F and MT-IL2I cells do not encode TRIMCyp. We then sequenced the exon eight of TRIM5 gene in these cells amplified by PCR to determine the allele (Fig. 5C). Because the polymorphism of cynomolgus TRIM5 allele has not been identified and reported yet, sequencing the exon eight in HSC-F cells was done only to confirm heterozygocity of TRIM5α and TRIMCyp shown in Fig. 5B. As expected from the result in Fig. 5B, HSR5.4S1 cells were homozygous for *Mamu7*. Sequence analysis also revealed that HSR1.4F cells are heterozygous for *Mamu3* and *Mamu4*, and that MT-IL2I cells have *Mamu1* and *Mamu3*. In total, the polymorphic genotypes of TRIM5 locus in the four macaque cell lines are as summarized in Fig. 5C.

Growth properties in various macaque cell lines of basic mt HIV-1s

We finally monitored comparatively the viral replication kinetics in HSC-F, HSR5.4S1, HSR1.4F, and MT-IL2I cells. Figure 6 shows the typical kinetics in human and macaque lymphocyte cells of NL4-3 (a standard HIV-1 virus), MA239N (a standard SIVmac virus) and three basic mt HIV-1s (NL-DT5R, NL-DT5R6/7S and NL-DT562). Athough SIVmac grew poorly in human cells (at an undetectable level in H9 [Fig. 6B] but at a distinct level in M8166 cells [2]) relative to HIV-1 (Fig. 6A), it did more rapidly than any other viruses in simian cells such as HSC-F (Fig. 6C) (2). As is clear in Fig. 6B and 6C, while the three mt HIV-1s did not grow in H9 cells, they grew considerably in HSC-F cells. Among the mt HIV-1s, the R5 virus NL-DT562 replicated most

