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1 **Title: Superior efficacy of an HIV vaccine combined with ARV prevention in**
2 **SHIV challenged non-human primates**

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27 **Running Title:** Superior Efficacy of Combined Vaccine and Microbicide in NHP

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29 **Key Words:** Microbicide, Vaccine, combination prevention, ART, HIV, prophylaxis

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32 **ABSTRACT:**

33 Although vaccines and antiretroviral (ARV) prevention have demonstrated partial success
34 against HIV infection in clinical trials, their combined introduction could provide more potent
35 protection. Furthermore, combination approaches could ameliorate potential increased risk of
36 infection following vaccination in the absence of protective immunity. We used a non-human
37 primate model to determine potential interactions of combining a partially effective ARV-
38 microbicide with an envelope-based vaccine. The vaccine alone provided no protection from
39 infection following 12 consecutive low dose intravaginal challenges with SHIVSF162P3, with
40 more animals infected compared to naïve controls. The microbicide alone provided a 68%
41 reduction in risk of infection relative to the vaccine group and a 45% reduction relative to naïve
42 controls. The vaccine-microbicide combination provided an 88% reduction in per exposure risk
43 of infection relative to vaccine alone, 79% reduction relative to controls. Protected animals in the
44 vaccine-microbicide group were challenged a further 12 times in the absence of microbicide and
45 demonstrated a 98% reduction in risk of infection. Taken together a total risk reduction of 91%
46 was observed in this group over 24 exposures ($P=0.004$). These important findings suggest that
47 combined implementation of new biomedical prevention strategies may provide significant gains
48 in HIV prevention.

49 **IMPORTANCE:**

50 There is a pressing need to maximize the impact of new biomedical prevention tools in the face
51 of 2 million HIV infections that occur each year. Combined implementation of complementary
52 biomedical approaches could create additive or synergistic effects that drive improved reduction
53 in HIV incidence. Therefore, we assessed combining an untested vaccine with an anti-retroviral
54 (ARV) based microbicide in a non-human primate vaginal challenge model. Vaccine alone
55 provided no protection (and may have increased susceptibility to SHIV challenge vaginal), while
56 the microbicide reduced infection risk compared to vaccinated and naïve animals. Importantly,
57 the combined interventions provided the greatest level of protection which was sustained
58 following withdrawal of the microbicide. The data suggests provision of ARV prophylaxis
59 during vaccination reduces the potential for unexpected increased risks of infection following
60 immunization and augments vaccine efficacy. These findings are important for potential
61 adoption of ARV-prophylaxis as the baseline intervention for future HIV/AIDS vaccines.

62 **INTRODUCTION:**

63 The Thai RV144 vaccine trial, based on a canary-pox vector prime (ALVAC)–protein boost
64 (AIDSVAX), is the first clinical trial to have shown moderate efficacy (31.2%) in cohorts at low
65 risk of HIV exposure [1]. Partial protection has also been observed with other new biomedical
66 approaches including the use of antiretroviral (ARV) drugs as oral (44-75%) or topical (vaginal,
67 39%) pre-exposure prophylaxis (PrEP) [2-5]. However, effectiveness was dependent upon
68 consistent product use and impacted by multiple factors influencing susceptibility and exposure
69 risk [6]. Three decades of research on combined implementation of structural and behavioural
70 interventions have indicated that combination approaches are more effective than any single
71 intervention alone [7]. Additional potential gains could be realised by assessing the impact of
72 combining new biomedical prevention strategies [8]. Indeed, positive impact would be seen if
73 combining ARV prevention and vaccines provides better protection than either intervention
74 alone. Here, reduction of the number of transmitted strains and/or delay in the initial viral
75 expansion phase might buy time for more effective immune clearance. Conversely systemic
76 immunity might curtail dissemination of virus that bypasses the activity of topically applied
77 ARVs. Furthermore, subjects protected from productive infection on repeat exposure to HIV
78 when using ARV-prevention, might evoke exposure-induced immunity. This could serve to
79 modify vaccine-induced immune responses to better recognize prevalent circulating virus.
80 Indeed, evidence from some non-human primate (NHP) studies indicates that animals exposed to
81 infectious virus when protected by PrEP demonstrate cellular immune responses to the challenge
82 virus [9, 10]. However, such immune responses in these non-vaccinated animals appeared
83 insufficient to protect animals from subsequent challenge in the absence of PrEP [10].
84 Conversely, combinations could also have potential negative interactions. Certain vaccine
85 induced immune activation may have potential to increase mucosal HIV-1 susceptibility [11, 12]

that in combination could reduce the efficacy of ARV-prevention. This has important implications given that increased sensitivity over the potential of novel vaccines to enhance the risk of HIV acquisition may drive the adoption of oral pre-exposure prophylaxis provision as the baseline intervention for future HIV/AIDS vaccine trials.

MATERIALS AND METHODS:

Ethics Statement

All 50 Mauritius-origin, outbred, young adult (4-6 years old) female cynomolgus monkeys (*Macaca fascicularis*) were housed in the CEA facilities ("Commissariat à l'Energie Atomique", Fontenay-aux-Roses, France, CEA accreditation no.: B 92-032-02) in compliance with Standards for Human Care and Use of Laboratory of the Office for Laboratory Animal Welfare (OLAW, USA). The study and procedures were approved by ethical committee "Comité Régional d'Ethique pour l'Expérimentation Animale Ile-De-France Sud" with notification number 10-062. All experimental procedures were carried out in the CEA animal facility and in strict accordance with European guidelines for NHP care (European directive 86/609, then, as for January 2013, EU Directive N 2010/63/EU) for protection of animal used in experimentation and other scientific purposes and the Weatherall Report. The monitoring of the animals was under supervision of veterinarians in charge of the animal facilities. All efforts were made to minimize suffering, including improved housing conditions with enrichment opportunities (e.g. 12:12 light dark scheduling, provision of treats as biscuits and supplemented with fresh fruit, constant access to water supply in addition to regular play interaction with staff caregivers and research staff). Experimental procedures were performed while animals were under anesthesia using 10mg/kg (body weight) of ketamine. Euthanasia was performed prior to development of symptoms of disease (indicated by a rapid decline in CD4⁺ T cells and or increase in viremia) and was

109 performed by IV injection of a lethal dose of pentobarbital. All 50 animals described were
110 experimentally naïve at the beginning of the study. Investigators were blind to the group
111 allocation while performing immunological and virological assessments.

112 **Cynomolgus macaque combined micorobicide, vaccine study**

113 Tenofovir gel (1%) or control placebo gel, a proprietary formulation containing purified water
114 with edetate disodium, citric acid, glycerin, methylparaben, propylparaben, and
115 hydroxyethylcellulose (pH 4.5) provided by CONRAD, (Arlington, VA) was transferred to 5-ml
116 syringes and administered in 2-ml volumes via a 10 FG soft catheter introduced ~2 cm into the
117 vagina. The process was atraumatic with no obvious leakage and carried out while the animals
118 were under anesthesia. Vaccine antigens, uncleaved gp140 TV1 and SF162, and MF59 adjuvant
119 were provided and manufactured by Novartis. For each intranasal (IN) immunization, 50µg each
120 of TV-1 and SF162 gp140 was given in solution in a volume of 0.2ml containing 500ug of
121 Resiquamod (R848) a TLR 7/8 agonist (Invivogen). The solution was dropped into each anterior
122 nares of sedated animals placed in a prone position with their heads tilted back. For
123 intramuscular (IM) immunizations 100µg each of TV-1 and SF162 gp140 was mixed with MF59
124 adjuvant and given in a volume of 0.4mls into the deltoid muscle of the upper arm. Vaccinated
125 cynomolgus macaques received three IN priming immunizations (0, 4, 8 weeks) followed by two
126 IM boosting immunizations (16 and 28 weeks). Challenge studies were commenced 11 weeks
127 after the final boost immunization.

128 50 Mauritius-origin, outbred, young adult (4-6 years old), female cynomolgus monkeys
129 (*Macaca fascicularis*) were utilized for this study. No randomization was used however groups
130 were balanced for susceptible and resistant MHC haplotypes (H2, H6 and H4) [13]. Recent
131 studies demonstrated that TRIM5a genotype has no impact on virus acquisition or vaccination

132 outcome [14]. Sample size was chosen knowing that 90% infection was expected in control
133 group based on previous titration of the challenge stock. A sample size of 16 for each of the M
134 and V+M groups was chosen with an 80% power to detect an increase in survival proportion of
135 0.53 with a significance level (alpha) of 0.05 (two-tailed log rank test). A sample size of 12
136 animals for the controls provided an 80% power to detect an efficacy of 67% in the other groups.
137 Only 8 animals available for inclusion in group V however this number was estimated sufficient
138 to prove that vaccine alone was ineffective in preventing infection. Investigators performing the
139 animal studies were not blinded as to group allocation. On the day of challenge, ~2-mL of the
140 microbicide gel, was applied atraumatically to the vagina (M and V+M groups), 1h before viral
141 challenge. SHIV_{162P3} was added in a 1-mL volume containing 0.5 AID₅₀ of *in vivo* titrated stock
142 of the R5 virus SHIV_{162P3} [15], derived from the HIV-1 SF162 primary isolate and propagated in
143 phytohemagglutinin (PHA)-activated rhesus macaque peripheral blood mononuclear cells
144 (PBMC). Stock was obtained through the National Institutes of Health (NIH) AIDS Research
145 and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious
146 Diseases, NIH (cat. no. 6526; contributors: Janet Harouse, Cecilia Cheng-Mayer, and Ranajit
147 Pal). Monkeys were bled weekly for viral loads, and infection status was determined by
148 measuring plasma viral load using an RT-PCR assay with a sensitivity limit of 60 RNA copies/
149 mL and a quantification limit of 300 RNA copies/mL [16]. No further vaginal treatments were
150 halted on detection of viremia. Animals were followed to determine set point viral loads. We
151 excluded macaque #25015 from group M because at autopsy 2 months post infection we found a
152 malformation of the genital tract: direct connection between vagina, uterus and peritoneal cavity.
153 We also excluded animal #28413 from M group due to a technical failure to deliver the full dose

154 of viral challenge. A schematic representation of the vaccination schedule, vaccination groups
155 and immunizations for part 1 and 2 is given in Fig. 1A.

