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CD4+ T Cells Support Production of Simian Immunodeficiency Virus Env Antibodies That Enforce CD4-Dependent Entry and Shape Tropism In Vivo

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CD4+ T cells rather than macrophages are the primary target cells infected by human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus (SIV) in vivo. Macrophage tropism has been linked to the ability to enter cells through CCR5 in conjunction with limiting CD4 levels, which are much lower on macrophages than on T cells. We recently reported that rhesus macaques (RM) experimentally depleted of CD4+ T cells before SIV infection exhibit extensive macrophage infection as well as high chronic viral loads and rapid progression to AIDS. Here we show that early-time-point and control Envs were strictly CD4 dependent but that, by day 42 postinfection, plasma virus of CD4+ T cell-depleted RM was dominated by Envs that mediate efficient infection using RM CCR5 independently of CD4. Early-time-point and control RM Envs were resistant to neutralization by SIV-positive (SIV+) plasma but became sensitive if preincubated with sCD4. In contrast, CD4-independent Envs were highly sensitive to SIV+ plasma neutralization. However, plasma from SIV-infected CD4+ T cell-depleted animals lacked this CD4-inducible neutralizing activity and failed to neutralize any Envs regardless of sCD4 pre-exposure status. Enhanced sensitivity of CD4-independent Envs from day 42 CD4+ T cell-depleted RM was also seen with monoclonal antibodies that target both known CD4-inducible and other Env epitopes. CD4 independence and neutralization sensitivity were both conferred by Env amino acid changes E84K and D470N that arose independently in multiple animals, with the latter introducing a potential N-linked glycosylation site within a predicted CD4-binding pocket of gp120. Thus, the absence of CD4 T cells results in failure to produce antibodies that neutralize CD4-independent Envs and CD4-pretriggered control Envs. In the absence of this constraint and with a relative paucity of CD4+ target cells, widespread macrophage infection occurs in vivo accompanied by emergence of variants carrying structural changes that enable entry independently of CD4.

Recently we showed that extensive macrophage infection developed in rhesus macaques (RM) experimentally depleted of CD4+ T cells from the blood and lymph nodes prior to infection with SIVmac251, and variants capable of using human CCR5 in the absence of CD4 emerged in plasma during chronic infection (21). Depleted animals also experienced high chronic viral load and progressed rapidly to AIDS. Thus, CD4+ T cell depletion prior to infection resulted in adaptation of SIV to decreased CD4 dependence and macrophage tropism in vivo and provides a model in which the forces that regulate tropism during infection can be elucidated.

In this study, we showed that very efficient CD4-independent use of rhesus macaque CCR5 arose in CD4+ T cell-depleted macaques during the postpeak phase of infection and was associated with sensitivity to neutralization by control SIV+ plasma but not by autologous plasma. A key distinguishing feature was the presence of antibody activity in control RM plasma, but not CD4+ T cell-depleted RM plasma, that neutralized control Envs if preincubated with sCD4 but not in the absence of sCD4 exposure. In
the absence of this CD4-inducible neutralization activity, and with
a paucity of CD4+ T cell targets in CD4+ T cell-depleted
animals, circulating SIV Envs acquired 2 amino acid changes in
gp120 that impart CD4-independent entry through CCR5. Thus,
CD4+ T cells contribute to the production of antibodies targeted
to conserved Env conformations that normally are induced only
by CD4 engagement. These antibodies were associated with strict
CD4 dependence of Env, maintenance of CD4+ T cell targeting,
and restrained tropism for CD4-low macrophages in vivo.

MATERIALS AND METHODS

Ethics statement. All animal experimentation was conducted following
guidelines established by the Animal Welfare Act and the NIH for housing
and care of laboratory animals and was performed in accordance with
institutional regulations after review and approval by the Institutional
Animal Care and Use Committees (IACUC) at the Yerkes National Pri-
mate Research Center (YNPRC) or the Tulane National Primate Research
Center (TPNPRC). Studies were also reviewed and approved by the Uni-
versity of Pennsylvania IACUC.

