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Derivation and characterization of a CD4-independent, non-CD4 tropic simian immunodeficiency virus

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Running Title: CD4-independent, non-CD4 tropic SIVmac239

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ABSTRACT

CD4 tropism is conserved among all primate lentiviruses and likely contributes to viral pathogenesis by targeting cells that are critical for adaptive anti-viral immune responses. Although CD4-independent variants of HIV and SIV have been described that can utilize coreceptors CCR5 or CXCR4 in the absence of CD4, these viruses typically retain their CD4 binding sites and can still interact with CD4. We describe the derivation of a novel CD4-independent variant of pathogenic SIVmac239, termed iMac239 that was used to derive an infectious R5-tropic SIV lacking a CD4 binding site. Of the seven mutations that differentiate iMac239 from wild-type SIVmac239, a single change (D178G) in the V1/V2 region was sufficient to confer CD4-independence in cell-cell fusion assays, although other mutations were required for replication competence. Like other CD4-independent viruses, iMac239 was highly neutralization sensitive, although mutations were identified that could confer CD4-independent infection without increasing its neutralization sensitivity. Strikingly, iMac239 retained the ability to replicate in cell lines and primary cells even when its CD4 binding site had been ablated by deletion of a highly conserved aspartic acid at position 385, that for HIV-1 plays a critical role in CD4 binding. iMac239, with and without the D385 deletion, exhibited an expanded host range in primary rhesus peripheral blood mononuclear cells that included $CCR5^{+}$, $CD8^{+}$ T cells. As the first non-CD4 tropic SIV, iMac239-ΔD385 will afford the opportunity to directly assess the in vivo role of CD4 targeting on pathogenesis and host immune responses.

46 **IMPORTANCE**

47 CD4 tropism is an invariant feature of primate lentiviruses and likely plays a key role in
48 pathogenesis by focusing viral infection onto cells that mediate adaptive immune
49 responses and in protecting virions attached to cells from neutralizing antibodies.
50 Although CD4-independent viruses are well described for HIV and SIV, these viruses
51 characteristically retain their CD4 binding site and can engage CD4 if available. We
52 derived a novel CD4-independent, CCR5-tropic variant of the pathogenic molecular
53 clone SIVmac239, termed iMac239. The genetic determinants of iMac239's CD4-
54 independence provide new insights into mechanisms that underlie this phenotype. This
55 virus remained replication competent even after its CD4 binding site had been ablated
56 by mutagenesis. As the first truly non-CD4 tropic SIV, lacking the capacity to interact
57 with CD4, iMac239 will provide the unique opportunity to evaluate SIV pathogenesis
58 and host immune responses in the absence of the immunomodulatory effects of $CD4^+$ T
59 cell targeting and infection.

INTRODUCTION

The primate lentiviruses, HIV-1, HIV-2 and SIV share a common mechanism of target cell entry by interacting with CD4 and a member of the chemokine receptor family (1–3). CD4 binding to the envelope glycoprotein (Env) trimer initiates a cascade of conformational changes resulting in formation and exposure of the coreceptor binding site on the gp120 subunit of Env. Following coreceptor binding, the gp41 subunit is released to interact with the target cell membrane, leading to formation of a fusion intermediate and, ultimately, the 6-helix bundle, which drives membrane fusion and viral entry (1, 3–9). While CCR5, CXCR4, and, less commonly, other coreceptors can be used by these viruses during entry, CD4 tropism, mediated by a highly conserved binding site on gp120, is an invariant feature (1, 10), indicating that it likely plays a major role in pathogenesis. CD4 binding enables HIV-1 to evade host neutralizing antibody responses by limiting antibody access to neutralizing epitopes once the virion has attached to CD4 on the cell surface (11, 12). In addition, CD4 tropism *in vivo* focuses viral infection onto $CD4^+$ T cell subsets that are critical in mediating adaptive anti-viral immunity (13–16). These cells include Th1, Th17, T follicular helper, and T regulatory cells that collectively contribute to the coordinated induction, maturation and maintenance of cellular and humoral immune responses (17–26) and (for Th17 cells) to the integrity of the epithelial barrier at mucosal surfaces (20, 27, 28).

Although CD4-tropism is conserved, rare examples of CD4-independent viruses have been described that can utilize coreceptors, either CCR5 or CXCR4, for entry in the absence of CD4. These viruses, through mutations in gp120 and/or gp41, pre-form and

83 expose a functional coreceptor binding site that is typically present only after CD4
84 binding occurs (29–40). By cryo-electron microscopy, Env trimers on CD4-independent
85 viruses exhibit more open conformations compared to CD4-dependent viruses and in
86 the absence of CD4 acquire conformations typically seen only after CD4 binding and
87 triggering occur (41, 42). Although CD4-independent viruses have been derived in vitro
88 (29–37, 43), they have only rarely been observed in vivo, as in rhesus macaques during
89 late stage disease or following depletion of $CD4^+$ T cells prior to infection with anti-CD4
90 antibodies (44–46). CD4-independent viruses are typically highly neutralization sensitive,
91 as a result of their more open Env trimer conformations and exposure of neutralization
92 epitopes on cell-free virions that are poorly accessible after binding to the cell surface
93 (12, 32, 39, 40, 47). Thus, CD4-independent viruses are likely strongly selected against
94 in vivo (32, 48, 49). Nonetheless, although not strictly CD4-independent, HIVs and SIVs
95 with the ability to utilize low levels of CD4 for entry are well-described, and this
96 phenotype has been proposed to contribute to infection of macrophages and microglial
97 cells, which exhibit a lower density of CD4 than T cells (32, 43–45, 50–57). For one
98 neuropathic SIV isolate, its ability to cause AIDS encephalopathy in macaques
99 correlated with infection of brain-derived endothelial cells that expressed CCR5 but not
100 CD4 (58). In addition, CD4-independent infection has been proposed to contribute to
101 SIV infection of macrophages in the context of cell-to-cell transmission (59, 60). Of note,
102 viruses that are CD4-independent, typically retain their CD4 binding site and the ability
103 to engage CD4, if present (30, 34, 58, 61, 62), and have been shown to exhibit faster
104 fusion kinetics in the presence of CD4 than CD4-dependent viruses (63).
105

106 For HIV-1, the CD4 binding site has been resolved at the atomic level and shown to be
107 a deeply recessed pocket on gp120 formed by regions within the inner and outer
108 domains that interact cooperatively with CD4 and gp41 during CD4 binding (1, 4, 41, 64,
109 65). Among HIV-1 and SIV isolates some variability exists in these interactions. For
110 SIVmac, the bulky side chain at Trp-375 has been shown to fill a space in the CD4
111 binding pocket reducing its dependency on CD4 binding, while HIV-1, containing a
112 serine at this position, requires additional contributions from a layer on the gp120 inner
113 domain (64). In HIV-1, residues Asp-368, Glu-370, and Trp-427 are highly conserved
114 and make multiple contacts with CD4, particularly amino acids Phe-43 and Arg-59 in its
115 outermost D1 domain. Among these, Arg-59 forms a salt bridge with Asp-368 on gp120,
116 and mutations in gp120 (10, 66) or CD4 (67, 68) that disrupt this bond ablate CD4
117 binding. Although crystallographic resolution of an SIV gp120 in complex with CD4 has
118 not been determined, an aspartic acid at the analogous position (i.e. amino acid 385 for
119 SIVmac) is conserved in all SIVs except for two SIV mandrill *env* clones that contain a
120 glutamic acid residue, suggesting that this aspartic acid is also critical for SIV gp120
121 interactions with CD4 (10) (Suppl. Fig. 1). Indeed, a brain-derived Env clone from a
122 SIVmac239-infected macaque with an asparagine at this position, exhibited a 40-fold
123 reduction in CD4 binding and CD4-independent use of CCR5 in a cell-cell fusion assay
124 (69).
125
126 We describe the in vitro derivation of a novel variant of SIVmac239 that is both CD4-
127 independent and unable to interact with CD4. This variant was adapted to replicate in a
128 CD4-negative clone of SupT1 cells that expressed rhesus CCR5. Four mutations in

gp120 and 2 in the gp41 ectodomain were associated with CD4-independence, and of these, a D178G mutation in the region analogous to the HIV-1 V1/V2 loops was shown to be necessary for this phenotype, while additional mutations were required to enable a spreading infection to occur. Although iMac239 like other CD4-independent viruses was highly neutralization sensitive, mutations in gp120 were identified that conferred CD4-independence in the absence of increased neutralization sensitivity. Notably, when Asp-385 was deleted to disrupt the CD4 binding site, fusion and infectivity of parental SIVmac239 were ablated while iMac239 remained fully replication competent on $CD4^+$, $CCR5^+$, and $CD4^-$, $CCR5^+$ cell lines and primary macaque lymphocytes. In addition, iMac239 with and without the Asp-385 deletion, exhibited an expanded host range in primary macaque peripheral mononuclear cells that included $CCR5^+$, $CD8^+$ T cells. Thus, iMac239 will provide a novel platform for exploring the molecular and structural determinants for CD4-independence and neutralization sensitivity, and has enabled a novel non-CD4 tropic SIV variant to be derived that can be used to directly explore the role of CD4 binding and tropism in pathogenesis and in modulating host immune responses.

