

1 **Defense-in-depth by mucosally administered anti-HIV dimeric IgA2**
2 **and systemic IgG1 mAbs: complete protection of rhesus monkeys**
3 **from mucosal SHIV challenge**

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33

34 **Abstract**

35 **Background:** Although IgA is the most abundantly produced immunoglobulin, its role
36 in preventing HIV-1 acquisition, which occurs mostly via mucosal routes, remains
37 unclear. Data from the RV144 trial implied that vaccine-induced plasma IgA antibodies
38 (Abs) counteracted the protective effector mechanisms of IgG1 Abs with the same
39 epitope specificity; mucosal samples were not available for study. We previously
40 performed passive mucosal immunizations in rhesus macaques (RMs) with the HIV-1
41 envelope (Env)-specific, neutralizing monoclonal antibody (mAb) HGN194. The dimeric
42 IgA2 (dIgA2) form of the mAb administered intrarectally (i.r.) protected only 17% of the
43 RMs, whereas the dimeric IgA1 (dIgA1) version also given i.r. prevented infection in
44 83% of the macaques after i.r. challenge with simian-human immunodeficiency virus
45 (SHIV). We hypothesized that mucosal dIgA2 might diminish the protection provided by
46 IgG1 mAbs targeting the same epitope.

47 **Results:** To test our hypothesis, we compared intravenously (i.v.) administered
48 HGN194 IgG1 either alone or in combination with the dIgA2 version given i.r.. Both
49 mAb forms used as single agents and the combination of the two neutralized the
50 challenge virus equally well in vitro. None of the RMs given i.v. HGN194 IgG1 alone
51 remained virus-free. In contrast, all RMs given the HGN194 IgG1+dIgA2 combination
52 were completely protected against high-dose i.r. SHIV-1157ipEL-p challenge.

53 **Conclusion:** Combining suboptimal defenses at the mucosal and systemic levels can
54 completely prevent virus acquisition in all animals. These data imply that active
55 vaccination should focus on defense-in-depth, a strategy that seeks to build up fortified
56 defensive fall-back positions well behind the armed frontline.

57

58 **Keywords:** IgA, dimeric IgA2, IgG, complete protection, macaque model, passive

59 immunization, non-human primate model, mucosal challenge, SHIV, HIV

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61

62 **Background**

63 The partially successful RV144 trial has opened new horizons for HIV-1 vaccine design
64 while posing new challenges for researchers [1]. Post-trial analyses revealed an
65 inverse correlation between IgG antibodies (Abs) specific for the variable loops 1 and 2
66 (V1V2) of the HIV-1 envelope (Env) and the risk of HIV-1 infection. A direct correlation
67 between plasma Env-specific IgA and the risk of HIV-1 infection was also observed [2],
68 although vaccinees with high Env-specific plasma IgA were not more likely to become
69 infected than placebo recipients. Additionally, antibody-dependent cellular cytotoxicity
70 (ADCC) responses in the presence of low plasma concentration of anti-Env IgA
71 correlated with reduced risk of infection. These findings suggested that Env-specific
72 circulating IgA impeded the protective effects of IgG Abs. Secondary analyses showed
73 that vaccinees with plasma IgA specific to the first conserved region (C1) of HIV-1 Env
74 gp120 had a higher risk of infection than vaccinees without C1-specific IgAs [2]. C1-
75 specific monoclonal Abs (mAbs) isolated from RV144 vaccinees and expressed as IgG1
76 showed HIV-1-specific ADCC-mediated cell killing [3]. Of note, two of these ADCC-
77 mediating IgG1 mAbs, namely CH29 and CH38, were originally of IgA2 and IgA1
78 isotypes, respectively. Later, Tomaras et al. demonstrated that the C1 epitope
79 recognized by total plasma IgA and mAbs CH29 and CH38 expressed as IgA2
80 overlapped with the epitopes of IgG1 mAbs within the same Env region [4].
81 Remarkably, mAb CH38 expressed as IgA2 (originally IgA1) inhibited ADCC activity of
82 C1-specific IgG1 mAbs isolated from RV144 vaccinees, while mAb CH29 expressed as
83 IgA2 (originally IgA2) did not [4]. Since mucosal samples had not been collected during

84 the RV144 trial, the question remains as to how IgA2 and IgG1 with the same epitope
85 specificity would interact in the mucosal compartment.