156 **Immunogenicity antibody analysis**

157 TV-1 and SF162 specific binding antibodies were analyzed in serum and mucosal secretions.
158 Briefly, 96 well plates were coated with a 1:1 ratio of α -Human κ and α -Human λ antibodies
159 (Southern Biotech) to capture the standard curves (IgG or IgA standards) and TV1 and SF162
160 protein (1 μ g/ml) to capture antigen specific antibodies. Negative controls consisted of normal
161 cynomolgus macaque serum and assay buffer. Standard curves for IgG and IgA consisted of
162 5fold serial dilutions of purified IgG or IgA (starting at 1 μ g/ml), macaque serum and mucosal
163 secretions samples were “screened” at 1:100 and 1:10 respectively with samples and controls
164 added in triplicate. Bound IgG was detected with goat anti-monkey IgG (Fc-specific) HRP
165 conjugate (Serotec) and bound IgA was detected with goat anti-monkey IgA (α -chain-specific)
166 HRP conjugate (Autogen Bioclear,). Following secondary antibody addition and development
167 plates were read at 450nm. Positive responses were according to pre-defined cut-off values.
168 Positive samples were titrated and concentrations determined by extrapolation of unknown
169 samples against standards and expressed as μ g/ml of specific IgG or IgA.

170 **Peptide array serum specificity mapping**

171 Serum epitope mapping of heterologous strains was performed essentially as previously
172 described [17, 18]. Briefly, a peptide library of overlapping peptides (15-mers overlapping by
173 12), covering 7 full-length HIV-1 gp160 Env consensus sequences (clades A, B, C, and D, group
174 M, CRF1, and CRF2) and 6 vaccine and laboratory strain gp120 sequences (A244_1, TH023_1,
175 MN_B, 1086_C, TV1_C, and ZM651_C), was printed onto epoxy glass slides (provided by JPT
176 Peptide Technologies GmbH [Germany]). Microarray binding was performed using the HS4800

177 Pro Hybridization Station (Tecan, Männedorf, Switzerland). All arrays were blocked with
178 Superblock T20 PBS blocking buffer for 0.5 hour at 30°C, followed by a 2 hr incubation at 30°C
179 with heat inactivated plasma diluted 1:250 in Superblock T20. Arrays were incubated for 45
180 minutes at 30°C with Goat Anti-Hu IgG conjugated with DyLight649 (Cat #109-495-098,
181 Jackson ImmunoResearch, PA) (1.5 µg/ml final concentration) diluted with Superblock T20.
182 Washes between all steps were with PBS containing 0.1% Tween. Arrays were scanned at a
183 wavelength of 635 nm using an Axon Genepix 4300 Scanner (Molecular Devices, Sunnyvale,
184 CA, USA) at a PMT setting of 540, 100% laser power. Images were analyzed using Genepix Pro
185 7 software (Molecular Devices). Binding intensity of the post-immunization serum to each
186 peptide was corrected with its own background value, which was defined as the median signal
187 intensity of the prebleed serum for that peptide plus 3 times the standard errors among the 3
188 subarray replicates present on each slide as described.

189 **Neutralization TZMbl assay analysis**

190 Viral titration and neutralization assays were performed as previously described [19, 20]. The
191 following Pseudotyped viruses (PSV) pCAGGS SF162gp160, BX08, 93MW965.26, TV1.21,
192 TV1.29, QH0692, DJ263.8, pSV7d-SHIVSF162-Qlc32 4014, the infectious molecular clone
193 (IMC) pNL-LucR.T2A-SHIV162P3.5.ecto and the culture supernatant of SHIV162P3 M623-
194 Derived were used. The JC53bl-13 (TZM-bl, Cat No 5011) cell line was obtained from NIBSC
195 Center for AIDS Reagents, UK and validated as mycoplasma free with MycoAlert (Lonza). Four
196 steps of 3-fold dilutions, starting with 1/20 of each serum or mucosal sample, were incubated
197 with virus supernatant (200 TCID₅₀) for 1 hour. Thereafter, 10⁴ TZMbl cells were added and
198 plates incubated for 48h, when luciferase activity was measured. Positive controls were sera of
199 HIV-1 infected individuals or macaques and monoclonal antibody known to neutralize the

200 viruses. Neutralization titers were defined as the sample dilution at which relative luminescence
201 units (RLU) were reduced by 50% compared to RLU in virus control wells after subtraction of
202 background RLU in control wells with only cells.

203 **ADCC activity**

204 ADCC was tested according to the protocol described in [21] using the IMC pNL-LucR.T2A-
205 SHIV162P3.5.ecto. IMC infected CEM.NKR.CCR5 cells (obtained from cell line was obtained
206 from NIBSC Center for AIDS Reagents (Cat No 0099), UK and validated as mycoplasma free
207 with MycoAlert (Lonza).) were incubated at 1:30 ratio with PBMCs and six four-fold dilutions
208 of each serum starting with 1:100 dilution. The percentage of the cells positive for the GzB
209 substrate are reported as percentage of Granzyme B activity. Positive control was the monoclonal
210 antibody b12.

211 **INF- γ and IL-2 T cell ELISpot assay**

212 ELISpot assays were performed using multiScreen 96-well filtration plates (Millipore,
213 Guyancourt, France) coated overnight at 4°C with monoclonal Ab against monkey IFN- γ (clone
214 GZ-4, Mabtech, Nacka, Sweden) and IL-2 (CT-611 kit, U-Cytech biosciences, Utrecht, the
215 Netherlands) following the manufacturer's instructions. Plates were washed 5 times with PBS
216 then blocked by incubation for 1h at 37°C with RPMI 1640 medium containing glutamax-1
217 (Gibco, Life technologies, UK) supplemented with 10% heat-inactivated fetal calf serum (FCS,
218 Lonza; culture medium). Fresh isolated PBMC (2×10^5 cells per well) were stimulated in
219 duplicate with 2 $\mu\text{g/ml}$ of HIV-1 gp120 SF162 recombinant protein (Novartis, batch N°
220 MID167d) or with SIVmac251 Gag peptide pools (15 mer overlapping of 11 aa). Control wells
221 (10^3 PBMC) were stimulated with medium alone or with PMA/neomycin (1 $\mu\text{g/ml}$). Plates were
222 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-IFN γ (clone 7-B6-1, Mabtech) or anti-IL-2 (CT-611 kit, U-Cytech biosciences) Ab were then added at a concentration of 1 μ g/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 μ g/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 analyses

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

$$\text{logit } P(\text{infection}=1) = \alpha_0 + \alpha_1 t + \alpha_2 G$$

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: 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).

$$\text{logit } P(\text{infection} = 1) = \alpha_0 + \alpha_1 t + \alpha_2 V + \alpha_3 M + \alpha_4 V * M$$

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

245 of the population, those being the most resistant being uninfected until the end. This analysis was
246 run on the dataset of Part 1 and Part 2. Then, we extended the analysis by dissociating the effect
247 of vaccination (V) and microbicide (M) and their interaction (V*M):
248 Second order interaction between M, V and time were tested but not significant (p=0.91).
249 However, in a final analysis:

$$\text{logit } P(\text{infection}=1) = \alpha_0 + \alpha_1 TP_2 + \alpha_2 V + \alpha_3 M + \alpha_4 V * TP_2 + \alpha_5 V * M$$

250 Time t, was categorized into an indicator of Part 1 or 2 $TP_2 = I(t > 22 \text{ weeks})$ and the effect of the
251 interaction $V * TP_2$ was kept (p=0.22). Results were used to compare effect of V+M in Part 1
252 versus Part 2. This analysis was run on the dataset in Part 1 and 2 pooled. All results are reported
253 in term of Odd-Ratio (OR), together with their 95% confidence interval and p-values for
254 significance. Results were presented as % of risk reduction but readers should keep in mind that
255 OR are only approximation of risk estimates. We compared the results of logistic regression with
256 the time-to-event Cox model analysis: Hazard ratios (HR) and OR give similar conclusions
257 (results not shown). Analyses were run using R software and packages “survival” for survival
258 and “lme4” for logistic mixed effects models.