SIVmac envelope clones, mutagenesis, and pseudotyped virus. The
Env genes from day 11 and day 42 SIV-infected rhesus macaque plasma
were PCR amplified using a procedure for endpoint diluted single ge-
nomes as previously described (21). Mutations were introduced into SIV
envelopes using a QuikChange II XL site-directed mutagenesis kit (Ag-
lent Inc., Santa Clara, CA) and verified by sequencing. SIVmac239 and
SIVmac251.6 Env clones were used as reference controls. Luciferase-ex-
pressing pseudotyped viruses carrying SIV Envs on an HIV-1 backbone
were generated as previously described (22) and were treated with DNase
prior to use in infection.

Virus infection and receptor function assays. Human 293T cells
were maintained in Dulbecco’s modified Eagle medium (DMEM) contain-
ing 10% fetal bovine serum (D10 media). Entry of pseudotyped viruses was
assayed in 293T target cells expressing CD4 and CCR5 or CCR5 alone.
Target cells were transfected with plasmids encoding rhesus macaque
CCR5 with or without rhesus macaque CD4, using pcDNA 3.1 as a “filler
plasmid (23). Cells transfected with pcDNA 3.1 only were used as a neg-
ative control. Target cells (2 × 10^5 per well in 96-well plates) were infected
with pseudotyped viruses (20 ng p24 antigen) by spinoculation at 1,200
rpm for 1 h, after which 25 μl was added to target cells (293T cells expressing
rmCD4 and rmCCR5) plated in 96-well flat-bottom plates (2 × 10^4 cells
in 100 μl medium). Cells were subject to spinoculation as described
above, incubated for 72 h, and lysed for measurement of luciferase activity
as an indication of infection. Neutralization was determined based on the
percentage of infection in the absence of antibody (using virus incubated
with or without sCD4, as appropriate, for the no-antibody treatment).
Neutralization data represent a minimum of at least three independent replicate experiments.

Plasma binding antibody. Binding antibodies to SIV Env protein
were assessed by an enzyme-linked immunosorbent assay (ELISA) as de-
scribed previously (29). Briefly, 96-well plates were coated with 1 μg/ml
SIVmac239 gp140 protein (Immune Tech, New York, NY) by overnight
incubation at 4°C and then incubated with serial dilutions of plasma for 2
h followed by detection using horseradish peroxidase (HRP)-conjugated
goat anti-monkey IgG (AlphaDiagnostic, Owings Mills, MD) (1:10,000
dilution) and 3,3’,5,5’-tetramethylbenzidine (TMB) substrate. A dilution
series of a SIVmac239 gp140-specific IgG standard with a known concen-
tration (generously provided by P. Kozlowski, Louisiana State University)
was included in parallel.

Nucleotide sequence accession numbers. The sequences of the SIV
env clones described in this study have been submitted to GenBank under
the following accession numbers: KF276976 to KF277047.

RESULTS

CD4-independent SIV Env emerges in CD4+ T cell-depleted
rhesus macaques. SIVmac251 infection of animals following an-
tibody-mediated depletion of CD4+ T cells from peripheral
blood, lymph node, and bone marrow but not mucosal sites re-
sulted in extensive in vivo macrophage infection, high postpeak
viremia, and rapid disease progression (21). We previously
showed that Env clones derived from postpeak (day 42) plasma of
these CD4-depleted SIV-infected rhesus macaques were able to
target cells expressing human CCR5 in the absence of CD4,
reaching levels approximately 50% of that seen in cells expressing
CCR5 together with CD4 (21). Here we asked whether CD4-inde-
pendent variants were present in plasma at an earlier time point,
during peak viremia (day 11), and if CD4 independence was
maintained using species-matched rhesus macaque CCR5 and
CD4 (6). Single-genome amplification (SGA)-derived Env clones
were generated from 4 depleted and 2 control animals and 6 Envs
from each animal at each time point (72 total Envs) and were used to
generate a panel of Env-pseudotyped reporter viruses. Pseu-
dotyped viruses were tested for their ability to enter 293T cells
transfected with CCR5 in the presence or absence of CD4 (Fig. 1).