METHODS

Cell Lines

Human SupT1 and BC7 cell lines (29) were transfected with a lentiviral vector to express rhesus CCR5 (RhCCR5)(70). SupT1/RhCCR5, BC7/RhCCR5, CEMx174, and HUT-78 cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal

bovine serum (FBS), 2 mM glutamine, and 2 mM penicillin-streptomycin (RPMI-Complete). The Japanese quail fibrosarcoma cell line QT6, the human embryonic kidney cell line 293T, and the human HeLa cell line TZM-bl engineered to express CD4 and CCR5 (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, from John C. Kappes), were cultured in Dulbecco's modified Eagle medium supplemented with 10% FBS, 2 mM glutamine, and 2 mM penicillin-streptomycin (DMEM-Complete).

Env Cloning and Mutagenesis

Adapted *env* clones from SIV-infected BC7/RhCCR5 cultures, were isolated as described previously (71). Mutant *env* genes were created using the QuikChange site-directed mutagenesis kit (Stratagene) following the manufacturer's protocol. To repair a premature stop codon in the cytoplasmic tail of *env* at position 734 this codon was reverted to the wild-type Q by QuikChange. To generate recombinant molecular clones of the SIVmac239 genome containing adapted and mutant *env* genes, *env* clones were cloned into the previously described pHVP-2 construct (also known as p239SpSp3') containing an open *nef* reading frame with a corrected HindIII site at position 602, and which contains the 3' half of the viral genome, following HindIII/SacI digestion (72). Full-length genome constructs were then generated by cloning pVP-2 with p239SpSp5' as previously described (71) (p239SpSp5' was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, from Ronald Desrosiers). The identities of the recombinant clones were confirmed using restriction analysis and DNA sequencing. For the generation of Env pseudotyped viruses, SIV *env* genes with a

premature stop codon in the cytoplasmic tail (CT) coding region (Q734Stop) in pCR2.1 were digested with KpnI and XbaI and cloned into the similarly digested pCDNA3.1(-) expression construct.

Fusion Assays

Env fusion was assessed quantitatively on quail QT6 cells using a cell-cell fusion assay, and expression constructs for CD4 and various coreceptors, and use of a reporter plasmid encoding luciferase under the control of a T7 promoter has been previously described (71, 73, 74). Rhesus CD4 and coreceptors CCR5, CXCR6, APJ, GPR1, GPR15, CCR2 and CCR8 were kindly provided by Drs. Ronald Collman and Robert Doms.

Pseudotype Viruses

Pseudotyped viruses were generated, as previously described (75) by cotransfecting 293T cells with 8 μg of plasmid encoding the SG3 Δenv-based virus backbone and 4 μg of the appropriate *env* expression vector for 3-8hrs with the FuGENE6 transfection agent (Promega). Cell supernatants were collected at 48hrs post-transfection and stored at -80°C.

Virus Production

To generate molecularly cloned viruses, 293T cells were transfected with full-length viral genome constructs for 5hrs using calcium phosphate. Cell supernatants were collected

48 or 72hrs post-transfection and stored at -80°C. The uncloned iMac239 swarm was generated from supernatants of acutely infected BC7RhR5 cells, and stored at -80°C.

Viral Replication Assays

Virus concentrations were determined by enzyme-linked immunosorbent assay for viral p27 Gag antigen (Advanced Bioscience Laboratories). SupT1/RhCCR5 and BC7/RhCCR5 were inoculated with equivalent amounts of p27-containing virus. Following overnight incubation at 37°C, infected cells were washed in RPMI supplemented with 5% FBS to remove excess virus, and then maintained in RPMI Complete media. Viral replication was monitored by viral reverse transcriptase (RT) activity in the culture supernatants (29, 76).

Neutralization Assays

The sensitivity of pseudotyped viruses bearing Envs of interest to neutralization by sera or plasmas from SIVmac251-infected rhesus macaques, soluble CD4, or monoclonal antibodies to SIVmac Envs, including murine antibodies 7D3, 11F2, 17A11, 5B11, 4E11, 171C2, and 36D5 (77), human antibodies 1.4H, 6.10F, 6.10B, 9.1A, and 1.7A, or rhesus antibodies 1.10A, 3.11H (78–81), and 4.10F (unpublished, produced as in (79)) was assessed in a TZM-bl pseudotype assay as previously described (75). Briefly, pseudotyped viruses were incubated for 1hr at 37°C with various dilutions of serum, plasma, sCD4, or monoclonal antibodies and then used to infect TZM-bl cells pretreated with DEAE-dextran. Cells were incubated at 37°C for 48hrs and then lysed with the BriteLite Plus luminescence reporter assay system (Perkin Elmer). A long terminal

repeat (LTR) driven luciferase reporter gene in TZM-bl cells generated the luciferase signal. Infection was quantified by measuring luciferase activity with a Thermo LabSystems Luminoskan Ascent luminometer. Neutralization was measured as the reduction in luciferase activity compared with that of untreated controls.

Infection of PBMCs

Purified peripheral blood mononuclear cells (PBMCs) from rhesus macaques stored at -140°C were thawed and stimulated for 3 days with 5 µg/mL ConA at a concentration of 10^6 cells/mL in RPMI Complete media. Cells (5 x 10^6) were then inoculated with viruses (125 ng of p27 Gag) and media supplemented with IL-2 (100 IU/mL). After 24hrs, cells were washed to remove the viral inoculum and cultured in fresh RPMI Complete media supplemented with IL-2 (100 IU/mL).

Antibody Reagents

Antibodies used for surface staining included anti-CD14 BV650, anti-CD20 BV605, anti-CD8 BV570 (Biolegend), anti-CD16 APC Cy7, anti-CD95 PECy5, anti-CCR5 PE (BD Biosciences), anti-CD4 PECy5.5 (Invitrogen Life Technologies), and anti-CD28 ECD (Beckman Coulter). Antibodies used for intracellular staining included anti-CD3 V450 (BD Biosciences) and anti-p27 FITC (kindly provided by E. Rakasz, WNPRC).

Flow Cytometry Staining Assay

At peaks of viral replication, infected rhesus PBMCs were identified by p27 Gag positivity and flow cytometry. Aliquots of cells (1 x 10^6 per sample) were washed once

with PBS and stained for viability with Aqua amine-reactive dye (Invitrogen) for 10 min in the dark at room temperature. A mixture of surface marker antibodies was added and kept at room temperature for 30 min in the dark. Cells were then washed with PBS containing 1% bovine serum albumin (BSA) and 0.1% sodium azide and permeabilized for 17 min at room temperature using the Cytofix/Cytoperm Kit (BD Biosciences). Immediately following permeabilization, cells were washed in Perm/Wash buffer (BD Biosciences) and a cocktail of antibodies for intracellular markers added and incubated in the dark for 1 hr at room temperature. Cells were then washed with Perm/Wash buffer, fixed with PBS containing 2% paraformaldehyde, and stored at 4°C until flow cytometric analysis. For flow cytometric analysis 3 x 10^5 events were acquired on an LSRII flow cytometer (BD Immunocytometry Systems) modified to detect up to 18 fluorophores. Antibody capture beads (BD Biosciences) were used to prepare compensation tubes for each individual antibody used in the experiment. Data analysis was performed using FlowJo Software version 9.0.1 (TreeStar).

Nucleotide Sequence Accession Number

The iMac239 *env* sequence has been deposited in GenBank under accession number KT959233.

RESULTS

Adaptation of SIVmac239 to CD4-negative BC7/RhR5 cells.

