Protection of rhesus macaques against vaginal SHIV challenges by VRC01 and an anti-$\alpha_4\beta_7$ antibody

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Running title: VRC01 and $\alpha_4\beta_7$ blockade against SHIV-AD8 infection

Short summary: A combination of VRC01 and Rh-$\alpha_4\beta_7$ significantly delayed SHIV acquisition, protected CD4 counts, decreased gut viral load and modified the immune response to the virus.

ABSTRACT

VRC01 protects macaques from vaginal SHIV infection after a single high-dose challenge. Infusion of a simianized anti-$\alpha_4\beta_7$ mAb (Rh-$\alpha_4\beta_7$) just prior to, and during repeated vaginal exposures to SIVmac251 partially protected macaques from vaginal SIV infection and rescued $CD4^+$ T cells. To investigate the impact of combining VRC01 and Rh-$\alpha_4\beta_7$ on SHIV infection, 3 groups of macaques were treated with a suboptimal dosing of VRC01 alone or in combination with Rh-$\alpha_4\beta_7$ or with control antibodies prior to the initiation of weekly vaginal exposures to a high dose (1000$TCID_{50}$) of $SHIV_{AD8\text{-}EO.}$ The combination Rh-$\alpha_4\beta_7$-VRC01 significantly delayed $SHIV_{AD8\text{-}EO}$ vaginal infection. Following infection, VRC01-Rh-$\alpha_4\beta_7$-treated macaques maintained higher $CD4^+$ T cell counts and exhibited lower rectal SIV-DNA loads compared to the controls. Interestingly, VRC01-Rh-$\alpha_4\beta_7$-treated macaques had less IL-17 producing cells in the blood and the gut during the acute phase of infection. Moreover, higher T cell responses to the V2-loop of the $SHIV_{AD8\text{-}EO}$ envelope in the VRC01-Rh-$\alpha_4\beta_7$ group inversely correlated with set point viremia.

The combination of suboptimal amounts of VRC01 and Rh-$\alpha_4\beta_7$ delayed infection, altered anti-viral immune responses and minimized $CD4^+$ T cell loss. Further exploration of the effect of combining bNAbs with Rh-$\alpha_4\beta_7$ on SIV/HIV infection and anti-viral immune responses is warranted and may lead to novel preventive and therapeutic strategies.

20 **INTRODUCTION**

21 Integrin $\alpha_4\beta_7$ ($\alpha_4\beta_7$) is expressed at high levels by $CD4^+$ T cells trafficking to the gut
22 associated lymphoid tissues (GALT) [1-3], a critical site for HIV replication and
23 dissemination after transmission [4-7]. $\alpha_4\beta_7^{high}$ $CD4^+$ T cells are highly susceptible to HIV
24 infection and are preferentially depleted during acute HIV-1 and SIV infection [8-10]. Higher
25 frequencies of $\alpha_4\beta_7^{high}$ $CD4^+$ T cells have been correlated with increased susceptibility to
26 HIV-1 infection in humans and SIV infection in macaques and with disease progression
27 in both humans and macaques [11,12]. The higher risk of HIV-1 acquisition due to prevalent
28 HSV-2 infection has also been associated with increased levels of $\alpha_4\beta_7$ expression [13-15].
29 Targeting $\alpha_4\beta_7$ with a simianized anti-$\alpha_4\beta_7$ monoclonal antibody (Rh-$\alpha_4\beta_7$; mAb) prior to
30 and during a repeated low-dose vaginal challenge (RLDC) study in rhesus macaques
31 prevented SIV acquisition in half of the animals and delayed disease progression in those
32 animals that did become infected [16]. Reportedly, simultaneous treatment with Rh-$\alpha_4\beta_7$
33 and cART led to sustained viral control after cessation of all forms of therapy in at least
34 one model of SIV infection [17]. The mechanism(s) underlying the anti-HIV-1 activity of the
35 Rh-$\alpha_4\beta_7$ mAb are poorly understood. Rh-$\alpha_4\beta_7$ does not block viral entry into $CD4^+$ T cells
36 and has weak anti-HIV-1 activity *in vitro* [8,18,19]. We have recently shown that signaling
37 through $\alpha_4\beta_7$ can promote HIV replication[20] and, in this regard, we previously
38 demonstrated that Rh-$\alpha_4\beta_7$ blocks $\alpha_4\beta_7$ from adopting an active conformation that is
39 critical for this signaling [21].

40 In addition, we determined that Rh-$\alpha_4\beta_7$ selectively alters trafficking of $CCR6^+$ $CD4^+$ T
41 cells to mucosal tissues [22] and impacts the antibody response to SIV infection when given
42 in combination with cART [17]. Thus, interference with both immune cell trafficking and

43 $\alpha_4\beta_7$-driven viral amplification may, at least in part, explain the decrease in gut tissue SIV
44 loads when Rh-$\alpha_4\beta_7$ is administered prior to, and throughout the acute phase of infection
45 [23].
46 Passive transfer of a number of broadly neutralizing antibodies (bNAbs) targeting HIV-1
47 envelope (Env) has been shown to protect rhesus macaques against a single high-dose
48 inoculation with simian-human immunodeficiency virus (SHIV) [24-27] and this strategy is
49 currently being evaluated to prevent HIV-1 acquisition in humans [28]. In particular, VRC01,
50 a bNAb against the CD4 binding site (CD4bs) on the HIV envelope [29,30], is the first bNAb
51 to be investigated clinically for the prevention of HIV infection in adult men and women
52 (AMP trial; NCT02716675 and NCT02568215). Moreover, VRC01 is being tested for
53 safety in HIV-exposed infants (NCT02256631) as a potential agent to prevent mother-to-
54 child transmission (MTCT) of HIV-1. In preclinical studies, VRC01 protected monkeys
55 against single high-dose vaginal and rectal SHIV challenge [27] and its protective activity
56 against repeated low-dose rectal challenges decreases after several weekly challenges
57 [31]. In this regard, bNAb protection against repeated rectal challenges was shown to be
58 dependent on the potency and half-life of bNAbs [31]. A mutation in the Fc domain of the
59 antibody, which was shown to increase VRC01 half-life in both plasma and tissues,
60 increased [32] and prolonged [31] its protective activity. Several other strategies to improve
61 the pharmacokinetics and function of bNAbs [28] as well as the use of combinations of
62 bNAbs or bi- and trispecific antibody-based molecules [33-35] are being tested with the
63 ultimate goal of generating new prevention and therapeutic options against HIV-1
64 infection.

65 In the present study, we investigated the combination of VRC01 and Rh-$\alpha_4\beta_7$ in a
66 repeated vaginal challenges model using the tier 2 $SHIV_{AD8-EO}$ [36]. This challenge virus
67 was chosen for its multiple properties typical of pathogenic HIV-1 isolates [37], allowing to
68 explore the impact of the VRC01-Rh-$\alpha_4\beta_7$ combination on $SHIV_{AD8-EO}$ infection and anti-
69 viral immune responses during the acute and early chronic phase of infection. In order to
70 detect an effect of this combination over the sterilizing protective effect of VRC01, we
71 chose a repeated high-dose model of infection and treatment with suboptimal amounts of
72 both antibodies. The VRC01-Rh-$\alpha_4\beta_7$ combination significantly delayed $SHIV_{AD8-EO}$
73 acquisition, protected blood $CD4^+$ T cells and altered anti-viral immune responses.
74

75 **RESULTS**

76 **The VRC01-Rh-$\alpha_4\beta_7$ combination significantly delays $SHIV_{AD8-EO}$ vaginal infection**

77 VRC01 has been shown to provide sterilizing protection against high-dose vaginal
78 challenge with $SHIV_{SF162P3}$ [27,38]. In order to study the VRC01-Rh-$\alpha_4\beta_7$ combination in a
79 setting of suboptimal VRC01 protection we employed an inoculum 100 fold higher and
80 half the dose of VRC01 (10mg/kg) that resulted in delayed $SHIV_{AD8-EO}$ acquisition for a
81 median of 8 weeks in a repeated rectal low-dose challenge model [31]. A VRC01-alone
82 group was used to monitor baseline VRC01 protection.