259 **RESULTS:**

260 **Study design**

261 In this study we use a NHP model to determine potential interactions of combining a microbicide
262 with an envelope-based vaccine over either intervention alone. We chose to study vaginal
263 transmission as most vaccine and microbicide efficacy trials will likely be dependent upon the
264 use of trial sites in sub-Saharan Africa where infection rates are highest among women [22]. We
265 chose to evaluate 1% tenofovir microbicide gel, based on reported efficacy from the CAPRISA
266 004 trial [5]. We focused on an HIV-1 envelope based vaccine reflecting the likely protective
267 role of antibody in RV144, and adopted a vaccine strategy previously shown to protect NHP

268 against vaginal challenge with Tier-1 SHIV_{SF162p4} [23]. We designed a two-part study to test the
269 potential interactions (positive or negative) between these two biomedical strategies over single
270 interventions. In Part 1 we compare the protective efficacy of the envelope based vaccine (V),
271 1% microbicide tenofovir gel alone (M), and their combination (V+M) against 12 repeat vaginal
272 challenges with the Tier-2 SHIV_{SF162p3} (Fig.1A). Critical to the experimental design was that
273 neither of the individual interventions could be fully protective by themselves, therefore we
274 elected to use SHIV_{SF162p3} over the more closely matched SHIV_{SF162p4}, where vaccination was
275 previously shown to provide 100% protection [23]. In Part 2, protected animals were challenged
276 a further 12 times in the absence of microbicide.

277 **Vaccination and vaginal SHIV challenge**

278 Cynomolgus macaques in the vaccine groups (V, and V+M groups) received three intranasal
279 priming immunizations (0, 4, 8 weeks) with a combination of two gp140 uncleaved trimers (TV-
280 1 clade C + SF162 clade B) co-administered with R848 (TLR 7/8) adjuvant, followed by two
281 intramuscular boosting immunizations (16 and 28 weeks) delivered with MF59 adjuvant. This
282 induced robust serum binding antibody responses (IgG and IgA) to both immunogens (TV-1 and
283 SF162 gp140) that remained stable through to week 39 the start of challenge (Fig.1B-E). Low
284 vaginal responses were observed in some animals, post intranasal priming and were boosted
285 following intramuscular immunizations (Fig. 2). Peptide array analysis demonstrated all animals
286 developed a strong cross-clade anti-V3 response, and responses against the gp41
287 immunodominant region (gp41 ID). Animals also developed cross-clade responses of lower
288 intensity against V2, C2, and C5 gp120 epitopes (Fig. 3). Autologous serum neutralizing
289 antibodies (NAbs) against HIV-1 SF162 were induced following intramuscular boosts (mean
290 7891±11728 SD) that decreased by approximately 1 log prior to challenge (Fig. 4A).

291 Neutralizing antibodies were absent or below the level of detection in vaginal secretions. Serum
292 neutralizing responses were also induced to clade C MW965.26 (mean 1757±2240 SD) but not
293 TV1 or Tier-2 viruses of other subtype week 30 (Fig. 4 B & D). As similar responses had been
294 fully protective against Tier-1 SHIV_{SF162p4} [23] we elected to use the variant SHIV_{SF162p3} that
295 differs by 22 amino acids and contains an additional glycan at the N-terminal base of the V2 loop
296 predicted to confer escape from autologous neutralization [24]. Pre-challenge sera and vaginal
297 samples were confirmed to have little or no neutralizing activity against SHIV_{SF162p3} (Fig. 4C).
298 As predicted based on neutralization, the vaccine alone (group V) showed no protection against
299 12 consecutive low dose intravaginal challenges with SHIV_{SF162p3} (Fig. 5A and Table 1A), where
300 50% of the animals in both the V and naïve control group (C) became infected after 2 challenges
301 (Table 2). Strikingly there were more infections with the vaccine than in naïve controls with an
302 odds ratio of 1.73, although this did not reach statistical significance (p=0.341, Table 1A), where
303 the baseline risk of infection was almost double that of controls (Table 2). Furthermore the
304 vaccine alone had no impact on viral load kinetics or control (Fig.5B).

305 **Tenofovir gel and vaginal SHIV challenge**

306 The microbicide regime was designed to be partially protective, in our case application of 1%
307 tenofovir gel applied vaginally 1 hour before each of 12 sequential vaginal challenges. We
308 confirmed in cynomolgus macaques that TDF levels measured in peripheral blood and genital
309 tissue at different time points following 1% Tenofovir gel application reached concentrations
310 compatible with local antiviral activity (Table 3). We also measured TDF-DP in genital tissues
311 as a means to quantify active phosphorylated drug by local exposed cells. In both cases, similar
312 levels to those reported in rhesus macaques [25] and in humans in the CAPRISA 004 trial were
313 observed [26]. To limit the number of animals included in this study (50 in total), we assumed

314 the risk of not detecting a benefit of the partially effective microbicide alone (group M) relative
315 to naïve animals (group C). This was indeed the case when compared to naïve controls (OR 0.55,
316 $p=0.263$, Table 1A). However, based on the Kaplan-Meier curves (Fig. 5A) 9.5 challenges would
317 be required to infect 50% of animals in group M compared to 2 challenges for groups C and V
318 (Table 2). The microbicide alone provided a 68% reduction in risk of infection relative to the
319 vaccine group (OR 0.32, $p=0.045$, Table 1B). There was however, no apparent impact of
320 tenofovir gel on viral load kinetics in those animals that became infected (Fig. 5B).

321 **Vaccine-microbicide combination provides enhanced protection**

322 As the primary objective was to assess the potential benefit of combining the vaginal microbicide
323 approach in previously vaccinated animals, the study was designed to detect efficacy of the
324 combination (group V+M, $n=16$) over either intervention alone. For the V+M group animals
325 were vaccinated in parallel to the vaccine only group (V), and challenge studies commenced at
326 week 39. In these animals, the microbicide was applied in an identical fashion to group M for
327 each of the 12 challenges in Part 1 Fig. 1A. The V+M combination provided a 79% reduction in
328 per-exposure probability of infection ($p=0.013$, Table 1A) relative to naïve controls, an 88%
329 reduction (OR 0.12, $p=0.001$, Table 1B), relative to vaccine alone, and a 63% reduction (OR
330 0.39) relative to microbicide alone, although this did not reach statistical significance ($p=0.114$)
331 (Fig. 5A). Only 4 animals were infected after 12 repetitive challenges, insufficient to predict the
332 number of challenges to reach 50% infection in the M+V group. Animals that remained
333 uninfected following 12 consecutive intravaginal challenges in presence of microbicide
334 (challenge Part 1) immediately progressed to challenge Part 2 (Fig. 1A). Here all protected
335 animals received a further 12 sequential challenges, irrespective of initial assignment to M ($n=6$)
336 or M+V ($n=12$) groups, to determine susceptibility in the absence of microbicide. By the end of

337 Part 2 (Fig. 5A) there was still no statistical difference between group M and untreated controls
338 in Part 1 (Table 1C), while for the V+M group there was a 84% reduction in per exposure
339 probability of infection ($p=0.010$) relative to untreated controls and a 86% reduction in per
340 exposure probability of infection relative to vaccine alone ($p=0.002$). Here the positive
341 interaction between microbicide and vaccine remained the same (interaction coefficient $p=0.13$).
342 In order to gain further insight, we discretized the time into parts 1 and 2 and re-ran the analysis.
343 This analysis indicated that the microbicide alone over the entire course of parts 1 and 2 showed
344 a trend toward protection [OR 0.26, (0.06:1.02) $p=0.054$, Table 1D] whereas microbicide and
345 vaccine combined provided significant protection with a 91% reduction in the per exposure
346 probability of infection ($p=0.004$, Table 1D). Further analysis was performed to investigate the
347 potential interaction between time and vaccination in the V+M group (discretized in Part 1 and
348 2). The effect of the V+M group compared to control increased in Part 2 providing a 98%
349 ($p=0.002$) reduction in per exposure probability of infection in Part 2 compared to 89%
350 ($p=0.010$) in Part 1, indicating a long term effect of vaccination even without microbicide (Table
351 1E). We excluded potential confounders that might have influenced differences in susceptibility.
352 Distribution of the MHC genotype was equal across the different groups [13]. Furthermore,
353 recent studies demonstrated that TRIM5 α genotype has very little variability in Mauritian
354 cynomolgus macaques and has no impact on virus acquisition or vaccination outcome [14]. To
355 more faithfully replicate the human condition animals were not treated with Depo-provera often
356 used to enhance susceptibility of infection. All animals were naturally cycling. Analysis of
357 progesterone levels showed no over representation in any group of animals in the follicular
358 phase, associated with heightened susceptibility.