We derived a CD4-independent strain of SIV by serially passaging SIVmac239 on a 1:10 mixture of CD4-positive SupT1 cells and a CD4-negative variant of this line, BC7 (62) each of which stably expressed rhesus CCR5 (designated SupT1/RhR5 and BC7/RhR5, respectively). After 13 passages the virus could infect a pure culture of BC7/RhR5 cells and was then passaged an additional eight times in this cell line. The resulting viral swarm was able to replicate with high efficiency both in SupT1/RhR5 and in BC7/RhR5, while parental SIVmac239 could only replicate in the $CD4^+$ SupT1/RhR5 cells (Figure 1A). Env clones PCR-amplified from genomic DNA of infected BC7/RhR5 cells were derived, and their ability to mediate CD4-independent fusion using rhesus CCR5 evaluated on quail QT6 cells (73) (Fig. 1B). One clone (p8cl18) mediated comparable levels of fusion in the presence or absence of rhesus CD4 and was selected for further characterization. When this Env was inserted into a SIVmac239 backbone, the resulting virus (iMac239 p8cl18 in Fig. 1C) was able to replicate with rapid growth kinetics in both SupT1/RhR5 (data not shown) and BC7/RhR5 cells (Fig. 1C), while parental SIVmac239 was only able to replicate in SupT1/RhR5 (Fig. 1A). CD4-independent replication in BC7/RhR5 cells was retained when the expected premature stop codon acquired during passaging of this SIVmac in human cell lines (82) was corrected (data not shown), and this Env was used to construct a virus containing a full length cytoplasmic tail, designated iMac239, that was employed in all subsequent experiments, except for the production of Env pseudotyped viruses, for which Envs with a short cytoplasmic tail were used.

Mutations required for iMac239 CD4-independence

Sequencing of the iMac239 p18cl8 *env* revealed seven coding changes from SIVmac239, four in gp120 and three in gp41 (Table 1, Suppl. Fig. 2). None of the gp120 mutations occurred in regions analogous to those of HIV-1 gp120 that contribute to the CD4 binding site in HIV-1, indicating that although the iMac239 Env and virus were CD4-independent, they likely retained the ability to interact with CD4. For gp120, three mutations (D178G, D337Y, and R427K) occurred within variable loop regions V1/V2, V3, and V4, respectively, while a single mutation (H224Q) occurred in a region analogous to the HIV-1 C2 region flanking the V1/V2 stem. In gp41, two mutations in the ectodomain (K573T and N673I) were located within regions comparable to HIV-1 heptad-repeat regions-1 and -2 (HR1 and HR2), respectively, while one (L820M) occurred in the cytoplasmic tail.

The contributions of these mutations to CD4-independence were first evaluated in a cell-cell fusion assay (Fig. 2A). In the absence of CD4 the iMac239 Env generated fusion levels on CCR5 that were comparable to or slightly greater than in the presence of CD4, while parental SIVmac239 exhibited <10% fusion. When iMac239 mutations were introduced singly into the SIVmac239 Env, D178G in the V1/V2 loop was sufficient to confer CD4-independent fusion at levels approximately 50% greater than in the presence of CD4, although gp41 mutations K573T and N673I each produced modest increases in fusion to levels 40-50% of fusion in the presence of CD4.

We next evaluated CD4-independence in an infection assay on SupT1/RhR5 and BC7/RhR5 cells using viruses containing Envs with varying combinations of iMac239

mutations (Fig. 2B). When all 4 gp120 mutations were introduced into the SIVmac239 Env (SIVmac239 D178G H224Q D337Y R427K in Fig. 2B), robust CD4-independent replication was observed in BC7/RhR5 cells with kinetics and levels that were comparable to a virus with the full iMac239 Env. However, a virus containing only the gp41 K573T and N673I mutations replicated poorly in both cell types (not shown). Interestingly, although the D178G mutation alone was sufficient to confer CD4-independence in the cell-cell fusion assay, a virus containing only this mutation replicated poorly in BC7/RhR5 cells and was noninfectious on $CD4^+$ SupT1/RhR5 cells (Fig. 2B). Virions from this virus exhibited similar levels of Env compared to both SIVmac239 WT and iMac239 virus, as measured by western blot, indicating that this defect was not the result of a failure of Env incorporation into virions (data not shown). However, when viruses contained the D178G in combination with the gp120 H224Q mutation, replication was restored in both SupT1/RhR5 and BC7/RhR5 cells, although CD4-independent replication occurred more slowly in the latter (Fig. 2B). This apparent rescue of infectivity for virus containing the D178G alone was not seen when the other gp120 mutations were inserted individually (not shown). Significantly, removal of the D178G mutation from the iMac239 Env with all four gp120 changes resulted in a virus (SIVmac239 H224Q D337Y R427K) that was replication competent on SupT1/RhR5 cells but no longer CD4-independent and unable to infect BC7/RhR5 cells (Fig. 2B). Thus, among the gp120 mutations that conferred CD4 independence to SIVmac239, while D178G in V1/V2 was critical, this mutation alone resulted in a virus that was noninfectious in both CD4-positive and -negative cell types but could be rescued by the H224Q change in gp120.

334

335 **Neutralization sensitivity of iMac239**

336 For HIV-1 and SIV, CD4-independent Envs are typically highly neutralization sensitive,
337 owing to their more open conformation of the Env trimer on virions (41, 42) and
338 formation of highly immunogenic epitopes that are typically induced only in the presence
339 of CD4 (11, 32, 39, 40). Given the well-described neutralization resistance of the
340 SIVmac239 Env, we were interested in determining the neutralization sensitivity of the
341 iMac239 Env to a panel of sera and plasmas from SIVmac-infected rhesus macaques
342 and to a panel of anti-SIVmac gp120 murine monoclonal antibodies, previously shown
343 to potently neutralize lab-adapted SIVmac251 but not SIVmac239 (77). Neutralization
344 assays were performed on TZM-bl cells using viral particles pseudotyped with Envs. In
345 addition to iMac239, we also evaluated SIVmac239 Envs containing the four gp120
346 changes that were sufficient to confer CD4 independence (Fig. 2B) in viral replication
347 assays, and an Env containing only the D178G, which was CD4-independent in the cell-
348 cell fusion assay (Fig. 2A).

349

350 As expected, whereas SIVmac239 was resistant to neutralization by anti-SIV sera or
351 plasma, with reciprocal 50% inhibitory dilutions (ID_{50}) ≤270, iMac239 was highly
352 sensitive with ID_{50} values >2 million for plasma and 8,793 for sera (Fig. 3A). We sought
353 to determine if iMac239's neutralization sensitivity could be mapped to particular
354 epitopes and thus utilized a panel of monoclonal antibodies to SIVmac variable loops
355 (V2, V3 and V4), and to the CD4 and CCR5 binding sites (77–81) in TZM-bl cell
356 neutralization assays. As with the plasma and serum samples, SIVmac239 was highly

resistant to all of the antibodies in the panel, while iMac239 was highly sensitive to 7 of 13 antibodies and resistant only to an anti-CD4 binding site (5B11), anti-CD4/CCR5 binding site (17A11), an anti-V2 (171C2), and an anti-V4 loop (1.7A) antibody (Fig. 3A). Surprisingly, iMac239 Envs containing the minimum number of mutations in gp120 that conferred CD4 independence in either cell-cell fusion or viral infection assays (Fig. 2) remained neutralization resistant at levels comparable to parental SIVmac239. These findings indicate that although typically associated, CD4-independence and increased neutralization sensitivity can be dissociated. Moreover, these findings also suggest that changes in the iMac239 gp41 that were selected for in vitro and not present in the SIVmac239 D178G H224Q D337Y R427K Env used in this assay were key determinants for its marked neutralization sensitivity.

CD4-independence of iMac239 is retained following ablation of the CD4 binding site

Although CD4-independent Envs are structurally altered and expose or form neutralization epitopes (32, 39–42, 47), as noted above, these Envs retain the ability to bind and use CD4. In order to determine if the iMac239 Env would remain competent for fusion and infection even after its CD4 binding site had been ablated, we introduced a 3 nucleotide deletion removing a codon for an aspartic acid at amino acid position 385 that is highly conserved throughout HIV and SIV phylogeny (10) (Suppl. Fig. 1). For HIV-1, the analogous aspartic acid at position 368 (HXB numbering) forms a salt bridge with arginine 59 of CD4 (1, 2), and a D368R mutation in HIV-1 gp120 ablates CD4 binding and most CD4 binding site antibody epitopes (10, 66).

