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Neutralization-Sensitive R5 SHIV-2873Nip Encoding *env* from a Infant with Recent HIV Clade C Infection

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HIV-C (HIV-C) accounts for >56% of all HIV infections worldwide. To investigate vaccine safety and efficacy in non-human primates, a pathogenic, R5-tropic, neutralization-sensitive simian-human immunodeficiency virus (SHIV) encoding HIV-C *env* would be desirable. We have constructed SHIV-2873Ni, an R5 SHIV encoding a primary pediatric HIV-C *env* isolated from a 2-month old Zambian infant, who progressed to death within one year of birth. SHIV-2873Ni was constructed using SHIV-1157ipd3N4 (Song et al., J. Viol. 80**:**8729-38, 2006**) as backbone since the latter contains additional NF-κB sites in the long terminal repeats (LTRs) to enhance viral replicative capacity. The parental virus, SHIV-2873Ni, was serially passaged through 5 rhesus monkeys (RM); SHIV-2873Nip, the resulting passaged virus, was reisolated from the 4th recipient about 1 year post-inoculation. SHIV-2873Nip was replication-competent in RM peripheral blood mononuclear cells (PBMC) of all random donors tested, was exclusively R5 tropic and its *env* gene clustered with HIV-C by phylogenetic analysis; its high sensitivity to neutralization led to a classification as Tier 1 virus. Indian-origin RM were inoculated by different mucosal routes, resulting in high peak viral RNA loads. Signs of virus-induced disease include depletion of gut CD4$^+$ T lymphocytes, loss of memory T cells in blood, and thrombocytopenia that resulted in fatal cerebral hemorrhage. SHIV-2873Nip is a highly replication-competent, mucosally transmissible, pathogenic R5 virus that will be useful to study viral pathogenesis and to assess the efficacy of immunogens targeting HIV-C Env.**

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Currently, 33 million people are living with HIV/AIDS (www.unaids.org), and the majority of them live in sub-Saharan Africa, South and Southeast Asia, including China and India, where HIV subtype C (HIV-C) circulates in >90% of the population (UNAIDS) (50). This distribution makes HIV-C the most prevalent subtype in the global pandemic, accounting for >56% of all HIV infections worldwide (www.unaids.org). Globally, HIV is one of the leading causes of childhood morbidity and mortality. Children account for 20% of all HIV-related deaths, 7% of individuals living with HIV, and 16% of new infections annually (reviewed in (26, 29, 38). In sub-Saharan Africa, HIV-C is responsible for approximately 50% of all infections, and a significant number of infections are in infants and children. HIV transmission from infected mothers to their infants is the primary mode of infection in children and can occur *in utero*, intrapartum, or postnatally through breast milk. The use of antiretroviral drugs has successfully reduced the rate of HIV infection in infants in the developed world to approximately 1%; nevertheless, such regimens have only recently become available in many of the developing nations where HIV mother-to-child transmission (MTCT) is most significant (reviewed in (26, 38).

Simian-human immunodeficiency viruses (SHIVs) are chimeric viruses that contain HIV envelope genes in the simian immunodeficiency virus (SIV) backbone. They have been used in a wide range of studies investigating lentiviral pathogenesis, antiviral immunity, virus-host interactions, mucosal transmission and vaccine- and drug efficacy (20). However, the majority of current SHIV strains utilize envelope genes derived from HIV clade B strains, which represent less than 10% of all global infections. Therefore, the available SHIV chimeras do not reflect the genetic diversity of the HIV epidemic, which is dominated by non-B clades, especially by HIV-C. Only a few studies have focused on developing anti-clade C Env vaccines (25, 27, 44, 49)

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with one efficacy study in primate models (44). To investigate lentiviral pathogenesis as well as anti-HIV-C vaccine safety and efficacy in non-human primate models, a pathogenic, CCR5-restricted, clade C SHIV (SHIV-C) would be very useful.

Previously, we have generated an R5 SHIV-C, SHIV-1157i (6, 51), which encodes *env* from a 6-month-old Zambian infant born to an HIV-positive mother. During prospective long-term follow-up, this infant turned out to be a long-term non-progressor (LTNP) who has remained asymptomatic at 8 years of age (61). The rhesus monkey (RM)-adapted strain, SHIV-1157ip, was pathogenic and caused AIDS in several monkeys thus far, but with a relatively slow rate of disease progression. AIDS developed in RM between 127 – 300 weeks post-inoculation. A late virus was reisolated and engineered to contain extra NF-kB sites in the long terminal repeats (LTRs) (51); follow-up times of monkeys infected with this late form are not yet sufficient to assess development to AIDS, although signs of disease have developed. A possible explanation is that the *env* gene used to construct the original SHIV-1157i is an important determinant of the disease progression rate. The fact that the *env* gene was derived from a LTNP may be linked to the relatively slow disease progression we observed in RM infected with SHIV encoding the corresponding *env* gene.

We sought to test whether constructing an R5 SHIV with an *env* gene derived from a rapid progressor would give rise to a more virulent R5 SHIV-C. Although HIV- or SIV-infected individuals with either typical rates of disease progression or with long-term non-progression have been studied extensively, few reports were focused on the virologic and immunologic characteristics of patients with rapid disease progression (9, 22). Patients who progress to AIDS within one to two years from the time of infection have been identified among infants and adults

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(7, 13, 34, 35, 46), with a higher frequency in infant populations. These patients demonstrate rapid loss of $CD4^+$ T cells and lack potent cellular and humoral immune responses.

Here we report the construction of SHIV-2873Ni, a chimera that encodes *env* of an R5 HIV-C strain isolated from a rapid progressor, a 2-month-old Zambian baby, who died of AIDS-related disease within one year of birth. SHIV-2873Ni was serially passaged through 5 RM; SHIV-2873Nip, the passaged virus, was reisolated and characterized from the 4^{th} recipient about 1 year post-inoculation when signs of disease were manifest. The RM-adapted virus caused T-cell depletion within a few months post-inoculation.

MATERIALS AND METHODS

Original virus isolates and nomenclature. HIV2873i is a biological isolate obtained from a Zambian infant at 2 months of age. The infant, born to an HIV-C-infected mother, was PCR negative at birth and rapidly progressed to AIDS-related death within one year. The designation "i" indicates a virus strain (or *env* gene) isolated from an infant. SHIV-2873Ni is the original, non-adapted infectious molecular clone that contains two NF-κB sites in the 3' long terminal repeat (LTR) instead of the usual single NF-kB site present in the SIVmac239 LTR. This duplicate NF-κB site is copied into the 5' LTR during subsequent reverse transcription steps of the retroviral life cycle (8). SHIV-2873Nip, a biological isolate obtained after passage of SHIV-2873Ni through four RM, was reisolated from a monkey systemically infected for approximately one year; "p" designates a passaged (or monkey-adapted) virus.

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Cell lines, antibodies and viruses. U87 or GHOST cell lines, which express CD4 only or CD4 with different chemokine receptors, as well as CEM.NKR.CCR5 cells, were provided by the NIH AIDS Research & Reference Reagents Program (ARRRP, Germantown, MD). Neutralizing monoclonal antibodies (nmAbs) 2F5 (36), 2G12 (56) and 4E10 (54) were provided by Dr. Hermann Katinger (Polymune Scientific, Vienna, Austria). MAb b12 (1) is an IgG1 isotype and was produced by expression in recombinant CHO cells (kindly provided by Dr. Dennis Burton, Scripps Research Institute, La Jolla, CA). CEMx174-GFP cells, provided by Dr. Barbara Felber (National Cancer Institute, Frederick, MD), contain the green fluorescent protein (GFP) gene under HIV-1 LTR regulation and express CXCR4 but not CCR5. TZM-bl cells (also called JC53-bl [clone 13] cells; ARRRP) (11) are derived from a HeLa cell line (JC.53) that stably expresses CD4 and CCR5. TZM-bl cells also express luciferase and β-galactosidase under control of the HIV-1 LTR.

Animals and animal care. RM (*Macaca mulatta*) of Indian origin were used in this study. The animals were kept according to National Institutes of Health guidelines on the care and use of laboratory animals at the Yerkes National Primate Research Center (YNPRC, Emory University, Atlanta, GA). These facilities are fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Animal experiments were approved by the Animal Care and Use Committees of YNPRC and the Dana-Farber Cancer Institute.