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

360 Subsequently we assessed potential immune parameters that might be associated with enhanced
361 protection in the V+M group relative to the V or M groups. Prior to challenge serum or mucosal
362 antibody titre in the V and V+M group were similar although serum antibody titres were slightly
363 raised for the V only group ($p=0.0045$, Fig.6A & Fig.1B-D). Induced serum binding antibodies
364 to SF162 and TV-1 gp140 were high pre-challenge and may have masked any potential boosting
365 effects of protected exposure in the V+M group (Fig. 1B). However there was no evidence of
366 boosting in the mucosal samples of protected animals over time. There was little or no
367 neutralization in sera and mucosal samples to SHIV_{SF162P3} prior to challenge in both V and V+M
368 groups (Fig. 4) and no evidence of induced response in protected animals at any point during
369 Parts 1 & 2. However, autologous neutralizing responses to HIV-1_{SF162} were equivalent in the
370 V+M group relative to the V group prior to challenge and post immunizations (Fig. 7A) with no
371 evidence of boosting in protected animals after 6, 9 or 12 challenges in Part 1 (Fig. B-E) or
372 change in epitope recognition assessed by peptide array analysis (data not shown). In addition,
373 there was no evidence of boosting neutralizing responses to HIV-1_{SF162} or _{MW965} in protected
374 animals in Part 1 in the presence of microbicide or Part 2 in the absence of microbicide (Fig. 8).
375 Furthermore, ADCC responses against SHIV_{SF162P3} were absent in all uninfected animals at all-
376 time points.

377 Robust cellular immune responses against SF162 gp120, were detected by ELISPOT in V
378 and V+M groups after the five immunizations. Responses were similar in both groups Fig. 9 with
379 mean numbers of spot forming cells (SPC) per million PBMC of 369 ± 246 and 274 ± 171 for IFN-
380 γ and IL-2, respectively (Fig. 9A & B). There was no correlation between pre-challenge vaccine
381 induced IFN- γ and IL-2 gp120 specific responses and protection observed after challenges in
382 Parts 1 and 2. We also measured T-cell responses to gp120 stimulation after the first sequence of

383 challenges. These had significantly decreased in group V, when compared to pre-challenge
384 measures at week 34 ($p=0.0058$ and $p=0.0002$ for IFN- γ and IL-2, respectively, Fig. 9C & D),
385 down to the level of control animals (group C). Repeated exposure of non-vaccinated animals in
386 group M were similar ($p=0.7315$ and $p=0.9027$ for IFN- γ and IL-2, respectively) to those
387 observed in control animals (group C) indicating no added benefit for naïve animals when
388 exposed to the virus in presence of microbicide.

389 Remarkably, responses in vaccinated animals appeared to be significantly increased when
390 exposed to SHIV_{SF162P3} following treatment with TDF gel (group V+M) by comparison to
391 animals of group V ($p=0.0058$ and $p=0.0002$ for IFN- γ and IL-2) and to animals of group M
392 ($p<0.0001$ and $p<0.0001$ for IFN- γ and IL-2), demonstrating that repeated challenges in pre-
393 immunized animals when using microbicides for prevention evoke exposure-induced immunity.
394 However, responses raised in protected animals in this group were similar to non-protected
395 macaques. All animals infected in Parts 1 or 2 demonstrated robust responses to SIV Gag peptide
396 pools irrespective of intervention group (Fig. 9G & H), however there were no detectable anti-
397 Gag responses in any of the protected animals following sequential challenges in Parts 1 or 2,
398 irrespective of the intervention group (M or V+M).

399 **DISCUSSION:**

400 The primary aim of this study was to determine any potential benefit from a microbicide and
401 vaccine combination over single prevention approaches. The lack of protection by the vaccine
402 alone, and lack of virologic control when challenged with a Tier-2 autologous escape variant
403 SHIV_{SF162p3} is unsurprising. This contrasts with the earlier observation of protection against a
404 high dose challenge with the SF162 immunogen matched Tier-1 SHIV_{SF162p4} [23]. These data
405 reflect the dominant role of neutralizing antibodies in sterilizing protection, while the absence of

406 induced ADCC activity likely accounts for the lack of impact on virologic control. However the
407 observation that 7/8 animals were infected in the vaccine group versus 4/12 in the controls,
408 although not statistically significant, is concerning given the potential for vaccination to increase
409 risk of acquisition in large cohort studies [11, 12]. Interestingly the observed level of protection
410 (43%) in the M group closely matched that reported for women that were highly compliant with
411 the dosing regime in CAPRISA 004 [5], although in our study with limited number of animals
412 this did not reach statistical significance. Gel alone had no impact on viral kinetics, thus at the
413 systemic level there was no evidence to suggest the microbicide delayed or blunted infection.
414 Indeed while protection appeared to be an all or nothing event, the per-exposure probability was
415 reduced by 68% relative to the vaccine group, with a predicted per exposure risk of infection of
416 0.068 relative to 0.087 for controls or 0.159 for vaccine only (Table 2). Interestingly there was
417 no evidence of seroconversion in exposed but protected animals in the M group despite repeated
418 viral challenge, (Fig. 5). These data concord with previous studies in humans and macaques
419 where repeat vaginal exposure to 500µg of recombinant gp140 failed to induce antibody
420 responses [27, 28]. The observed lack of seroconversion contrasts to those reported in a small
421 number of subjects from the CAPRISA 004 trial [29], but concords with that seen in the Partners
422 PrEP study [30]. It is unclear if induced antibody responses in CAPRISA 004 were dependent
423 upon limited replication that was insufficient to establish infection.

424 The V+M combination was the only group to show a statistical difference to the naïve
425 controls and to the V only group. Thus any potential vaccine related enhancement in per
426 exposure risk of infection was mitigated by the combination of V+M. Furthermore, there was a
427 63% reduction (OR 0.39) in the V+M group relative to M alone, although this did not reach
428 statistical significance. Nevertheless, the Kaplan-Meier curves from Part 1 predict that 9.5

429 challenges would be required to infect 50% of animals in group M, while too few animals (4/16)
430 were infected in group V+M to reliably calculate the same estimation for the combination group.
431 Larger studies would be needed to confirm or refute a difference between V+M and M groups.
432 However the positive interaction between microbicide and vaccine appeared to have a sustained
433 effect in Part 2 where animals were challenged in the absence of microbicide (interaction
434 coefficient $p=0.13$). This long-term benefit may indicate a durable effect of vaccination in this
435 group or reflect that repetitive challenge selects animals with higher or increasing resistance to
436 infection over time. The only observable impact of protected viral exposure on vaccine induced
437 immunity in the V+M group were increased cellular responses to Env relative to the V group
438 after 6 repetitive challenges. The significance of this finding is unclear as the level of cellular
439 responses were not predictive of resistance to infection. Nevertheless, this echoes studies with
440 the rhesus CMV vector encoding SIV Gag, Rev-Tat-Nef and Env that protected 50% of animals
441 from productive infection despite inducing equivalent cell mediated responses in protected and
442 unprotected animals [31]. By contrast, antibody levels were too high to ascertain any boost
443 effects of protected exposure in the V+M group. Irrespective of the mechanism, the persistent
444 positive impact for the V+M combination is encouraging.

445 Previous studies in NHP have suggested potential benefit from combining T cell based
446 vaccine approaches with vaginal microbicides, however sample sizes were small and the
447 microbicide approaches used have yet to be tested in human efficacy studies [32, 33]. This study
448 is the first to assess the potential benefit of combining a microbicide with a humoral vaccine. We
449 believe our data suggest that a microbicide-vaccine combination might provide greater efficacy
450 than either intervention alone. The observed benefits are likely to be improved with a vaccine
451 that contains optimal B and T cell immunogens. Perhaps more importantly, these data indicate

452 that provision of ARV-prophylaxis ameliorates the potential for vaccine associated increased risk
453 of infection. Although we assessed the efficacy of tenofovir gel, the protective effects would
454 likely be similar or higher with oral prophylaxis, where compliance levels may be more reliable
455 [34]. This has important implications given that oral pre-exposure prophylaxis could be adopted
456 as the baseline intervention for future HIV/AIDS vaccine trials. Our findings are
457 contemporaneous with plans to evaluate a similar regimen to RV144 in South Africans, a
458 partially effective vaccine thought to be mediated by humoral immunity in the absence of Tier-2
459 neutralization [35, 36]. This has important implications given that oral pre-exposure prophylaxis
460 could be adopted as the baseline intervention for future HIV/AIDS vaccine trials.

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477 DD, XS, GT, MP, AC, GS, RT & RJS analysed data. CO, SB provided reagents. RJS, RLG, GS
478 and AC wrote the manuscript.

479 **Conflict of interest statement:** The authors have declared that no conflict of interest exists.

480 **TABLES:**

481 **Table 1. Odds ratios for Group effects on probability of infection Parts 1 and 2**

482 **Table 1A:** Odds ratio for group effects on the probability of infection in Part 1(ref=group C).

483

Group	OR	IC 95%	p-value
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

484

485 **Table1B:** Odds ratio for group effects on the probability of infection in Part 2 (ref=group V).

486

Group	OR	IC 95%	p-value
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

487

488 **Table 1C:** Odds ratio for treatment type on the probability of infection in Parts 1 and 2

489 combined.