380

381 The effects of the D385 deletion (ΔD385) on SIVmac239 and iMac239 Envs were
382 assessed in cell-cell fusion assays and on viral replication on CD4-positive and CD4-
383 negative cell lines. Remarkably, whereas the ΔD385 largely ablated fusion of
384 SIVmac239 Env on target cells bearing CD4 and CCR5 to levels <10% of wildtype,
385 iMac239 fusion was unaffected and was actually enhanced in the presence of this
386 mutation (Fig. 4A). When viral replication was assessed in SupT1/RhR5 and BC7/RhR5
387 cells, SIVmac239 containing the ΔD385 mutation was unable to replicate in either cell
388 type, whereas iMac239 with or without the ΔD385 mutation replicated in both cell types
389 with similar kinetics (Figs. 4B Left and Right panels). We confirmed that viruses used in
390 these infection assays expressed comparable amounts of gp120 relative to p27 Gag
391 (not shown).

392

393 The sensitivity of viral pseudotypes bearing these Envs to neutralization by soluble CD4
394 (sCD4) was also assessed as an indicator of CD4 binding to Env trimers on virions.
395 Infectivity of pseudotypes containing SIVmac239, SIVmac251.6 (a lab-adapted SIVmac),
396 iMac239, or iMac239-ΔD385 Envs was evaluated on TZM-bl cells in the presence of
397 varying concentrations of sCD4. While sCD4 sensitivity was observed for SIVmac239
398 (IC_{50}, 7.8 μg/ml) and markedly enhanced for SIVmac251.6 (IC_{50}, 0.1 μg/ml) and
399 iMac239 (IC_{50} <0.01 μg/ml), iMac239 containing the ΔD385 mutation was highly
400 resistant (IC_{50}, >20 μg/ml) (Fig. 4C). Collectively these findings indicate that although
401 the iMac239 Env retained a CD4 binding site and was highly sensitive to sCD4
402 neutralization, this Env could mediate entry while lacking a CD4 binding site.

Identifying neutralization epitopes on iMac239 and iMac239-ΔD385.

Given the exquisite sensitivity of iMac239 Env to sera from SIVmac-infected macaques and monoclonal antibodies to gp120 epitopes (Fig. 3A) and the ability of iMac239 virus to replicate without a functional CD4 binding site (Fig. 4B), we sought to determine if conformational changes associated with its altered antigenicity were affected by the loss of the CD4 binding site. Neutralization of viral particles pseudotyped with Envs from iMac239 or iMac239-ΔD385 were assessed on TZM-bl cells using the same panel of monoclonal antibodies shown in Figure 3A**.** As seen previously (Fig. 3A), The iMac239-ΔD385 Env was also highly neutralization sensitive at levels that were comparable to or greater than iMac239. Thus, relative to SIVmac239, CD4-independent iMac239 was globally neutralization sensitive to multiple antibodies, and this sensitivity was further enhanced by the ΔD385 mutation. These findings also indicate that while the ΔD385 mutation largely ablated CD4 binding function, it did not disrupt the antigenicity of the CD4 binding site, as determined by the antibodies in our panel.

Replication of iMac239 and iMac239-ΔD385 in primary rhesus macaque PBMC.

Given the ability of CD4-independent iMac239 to replicate in T cell lines with or without a CD4 binding site, we assessed the infectivity of iMac239 and iMac239-ΔD385 on ConA/IL-2 stimulated primary rhesus PBMCs. Similar to T cell lines, parental SIVmac239 containing the ΔD385 mutation was completely noninfectious. However, both iMac239 and iMac239-ΔD385 replicated to levels identical to SIVmac239. Peak intracellular viral p27 Gag expression occurred at different days post inoculation with

SIVmac239 infection peaking at 4 dpi and iMac239 and iMac239-ΔD385 peaking at 10 dpi (Fig. 5A). These data indicate that, while CD4 binding could facilitate iMac239 replication, a CD4 binding site was not required for this virus to infect primary cells.

Next, given the potential for CD4-independent iMac239 and iMac239-ΔD385 to have an expanded cellular tropism, we assessed their infectivity for different cell types in the stimulated rhesus macaque PBMC population using flow cytometry with a panel of antibodies to T, B and monocyte subsets and to CCR5. Gating strategies are shown on uninfected cells (Suppl. Fig. 3). Among $CD3^+$ T cells assayed at the viral peak, iMac239 infection produced a significant increase in p27 Gag-positive cells compared to both SIVmac239 and iMac239-ΔD385 (i.e., 25.4% vs. 5.56% and 6.46%, respectively, in the representative experiment shown in Fig. 5B). Among p27 Gag^+, $CD3^+$ T cells, the vast majority (>90%) of SIVmac239-infected cells were negative for CD4 and CD8, most likely reflecting $CD4^+$ T cells from which CD4 was downregulated by the effects of Nef and Env expression (83–86), and only rare cells (<1%) expressed CD8. In marked contrast, for iMac239 and iMac239-ΔD385 infections, on average 60% and 40% of p27 Gag^+ cells, respectively, expressed CD8 (Fig. 5C). Infection of monocytes ($CD16^+$, $CD14^+$) or B cells ($CD20^+$) was not observed (data not shown), although we note that culture conditions did not support expansion of these cell types. Further analysis will be required to determine if any additional CD4-negative cell types in rhesus PBMC populations are infected by iMac239 and iMac239-ΔD385. Collectively, these data indicate that both CD4-independent iMac239 and iMac239-ΔD385 have expanded tropism on primary cells, which includes $CD8^+$ T cells.

No use of alternative coreceptors by iMac239 and iMac239-ΔD385.

SIVmac239 and other SIVs have been shown to use coreceptors in addition to CCR5, including CXCR6, APJ, GPR1, GPR15, CCR2 and CCR8 (87–91). To determine if CD4-independent use of CCR5 by iMac239 and iMac239-ΔD385 affected CD4-dependent or -independent use of alternative coreceptors, a cell-cell fusion assay was used to assess fusion on target cells expressing rhesus CXCR6, APJ, GPR1, GPR15, CCR2 and CCR8 with or without rhesus CD4. In the presence of CD4, SIVmac239, iMac239, and iMac239-ΔD385 exhibited some capacity to use CXCR6, GPR1 and GPR15, although levels of fusion were less than for CCR5 (Fig. 6A Left panel). However, in the absence of CD4, only iMac239 and iMac-ΔD385 exhibited CD4-independent fusion, and only on CCR5 (Fig. 6A Right panel). We also assessed infection of two $CD4^+$, CCR5-negative cell lines, CEMx174 and HUT-78, previously shown to be permissive for SIVmac infection, most likely through their expression of GPR15 (91–96). In contrast to SIVmac239, both iMac239 and iMac239-ΔD385 were unable to replicate in these cell lines (Fig. 6B). Thus, while adapted for CD4-independent use of rhesus CCR5, these findings suggest that iMac239 and iMac239-ΔD385, are strictly CCR5 tropic and unable to use alternative coreceptors for infection in the presence or absence of CD4.

DISCUSSION

CD4 tropism is conserved among all primate lentiviruses and has been proposed to play a key role in protecting viruses from neutralizing antibodies that are sterically restricted

from accessing the Env trimer once virions have bound to the cell surface (11, 12). However, by focusing infection onto cells that are critical to host adaptive immune responses, CD4 tropism likely also exerts potent immunomodulatory effects that contribute to disease progression and/or viral persistence. Although CD4-independent viruses have been observed in vivo, particularly in nonhuman primate models of pathogenic SIV infection, they typically appear in the setting of highly immunocompromised hosts with advanced neurological or pulmonary complications (44, 58, 69) at sites where non-lymphoid cells (primarily macrophages) with little or no CD4 are infected. In rhesus macaques depleted of CD4 T cells with anti-CD4 antibodies prior to SIVmac infection, CD4-independent viruses rapidly appeared, in association with SIV encephalitis and macrophage infection (46, 53), indicating that this phenotype can readily emerge in vivo. Because CD4-independent viruses are characteristically neutralization sensitive, it is likely that they are strongly selected against during typical pathogenic infection (32, 48, 49, 97). Recent publications by Yen, et al. have suggested that CD4-independent entry may serve as a mechanism of cell-cell viral spread in tissue macrophages, which is more efficient and can shield virus from neutralizing antibodies compared to cell-free transmission, thereby allowing CD4-independent variants to circulate in compartments such as the brain (59, 60). Interestingly, primary isolates of HIV-2, which is less pathogenic than HIV-1 (reviewed in (98)), have been reported to exhibit CD4-independence in vitro (61, 99). However, for HIV-1 (and as we show for SIVmac239) extensive passaging is required to derive CD4-independent viruses in vitro, indicating that for these viruses there are likely to be additional barriers to their emergence (29, 30, 34, 36, 37, 39, 47). Of note, CD4-independent Envs typically retain

their CD4 binding site, and their infectivity is generally enhanced in the presence of CD4 (30, 32, 46, 61). Thus, while CD4-independent viruses arising in vivo or in vitro provide potentially useful tools to understand conformational changes associated with coreceptor engagement and viral entry (34, 35, 39, 40, 63), their applicability to questions of what role CD4 interactions play in pathogenesis and on host immune responses has been limited.