Construction of SHIV-2873Ni molecular clones. Peripheral blood mononuclear cells (PBMC) of Zambian infant 2873i were collected two months after it was born to an HIV-positive mother (62) and briefly cocultured with normal human donor PBMC. DNA from this coculture was extracted for PCR amplification. A pair of specific primers, designed to amplify the entire HIV-1 *env*, incorporated the *Hind*III or *Xho*I restriction enzyme sites and had the following

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sequence: 2873-*Hind*III, 5'-GGGGGAAGCTTATGAGAGTGATGGGGATACAGAGG-3' and 2873-*Xho*I, 5'-CCCCCTCGAGTTATTGCAAAGCTGCTTCAAAGCCC-3'. The full-length HIV2873i *env* was digested with the restriction enzymes *Hind*III and *Xho*I and cloned into vector pcDNA6/myc-His B (Invitrogen, Carlsbad, CA). SHIV-1157ipd3N4 (51) encodes *env* of a late stage SHIV isolated from a RM that developed AIDS 137 weeks after inoculation of SHIV-1157i. The 2.2 kb *Kpn*I (K)-*BamH*I (B) fragment of HIV2873i (spanning most of gp120, the entire gp41 extracellular domain, the transmembrane region (TM), and a part of the cytoplasmic domain) was amplified to replace the corresponding region of SHIV-1157ipd3 *env*. The modified 3'-half was ligated with the 5' half of SHIV-vpu$^+$ (23) proviral DNA to generate full-length SHIV-2873Ni (51).

Co-receptor usage of SHIV constructs. The U87 or GHOST cell lines expressing CD4 alone or CD4 and HIV-1 or SIV coreceptors were used to study virus tropism. U87.CD4, U87.CD4.CCR1, U87.CD4.CCR2, U87.CD4.CCR3, U87.CD4.CXCR4, U87.CD4.CCR5, GHOST.BOB and GHOST.BONZO were infected with virus stock. Cells were washed and resuspended in 1 ml of fresh medium. On days 0, 2, 4, and 6, supernatants were collected for p27 titration. The molecular clones $SHIV_{SF162P3}$ (28) (clade B, R5), and SHIV-vpu$^+$ (23) (clade B, X4) were used as controls. These experiments were carried out with both SHIV-2873Ni and SHIV-2873Nip.

Serial passage of SHIV-2873Ni. Rhesus macaque RBl-9 was inoculated intravenously (i.v.) with 10 ml of a SHIV-2873Ni stock prepared from RM PBMC. After RBl-9 was confirmed virus positive by real-time RT-PCR (17), 10 ml of blood from RBl-9 was transferred i.v. to RAg-9 at week 2 post-inoculation of the donor. Three additional animals, RAi-8, RNt-9 and RGc-9, received serial blood transfers. Animal RWa-9, which had been previously exposed

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to parental SHIV-2873Ni but had remained uninfected, received blood from donor RAi-8. All animals were monitored for viral loads, antibody responses, and T-cell subsets.

PCR and sequencing analysis. Chromosomal DNA was extracted from 10^6 PBMC from animal RNt-9 using a DNAzol genomic DNA isolation kit (Molecular Research Center Inc., Cincinnati, OH). To analyze the molecular evolution of SHIV-2873Nip *env* during in vivo passage, two different primers were synthesized to amplify the entire *env* gene (approximately 2.5 kb) of SHIV-2873Nip isolated from the last animal RNt-9 about 1 year post-inoculation after it had developed signs of disease. The *env* gene of SHIV-2873Nip was amplified using the pair of primers: forward (5'-CCCCCAAGCTTCCACCATGAGAGTGAAGGAGAAATATC-3') and reverse (5'-CCCCCGAATTCCATCTTCCTCATCTATATCATCC-3'); the PCR was carried under end-point dilution conditions. The amplified fragment was cloned into the *Hind*III and *EcoR*I sites of pcDNA6/myc-His B vector for sequencing. Eight clones encoding an infectious *env* gene were randomly picked for DNA sequencing.

Phylogenetic analysis. The sequences of the *env* genes of SHIV-2873Ni, SHIV-2873Nip, other clade C SHIVs generated by us (SHIV-1157i, SHIV-1157ip, SHIV-1157ipd3N4) and HIV1084i (14) were aligned with full-length reference sequences of several Group M viruses obtained from the Los Alamos sequence database (http://hiv.lanl.gov/content/hiv-db/SUBTYPE_REF/align.html). Nucleotide sequences were gap-stripped and aligned using CLUSTAL X (55) and Neighbor-joining trees were generated with the Kimura 2 parameter substitution model using MEGA3.2 software (21). Pairwise evolutionary distances were estimated using DNADIST from the PHYLIP 3.6 package (12), and the reliability of the topologies was evaluated by bootstrap analysis with 100 replicates using DNADIST, NEIGHBOR and CONSENSE.

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Generation of a large-scale SHIV-2873Nip stock. A large-scale stock of the infectious, uncloned biological isolate was prepared by infecting concanavalin A (ConA)-stimulated naïve RM PBMC in the presence of human interleukin-2 (IL-2) (20 U/ml) and tumor necrosis factor alpha (10 ng/ml) using virus harvested from co-cultured, infected PBMC from monkey RNt-9 approximately 1 year post-inoculation. The rhesus PBMC-grown stock has a p27 concentration of 140 ng/ml and 4 x 10^6 50% tissue culture infectious doses ($TCID_{50}$) per ml as titrated in TZM-bl cells according published protocols (11).

Neutralization assays. The neutralization sensitivity of SHIV-2873Nip was determined using both PBMC-based and TZM-bl reporter cell line-based neutralization assays, as described previously (11, 19, 24, 44, 51). In both cases, serial dilutions of either RM sera or mAbs were set up in triplicate in 96-well plates, virus was added (50-200 $TCID_{50}$) and incubated for 1 h at 37°C. Either PBMC or TZM-bl cells were then added. For assays employing immune sera, neutralization titers were calculated based on virus production in wells containing sera pooled from 4 naive RM as negative controls. In both assays, the concentration of serum giving 50% neutralization of virus production (IC_{50}) was calculated using the level of virus production in control wells containing the same dilution of pooled naive sera; for mAb titers, IC_{50} was calculated based on control wells containing virus plus cells only.

For the PBMC-based assays, human PBMC were stimulated overnight with PHA (5 μg/ml), washed and added to wells at 2 x 10^5/well. In assays testing sera, PBMC were washed in assay plates after 1 day of culture and fresh IL-2 (10 U/ml) - containing media added; alternatively, in PBMC-based assays testing mAbs, the mAbs were not washed away but were diluted 1:1 with fresh medium daily, starting on day 3 of the experiment. Because this latter

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assay condition takes into account the long half-lives of antibodies, neutralization titers may differ slightly from titers measured by other methods (2, 32). Aliquots of supernatants were harvested every other day, assayed for p27 levels in wells containing only virus plus cells, and neutralization activity was measured on the culture day showing linear phase of increase. For TZM-bl based assays, cells were added in the presence of DEAE dextran (40 μg/ml), washed 1x on day 1, luciferase substrate (Bright-Glo, Promega, Madison, WI) was added on day 2, and luciferase activity was measured in a luminometer.

Measurement of plasma viral RNA levels. Plasma viral RNA was isolated by use of the QiaAmp Viral RNA Mini-Kit (Qiagen), and viral RNA levels were measured by quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) for SIV *gag* sequences (17) at weeks 0, 1, 2, 4, 8 and monthly thereafter. The assay sensitivity was 50 viral RNA copies/ml.

Oral and intrarectal inoculation of SHIV-2873Nip. Indian-origin RM received 3 ml or 1 ml of the large-scale virus stock by the oral or intrarectal (i.r.) routes, respectively. Six additional animals received repeated weekly low-dose i.r. inoculations (up to a maximal number of 5 inoculations): 1,500 $TCID_{50}$ (two monkeys) and 5,000 $TCID_{50}$ (four monkeys). Our protocol stipulated that animals remaining aviremic or failing not reach plasma viral RNA levels of $\geq 10^4$ copies/ml at the 2-week time point after the 5^{th} low-dose virus exposure would receive a single high-dose i.r. challenge (30,000 $TCID_{50}$). Blood was collected at 0, 1, 2, 4, 8, and 16 weeks and at 3-month intervals post-inoculation to determine viral RNA loads and to measure T-cell subsets.