490

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

491

492 **Table 1D:** Odds ratio for treatment type on the probability of infection in Parts 1 and 2

493 combined (discretized time).

494

Group	OR	IC 95%	p-value
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
V+M (vs. control)	0.09	[0.02;0.46]	0.004
V+M (vs. vaccine)	0.09	[0.02;0.45]	0.003

495

496 **Table 1E:** Odds ratio for treatment type on the probability of infection in Parts 1 and 2 combined
497 depending on time (interaction of order 2 vac*mic*time).
498

Group	OR	IC 95%	p-value
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
Microbicide (vs. control)	0.37	[0.09;1.59]	0.185
V+M phase A (vs. control)	0.11	[0.02;0.59]	0.010
V+M phase B (vs. control)	0.02	[0.001;0.22]	0.002

499

500 **Table 2. Summary analysis of infection risk in Part 1**

Animal Group	Number of Protected/ Total number of animals	Percentage Protected animals	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

501

502 **Table 3. Pharmacokinetics of vaginally applied 1% Tenofovir gel**

503

504

TDF			
Tissue	C_{max} (ng/g)	T_{max} (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

509

TDF-DP

Tissue	C_{max} (ng/g)	T_{max} (h)	AUC₀₋₁₂ (ngxh/g)
Vagina	94 ± 4	4	668 ± 142
Exocervix	64 ± 16	4	883 ± 571
Endocervix	<10	-	-
Uterus	<10	-	-

513 **Foot Note Table 3:** Pharmacokinetics of tenofovir (TDF) and tenofovir-diphosphate (TDF-DP) in female genital
 514 tract tissues after intravaginal administration of 1% tenofovir gel (mean ± SD). Tissues were sampled at necropsy (2
 515 animals per time point) which were performed at 0, 1, 4 and 12 hours after gel administration. Tenofovir was
 516 quantified in serum by HPLC-MS/MS. Lower limit of quantification: 10 ng/g in vaginal, Exocervix and uterus and
 517 25 ng/g in Endocervix. Tenofovir-diphosphate was quantified in serum by HPLC-MS/MS. Lower limit of
 518 quantification: 10 ng

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673

674 **Figure Legends:**

675 **Fig 1: Fig 1. Vaccination and challenge schedule with longitudinal serum antibody**
676 **responses in V+M group pre-challenge. (A)** Schematic representation of vaccination and
677 challenge schedules V – vaccine alone, V+M – vaccine + microbicide, M- microbicide alone, C-

678 control Part 1 & Part 2. Green vertical arrows indicate intranasal vaccinations with R848 at
679 weeks 0,4 & 8, red vertical arrows intramuscular vaccinations at weeks 16 &28 with MF59. Blue
680 vertical arrows indicate 12 challenges in the absence of microbicide (weeks 39-59), black
681 vertical arrows 12 challenges in the presence of microbicide (weeks 60-80)). **(B)** SF162 serum
682 IgG responses in V+M group, SF162 serum IgA in **(C)** TV-1 serum IgG responses in **(D)** and
683 serum IgA TV-1 responses in E all pre-challenge

684
685
686 **Fig 2: Vaginal binding antibody responses for V+M group only post immunizations, prior**
687 **to challenge.** Antibody responses measured in vaginal secretion samples collected post –
688 intranasal immunization (post IN at week 12) and after intramuscular immunizations (post IM) at
689 week 34 prior to challenge. SF162 and TV-1 specific IgG antibodies are indicated as black
690 symbols, red symbols show SF162 and TV-1 specific IgA responses (ng/ml). Lines indicate
691 mean and +/- SEM for each group

692
693 **Fig 3: Mapping of serum IgG binding to gp120 linear epitopes by peptide microarray: (A)**
694 **& (B)** Representative gp120 binding plots in serum of an immunized animal binding to either
695 consensus gp120 sequences or vaccine strain gp120 at peak serum IgG response (2 weeks post
696 last immunization). Numbers on X-axis are peptide numbers in the array library. Y-axis are
697 signal intensity values (baseline subtracted). Different color bars represent different
698 strains/clades as indicated. Variable –V and constant - C domains of gp120 are labelled in each
699 panel. **(C)** Binding intensity to each epitope identified for the animals. The definition of each
700 epitope as the range of peptide numbers in the array library is listed under each epitope. Color
701 coding highlights higher intensity in darker red. Pie chart shows average percentage of binding to
702 each epitope in the total. Each pie slice represents the mean values of all animals for maximum
703 binding to the specified epitope/sum of maximum binding of all epitopes

704 **Fig 4: Neutralizing antibody titers groups V+M and V:** **(A) & (B)** show kinetics of
705 neutralizing response after each immunization for the V+M group. **(C)** V - group. Sera were
706 tested in TZMbl assay against pseudotype viruses (PSV) SF162 or MW965.26. Dotted lines
707 represent vaccinations at weeks 0, 4, 8 (intranasal immunizations with R848), 16 and 28 weeks
708 (intramuscular immunizations with MF59). **(D)** Shows neutralizing antibody titers of NHP of

709 group V+M 30 weeks after the first immunization against a panel of PSVs of Tier 1 and Tier 2
710 tested in TZMbl assay. Solid horizontal lines in panel D indicate mean neutralizing titers

711

712 **Fig 5: Time to event survival analysis Kaplan-Meier curves and infection of macaques**
713 **determined by RT-PCR in plasma;** V – vaccine alone, V+M – vaccine + microbicide, M-
714 microbicide alone, C- control; (A) Kaplan-Meier plots of animals confirmed infected by RT-
715 PCR. Red – Control animals, Purple – V group, Blue – V+M group, green – M group; (B)
716 Plasma viral load of individual macaques during challenge phases; vertical dotted lines-date of
717 challenges; horizontal lines – limit of quantification 300 copies /mL.

718

719 **Fig 6. : Vaccine induced binding antibodies to SF162 in serum:** V – vaccine alone, V+M –
720 vaccine + microbicide, M- microbicide alone, C- control. (A) Serum response antibodies as
721 ng/ml pre-challenge at week 34. (B) post-6 challenges. (C) post-9 challenges, (D) post-12
722 challenges. Colors are indicative of the time infection was detected by plasma viremia: Red
723 during the first 6 challenges (till week 9 post-challenge), blue during challenges 7 to 12 (weeks
724 11 to 21 post-challenge), black during challenges 13 to 24 (from week 22, in absence of
725 microbicide) and empty symbol for animals which did not show sign of infection.

726

727 **Fig 7: Neutralizing antibody titres in serum against SF162:** Sera were analyzed by TZMbl
728 assay, at 12 (post IN) and 34 weeks (post IM) immunization (A). Pre-challenge (B) post-6
729 challenges (C), post-9 challenges (D) and post-12 challenges (E). Each symbol represents one
730 animal, colors are indicative of the time infection was detected by plasma viremia: Red during
731 the first 6 challenges (up to 9 weeks post-challenge), blue, challenges 7 to 12 (weeks 11 to 21
732 post-challenge), black challenges 13 to 24 and empty symbols for animals which did not get
733 infected. P = n.s. between V and V+M at each time-point.

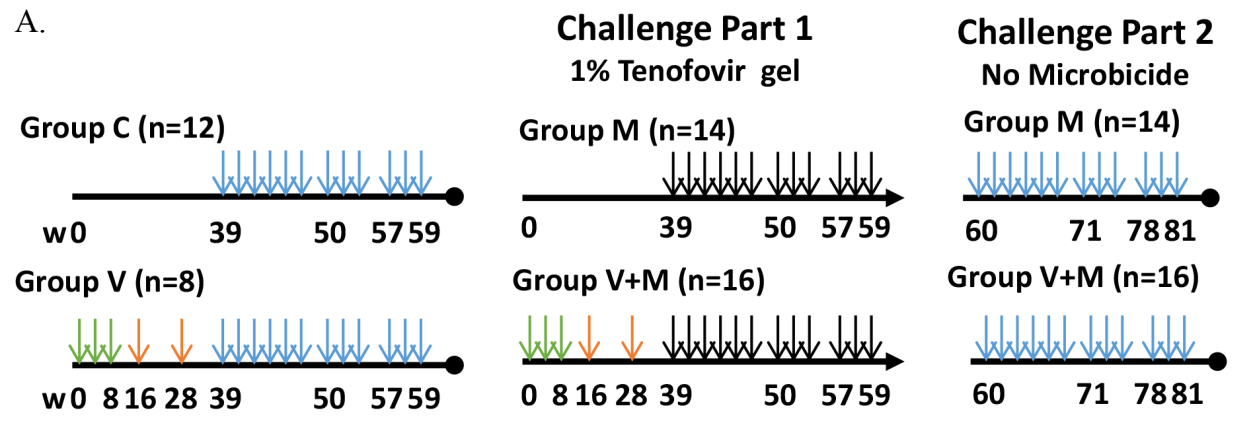
734 **Fig 8. Neutralizing antibody responses against SF162 during challenge phases Part 1 &**
735 **Part 2:** TZMbl assay neutralization data 5 weeks before challenge (0 wk), 9 weeks, 17 weeks
736 and 21 weeks after the first challenge with microbicide, and one week after the 12 challenges
737 without microbicide (week 42) and 5 weeks thereafter (week 47). Each symbol represents one
738 animal. Colors are indicative of the time infection was detected by plasma viremia: Red during
739 the first 6 challenges (till week 9 post-challenge), blue during challenges 7 to 12 (weeks 11 to 21