Envs from both the CD4-depleted and control animals re-
quired CD4 at day 11 for efficient entry, both with rhesus CCR5
(Fig. 1A) and with human CCR5 (data not shown). A few Envs
mediated low-level entry in the absence of CD4, and while this
appeared to be slightly greater among CD4-depleted than control
day 11 Envs, the level was variable and did not reach statistical
significance. In contrast, nearly all day 42 Envs from the CD4-
depleted group mediated robust CD4-independent entry (Fig.
1B). In fact, using these rhesus-derived receptors, most clones
were essentially indifferent to the presence of CD4 (mean CD4-
independent entry was 94% ± 7% compared with CD4-depen-
dent entry). In contrast, day 42 Envs from the control animals all
required CD4 for entry through rhesus CCR5. These data indicate
that in animals depleted of CD4+ T cells, CD4-independent vari-
ants emerged in vivo during the postpeak phase of SIV infection
and dominated in plasma during chronic infection.

Emergence of SIV Env D470N and E84K confers CD4 inde-
pendence. We next asked what molecular determinants conferred
the CD4-independent phenotype that arose in vivo in multiple CD4-depleted animals. We aligned the amino acid sequences of the 72 day 11 and day 42 SGA Envs and found that 3 amino acid changes in gp120, G62N, E84K, and D470N (based on SIVmac239 numbering [24]) were enriched in CD4-depleted RM Envs compared to controls (Fig. 1). E84K and D470N were found exclusively in day 42 CD4-depleted RM Envs, and both were significantly associated with CD4-independent entry (P < 0.001; McNemar test for associations). In contrast, G62N was found in CD4-depleted RM Envs at both the day 11 and day 42 time points,
as well as in a few control Envs, and was not significantly associated with CD4-independent entry \((P = 0.14)\). One partially CD4-independent Env clone, RU6.d42.6.1, carried neither E84K nor D470N but did contain D385N, G383R, and D180N, which had each been previously described in CD4-independent SIV Envs \((30)\). Only one day 42 Env from a CD4-depleted animal did not mediate appreciable CD4-independent entry (RZj5.D42.9.2) and did not carry D470N, E84K, or any previously described polymorphism(s) associated with CD4 independence. G62N, E84K, and D470N have not been explicitly characterized in previous studies, but an analysis of SIVmac sequences revealed that G62N has \(\sim 5\%\) frequency within SIVmac251 viral stocks \((31–34)\), while D470N and E84K are extremely rare and present mainly in \textit{in vivo}-passaged SIVmac251-related isolates \((34–38)\). Of note, all instances of G62N, E84K, and D470N that arose in these animals were the result of G-to-A nucleotide changes \((\text{Fig. 1C})\).

To determine whether G62N, E84K, and D470N regulate CD4-independent entry, we introduced each of these mutations, as well as E84K plus D470N, into a representative control Env, RZu4.d42.7.1 \((\text{Fig. 2A})\). G62N had negligible effects on
RZu4.d42.7.1 entry and did not enable CD4 independence. E84K and D470N each severely attenuated total entry, although the signal remained significantly above the assay background and represented bona fide Env-mediated entry. Despite the overall low entry levels, however, levels of D470N entry were similar whether or not CD4 was present, while E84K remained CD4 dependent. When E84K and D470K were simultaneously introduced into RZu4.d42.7.1, robust entry was restored and the levels of entry were equivalent in the presence and absence of CD4. Thus, D470N appears to play a central role in CD4 independence but results in attenuation, while E84K restores overall efficiency of entry in this Env background.

We also introduced the reciprocal mutations, including N62G, K84E, N470D, and K84E plus N470D, into a representative CD4-independent Env, RP6.e.d42.7.2 (Fig. 2B). Similarly, N62G had little effect. K84E severely attenuated overall entry and also modestly reduced relative CD4-independent entry. N470D also reduced CD4-independent entry, although again only modestly (76% reduction). However, the combination of mutations in K84E plus N470D restored the level of overall entry to that seen with the parental Env and dramatically reduced CD4-independent entry (95% reduction). Thus, in the CD4-independent background, both N470D and E84K contribute to CD4 independence and interact to maintain overall efficiency of entry.

