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Early short-term treatment with neutralizing human monoclonal antibodies halts SHIV infection in newborn macaques

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Abstract

Prevention of mother to child transmission (MTCT) of HIV remains a major objective where antenatal care is not readily accessible. We tested anti-HIV-1 human neutralizing monoclonal antibodies (NmAb) as post-exposure therapy in an infant macaque model for intrapartum MTCT. One-month-old rhesus macaques were inoculated orally with $SHIV_{SF162P3}$. On days 1, 4, 7, and 10 after virus exposure, we injected animals subcutaneously with NmAbs and quantified systemic distribution of NmAbs in multiple tissues within 24 h following administration. Replicating virus was found in multiple tissues by day 1 in animals without treatment. All NmAb-treated macaques were free of virus in blood and tissues at 6 months post-exposure. We detected no anti-SHIV T cell responses in blood or tissues at necropsy, and no virus emerged following $CD8^+$ T cell depletion. These results suggest early passive immunotherapy can eliminate early viral foci and thereby prevent the establishment of viral reservoirs.

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AUTHOR CONTRIBUTIONS

Studies were designed and planned by N.L.H. and A.J.H.; experimental work was done by A.J.H, J.P.J., E.E., K.M., S.P., J.R., W.F.S., K.B.H., T.A.C., P.T.B., A.L., S.P., X.C., K.W., D.S., D.B.; pathology was described by A.L.; veterinary care was provided by J.S.; A.J.H., N.L.H, J.B.S, J.R.M., and B.S.G. wrote the manuscript; A.J.H., J.P.J., E.E., C.K, V.M.H., A.P., J.B.S., N.L.H. analyzed the data; B.S.P. performed statistical analyses.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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Recent advances in the discovery of human HIV neutralizing monoclonal antibodies (NmAbs) with high potency and breadth of coverage have rekindled an interest in their use as pre-exposure prophylaxis as well as therapeutic agents, including in the setting of mother-to-child transmission (MTCT), where the time of exposure is known[1,2]. A combination of measures, including antiretroviral treatment (ART) of the mother and the infant, Caesarean section, and formula feeding, have diminished the rate of MTCT from 35% to less than 3%[3]. Despite this reduction, HIV infects approximately 200,000 children yearly, primarily where ART is not available[4]. Treatment of babies with ART during both the early peripartum and breastfeeding timeframes is recommended[5], but risks remain, including toxicities associated with long-term use and the development of drug resistant viral variants[6]. Therefore, discovering less toxic methods to limit transmission to newborns would be advantageous[2].

In mucosal HIV and SIV transmission, the virus establishes a small founder population of infected cells once it has traversed the vaginal mucosal barrier[7]. This localized infection undergoes rapid expansion and spreads to local draining lymph nodes (LN), before disseminating systemically by 1 week post exposure[8,9]. Similarly in NHP models of oral SIV exposure, the oral and esophageal mucosa and tonsils are sites of early viral infection within 1 day post-exposure, with rapid systemic dissemination *via* the regional lymphatics occurring within 1 week post-exposure[10,11]. Because IgG from the circulation contributes significantly to the immunoglobulin pool in tissue and genital tract secretions, passively transferred neutralizing antibodies (NAbs) may exhibit their protective effect by interaction with the virus at the mucosal level[12], thus preventing systemic spread. In adult nonhuman primate (NHP) models of mucosal SHIV transmission, there is abundant evidence for protective prophylactic efficacy with passively transferred human NmAbs[13–18]. *In vitro*, NmAbs have been shown to block HIV infection of dendritic cells and subsequent transmission to T cells[19]. Direct vaginal application of NAbs prior to challenge is protective in macaques[20], and in HIV exposed but uninfected humans mucosal IgA can block transcytosis *in vitro*[12]. Vaccine-induced protection from vaginal challenge correlated with levels of gp41-specific cervicovaginal IgA and IgG with antiviral and transcytosis blocking activities[21]. However, tissue localization and kinetics of passively transferred antibodies are still not well-defined[13,22].

There is evidence for impact of NmAbs on the plasma virus in established infections in NHP models[23–25] and in humans[25,26]. In NHP models, post-exposure prophylaxis using cocktails of first generation human NmAbs b12, 2G12, 2F5 and 4E10 partially prevented oral SHIV infection in newborns[24]. A single dose combining the newer, more potent NmAbs VRC07-523 and PGT121 delivered 10 days after intravenous SHIV infection suppressed acute viremia and limited seeding of viral reservoirs in adult macaques [27]. We have shown that neutralizing polyclonal IgG purified from SIV- or SHIV-infected macaques and given subcutaneously (s.c.) can effectively control viremia and accelerate B cell responses, resulting in reduced pathogenesis in SIV-infected adults[28] and in SHIV-infected newborn macaques[29,30]. We hypothesized that a cocktail of two potent and broadly cross-reactive NmAbs, VRC07-523 and PGT121, would slow the initial virus expansion and reduce the chance of rapid escape in newborn macaques exposed to pathogenic SHIV. We show that combined doses as low as 10 mg/kg beginning at 24 h post-exposure can intercept

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replicating viral foci established by day one and prevent orally administered virus from establishing permanent viral reservoirs.

RESULTS

Titration and biodistribution of s.c. antibodies in macaques

We initially conducted studies: (1) to define the protective dose and kinetics of the CD4 binding site-directed NmAb VRC01 to block oral $SHIV_{SF162P3}$ infection in newborn macaques after s.c injection, and (2) to determine the kinetics of passively transferred IgG in naive and infected macaques. First, we administered VRC01 to a total of seven male and female one-month-old macaques at 20 mg/kg ($n = 2$) or 5 mg/kg ($n = 5$) 24 h before SHIV exposure. We measured Envelope-specific binding and neutralizing Ab kinetics *in vivo*. The time to maximal concentration in the plasma was 24 h, independent of dose, and the serum (plasma) half-life of VRC01 was 3.9 – 4.2 days (Supplementary Fig. 1). Neither of the two 20 mg/kg dose animals and only one of five 5 mg/kg macaques became infected. In this macaque, the magnitude and kinetics of plasma virus, termed plasma virus load (PVL), were indistinguishable from that of control animals treated with IgG purified from naive macaques[30] (Supplementary Fig. 1). These data are consistent with results from VRC01 passive protection studies in juvenile and adult macaques[18] and guided the therapeutic range for infant macaques.

