**Superior efficacy of a microbicide and vaccine combination over single prevention approaches against vaginal SHIV challenge in cynomolgus monkeys.**

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**Abstract**
Although vaccines and microbicides have demonstrated partial success against HIV infection in clinical trials, their combined introduction could provide more potent protection. We used a non-human primate model to determine potential interactions of combining a partially effective microbicide with an envelope based vaccine. The vaccine-microbicide combination provided an 88% reduction in per exposure risk of infection, following 12 consecutive low dose intravaginal challenges with $SHIV_{SF162P3}$ relative to vaccine alone, which provided no protection compared to controls. The microbicide alone provided a modest reduction against naïve controls (45%). Protected animals in the vaccine-microbicide group were challenged a further 12 times in the absence of microbicide and demonstrated a 98% reduction in risk of infection. Taken together a total risk reduction of 91% was observed in this group over 24 exposures (P=0.004). These important findings suggest that combined implementation of new biomedical prevention strategies may provide significant gains in HIV prevention.

The Thai RV144 vaccine trial, based on a canary-pox vector prime (ALVAC)–protein boost (AIDSVAX), is the first clinical trial to have shown moderate efficacy (31.2%) in cohorts at low

risk of HIV exposure (1). Partial protection has also been observed with other new biomedical approaches including the use of antiretroviral (ARV) drugs as oral (44-75%) or topical (vaginal, 39%) pre-exposure prophylaxis (PrEP) (2-5). However effectiveness was dependent upon consistent product use and impacted by multiple factors influencing susceptibility and exposure risk (6). Three decades of research on combined implementation of structural and behavioural interventions have indicated that combination approaches are more effective than any single intervention alone (7). Additional potential gains could be realised by assessing the impact of combining new biomedical prevention strategies (8). Indeed, positive impact would be seen if combining ARV prevention and vaccines provides better protection than either intervention alone. Here, reduction of the number of transmitted strains and/or delay in the initial viral expansion phase might buy time for more effective immune clearance. Conversely systemic immunity might curtail dissemination of virus that bypasses the activity of topically applied ARVs. Furthermore, subjects protected from productive infection on repeat exposure to HIV when using ARV-prevention, might evoke exposure-induced immunity. This could serve to modify vaccine-induced immune responses to better recognize prevalent circulating virus. Indeed, evidence from some non-human primate (NHP) studies indicates that animals exposed to infectious virus when protected by PrEP demonstrate cellular immune responses to the challenge virus (9, 10). However, such immune responses in these non-vaccinated animals appeared insufficient to protect animals from subsequent challenge in the absence of PrEP (10). Conversely, combinations could also have potential negative interactions. Certain vaccine induced immune activation may have potential to increase mucosal HIV-1 susceptibility (11) that in combination could reduce the efficacy of ARV-prevention.

## Results

### Study design

In this study we use a NHP model to determine potential interactions of combining a partially effective microbicide with an envelope-based vaccine. We chose to study vaginal transmission as most vaccine and microbicide efficacy trials will likely be dependent upon the use of trial sites in sub-Saharan Africa where infection rates are highest among women (12). We chose to evaluate 1% tenofovir microbicide gel, based on reported efficacy from the CAPRISA 004 trial and its potential early introduction into a sub-Saharan African setting, should efficacy be confirmed in the ongoing FACTS 001 phase III trial (13). We focused on an HIV-1 envelope based vaccine reflecting the likely protective role of antibody in RV144, and adopted a vaccine strategy previously shown to protect NHP against vaginal challenge with Tier-1 $SHIV_{SF162p4}$ (14). We designed a two-part study to test the potential interactions (positive or negative) between these two biomedical strategies. In Part 1 we assessed the protective efficacy of the envelope based vaccine (V), 1% microbicide tenofovir gel alone (M), and their combination (V+M) against 12 repeat vaginal challenges with the Tier-2 $SHIV_{SF162p3}$ (Fig 1a). Critical to the experimental design was that neither of the individual interventions could be fully protective by themselves. In Part 2, protected animals were challenged a further 12 times in the absence of microbicide.

### Vaccination and vaginal SHIV challenge

Cynomolgus macaques in the vaccine groups (V, and V+M groups) received three intranasal priming immunizations (0, 4, 8 weeks) with a combination of two gp140 uncleaved trimers (TV-1 clade C + SF162 clade B) co-administered with R848 (TLR 7/8) adjuvant, followed by two intramuscular boosting immunizations (16 and 28 weeks) delivered with MF59 adjuvant. This induced robust serum binding antibody responses (IgG and IgA) to both immunogens (TV-1 and SF162 gp140) that remained stable through to week 39 (the start of challenge, Supplementary Fig 1). Lower vaginal responses were observed in some animals post intranasal priming and were boosted following intramuscular immunizations (Supplementary Fig 2). Peptide array analysis demonstrated all animals developed a strong cross-clade anti-V3 response, and responses against the gp41 immunodominant region (gp41 ID). Animals also developed cross-clade responses of lower intensity against V2, C2, and C5 gp120 epitopes (Supplementary Fig 3). Autologous neutralizing antibodies (NAbs) against HIV-1 SF162 were induced following intramuscular boosts (mean 7891±11728 SD) that decreased by approximately 1 log prior to challenge (Supplementary Fig 4a). Neutralizing responses were induced to clade C MW965.26 (mean 1757±2240 SD) but not TV1 or Tier-2 viruses of other subtype (week 30, Supplementary Fig 4b & d). As similar responses had been fully

protective against Tier-1 $SHIV_{SF162p4}$ (14) we elected to use the variant $SHIV_{SF162p3}$ that differs by 22 amino acids and contains an additional glycan at the N-terminal base of the V2 loop predicted to confer escape from autologous neutralization (15). Pre-challenge sera and vaginal samples were confirmed to have little or no neutralizing activity against $SHIV_{SF162p3}$ (Supplementary Fig 4c). As predicted based on neutralization, the vaccine alone (group V) showed no protection against 12 consecutive low dose intravaginal challenges with $SHIV_{SF162P3}$ (Fig 1b and Supplementary Table 1a), where half the animals in both the V and naïve control group (C) became infected after 2 challenges (Supplementary Table 2). Furthermore the vaccine alone had no impact on viral kinetics (Fig 1c and Supplementary Fig 5).