86 Most existing vaccines are administered intramuscularly or subcutaneously and
87 induce both systemic IgG and IgA antibody responses. However, robust mucosal IgA
88 responses with such vaccines are rarely generated [reviewed in [5]]. In contrast,
89 intranasal and oral vaccination strategies induce strong mucosal IgA as well as serum
90 IgG responses and have been successfully implemented against the number of
91 infectious agents [reviewed in [5, 6]]. Mucosal immunization of rhesus monkeys (RMs)
92 with HIV or SIV antigens led to the development of specific IgA responses in vaginal
93 and rectal fluids [7-9]. RMs immunized via both the intramuscular and intranasal routes
94 with HIV-1 gp41-subunit antigens grafted on virosomes were completely protected from
95 persistent systemic infection with SHIV-SF162P3 and showed gp41-specific vaginal
96 transcytosis-blocking IgAs as well as vaginal IgGs with neutralizing and/or antibody-
97 dependent cellular-cytotoxicity activities [10].

98 IgA is the most abundant immunoglobulin (Ig) class: each day, the human body
99 generates more IgA than all other Ig classes combined [reviewed in [11]]. IgA is mostly
100 produced in lymphoid tissues associated with mucosal surfaces; IgA production in the
101 gut amounts to 40–60 mg kg⁻¹ day⁻¹ [reviewed in [12]]. Humans have two IgA isotypes –
102 IgA1 and IgA2 – and both are presented as dimers or polymers at mucosal sites. HIV-1
103 Env-specific IgA1 has been found in cervicovaginal secretions of highly exposed
104 persistently seronegative (HEPS) women [13, 14]. Moreover, HEPS men who had sex
105 with men developed serum IgA1 recognizing HIV-1 Env after oral exposure to HIV-1
106 [15] and HIV-1-specific IgA was found in cervicovaginal fluids of repeatedly exposed sex

107 workers [16, 17]. Furthermore, mucosal IgA Abs specific to HIV-1 gp41 were
108 associated with protection against HIV-1 infection in seronegative partners of HIV-1-
109 infected individuals [18].

110 The RM/primate immunodeficiency virus model is widely used for HIV-1 vaccine
111 research to reflect vaccination of HIV-1-naïve individuals. However, it is likely that most
112 people are exposed to live HIV-1 without becoming systemically infected. Thus, the
113 ability of the naïve RM model to predict possible outcomes in live-HIV-experienced
114 humans is unknown. In this regard, a non-human primate model using simian-human
115 immunodeficiency virus (SHIV)-exposed but uninfected macaques will reflect the real-
116 life situation where prospective recipients of an AIDS vaccine are not naïve but have a
117 history of HIV-1 exposure that did not result in seroconversion.

118 In our recent RM study, passive intrarectal (i.r.) immunization with a dimeric IgA1
119 (dlgA1) version of the anti-V3 loop crown mAb, HGN194 [19], completely protected five
120 out of six RMs against i.r. SHIV challenge [20]. In contrast, the dIgA2 form of the same
121 mAb protected only one out of six RMs. In the same study, the IgG1 version of
122 HGN194 given i.r. prevented infection in two out of six passively immunized animals.
123 The proposed mechanism of differential protection was better virion capture by dIgA1
124 compared with dIgA2 due to the longer hinge region of dIgA1.

125 It is worth mentioning that the ratio of IgA1 and IgA2 varies in different human
126 mucosal fluids, with IgA1 percentages in male genital secretions and nasal fluids
127 reaching 80-90% and 60% in saliva. Female genital secretions and rectal fluids contain
128 approximately 60% IgA2 [reviewed in [21]]. Among primates, only some great apes
129 have two IgA isotypes, and all macaques, including RMs, possess only one IgA version,

130 which is structurally similar to human IgA2 [22]. In this regard, assessing the
131 relationship between mucosal IgA2 and systemic IgG with the same epitope specificity
132 and elucidating the role of this combination against immunodeficiency virus acquisition
133 is important and timely.

134 The potentially negative role of IgA responses in the RV144 trial mentioned
135 above led us to hypothesize that mucosal dimeric IgA2 could compromise the protective
136 effect of IgG1 of the same epitope specificity. Here, we present the results of a study
137 using systemic infusion of IgG1 and i.r. application of dIgA2 mAbs with the same
138 epitope specificity performed in RMs that had been previously exposed to SHIV but
139 remained aviremic and seronegative. Unexpectedly, all RMs treated with the
140 combination of HGN194 IgG1+dIgA2 were completely protected against mucosal SHIV
141 challenge.

142

143 **Results**

144 **Animal selection and analysis of immune responses**

145 The current study used RMs that had remained aviremic and seronegative during two
146 separate, earlier experiments involving passive immunization with mAb HGN194
147 followed by i.r. SHIV challenge. The human IgG1 neutralizing mAb (nmAb) HGN194,
148 isolated from a long-term non-progressor infected with HIV-1 clade AG, targets the V3-
149 loop crown and protects against cross-clade SHIV challenge *in vivo* [19, 23]. The use of
150 previously exposed animals recapitulates the common scenario in humans, where any
151 given HIV-1 exposure results in a low incidence of systemic infection and where non-

152 transmitting exposures result in local and systemic immune responses in some
153 individuals.