Next, in a separate study designed to determine if the kinetics of passively transferred IgG is altered in the presence of viral antigen, we assessed the distribution of purified SIVIG (polyclonal Ig from SIV-infected macaques) in four male and female macaques, comparing SIVIG kinetics in SIV-infected macaques to naive macaques. SIVIG was rapidly distributed in plasma and tissues of infected and naive animals. (Supplementary Fig. 2). We used *in situ* hybridation to localize SIV in tissue samples collected at 24 h and at 2 weeks after SIV_{smE660} oral challenge. SIV was undetectable in 24 h tissue samples, but was detectable by 2 weeks in tissues both adjacent to and distant from the site of challenge (Supplementary Fig. 3). Thus, IgG delivered s.c. is rapidly and widely distributed and is unimpeded by viral antigen.

NmAb cocktail immunotherapy in the presence of SHIV

We next assessed the effectiveness of HIV-1 NmAbs as post-exposure prophylaxis in one-month-old newborns inoculated orally with $SHIV_{SF162P3}$. For *in vivo* therapy, we tested a cocktail of VRC07-523 and PGT121, two potent NmAbs that target different regions of the HIV-1 Envelope and that have been shown to be additive *in vitro*[31]. VRC07-523 is an engineered clonal relative of VRC01 with increased neutralization of most HIV strains and improved *in vivo* protection capabilities[32]. Therefore we used VRC07 in lieu of VRC01 for these therapeutic studies. PGT121 interacts with variable regions and glycans of HIV-1 gp120[33,34] and protected adult macaques from mucosal challenge at very low plasma titers[35]. Cocktails of PGT121 and VRC07-523 were prepared at total doses of 10 mg/kg (5 mg/kg each) and 40 mg/kg (20 mg/kg each) and delivered s.c.

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We inoculated twenty one-month-old rhesus macaques orally with $SHIV_{SF162P3}$ on day 0 and followed them for up to 28 weeks to assess virological, immunological, and disease outcomes with or without NmAb treatment starting on day 1. Pairs of animals were killed at days 1, 2, or 14 post-exposure to monitor the development of SHIV $_{SF162P3}$ infection in blood and tissues of treated and untreated macaques (Table 1, Groups 1–4). We delivered NmAbs on days 1, 4, 7, and 10 after SHIV exposure (Fig. 1a and Table 1, Groups 4–6). $SHIV_{SF162P3}$ infection by the oral route in one-month-old macaques results in reproducible, sustained PVL at $>10^7$ copies/ml plasma and $\sim 10^4$ copies per μg DNA for at least 24 weeks in all animals[30]. To conserve animals, historical controls were used as a comparison group for the 24-week follow-up (Table 1, Group 7).

We evaluated the kinetics of the individual NmAbs and the cocktail in plasma from all twelve treated infants that were on study for at least 2 weeks (Table 1, Groups 4, 5, and 6). Peak NmAb cocktail concentrations in plasma occurred by 24 h post s.c. injection in all animals at both NmAb doses. In the four animals that received 10 mg/kg, the average cocktail concentration during the first 2 weeks was 44 μg/ml, and in the eight animals that received 40 mg/kg, it was 113 μg/ml (Fig. 1b). Using reagents designed to specifically detect each NmAb independently, we found that PGT121 concentrations in the plasma were consistently higher at both doses than those of VRC07-523. Multiple dosing prevented calculation of *in vivo* half-lives of each NmAb, but PGT121 was detectable in plasma for 2 weeks longer than VRC07-523 in several macaques. PGT121, administered at 5 mg/kg, was maintained for >20 weeks in the plasma from a single macaque, 33537 (Fig. 1b, bottom left). An unusually slow decay from plasma of passively infused PGT121 has been recently reported where plasma concentrations of 5–20 μg/ml were still present after 10 weeks [27].

We assessed $SHIV_{SF162P3}$ neutralization activity in the plasma of all infant macaques and found it decayed by 6–7 weeks in all animals except 33537, where declining neutralization of $SHIV_{SF162P3}$ was detected at titers of 10^2–10^3 before becoming undetectable at week 20 (Supplementary Fig. 4). Calculation of the average plasma IC_{50} concentration of the NmAb cocktail during the first 2 weeks after SHIV exposure was 0.0134 and 0.0120 μg/mL in the 10 mg/kg and 40 mg/kg groups, respectively, which is close to the IC_{50} (0.0128 μg/mL) obtained from purified NmAbs against $SHIV_{SF162P3}$ in TZM-bl cells (Supplementary Fig. 4).

To measure transudation of NmAb into tissues and to assess neutralization potency within tissues and organs during the first 2 weeks, we extracted specimens from six macaques at different necropsy timepoints. Analysis of the antibody extracted from two macaques (Group 4) that were sacrificed at 14 days after four doses of NmAbs showed that NmAbs were systemically distributed at concentrations from 48 to 700 ng/ml of tissue lysate (Supplementary Table 1). Two macaques (Group 2a) exposed to $SHIV_{SF162P3}$, treated once with a NmAb cocktail dose of 10 mg/kg one day later, and sacrificed on day 2 had NmAbs in tissue lysates in concentrations up to 791 ng/ml.. Two macaques (Group 2b) treated once with 10 mg/kg without SHIV exposure had NmAbs in tissues at levels similar to macaques 34263 and 34290 that were pre-exposed to SHIV. We assessed the neutralizing activity against $SHIV_{SF162P3}$ in tissue homogenates from ~100 mg of necropsy samples from the animals sacrificed at 1, 2, and 14 days post-exposure (Groups 2 and 4). The 50%

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neutralization titers (ID_{50}) against $SHIV_{SF162P3}$ of tissue lysates averaged ~1:50 in the tested samples at 1 day after s.c. NmAb injections, increased to an average of ~1:100 by day 2, and were similar at day 14, with good agreement among titers of animals sacrificed at the same timepoints (Table 2). However, NmAb titers in the colon and reproductive tract from animal 34290 were about three to five times higher than animal 34263, necropsied at the same time point. Tissue-associated IC_{50} concentrations of the NmAb cocktail in samples tested ranged from 0.5 ng/ml to 10.0 ng/ml, near the IC_{50} of the NmAb cocktail (Supplementary Fig. 4). We conclude that the presence of SHIV during the first day after oral exposure did not affect NmAb distribution or levels *in vivo* and that the NmAb cocktail was rapidly distributed to tissues.