**Tenofovir gel alone provides partial protection against SHIV challenge**

The microbicide regime was designed to be partially protective, in our case application of 1% tenofovir gel applied vaginally 1 hour before each of 12 sequential vaginal challenges. We confirmed in cynomolgus macaques that TDF levels measured in peripheral blood and genital tissue at different time points following 1% Tenofovir gel application reached concentrations compatible with local antiviral activity (Supplementary Table 3). We also measured TDF-DP in genital tissues as a means to quantify active phosphorylated drug by local exposed cells. In both cases, similar levels to those reported in rhesus macaques (16) and in humans in the CAPRISA 004 trial were observed (17). To limit the number of animals included in this study (50 in total), we assumed the risk of not detecting a benefit of the partially effective microbicide alone relative to naïve animals (group M). This was indeed the case when compared to naïve controls (OR 0.55, p=0.263, supplementary Table 1a). Only 8 animals were infected after 12 repetitive challenges, where based on the Kaplan-Meier curves (Fig 1b) 9.5 challenges would be required to infect 50% of animals in group M compared to 2 challenges for groups C and V (Supplementary Table 2). Furthermore, the microbicide alone provided a 68% reduction in risk of infection relative to the vaccine group (OR 0.32, p=0.045, Supplementary table 1b). As with the V group there was no apparent impact on viral load kinetics in those animals that became infected (Fig 1c and Supplementary Fig 5).

**Vaccine-microbicide combination provides enhanced protection**

As the primary objective was to assess the potential benefit of combining the vaginal microbicide approach in previously vaccinated animals, the study was designed to detect efficacy of the combination (group V+M, n=16) over naive controls. For the V+M group animals were vaccinated in parallel to the vaccine only group (V), and challenge studies commenced at week 39. In these animals, the microbicide was applied in an identical fashion to group M for each of the 12 challenges in Part 1 (Fig 1). The V+M combination provided a 79% reduction in per-exposure probability of infection (p=0.013, supplementary Table 1a) relative to naïve controls and an 88% reduction (OR 0.12, p=0.001, supplementary Table 1b), relative to vaccine alone (Fig 1b). Only 4 animals were infected after 12 repetitive challenges, insufficient to predict the number of challenges to reach 50% infection in the M+V group.

Animals that remained uninfected following 12 consecutive intravaginal challenges in presence of microbicide (challenge Part 1) immediately progressed to challenge Part 2 (Fig 1a). Here all protected animals received a further 12 sequential challenges, irrespective of initial assignment to M (n=6) or M+V (n=12) groups, to determine susceptibility in the absence of microbicide. By the end of Part 2 (Fig 1b), there was still no statistical difference between group M and untreated controls (Supplementary table 1c) while for the M+V group there was a 84% reduction in per exposure probability of infection (p=0.010) relative to untreated controls and a 86% reduction relative to vaccine alone (p=0.002, supplementary table 1c). Here the positive interaction between microbicide and vaccine remained the same (interaction coefficient p=0.13). In order to gain further insight, we discretized the time into parts 1 and 2 and re-ran the analysis. This analysis indicated that the microbicide alone over the entire course of parts 1 and 2 showed a trend toward protection (OR 0.26 [0.06;1.02] p=0.054) whereas microbicide and vaccine combined significantly reduce the per exposure probability of infection by 91% (p=0.004 (Supplementary table 1d). Further analysis was performed to investigate the potential interaction between time and vaccination in the V+M group (discretized in Part 1 and 2). The effect of the V+M group compared to control increased in Part 2 providing a 98% (p=0.002) reduction in per exposure probability of infection in Part 2 compared to 89% (p=0.010) in Part 1, indicating a long term effect of vaccination even without microbicide (Supplementary table 1e). We excluded potential confounders that might have

influenced differences in susceptibility. Distribution of MHC genotype was equal across the different groups (Supplementary Fig 6) (18). Furthermore, recent studies demonstrated that TRIM5α genotype has very little variability in Mauritian cynomolgus macaques and has no impact on virus acquisition or vaccination outcome (19). To more faithfully replicate the human condition animals were not treated with Depo-provera often used to enhance susceptibility of infection. All animals were naturally cycling. Analysis of progesterone levels showed no over representation in any group of animals in the follicular phase, associated with heightened susceptibility (Supplementary Fig 7).

**Immune parameters modulated by protected exposure to infectious SHIV**

Subsequently we assessed potential immune parameters that might be associated with enhanced protection in the V+M group relative to the V or M groups. Prior to challenge serum or mucosal antibody titre in the V and V+M group were similar although serum antibody titres were slightly raised for the V only group ($p=0.045$) (Fig 2). Induced serum binding antibodies to SF162 and TV-1 gp140 were high pre-challenge and may have masked any potential boosting effects of protected exposure in the V+M group (Supplementary Fig 1). There was no evidence of boosting in the mucosal samples of protected animals over time (Fig 2). However, protected animals in the M group developed low, but detectable systemic binding and neutralizing antibodies to SF162 following 6 challenges in Part 1 (Fig 2 and 3). There was little or no neutralization in sera and mucosal samples to $SHIV_{SF162P3}$ prior to challenge in both V and V+M groups (Supplementary Fig 4 and data not shown) and no evidence of induced response in protected animals at any point during Parts 1 & 2. However, autologous neutralizing responses to $HIV\text{-}1_{SF162}$ were equivalent in the V+M group relative to the V group prior to challenge (Supplementary Fig 8) with no evidence of boosting in protected animals after 6, 9 or 12 challenges in Part 1 (Fig 3). In addition, there was no evidence of boosting neutralizing responses to $HIV\text{-}1_{SF162}$ or $_{MW965}$ in protected animals in Part 1 in the presence of microbicide or Part 2 in the absence of microbicide (Supplementary Fig 9 and 4b). Furthermore, ADCC responses against $SHIV_{SF162P3}$ were absent in all uninfected animals at all time points (Supplementary Fig 10).