In this report we describe the derivation and characterization of a CD4-independent and truly non-CD4 tropic variant of SIVmac239 that lacks the ability to interact with CD4. A CD4-independent virus, iMac239, was first derived in vitro and shown to be highly competent in mediating fusion and infection of cells bearing rhesus CCR5 in the absence of CD4. Unlike SIVmac316, a macrophage tropic variant of SIVmac239 that was CD4-independent in cell-cell fusion assays (32, 44), the determinants for iMac239's altered tropism resided solely within gp120, and this virus did not require a truncated cytoplasmic tail to exhibit this phenotype. Notably, after deletion of the codon for a highly conserved aspartic acid in the CD4 binding loop on gp120, shown for HIV-1 to be critical for CD4 binding, iMac239 remained fully infectious on CD4-negative cell lines expressing rhesus CCR5 and on primary peripheral blood lymphocytes. This mutation in parental SIVmac239 completely ablated its function in cell-cell fusion and infection assays. Moreover, whereas SIVmac239 and especially iMac239 were sensitive to neutralization by soluble CD4, iMac239 containing the ΔD385 deletion was completely resistant, consistent with the view that CD4 binding for this virus was ablated or at least markedly reduced (Fig. 4).

518

519 Among the seven mutations in the iMac239 Env, four changes in gp120 were sufficient
520 to confer CD4-independent infection of CCR5-expressing cells. A D178G in the
521 iMac239 V1/V2 loop was critical in that this change alone conferred CD4-independent
522 fusion to the SIVmac239 Env, and correction of this change alone, ablated CD4-
523 independent infection by a virus bearing the minimum set of gp120 mutations required
524 for CD4-independence. For HIV-1 and SIV, changes in V1/V2 are frequently associated
525 with CD4-independence (30, 33–35, 43, 100–102) and/or an enhanced ability to infect
526 cells that express low levels of CD4 (60, 99). Structural studies of HIV-1 soluble SOSIP
527 timers (7, 9, 103–105) and cryo-electron microscopic analyses of virion-associated
528 trimers have shown, in the absence of CD4, V1/V2 loops to be oriented towards the
529 apex of the trimer, in contrast to their more lateral positioning upon CD4 activation (8,
530 41). Given that soluble gp120 even in the absence of CD4 is thermodynamically favored
531 to assume a CD4 bound conformation as an apparent default structure (106) but that its
532 conformation is likely restrained by the V1/V2 and V3 variable loops (104–106),
533 changes in V1/V2 that perturb its quaternary interactions within or between adjacent
534 protomers could favor the spontaneous opening of the trimer to a CD4 bound
535 conformation and promote CD4-independent function. Interestingly, although D178G
536 alone enabled the SIVmac239 Env to fuse independently of CD4 and a viral pseudotype
537 bearing this Env could permit infection in a single round entry assay, SIVmac239 virus
538 containing only this change was not replication competent on either CD4-positive or -
539 negative cells. However, this Env could be rescued by iMac239's H224Q mutation distal
540 from the V1/V2 stem. Because the Env trimer has been modeled as a metastable

structure with the potential to assume conformations that are either favorable or non-permissive for fusion (107, 108), we interpret these results to indicate that D178G, while necessary for CD4-independent fusion and entry, requires the H224Q to guide conformational changes and/or promote Env stability during fusion, and to prevent fusion incompetent conformations from being formed, as described for HIV-1 Envs after cold treatment (107) and/or small molecule CD4 binding site agonists (108–110).

As noted, it is likely that the enhanced neutralization sensitivity of CD4-independent viruses results from a more open structure as their Env trimers assume conformations that typically only occur in the presence of CD4, exposing epitopes that are shielded on resting virions (41). In addition, CD4-induced epitopes that contribute to the coreceptor binding site and are highly immunogenic (11) are poorly formed in the absence of CD4 binding and inaccessible to antibodies on cell-bound virions, but are able to be targeted on CD4-independent viruses on which they are formed and exposed (39) or sampled more frequently in the absence of CD4 (111). As we demonstrated, iMac239 as well as its non-CD4 binding derivative, iMac239-ΔD385, were globally neutralization sensitive to sera from SIVmac-infected animals and to monoclonal antibodies to CD4-induced and non-induced epitopes (Fig. 3). However, an Env containing only the iMac239 gp120 changes, while CD4-independent, remained highly neutralization resistant, similar to parental SIVmac239. Similar findings were reported by Yen and coworkers, in which the loss of a glycosylation site in the SIVmac239 V2 loop (Asn-173) conferred the ability to infect macrophages in the context of cell-to-cell transmission while retaining the neutralization resistant phenotype of SIVmac239 (59, 60). In addition to indicating that

564 CD4-independence and enhanced neutralization sensitivity can be dissociated, our
565 findings also suggest that changes in the gp41 ectodomain that arose with iMac239's
566 CD4-independence contribute to its neutralization sensitivity. The effect of gp41
567 mutations on gp120 neutralization sensitivity has been more extensively characterized
568 in HIV-1 (112–115). Our finding is also consistent with the model of "intrinsic reactivity"
569 of the Env trimer proposed by Haim, et al. (107), in which changes in gp41 enhanced
570 the spontaneous formation/exposure of the HR1 coiled coil, decreasing the threshold for
571 Env to transition upon activation from a high to a lower-energy state.
572
573 As described, CD4-independent iMac239 virus, following deletion of Asp-385, remained
574 fully infectious on CCR5-expressing cell lines and on primary lymphocytes. Although the
575 structure of the SIVmac gp120 in complex with CD4 has not been resolved at the
576 crystallographic level, for HIV-1 this residue forms a covalent bond with Arg-59 on
577 human CD4 (corresponding to Lys-59 on rhesus CD4), and is highly conserved across
578 nearly all HIV and SIV isolates ((10) and see Suppl. Fig. 1). While we cannot rule out
579 the possibility that iMac239 containing this mutation maintained some low level
580 interactions with CD4, the finding that it became completely resistant to soluble CD4
581 while iMac239 was exquisitely sensitive, strongly supports the view that CD4 binding
582 was markedly impaired (Fig. 4). We chose to introduce a deletion rather than a point
583 mutation at this position to create a CD4-binding site mutant that would be less likely to
584 revert in vivo in macaques. In vitro, when iMac239-ΔD385 was serially passaged up to
585 20 times in $CD4^+$ SupT1/RhR5 cells, this mutation remained stable (not shown),

indicating that loss of CD4 binding function, at least in cell lines, did not confer a major fitness cost during long-term propagation in vitro.

In rhesus PBMCs cultured with T cell mitogens, SIVmac has been shown to infect CD4 effector and central memory T cells, consistent with expression of CCR5 on these cells and SIVmac's highly efficient use of this coreceptor for entry (49, 116, 117). Although alternative coreceptors can be used by SIVs in vivo (87, 118), it is likely that levels of CCR5 expression are a key determinant of tropism and pathogenicity. Consistent with this hypothesis, sooty mangabeys, a natural host for nonpathogenic SIVsm infection, exhibit low CCR5 expression on central memory CD4 T cells, likely accounting for sparing of this subset in the context of SIVsmm infection (117, 119). Among peripheral blood cells stimulated with T cell mitogens, iMac239 with and without the ΔD385 deletion, exhibited an expanded host range that included $CD8^+$ T cells, most likely through their expression of CCR5 (Fig. 5). We observed that 30 - 65% of $CD8^+$ T cells in these cultures expressed CCR5 (not shown), which was associated with infection of approximately 20% and 4% of $CD3^+$, $CD8^+$ T cells by iMac239 and non-CD4 tropic iMac239-ΔD385, respectively, in contrast to <0.15% for SIVmac239. Adaptation of SIVmac239 for CD4-independent use of CCR5, led to a reduced capacity to utilize alternative coreceptors (Fig. 6), suggesting that its expanded tropism in vitro was largely driven by CCR5 expression. Collectively, these findings clearly show that the tropism of SIVmac239 on primary cells can be altered and redirected from its exclusive infection of $CD4^+$ target cells. Whether these viruses can infect additional cell types such as NK, B cells or monocytes remains to be determined.

