



## Topical gel formulation of broadly neutralizing anti-HIV-1 monoclonal antibody VRC01 confers protection against HIV-1 vaginal challenge in a humanized mouse model

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### ABSTRACT

The new generation broadly neutralizing antibody VRC01 against HIV-1 shows great potential as a topically administered microbicide to prevent sexual transmission. We evaluated its efficacy in a RAG-hu humanized mouse model of vaginal HIV-1 transmission. Mice were challenged vaginally with R5 tropic HIV-1 BaL an hour after intravaginal application of the VRC01 (1 mg/ml concentration) gel. A combination of four first generation bNAbs, namely b12, 2F5, 4E10 and 2G12, was used as a positive efficacy control whereas a non-specific dengue MAb 4G2 was used as negative control. Our results showed that seven out of nine VRC01 antibody administered mice and all of the mice receiving the four bNAb antibody combination were protected against HIV-1 challenge. These findings demonstrate the efficacy of the new bNAb VRC01 as a topical microbicide to protect against HIV-1 vaginal transmission and highlight the use of the RAG-hu mouse model for testing HIV prevention strategies.

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### Introduction

HIV incidence continues to be unabated worldwide in the absence of an effective vaccine (UNAIDS (Website)). Therefore there is an urgency to develop alternative prevention methods to contain this epidemic. In this regard, topically applied microbicide gels to prevent sexual transmission show considerable promise (Lederman et al., 2008; Omar and Bergeron, 2011; Shattock and Rosenberg, 2012). While a number of early clinical trials using non-specific compounds such as nonoxynol-9 failed to show protection, the recent success achieved with the RT inhibitor tenofovir gel in the CAPRISA004 trial provided renewed optimism in the microbicide field (Abdool Karim et al., 2010; Abdool Karim and Baxter, 2012). Based on this success, the current strategies are focused on using HIV specific compounds that act against different stages of the viral life cycle. These include entry inhibitors such as maraviroc, nucleoside and non-nucleoside reverse transcriptase inhibitors like tenofovir and TMC 120 and integrase inhibitors exemplified by raltegravir, to name a few (Shattock and Rosenberg, 2012). Successful development and deployment of an

effective anti-HIV microbicide will empower women to protect themselves against contracting the infection and thus is estimated to prevent millions of new cases of HIV.

In practice, prevention of HIV infection by the use of microbicides involves repeated applications of the gels that contain high concentrations of anti-HIV compounds of various chemistries (Hladik and Doncel, 2010; Kelly and Shattock, 2011; Klasse et al., 2006). It is possible that such a repeated exposure of sensitive cervico-vaginal mucosal tissues to these drugs during long-term use may result in toxicities causing epithelial damage eventually undermining the protective effects. In this regard, use of biomolecules such as neutralizing antibodies may circumvent potential damage to the mucous membranes thus affording long-term safety. Additionally, combining these with that of other anti-HIV compounds may permit reducing the concentrations of the respective compounds to lower and much safer levels. Given the extensive antigenic variability of HIV-1, any candidate antibody has to be broadly neutralizing to qualify as an effective microbicide to prevent infection. Until recently, studies in this area have been limited due to sparse numbers of available potent broadly neutralizing antibodies (bNAbs) (Hessell et al., 2009a, 2010; Parren et al., 2001; Veazey et al., 2003). This limitation is now overcome by the discovery of several new more potent bNAbs (Mascola and Montefiori, 2010; Walker et al., 2009;

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Wu et al., 2010). Early evidence that bNAbs do indeed have microbicide potential was derived by the studies of Veazey et al. (2003) in a monkey model. Animals were administered a bNAb antibody b12 vaginally in either saline or hydroxyethyl cellulose gel. Only three out of 12 treated and SHIV challenged animals became infected and it was found that the duration of protection lasts for at least two hours after administration. These results form a sound basis that neutralizing antibodies have good microbicide potential.