Robust cellular immune responses against SF162 gp120, were detected by ELISPOT in V and V+M groups after the five immunizations. Responses were similar in both groups (Fig 4) with mean numbers of spot forming cells (SPC) per million PBMC of 369±246 and 274±171 for IFN-γ and IL-2, respectively (Fig 4a.). There was no correlation between pre-challenge vaccine induced IFN-γ and IL-2 gp120 specific responses and protection observed after challenges in Parts 1 and 2. We also measured T-cell responses to gp120 stimulation after the first sequence of challenges. These had significantly decreased in group V, when compared to pre-challenge measures at week 34 ($p=0.0078$ and $p=0.0078$ for IFN-γ and IL-2, respectively), down to the level of controls (group C) animals. Repeated exposure of non-vaccinated animals in group M were similar ($p=0.7315$ and $p=0.9027$ for IFN-γ and IL-2, respectively) to those observed in control animals (group C) indicating no added benefit for naïve animals when exposed to the virus in presence of microbicide. Remarkably, responses in vaccinated animals appeared to be significantly increased when exposed to $SHIV_{SF162P3}$ following treatment with TDF gel (group V+M) by comparison to animals of group V ($p=0.0058$ and $p=0.0002$ for IFN-γ and IL-2) and to animals of group M ($p<0.0001$ and $p<0.0001$ for IFN-γ and IL-2), demonstrating that repeated challenges in pre-immunized animals when using microbicides for prevention evoke exposure-induced immunity. However, responses raised in protected animals in this group were similar to non-protected macaques. All animals infected in Parts 1 or 2 demonstrated robust responses to SIV Gag peptide pools irrespective of intervention group (Supplementary Fig 11), however there were no detectable anti-Gag responses in any of the protected animals following sequential challenges in Parts 1 or 2, irrespective of the intervention group (M or M+V).

**Discussion**

The primary aim of this study was to determine any potential benefit from a microbicide and vaccine combination over single prevention approaches. The lack of protection by the vaccine alone, and lack of virologic control when challenged with a Tier-2 autologous escape variant $SHIV_{SF162p3}$ is unsurprising. This contrasts with the earlier observation of protection against a high dose challenge with the SF162 immunogen matched $SHIV_{SF162p4}$ (14). These data reflect the dominant role of neutralizing antibodies in sterilizing protection, while the absence of induced ADCC activity likely accounts for the lack of impact on virologic control. Interestingly

the observed level of protection in the M group closely matched that reported for women that were highly compliant with the dosing regime in CAPRISA 004 (5). In this study the gel alone had no impact on viral kinetics, thus at the systemic level there was no evidence to suggest the microbicide delayed or blunted infection. Indeed while protection appeared to be an all or nothing event, the per-exposure probability was reduced by 68% relative to the vaccine group, with a predicted per exposure risk of infection of 0.068 relative to 0.087 for controls or 0.159 for vaccine only (Supplementary table 2). Interestingly exposed but protected animals in the M group seroconverted to HIV-1 SF162. This contrasts to previous studies in humans and macaques where repeat vaginal exposure to 500μg of recombinant gp140 failed to induce antibody responses (20, 21). These data suggest induced response likely requires exposure to envelope in the context of viral particles. It is unclear if induced antibody responses were dependent upon limited replication that was insufficient to establish infection. It is noteworthy that only 1 of 4 uninfected macaques from the control group had detectable responses while all uninfected animal in the M group seroconverted after 6 challenges. These responses echo those reported in a small number of subjects from the CAPRISA 004 trial (22)

The V+M combination was the only group to show a statistical difference to the naïve controls and to the V only group. Furthermore, while the Kaplan-Meier curves from Part 1 predict that 9.5 challenges would be required to infect 50% of animals in group M, too few animals (4/16) were infected in group V+M to reliably calculate the same estimation for the combination group. The positive interaction between microbicide and vaccine appeared to have a sustained effect in Part 2 where animals were challenged in the absence of microbicide (interaction coefficient $p=0.13$). This long-term benefit may indicate a durable effect of vaccination in this group or reflect that repetitive challenge selects animals with a higher resistance to infection over time. The only observable impact of protected viral exposure on vaccine induced immunity in the V+M group were increased cellular responses to Env relative to the V group after 6 repetitive challenges. The significance of this finding is unclear as the level of cellular responses were not predictive of resistance to infection. Antibody levels were too high to ascertain any boost effects of protected exposure in the V+M group, but low level serum responses were observed in protected animals in the M group, suggesting that envelope in the context of virions can modulate humoral immune response. Irrespective of the mechanism, the persistent positive impact for the V+M combination is encouraging.

Previous studies in NHP have suggested potential benefit from combining T cell based vaccine approaches with vaginal microbicides, however sample sizes were small and the microbicide approaches used have yet to be tested in human efficacy studies (23, 24). This study is the first to assess the potential benefit of combining a microbicide with a humoral vaccine. We believe our data provide the strongest evidence to date that a microbicide and vaccine combination might provide greater efficacy then either intervention alone. The observed benefits are likely to be improved with a vaccine that contains optimal B and T cell immunogens. This has important implications given that, should tenofovir gel be licensed for use by women in sub-Sahran Africa, this may become the baseline intervention for future HIV/AIDS vaccine trials. This is likely to be contemporaneous with plans to evaluate a similar regimen to RV144 in South Africans, a partially effective vaccine thought to be mediated by humoral immunity in the absence of Tier-2 neutralization (25). Data presented in this study would suggest a positive benefit in combining these two approaches, and argue that assessment of potentially beneficial combinations be factored into the design of planned randomised controlled clinical efficacy trials.

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## Online Materials and Methods

### Microbicide

Tenofovir gel (1%) or control placebo gel, a proprietary formulation containing purified water with edetate disodium, citric acid, glycerin, methylparaben, propylparaben, and hydroxyethylcellulose (pH 4.5) provided by CONRAD, (Arlington, VA) was transferred to 5-ml syringes and administered in 2-ml volumes via a 10 FG soft catheter introduced ~2 cm into the vagina. The process was atraumatic with no obvious leakage.