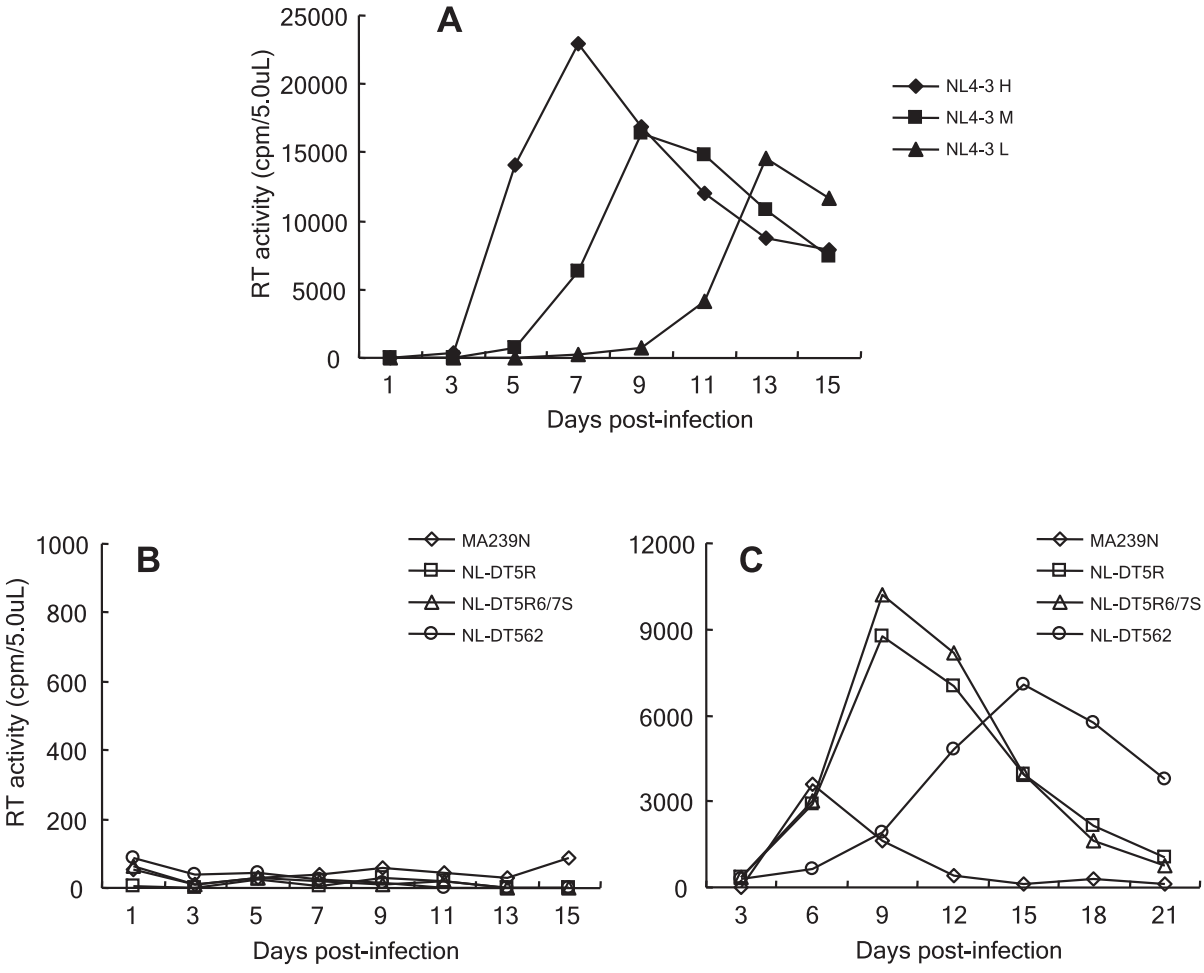


Figure 6 : Growth kinetics of SIVmac and various HIV-1s. Input virus samples were prepared from 293T cells transfected with proviral clones indicated, and infected into human and cynomolgus macaque cells. In (A), human H9 cells (10^6) were infected with 1.1×10^6, 1.1×10^5, or 1.1×10^4 RT units of NL4-3 (NL4-3 H, NL4-3 M, and NL4-3 L, respectively). In (B), H9 cells (10^6) were infected with 2.4×10^6 RT units of MA239N or 6.6×10^6 RT units of the three mt HIV-1s indicated. In (C), cynomolgus macaque HSC-F cells (10^6) were infected with 4.7×10^4 RT units of MA239N or 7.6×10^6 RT units of the three mt HIV-1s indicated. After infection, virus replication was monitored at the indicated intervals by RT activity in the culture medium.

poorly in HSC-F cells. This was probably due to its protein(s) encoded by its unique genome (see the genomic structure in Fig. 1) (5), not to a CCR5 level on the cell surface (Fig. 2). In rhesus macaque cells, noteworthy results in respect to the TRIM5 alleles and virus susceptibility were obtained (Fig. 7). While SIVmac and all the mt HIV-1 grew quite well in HSR5.4S1 cells (*Mamu7*) (Fig. 7A), viruses except for SIVmac did not in HSR1.4F (*Mamu3* and *Mamu4*) and MT-IL2I (*Mamu1* and *Mamu3*) (Fig. 7B and 7C). Moreover, SIVmac replicated better in HSR1.4F cells than in MT-IL2I. These data were perfectly consistent with the reported observations described above (13, 14, 21, 22). We were interested in determining the molecular basis for different replication potentials of mt HIV-1s and SIVmac. Our functional and structural analyses have suggested that viral CA protein, as expected, is the major determinant for the observed viral growth property in these cells (data not shown). Another point worth mentioning here is that NL-DT562 grew comparably with NL-DT5R and better than NL-DT5R6/7S in HSR5.4S1 cells (Fig. 7A), different in HSC-F cells (Fig. 6C). These results might be explained by relative expression levels of CXCR4 and CCR5 on target cells (Figs. 2 and 3).

Conclusions

In this study, we have characterized and analyzed four macaque cell lines with respect to the expression of HIV-1 receptors (Figs. 2-4), genotype of antiretroviral TRIM5 locus (Fig. 5), and susceptibility to SIV/HIV-1 viruses (Figs. 6 and 7). All cynomolgus and rhesus macaque cell lines examined here were found to be positive for the primary and secondary HIV-1 receptors, i.e., CD4, CXCR4, and CCR5. Particularly, a high expression level of CCR5 observed in some cells would be an important property to study R5 viruses that are critical for the disease progression. Furthermore, to be good for virological