$SHIV_{SF162P3}$ dissemination with and without NmAb therapy

To determine the kinetics of $SHIV_{SF162P3}$ in blood and tissues early in infection with and without post-exposure NmAb cocktail therapy, we measured virus in blood from macaques killed on days 1, 2, and 14 after oral $SHIV_{SF162P3}$ exposure (Table 1, Groups 1–4). The two-week timepoint was anticipated to be nearest to time of peak PVL. Plasma viremia in animals necropsied at day 14 without NmAbs was detected by day 4, increased rapidly, and peaked between $1–5 \times 10^8$ copies/ml of plasma (Fig. 2a,), consistent with the VRC01 study (Supplementary Fig. 1) and prior studies[29,30]. In stark contrast, no virus was detected in plasma or peripherial blood mononuclear cells (PBMC) from NmAb-treated animals sacrificed at day 14 (Fig. 2a, b).

We collected multiple tissues at necropsy (Fig. 2c), and in samples collected within 2 days of SHIV $_{SF162P3}$ exposure, we measured low levels of SHIV $_{SF162P3}$ DNA in mucosa and LN, proximal and distal to the oral exposure site (Fig. 2d, e). In comparison, virus was widespread at day 14 in untreated animals (Fig. 2f) and peaked at >3,000 copies/μg of DNA throughout the LN and gut, consistent with levels of DNA in the tissues of adult and six-month-old *M. nemestrina* with high levels of plasma viremia[36]. As seen in the blood, following NmAb treatment on days 1, 4, 7, and 10 at 40 mg/kg, virus was undetectable in any tissues at day 14 (Fig. 2g and Supplementary Table 2). To determine whether the viral DNA-positive tissues contained replicating SHIV, we measured viral RNA in several tissue samples taken from these same macaques sacrificed on 1, 2, or 14 days after virus exposure. Viral DNA and RNA levels, tested as blinded samples from these tissues, show that productive SHIV infection has begun in multiple tissues by day 1, increasing significantly by day 14 (Table 2). However, in two macaques treated with 10 mg/kg on day 1 (Group 2a, sacrificed on day 2) there was no viral RNA detected in the samples tested. Importantly, NmAbs were co-localized in these virus-positive tissues, suggesting the potential for antibody effects as early as 1 day post-treatment. Moreover, the results suggest that virus present early after exposure can be intercepted and cleared by NmAb present in the same tissues (Table 2).

Prevention of productive infection, viral rebound, and pathogenesis

To evaluate the effect of early short-term NmAb therapy on viral control, we monitored SHIV in blood, LN, and tissues in animals followed for 24–28 weeks. PVL in controls routinely peaked at 2 weeks post infection and persisted at levels that ranged from $10^6–10^8$

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copies/ml. In newborns treated with 10 mg/kg (Fig. 3a) or 40 mg/kg (Fig. 3c), there was no plasma viremia detected in any of the samples collected over the course of the study. A single time point in the 40 mg/kg group was positive for only one of two replicates, and additional material to retest this sample was not available. Longitudinal cell-associated viral loads (CAVL) in PBMC DNA were negative for each of the >300 samples tested from the 10 animals in Groups 5 and 6 (Fig. 3b, d). In short, all of the NmAb-treated infants had undetectable PVL or CAVL in blood.

We also measured the levels of SHIV $_{SF162P3}$ DNA in >300 homogenized tissue samples obtained at 24–28 weeks post-exposure (w.p.i) and in inguinal LNs collected at week 12 from all ten macaques using ultrasensitive QPCR[37]. Tissue samples from all SHIV-exposed infants that received the two-week course of NmAb cocktail were tested as coded samples and were negative for virus in both dosage groups (Supplementary Table 2). As discussed above, only very low levels of virus were detected in tissue specimens of Group 2a animals 34263 and 34290, sacrificed at 2 days post-exposure (d.p.i.) after a single NmAb dose (Fig. 2e). As was seen in the blood, virus was widespread in tissues at 14 d.p.i. in Group 3 control animals that did not receive NmAbs (Fig. 2f). As early as 1 d.p.i., tissue-associated virus in mucosal tissue adjacent to exposure site, draining LN and gut tissue was evident in Group 1 untreated controls (Fig. 2d; Supplementary Table 2). When compared, NmAb-treated (Group 2a) versus non-treated (Group 1) groups that were sacrificed on 2 d.p.i and 1 d.p.i., respectively, Group 2a macaques had significantly lower amounts of tissue-associated virus detected ($P = 0.0061$; Fig. 4). The discovery of traces of virus detected 2 d.p.i (Fig. 2e) and none at 14 d.p.i. (Fig. 2g) implies that NmAbs intercepted, neutralized and disrupted SHIV in these animals and in all ten NmAb-treated infants that were followed for 6 months. Consistent with these data, pathology results also showed the absence of organ or tissue pathology (Supplementary Fig. 5).

No evidence for T cell immunity or viral rebound

To evaluate T cell immunity in the SHIV-exposed macaques, we used intracellular cytokine staining (ICS) to measure Gag and Vif responses in the PBMC, spleen and mesenteric LN for the ten macaques studied for 6 months. No anti-SHIV T cell responses were detected in PBMCs at week 20 or in necropsy tissue samples (Supplementary Fig. 6). To determine whether there were any reservoirs controlled by $CD8^+$ T cell-mediated suppression, we depleted $CD8^+$ cells in the four animals in the 10 mg/kg group to undetectable levels and monitored for viremia for 4 weeks (Supplementary Fig. 6). There was no evidence of plasma virus rebound in these animals during the $CD8^+$ depletion phase (Fig. 3a, b), further supporting the concept that early passive NmAb therapy with the cocktail of VRC07-523 and PGT121 disrupted establishment of virus reservoirs preventing exposure to antigen and the development of cellular immunity.

DISCUSSION

Pre-Exposure Prophylaxis (PrEP) with ART is effective in limiting transmission in the setting of MTCT as well as in healthy adults[38]. One of the major goals in treating HIV-1 infection is to discover methods that can clear the established viral reservoir[39]. To date, only

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a single case of a "functional cure" has been documented following bone marrow transplantation[40]. Vaccine-induced persistent T cell responses cannot prevent infection but can reduce established SIV reservoirs to undetectable levels in about half of vaccinated macaques[41]. NHP studies with ART suggest that treatment as early as 3 days post-infection is too late to prevent establishment of the reservoir, as virus rebounded upon drug cessation[42]. These data are consistent with the case of the "Mississippi baby", where ART therapy begun within 30 hours of birth did not prevent HIV infection[43,44]. Thus, the time for intervention is extremely limited, and ART alone may not be effective at eliminating a founder infection.