83 A total of 27 animals were infused with VRC01 (10mg/kg) and Rh-$\alpha_4\beta_7$ (25mg/kg; n=9) or
84 with 10mg/kg of VRC01-alone (n=9) or with control human and rhesus IgGs (n=9), 3 days
85 before weekly vaginal challenges with a high-dose inoculum of $SHIV_{AD8-EO}$ (1000$TCID_{50}$)
86 until all animals became infected (Fig 1A). Rh-$\alpha_4\beta_7$ infusions were repeated every 3 weeks
87 for a total of 6 infusions. A Rh-$\alpha_4\beta_7$-alone group was not included because Rh-$\alpha_4\beta_7$ does

88 not protect from high-dose challenge[39] and the levels of VRC01 rapidly decrease[27]. Thus,
89 the impact of Rh-$\alpha_4\beta_7$ on SHIV-AD8 infection can be inferred from comparison with the
90 VRC01-only and control groups.
91 The peak concentrations of VRC01 on the day of the first challenge in the VRC01-only
92 group and in the VRC01-Rh-$\alpha_4\beta_7$ group were 98.31 ± 25.83μg/ml and 97.66 ± 55.29 μg/ml
93 (mean±SD), respectively (Fig 1B). Of note, for reasons that may be attributed to problems
94 during the infusion, 2 macaques in the VRC01-Rh-$\alpha_4\beta_7$ group had peak concentrations of
95 VRC01 at the time of the first challenge about 10 fold lower than the mean and below the
96 protective concentration against $SHIV_{SF162P3}$ high-dose challenge [27,38]. (KT57:
97 19.145 μg/ml and GH63: 3.350 μg/ml; respectively green and orange in Fig 1B). Thus,
98 these 2 animals were excluded from the SHIV acquisition analysis, but not from other
99 post-infection analysis since VRC01 levels were undetectable in all animals by week 6
100 post-infection (Fig 1B). Consistent with previous studies, antibodies against VRC01
101 (ADA) developed around week 3 after infusion in all VRC01-treated animals and levels
102 were similar in both treatment groups (Fig S1). The peak concentration of Rh-$\alpha_4\beta_7$ in the
103 VRC01-Rh-$\alpha_4\beta_7$ group at the time of the first challenge was 112±37 μg/ml (mean±SD; Fig
104 1C). One animal with peak concentrations 10 times higher than the average of the other
105 animals was excluded from the calculation of the average. None of the animals had
106 plasma Rh-$\alpha_4\beta_7$ concentrations below the expected range and all 9 animals were included
107 in the analysis of the impact of Rh-$\alpha_4\beta_7$ on acute and early infection parameters. As shown
108 in Fig 1D, all 9 animals in the control group became viremic after 1 to 5 challenges
109 (median of 2 weekly exposures required for infection).

110 Suboptimal dosing of VRC01 resulted, as expected, in a non-significant delay in SHIV
111 acquisition (Log-rank p=0.074). Nonetheless, the cumulative number of challenges to
112 infect in the VRC01 group was significantly higher than in the control group (Fisher exact
113 test: p=0.02). In the VRC01-Rh-$\alpha_4\beta_7$ combination treatment, it was possible to detect a
114 significant delay in SHIV acquisition (Log-rank p=0.016; Fig 1E) and the difference with
115 the control group in cumulative number of challenges to infect was highly significant
116 (Fisher exact-test: p<0.001). The median number of challenges needed to infect the two
117 treatment groups was similar (n=4) and the VRC01-Rh-$\alpha_4\beta_7$ combination did not
118 significantly delay acquisition compared to VRC01 alone (Log-rank p=0.6). Peak plasma
119 viral load (VL) did not significantly differ between the treatment groups and when
120 comparing the treatment groups with the control group (Fig 1F and Fig S2). Set-point VL
121 was ~1 Log_{10} lower in the VRC01-Rh-$\alpha_4\beta_7$ group compared with the other 2 groups (Fig
122 1F). However, the difference did not reach statistical significance (p=0.072 at week 18
123 p.i.). Confirming previous reports on the effects of Rh-$\alpha_4\beta_7$ [23,40], peripheral $CD4^+$ T cells
124 were protected and CD4 counts were significantly higher in the VRC01-Rh-$\alpha_4\beta_7$ when
125 compared with the other 2 groups (treatment adjusted for time; 2-way Anova p=0.005;
126 Fig 1G). This is at least partially due to Rh-$\alpha_4\beta_7$-driven lymphocytosis, as we
127 demonstrated in naïve macaques [22], and explains the higher baseline CD4 count of the
128 VRC01-Rh-$\alpha_4\beta_7$ group.
129 Although gut SIV DNA loads were slightly higher in both treatment groups compared with
130 controls at week 3-4 p.i. (2-3 weeks after 1st virus detection in plasma), by week 20 p.i.,
131 VRC01-Rh-$\alpha_4\beta_7$ treated animals had more than 10-fold lower amounts of cell-associated
132 SIV DNA in the gastrointestinal (GI) tract compared to the controls (Fig 2A). SIV RNA

133 loads in the GI tract of the VRC01-Rh-$\alpha_4\beta_7$ treated animals were also 100 times lower, on
134 average, than in the control group during the post-acute phase (week 7-8 p.i.; Fig 2B).
135 However, by week 20 p.i. several animals in the control group had undetectable SIV-RNA
136 levels in the GI tract and the difference with the VRC01-Rh-$\alpha_4\beta_7$ lost its significance.
137 Interestingly, no significant differences in SIV loads were found by directly comparing the
138 VRC01-Rh-$\alpha_4\beta_7$ combination group and the VRC01-alone group and the SIV loads in the
139 VRC01-only group often averaged between the other 2 groups. Vaginal SIV DNA and
140 RNA loads did not differ in the acute nor in the early chronic phase of the infection among
141 the treatment groups (Fig S3). Finally, although the SIV-DNA loads at necropsy (around
142 20 weeks p.i.) were on average slightly lower in the jejunum and iliac lymph nodes of the
143 VRC01-Rh-$\alpha_4\beta_7$ group compared with the other 2 groups, the differences were not
144 significant (Fig S4). No differences were detected in other tissues and lymph nodes at
145 necropsy (Fig S4).
146
147 **VRC01-Rh-$\alpha_4\beta_7$ decreases IL-17-producing cells in acute infection and increases**
148 **IFNγ-producing cells in the chronic phase.**
149 Mononuclear cells isolated from blood and colorectal biopsies in the acute phase of
150 infection (week 3-4 p.i.) and from ileum, jejunum and colorectal tissue at necropsy
151 (chronic phase) were stimulated with PMA/ionomycin to determine the frequency of IL-17
152 (acute and chronic phase samples) and IFNγ, IL-2, TNFα and IL21-producing cell subsets
153 (chronic phase samples). VRC01-Rh-$\alpha_4\beta_7$ treated animals had significantly lower
154 frequencies of IL-17 producing $CD8^+$ T cells in blood and of IL-17 producing $CD4^+$ T cells
155 and $CD3^-$ $NKp44^+$ NK cells in the colorectal tissue compared to the control group (Fig 3A-