### Vaccine Delivery

Vaccine antigens, uncleaved gp140 TV1 and SF162, and MF59 adjuvant were provided and manufactured by Novartis. For each intranasal (IN) immunization, 50μg each of TV-1 and SF162 gp140 was given in solution in a volume of 0.2ml containing 500ug of Resiquamod (R848) a TLR 7/8 agonist (Invivogen). The solution was dropped into each anterior nares of sedated animals placed in a prone position with their heads tilted back. For intramuscular (IM) immunisations 100μg each of TV-1 and SF162 gp140 was mixed with MF59 adjuvant and given in a volume of 0.4mls into the deltoid muscle of the upper arm. Vaccinated cynomolgus macaques received three IN priming immunizations (0, 4, 8 weeks) followed by two IM boosting immunizations (16 and 28 weeks). Challenge studies were commenced 11 weeks after the final boost immunization.

### Microbicide Delivery and Virus Challenge.

50 Mauritius-origin, outbred, young adult, female cynomolgus monkeys (*Macaca fascicularis*) were utilized for this study. Groups were balanced for susceptible and resistant MHC haplotypes (Supplementary Fig 6). Recent studies demonstrated that TRIM5a genotype has no impact on virus acquisition or vaccination outcome (1). On the day of challenge, ~2-mL of the microbicide gel, was applied atraumatically to the vagina, 1h before $SHIV_{162P3}$ was added in a 1-mL volume containing 0.5 $AID_{50}$ of *in vivo* titrated stock of the R5 virus $SHIV_{162P3}$ (2), derived from the HIV-1 SF162 primary isolate and propagated in phytohemagglutin (PHA)-activated rhesus macaque peripheral blood mononuclear cells (PBMC). Stock was obtained through the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, NIH (cat. no. 6526; contributors: Janet Harouse, Cecilia Cheng-Mayer, and Ranajit Pal). Monkeys were bled weekly for viral loads, and infection status was determined by measuring plasma viral load using an RT-PCR assay with a sensitivity limit of 60 RNA copies/ mL (3). Animals were followed to determine set point viral loads. All protocols were approved by the institutional animal care and use committees. The animals were housed in CEA facilities (accreditation no.: B 92-032-02) and handled in accordance with European guidelines for NHP care (European directive 86/609, then, as for January 2013, EU Directive N 2010/63/EU). All 50 animals described were experimentally naïve at the beginning of the study. This study was part of the Europrise project which NHP studies were accredited by ethical committee “Comité Régional d'Ethique pour l'Expérimentation Animale Ile-De-France Sud” under statement number 10-062.

### Humoral Immune assays

TV-1 and SF162 specific binding antibodies were analysed in serum and mucosal secretions. Briefly, 96 well plates were coated with a 1:1 ratio of α-Human κ and α-Human λ (1:1 ratio) capture antibodies to capture the standard curves (IgG or IgA standards) and TV1 and SF162 protein (1μg/ml) to capture antigen specific antibodies. Negative controls consisted of normal cynomologous macaque serum and assay buffer. Standard curves for IgG and IgA consisted of 5-fold serial dilutions of purified IgG or IgA (starting at 1μg/ml), macaque serum and mucosal secretions samples were “screened” at 1:100 and 1:10 respectively with samples and controls added in triplicate. Following secondary antibody addition and development plates were read at 450nm. Positive responses were according to pre-defined cut-off values. Positive samples were titrated and concentrations determined by extrapolation against standards of expressed as μg/ml of specific IgG or IgA.

### Peptide array serum specificity mapping

Serum epitope mapping of heterologous strains was performed essentially as previously described (4,5). Briefly, a peptide library of overlapping peptides (15-mers overlapping by 12), covering 7 full-length HIV-1 gp160 Env consensus sequences (clades A, B, C, and D, group M, CRF1, and CRF2) and 6 vaccine and laboratory strain gp120 sequences (A244_1, TH023_1, MN_B, 1086_C, TV1_C, and ZM651_C), was printed onto epoxy glass slides (provided by JPT Peptide Technologies GmbH [Germany]). Microarray binding was performed using the HS4800 Pro Hybridization Station (Tecan, Männedorf, Switzerland). All arrays were blocked with Superblock T20 PBS blocking buffer for 0.5 hour at 30°C, followed by a 2 hr incubation at 30°C with heat inactivated plasma diluted 1:250 in Superblock T20. Arrays were incubated for 45 minutes at 30°C with Goat Anti-Hu IgG conjugated with DyLight649 (Jackson ImmunoResearch, PA) (1.5 µg/ml final concentration) diluted with Superblock T20. Washes between all steps were with PBS containing 0.1% Tween. Arrays were scanned at a wavelength of 635 nm using an Axon Genepix 4300 Scanner (Molecular Devices, Sunnyvale, CA, USA) at a PMT setting of 540, 100% laser power. Images were analyzed using Genepix Pro 7 software (Molecular Devices). Binding intensity of the post-immunization serum to each peptide was corrected with its own background value, which was defined as the median signal intensity of the prebleed serum for that peptide plus 3 times the standard errors among the 3 subarray replicates present on each slide as described.

**TZMbl neutralization assay**

Viral titration and neutralization assays were performed as previously described (6,7). The following Pseudotyped viruses (PSV) pCAGGS SF162gp160, BX08, 93MW965.26, TV1.21, TV1.29, QH0692, DJ263.8, pSV7d-SHIVSF162-Qlc32 4014, the infectious molecular clone (IMC) pNL-LucR.T2A-SHIV162P3.5.ecto and the culture supernatant of SHIV162P3 M623-Derived were used. Four steps of 3-fold dilutions, starting with 1/20 of each serum or mucosal sample, were incubated with virus supernatant (200 TCID50) for 1 hour. Thereafter, $10^4$ TZMbl cells were added and plates incubated for 48h, when luciferase activity was measured. Positive controls were sera of HIV-1 infected individuals or macaques and monoclonal antibody known to neutralize the viruses. Neutralization titers were defined as the sample dilution at which relative luminescence units (RLU) were reduced by 50% compared to RLU in virus control wells after subtraction of background RLU in control wells with only cells.