740 post-challenge), black during challenges 13 to 24 (from week 22, in absence of microbicide) and
741 empty symbols for animals which did not get infected.. The mean is indicated for each group
742 with a solid line. V+M vs. V $p < 0.0001$; Mann-Whitney

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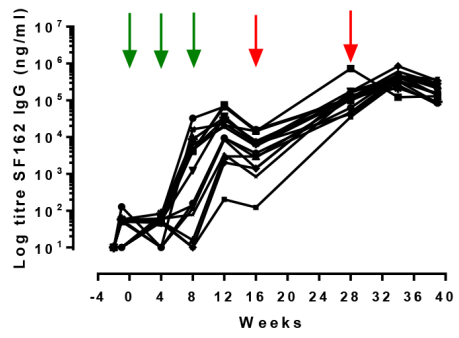
744 **Fig 9. T-cell responses measured by Elispot assay pre and post challenge:** gp120 specific T
745 cell Elispot responses as spot forming cells (SFU)/ 10^6 PBMC pre-challenge, (A) & (B), post 6
746 challenges (C) & (D). SIV-gag specific T cell Elispot responses pre-challenge (E) & (F) and
747 post 6 challenges in (G) & (H). Red symbols indicate infection during the first 6 challenges, blue
748 during challenges 7 to 12 (weeks 11 to 21 post-challenge), black during challenges 13 to 24,
749 empty symbols for animals which did not get infected. Solid lines indicate means and p-values
750 where relevant following statistical analysis.

A.



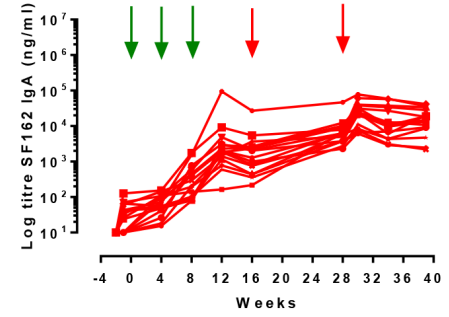
V+M Group Serum IgG SF162 Pre-challenge

B.



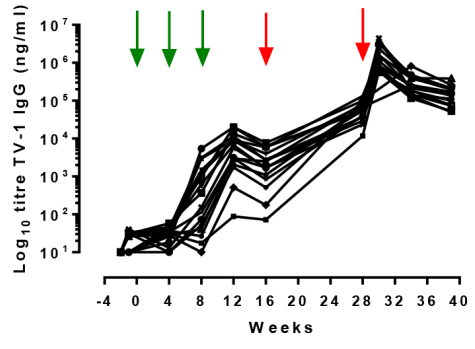
V+M Group Serum IgA SF162 Pre-challenge

C.



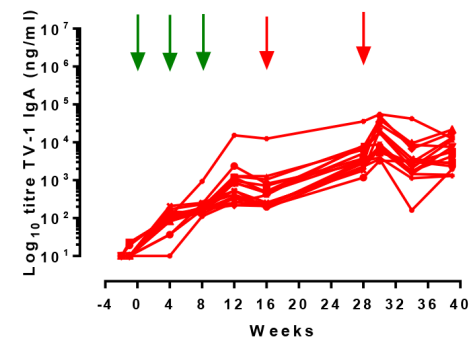
D.

V+M Group Serum IgG TV-1 Pre-challenge

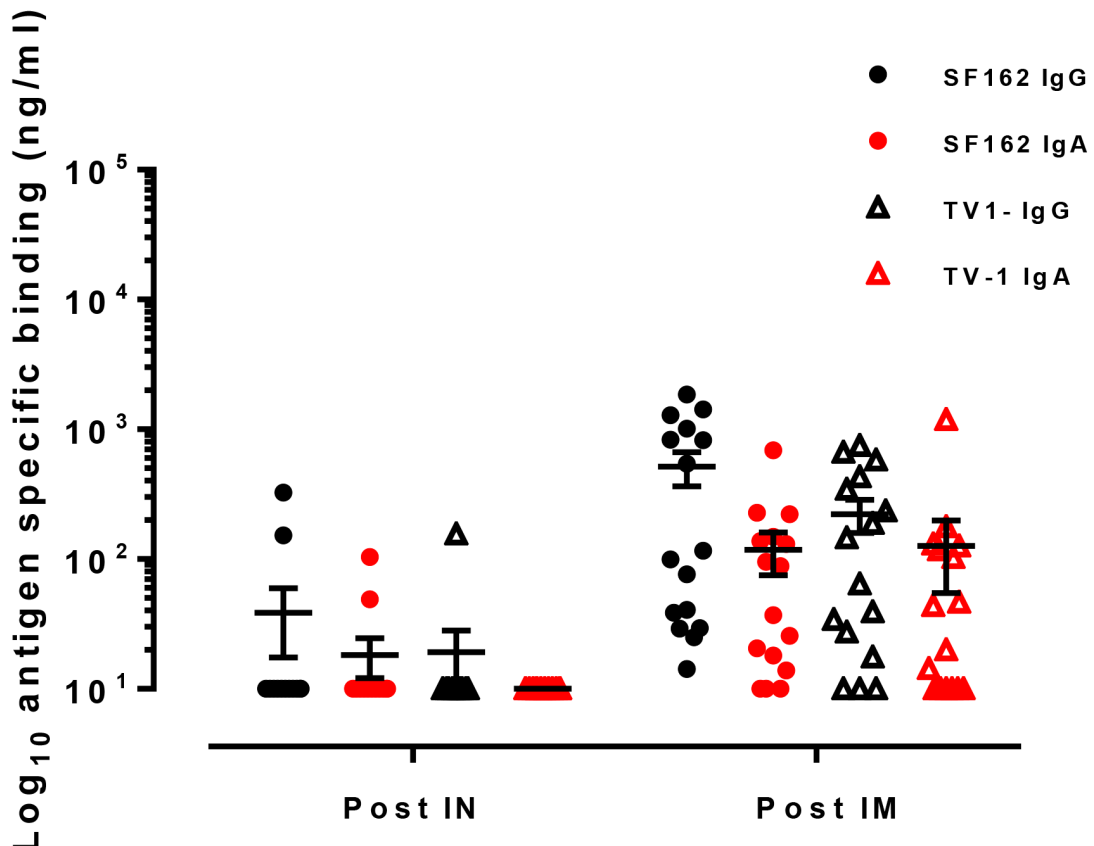


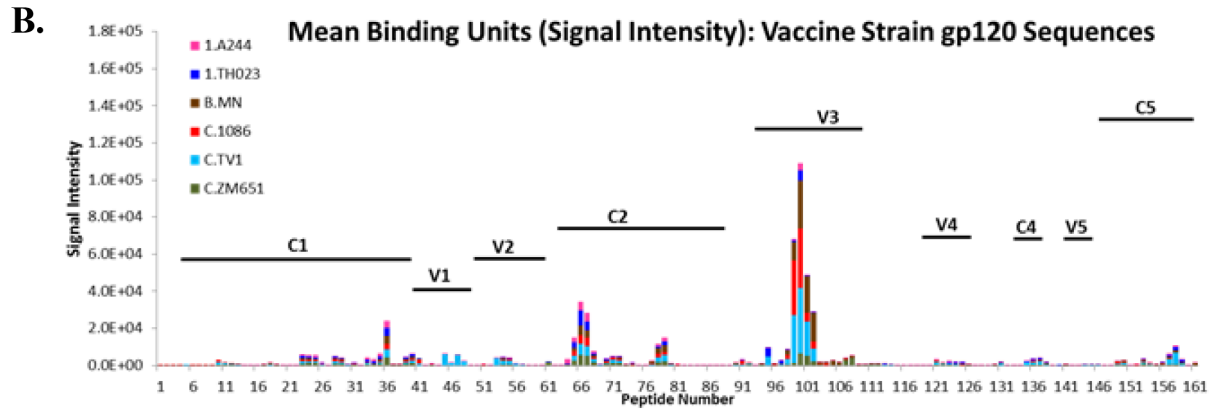
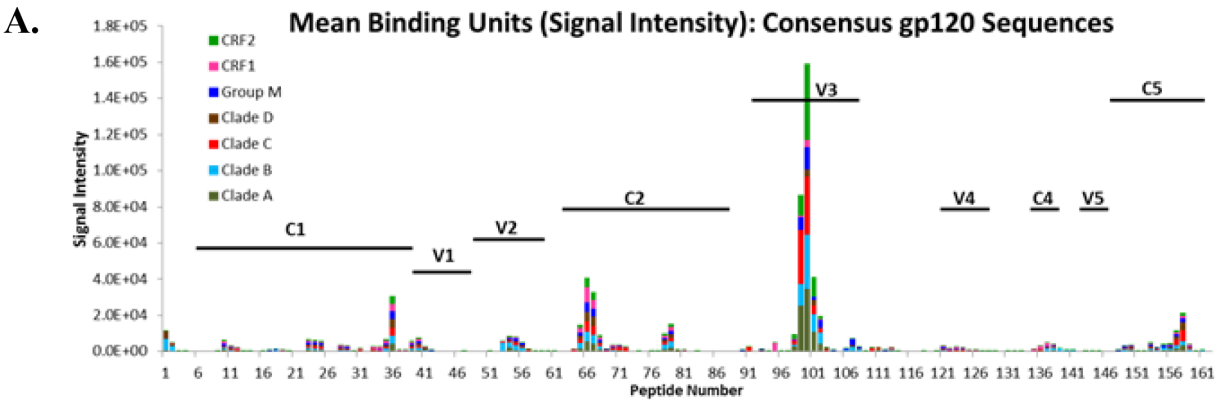
E.