156 B). For these subsets, the difference did not reach significance when compared with the
157 VRC01-only group. However, the frequency of IL-17-producing NKG2A$^+$ NK cells in blood
158 was significantly lower in the VRC01-Rh-$\alpha_4\beta_7$ group compared with the VRC01-only group
159 (Fig 3A). In contrast, in the chronic phase, the frequency of IFNγ-producing CD4$^-$ T cells
160 was higher in the VRC01-Rh-$\alpha_4\beta_7$ group compared with the control group in the small
161 intestine (Fig 3C). No significant differences in IFNγ-producing cells were noted in blood
162 or upon direct comparison of the VRC01-only group with the VRC01-Rh-$\alpha_4\beta_7$ group nor
163 between the VRC01-only group and the control. No significant differences in IL-17, IL-2,
164 TNFα and IL21-producing cells were noted in these tissues in the chronic phase of
165 infection.
166 Interestingly, when we investigated how the treatments had impacted immune cells in the
167 lymph nodes at necropsy, we found that in the VRC01-only group had a lower frequency
168 of CD25$^+$ T cells (both CD4$^+$ and CD4$^-$, Fig 4) compared with the control group. Rh-$\alpha_4\beta_7$
169 in the VRC01-Rh-$\alpha_4\beta_7$ may have interfered with this decrease since the difference was
170 not significant between the VRC01-Rh-$\alpha_4\beta_7$ group and the controls. Moreover, we found
171 that the VRC01-treated animals had lower frequencies of CXCR3 CCR6 double positive
172 CD4$^-$ and CD4$^+$ T cells compared with both the VRC01-Rh-$\alpha_4\beta_7$ and the control groups
173 (Fig 4A and B). Finally, the VRC01-only treated animals had lower CXCR5$^+$ CD4$^+$
174 follicular T cells in the lymph nodes compared with the controls (Fig 4A). No other
175 differences were noted among CD103$^+$, CD69$^+$, and Treg-like CD127$^-$CD25$^+$ cells within
176 the CD4$^+$ or CD4$^-$ cell subsets in the lymph nodes between the groups (not shown).
177 Phenotyping of mononuclear cells isolated from blood during the chronic phase of

178 infection showed that Rh-$\alpha_4\beta_7$ treatment was associated with an increase of $CCR6^+CD95^-$
179 $CD4^-$ cells compared to both control groups (Fig S5).
180
181
182 **T cell responses in the VRC01-Rh-$\alpha_4\beta_7$ are primarily directed against the V2-loop of**
183 **the $SHIV_{AD8\text{-}EO}$ envelope protein.**
184 Blood T cell responses were analyzed against pooled 15-mer overlapping peptides from
185 the consensus B envelop and gag proteins around week 18 post-infection. Interestingly,
186 T cell responses against the envelope peptides in the VRC01-Rh-$\alpha_4\beta_7$ group were virtually
187 undetectable and significantly lower than the T cell responses in the control group (Fig
188 S6). T cell responses in the VRC01 group were more similar to the control group, but the
189 difference between the VRC01-only and VRC01-Rh-$\alpha_4\beta_7$ groups reached statistical
190 significance only for TNFα and IL22-secreating $CD4^+$ T cells (Fig S6A). The responses to
191 gag peptides were generally lower than those to the envelope peptides at this stage of
192 infection in all groups and differences between groups could not be determined.
193 Since the sequence of the consensus B envelope differs substantially from the sequence
194 of the $SHIV_{AD8\text{-}EO}$ envelope in the V1V2 region (Fig S7) and a specific response against
195 the V2-loop was found in the Rh-$\alpha_4\beta_7$-treated animals of the cART-Rh-$\alpha_4\beta_7$ study [17], we
196 synthetized seven 20-mer, 14aa overlapping peptides spanning the V1V2 region that
197 differs between the $SHIV_{AD8\text{-}EO}$ and the consensus B envelope and used them to probe T
198 cells and antibody responses. Interestingly, the VRC01-Rh-$\alpha_4\beta_7$ macaques had higher
199 frequencies of IFNγ-producing $CD4^+$ T cells in response to the V1V2 peptide pool than
200 both the VRC01 and control groups (Fig 5A). In contrast, TNFα-producing $CD4^+$ T cells

201 and IFN-γ-producing $CD8^+$ T cells were higher in both treatment groups compared with
202 the control group (Fig 5A-B). A higher IL-17 response in the $CD8^+$ T cell subset was also
203 noted in the VRC01-Rh-$\alpha_4\beta_7$ group compared to the controls (Fig 5B). Finally, a higher
204 frequency of TNF-α-$CD8^+$ T cells was present in the VRC01-Rh-$\alpha_4\beta_7$ group compared
205 with the VRC01-only group. In summary, higher T cell responses were detected against
206 the V1V2 loop in the VRC01-Rh-$\alpha_4\beta_7$ group particularly compared to control animals.
207 Interestingly, the frequency of $CD8^+$ T cells secreting TNF-α in response to the V1V2
208 peptides inversely correlated with viral load (Fig S8), suggesting that a significant role is
209 played by V1V2 responses in controlling viral replication.
210 Total anti-HIV envelope antibodies were tested against the HIV-Bal envelope protein and
211 no differences were noted between the groups. Moreover, a peptide scan against
212 consensus B envelope peptides (with the 8 peptides corresponding to the V1V2 loop
213 replaced by the 7 SHIV-AD8-specific peptides that we had synthetized) was carried out
214 on sera from 4 animals in each group with the highest antibody responses. No clear
215 differences in the response to specific regions of the envelope were noted between the
216 groups (Fig S8).
217

218 **DISCUSSION**

219 bNAbs are being tested in the clinic for the prevention and therapy of HIV infection.
220 VRC01 is the first to reach efficacy testing and other bNAbs will soon follow [41,42]. However,
221 it is clear that individual bNAbs cannot be used alone as a single intervention.
222 Combinations of more bNAbs or bi- or tri-specific molecules need to be employed to
223 achieve better and more durable protection from HIV acquisition [33,43-45]. Moreover, recent

224 data suggest that bNAbs treatment may impact immune responses to infection [46-48]. This
225 feature represents a potential new therapeutic approach toward an HIV-1 cure. Rh-$\alpha_4\beta_7$
226 has also demonstrated the ability to partially prevent SIV infection in macaques [16] and
227 treatment of SIV infected macaques with Rh-$\alpha_4\beta_7$ in combination with cART has shown
228 its potential utility in inducing long-term control of SIV replication without eradicating the
229 virus [17]. Nonetheless, much more needs to be understood about the ability of bNAbs and
230 $\alpha_4\beta_7$-blockage to impact immune responses against SIV/HIV.
231
232 The present study represents the first investigation of the combination of a bNAb and Rh-
233 $\alpha_4\beta_7$. We aimed to determine how this dual-treatment might alter key features of acute
234 and early-chronic infection, including anti-viral immune responses. In order to observe
235 such effects beyond the powerful protective activity of VRC01, we performed the study in
236 a setting of suboptimal amounts of VRC01 against repeated challenges with a very high
237 viral inoculum of a pathogenic SHIV.
238 Overall, we determined that Rh-$\alpha_4\beta_7$ has the potential to increase the protective activity
239 of suboptimal dosing of VRC01. However, larger studies with a more physiologically
240 relevant, low-dose viral challenge will be needed to precisely quantify the incremental
241 effect of combining Rh-$\alpha_4\beta_7$ and VRC01 over VRC01 on SHIV acquisition. Interestingly,
242 we were able to determine that some of the key effects of the Rh-$\alpha_4\beta_7$ that were previously
243 described [16,23,40] are retained in the SHIV model. They include the ability of the Rh-$\alpha_4\beta_7$
244 to protect circulating $CD4^+$ T cells, a modest effect on the viral set-point and a profound
245 effect on the gut viral load. However, we did not find a decrease viral load in any other
246 tissue or lymph nodes as it was described in the SIV model [23].