To see if the effects of G62N, E84K, and D470N in these 2 representative Envs were generalizable to other viruses from additional animals, we introduced D470N and E84K plus D470N into a larger panel of CD4-dependent Envs from the animals in this study, including Envs from day 11 control, day 11 CD4-depleted, and day 42 control animals (n = 7). We also tested the reciprocal mutations in an expanded panel of CD4-independent Envs from day 42 CD4 + T cell-depleted animals (n = 4). Similar to the representative Env results, D470N and, more potently, D470N plus E84K regulated CD4-independent entry of multiple Envs, including CD4-independent variants that arose independently in different animals (Fig. 2C and D).

Structural significance of CD4-independent mutations that arise in vivo. Using Jmol (25), we mapped predicted CD4-binding residues onto the previously described core structure of SIVmac32H gp120 (24) based on homology with known HIV gp120-binding residues (24, 26) and highlighted residues E84 and D470 (Fig. 2E) (the published core structure of SIV gp120 is N-terminally truncated and excludes G62). This analysis indicates that D470 is a predicted CD4 contact residue residing in a cavity flanked by other CD4 contact residues, while E84 is proximal to the predicted gp120 CD4-binding surface, situated on the first alpha helix, and at a distance of 13.3Å to 19.2Å from D470. In addition, owing to its linear position upstream from a threonine residue in SIVmac251 gp120, D470N introduces a potential N-linked glycosylation site (PNLG) within a predicted CD4-binding pocket (Fig. 2F).

To address whether the PNLG introduced into SIV Env by D470N was important in CD4-independent entry (Fig. 2F), we introduced D470Q and N470Q changes into our representative CD4-dependent and CD4-independent Envs (Fig. 2A and B) as well as the extended panel of Envs (Fig. 2C and D). Q was chosen as a conservative change compared to N that would maintain the charge but abrogate glycosylation, as described previously (39). D470Q in control Envs did not substantially increase CD4-independent entry (Fig. 2A and C), and N470Q in CD4-independent Envs reduced CD4 independence similarly to N470D (Fig. 2B and D). These findings suggest that the PNLG in this deep pocket on the CD4-binding surface is an important component of CD4-independent entry.

Constitutive sensitivity of CD4-independent Envs to MAbs. We then tested a panel of additional SIV Env monoclonal antibodies with differing target epitopes (27) for the ability to neutralize control macaque CD4-dependent Envs without and with sCD4 pre-exposure and neutralization of these Envs carrying E84K, D470N, and D470Q mutations (Fig. 5). Both a V3-specific MAb (Fig. 5A and B) and an anti-gp120 conformational epitope MAb (Fig. 5C and D) showed patterns similar to those seen with 7D3, in which Env was neutralized only if pre-exposed to sCD4 or if mutated to carry D470N (with or without E84K) but not
In contrast, a V1/V2-specific MAb was poorly neutralizing regardless of sCD4 pretreatment but did inefficiently neutralize the mutant Envs (Fig. 5E and F), whereas a gp41 MAb failed to neutralize either the sCD4-treated or mutant Envs (Fig. 5G and H). Thus, both sCD4 pretriggering and the D470N mutation that emerged in CD4-depleted RM render SIV Envs sensitive to antibodies with several distinct, but not all, epitope targets.

FIG 3 A CD4-inducible epitope monoclonal antibody neutralizes CD4-independent Envs. (A) Pseudotype viruses carrying a representative CD4-dependent Env from a control animal (RZu4.d11.2.1) and CD4-independent Env from a CD4-depleted animal (RP6.42.7.2) were incubated with or without sCD4 followed by various concentrations of monoclonal antibody 7D3 prior to infection of 293T cells transfected with rmCD4 plus rmCCR5. (B) The parental and E84K-plus-D470N mutant forms of the CD4-dependent Env were similarly tested for neutralization by 7D3. (C) The parental and K84E-plus-N470D mutant forms of the CD4-independent Env were tested for 7D3 neutralization.

FIG 4 Molecular determinants of 7D3 neutralization are consistent across the extended panel of Envs. (A) CD4-dependent Envs from either control animals or day 11 CD4-depleted animals (n = 6) were mutagenized to introduce E84K or D470N or both or non-PNLG mutant D470Q and then tested for neutralization by monoclonal antibody 7D3. Data show the means ± SEM of the results from all Envs analyzed. (B) Means ± SEM for 7D3 neutralization of CD4-independent Envs from CD4-depleted day 42 animals and Env mutants are shown.