Isolation of cells from blood and rectal biopsies. PBMC were isolated using standard procedures; lymphocytes from rectal biopsies were obtained by digestion with collagenase followed by a separation step using Percoll gradients as described (58). Briefly, 10-20 pinch

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biopsies were collected in complete RPMI and washed two times with ice-cold Hanks Balanced Salt Solution (HBSS). Biopsies were digested with collagenase type IV (Worthington, Lakewood, NJ) and DNase I (Roche, Indianapolis, IN), passed through decreasing size of needles (16G, 18G and 20G, 5-6 times with each needle), and filtered through a 100 μm filter. Cells were suspended in 35% Percoll in PBS, underlayed with 60% Percoll and centrifuged at 2,500 rpm for 30 min. Cells from the interface were collected, washed and resuspended in complete RPMI for analysis.

Phenotypic analysis of T cells from blood and rectum. For T-cell subset analyses, approximately 1 x 10^6 PBMC or lymphocytes from rectal biopsies were surface stained with the following mAbs (BD Pharmingen, San Jose, CA): anti-CD3 conjugated to Alexa 700 (clone SP34-2), anti-CD4 conjugated to PerCp (clone L-200), and anti-CD95 conjugated to APC (clone DX2). The following mAbs were from eBiosciences (San Diego, CA): anti-CD28 conjugated to PeCy7 (clone CD28.2), anti-CCR5 conjugated to PE (clone 3A9), and anti-CD45RA (clone ALB11). Following staining, cells were acquired using LSRII (BD Biosciences, San Jose, CA) and analyzed using FlowJo software (Treestar, Inc., San Carlos, CA). Lymphocytes were identified based on scatter pattern and $CD3^+$, $CD8^-$, $CD4^+$ cells were considered as $CD4^+$ T cells, while $CD3^+$, $CD8^+$, $CD4^-$ cells were considered as $CD8^+$ T cells. $CD3^+CD4^+$ T cells were gated based on CD28 and CD95 expression to define memory $CD4^+$ T-cell subpopulations: naive ($CD28^+CD95^-$), central memory ($CD28^+CD95^+$), and effector memory ($CD28^-CD95^+$).

Statistical analysis. Statistical analysis was performed using Prism® (GraphPad Software, version 4). Differences in percentages and numbers of lymphocytes were compared between groups of uninfected versus SHIV-2873Nip-infected animals using the Mann-Whitney test.

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Nucleotide sequence accession number: The complete nucleotide sequence of the *env* gene of SHIV-2873Nip has been submitted to Genbank; an accession number is pending.

RESULTS

Construction of a SHIV-C encoding *env* from a pediatric rapid progressor. PBMC were collected from infant 2873i at 2 months of age and cocultured briefly with normal human donor PBMC. This infant, born to an HIV-C-positive Zambian mother, had been PCR negative at birth and became infected either intrapartum or through breast feeding. A rapid progressor, the infant died of tuberculosis within one year. Tuberculosis, an AIDS-defining illness, is a frequent diagnosis among HIV-infected children in Zambia. At four months of age, this child's PBMC contained a high copy number of HIV proviral DNA (>750 copies per 10^6 PBMC), indicative of a relatively high viral load. Of note, neither CD4 T-cell subset nor viral RNA load determinations were available at the clinic when the child presented with Tb.

Primary full-length *env* genes were PCR amplified from cocultured PBMC DNA of infant 2873i and cloned into the expression vector pcDNA6/B. To identify an infectious envelope, the resultant constructs were co-transfected into 293T cells with HIV-1 ΔEN, a proviral clone deleted in *env* and *nef* that encodes GFP in lieu of *nef.* Cell-free supernatants containing pseudotyped viruses were used to infect CEM.NKR.CCR5 cells, which were screened for GFP expression; several infectious HIV2873i *env* clones were identified (Fig. 1A, right panel). SHIV-2873Ni was constructed using a previously identified late stage virus, SHIV-1157ipd3N4, as backbone (Fig. 1B). After exchanging *env* inserts, the resulting chimera, SHIV-

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2873Ni, contained most of gp120, the entire extracellular domain, the transmembrane region and part of the cytoplasmic tail of gp41 of the primary isolate HIV2873i.

Next, we assessed coreceptor usage of SHIV-2873Ni and SHIV2873Nip; as control, we also included SHIV-2873i, a virus that encodes the standard SIVmac239 LTRs with only a single NF-κB site/LTR. These viruses did not replicate in any cell line lacking CCR5, including CEMx174-GFP, U87.CD4, U87.CD4.CCR1, U87.CD4.CCR2, U87.CD4.CCR3, U87.CD4.CXCR4, GHOST-BOB and GHOST-BONZO cells (Fig. 2A). We observed productive infection only in U87.CD4.CCR5 cells (Fig. 2B), suggesting that SHIV-2873Ni exclusively used CCR5 as coreceptor for entry.

Replication of SHIV-2873Ni in RM PBMC. We next sought to evaluate the growth of parental SHIV-2873Ni in PBMC from six randomly selected naïve Indian RM donors. This virus replicated in PBMC from three donors (RQz, N713, and RCa-3) out of the six naïve donors tested (data not shown) with peak p27 production in supernatant observed between days 17-21. These data implied that despite the introduction of extra NF-kB binding sites into the LTRs to increase viral replicative capacity (51), the new SHIV strain still needed to undergo adaptation for optimal replication in RM.

Adaptation of SHIV-2873Ni to RM and generation of SHIV-2873Nip. Rapid animal-to-animal passage of whole blood at the time of peak viremia (week 2 post-inoculation) was used to adapt the new SHIV-C strain. This adaptation strategy selects viruses for improved replication fitness in the new host species without favoring neutralization escape variants since neutralizing antibody responses typically take many weeks to mature. SHIV-2873Ni was passaged in five Indian-origin RM (Fig. 3A). The initial cell-free SHIV-2873Ni viral stock was prepared in rhesus monkey PBMC that were exposed to cell-free supernatant of 293T cells

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transfected with proviral DNA. The first macaque, RBl-9, was inoculated intravenously with 10 ml of SHIV-2873Ni stock; peak viremia reached 2.5 x 10^5 RNA copies/ml at week 2 post-inoculation (Fig. 3B). Four additional animals were subjected to serial blood transfer (Fig. 3A), in which 10 ml of whole blood collected at week 2 post-inoculation was directly transferred into the following recipients: RAg-9, RAi-8, RWa-9, RNt-9 and RQc-9. Animal RWa-9 had been previously exposed to supernatant of 293T cells transfected with SHIV-2873Ni proviral DNA, but had remained uninfected. After receiving infected blood from RM RAi-8, animal RWa-9 became infected as did all monkeys enrolled in the serial virus passage described in Fig. 3A. All six animals seroconverted (data not shown). The passaged virus reached the highest peak viremia level in the last recipient, monkey RQc-9. We reisolated a virus about 1 year later from monkey RNt-9, the penultimate virus recipient in the adaptation schema. This animal was persistently viremic and had signs of disease progression at that time. The passaged virus, SHIV-2873Nip, is an uncloned biological isolate that was able to replicate in PBMC of 10 out of 10 RM donors (data not shown), indicating its adaptation to the new host species, although there was donor-to-donor variability in virus replication expected for outbred animals.

Phylogenetic analysis. Primary full-length *env* genes were amplified from genomic DNA by PCR, cloned into the expression vector pcDNA6/B, and tested for infectivity. Infectious *env* genes of the various SHIV strains were sequenced and phylogenetic analysis was performed using Clustal W and Paup. The *env* genes of the newly created SHIV strains clustered with HIV-C; among the strains tested, the closest relationship was found with our other set of SHIV-Cs derived from a pediatric HIV-C strain isolated from an infant of the same cohort of HIV-infected mothers/infants followed prospectively at the University Hospital in Lusaka, Zambia. Thus, the proximity of the SHIV-2873Nip and SHIV-1157ipd3N4 *env* genes on the

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phylogenetic tree reflects the closeness of HIV-C strains circulating within the same community (Fig. 4A). During *env* evolution in the SHIV-infected RM, the genes diverged as expected for chronically infected hosts (Fig. 4B).

Evolution of SHIV-2873Nip Env during adaptation. Sequence analysis of SHIV-2873Nip gp160, cloned from genomic DNA of RNt-9 PBMC collected at about 1 year post-blood transfer, demonstrated a number of mutations. Compared with the parental Env sequence, SHIV-2873Ni, the SHIV-2873Nip consensus sequence revealed 14 point mutations throughout gp160, a 5-amino acid (aa) deletion at the end of V4, as well as a 3 aa deletion at the beginning of the V5 region (Fig. 4B).