247 Interestingly, most of the differences that we noted are significant only when the VRC01-
248 Rh-$\alpha_4\beta_7$ is compared with the control group and the data from the VRC01-only group fell
249 in between the other 2 groups. This suggests that the presence of minimal quantities of
250 VRC01 at the time of infection may have facilitated the impact of Rh-$\alpha_4\beta_7$ on virologic and
251 immune parameters, hinting at the possibility of a synergistic effect of the two antibodies.
252 This, also, will need further investigation.
253 Notably, we found that during the acute phase of infection (which coincided with the Rh-
254 $\alpha_4\beta_7$-treatment) the VRC01-Rh-$\alpha_4\beta_7$ group had a lower frequency of IL-17 producing T
255 cells in the gut. This may be due to the profound impact of Rh-$\alpha_4\beta_7$ on CCR6$^+$ T cells that
256 we noted in absence of infection [22] and may help explain the overall decrease in T cell
257 responses that was observed in the VRC01-Rh-$\alpha_4\beta_7$ group when pools for the entire
258 envelope and gag proteins were used. Perhaps because the Rh-$\alpha_4\beta_7$ was administered
259 during the earliest stages of the infection, we did not see an impact on the antibody
260 responses as was described in the ART-Rh-$\alpha_4\beta_7$ combination study [17]. However,
261 treatment with Rh-$\alpha_4\beta_7$ increased the T cell responses against the V2 region of the
262 envelope and our data suggest a role for these responses in maintaining virologic control.
263 How and why this happens requires further investigation. It is possible to speculate that
264 in the presence of Rh-$\alpha_4\beta_7$ immune cell priming occurs more efficiently outside the
265 mucosal tissues and/or that the Rh-$\alpha_4\beta_7$ changes the antigenicity of the envelope or
266 antigen processing.
267
268 Interestingly, the only VRC01-specific effect we observed appeared in the lymph nodes,
269 where in the VRC01-only treated animals we detected significantly lower frequencies of

270 CD25$^+$ and CXCR5$^+$ CD4$^+$ T cells. In the VRC01-Rh-$\alpha_4\beta_7$ group this effect was still
271 present, but less pronounced. This further supports the published observations that
272 suggest a direct impact of bNAbs on the antiviral immune response [47,49]. Nonetheless,
273 the amount of time the animals were exposed to meaningful concentrations of VRC01
274 during infection was very short and the data should be interpreted with caution.
275 Importantly, the VRC01-Rh-$\alpha_4\beta_7$ group had higher frequencies of IFNγ producing T cells
276 in both the large and small intestine. This increase suggests a protective activity of the
277 Rh-$\alpha_4\beta_7$ on the gut immune system and helps explain the long-term survival of SIV
278 infected animals treated with Rh-$\alpha_4\beta_7$ during acute infection (Aftab Ansari, unpublished
279 and reported at CROI 2018 by J. Arthos). Boosting effective mucosal immune responses
280 may also contribute to explain the virologic control seen when the Rh-$\alpha_4\beta_7$ was used in
281 combination with cART as therapeutic approach in Byrareddy et. al, Science 2016 [17]. This
282 control was not replicated in other similar studies (as reported by Di Mascio and Fauci at
283 AIDS2018). Nonetheless, our study adds to the considerable amount of work that
284 supports a profound effect of the Rh-$\alpha_4\beta_7$ on immune cells and the immune responses.
285
286 In conclusion, a suboptimal dose of VRC01 in combination with the Rh-$\alpha_4\beta_7$ significantly
287 delayed infection and impacted the availability and distribution of immune cell subsets as
288 well as the T cell responses to the virus. VRC01 and Rh-$\alpha_4\beta_7$ impact HIV infection by
289 distinct mechanisms, neither of which is fully understood. This study gives the first insight
290 into the combination of these antibodies and contributes to our understanding their effect
291 on HIV infection. Future studies should address how other bNAbs combinations with Rh-

292 $\alpha_4\beta_7$ could be harnessed in different therapeutic and preventive settings to fight HIV
293 infection.
294
295
296 **MATERIALS AND METHODS**
297 **Macaques and treatments**
298 A total of 27 adult female Indian rhesus macaques (average weight 8.1Kg, range: 4.5,
299 13.05 and age 9.5 years, range: 3.9, 18) were socially housed, indoors, in climate
300 controlled conditions at the Tulane National Primate Research Center (TNPRC) in
301 compliance with the regulations under the Animal Welfare Act, the Guide for the Care and
302 Use of Laboratory Animals [50]. All procedures were approved by the Animal Care and Use
303 Committee of the TNPRC and in compliance with animal care procedures. The animals
304 were euthanized at the end of the study using methods consistent with recommendations
305 of the American Veterinary Medical Association (AVMA) Panel on euthanasia and per the
306 recommendations of the IACUC.
307 Macaques were divided in three groups of 9 animals each. Animals were administered
308 intravenously 1) 1 injection of 10mg/Kg of VRC01 + 1 injection of 25mg/Kg of Rh-$\alpha_4\beta_7$
309 mAb; followed by 5 additional injections of 25mg/Kg of Rh-$\alpha_4\beta_7$ mAb every 3 weeks 2) 1
310 injection of 10mg/Kg of VRC01; 3) 1 injection of 10mg/Kg of control Human IgG and
311 25mg/Kg of control Rhesus IgG. Starting 3 days after treatment, macaques were
312 challenged weekly with intravaginal administration of $SHIV_{AD8\text{-}OE}$ (1000 $TCID_{50}$/challenge)
313 for 8 weeks. Blood was collected weekly to monitor infection as described below. After
314 two consecutive positive SIV PCRs (3-4 weeks post infection; acute phase) fresh rectal

315 biopsies and blood were used for cell isolation as described below. Subsequently, blood
316 was collected every two weeks throughout the study. Rectal and vaginal samples were
317 also collected at 7-8 weeks post infection (post-acute phase). At necropsy, blood, lymph
318 nodes, gut, brain, vaginal and cervical tissues were harvested and used for cell isolation.
319

320 **Measurement of plasma levels of VRC01 and Rh-$\alpha_4\beta_7$**

321 Levels of VRC01 were measured as described in [27]. Levels of rhesus Rh-$\alpha_4\beta_7$ antibody
322 in macaque plasma were measured using the $\alpha_4\beta_7$-expressing human T cell line HuT-78
323 (NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: HUT 78 from Dr. Robert
324 Gallo) in a flow cytometry-based assay as described in (Calenda 2018, Byrareddy 2014)
325 using the standard curve method. Briefly, HuT78 cells were first incubated for 2-3 days in
326 complete RPMI 1640 media containing 100nM retinoic acid to increase the surface
327 expression of $\alpha_4\beta_7$. Cells (150,000/condition) were stained with LIVE/DEAD Aqua dye
328 (Thermo Fisher Scientific, Waltham, MA) for live/dead discrimination, incubated for 30min
329 at 4°C with the plasma to be tested (1:10 diluted in PBS) obtained from macaques from
330 the VRC01-$\alpha_4\beta_7$ treatment group before (baseline, BL) and up to 6 weeks after treatment.
331 Cells were then washed and incubated for 30 min at 4°C with anti-rhesus IgG1 (NHP
332 resource center, antibody 7H11, in house biotinylated with EZ-link NHS-biotin (Thermo
333 Fisher Scientific) following the manufacturer's instructions), washed again and
334 resuspended in neutravidin-PE (Thermo Fisher Scientific) for 20 min at 4°C. PE
335 fluorescence was analyzed on a flow cytometer. For the standard curve, baseline plasma
336 was pooled and spiked with serial dilutions of Rh-$\alpha_4\beta_7$ (2500 μg/ml – 0 μg/ml).
337

338 **Anti-VRC01 and anti-Rh-$\alpha_4\beta_7$ antibodies**

339 Levels of anti-VRC01 antibodies were measured as described in [32]. Levels of anti-Rh-
340 $\alpha_4\beta_7$ antibodies were measured via lamda light chain detection assay. ELISA plates were
341 coated with Rh-$\alpha_4\beta_7$ (10μg/ml) overnight at 4°C, washed and blocked with TBS 2% BSA
342 0.1% Tween20 for 2 hours at room temperature (RT). Test plasma was serially diluted in
343 dilution buffer (starting at 1:10 then serial 1:4 dilutions), 100 μl applied to the plates and
344 incubated 1 hour at RT. Plates were washed and incubated with anti-Ig human lambda
345 light chain-biotin (Miltenyi), which does not recognize the kappa chain of the Rh-$\alpha_4\beta_7$ 1
346 hour at RT. Plates were washed and incubated with diluted streptavidin-HRP (Invitrogen)
347 1 hour at RT. Plates were washed and enzymatic activity detected by adding TMB
348 substrate and read on a luminometer at 450nm. Endpoint was the highest dilution with
349 OD 2-fold higher the pre-treatment sample.