While the previous generation bNAbs such as b12, 2G12, 2F5, and 4E10 were shown to be capable of neutralizing many primary HIV-1 isolates belonging to multiple genetic subtypes, they are not pan-reactive (Mascola and Montefiori, 2010). In recent studies directed towards finding more bNAbs with an ultimate goal of designing better vaccines, newer high throughput strategies were used (Walker et al., 2009; Wu et al., 2010). These studies have led to the discovery of more highly potent neutralizing antibodies. These include bNAbs PG9, PG16, HJ16 and VRC01, among others. While b12 and HJ16 can neutralize about 40% of known viral isolates, PG9 and PG16 bNAbs neutralized 73% and 79% of viral isolates tested respectively (Corti et al., 2010; Walker et al., 2009). The bNAb VRC01 that recognizes the CD4 binding site on HIV gp120 showed even more potency and breadth with a capacity to neutralize 91% of viral strains tested (Wu et al., 2010). With these successes as a background, a more intensified search recently yielded even more potent bNAbs such as multiple PGT bNAbs recently discovered by Walker et al. (2011). The availability of these antibodies opened up many new avenues of HIV therapies and prevention including microbicides.

With regard to testing microbicide strategies in vivo using bNAbs as well as other anti-HIV compounds, macaque models have been the leading standard (Veazey et al., 2012). They were instrumental for the basic groundwork and provided the preliminary efficacy data for subsequent human clinical trials. However, they are expensive, and cannot use HIV-1 itself for testing and therefore rely on SIV and/or varieties of hybrid SHIV viruses for viral challenge (Denton and Garcia, 2009, 2011; Hladik and Doncel, 2010; Klasse et al., 2008). Furthermore, it is not possible to test against drug resistant and genetically diverse HIV-1 strains that exist in the field. Some of these deficiencies can now be overcome by the newer generation humanized mouse models that have emerged (Berges and Rowan, 2011; Denton and Garcia, 2009; Shultz et al., 2011). These include RAG-hu mice and BLT mice which were found to have HIV susceptible human cell reconstitution in cervico-vaginal and rectal mucosal compartments (Berges et al., 2008; Sun et al., 2007). A number of recent studies have demonstrated their susceptibility to HIV-1 infection by both vaginal and rectal routes (Akkina et al., 2011; Berges et al., 2008; Denton et al., 2008; Neff et al., 2011). Exploiting this property, indeed both pre-exposure prophylaxis and microbicide-based prevention of HIV-1 mucosal transmission has recently been shown using these models by us and others (Denton et al., 2011; Neff et al., 2011, 2010). More recently we validated the RAG-hu mouse model for vaginal microbicide testing using maraviroc which showed good efficacy (Neff et al., 2011).

With the advent of new discoveries that continuously yield a stream of newer and more potent bNAbs and other new biological molecules for potential use as microbicides, it is important that promising candidates need to be evaluated quickly and inexpensively for generating preliminary data for later macaque studies and human clinical trials. With these questions as a background, we conducted the following proof-of-concept studies to evaluate bNAb VRC01 as a potential microbicide. Our results show that these antibodies confer significant protection against HIV-1 vaginal challenge and that RAG-hu mice provide a suitable in vivo system to test biomolecules as potential anti-HIV microbicides.