SHIV-2873Nip exclusively uses CCR5 as coreceptor. We assessed the coreceptor usage of SHIV-2873Nip as described in Fig. 2B. We observed productive infection only in the CCR5-expressing cell line suggesting that SHIV-2873Nip maintained R5 tropism after rapid animal-to-animal passage.

Susceptibility of SHIV-2873Ni and SHIV-2873Nip to neutralization by human nmAbs. If SHIV-2873Nip is to become a useful tool to assess vaccine efficacy against HIV-C, maintaining a neutralization-sensitive Env structure will be important for its use as challenge virus. First, we determined the susceptibility of SHIV-2873Ni and the passaged SHIV-2873Nip to the broadly reactive human nmAbs IgG1b12, 2G12, 2F5 and 4E10 in human PBMC; the 50 percent inhibitory concentrations (IC_{50}) were compared to those of SHIV-1157ipd3N4 (45), an infectious molecular clade C SHIV clone created by our group earlier. These nmAbs recognize conserved epitopes on HIV gp120 and on the extracellular domain of gp41. IgG1b12 targets the CD4 receptor-binding site (63), 2G12 recognizes conserved mannan residues on gp120 (48),

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whereas 2F5, and 4E10 recognize a coiled-coil region on gp41 that plays a crucial role during virus fusion with the cell membrane (41, 54, 64, 65).

SHIV-2873Ni and SHIV-2873Nip were effectively neutralized by IgG1b12, 2F5 and 4E10, but not by 2G12 (Table 1) in PBMC-based assays. The parental SHIV-2873Ni had a neutralization profile that was similar to that of SHIV-2873Nip. The IC_{50} values for SHIV-2873Ni and SHIV2873Nip obtained with nmAbs IgG1b12, 2F5 and 4E10 were generally lower compared to SHIV-1157ipd3N4 (Table 1). Similar results were obtained using RM PBMC in the neutralization assays. We could not assess the neutralization sensitivity of SHIV-2873Ni in RM PBMC, since this parental, non-adapted virus did not replicate in the RM donor PBMC pool tested. Of note, none of the SHIV-Cs tested were susceptible to nmAb 2G12. Its epitope includes N-linked mannan moieties associated with the five residues N295, N332, N339, N386 and N392, with glycans attached to N295, N332 and N392 contributing to a core epitope (47, 48). Only four of these five residues are present in SHIV-2873Ni and SHIV-2873Nip Env sequences, and the crucial N295 residue was substituted by T295. The 2G12 epitope has been found to be missing in many primary HIV-C isolates (24). Compared with the linear motif NWFDIT recognized by 4E10, two aa (S vs D and S vs T) of this epitope were different in the predicted SHIV-2873Ni and SHIV-2873Nip Env sequences. In addition, these residues are not among the crucial residues of this epitope (64). With regards to the linear epitope ELDKWA recognized by 2F5, ALDSWN was found in both SHIV-2873Ni and SHIV-2873Nip with three residue substitutions compared to the standard epitope (4). The DSW motif, instead of DKW, may affect 2F5 binding efficiency.

Susceptibility of SHIV-2873Nip to neutralization by autologous and heterologous RM plasma/sera. To test the susceptibility of SHIV-2873Nip to neutralization by polyclonal

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antibodies, we performed a series of PBMC and TZM-bl-based neutralization assays with autologous and heterologous RM plasma or serum samples. Autologous RM plasma samples were tested from monkey RNt-9 at the time of virus isolation and 10 months later. Virus was tested in the presence of plasma from naïve controls and experimental animals; the ratio of the two values was used to calculate the percent inhibition. SHIV-2873Nip could be neutralized by autologous plasma (Table 1) and the neutralizing antibody (nAb) titers in the RM increased with time. Sera from RM chronically infected with an earlier form of SHIV-1157ipd3N4 potently neutralized SHIV-2873Nip (Table 1, data shown only for 2 RM). Similar results were obtained using the TZM-bl neutralization assay (data not shown).

Neutralization Tier assignment. Recently, a Tier system has been developed to differentiate the neutralization sensitivity of primary HIV or SHIV strains (30). Tier 1 strains are noticeably neutralization sensitive; Tier 2 strains are more difficult to neutralize and represent average sensitivity for primary isolates. To assess the neutralization sensitivity, we have tested SHIV-2873Nip against a panel human nmAbs and polyclonal sera collected from HIV^+ individuals in TZM-bl cells. According to the data in Table 2, SHIV-2873Nip was classified as Tier 1 virus and our "late-stage" virus, SHIV-1157ipd3N4, falls into Tier 2. $SHIV_{SF162P3}$ and $SHIV_{SF162P4}$, which had been classified previously as Tier 2 and Tier 1 viruses, respectively, were used as comparison in the assay.

Mucosal transmissibility of SHIV-2873Nip. Since approximately 90% of all new HIV infections are acquired by mucosal exposure during sexual contact or via MTCT, candidate AIDS vaccines should protect against mucosal virus challenge. Preclinical vaccine safety and efficacy studies in primate models should thus focus on mucosal virus challenges (reviewed in (59). We sought to test whether SHIV-2873Nip could be transmitted mucosally. A large stock

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of SHIV-2873Nip was generated in RM PBMC. To demonstrate mucosal transmissibility, we inoculated one monkey each by the oral and i.r. routes. Both animals showed robust viral replication during the first two weeks of post-inoculation (Fig. 5A). Next, we sought to determine whether SHIV-2873Nip could lead to systemic infection after repeated weekly low-dose i.r. challenges; we set 5 weekly inoculations as maximum. Of two RM exposed to a weekly dose of 1,500 $TCID_{50}$, one animal became viremic after 3 inoculations, whereas the second one did not and was subsequently given a single dose of 30,000 $TCID_{50}$, which promptly led to high viremia. Another four RM were challenged weekly with 5,000 $TCID_{50}$; all became viremic after 1 or 4 inoculations, respectively (Fig. 5B). These data indicate that SHIV-2873Nip can be transmitted reproducibly by mucosal challenge.

Signs of SHIV-2873Nip pathogenicity. Animal RNt-9, the penultimate recipient during serial passage, developed thrombocytopenia at 36 weeks post-inoculation; the platelet count never recovered (Fig. 3C). At 92 weeks post-inoculation, the monkey was found unresponsive with epistaxis, tachypnea, and an abnormal pulmonary exam by auscultation. At necropsy, severe multifocal cerebral hemorrhages were noted as well as extensive petechial or multifocal ecchymotic hemorrhages involving the myocardium, stomach, liver, cecal mucosa, both lungs and the bladder. The stomach also showed severe ulceration. Clearly, the monkey had developed a fatal thrombocytopenia. Although the absolute number of peripheral blood $CD4^+$ T cells remained normal, the $CD4^+CD29^+$ memory T-cell subset was persistently low from 26 weeks post-inoculation onwards (Fig. 3D). Although most lymph nodes were normal at necropsy, splenomegaly was observed.

To assess the effect of SHIV-2873Nip on gut lymphocytes, all six of the mucosally inoculated RM underwent rectal biopsies between weeks 6 and 12 p.i; animal RNt-9 was

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subjected to rectal biopsy at week 84 p.i. (eight weeks before the fatal cerebral hemorrhage). We observed significant depletion of gut $CD4^+$ T cells in all monkeys compared to uninfected controls (n = 7; $P = 0.0006$; Mann-Whitney test, Fig. 5C). In contrast, no statistically significant differences were noted in the blood (Fig. 5D); thus far, all of the SHIV-2873Nip-infected monkeys have maintained normal absolute $CD4^+$ T-cell counts. However, four of the SHIV-2873Nip-infected animals (RWl-11, RAb-11, RTb-11, RMs-11) demonstrated loss of peripheral blood $CD4^+$ memory T cells as assessed by $CD4^+CD29^+$ double staining (data not shown).

DISCUSSION

Here, we describe the development of a new R5 SHIV-C that encodes an African pediatric HIV-C *env*. The newly constructed molecular clone, SHIV-2873Ni, and the biological isolate, SHIV-2873Nip, have a number of relevant characteristics: 1) SHIV-2873Ni encodes an HIV-C *env* from a pediatric rapid progressor; 2) SHIV-2873Ni was cloned using the SHIV-1157ipd3N4 backbone, which has a deletion in the 3' end of HIV *env* that restored the original SIV *env* C-terminus extending into *nef;* 3) SHIV-2873Ni and its passaged progeny contain extra NF-kB sites; 4) SHIV-2873Nip retained exclusive R5 tropism; 5) SHIV-2873Nip showed signs of pathogenicity (memory T-cell depletion, loss of $CD4^+$ T cells in rectal tissues, and severe, fatal thrombocytopenia); and 6) SHIV-2873Nip was mucosally transmissible and neutralization sensitive with a Tier 1 profile.