Viral RNA has been detected as early as 1 day in macaques following vaginal exposure to SIV[45], but there is a common view that HIV and SIV may have a short 'eclipse phase' of limited, localized viral replication lasting a number of days, wherein the spread of the founder infection is dependent upon target cell availability in order to spread to lymphatic tissues [45]. Our data show that, at least in this model system of oral SHIV exposure in newborn macaques, virus replication is detected in lymphatic tissues by 24 hours and is not locally restricted. In this study, we found evidence of immediate impact of a single dose of passively transferred NmAbs on seeding of virus, with a significant difference in early tissue-associated viral RNA and DNA in treated versus non-treated infants. Early post-exposure short-term administration of powerful NmAbs effectively cleared the virus *in vivo* by 14 days and prevented rebound after decay of the passive NmAbs. We present three lines of evidence that the ten macaques studied for >24 weeks were clear of virus. First, all of the macaques failed to develop adaptive immune responses. Second, we showed that over 300 coded tissue samples from these macaques were virus negative by an ultrasensitive PCR methodology based on SIV *gag*. Third, we depleted the $CD8^+$ T cells in the four lower-dose macaques (Group 6), and observed no viral rebound.

These experiments show that NmAbs delivered s.c. are swiftly distributed to blood and tissues and that they maintain neutralizing activity at distal sites. They further indicate that NmAbs are effective at clearing viral foci in blood and tissues during the earliest stages of HIV penetration of tissues, a different mechanism from that of ART. We hypothesize that Fc-mediated functions are required for killing infected cells that express gp160 on their surface[46]. If so, then NmAbs present during an extended period of time after exposure would have the capability to neutralize virus emanating from infected cells that were established within the first few hours following an exposure event. In this setting, post-exposure therapy using a NmAb cocktail administered 24 h after SHIV exposure and continuing for 2 weeks resulted in maintenance of high concentrations *in vivo* for at least 2 weeks. The importance of repeated dosing is not known, but in the case of breastfeeding mothers, there is continued opportunity for transmission of HIV-1. It will be important to understand if repeated NmAb dosing in babies could expand the protective window.

Several relevant questions remain unanswered for treatment of HIV-infected newborns and children born to HIV-positive mothers, including practical and cultural issues of treating breastfeeding mothers and babies, as well as a determination of optimal antibody cocktail formulations. Any future use of human NmAbs in the clinic will likely require several antibodies or engineered antibodies with multiple specificities in order to avoid the potential

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for escape. Because ART has a short half-life and requires strict adherence to the drug regimen to be effective, supplementation with passive NmAbs with relatively long half-lives may widen the therapeutic window. Identifying human NmAbs that can impact infection in a macaque model for MTCT can provide proof-of-principle of the value of using antibodies to augment ART. In fact, safety trials of HIV-exposed newborns with the NmAb VRC01 have begun in the US and South Africa (http://impaactnetwork.org/studies/P1112.asp.), following a safety trial in adults[47]. Our findings begin to define the window of opportunity for effective treatment after intrapartum exposure. If these results can be applied to clinical settings, there is optimism that early passive immunotherapy may provide protection from viral infection, even in the absence of ART.

ONLINE METHODS

Animal models and humane care guidelines

The Oregon Health & Science University West Campus Institutional Animal Care and Use Committee approved all macaque studies. Studies were performed at the Oregon National Primate Research Center in Beaverton, Oregon, U.S.A. (ONPRC). The ONPRC is accredited by the American Association for the Accreditation of Laboratory Animal Care International and adheres to the Guide for the Care and Use of Laboratory Animals and the United States Public Health Service Policy on the Humane Care and Use of Laboratory Animals. The initial study with SIVIG utilized four *M. mulatta* (male and female) of varying ages that were obtained from the breeding colony. For the studies in one-month-old macaques, 27 seven day-old *M. mulatta* (rhesus macaques, male and female) were obtained from the breeding colony and raised for 3 weeks in the ABSL-2 infant nursery. Time of birth determined animal allocation, so that animals were randomly assigned to the study groups as they accrued. Protection studies with VRC01 ($n = 7$ infants) were pilot studies and were not designed for statistical analyses (all or none effects of virus acquisition). Group sizes of six had been previously shown to allow statistically distinguishable measurements in plasma and cell associated virus loads at 6 months as the primary study outcome for antibody treatment. Serial sacrifice studies included groups of two animals each and viral quantification analyses of ~30 tissue samples per animal. Infants were excluded if sire or dam could not be confirmed to be absent of *Mamu* B*08 and B*17 MHC Class I alleles. At 1 month of age, after adaptation to formula feeding, animals were transferred to ABSL-2+ containment for study procedures. Infants were paired with another macaque of the same age for nursery care and containment housing. In all studies, infants were monitored for clinical signs of disease. Clinical evaluation included measurement of peripheral LN size and body weight, as well as evaluation of appetite, attitude and stool quality. All animals were euthanized under IACUC guidelines and consistent with the recommendations of the American Veterinary Medical Association (AVMA) Guidelines for Euthanasia.

IgG and NmAb preparations

Normal IgG was purified from 1 L of pooled plasma from SRV and SIV negative adult rhesus macaques as previously described[30]. VRC01, VRC07-523 and PGT121 were expressed as IgG1 antibodies by transient transfection of Expi293F™ cells (ThermoFisher Scientific Inc.), and purified over protein A columns.[32,49] The V_H and V_L regions of

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PGT121 were synthesized based on the published sequence[33]. Purified polyclonal anti-SIV_{smE660} IgG (SIVIG) was pooled from two SIV_{smE660} infected animals from a prior experiment[28]. All antibody preparations were delivered s.c. at multiple sites around the dorsal cervical and thoracic regions of the animals in the doses described in the text.

Virus inoculations

Titrated stocks of $SHIV_{SF162P3}$ (passage 3) virus[50] were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, US National Institutes of Health (catalog number 6526; contributors J. Harouse, C. Cheng-Mayer and R. Pal) for each study. Infant macaques were administered a 50% animal infectious dose (AID_{50}) (~7×10^8 viral RNA copies) of a macaque cell-grown stock of $SHIV_{SF162P3}$, divided into two 1 ml oral doses given ~15 min apart. AID_{50} was determined in a titration experiment described previously.[36]

Virus detection in plasma, PBMC, and tissue homogenates

Nucleic acid from plasma and cell culture supernatant or PBMC was purified using a Maxwell 16 instrument (Promega, Madison, WI) according to the manufacturer's protocol, using the LEV Viral Nucleic Acid Kit and the LEV Whole Blood Nucleic Acid Kit, respectively. SHIV viral loads in plasma and cell culture supernatant were determined by quantitative RT-PCR using the methods developed by Piatak et. al.[51], except for a slightly modified master mix to increase sample input per reaction. SHIV viral loads in PBMC DNA were determined by quantitative PCR using Fast Advanced Mastermix on an Applied Biosystems QuantStudio 6 Flex instrument (Life Technologies, Carlsbad, CA). Reactions were performed with 2 μg nucleic acid input for 45 cycles using the FAST cycling protocol (95°C for 1 s, 60°C for 20 s) in a 30 μl reaction volume. Virus copy numbers were estimated by comparison to a linearized pBSII-SIVgag (gift from Michael Piatak, NCI) standard curve and calculated per cell equivalent using the input nucleic acid mass and by assuming a DNA content of 6.5 μg per million cells. Primers and probe used for plasma and PBMC assays were those described by Piatak et. al.: SGAG21 forward (GTCTGCGTCATPTGGTGCATTC), SGAG22 reverse (CACTAGKTGTCTCTGCACTATPTGTTTTG), and pSGAG23 (5'-(FAM)-CTTCPTCAGTKTGTTTCACTTTCTCTTCTGCG-(BHQ1)-3').