350

351 **$SHIV_{AD8\text{-}EO}$ stock generation and titration**

352 The full-length proviral plasmid pSHIVAD8-EO was a gift from Dr. Malcom Martin. Virus
353 stocks were prepared by transfecting 293T cells with 5μg of the $pSHIV_{AD8\text{-}EO}$ molecular
354 clone using Lipofectamine 2000 (Invitrogen). Culture supernatant was collected 60 hours
355 later, clarified by centrifugation (300g 10 minutes, 4°C) and used to infect CD8-depleted,
356 PHA-activated rhesus macaque PBMC. Cells were incubated overnight in 293T
357 supernatants, washed and resuspended in RPMI 10% FBS medium for 10 days.
358 Supernatants from parallel cultures were pooled on day 7, clarified by centrifugation
359 (10,000g, 15minutes, 4°C), aliquoted, and stored at -80°C. The resulting stock was
360 titrated in PHA-activated rhesus macaque PBMC.

361

362 **SIV viral loads**

363 Macaque infection was confirmed by SIVgag nested PCR on PBMC as described [49].
364 Plasma samples were obtained from EDTA-treated whole blood and used for the
365 determination of plasma VL by SIVgag qRT-PCR [50] (quantitative Molecular Diagnostics
366 Core, AIDS and Cancer Virus Program Frederick National Laboratory). DNA and RNA
367 were extracted from snap frozen tissues using DNeasy/RNeasy blood and tissue kits
368 (Qiagen) following the manufacturer's instructions. Tissue viral DNA loads were quantified
369 using the standard curve method and normalized by albumin copy numbers by gag-qPCR
370 as described in [51]. For tissue RNA loads, 1μg of total RNA was retrotranscribed to DNA
371 using the VILO Kit (Thermo Fisher) quantified by gag-qPCR [51].

372

373 **Cell isolation and flow cytometry**

374 3-4 weeks post infection, PBMCs were isolated using Ficoll-Hypaque density gradient
375 centrifugation and cells from rectal biopsies were isolated by enzymatic digestion in HBSS
376 containing 2mg/mL Collagenase IV (Worthington Biochemical) and 1mg/ml of Human
377 Serum Albumin (Sigma-Aldrich), shaking at 37°C for 50 minutes. The resulting cell
378 suspension was passed through a 40μm cell strainer and washed with PBS. PBMCs and
379 rectal cells were then stimulated with 60ng/ml PMA, 0.5μg/ml Ionomycin and 5μg/ml
380 Brefeldin A (BFA) for 4 hours at 37°C, stained with LIVE/DEAD Aqua viability dye (Thermo
381 Fisher Scientific) and incubated with a cocktail of different panels of monoclonal
382 antibodies as listed in the tables in Fig S9.

383 At the time of necropsy, PBMCs were isolated by Ficoll-Hypaque density gradient
384 centrifugation. Enzymatic digestion was used to isolate cells from jejunum, ileum,
385 colorectal, vaginal and cervical tissues as described above. Spleen and LNs (axillary,
386 mesenteric, inguinal, iliac) were cut in small pieces and passed directly through a 40µm
387 cell strainer. Isolated cells were washed, frozen for phenotyping and stimulation
388 experiments at a later time. For PBMC stimulation experiments, up to $3x10^6$ cells/sample
389 were thawed, plated on a plate pre-coated with 2.5µg/ml goat anti mouse (GAM) IgGs
390 and cross linked with 10µg/ml anti-CD28 and anti-CD49d antibodies (Sigma Aldrich).
391 Cells were stimulated either with SIVMAC239 GAG peptide pool (1µg/ml; 125 15mers
392 with 11 aa overlap, AIDS reagents program, Division of AIDS, NIAID, NIH), HIV-1
393 Consensus B Env Peptide Set (2µg/ml; 211 15mers with 11 aa overlap, AIDS reagents
394 program, Division of AIDS, NIAID, NIH) or a $SHIV_{AD8OE}$ specific Env V1-V2 peptide pool
395 (2µg/condition; 7 20mers with 14 aa overlap, Peptide 2.0, Chantilly, VA). Pooled cells
396 stimulated with 1µg/ml PMA/Ionomycin were used a positive activation control. One hour
397 later 10µg/ml BFA and monensin (GolgiStop, BD Biosciences) were added to each well.
398 After 5 hours, cells were transferred to a FACS plate and stained with the panels listed in
399 table 1. To maximize CD107a detection, antibody staining was performed during
400 stimulation. For gut tissue stimulation experiments, cells isolated from colorectum,
401 jejenum and ileum where thawed and stimulated with 60ng/ml PMA, 0.5µg/ml Ionomycin
402 and 5µg/ml Brefeldin A (BFA) for 4 hours at 37°C, stained with LIVE/DEAD Aqua viability
403 dye (Thermo Fisher Scientific) and incubated with a cocktail of different panels of
404 monoclonal antibodies as listed in the tables in Fig S9. For phenotyping experiments,
405 cells were thawed and stained with the panels listed in tables in Fig S9.

406

407 **Pepscan**

408 Peptide scan was performed against consensus B envelope peptides with the 8 peptides
409 corresponding to the V1-V2 loop replaced by the 7 $SHIV_{AD8OE}$-specific peptides that we
410 had synthetized was performed on serum samples from the 4 animals from each group
411 showing the highest antibody response by ABL Inc.

412

413 **Statistics**

414 To analyze the differences in SHIV acquisition, the survival curves generated with time to
415 first viral detection in plasma from each treatment group were compared to each other
416 directly and each with the curve from the control group using the Log-rank (Mantel-Cox)
417 test. The cumulative number of challenges needed to infect in each group was also
418 compared to the cumulative number in each other group by Fisher exact test. Viral loads
419 and CD4 counts were compared by two-way ANOVA for repeated measures. To address
420 whether either treatment had a significantly different effect on infection and immunological
421 parameters than the control group, data was analyzed using Kruskal-Wallis non-
422 parametric test adjusted for multiple comparisons followed by the Dunn's multiple
423 comparisons post-hoc test. To address whether treatment groups differed from each
424 other a Mann-Whitney unpaired t-test was performed. All analyses were performed using
425 the GraphPad Prism software V7. Significant *p*-values of $\alpha<0.05$ (*), $\alpha<0.01$ (**) and α
426 <0.001 (***) are indicated.

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428

429

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444 in this report.

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Author contributions

EM, JA, conceived and designed the study; GC, IF, GAB coordinated the receipt and processing of macaque samples, run the assays and analyzed the data; AP and KW measured VRC01 concentrations and anti-VRC01 antibodies; SM processed samples and run virologic assays; BJ, JLB and AG coordinated the macaque studies, sample collection and animal welfare; EM and KR performed statistical analysis; EM, JA, CC, ASF, JRM interpreted the data and wrote the manuscript.

Disclosure

The authors declare no competing interests.

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617 **Figure Legends**

618

619 **Figure 1. The Rh-$\alpha_4\beta_7$-VRC01 combination significantly delays $SHIV_{AD8-EO}$**
620 **acquisition.**

621 (A) Schematic of the study procedures (B-C) The concentrations of VRC01 (B) and Rh-
622 $\alpha_4\beta_7$ (C) were measured in plasma for the first 8 weeks after initiation of treatment. VRC01
623 levels were below the protective concentration in GH63 and KT57 (orange and green in
624 B, respectively). (D) Copies of viral RNA copies in plasma are shown from the time of the
625 first challenge (week 0). (E) Kaplan-Meier curves generated with time to first viral
626 detection in plasma are shown. Curves were compared with the Log-rank test (* *p*-value
627 of $\alpha<0.05$ was considered significant). (F-G) Mean ± SEM of the plasma viral loads (F)
628 and $CD4^+$ T cell counts (G) are shown.