SHIV-2873Nip encodes the envelope gene of a pediatric HIV-C, the most prevalent strain in the worldwide. Although other SHIV strains ($SHIV_{CHN19}$, $SHIV_{MJ4}$, SHIV-MCGP1.3

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and SHIV-XJ02170) encoding clade C envelopes have been created, they are either dual-tropic (SHIV-MCGP1.3) (4), unable to replicate in rhesus macaque PBMC ($SHIV_{CHN19}$) (5), or were difficult to reisolate post adaptation ($SHIV_{MJ4}$ and SHIV-XJ02170) (37, 60). In contrast, SHIV-2873Nip was mucosally transmissible and was able to replicate vigorously in PBMC of all RM donors tested, indicating effective adaptation to the new host species. Only one other SHIV-C strain, SHIV-1157ipd3N4 (51), is exclusively R5 tropic and highly replication-competent in RM.

Genetic analysis of SHIV-2873Nip *env* showed a 5-aa deletion in the V4 region, a 3 aa deletion at the beginning of V5, and fourteen point mutations throughout gp160. Interestingly, Env was still functional in the pseudotype virus assays in *vitro* despite these deletions. In all monkeys infected mucosally, SHIV-2873Nip replicated vigorously (peak vRNA levels 2.1×10^5 to 9.5×10^6 copies/ml). Since most new HIV infections worldwide are acquired mucosally, mucosal transmission should be employed to assess vaccine efficacy. Clearly, SHIV-2873Nip showed reproducible mucosal transmissibility.

SHIV-2873Nip showed clear signs of pathogenicity within a few months post-inoculation. Studies with SIV-infected RM and HIV-infected humans have documented that acute infection is accompanied by a marked depletion of $CD4^+$ memory T cells, primarily in mucosal tissues (3, 31, 57). We observed loss of memory $CD4^+CD29^+$ T cells in peripheral blood and depletion of $CD4^+$ T cells in the gut tissues of all SHIV-2873Nip-infected RM. This SHIV-C targets memory $CD4^+$ T cells, whereas the acutely pathogenic, X4- or dual tropic SHIVs that have been used frequently in vaccine efficacy studies in non-human primate models predominantly affect naïve $CD4^+$ T cells (16, 39) and induce precipitous drops in peripheral blood $CD4^+$ T cells that are irreversible in most RM (43, 45, 53). In contrast, SHIV-2873Nip does not induce acute, severe lymphocyte depletion, suggesting that this new R5 SHIV-C

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exhibits biological characteristics that mimic HIV disease progression in humans. Another R5 SHIV that encodes an HIV clade B *env,* $SHIV_{SF162P3}$, also induces gradual $CD4^+$ T-cell loss and causes AIDS in some but not all rhesus macaques (15). Recently, Pahar et al. (40) using repeated low-dose vaginal $SHIV_{SF162P3}$ challenges observed control of viremia in most animals with modest depletion of the memory $CD4^+$ T-cell subsets. In our repeated low-dose i.r. challenge approach, SHIV-2873Nip led to statistically significant depletion of $CD4^+$ T cells in the gut.

SHIV-2873Nip induced fatal thrombocytopenia in one RM. Thrombocytopenia is a known complication of lentiviral infection and has been associated with all stages of HIV infection in humans (reviewed in (18) as well as SIV and SHIV infection in macaques (reviewed in (17, 33, 51). A relatively recent population-based study examined the association between AIDS and strokes; a strong link was found with both intracerebral hemorrhages and ischemic strokes (52). An earlier report described an association between thrombocytopenia and the development of intracerebral hemorrhages in patients with AIDS (42).

Our data indicate that SHIV-2873Nip is sensitive to neutralization by human nmAbs, polyclonal sera of SHIV-C-infected RM and HIV-C-infected humans as well as HIVIG, which had been generated from HIV clade B-infected individuals. SHIV-2873Nip was more neutralization sensitive than SHIV-1157ipd3N4. This is probably due to the fact that we reisolated SHIV-2873Nip approximately a year after RM RNt-9 became infected. In contrast, SHIV-1157ipd3N4 was generated from a virus reisolated after a significantly longer period of time from an infected RM that had progressed to AIDS 2.7 years post-infection; SHIV-1157ipd3N4 was clearly a neutralization escape virus (51). According to an intriguing study, recently transmitted HIV-C isolates were surprisingly neutralization sensitive compared to donor

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virus strains (10, 24). Among discordant couples, newly infected individuals harbored more neutralization-sensitive viruses compared with the strains that predominated in their infected partners when tested against contemporaneous donor plasma, suggesting that a bottleneck effect during or shortly after sexual transmission favored neutralization-sensitive HIV-C quasispecies. The newly transmitted HIV-C strains had significantly fewer N-linked glycosylation sites and shorter variable loops compared to the strains that predominated in the infected source persons (10, 24).

Of note, SHIV-2873Nip was classified as a Tier 1 virus based upon its susceptibility to neutralization by polyclonal sera collected from HIV-C-infected individuals, HIVIG and nmAbs; this high neutralization sensitivity will be useful in the development of nAb-response-based AIDS vaccine concepts. To date, the induction of sufficient nAb levels with extended breadth has been a major hurdle. We suggest that initial vaccine efficacy testing should make use of a Tier 1 SHIV challenge virus in primate models. If protection is achieved, subsequent vaccine development steps could then use progressively more difficult-to-neutralize SHIV strains in primates. SHIV-2873Nip will be useful tool to evaluate vaccine candidates that seek to induce anti-HIV-C nAb responses, and as of today, it is the only SHIV-C Tier 1 virus described.

Our in vivo data indicate that SHIV-2873Nip is a highly replication-competent R5 SHIV-C. We have previously generated SHIV-1157ipd3N4, a Tier 2 R5 SHIV-C that is pathogenic and induces AIDS (51), although disease progression has been slow. Nevertheless, SHIV-1157ipd3N4 has been used successfully to assess the efficacy of a multigenic DNA prime/protein boost and a multigenic protein-only vaccine (44). The following parameters have been used as read-outs of vaccine efficacy: complete protection from systemic viral infection, as well as delay and lowering of peak viremia. We posit that the same strategy can be applied to challenge

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studies involving the Tier 1 SHIV-2873Nip. In addition, protection from depletion of gut $CD4^+$ lymphocytes could serve as measurement of vaccine efficacy. Prolonged, prospective follow-up will reveal whether SHIV-2873Nip is more pathogenic than SHIV-1157ipd3N4.

In summary, the R5 SHIV-2873Nip is a highly replication-competent, mucosally transmissible Tier 1 SHIV-C that will be a useful tool to study viral pathogenesis and to assess the efficacy of immunogens targeting HIV-C Env and testing of vaccine candidates that seek to induce anti-HIV-C nAb responses.

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Figure legends

FIG. 1. Construction of SHIV-2873Ni. (A) Identification of an infectious *env* clone. Primary full-length *env* genes were PCR amplified from genomic DNA of normal human donor PBMC cocultured with infected PBMC of Zambian infant 2873i collected at 2 months of age and cloned into the expression vector pcDNA6/B. Infant 2873i was a rapid progressor who died of AIDS-related disease within 1 year of birth to an HIV-C-positive mother. To identify an infectious envelope, the resultant constructs were cotransfected into 293T cells with HIV-1 ΔEN, an infectious HIV provirus deleted in *env* and *nef* and encoding GFP in lieu of *nef*. The resulting pseudovirus released into cell supernatants was used to infect CEM.NKR.CCR5 cells, which were screened for GFP expression. HIV-1 ΔEN cotransfected with ADA *env* was used as positive control. (B) Construction of SHIV-2873Nip. SHIV-1157ipd3N4 (51) was used as backbone. The 2.2 kb KpnI (K) - BamHI (B) fragment of HIV2873i (spanning most of gp120, the entire gp41 extracellular domain, the transmembrane region (TM), and part of the cytoplasmic domain) was amplified to replace the corresponding region of the proviral backbone. The modified 3'-half was ligated with the 5' half of SHIV-vpu$^+$ proviral DNA to form full-length SHIV-2873Ni. NN, 2 NF-κB sites are present in the 3'LTR; during viral replication, this duplication is copied into the 5'LTR also.