**ADCC**

ADCC was tested according to the protocol described in (8) using the IMC pNL-LucR.T2A-SHIV162P3.5.ecto. IMC infected CEM.NKR.CCR5 cells were incubated at 1:30 ratio with PBMCs and six four-fold dilutions of each serum starting with 1:100 dilution. The percentage of the cells positive for the GzB substrate were reported as percentage of Granzyme B activity. Positive control was the monoclonal antibody b12..

**INF-γ and IL-2 ELISpot assay**

ELISpot assays were performed using multiScreen 96-well filtration plates (Millipore, Guyancourt, France) coated overnight at 4°C with monoclonal Ab against monkey IFN-γ (clone GZ-4, Mabtech, Nacka, Sweden) and IL-2 (CT-611 kit, U-Cytech biosciences, Utrecht, the Netherlands) following the manufacturer's instructions. Plates were washed 5 times with PBS then blocked by incubation for 1h at 37°C with RPMI 1640 medium containing glutamax-1 (Gibco, Life technologies, UK) supplemented with 10% heat-inactivated fetal calf serum (FCS, Lonza; culture medium). Fresh isolated PBMC ($2x10^5$ cells per well) were stimulated in duplicate with 2 µg/ml of HIV-1 gp120 SF2 recombinant protein (Novartis, batch N° MID167d) or with SIVmac251 Gag peptide pools (15 mer overlapping of 11 aa). Control wells ($10^3$ PBMC) were stimulated with medium alon or with PMA/ionomycin (1µg/ml). Plates were incubated for 24h (gp120 glycoprotein) or 18h (Gag peptide pools) at 37°C in 5% CO2 atmosphere. They were then washed 5 times with PBS. Biotinylated anti-IFNγ (clone 7-B6-1, Mabtech) or anti-IL-2 (CT-611 kit, U-Cytech biosciences) Ab were then added at a concentration of 1 µg/ml in 0.5% FCS in PBS and the plates were incubated overnight at 4°C. Plates were then washed 5 times with PBS, incubated with 0.25 µg/ml alkaline phosphatase-streptavidin conjugate (Sigma-Aldrich, St-Quentin Fallavier, France) for 1h at 37°C, washed 5 times with PBS. Spots were developed by adding NBT/BCIP substrate (Sigma-Aldrich) and counted using an Automated Elispot Reader ELR04 XL (Autoimmun Diagnostika GmbH, Strassberg, Germany).

**Statistical Analysis**

A time-to-event analysis was conducted with first Kaplan Meir estimates with log-rank test comparing groups for datasets in Phase A and dataset in Phase B. Then, we used a logistic model with random effects taking into account repeated measures in each macaque. This type of model allows taking into account the discrete exposure to the infection due to the challenges at given times. Preliminary analyses showed that the variance of the random effect was not significantly different from zero (p=0.25), indicating a low variability of response between monkeys. The first analysis of interest was: ,

$$logit P infection=1=\alpha 0+\alpha 1t+\alpha 2G$$

where t represents the time since vaccination and G represents the group of treatment. The time of infection was taken as the date of previous challenge prior to first positive SIV test. However, sensitivity analysis, taking time of infection as previous challenge or ante-previous challenge did not change qualitatively the results (results not shown). The rationale of including t in the regression is to take into account any residual confounding associated to a change of the probability of infection over time. This could be due to the selection of the population, those being the most resistant being uninfected until the end. This analysis was run on the dataset of Part 1 and Part 2. Then, we extended the analysis by dissociating the effect of vaccination (V) and microbicide (M) and their interaction (V*M):

$$logit P infection =1=\alpha 0+\alpha 1t+\alpha 2V+\alpha 3M+\alpha 4V*M$$

Second order interaction between M, V and time were tested but not significant (p=0.91).

However, in a final analysis:

$$logit P infection=1=\alpha 0+\alpha 1TP2+\alpha 2V+\alpha 3M+\alpha 4V*TP2+\alpha 5V*M$$

, time was categorized into an indicator of Part 1 or 2 $T_{P2}$=I (t>22weeks) and the effect of the interaction V* $T_{P2}$ was kept (p=0.22). Results were used to compare effect of V+M in Part 1 versus Part 2. This analysis was run on the dataset in Part 1 and 2 pooled. All results are reported in term of Odd-Ratio (OR), together with their 95% confidence interval and p-values for significance. Results were presented as % of risk reduction but readers should keep in mind that OR are only approximation of risk estimates. We compared the results of logistic regression with the time-to-event Cox model analysis: Hazard ratios (HR) and OR give similar conclusions (results not shown). Analyses were run using R software and packages “survival” for survival and “lme4” for logistic mixed effects models.

**References for Methods**

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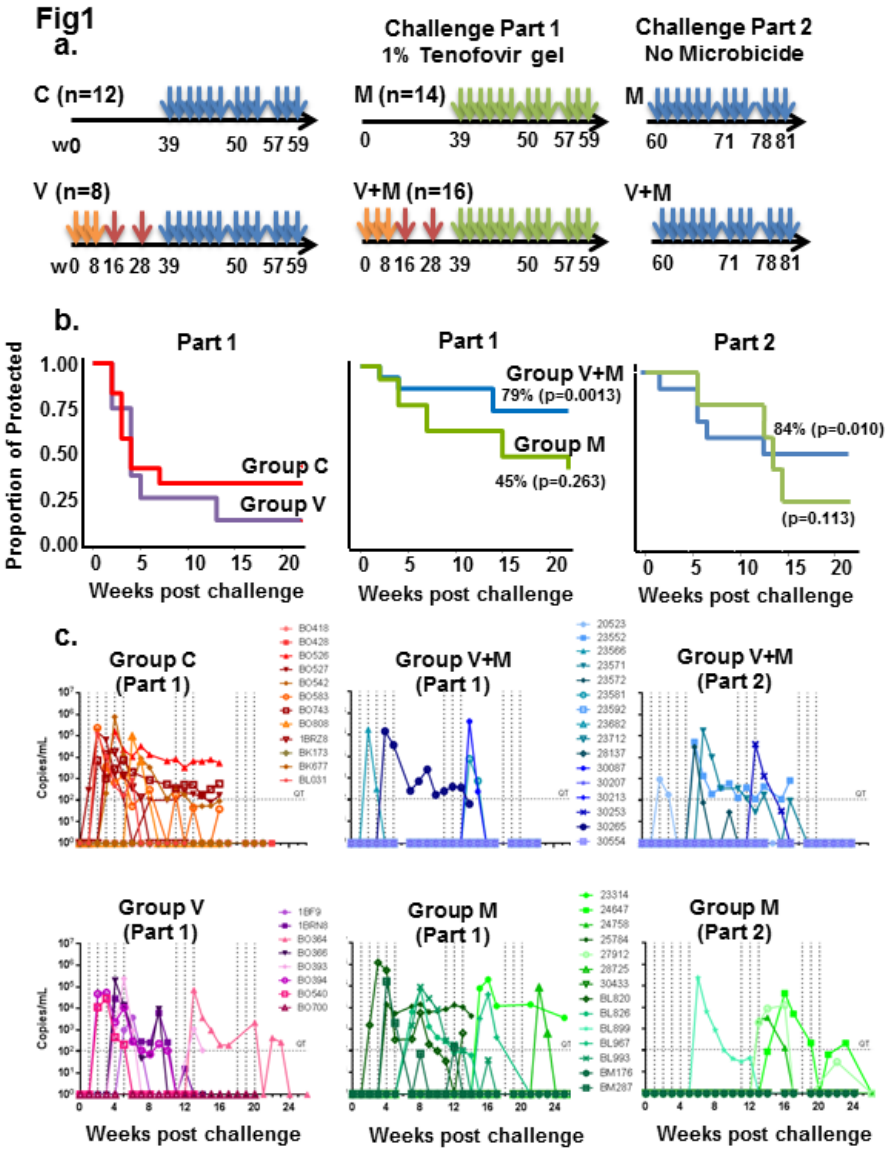