154 The first study involved topical (i.r.) application of HGN194 dIgA1, dIgA2 or IgG1
155 [20]. A second, unpublished experiment sought to elucidate the role of nmAb effector
156 functions in protection against i.r. SHIV challenge. In this second study, RMs had been
157 treated intravenously (i.v.) with wild-type HGN194 IgG1 (IgG1_{wt}), its LALA mutant
158 (IgG1_{LALA}) in which binding to the Fcγ receptor (FcγR) was abrogated thereby deleting
159 effector functions, or with an afucosylated version (IgG1_{kif}) of HGN194 IgG1 that had
160 increased binding to FcγRIII, respectively (unpublished data). In both studies, the
161 macaques had been challenged i.r. with 31.5 50% animal infectious doses (AID₅₀) of the
162 R5 clade C SHIV-1157ipEL-p [24].

163 All RMs selected for the current study were persistently aviremic (as measured
164 by a sensitive RT-PCR assay [25]) and were seronegative by SIV Gag ELISA (data not
165 shown). We assessed preselected animals for cellular and humoral immune responses
166 to viral proteins. SIV Gag-specific proliferative CD4⁺ and CD8⁺ T-cell responses were
167 measured at four to eight weeks after the earlier viral challenge (Figure 1). Although
168 persistently aviremic, 9 out of 14 macaques had measurable proliferation of CD4⁺ cells
169 and 11 animals showed CD8⁺ proliferation in responses to stimulation with SIV Gag
170 peptides; three RMs had marginal responses and animal RBk-14 showed no reaction.
171 In general, proliferation of CD8⁺ cells was weaker than CD4⁺ lymphocytes.

172 To analyze possible humoral immune responses among the preselected RMs,
173 we first analyzed the residual plasma concentration of HGN194 IgG1 in all animals that
174 had received the HGN194 mAbs systemically in a previous, unpublished study (Figure

175 2A). At week 8 after the mAb passive transfer and virus challenge, the concentration of
176 HGN194 IgG1_{wt} was about 2 µg/ml and the IgG1_{kif} concentration was marginally above
177 background. Four weeks later, IgG1_{wt} was detected as low as at 0.14, 0.07 and 0.08
178 µg/ml in the plasma of RMs RBk-14, REo-14, and Rlk-14, respectively. At the same
179 time, the mAb concentration in the plasma of HGN194 IgG1_{kif} -treated macaques fell
180 below the detection limit. Of note, the average *in vivo* 90% plasma inhibitory
181 concentration (IC₉₀) of HGN194 IgG1 was estimated at 2.15 µg/ml [23]. To allow
182 complete clearance of previously infused mAbs, the current experiment was scheduled
183 at 16 - 18 weeks after the initial mAb administration. Additionally, we tested plasma
184 samples of animals RBk-14, REo-14 and Rlk-14 collected on the day of new mAb
185 administration for any remaining HGN194 IgG1. As expected, no mAb was detected
186 just before re-administration (not shown).

187 Next, we analyzed the RMs that had received the human mAbs HGN194 IgG1_{wt}
188 and IgG1_{kif} systemically for possible anti-human IgG antibody responses. During
189 recurrent administrations of human IgG, these anti-species Abs, if developed, might
190 cause adverse reactions and rapid elimination of human mAbs from the circulation.
191 Importantly, none of the HGN194-treated RMs had developed any RM anti-human IgG
192 Ab responses (Figure 2B) as a consequence of their prior treatment with HGN194 IgG1.

193 Using ELISA, we next confirmed that the passively immunized, protected RMs
194 had not mounted any anti-HIV-1 Env Ab responses of their own (Figures 2C and D).
195 The secondary Ab in the ELISA was specific for RM IgGs only. No reactivity was seen,
196 as expected from the negative SIV Gag ELISA data (not shown). Thus, the animals did

197 not have any Ab responses that may have skewed the new passive immunization/SHIV
198 challenge study.

199

200 **Group assignment and study design**

201 Animals that had earlier received different versions of HGN194 mAb through different
202 routes were distributed evenly between two new experimental groups (Table 1). Each
203 group contained two RMs that had received HGN194 IgG1 systemically and four RMs
204 treated topically. The control group consisted of two macaques that had received
205 HGN194 IgG1 systemically. MHC alleles and TRIM5 α genotypes also were distributed
206 evenly among the new groups (Table 1).

207 The experimental timeline of the current study is depicted in Figure 3. RMs in
208 both Groups A and B received HGN194 IgG1 i.v. at 1.45 mg/kg 24 h before the viral
209 challenge. RMs of Group A were additionally treated with 1.25 mg of HGN194 dIgA2
210 applied i.r. (1.25 mg of mAb in 2.1 ml of phosphate-buffered saline (PBS)) 30 min before
211 the virus challenge. Control Group C macaques were left untreated. All animals were
212 challenged i.r. with 31.5 AID₅₀ of SHIV-1157ipEL-p [24], an R5 clade C SHIV, and
213 monitored prospectively by measuring of plasma viral RNA (vRNA) loads.