For viral RNA and DNA reservoir detection in tissues, a recently developed ultrasensitive nested quantitative PCR and RT-PCR approach[37] targeting a highly conserved region in SIV and SHIV gag was employed. Primers used for DNA pre-amplification were SIVnestF01 (GATTTGGATTAGCAG AAAGCCTGTTG) and SIVnestR01 (GTTGGTCTACTTGTTTTTGGCATAGTTTC). The reverse transcription step in the RNA assay used the SIVnestR01 primer instead of random hexamers, in order to facilitate priming of specific target sequences. Primers used for quantitative PCR were SGAG21 forward, SGAG22 reverse, and pSGAG23 as described above. PCR reaction conditions for both rounds were as described with minor modifications [52]. Briefly, samples were heated at 95°C for 5 min, and then put on ice. Each sample was assayed in 12 replicates (5 μg each), with two of the reactions including a spike of 10 or 20 copies of DNA or RNA, respectively, containing the SIVgag target sequence in order to assess PCR reaction efficiency. None of

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the tested RNA and DNA samples showed significant amplification inhibition, which was defined as a 5 cycle amplification delay compared to the amplification kinetics of reactions containing solely 10 copies of standard. First round amplification involved 12 cycles (95°C for 30 s and 60°C for 1 min) in 50 μl reactions. Then, 5 μl of each pre-amplified replicate was assayed by quantitative PCR using Fast Advanced Mastermix in a 30 μl reaction volume in the QuantStudio 6 Flex instrument. Reactions were performed for 45 cycles using the FAST cycling protocol. Virus copy numbers were derived from the frequency of positive replicates using the Poisson distribution and calculated as copies per μg of DNA. Plasma and tissue samples tested for virus were blinded to staff members performing the RNA and DNA assays.

Antibody detection in tissues and secretions

Tissue samples were sectioned and transferred into Radio-Immunoprecipitation Assay (RIPA) buffer with protease inhibitor cocktail. Tissue disruption was accomplished with zirconia/silica beads (1.0 mm, Biospec Products) in a Beadbeater (Biospec Products) device with 2 cycles of 2-min intervals with brief incubations on ice between each cycle. Supernatants were aspirated and centrifuged for 5 min to pellet residual debris. Mucosal secretions were collected on Weck-cel spears and extracted as previously described.[53] Secretions were stored at −80° C until assayed. Homogenates and secretions containing transudated antibody were used in ELISA and neutralization assays as described below.

$CD8^+$ T cell depletion and staining

Four animals were given the CD8α-depleting antibody M-T807R1 (US NIH, Nonhuman Primate Reagent Resource). Peripheral blood was monitored for the presence of $CD8^+$ T cells for four weeks. 1×10^5 PBMC were stained with CD3 AlexaFluor 700, CD8 Pacific Blue (BD Biosciences), and CD4 PE-Cy7 (Biolegend).

Intracellular cytokine staining (ICS)

$CD4^+$ and $CD8^+$ T cell responses were measured from blood and tissues by flow cytometric ICS, as previously described.[54] Briefly, 1×10^6 mononuclear cells were incubated with Gag or Vif open-reading frame pools and the co-stimulatory molecules CD28 and CD49d (BD Biosciences) for 1 hour, followed by addition of Brefeldin A (Sigma-Aldrich) for an additional 8 h. Co-stimulation without antigen served as a background control, while incubation with Staphylococcal Enterotoxin B (Toxin Technology) served as the positive control. The cells were then labeled with CD4 PE-Cy7 (Biolegend) and CD8 PerCP-Cy5.5 (BD Biosciences) and fixed with 2% paraformaldehyde. After permeabilization, the cells were stained with CD3 Pacific Blue, IFN-γ APC, TNF-α FITC (BD Biosciences, all), and CD69 PE-Texas Red (Beckman Coulter). The cells were fixed and flow cytometric analysis was performed on an LSR-II instrument (BD Biosciences). Analysis was done using FlowJo software (Tree Star, Ashland, OR). In some cases, cells were CD25-depleted prior to setting up the ICS experiment to remove T regulatory cells (Miltenyi Biotec).

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In-situ hybridization (ISH)

In the SIVIG transudation experiments, measurement of *in-situ* hybridization for SIV antigen was performed on tissues collected from the two animals that underwent oral SIV challenge. Formalin-fixed, paraffin-embedded tissues were assayed for SIV viral RNA expression by ISH as previously described.[55] Briefly, following deparaffinization, the sections were hybridized overnight at 45°C with either a sense or an antisense SIVmac239 digoxigenin-UTP-labeled riboprobe. The hybridized sections were blocked with 3% normal sheep and horse serum in 0.1 M Tris, pH 7.4, and then incubated with sheep anti-digoxigenin-alkaline phosphatase (Roche Molecular Biochemicals) and nitroblue tetrazolium-5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (BCIP; Vector Labs). ISH-stained tissues from submandibular LN, tonsil and the ileum were visualized and photographed with a Zeiss Axiophot microscope.