***Figure 1:*** **Study design and infection of macaques as determined by detection by RT-PCR of SHIV in plasma** . V – vaccine alone, V+M – vaccine + microbicide, M- microbicide alone, C-control; a. Blue arrows – date of challenge in absence of microbicide, green arrows – date of challenge in presence of 1%TDF gel, orange arrows – intranasal administration of the vaccine, red arrows – intra-muscular administration of the vaccine; b. Kaplan-Meyers of animals confirmed infected by RT-PCR. Red – Control animals, Purple – V group, Blue – V+M group, green – M group; c. Plasma viral load of individual macaques during challenge phases; vertical dotted lines-date of challenges; horizontal lines – limit of quantification

## Fig 2: B-cell responses

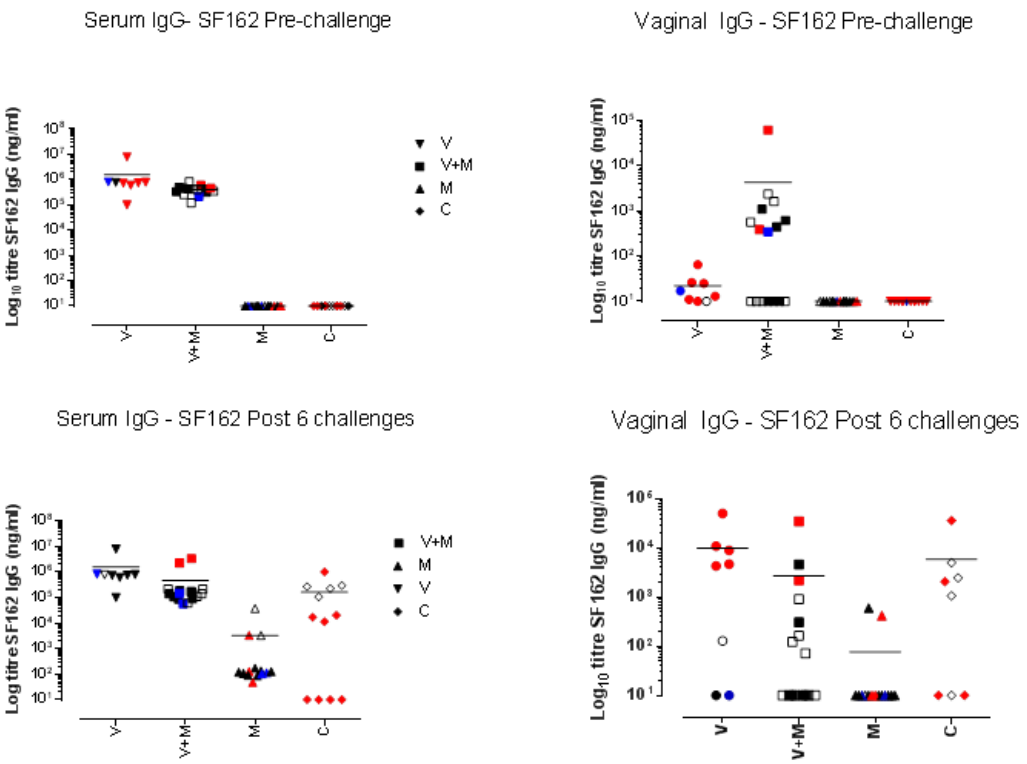


***Figure 2:*** Vaccine induced binding SF162 antibody responses (ng/ml) in serum and vaginal secretions according to animal group; V – vaccine alone, V+M – vaccine + microbicide, M- microbicide alone, C- control. Red symbols denote animals that became infected after 6 challenges , blue symbols denote animals that became infected after 12 challenges. The solid line denotes mean for each group