609

610 The ability to remove CD4 tropism from SIVmac creates new opportunities to assess
611 the role of CD4 in pathogenesis. Non-human primate models of AIDS have clearly
612 shown that during early SIV infection, $CD4^+$ T cells that express CCR5 and reside in
613 mucosal tissues are selectively and rapidly depleted (116, 120, 121), associated with a
614 disruption in the epithelial barrier that contributes to microbial translocation and
615 systemic immune activation (20, 27, 28, 122, 123). In addition, by focusing infection
616 onto T cell subsets that provide help for adaptive immune responses, including Th1,
617 Th17, and Tfh cells, it is likely that CD4 tropism has profound effects on antiviral
618 immune responses, which are ultimately inadequate to contain viral replication and
619 disease progression. Binding of gp120 to CD4 also has the potential to disrupt CD4's
620 physiologic interaction with HLA class-II on antigen presenting cells, which underlies T-
621 cell immunologic helper functions. Although iMac239-ΔD385 exhibited expanded cell
622 tropism in vitro, its inability to selectively target $CD4^+$ T cell subsets raises the possibility
623 that T cell help for cytotoxic CD8 and CD4 cellular responses will be qualitatively or
624 quantitatively altered and that B cell maturation and memory responses, which are
625 dependent on interactions with Tfh cells, may lead to improved antibody responses.
626 Future studies that assess the quality of anti-SIV responses in the context of a CD4
627 sparing infection will provide new insights into pathogenesis and possibly inform
628 interventions that can be directed to improve host immune responses to infection and
629 vaccines.

630

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656

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Figure Legends

Figure 1: Replication and fusion of CD4-independent variants of SIVmac239. (A) Replication of parental SIVmac239 and an uncloned CD4-independent viral swarm is shown in $CD4^+$ SupT1/RhR5 cells (Left panel) and $CD4^-$ BC7/RhR5 cells (Right panel) each of which stably expressed rhesus CCR5. RT, (reverse transcriptase activity). **(B)** Fusion activity of SIVmac239 and four iMac239 *env* clones is shown on QT6 cells using a cell-cell fusion assay. For each *env*, the level of CD4-independent fusion on rhesus CCR5 is shown as a percentage of fusion (luciferase activity) in the presence of rhesus CD4. Background fusion levels on cells expressing only GFP were subtracted. The data shown are the means of three experiments plus the standard errors of the means (S.E.M.). **(C)** Growth curves in CD4-negative; BC7/RhR5 cells are shown for wildtype (WT) SIVmac239, the iMac239 viral swarm, and four recombinant SIVmac239-based viruses bearing the indicated iMac239 *env* clones. RT activity in culture supernatants was measured at the indicated time points. Results from a representative experiment are shown.

Figure 2: Determinants for iMac239 Env CD4-independence in cell-cell fusion and viral replication assays. (A) Fusion activity on rhesus CCR5 in the presence or absence of rhesus CD4 is shown for SIVmac239 Envs containing the indicated single mutations from iMac239. Data from 3 experiments + S.E.M are shown as in Fig. 1B. **(B)** Replication of SIVmac239-based viruses bearing the indicated Envs is shown in $CD4^+$ SupT1/RhR5 (Left panel) and $CD4^-$ BC7/RhR5 cells (Right panel). Four changes in gp120 are sufficient to confer CD4-independent replication. A virus with D178G alone

1135 was unable to replicate in either cell type, but rescued for replication in SupT1 by
1136 H224Q. RT activity was measured at the indicated time points. Results from a
1137 representative experiment are shown.
1138
1139 **Figure 3: Neutralization sensitivity of Envs with iMac239 mutations.** Viral
1140 pseudotypes containing the indicated Envs were preincubated with varying dilutions of
1141 plasma, serum, or monoclonal antibodies prior to infection of TZM-bl cells. Reciprocal
1142 50% inhibitory dilutions (ID_{50}) for plasma and serum are color-coded (<100, green; 100-
1143 1,000, yellow; 1,000-100,000, orange; >100,000 red). 50% inhibitory concentrations of
1144 monoclonal antibodies (IC_{50}) are color-coded (>2 ug/mL, green; 0.2-2 ug/mL, yellow;
1145 <0.01-0.2 ug/mL, red). **(A)** Neutralization of SIVmac239 and iMac239 are shown relative
1146 to two viruses (SIVmac239 D178G and SIVmac239 D179G, H224Q, D337Y, R427K)
1147 each containing indicated mutations in gp120. **(B)** Neutralization of iMac239 and
1148 iMac239-ΔD385, containing a deletion of Asp-385 within the CD4 binding site. Gray
1149 shaded areas indicate assays that were not tested.
1150
1151 **Figure 4**: **Effect of the ΔD385 mutation in cell-cell fusion, viral replication, and**
1152 **neutralization assays. (A)** Fusion activities of SIVmac239, SIVmac239-ΔD385,
1153 iMac239, and iMac239-ΔD385 on rhesus CCR5 only are shown for each Env as the
1154 percentage of fusion in the presence of rhesus CD4. Background was subtracted as in
1155 Fig. 1B. The data shown are the means of four experiments + S.E.M. **(B)** Replication of
1156 SIVmac239, SIVmac239-ΔD385, iMac239, and iMac239-ΔD385 viruses in $CD4^+$
1157 SupT1/RhR5 cells (Left panel) and $CD4^-$ BC7/RhR5 cells (Right panel). RT activity in

culture supernatants was measured at the indicated time points. Results from a representative experiment are shown. **(C)** Soluble CD4 (sCD4) neutralization of viral pseudotypes containing SIVmac239, SIVmac251.6, iMac239, and iMac239-ΔD385 Envs is shown on TZM-bl cells. Percent neutralization was calculated using luciferase activity normalized to infection in the absence of sCD4. Results from a representative experiment are shown.

Figure 5: **Replication of SIVmac239 and iMac239 with and without the ΔD385 mutation in rhesus PBMCs.** Replication of SIVmac239, SIVmac239-ΔD385, iMac239, and iMac239-ΔD385 in ConA/IL-2 stimulated rhesus PBMCs is shown. p27 Gag in culture supernatants was quantified by ELISA at the indicated time points. Results from a representative experiment are shown **(A)**. **(B)** For each virus, the percentage of total $CD3^+$ T cells that are positive for p27 Gag are indicated (Left panel). Flow cytometry cytograms from a representative experiment show the percentage of p27 Gag^+, $CD3^+$ T cells (Right panel). **(C)** The percentage of p27 Gag^+, $CD3^+$ T cells that express CD4 and/or CD8 at peak infection is shown (Left panel). Cytograms from a representative experiment show that for iMac239 and iMac239-ΔD385, a marked increase in p27 Gag is detectable in $CD8^+$ T cells (Right panel).

Figure 6: **Use of alternative coreceptors by SIVmac239, iMac239, and iMac239-ΔD385. (A)** Fusion activity of the indicated Envs on rhesus coreceptors in the presence (Left panel) or absence (Right panel) of rhesus CD4 was assessed in a cell-cell fusion assay. In each panel luciferase activity was normalized to values for rhesus CCR5.

1181 Background fusion levels were subtracted prior to normalization. Data shown are the
1182 means of three experiments + S.E.M. **(B)** Replication of SIVmac239, iMac239, and
1183 iMac239-ΔD385 viruses in $CD4^+$, $CCR5^-$ CEMx174 (Left panel) and HUT-78 (Right
1184 panel) cells. RT activity in culture supernatants was measured at the indicated time
1185 points. Results from a representative experiment are shown.