629

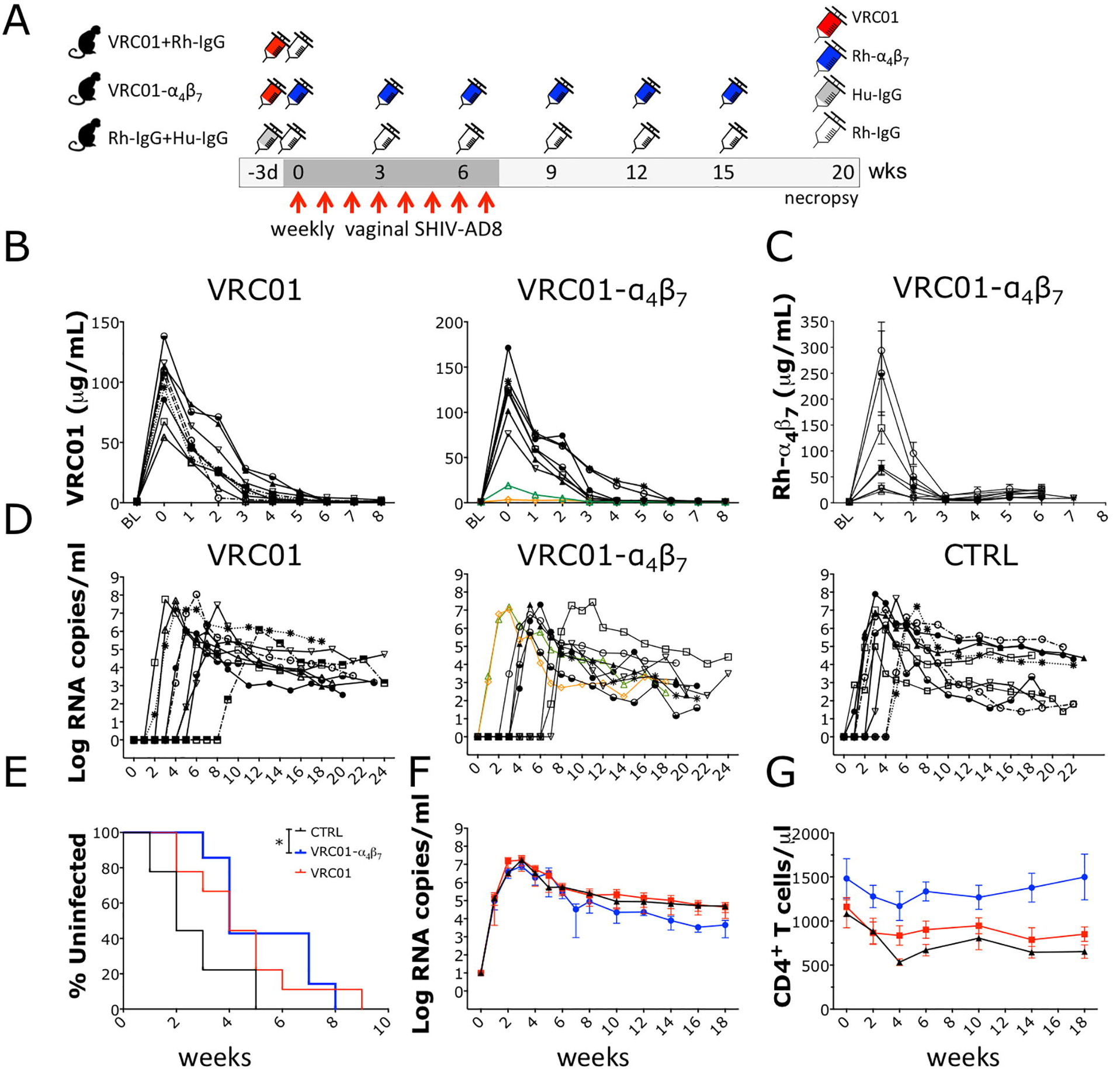
630 **Fig 2. The Rh-$\alpha_4\beta_7$-VRC01 combination reduces viral DNA and RNA in the gut.**
631 Copies of SIV DNA (A) and RNA (B) from colorectal biopsies at the indicated times after
632 infection were quantified by gag-qPCR (normalized on albumin content) and by RT-qPCR
633 (normalized on RNA content) respectively. The dotted line indicates the lower limit of
634 detection (LLOD) of the assay. Data from the treatment groups were compared with the
635 control by Kruskal-Wallis test and the results of the Dunn's multiple comparisons post-
636 hoc test are shown (*p*-value of * $\alpha<0.05$ and ** $\alpha<0.01$ were considered significant).

637

638

639 **Fig 3. Decreased IL-17 and increased IFN□ producing cells in the gut of Rh-$\alpha_4\beta_7$-**
640 **VRC01-treated macaques.** (A-B) The frequency of IL-17-secreting cells within the
641 indicated subsets in blood (A) and colorectal biopsies (B) collected 2 weeks after the first
642 detection of virus in plasma (3-4 weeks post-infection) are shown. (C) The frequency of
643 IFN-□-secreting cells within $CD8^+$ T cells in the indicated tissues are shown. (A-C) NK-
644 like cells were defined as $CD3^-NKG2A^+$ in the blood and $CD3^-NKp44^+$ in the colorectal
645 tissue. Data from the treatment groups were compared with the control by Kruskal-Wallis
646 test and the results of the Dunn's multiple comparisons post-hoc test and the Mann-
647 Whitney test to compare the treatment groups between each other are shown (*p*-value of
648 * $\alpha<0.05$ and ** $\alpha<0.01$ were considered significant).
649
650 **Fig 4. Decreased frequencies of $CD25^+$, $CCR6^+$ $CXCR3^+$ and $CXCR5^+$ T cells in the**
651 **lymph nodes of VRC01 pretreated, SHIV-infected macaques.** (A-B) At necropsy, cells
652 were isolated from inguinal lymph nodes and analyzed by flow cytometry. The frequencies
653 of subsets within the $CD4^+$ (A) and $CD8^+$ (B) T cells that were significantly different in any
654 of the treatment groups compared to the controls by Kruskal-Wallis test are shown. The
655 results of the Dunn's multiple comparisons post-hoc test and the Mann-Whitney test to
656 compare the treatment groups between each other are shown (*p*-value of * $\alpha<0.05$, **
657 $\alpha<0.01$ and *** $\alpha<0.001$ were considered significant).
658
659
660 **Fig 5. T cell responses to the $SHIV_{AD8-EO}$ V2-loop peptides are higher in Rh-$\alpha_4\beta_7$-**
661 **VRC01 treated macaques.** PBMCs isolated around 18 weeks post infection were