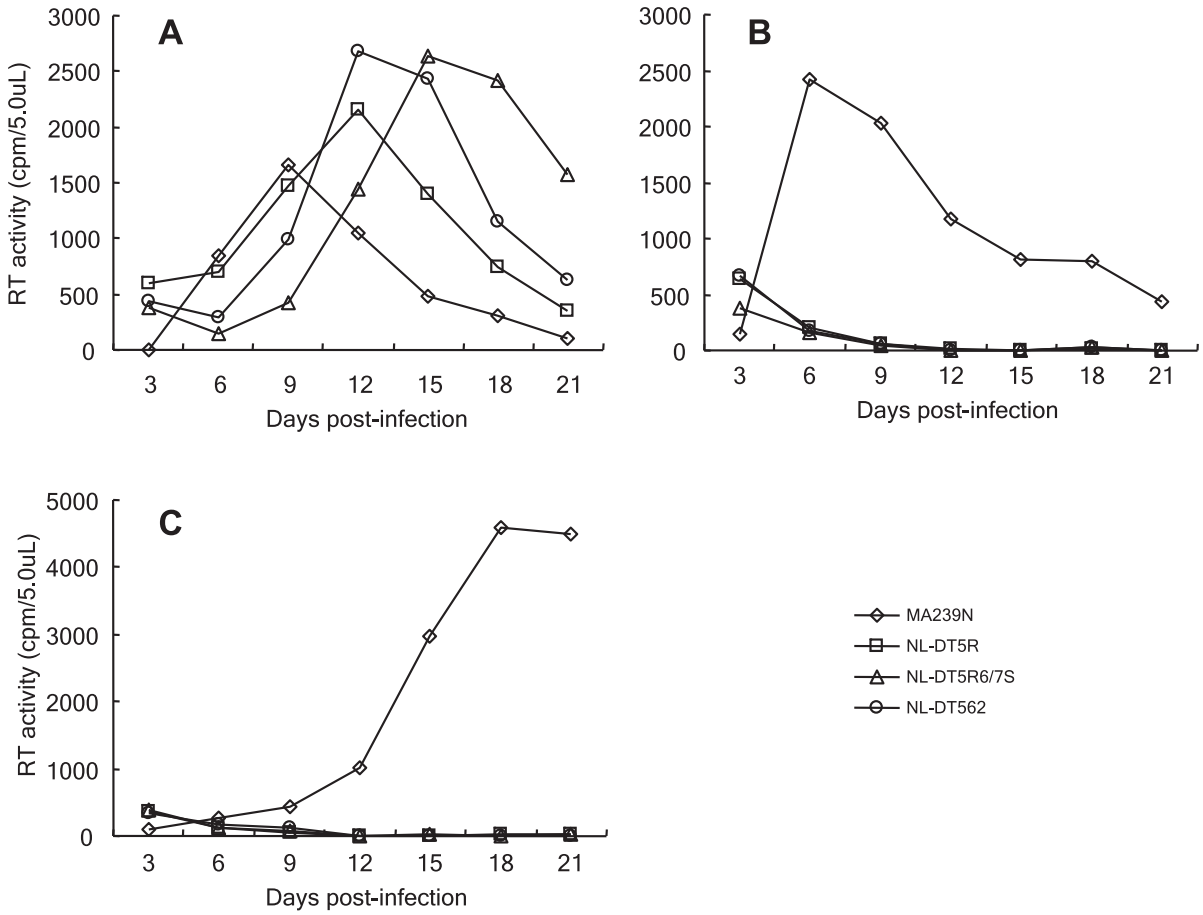


Figure 7 : Growth kinetics of SIVmac and various mt HIV-1s. Input virus samples were prepared from 293T cells transfected with proviral clones indicated, and inoculated into rhesus macaque cells. In (A), HSR5.4S1 cells (3×10^6) were infected with 4.7×10^4 RT units of MA239N or 7.6×10^6 RT units of the three mt HIV-1s indicated. In (B), HSR1.4F cells (3×10^6) were infected with 1.5×10^6 RT units of MA239N or 1.3×10^7 RT units of the three mt HIV-1s indicated. In (C), MT-IL2I cells (10^6) were infected with 1.6×10^6 RT units of MA239N or 1.4×10^7 RT units of the three mt HIV-1s indicated. After infection, virus replication was monitored at the indicated intervals by RT activity in the culture medium.

studies, these cell lines were demonstrated to be unique in their TRIM5 genotype having four distinct allele combinations. In parallel with these results, the cell lines responded uniquely to infection with SIVmac or the three mt HIV-1s (Fig. 1). In conclusion, HSC-F, HSR5.4S1, HSR1.4F and MT-IL2I cells were useful for biological and molecular biological studies on mt HIV-1.

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