1186 **Table 1**: Amino acid differences in envelope glycoproteins between SIVmac239 and

1187 CD4-independent iMac239 (see Supplemental Figure 2).

1188

Region of Env	gp120				gp41		
	V1/V2	C2	V3	V4	HR1	HR2	CT
A.A. Position	*178*	*224*	*337*	*427*	*573*	*673*	*820*
SIVmac239	D	H	D	R	K	N	L
iMac239	G	Q	Y	K	T	I	M

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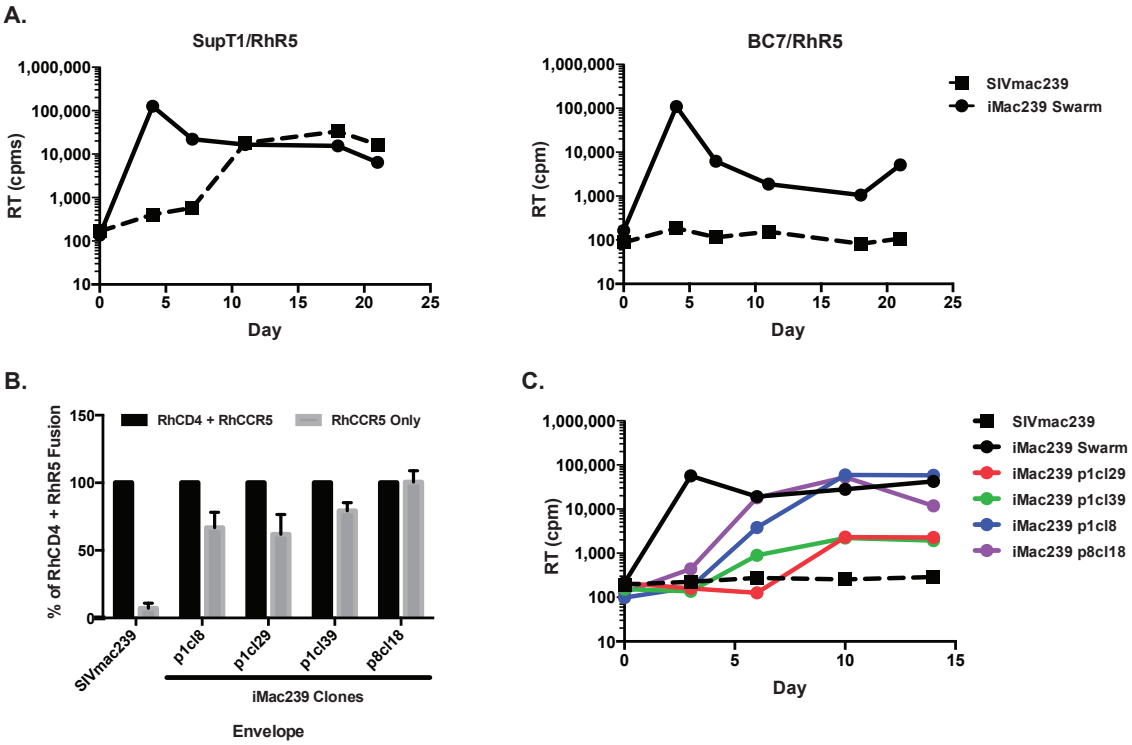

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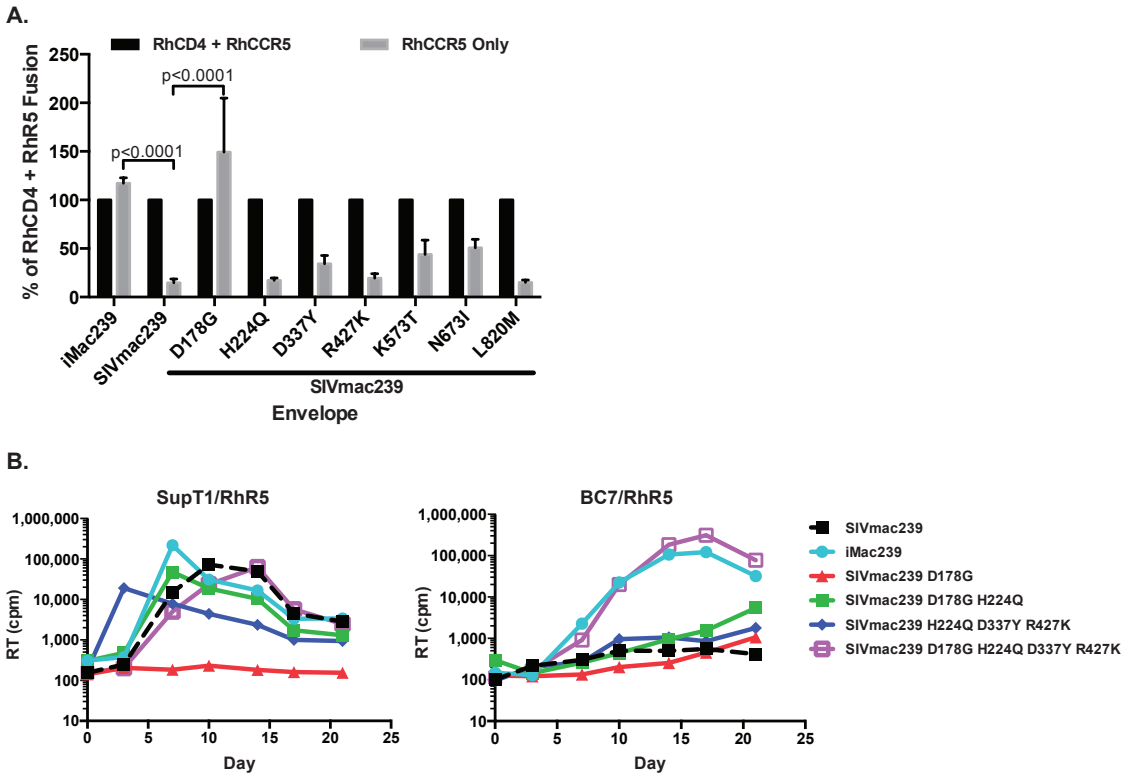

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A.

			SIVmac239	iMac239	SIVmac239 D178G	SIVmac239 D178G H224Q D337Y R427K
Plasma/Serum		23696 Pool A	270	>2,343,750	358	442
		23166 Pool A	<30	>2,343,750	46	47
		24724 Pool B	<30	>2,343,750	58	69
		P309 Serum	<30	8,703	<30	<30
mAb	CD4 Binding Site	6.10B	>34.5	0.09	>34.5	>34.5
	CD4 Binding Site	5B11	>25	3.72	>25	>25
	CD4/CCR5 Binding Site	17A11	>25	18.1	>25	>25
	CD4/CCR5 Binding Site	4E11	>25	0.44	24	>25
	CCR5 Binding Site	7D3	>25	<0.01	13.04	8.36
	V2 Loop	171C2	>25	>25	>25	>25
	V3 Loop	6.10F	>17.5	<0.01	>17.5	10.28
	V3 Loop	36D5	>9.5	0.1	>9.5	>9.5
	V3 Loop	3.11H	>25	<0.01	>25	19.96
	V4 Loop	1.7A	>18.5	7.36	17.79	>18.5
	V4 Loop	4.10F	>20.5	1.53	19.18	>20.50
	V4 Loop	1.10A	>25	<0.01	15.2	4.84
	V4 Loop	9.1A	>22.5	<0.01	>22.5	>22.5

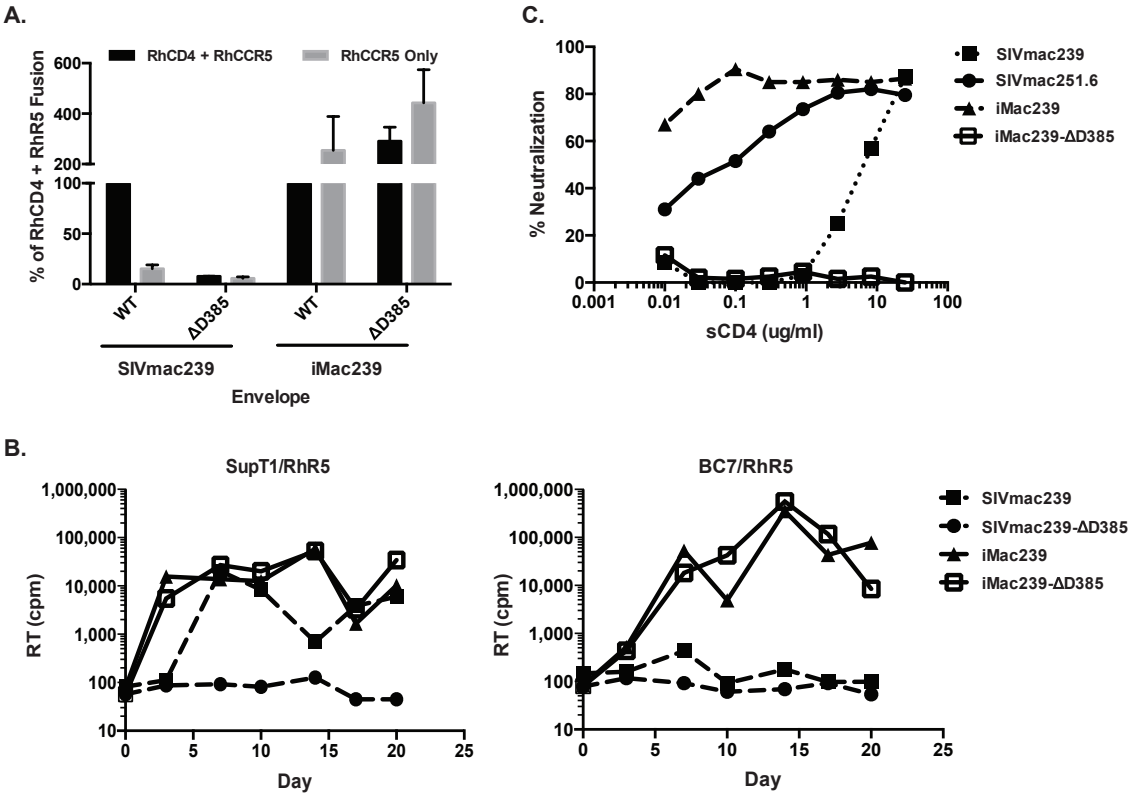
B.