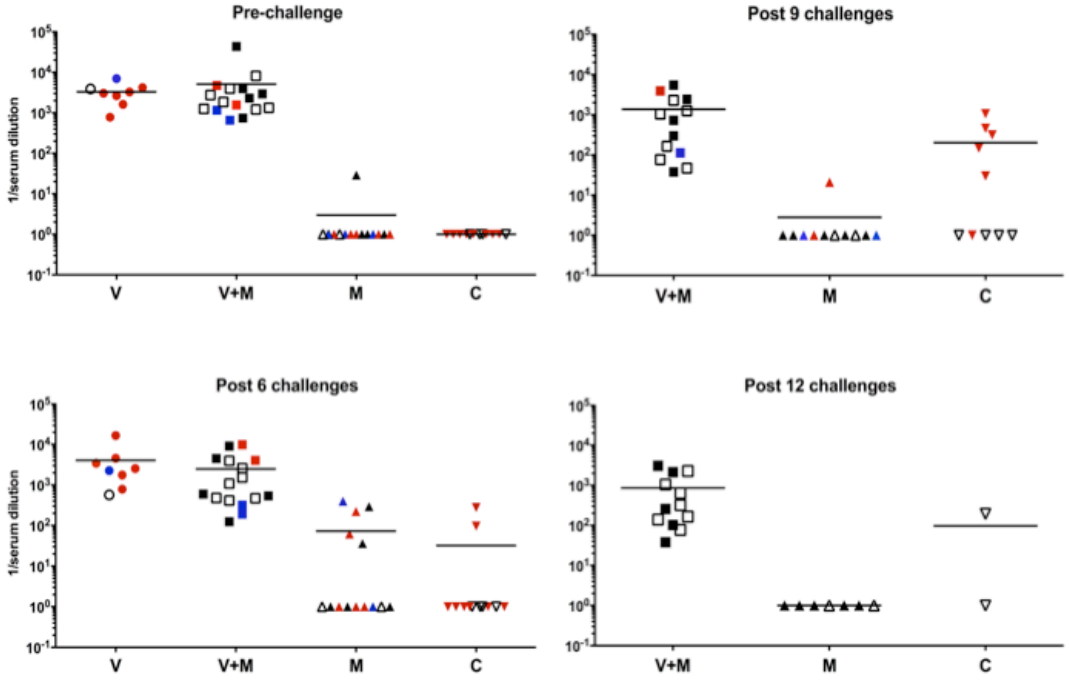


***Figure 3*: Neutralizing antibody responses against PSV SF162 in NHP.** Sera, tested with Tzmbl assay, were obtained 5 weeks before challenge (Pre-challenge), 9 weeks after the first challenge (Post 6 challenges), 17 weeks after the first challenge (Post 9 challenges) and 21 weeks after the first challenge (Post 12 challenges). Each symbol represent one animal. Colors are indicative of the time infection was detected by plasma viremia: Red during the first 6 challenges (till week 9 post-challenge), blue during challenges 7 to 12 (weeks 11 to 21 post-challenge), black during challenges 13 to 24 (from week 22, in absence of microbicide) and empty symbol for animals which did not show sign of infection. P = n.s. between V and V+M at each time-point.

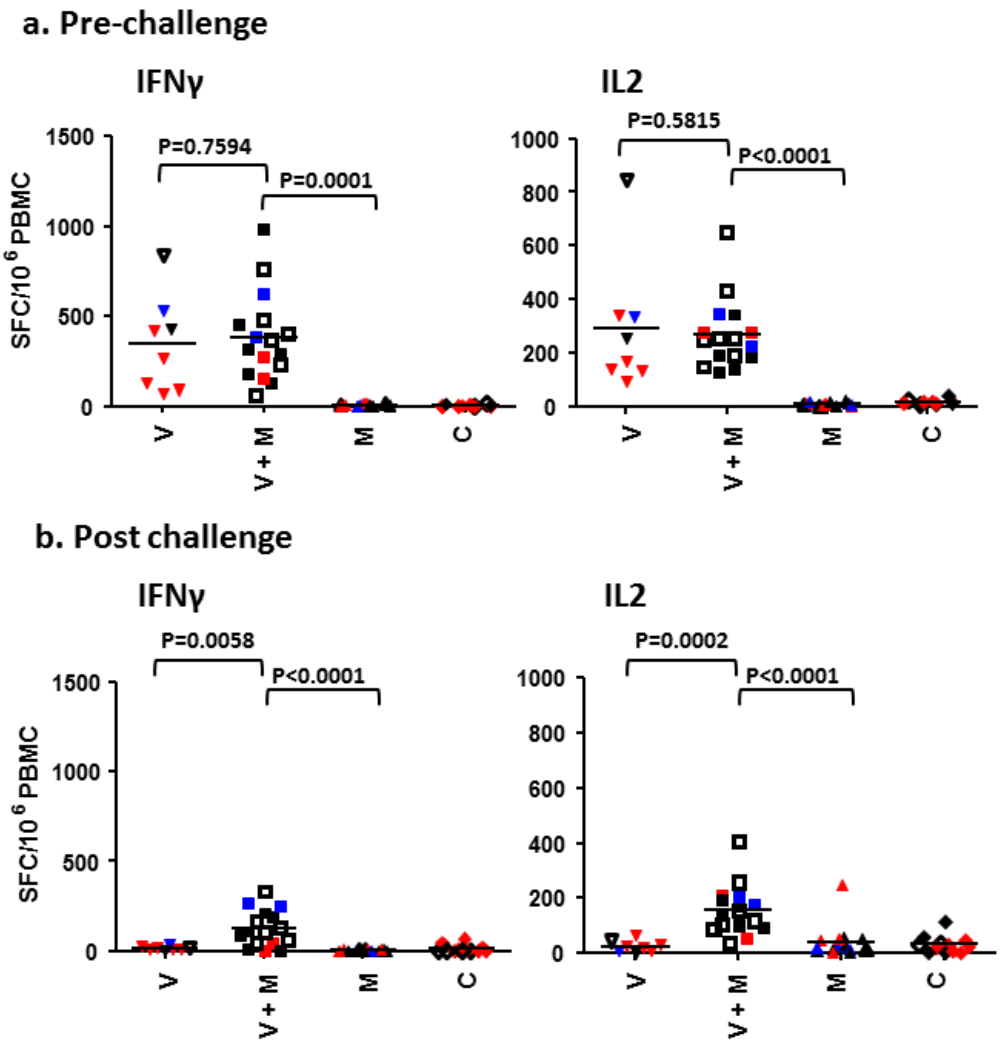


***Figure 4:*** **gp120 T-cell responses as measured by ELISPOT detection of IFNγ and IL2 spot forming cells (SFC) per $10^6$ PBMC ;**a. Analysis at week 34 post first vaccine injection to groups V and V+M, corresponding to 5 weeks before first challenge in part 1. b. responses after six challenges in part 1 ; V – vaccine alone, V+M – vaccine + microbicide, M- microbicide alone, C- control. Colors are indicative of the time infection was detected by plasma viremia: Red during the first 6 challenges (till week 9 post-challenge), blue during challenges 7 to 12 (weeks 11 to 21 post-challenge), black during challenges 13 to 24 (from week 22, in absence of microbicide) and empty symbol for animals which did not show sign of infection.