CD4-independent Envs are sensitive to neutralization by CD4-i activity in SIV+ plasma. Previous studies have shown that commonly used SIV strains, such as mac239, mac251, and SM-E660, are highly resistant to neutralization by most SIV+ plasma (21, 42–44). However, it has also been shown that HIV+ plasma contains antibodies that target broadly conserved CD4-i binding-structures and can neutralize viruses if pretriggered with sCD4 (9). Therefore, we asked if SIV+ plasma contains antibodies with similar activities and whether these SIV Envs could be neutralized by pooled plasma from chronically SIVmac251-infected macaques (Fig. 6).

As expected, CD4-dependent control Env RZu4d11.2.1 was resistant to plasma neutralization (Fig. 6A). However, preincubation with sCD4 rendered this Env sensitive to neutralization, confirming that SIV+ plasma contains CD4-i neutralizing activity. In contrast, CD4-independent Env RU5d42.4.3 from a day 42 CD4-depleted animal was sensitive to neutralization regardless of CD4 pretriggering. The D470N mutation, either alone or in combination with E84K, rendered the control CD4-dependent Env sensitive to neutralization by SIV+ plasma without sCD4 pre-exposure (Fig. 6B and data not shown). Similarly, K84E/N470D rendered the CD4-independent Env resistant to plasma neutralization unless pre-exposed to sCD4 (Fig. 6C), while, like MAb 7D3, neither N470D nor K84E alone rendered CD4-independent Envs plasma neutralization resistant (data not shown).
To determine whether this sensitivity to SIV$^+$ plasma neutralization was common among the viruses that arose in CD4-depleted animals, we tested a larger panel of Envs from our study along with SIVmac239 and SIVmac251.6 reference Envs for comparison. As shown in Fig. 7A, day 11 Envs from both control RM (solid lines) and CD4$^+$ T cell-depleted RM (dotted lines) were resistant to pooled plasma neutralization but became sensitive if first exposed to sCD4. SIVmac239 and SIVmac251.6 showed similar patterns of CD4-induced neutralization sensitivity (Fig. 7A). At day 42, all Envs from CD4$^+$ T cell-depleted animals were sensitive to plasma neutralization regardless of sCD4 pretreatment (Fig. 7B). Control animal day 42 Envs were resistant to plasma neutralization but became more sensitive with sCD4 pretreatment (Fig. 7B). Some Envs exhibited a drop in infectivity at the highest
concentrations of plasma, but this was also observed with uninfected control plasma (data not shown) and likely due to nonspecific effects of high concentrations of plasma as has been previously described (28).

Thus, the CD4-independent Envs that arose in CD4-depleted macaques are constitutively sensitive to CD4-inducible neutralization by SIV+ plasma, which targets Env conformations regulated by D470N and E84K. These findings also suggested that this antibody activity generated during normal SIV infection could restrict the emergence of CD4-independent variants \textit{in vivo}.

**CD4+ T cell-depleted macaques lack robust plasma CD4-independent neutralization activity.** Both the CD4+ T cell-depleted and control animals in this study seroconverted by Western blot analysis and ELISA (21). Having observed the marked sensitivity to neutralization by SIV+ plasma of CD4-independent Envs that emerged in CD4+ T cell-depleted animals, we hypothesized that these animals might lack CD4-i neutralization activity. To address this, we used chronic infection plasma samples (day 56) from the CD4+ T cell-depleted and control animals and tested their ability to neutralize autologous and heterologous Envs (Fig. 8). As expected, plasma from control animals did not neutralize autologous day 11 or day 42 Envs in the absence of sCD4 but did neutralize Envs pre-exposed to sCD4, confirming the presence of CD4-i activity (Fig. 8A to D; black lines). In addition, plasma from control animals also neutralized heterologous CD4-independent Envs from day 42 CD4-depleted animals even in the absence of sCD4 (Fig. 8E to H; black lines). In sharp contrast, plasma from CD4-depleted animals showed a markedly reduced ability to neutralize heterologous CD4-dependent Envs (Fig. 8A to D; red lines), autologous day 11 CD4-dependent Envs (Fig. 8E and G; red lines), or autologous day 42 CD4-independent Envs (Fig. 8F and H; red lines), regardless of sCD4 treatment (although the possibility of the occurrence of very weak neutralization at high plasma concentrations cannot be completely excluded). Thus, plasma from CD4+ T cell-depleted animals in which CD4-independent viruses emerged lacks robust CD4-i neutralizing activity. Plasma from earlier time points, day 14 and day 25, had no detectable CD4-i neutralizing activity (Fig. 8A to D; red lines), indicating that CD4-i neutralizing antibody activity emerges between days 25 and 56 after infection in normal SIV infection.