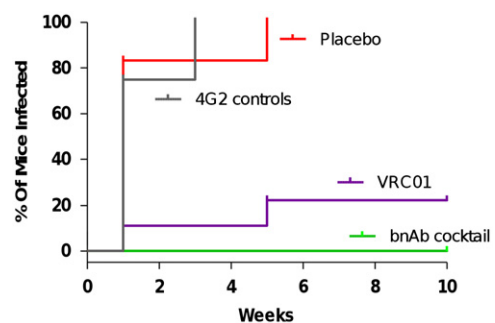
## Results

### *Vaginal application of VRC01 gel confers protection against HIV-1 vaginal challenge in RAG-hu mice*

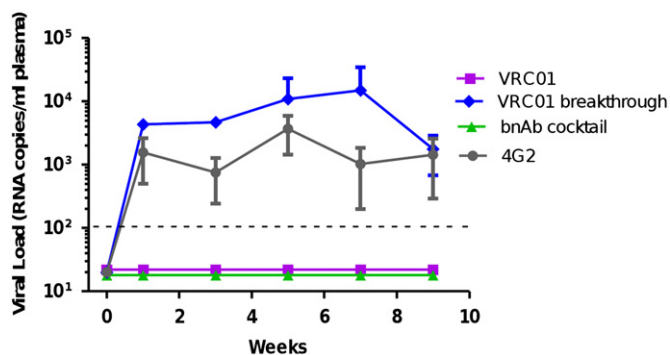
We have previously shown that RAG-hu mice are susceptible to vaginal HIV-1 transmission and can be used to test both oral PrEP and topically administered anti-HIV compounds, namely the CCR5 inhibitor maraviroc and integrase inhibitor raltegravir (Neff et al., 2011, 2010). Here we evaluated a biological molecule VRC01, a broadly neutralizing monoclonal antibody, for its ability to protect humanized mice against HIV-1 vaginal challenge. Mice were monitored by qRT-PCR on a bi-weekly basis for ten weeks to detect virus in the plasma of virally challenged mice. As a positive effective control, a gel containing a cocktail of four bNAbs, b12, 4E10, 2F5 and 2G12 antibodies, was employed. Our results (Fig. 1) showed that all the mice (6/6) receiving either a placebo gel (no antibodies) or a gel containing an irrelevant anti-dengue MAB (4/4) became virus positive within five and three weeks post-viral challenge, respectively. In contrast, in the VRC01 treated group only two out of nine mice (2/9) became virus positive, showing significant protection. In mice receiving the bNAb cocktail of four antibody combination, none were infected (0/5) thus showing complete protection. In mice which were not virally challenged, as expected, no virus could be detected (0/2). With regard to viremia, unprotected mice receiving placebo gel, irrelevant non-specific antibody gel as well as non-protected mice in the VRC01 treated group exhibited persistent viremia after becoming virus positive (Fig. 2). Overall, these data indicate that VRC01 bNAb confers significant protection against HIV-1 vaginal challenge when compared to placebo and 4G2 non-specific antibody treated RAG-hu mice, with *P* values of 0.0013 and 0.002, respectively. Additionally, when comparing VRC01 and bNAb cocktail treated mice, a *P* value of 0.2774 indicates that there is no significant difference between these two bNAb treated groups. These data taken together suggest that VRC01 bNAb conferred partial but significant protection and the combinatorial antibody gel provided full protection.

### *CD4 T cell loss in placebo-gel administered and non-protected versus VRC01 protected RAG-hu mice*

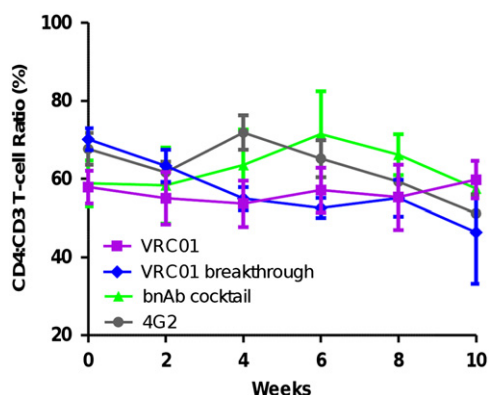
The RAG-hu mouse model mimics the immunopathology of HIV-1 infection in humans, as demonstrated by virally mediated depletion of CD4 T cells. While the qRT-PCR data detected no viremia in microbicide gel protected mice, we wanted to further confirm that there is no evidence of HIV induced pathology as can



**Fig. 1.** Vaginal application of VRC01 gel protects humanized mice against vaginal HIV-1 challenge. RAG-hu mice were challenged by vaginal route 1 h after vaginal administration of VRC01, combinatorial bNAb, irrelevant antibody or placebo gels as described in *Materials and methods*. Blood was collected bi-weekly from infected mice and the status of HIV-1 infection was determined by qRT-PCR. Kaplan–Meier plots of time course of appearance of viremia in antibody treated versus non-treated virus challenged mice.



**Fig. 2.** Viral RNA loads in mice administered with different bNAb gels. RAG-hu mice were challenged by vaginal route an hour after vaginal application of bNAb gels as described in Materials and methods. Blood was collected bi-weekly. Viral RNA was extracted from plasma and viral RNA loads were determined by qRT-PCR as described in Materials and methods. The dotted line represents limit of PCR detection.



**Fig. 3.** CD4 T cell decline in non-protected mice versus protected mice. Levels of CD4 T cells were monitored bi-weekly by FACS to determine their decline in treated versus non-treated mice. Baseline values for each of the mice were established prior to infection as described in Materials and methods and are shown as week 0 values.

be seen by stable levels of CD4 T cells. Accordingly, peripheral blood from virally challenged mice was evaluated periodically by FACS analysis for 10 weeks (Fig. 3). The baseline CD4 T cell levels were established prior to viral challenge in each of the mice. Typical of HIV-1 infection of these mice, there was a trend of gradual CD4 T cell decline in the irrelevant antibody (4G2) gel treated mice in which viremia was seen. On the contrary, no discernible CD4 T cell loss could be seen in either VRC01 protected or bnAb cocktail gel treated mice. Consistent with the presence of viremia in the two VRC01 mice that became virus positive and were thus deemed non-protected, there was significant CD4 T cell loss. Collectively these data showed that the mice which did not become virus positive also did not show any HIV associated CD4 T cell loss.