FIG. 2. Coreceptor usage of SHIV-2873i, SHIV-2873Ni and SHIV-2873Nip. U87.CD4.CCR5 cells and U87.CD4.CXCR4 were exposed to SHIV-2873Ni, SHIV-2873Nip, $SHIV_{SF162}$ (HIV clade B *env*, R5), and SHIV-vpu$^+$ (HIV clade B *env,* X4). The levels of p27 Gag were measured in the supernatants as indicated. SHIV-2873i contains the standard SIVmac239 LTRs with only a single NF-κB site/LTR; it was built using SHIV-vpu$^+$ as backbone.

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FIG. 3. Serial passage of SHIV-2873Ni in rhesus macaques. (A) SHIV-2873Ni was passaged in five Indian-origin RM through serial blood transfer. Animal RWa-9 had been exposed previously to SHIV-2873Ni but had remained uninfected. After receiving blood from donor RAi-8, RM RWa-9 and all monkeys shown in Fig. 2A became infected. (B) Viral loads were measured after serial passage at the time points indicated. †, monkey RNt-9 developed fatal cerebral hemorrhages due to severe thrombocytopenia at week 92 post-inoculation. (C and D) absolute $CD4^+$ T-cell numbers, platelet counts and $CD4^+CD29^+$ memory T cells were assessed during the course of the infection. The dashed line denotes the lowest normal value (10%) for the $CD4^+CD29^+$ T cells.

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FIG. 4. Phylogenetic tree showing the relationship between SHIV-2873Ni and SHIV-2873Nip Env sequences and those of other primary strains of HIV. Phylogenetic trees were constructed from full-length Env sequences by using the Neighbor-joining method. Major subtypes of HIV group M were used as reference sequences; sequences from SHIV-1157i, SHIV-1157ip, SHIV-1157ipd3N4 (51) and HIV1084i (14) were also included, since HIV1084i and the *env* genes in these SHIVs had been derived from the same cohort of infected mothers and their infants in Lusaka, Zambia. The scale bar indicates the genetic distance along the horizontal branches, and the numbers at the nodes are bootstrap values. B. Evolution of SHIV-2873Nip Env during passage and replication in monkey RNt-9. The deletions of 5 and 3 aa at the end and beginning of V4 and V5 domains of gp120, respectively, in the adapted SHIV-2873Nip are shown. The Env consensus sequence for SHIV-2873Nip was derived by sequencing 8 individual clones encoding infectious *env* genes.

FIG. 5. Oral and intrarectal inoculation of SHIV-2873Nip. Two monkeys (RBg-10 and RUf-10) were inoculated orally or intrarectally, respectively, with SHIV-2873Nip stock. (B) Six monkeys were used in a repeated low-dose i.r. titration; the aim was to find a virus dose leading to systemic infection (defined as viral RNA $\geq 10^4$ copies/ml) after a maximum of five weekly i.r. inoculations. Monkeys remaining uninfected at week 2 after the 5^{th} weekly low-dose virus challenge were given a single high-dose SHIV-2873Nip dose (30,000 $TCID_{50}$). Viral loads were measured at the time points indicated. The horizontal dotted line indicates lower limit of detection (<50 viral RNA copies/ml). (C and D) Estimation of $CD4^+$ T-cell loss in blood and gut: comparison of $CD4^+$ T cells in rectal biopsy specimens (collected between weeks 6 and 12 after the last, successful inoculation) and blood of RM inoculated with SHIV-2873Nip compared with

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uninfected controls. The asterisk (∗) in panel C designates the percent $CD4^+$ T cells in rectal mucosa of monkey RNt-9 collected at week 84 post-inoculation.

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TABLE 1. Neutralization of clade C SHIV strains in human and rhesus monkey PBMC

Source of Abs (human nmAb or RM name)	IC_{50} (µg/ml or 1/x dilution)					
	SHIV-2873Ni*		SHIV-2873Nip*		SHIV-1157ipd3N4*	
	Human PBMC	RM PBMC	Human PBMC	RM PBMC	Human PBMC	RM PBMC
Human nmAbs						
IgG1b12	0.36	ND	0.4	0.03	1.3	0.48
2G12	>40	ND	>40	>40	>40	>40
2F5	0.32	ND	0.3	0.18	0.67	2.0
4E10	0.035	ND	0.035	0.05	0.15	0.43
4x	0.40	ND	0.09	0.05	0.15	0.19
Polyclonal RM plasma/sera						
autologous plasma:						
RNt-9 (time of virus isolation)	ND	ND	662	ND	ND	ND
RNt-9 (10 mos post virus isolation)	ND	ND	1,036	ND	ND	ND
heterologous sera:						
RHy-9 (SHIV-1157ipd3N4-infected RM)†	ND	ND	3,722	ND	>10,240	ND
RJa-9 (SHIV-1157ip-infected RM)	ND	ND	2,048	ND	>10,240	ND

*All three viruses lack the 2G12 epitope; 4x, quadruple combination of IgG1b12, 2G12, 2F5 and 4E10 at a 1:1:1:1 ratio.
ND, not determined; †, RHy-9 was described by Rasmussen et al. (44); RM, rhesus monkey.

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TABLE 2. Sensitivity of R5 SHIV strains to soluble CD4, human nmAbs and serum samples

SHIV strain	IC_{50} (μg/ml) in TZM-bl cells[1]						IC_{50} (reciprocal serum dilution) in TZM-bl cells[1]							***Tier***
	clade	sCD4	IgG1 b12	2G12	2F5	4E10	HIVIG	BB7	BB55	BB68	BB75	BB80	BB81	BB87	
SHIV-2873Nip (Zambian env; early isolate	C	0.1	0.5	>25	>25	>25	16.8	340	876	509	716	1,273	157	876	1
SHIV-1157ipd3N4 (Zambian env; late isolate	C	0.4	7.0	>25	>25	>25	1,160	105	131	86	79	72	47	260	2
$SHIV_{SF162P4}$	B	<0.01	15.7	15.7	1.5	0.7	25	363	2,543	328	615	828	211	360	1
$SHIV_{SF162P3}$	B	6.0	>25	>25	>25	>25	1,505	24	65	22	27	25	<20	180	2

Spectrum of neutralization sensitivity of R5 SHIV strains encoding HIV clade B or C *env*. [1]Values represent the concentration (μg/ml for soluble CD4 (sCD4) and human nmAbs IgG1b12, 2G12, 2F5, 4E10, or HIVIG) or the dilution (for serum samples) at which relative luciferase units (RLU) were reduced 50% compared to virus control wells. BB47, BB55, BB68, BB75, BB80, BB81, and BB87 are serum samples from individuals infected with HIV-1 clade C. HIVIG, polyclonal high-titer anti-HIV Ig preparation.