Finally, we used an ELISA against SIVmac230 gp140 to ask if total anti-Env IgG was reduced in the plasma of CD4 T cell-depleted animals. As shown in Fig. 9, Env-binding antibodies were present in all macaques, but titers were significantly lower in the CD4 T cell-depleted animals. Thus, CD4 T cell depletion results in global reduction of antigen-specific antibody production, consistent with the poor CD4-inducible neutralization capacity of CD4 T cell-depleted plasma.

**DISCUSSION**

SIV infection of rhesus macaques that were largely depleted of CD4+ T cells, as described here and in reference 21, reveals that CD4-independent viral variants emerge and consistently dominate circulating virus in this setting. Emergence of CD4-independent Env variants corresponded to extensive \textit{in vivo} infection of macrophages (21), a relationship which has been previously described \textit{in vitro} (6, 45–47) and in the CNS or lung compartments (48–54). Here we demonstrate for the first time the factors governing CD4 dependence and target cell tropism in the systemic compartment \textit{in vivo}. Animals that were not CD4+ T cell depleted before infection produced a robust antibody response which likely contributes to maintaining strict CD4 dependence and CD4+ T cell tropism. Such antibodies, which are nonneutralizing against control virus unless pretriggered with sCD4, are also abundantly present in chronic HIV-1 infection (9). The absence of such anti-
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FIG 7 SIV+ plasma neutralization of the extended panel of CD4-independent and control Envs and prototype SIV Envs. Pseudotype viruses carrying Envs from day 11 animals (A) and day 42 animals (B) were preincubated without (left) or with (right) sCD4, exposed to various dilutions of pooled SIV+ plasma, and used to infect 293T cells expressing rmCD4 and rmCCR5. Envs from CD4-depleted animals are represented by dotted lines, and Envs from control animals are represented by solid lines. Reference Envs from SIVmac239 and SIVmac251.6 were tested in parallel and are included in panel A.

bodies in the CD4+ T cell-depleted animals was associated with amino acid changes in Env gp120 that confer entry through CCR5 independently of CD4, enabling a shift in tropism to macrophages. These findings highlight the critical role that CD4-inducible neutralizing antibody activity has in vivo in shaping Env function, regulating CD4 use, and defining cell targeting early after infection.

We mapped the molecular determinants of CD4 independence to two amino acids in Env gp120, D470N and E84K. These changes were strongly associated with CD4 independence among Env variants, arose independently in multiple animals, and conferred the phenotype as shown by mutagenesis studies. Several pathways to CD4 independence in HIV and SIV have previously been described (7, 30, 48, 55–57), but our observation suggests that this particular pathway is especially favored in vivo, at least in the context of this SIVmac251 infection. SIVmac251 is a heterogeneous swarm, so it is conceivable that E84K and D470N were present as polymorphisms in the inoculum used to infect the animals in this study. However, our review of database sequences indicates that these amino acids are exceedingly uncommon, and the fact that D470N and E84K were found neither in Envs from control animals nor in those from day 11 CD4-depleted animals demonstrates that these changes are not favored until postpeak infection in the relative absence of CD4+ T cells. In contrast to D470N and E84K, G62N was enriched in CD4-depleted animals at both day 11 and day 42 but was not significantly associated with CD4-independent entry. G62N is present at an overall frequency of 4% to 5% among SIVmac sequences and occasionally represents the dominant sequence among some SIVmac251 swarms (33, 34). Thus, additional selective forces in the CD4-depleted animals other than CD4 independence per se appear to be responsible for selection at this residue. Of note, all occurrences of G62N, E84K, and D470N resulted from G-to-A nucleotide changes, implicating the APOBEC3 family of proteins (38) and suggesting an important role for this mechanism in achieving diversity rapidly in the context of this selective environment in vivo.