## Discussion

A safe and effective microbicide which women can use to protect themselves against contracting HIV infection would greatly help diminish the disease incidence. If found to be effective, use of naturally occurring biological molecules such as antibodies will have considerable edge over other chemical compounds in terms of assuring long term safety and reducing side effects. Here in these preliminary studies we have shown that a new generation potent broadly neutralizing monoclonal

antibody VRC01 confers significant protection against HIV-1 vaginal transmission. To our knowledge this is the first report to show the efficacy of the new generation bNAb as potential HIV microbicides in an in vivo system using HIV-1 itself as the challenge virus. While a number of previous macaque studies using previous generation of bNAb have shown their utility as microbicides (Ferrantelli et al., 2004; Hessel et al., 2009a,b, 2010; Hofmann-Lehmann et al., 2002; Ruprecht et al., 2001; Veazey et al., 2003), the humanized mouse system we used here permitted efficacy testing in the context of human target cells. Also, unlike in macaque studies, there was no need to condition the animals by progesterone hormonal treatment with Depo-Provera to induce vaginal thinning.

The new generation microbicide compounds are being chosen for their specific inhibitory effects on various stages of the viral life cycle in contrast to previous generation generic non-specific agents (Ferguson and Rohan, 2011; Omar and Bergeron, 2011). The recently field tested RT inhibitor microbicide compounds such as tenofovir showed promising efficacy but still fall short of the desired full protection level (Abdool Karim et al., 2010). The inhibitors such as RT and integrase inhibitors would act after viral entry (Klasse et al., 2008; Omar and Bergeron, 2011). If the drug levels are insufficient intracellularly in a small sub-population of cells, virus breakthrough is likely. Therefore, if possible it is highly desirable to prevent the first step of viral entry in the viral life cycle. In this regard, compounds such as maraviroc, a CCR5 antagonist, have been shown to prevent vaginal transmission of SHIV virus in macaque studies (Veazey et al., 2010). We have recently shown its anti-HIV microbicide potential in a humanized mouse system similar to the one we used here (Neff et al., 2011). While the inhibitory activity of RTIs is not co-receptor dependent, use of a single entry inhibitor like CCR5 antagonist maraviroc alone will be ineffective against X4 and dual tropic viruses which are transmitted, albeit at a low level. An advantage of bNAb in the context of entry inhibitors is that they are effective against all HIV-1 strains irrespective of their co-receptor tropism.

Among bNAb, VRC01 antibody is the most potent and most broadly neutralizing with activity against 91% of viruses tested (Wu et al., 2010). In our present study seven out of nine vaginally challenged mice were protected against HIV-1 infection at 1 mg/ml concentration of the topically applied gel. While the protection conferred is significant, it is not complete. It is possible that a higher concentration of VRC01 may be needed for complete protection. Alternatively an improved formulation that reduces possible biodegradation in the cervico-vaginal environment may improve its efficacy. Moreover, if better retention can be achieved using alternative gel formulations, lower doses of VRC01 might offer the same or higher level of protection, compared to the dose tested here. A recent preliminary report by Pegu et al. (Meeting Abstract Supplement, J. Immunol. April 2011, 186, 155.11) described the protective effect of passively transferred VRC01. In this study, macaques were administered with VRC01 systemically by i.v. route (dose 20 mg/kg). Two days later they were either challenged by vaginal or rectal routes with a SHIV virus. Whereas 3/4 animals got infected in the control groups by both the routes none of the animals (4/4) that received VRC01 were infected by either route thus showing full protection. This data demonstrated that passively administered VRC01 antibody provides sterilizing mucosal immunity.