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Fig. 1

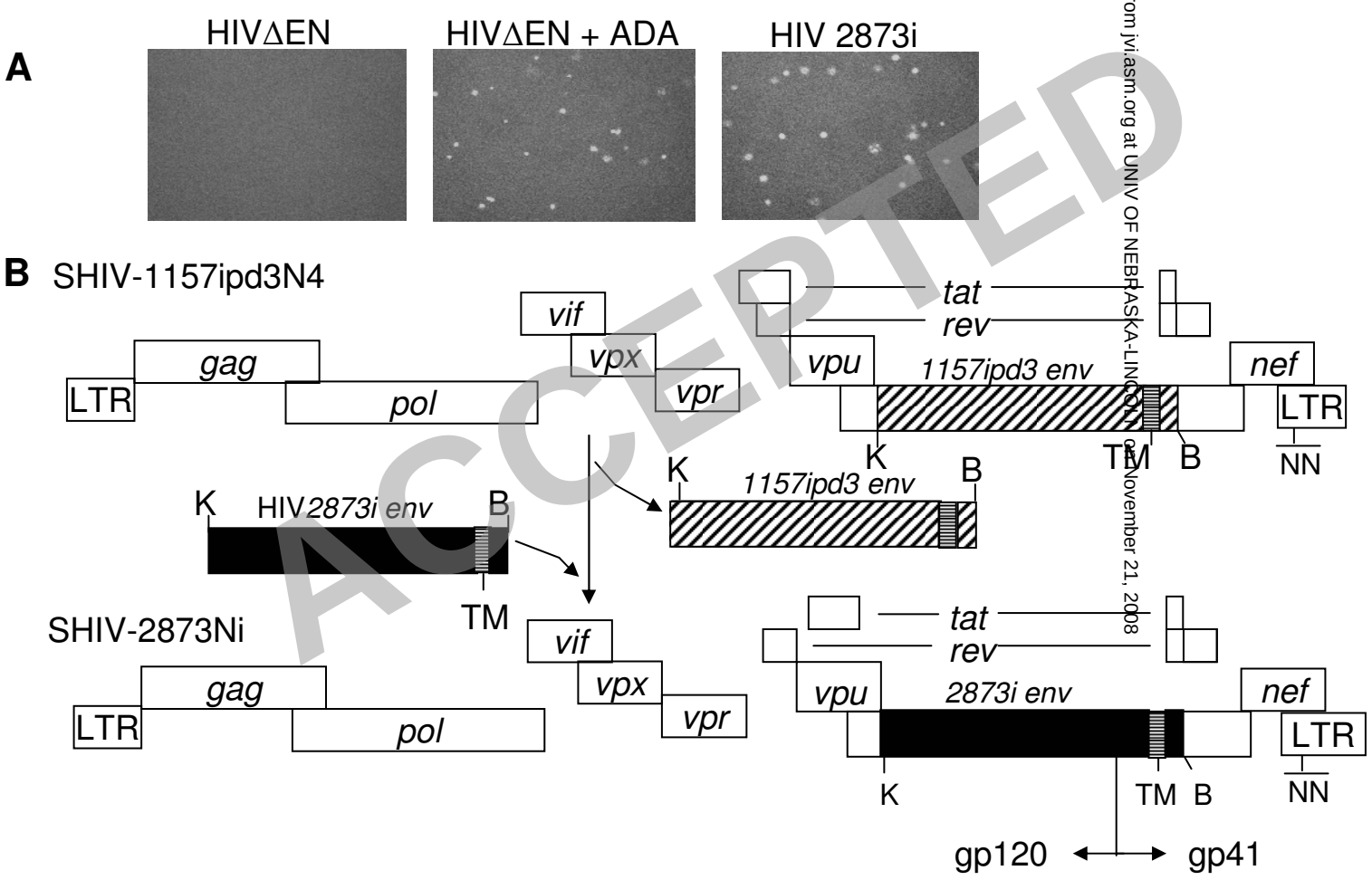

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Fig. 2

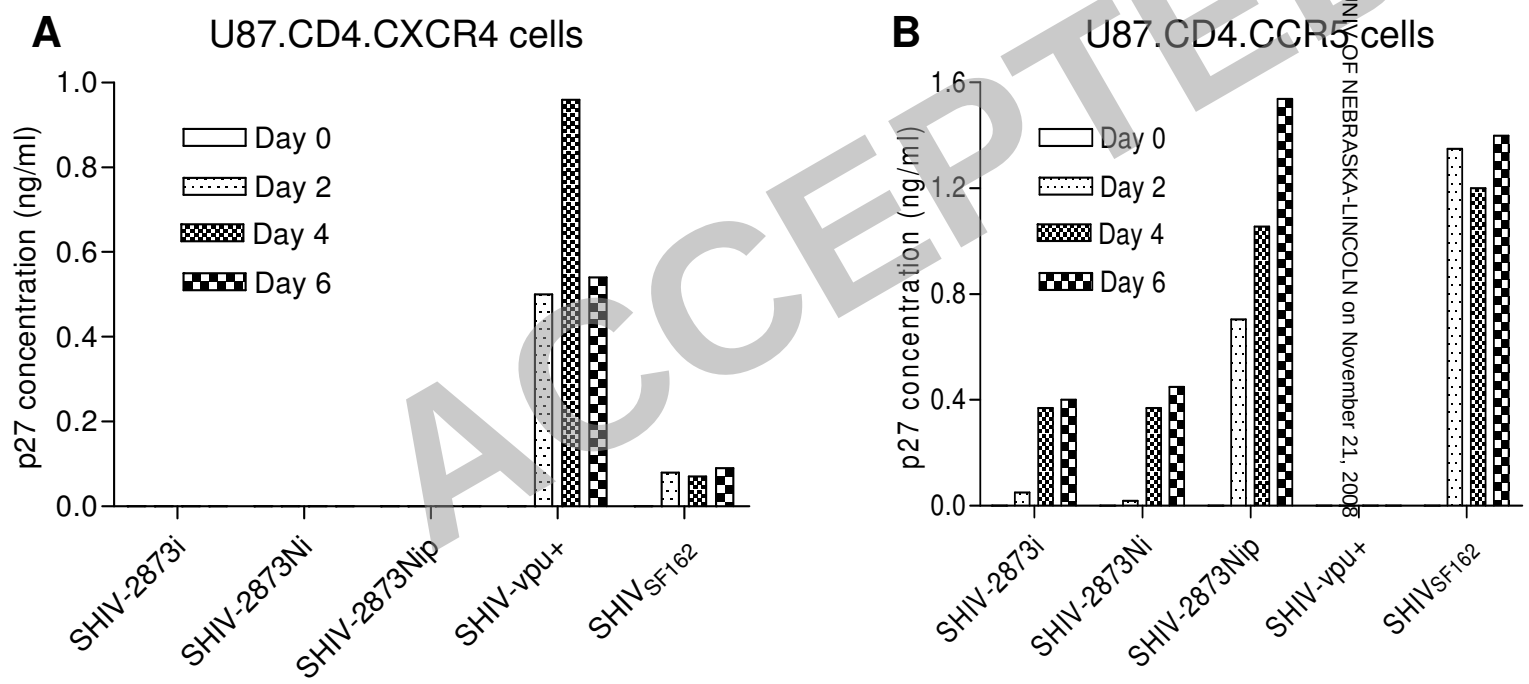

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Fig. 3

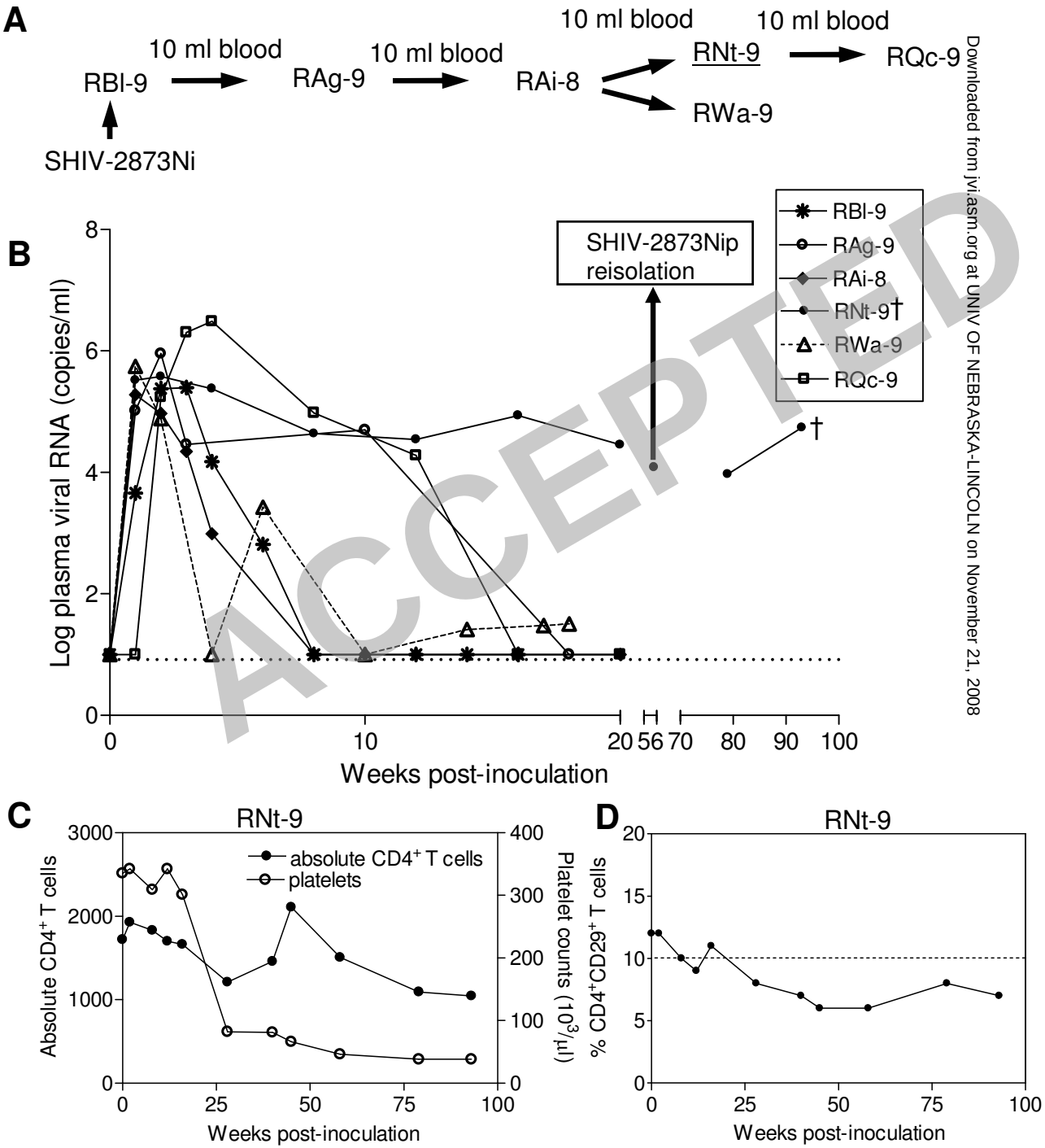

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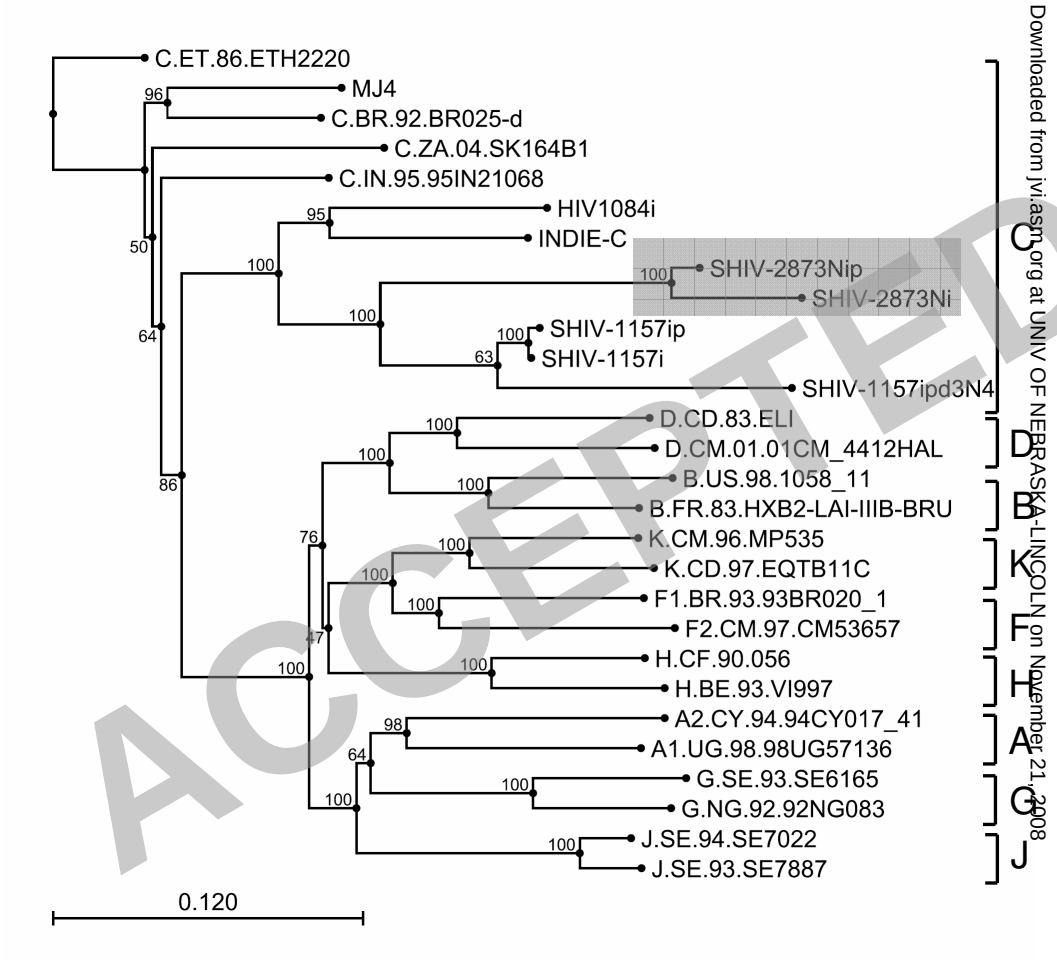