V+M Group Serum IgA TV-1 Pre-challenge



V+M Group Vaginal responses Pre-challenge

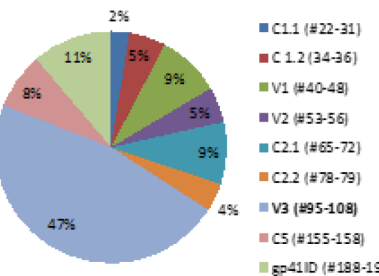


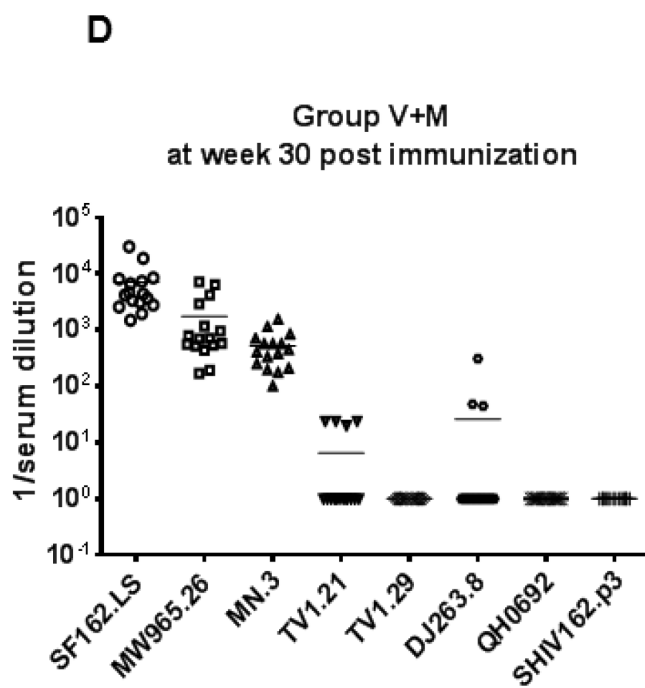
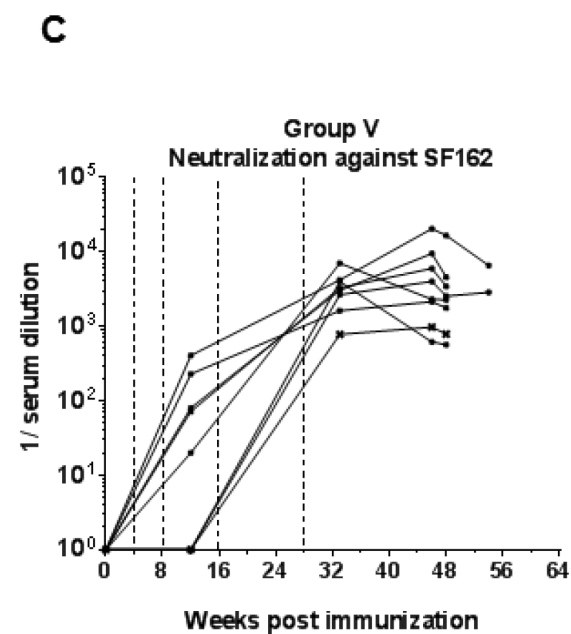
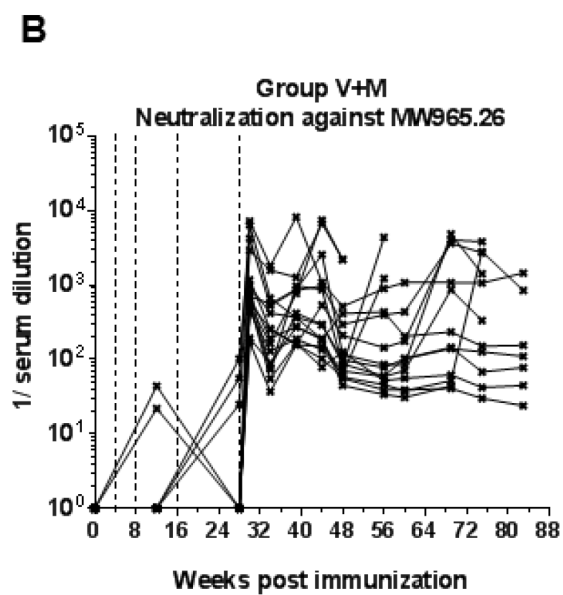
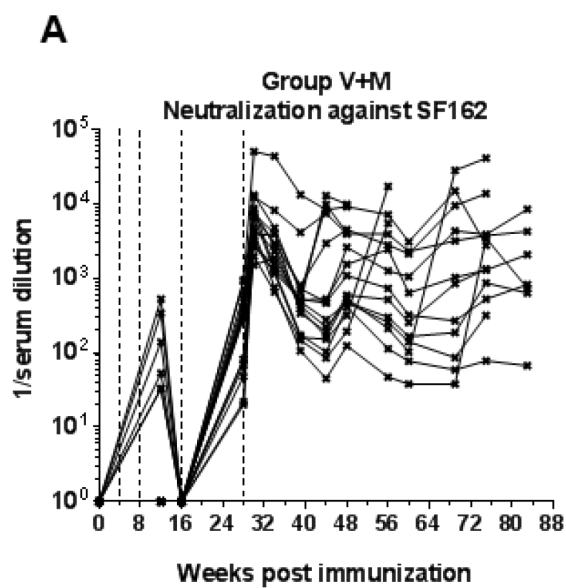


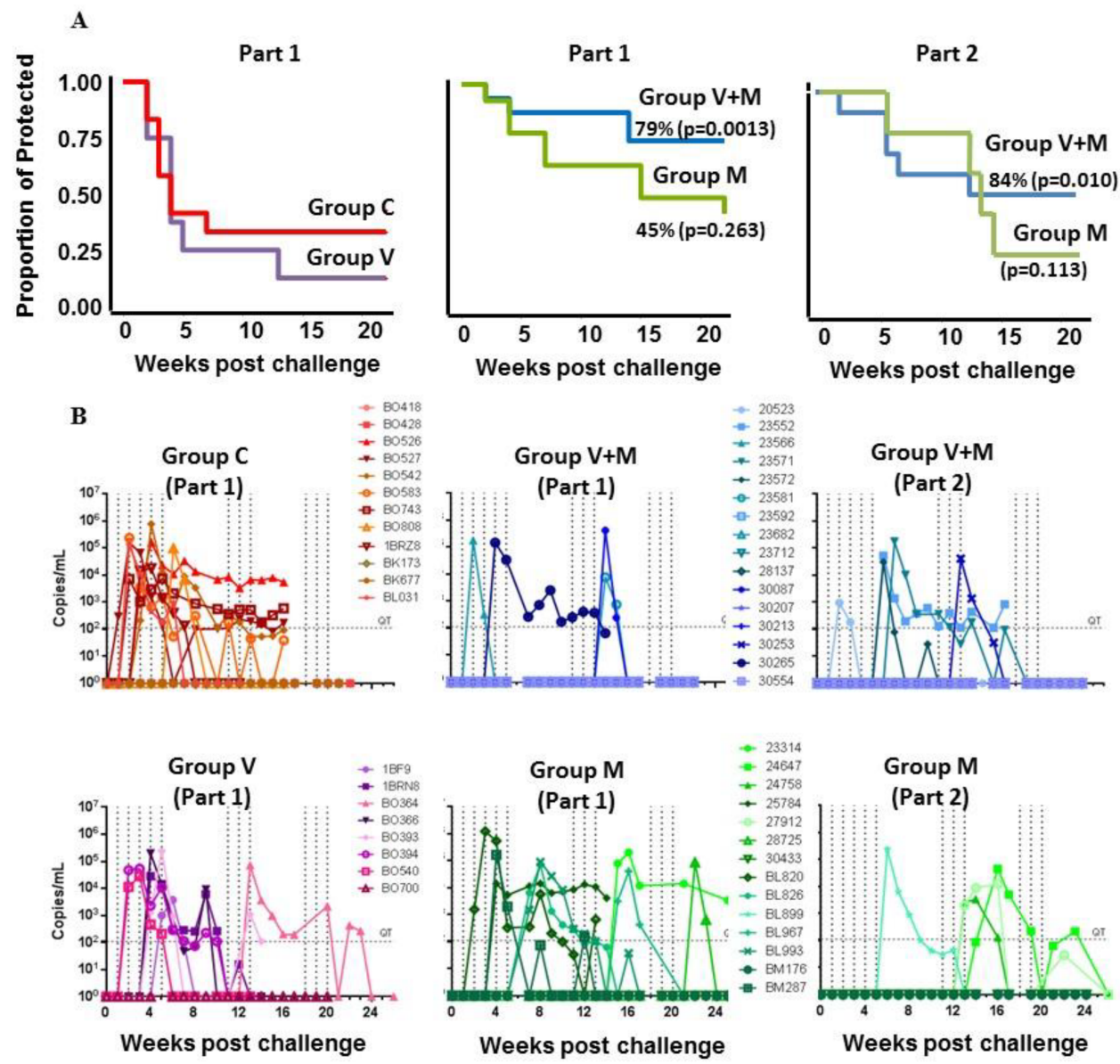
C.