The microbicide formulation containing a mix of bNAb 2G12, 4E10, 2F5 and b12 which we used as positive efficacy control in this study provided complete protection in which five out of five animals were protected. This result is consistent with other studies that showed high level of protection by the combination of the bNAb listed above in different in vivo and in vitro settings (Ferrantelli et al., 2004; Gauduin et al., 1997; Hessel et al.,

2009a,b, 2010; Hofmann-Lehmann et al., 2002; Luo et al., 2010; Parren et al., 2001; Ruprecht et al., 2001; Veazey et al., 2003). This complete protection offered by the combination of four bNABs with different target epitopes points to the important fact already recognized in the field—combinatorial approaches are needed for future microbicide formulations and the principle of using multiple antiviral agents must be adopted, keeping in mind the success of HAART therapy for already established HIV-1 infections. In a study pointing to the combinatorial approach by Euler et al. (2011) in which viral isolates from the early stages of HIV-1 epidemic as well as from recent years were tested against VRC01, PG16 and PG8 bNABs it was found that, even though resistance developed over time for all the bNABs tested, every strain included in the panel showed high sensitivity to at least one of the bNABs. A new study by West et al. (2011) showed that a construct which combines VRC01 scFv (single chain Fragment variable) and PG16 IgG yields a potent chimera with greater neutralization breadth thus opening the door for new modes of combining bNABs with different gp120 epitopes in the future. In addition to combinatorial approaches, efforts to engineer newer bNABs with increased potency and breadth based on the structure of VRC01 are already under way (Scheid et al., 2011; Wu et al., 2011).

Since the present data demonstrated the efficacy of the new generation bNABs as potential microbicides against HIV-1 infection, a number of other important questions need to be evaluated. Among these are—if combining these with other antibodies and/or with other ARVs will increase the efficacy and breadth of action. For these antibodies to reach wider application in the field as microbicides and to be used by millions of people at risk, it is necessary that they need to be mass produced in the required quantities. This is being currently addressed by newer mass production methods such as utilizing genetically engineered plants (Fox, 2011). However, possible immunogenicity of plant-expressed molecules and concerns regarding genetically engineered plants in some quarters would need to be resolved for their wider use and acceptance. With regard to large scale testing of a wide variety of biological molecules and chemical ARDs in the future to derive preclinical data, the humanized mouse model we employed here will offer a rapid cost effective *in vivo* model.

## Materials and methods

### Generation of humanized *Rag1*<sup>-/-</sup> *γc*<sup>-/-</sup> and *Rag2*<sup>-/-</sup> *γc*<sup>-/-</sup> mice (RAG-hu mice)

Humanized BALB/c- *Rag1*<sup>-/-</sup> *γc*<sup>-/-</sup> and BALB/c- *Rag2*<sup>-/-</sup> *γc*<sup>-/-</sup> (RAG-hu) mice were generated using human fetal liver-derived CD34<sup>+</sup> hematopoietic progenitor cells as previously described (Akkinä et al., 2011; Berges et al., 2008, 2010, 2006). Mice were maintained at the Colorado State University Painter Animal Center and all studies have been reviewed and specifically approved by the CSU Institutional Animal Care and Use Committee (Protocol 09-085A). Newborn mice were preconditioned by irradiating with a sublethal dose of 350 rad and then injected intrahepatically with 0.5–1 × 10<sup>6</sup> human CD34<sup>+</sup> cells. Mice were screened for human cell engraftment at 10–12 weeks post-reconstitution. Peripheral blood was collected by tail bleed and red blood cells were lysed by the Whole Blood Erythrocyte Lysing Kit (R&D Systems, Minneapolis, MN). The white blood cell fraction was stained against the human pan-leukocyte marker CD45 using hCD45-R-PE (Invitrogen) and FACS analyzed to determine the levels of human cell engraftment as previously reported (Berges et al., 2006). All mice included in the study were monitored for level of engraftment prior to the beginning of the experiment. Female mice prepared with different donor CD34<sup>+</sup> cells with over 50% engraftment were randomly distributed into experimental and control groups.

### Vaginal application of bNAB VRC01 gel and HIV-1 challenge by vaginal route

VRC01 antibody was kindly provided by Vaccine Research Center (VRC), NIAID, NIH, and older generation bNABs 2G12, b12, 4E10 and 2F5 were obtained from NIH AIDS Research & Reference Reagent Program. To prepare a topical gel formulation VRC01 antibody was incorporated into a 2.2% hydroxy ethyl cellulose (HEC) universal placebo gel at a concentration of 1 mg/ml. As a positive effective control, a four bNAB antibody mix was employed. This combinatorial bNAB cocktail gel formulation was prepared by using old generation bNAB antibodies of different

**Table 1**

Summary of human cell engraftment levels in humanized mice. Peripheral blood was collected from human CD34 cell reconstituted RAG-hu mice (BALB/c-RAG2<sup>-/-</sup>γc<sup>-/-</sup> or BALB/c-RAG1<sup>-/-</sup>γc<sup>-/-</sup>, the prefix J is indicative of RAG1) at 10–12 weeks post-engraftment. White blood cell fraction was stained with CD45 FITC conjugated antibody and analyzed by FACS to confirm human cell engraftment prior to treatment and vaginal HIV-1 challenges.