While both E84K and D470N contribute to the full CD4-independent phenotype, D470N appears to have a primary role, with E84K reflecting at least in part a compensatory change that reversed the degree of attenuation induced by D470N. Structural mapping of D470N on the unliganded core crystal structure of SIV gp120 (24) places a potential glycosylation site deep within a pocket of CD4-binding residues. In contrast to N at that location, Q470 Envs were not able to efficiently use CCR5 independently of CD4, suggesting that glycosylation rather than simply loss of a negative charge may be responsible for allowing CD4-independent entry. It is possible that placement of a bulky carbohydrate within that pocket could affect gp120 structure in a manner similar to that seen with docking of CD4 itself, causing Env to adopt a CD4-bound conformation and expose the conserved coreceptor-binding site. Whether differences in the structure of an intact SIV gp120 compared to the core structure employed here (as has been suggested for HIV-1 gp120 [40, 41, 59–61]) would affect the position of this residue is unknown. The homologous HIV-1 gp120 amino acid (D457) maps to the CD4-binding region in the HIV-1 gp120 core crystal structure and trimeric cryo-electron microscopy (cryo-EM) models (26, 59, 60), suggesting that the structural significance of D470 modeled here may be generalizable. How E84K might contribute to CD4 independence or to reverse D470N attenuation is unclear. This residue is near the predicted CD4-
FIG 8 Reduced CD4-inducible SIV Env neutralization activity in plasma from CD4− T cell-depleted animals. Chronic infection plasma (day 56) from control animals (KKE, RZu4; black lines) or CD4-depleted animals (RUf6, RU15; red lines) was used in neutralization studies with pseudotyped viruses carrying Envs from control animals (A to D) or CD4-depleted animals (E to H), with or without preincubation with sCD4. Black lines in panels A to D represent neutralization by autologous non-CD4-depleted plasma, while red lines represent neutralization by heterologous plasma from CD4-depleted animals. Red lines in panels E to H represent neutralization by autologous CD4-depleted plasma, while black lines represent neutralization by heterologous non-CD4-depleted plasma.
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binding region but not a predicted contact point and lies within an alpha-helix thought to move with CD4 engagement (24, 62, 63). This drastic charge change may have indirect effects that affect the structure or perhaps may stabilize inter- or intramolecular interactions induced by the D470N effects that mimic CD4 engagement.

Although these residues have not been explicitly characterized in previous studies, analysis of database sequences reveals that both E84K and D470N are very rare. E84K is present at a low frequency in some stocks of SIVmac239 and SIVmac251 (33, 34, 36), and D470N is present in a few brain and plasma sequences in a model of rapid disease progression (37). E84K is also present in an in vitro-derived CD4-independent variant of SIVmac239 (35, 64), although its specific contribution to CD4 independence was not assessed. In addition, both E84K and D470N were found in Envs from experimentally CD8-depleted, SIVmac251-infected rhesus macaques experiencing brain disease (38). CNS disease is driven largely by macrophage-tropic infection of myeloid cells in the brain, in the overall immune-privileged CNS environment (52). The presence of these mutations in the brain and in highly immunodeficient animals in previous studies is consistent with our observation of macrophage infection in vivo in CD4 T cell-depleted animals (21).

Recently, it has been suggested that the generally greater sensitivity of CD4-independent viruses to antibody neutralization is due to greater “intrinsic reactivity” of these Env variants (10). This property enables them to spontaneously sample a range of conformations in the absence of CD4 binding, leading to both greater efficiency of coreceptor engagement and greater sensitivity to structural perturbation (and thus neutralization) by antibodies with a range of epitope specificities and not just those reflecting the coreceptor-binding site exposed upon binding to CD4. Our CD4-independent variants were sensitive to antibodies targeting several (although not all) SIV gp120 epitopes (Fig. 4 and 5), which is consistent with this model. Thus, the mutations that arose in these Envs may not only expose discrete CD4-i coreceptor-binding-site epitopes on gp120 but may also increase the intrinsic reactivity, rendering them more sensitive to neutralization by multiple epitope antibodies. This is the first report to directly demonstrate in vivo the functional constraints on Env imposed by this mechanism and the consequences for coreceptor use and target cell tropism that are governed by such constraints.