## Supplementary Table 1a

Odd ratio for group effects on the probability of infection in Part 1(ref=group C).

| Group | OR | IC 95% | p-val |
|---|---|---|---|
| Intercept | 0.22 | [0.09;0.53] | <0.001 |
| Time | 0.94 | [0.87;1.00] | 0.077 |
| Group V+M vs C | 0.21 | [0.06;0.71] | 0.013 |
| Group M vs C | 0.55 | [0.20;1.56] | 0.263 |
| Group V vs C | 1.73 | [0.56;5.32] | 0.341 |

## Supplementary Table 1b

Odd ratio for group effects on the probability of infection in Part 2 (ref=group V).

| Group | OR | IC 95% | p-val |
|---|---|---|---|
| Intercept | 0.38 | [0.15;0.95] | 0.039 |
| Time | 0.94 | [0.87;1.01] | 0.077 |
| Group V+M vs V | 0.12 | [0.03;0.44] | 0.001 |
| Group M vs V | 0.32 | [0.11;0.98] | 0.045 |
| Group C vs V | 0.58 | [0.19;1.78] | 0.341 |

## Supplementary Table 1c

Odd ratio for treatment type on the probability of infection in Parts 1 and 2 combined.

| Group | OR | IC 95% | p-val |
|---|---|---|---|
| Intercept | 0.23 | [0.11;0.47] | <0.001 |
| Time | 0.95 | [0.92;0.98] | 0.004 |
| V vs C | 1.17 | [0.49;2.82] | 0.720 |
| M vs C | 0.46 | [0.18;1.20] | 0.113 |
| M+V vs C | 0.16 | [0.04;0.65] | 0.010 |
| M+V vs V | 0.14 | [0.04;0.50] | 0.002 |

## Supplementary Table 1d

Odd ratio for treatment type on the probability of infection in Parts 1 and 2 combined (discretized time).

| Group | OR | IC 95% | p-val |
|---|---|---|---|
| Intercept | 0.22 | [0.09;0.57] | 0.001 |
| Time in phase B | 0.35 | [0.10;1.19] | 0.093 |
| Vaccine (vs. control) | 0.97 | [0.28;3.28] | 0.958 |
| Microbicide (vs. control) | 0.26 | [0.06;1.02] | 0.054 |
| M+V (vs. control) | 0.09 | [0.02;0.46] | 0.004 |
| M+V (vs. vaccine) | 0.09 | [0.02;0.45] | 0.003 |

## Supplementary Table 1e

Odd ratio for treatment type on the probability of infection in Parts 1 and 2 combined depending on time (interaction of order 2 vac*mic*time).

| Group | OR | IC 95% | p-val |
|---|---|---|---|
| Intercept | 0.17 | [0.06;0.47] | 0.001 |
| Time in phase B | 0.75 | [0.14;4.09] | 0.738 |
| Vaccine (vs. control) | 1.91 | [0.39;9.28] | 0.425 |
| Microb. (vs. control) | 0.37 | [0.09;1.59] | 0.185 |
| M+V phase A (vs. control) | 0.11 | [0.02;0.59] | 0.010 |
| M+V phase B (vs. control) | 0.02 | [0.001;0.22] | 0.002 |

## Supplementary Table 2: Summary analysis of infection risk in Part 1

| Group | Number of Protected/ Total number of animals | Percentage of protected | Number of challenges | Nb of challenges to infect 50% of animals (95% CI) | P-value vs group C (Mantel-Cox test) Overall p=0.006 | Hazard ratio (95%IC) (Cox model for time to infection) | Baseline risk of infection |
|---|---|---|---|---|---|---|---|
| C | 4/12 | 33% | 12 | 2.0 (1.0 - Undetermined) | - | - | 0.087 |
| V | 1/8 | 13% | 12 | 2.0 (2.0 - Undetermined) | 0.568 | 1.53 (0.55-4.26) | 0.159 |
| M | 6/14 | 43% | 12 | 9.5 (5.0 - Undetermined) | 0.272 | 0.56 (0.21-1.49) | 0.068 |
| V+M | 12/16 | 80% | 12 | Undetermined | 0.014 | 0.22 (0.06-0.72) | 0.024 |

## Supplementary Table 3: Pharmacokinetics of vaginally applied 1% Tenofovir gel

| TDF | | | |
|---|---|---|---|
| **Tissue** | Cmax (ng/g) | Tmax (h) | AUC0-12 (ngxh/g) |
| Serum | 349 ± 196 | 1 | 793 ± 388 |
| Vagina | 151533 ± 89312 | 1 | 877034 ± 133061 |
| Exocervix | 286960 ± 169805 | 1 | 756546 ± 277502 |
| Endocervix | 39472 ± 29670 | 1 | 98139 ± 36024 |
| Uterus | 14449 ± 6068 | 1 | 38967 ± 13847 |
| **TDF-DP** | | | |
| **Tissue** | Cmax (ng/g) | Tmax (h) | AUC0-12 (ngxh/g) |
| Vagina | 94 ± 4 | 4 | 668 ± 142 |
| Exocervix | 64 ± 16 | 4 | 883 ± 571 |
| Endocervix | <10 | - | - |
| Uterus | <10 | - | - |

**Supplementary Table 3:** Pharmacokinetics of tenofovir (TDF) and tenofovir-diphosphate (TDF-DP) in female genital tract tissues after intravaginal administration of 1% tenofovir gel (mean ± SD). Tissues were sampled at necropsy (2 animals per time point) which were performed at 0, 1, 4 and 12 hours after gel administration. Tenofovir was quantified in serum by HPLC-MS/MS. Lower limit of quantification: 10 ng/g in vaginal, exocol and uterus and 25 ng/g in endocol. Tenofovir-diphosphate was quantified in serum by HPLC-MS/MS. Lower limit of quantification: 10 ng/g