ELISAs

Enzyme-linked immunoabsorbent assay (ELISA) was used to detect total IgG and SIV gp130-specific antibodies in the SIVIG transudation experiments. Briefly, half-well EIA plates (Costar) were coated with either a goat anti-rhesus IgG (H+L)-unlabeled (Southern Biotech) (for total IgG ELISA) or recombinant SIV_{smE660} gp130 purified as described[28] (for gp130 ELISA) at 2 μg/ml in carbonate/bicarbonate buffer and incubated overnight. Plates were washed three times (0.1% triton X-100 in 1× PBS) and blocked with 1% normal goat serum/5% non-fat dried milk in PBS for 1 h at RT. SIVIG standards and homogenates were diluted in 1% triton X-100/2% bovine serum albumin/5% fetal bovine serum in PBS. After washing, a 1:4,000 dilution of goat anti-rhesus IgG (H+L)-HRP (Southern Biotech) was added and incubated for 1 h at RT followed by TMB substrate (Southern Biotech). Plates were read on a SpectraMax 190 at absorbance at 650 nm. Data were reported as the slope of absorbance over time. Concentrations of SIVIG in tissue disruption supernatants were calculated by comparing the average slope numbers to the SIVIG standard curve. ELISA was used to assess for the presence of gp140-specific antibodies as previously described[54] in plasma and tissue homogenates. Plasma NmAb levels were quantified using plates coated with either RSC3 (VRC07-523) or ST0A9 (PGT121).[56] Briefly, Nunc MaxiSorp (Thermo Fisher) plates were coated overnight with 200 ng/well of RSC3 in PBS, washed with PBST five times, and blocked with TBST with 5% milk and 2% BSA for 1 h at RT. Serial dilutions of all samples were plated in duplicate. Each NmAb (for standard curves) and positive and negative controls were included on each plate. Plasma was incubated for 1 h at RT, followed by a PBST wash. Bound NmAbs were probed with a horseradish peroxidase-labeled goat anti-human IgG (1:5,000 dilution; Jackson Laboratories) for 30 min at RT. The plate was washed and TMB (Pierce) substrate was added. Once color was developed stopping buffer was added and the optical density at 450 nm was read. GraphPad Prism and Microsoft Office software was used to calculate NmAb concentrations.

TZM-bl neutralization assay

Plasma samples from each animal were tested at all available time-points for neutralizing activity using the 96-well TZM-bl neutralization assay described previously[57].

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Statistics

The data in Figure 4 shows SIV DNA copies measured in 17 anatomical sites in 2 paired groups of 4 control animals. A two-stage sequential approach (1) calculated the averages of SIV DNA copies of two biological replicates for each site in each group, and (2) applied a Wilcoxon signed rank test to account for the matched pair nature of anatomical sites.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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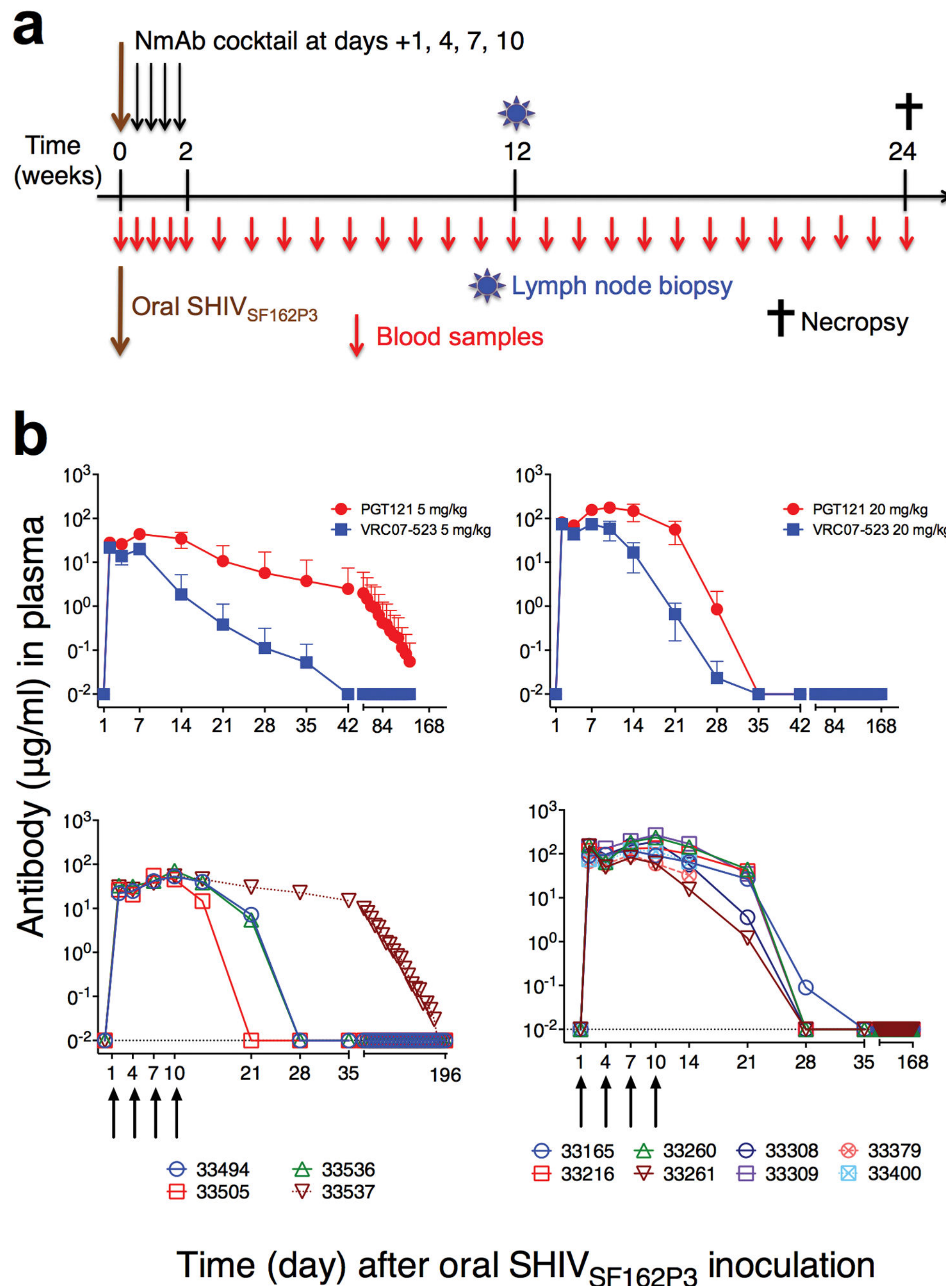

Figure 1. NmAb cocktail dosing and kinetics in plasma

(**a**) Experimental design of the early NmAb therapy experiment is shown and symbols are defined. (**b**) VRC07-523 and PGT121 were combined in a 1:1 mass ratio (µg/ml) to generate a cocktail for s.c. injection at doses of 10 mg/kg and 40 mg/kg. **(b,** top) Recombinant proteins RSC3 [48] and ST09AA [18] were used in ELISA for specific detection of VRC07-523 and PGT121 (5 mg/kg and 20 mg/kg each NmAb, respectively), and (**b,** bottom) the NmAb cocktail was assayed by an SF162 gp140 ELISA (left: 10 mg/kg cocktail; right: 40 mg/kg). Data shown are NmAb concentrations in the plasma of twelve macaques. Concentrations were determined using non-linear regression and the EC_{50} of the NmAb cocktail or the

individual NmAb and were graphed in GraphPad Prism. Error bars indicate Standard Deviation (SD). Individual NmAbs and NmAb cocktail served as standard curves.