VRC01 mouse no.	Engraftment (%)	Placebo mouse no.	Engraftment (%)
J961	58	1000	78.1
J975	59.3	997	70.4
J977	67.3	944	74.7
J978	63.6	945	63.7
J996	54.1	J1008	67.6
J997	50.9	J1009	87
J998	52.1		
J1033	72.9		
J1035	77		
bNAB cocktail mouse no.	Engraftment (%)	4G2 control mouse no.	Engraftment (%)
J909	69.5	J1010	63.2
J911	78.5	J1011	69.2
J981	54.2	J1012	78.4
J985	73.8	J1013	59.8
J989	55.4		
Negative control mouse no.	Engraftment (%)		
932	62.9		
933	68.2		

specificities that consisted of b12, 4E10, 2F5 and 2G12 antibodies, all incorporated into the 2.2% HEC gel at a final concentration of 0.125 mg/ml for each antibody in the mix (0.5 mg/ml total antibody final concentration). We also prepared a gel with a negative control bNAb (1 mg/ml) using an irrelevant non-HIV-1 antibody 4G2 (a mAb against dengue virus). For these experiments we used mice that have human CD45 cell engraftments of 50% or more (Table 1). Female RAG-hu mice were topically administered with VRC01 gel (nine mice), bNAb cocktail gel (five mice) or control 4G2 gel (four mice) an hour before the viral challenge. A 25  $\mu$ l volume of each of the gel formulations was carefully applied into the vaginal vault of RAG-hu mice. During the whole procedure mice were anesthetized by isoflurane inhalation (duration 7–10 min). An hour post-gel application, mice were challenged vaginally with HIV-1 BaL (3000 TCID) in a 25  $\mu$ l volume. Gel and viral inoculums were applied by using the bulbous end (1.25 mm in diameter) of a gavage needle (VWR, POPPER, NY) to assure no mucosal abrasions and tearing would occur (Neff et al., 2011). Anesthetized mice were held in an inverted position for 4 min post-inoculation to allow the virus to adsorb and to prevent immediate discharge of virus, as described previously (Berges et al., 2008; Neff et al., 2010). Control non-treated mice received 2.2% HEC placebo gel. Animals were observed daily and blood samples collected on a weekly basis to assess plasma viremia and CD4 T cell counts.

#### Measurement of viral loads

HIV-1 infection status and viral loads were assessed by qRT-PCR. RNA was extracted from 25 to 50  $\mu$ l of EDTA-treated plasma using the QIAamp Viral RNA kit (Qiagen, Valencia, CA). qRT-PCR was performed by using a primer set specific for the HIV-1 LTR sequence and a corresponding LTR specific probe as described previously (Berges et al., 2008, 2010). C1000 Thermal Cycler (CFX96™ Real-Time System, BIO-RAD) and iScript™ One-Step RT-PCR kit with SYBR® Green (BIO-RAD) were used to perform the real-time qPCR analysis and viral load was expressed as the number of HIV-1 RNA copies per milliliter plasma.

#### Flow cytometry

Mice were monitored biweekly to analyze the levels of CD4 T cells in the peripheral blood. Whole blood was collected and red blood cells lysed as described previously (Berges et al., 2010, 2006). Peripheral lymphocytes were stained for hCD45-FITC, hCD3-R-PE and hCD4-PE-Cy5 surface markers (Invitrogen, BD Biosciences) and monitored using a Coulter EPICS XL-MCL FACS analyzer (Beckman Coulter, Fullerton, CA). CD4+ T cell levels were calculated as a ratio of the entire CD3 population (CD4+CD3+:CD4-CD3+). All mice were screened prior to the gel application and HIV-1 challenge to establish baseline CD4 T cell ratios.

#### Statistics

Data regarding the number of viral RNA copies, CD4 T cell levels, and infection rates was analyzed using GraphPad Prism version 5 (GraphPad Software, USA). To compare the infection rates between the groups, Fisher's Exact test was applied for analysis of the percent infected curves. *P* values less than 0.05 were considered to be significant.

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