iMac239	iMac239-ΔD385
0.1	0.08
0.35	0.08
6.61	0.29
0.12	0.02
0.0004	0.001
22	0.8
0.002	0.005
0.013	0.02
0.01	0.02
20.85	5.84
2	0.09
0.0003	0.001
0.001	0.001

Plasma Dilutions (ID_{50})	<100	100 - 1,000	1,000 - 100,000	>100,000

IC_{50} Values (ug/mL)	>2	0.2 - 2	<0.01 - 0.2

Figure 3: Neutralization sensitivity of Envs with iMac239 mutations. Viral pseudotypes containing the indicated Envs were preincubated with varying dilutions of plasma, serum, or monoclonal antibodies prior to infection of TZM-bl cells. Reciprocal 50% inhibitory dilutions (ID_{50}) for plasma and serum are color-coded (<100, green; 100-1,000, yellow; 1,000-100,000, orange; >100,000 red). 50% inhibitory concentrations of monoclonal antibodies (IC_{50}) are color-coded (>2 ug/mL, green; 0.2-2 ug/mL, yellow; <0.01-0.2 ug/mL red). **(A)** Neutralization of SIVmac239 and iMac239 are shown relative to two viruses (SIVmac239 D178G and SIVmac239 D178G, H224Q, D337Y, R427K) each containing indicated mutations in gp120. **(B)** Neutralization of iMac239 and iMac239-ΔD385, containing a deletion of Asp-385 within the CD4 binding site. Gray shaded areas indicate assays that were not tested.



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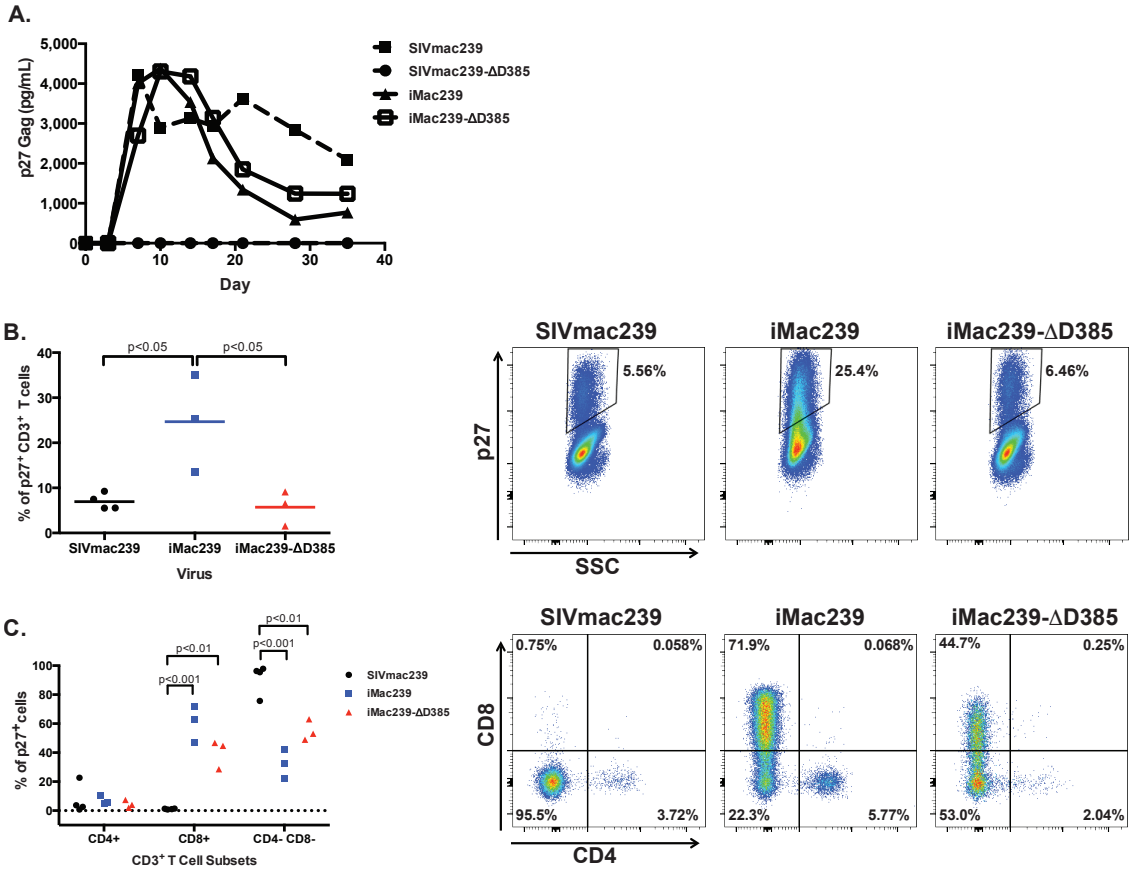

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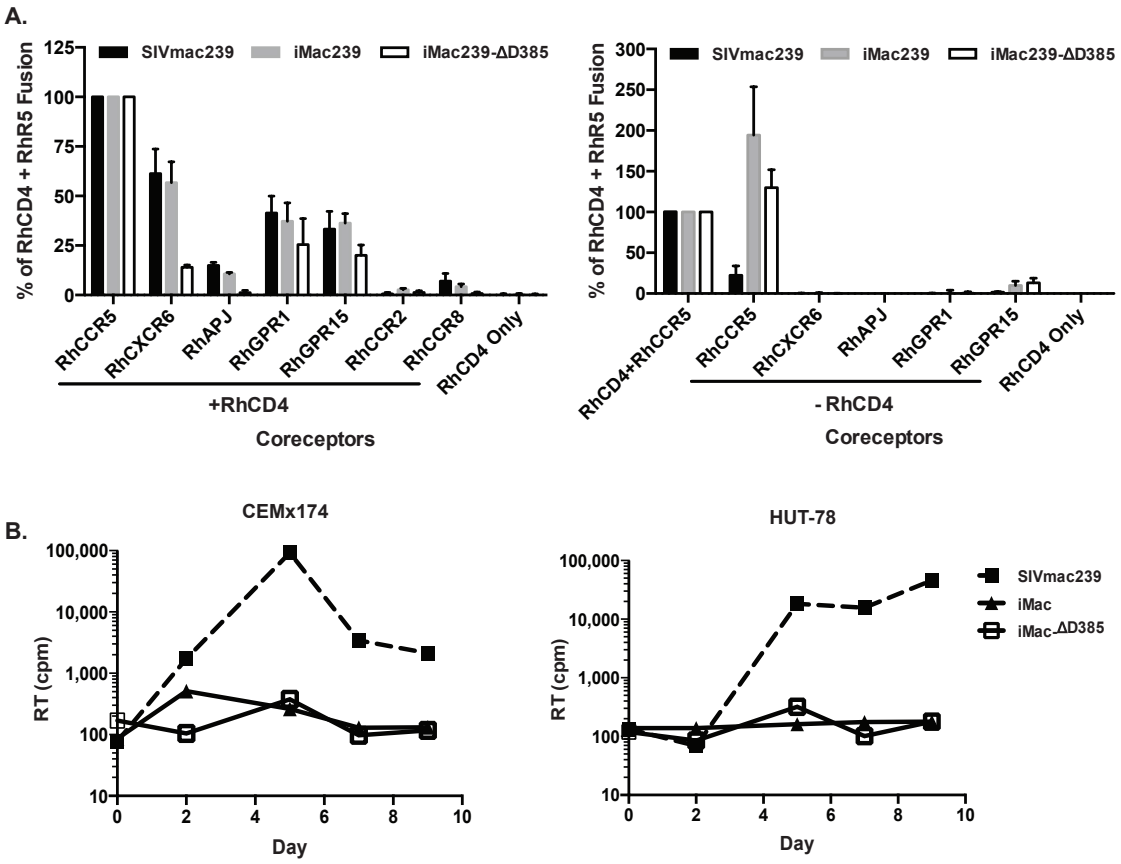

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