Fig. 4



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B gp120

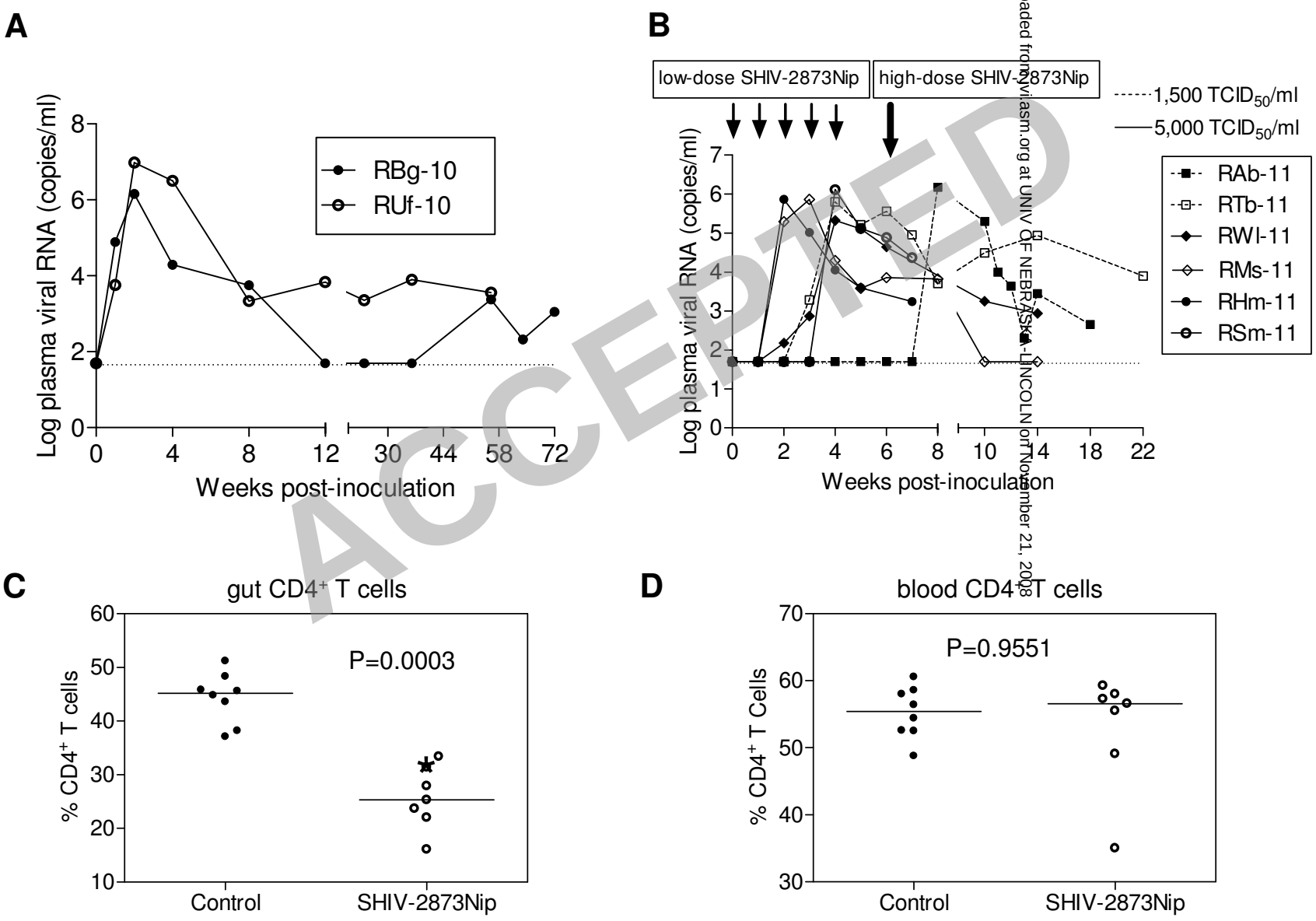
V4

SHIV-2873Ni EFFYCNTSSLFNHIYNSSGRDPYNVSHSNASET
SHIV-2873Nip .....EK.....-----..

V5

SHIV-2873Ni PIAGDITCTSNITGLLLTRDGGNNSNTEENSTQ
SHIV-2873Nip .....---.....

Fig. 5



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