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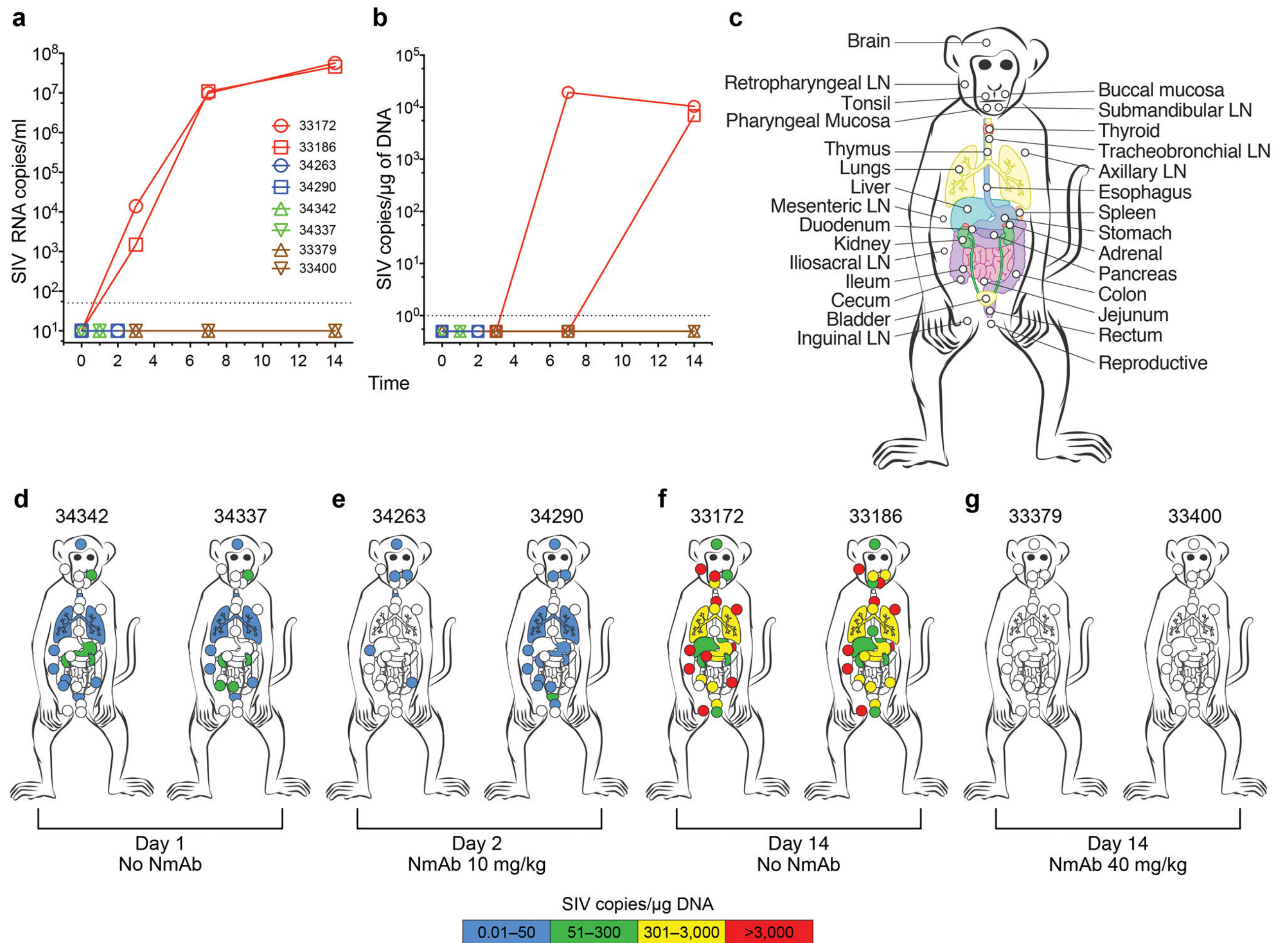

Figure 2. Viral kinetics and tissue distribution during the first two weeks after oral SHIV exposure

$SHIV_{SF162P3}$ viremia was quantified in eight male and female treated and untreated control animals. (**a**) Plasma viral loads assessed by measurements of SIV viral RNA in blood using a quantitative reverse-transcription PCR (QRT-PCR) assay and in (**b**) PBMC by quantitative PCR (QPCR). (**c**) Anatomic locations of tissues collected at necropsy following oral inoculation. (**d–g**) Viral DNA in tissues was detected by ultrasensitive nested quantitative PCR and RT-PCR[37] targeting a highly conserved region in SIV and SHIV *gag*. Each sample was assayed in 12 replicates (5 μg each). Virus copy numbers were derived from the frequency of positive replicates using the Poisson distribution and calculated as copies per μg of DNA or copies per 10^6 cell equivalents using the input nucleic acid mass and by assuming a DNA content of 6.5 μg per million cells. Infected tissues are colored to indicate quantified virus according to the scale shown in SIV *gag* copies/μg of DNA.

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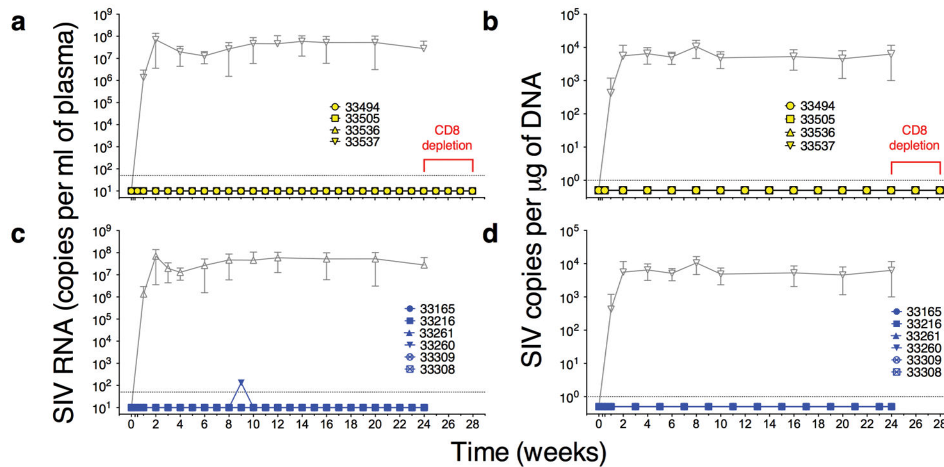

Figure 3. SHIV$_{SF162P3}$-associated viremia is not established in plasma or PBMC of NmAb-treated infants

(**a,c**) Quantified virus in blood and (**b,d**) peripheral blood cells in both NmAb dosing groups of male and female infant rhesus macaques (*n* = 10). Plasma viral loads were assessed by measurements of SIV viral RNA in blood using a quantitative reverse-transcription PCR (QRT-PCR) assay and in (**b**) PBMC by quantitative PCR (QPCR). $CD8^+$ T cell depletion study timeline is shown in red. Data shown in gray indicate mean plasma virus (+/− SD) from eight historical controls from an earlier study[18,30].