Sample ID	C1.1 (#22-31)	C1.2 (#34-36)	V1 (#40-48)	V2 (#53-56)	C2.1 (#65-72)	C2.2 (#78-79)	V3 (#95-108)	C5 (#155-158)	gp41 ID (#188-194)
23552	2,101	2,030	1,817	1,900	11,341	2,062	14,757	4,680	7972
23566	562	1,251	5,410	8,200	11,115	1,343	54,475	6,082	23077
23571	1,997	12,610	819	3,615	8,566	2,715	56,100	13,192	11790
23572	1,698	3,255	169	2,988	4,223	1,455	49,248	2,794	8323
23581	394	4,651	7,628	1,825	6,948	3,529	31,643	3,206	3557
23592	16,330	1,648	26,785	2,959	5,788	2,507	31,899	7,960	14373
23682	2,086	1,635	22,439	15,123	5,929	2,098	60,280	21,149	29259
23712	7,506	24,333	1,715	9,978	13,194	8,456	36,350	8,760	23296
28137	2,606	12,914	18,019	27,949	21,886	8,722	56,154	10,078	20994
30265	6,200	3,136	11,220	5,878	8,987	4,038	48,659	5,535	7136
30087	1	2,492	13,050	678	6,422	703	33,543	4,756	2046
30207	1,117	2,979	4,236	4,015	3,232	4,009	48,786	2,210	4982
30213	1	1,471	10,346	1,671	2,666	5,269	63,211	3,190	3767
30253	1	628	5,183	2,412	9,117	2,999	46,190	7,645	5202
30554	1	1,125	3,845	1,510	1,248	3,260	15,119	5,937	7985
20523	198	11,747	6,577	2,049	10,489	6,152	57,944	15,602	8530

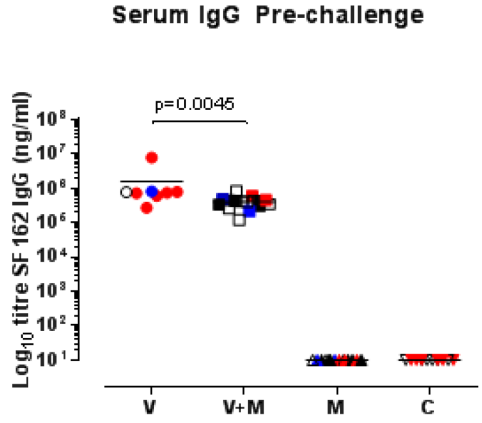
Average Percentage of Binding



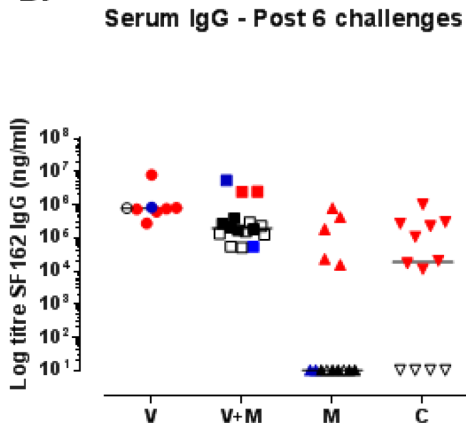




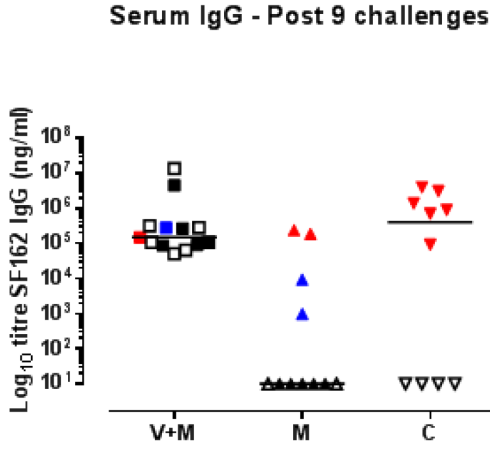
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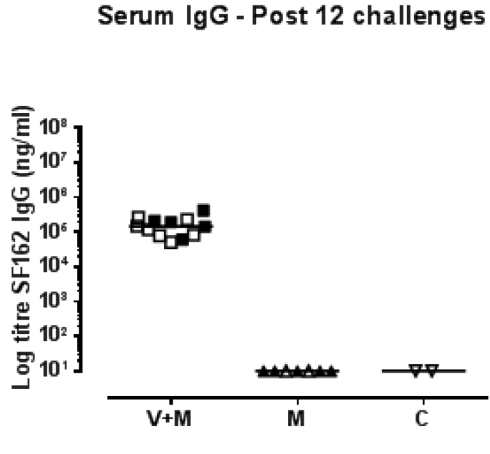
B.



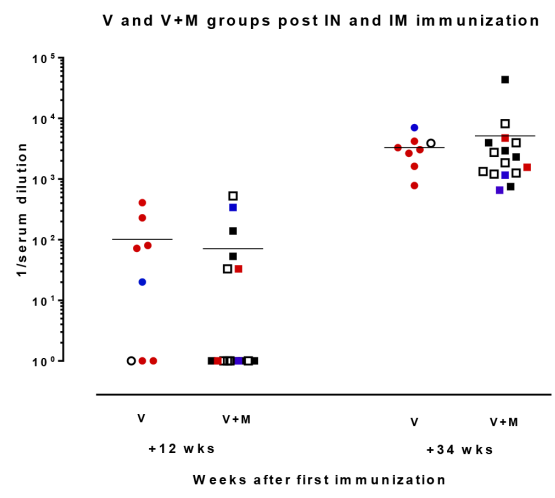
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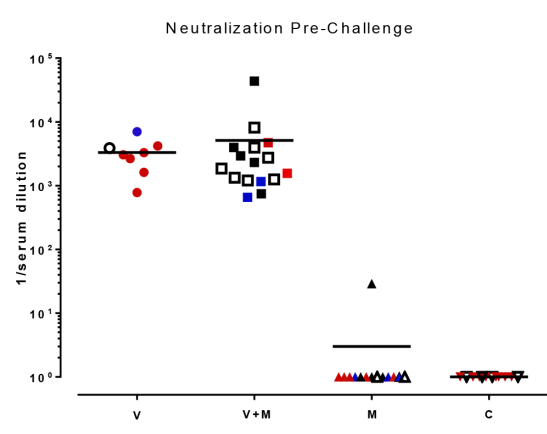
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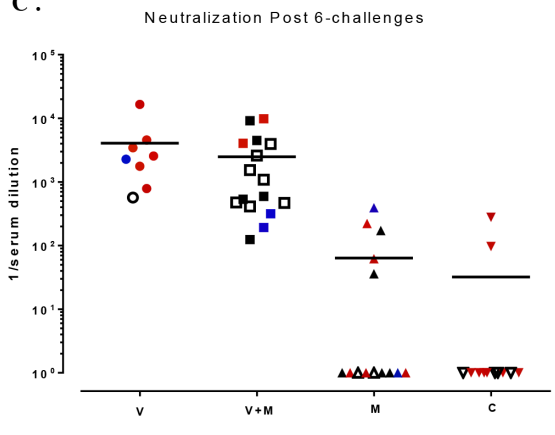
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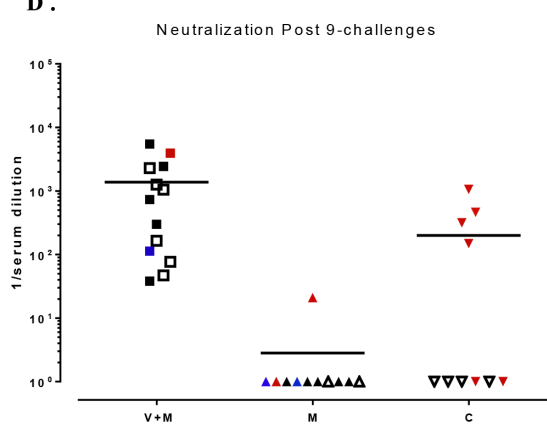
B.



C.



D.



E.