In our model, CD4-i T cells were profoundly depleted from blood and inductive lymphoid compartments, although incompletely from mucosal sites (21). It has been proposed that CD4-i epitope antibodies arise in vivo through B cell recognition of Env complexed with CD4, which exposes the conserved binding-binding site to the B cell receptor (65). Our observation that CD4 + T cell depletion leads to decreased CD4-inducible antibody activity, despite the availability of these epitopes directly on these CD4-independent Envs for potential recognition by B cells, suggests that it is the loss of CD4 + T helper function in these animals that leads to the dysfunctional antibody response.

Along with the in vivo tropism shift toward macrophage target cells, CD4 + T cell-depleted animals exhibited rapid disease progression and an absence of postpeak decline in plasma viremia. While the role of antibody activity in shaping altered tropism in the CD4-depleted animals seems clear, the mechanisms responsible for sustained high-level plasma viremia are less clear. Robust autologous or heterologous neutralizing activity was not detected in the control animals even at the day 56 time point (21) (Fig. 8). In addition, studies differ on whether direct B cell depletion prior to infection of rhesus macaques affects the postpeak viral setpoint (66–68). Similarly, although CD8 + T cells clearly play a central role in postpeak viral decline (69–73), the current paradigms are that CD4 + T cell help is dispensable for primary CD8 T cell responses (74–76), and indeed no differences in CD8 + T cell responses were seen between control and CD4-depleted animals (21). In contrast, a variety of direct HIV- or SIV-specific antiviral activities have been ascribed to CD4 + T cells (77), including a role in postpeak decline following acute infection (21, 78). CD4 + T cells have also been reported to have particular antiviral activity against SIV-infected macrophages (79). Thus, loss of direct CD4 + T cell antiviral mechanisms may be responsible for the sustained viremia. At the same time, loss of strict CD4 + T cell dependence with consequent expansion to macrophages as a second robust target cell population may be an additional factor favoring sustained postpeak viremia, particularly in this setting of limited CD4 + T cells.

Some SIV-infected macaques that progress rapidly to AIDS fail to seroconvert (80, 81), a phenomenon that has been linked to early B cell defects following infection (82). In addition, a few CD4-independent SIV Env variants have previously been reported to arise spontaneously in SIV rapid progressors (30). Brain disease in SIV infection is also far more common in rapid- than normal-progressor animals (83), and macrophage infection within the CNS was also seen in these CD4 T cell-depleted animals (data not shown). Our results suggest that failure to develop CD4-inducible neutralization activity, whether as a consequence of direct virus-induced B cell defects as in spontaneous rapid progressive disease or of experimental abrogation of CD4 + T cell help as shown here, may be a common factor that enables emergence of these macrophage-tropic variants with an expanded host range and a propensity for CNS infection. Furthermore, while most HIV and SIV blood variants exhibit modest macrophage tropism, highly macrophage-tropic variants may emerge in very-late-stage disease when profound CD4 + T cell loss has developed (18–20). Infection with X4-tropic simian-human immunodeficiency virus (SHIV) also results in rapid profound CD4 depletion, with emergence of macrophage-tropic variants (19, 20). Furthermore, brain disease developed in all animals in another recent study that involved the use of CD4-depleted macaques prior to SIV infection using an approach slightly different from ours, although CD4 independence was not assessed (76). Future studies will help eluci-
In summary, our findings reveal a critical role for CD4 T cell-dependent production of CD4-inducible neutralizing activity early after infection in vivo in shaping Env function and target cell tropism. These data suggest that, normally, there is a balance in which CD4+ T cells support the production by B cells of antibodies that, paradoxically, enforce strict CD4+ T cell tropism for the virus. In this model, CD4+ T cell depletion results in both a profound reduction of levels of CD4+ T cell targets and impaired production of these antibodies. As a result, strict CD4 dependence is lost and expanded tropism for CD4-low macrophages emerges. In addition to the forces regulating normal infection, these findings have implications as well for infection in the CNS and, possibly, late-stage disease upon severe CD4+ T cell depletion.

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