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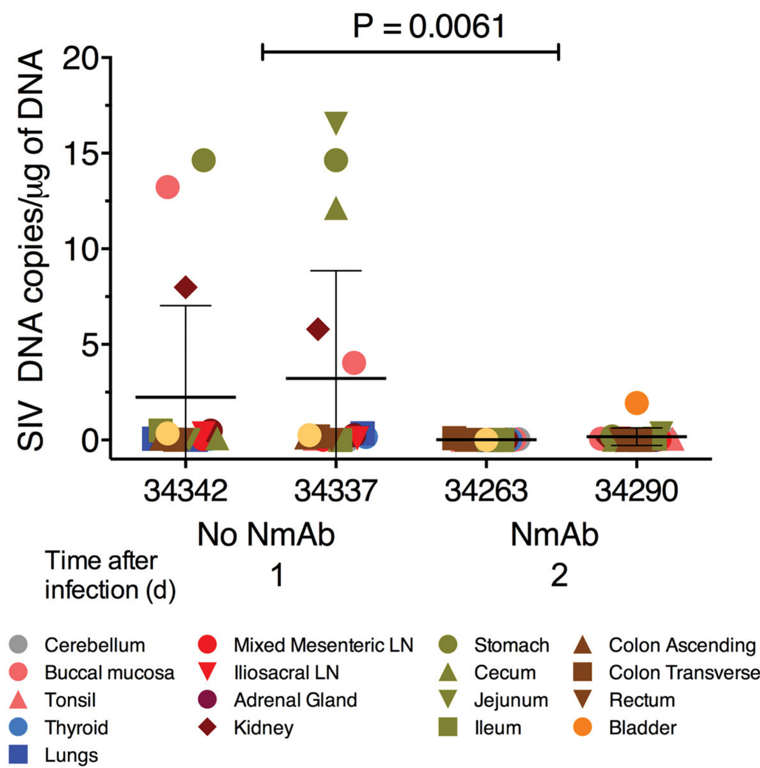

Figure 4. NmAb cocktail lowers tissue-associated viremia within 24 h after s.c. delivery

SHIV DNA quantified by ultrasensitive nested quantitative PCR and RT-PCR[37] in each tissue sample shown in four control animals (Table 1, Groups 1 and 2a) at either 1 day after SHIV exposure with No NmAb treatment or 1 day after s.c. injection of 10 mg/kg NmAb cocktail and 2 days after SHIV inoculation. Wilcoxon signed rank test (statistics performed in SAS 9.4 software).

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Table 1

Experimental design for testing a human NmAb cocktail in the therapeutic mode.

Group	Size	Animal ID	Virus exposure (day)	NmAb treatment (day)	NmAb dose (mg/kg)	CD8 depletion (day)	Necropsy (day)
1	2	34342, 34337	0	--	--	--	1
2a	2	34263, 34290	0	1	10	--	2
2b	2	34365, 34345	-- [a]	1	10	--	2
3	2	33172, 33186	0	--	--	--	14
4	2	33379, 33400	0	1, 4, 7, 10	40	--	14
5	6	33165, 33216, 33260, 33261, 33308, 33309	0	1, 4, 7, 10	40	--	168
6	4	33494, 33505, 33536, 33537	0	1, 4, 7, 10	10	168–196	196
7[b]	8	28792, 28785 29003, 29010 29012, 29077 29079, 29081	0	--	--	--	168

[a]Indicates none or not done.

[b]published previously[30]

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Table 2

Neutralizing activity in tissue homogenates of infant rhesus macaques co-localizes with virus

NmAb cocktail dose				10 mg/kg					No NmAb		40 mg/kg	
Day of sample collection	1 [a,b]				2 [c]				14 [d,e]			
Animal ID	**34342**	**34337**	**34365**	**34345**		**34263**		**34290**	**33172**	**33186**	**33379**	**33400**
	vRNA	**vRNA**		**ID_{50}**	**ID_{50}**	**vRNA**	**ID_{50}**	**vRNA**	**vRNA**	**vRNA**	**ID_{50}**	**ID_{50}**
Cerebellum									1,936.7	1,334.7		
Buccal mucosa			<20	<20	<20		32				72	88
Pharyngeal mucosa			<20	<20	<20		<20					54
Mixed Mesenteric LN	1.3	0.2	44	56	66		32	0	417,832.3	671,839.9	36	175
Spleen	0	0	74	34	71		52	0	887,585.7	392,031.7	145	157
Iliosacral LN	0.7	65.1										
Inguinal LN								0				
Colon	0.2	0	72	34	96		289	0	1,410,029.7	105,623.6	33	245
Rectum			<20	28	58		71				<20	42
Repro Tract			71	82	79		386				69	110

[a] SHIV day 0. No NmAb. Necropsy day 1. (34342, 34337). Quantified vDNA (Supplementary Table 2).

[b] NmAbs day 0. No SHIV. Necropsy day 1. (34365, 34345)

[c] Oral SHIV day 0 and s.c. NmAbs day 1. No vDNA (Supplementary Table 2). Necropsy day 2. (34263, 34290)

[d] Oral SHIV day 0. No NmAb. Necropsy day 14. (33172, 33186)

[e] Oral SHIV day 0 with s.c. NmAbs day 1, 4, 7 and 10. No vDNA (Supplementary Table 2). (33379, 33400)

ID_{50} = Dilution of plasma that results in 50% neutralization of $SHIV_{SF162P3}$ in TZM-bl cells. SIV*gag* viral RNA (copies/1 × 10^6 cell equivalents); coded samples; ultrasensitive nested QPCR and RT-PCR in 12 replicates Blank = No data