662 stimulated with a pool of 7 20mers with 14aa overlap peptides spanning the V2 loop of
663 the $SHIV_{AD8-EO}$ envelope for 5hrs. The frequency of cells secreting the indicated cytokines
664 are shown for the $CD4^+$ and $CD8^+$ T cell subsets after subtraction of the baseline values
665 (in absence of peptides). The results of the Dunn's multiple comparisons post-hoc test
666 (after the Kruskal-Wallis test controlled for multiple comparisons) and the Mann-Whitney
667 test to compare the treatment groups between each other are shown (*p*-value of * $\alpha<0.05$,
668 ** $\alpha<0.01$ and *** $\alpha<0.001$ were considered significant).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VRC01+Rh-IgG
VRC01-α4β7
Rh-IgG+Hu-IgG
VRC01
Rh-α4β7
Hu-IgG
Rh-IgG
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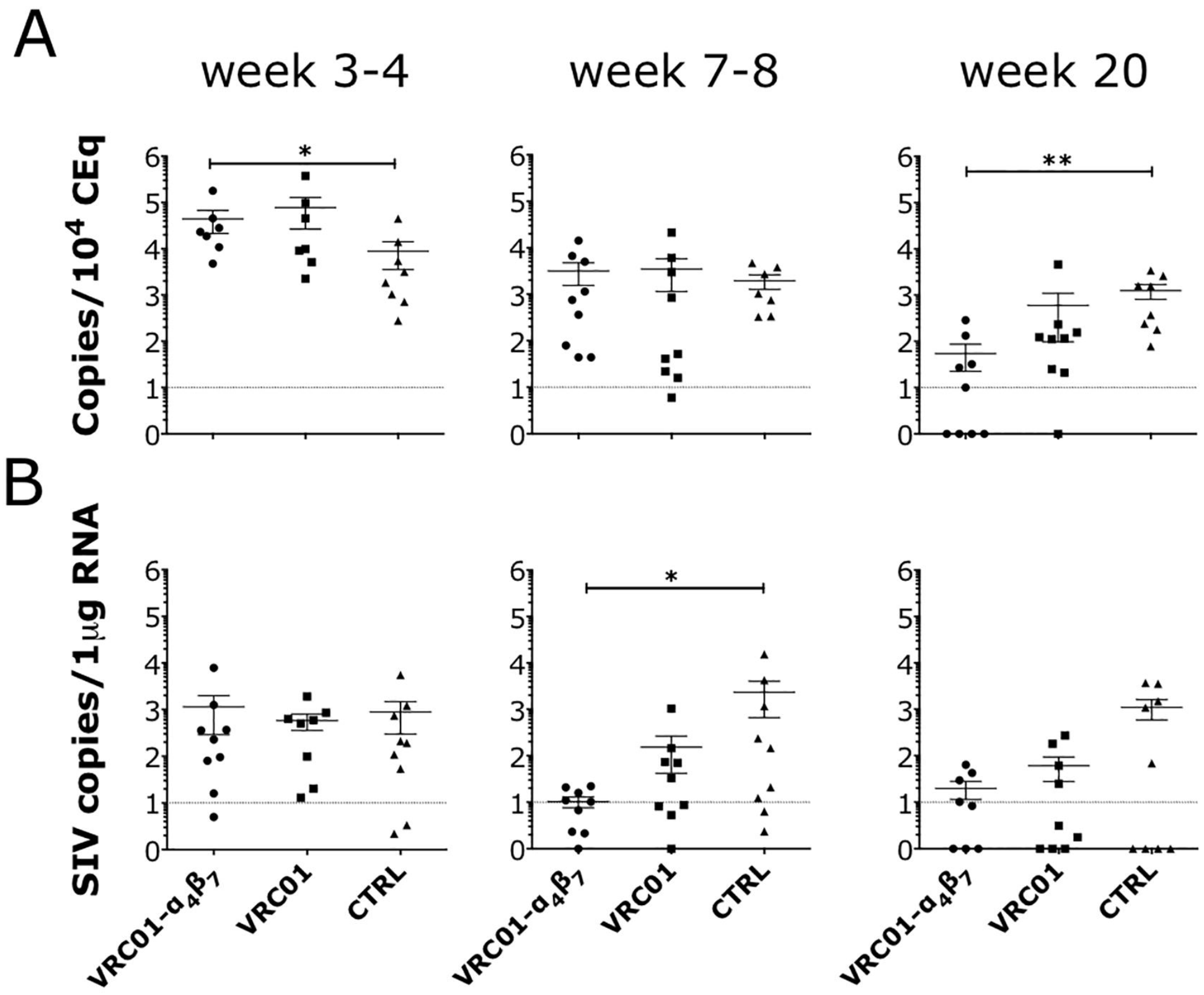
wks
necropsy
weekly vaginal SHIV-AD8
B
VRC01
VRC01 (μg/mL)
VRC01-α4β7
C
VRC01-α4β7
Rh-α4β7 (μg/mL)
D
VRC01
Log RNA copies/ml
VRC01-α4β7
CTRL
E
% Uninfected
CTRL
VRC01-α4β7
VRC01
weeks
F
Log RNA copies/ml
weeks
G
CD4+ T cells/μl
weeks


A
week 3-4
week 7-8
week 20
Copies/10^4 CEq
*
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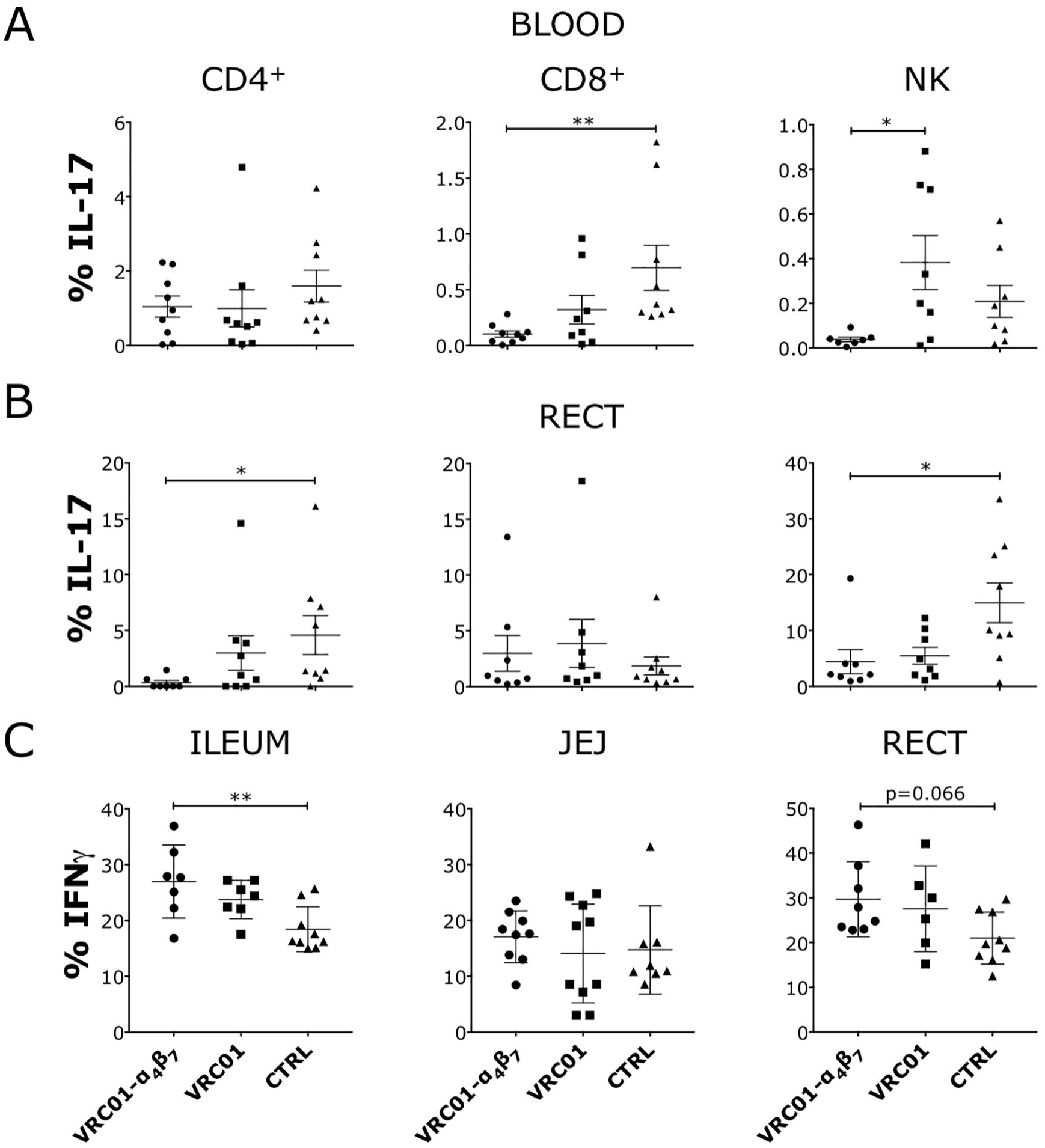
B
SIV copies/1μg RNA
*
VRC01-α4β7
VRC01
CTRL


A
BLOOD
CD4+
CD8+
NK
% IL-17
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B
RECT
% IL-17
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C
ILEUM
JEJ
RECT
% IFNγ
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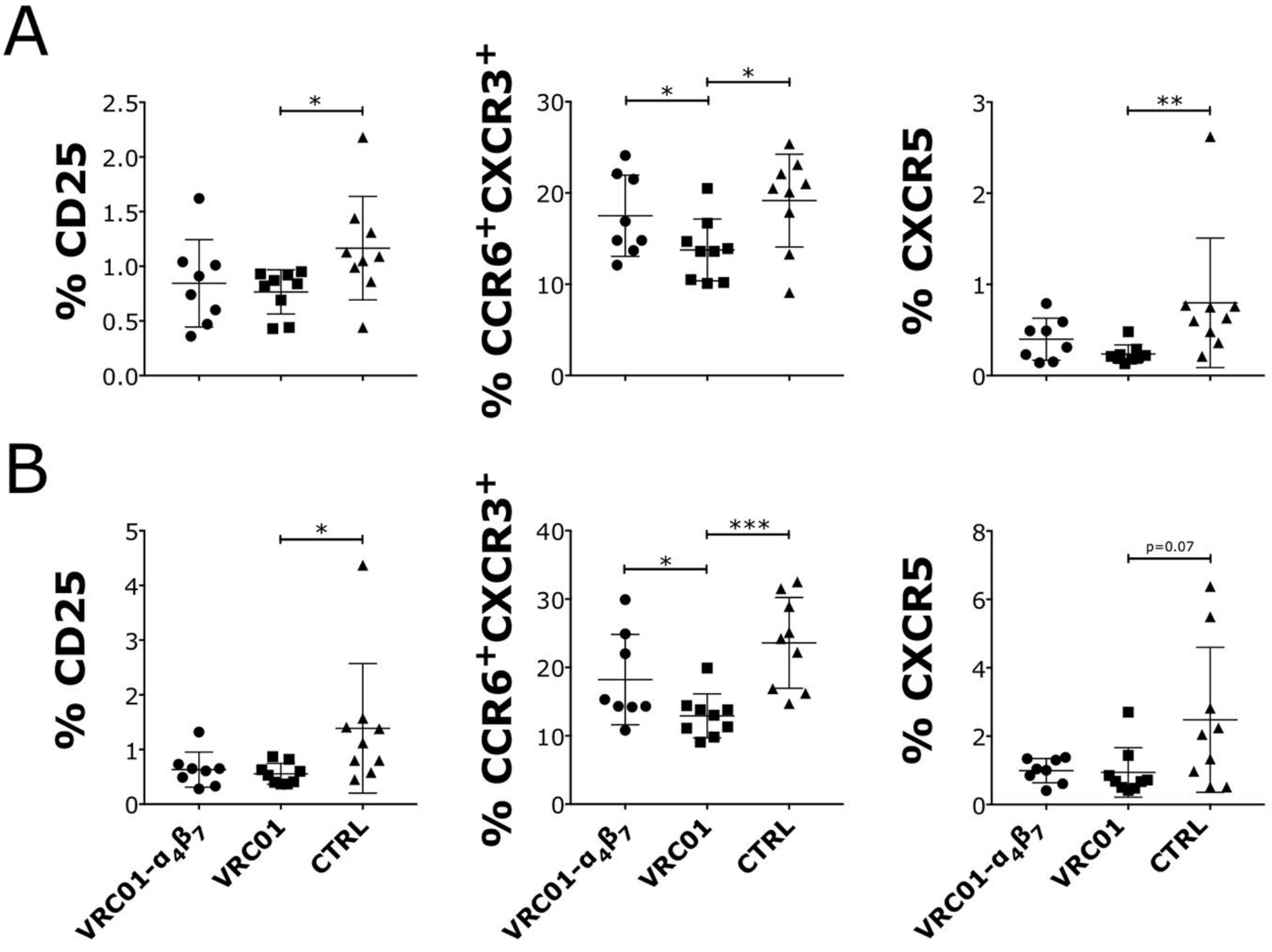
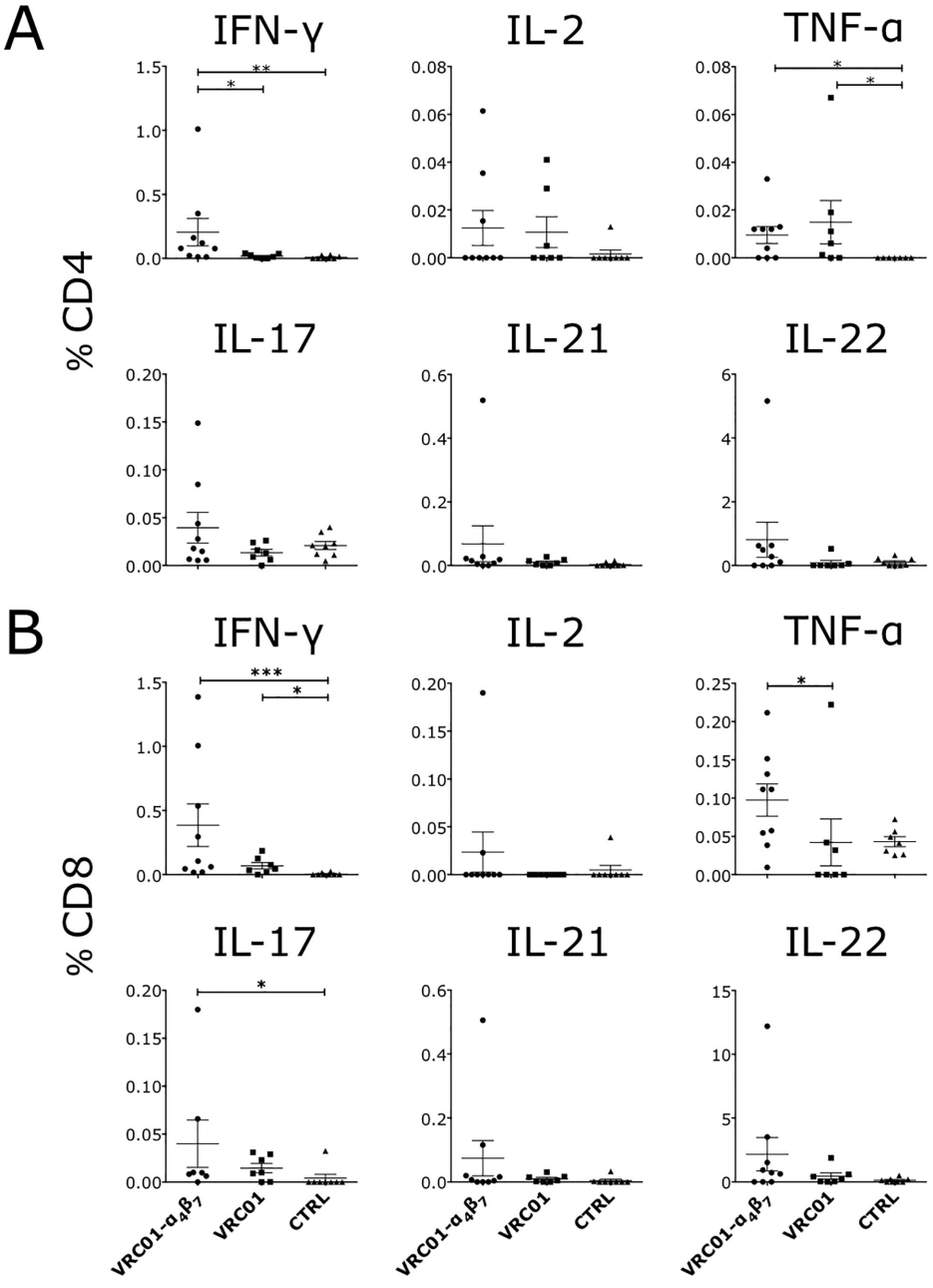
p=0.066
VRC01-α4β7
VRC01
CTRL


A
% CD25
% CCR6+CXCR3+
% CXCR5
B
% CD25
% CCR6+CXCR3+
% CXCR5
VRC01-α4β7
VRC01
CTRL
p=0.07


A
IFN-γ
IL-2
TNF-α
IL-17
IL-21
IL-22
% CD4
B
IFN-γ
IL-2
TNF-α
IL-17
IL-21
IL-22
% CD8
VRC01-α4β7
VRC01
CTRL