214

215 **The combination of IgG1+dIgA2 versions of HGN194 completely protects RMs** 216 **from single high-dose SHIV challenge**

217 The single mucosal high-dose challenge with SHIV-1157ipEL-p resulted in systemic
218 infection of all macaques of Group B (HGN194 IgG1 only) by week 3 (Figure 4A).

219 Control animals (Group C) were viremic as well. Surprisingly, all Group A RMs, which

220 had received the combination of IgG1+dlgA2, remained aviremic. The time to vRNA
221 load >50 copies/ml for Groups A and B animals was compared by Kaplan-Meier
222 analysis using the log-rank test with two-sided P-values (Figure 4B). The combination
223 of IgG1+dlgA2 demonstrated significantly better protection against mucosal SHIV-
224 1157ipEL-p challenge compared with IgG1 alone ($P = 0.0005$). The shorter half-life of
225 IgG1 in Group B RMs can be explained by absorption and removal of IgG1 from the
226 circulation by newly replicating virus.

227 In our previous experiment, the same virus challenge caused systemic infection
228 of five out six RM treated with the same dose of dlgA2 i.r. [20]. The results of another,
229 yet unpublished study with different IgG1 versions of HGN194 demonstrated infection of
230 four out seven macaques infused i.v. with 1 mg/kg of IgG1_{wt}. Taken together, these
231 results indicate that the combination of systemic IgG1 and topical dlgA2 treatments
232 yielded better protection compared with individual mAb treatment alone.

233

234 **HGN194 IgG1 pharmacokinetics and plasma neutralization capacity**

235 The IgG1 pharmacokinetics were analyzed by ELISA. The infused IgG1 mAb showed
236 the classical circulation profile in both groups of macaques (Figure 5A) following virus
237 challenge. RMs of Group B cleared IgG1 faster compared with animals of Group A
238 (Figure 5B). Mean half-lives of HGN194 IgG1 were calculated at 15.8 ± 3.9 days for
239 Group A and 8.2 ± 2.6 days for Group B RMs ($P = 0.0087$, Mann-Whitney test).

240 Although HGN194 IgG1 demonstrated shorter half-life in Group B RMs, mAb
241 concentrations on the day 0, the day of virus challenge, were similar for Groups A and B
242 animals (Table 2). These concentrations were comparable to that reported previously

243 [23] as well as to the IgG1 concentration observed during the previous experiment
244 (unpublished data). Mean HGN194 IgG1 concentrations were 3.3 ± 0.9 $\mu\text{g/ml}$ for Group
245 A and 3.1 ± 0.5 $\mu\text{g/ml}$ for Group B, respectively.

246 Plasma samples of Group A and B RMs collected on the day of virus challenge
247 were able to neutralize SHIV-1157ipEL-p, the challenge virus, with the same efficiency
248 as demonstrated by TZM-bl cell-based neutralization assays (Table 2). There was no
249 difference between mean 50% plasma inhibitory concentration (IC_{50}) values of Groups
250 A (0.5 ± 0.2 $\mu\text{g/ml}$) and B (0.4 ± 0.08 $\mu\text{g/ml}$) measured in RM plasmas on the day of
251 virus challenge. These results are in line with *in vivo* IC_{50} values observed previously
252 for HGN194 IgG administered to infant RMs at 1 mg/kg dose [23]. Also, these data
253 clearly demonstrate that, in spite of faster clearance of mAb by RMs of Group B, RMs of
254 Groups A and B maintained equal concentrations of IgG1 in plasma on the day of SHIV-
255 1157ipEL-p challenge and that mAb was able to neutralize the challenge virus with the
256 same efficiency. Of note, all RMs in Group B with the shorter half-life were viremic,
257 which probably resulted in faster clearance post-challenge due to immune complex
258 formation.

259

260 **Individual mAbs and combination of IgG1+dIgA2 show the same neutralization** 261 **profiles *in vitro***

262 To understand why the combination was more protective than treatment with individual
263 mAbs, we examined the neutralization of the challenge virus *in vitro* by the combination
264 of IgG1+dIgA2. Toward this end, we used TZM-bl, A3R5 and human peripheral blood
265 mononuclear cell (PBMC)-based assays (Figure 6A-C). For all three assays, the

266 differences between neutralization curves were not significant as evaluated by multiple
267 *t*-tests and two-way ANOVA test for multiple comparisons (not shown).

268 It should be noted that in the neutralization assays, dIgA2 and IgG1 were used at
269 the same mass concentrations. The concentration of the IgG+dIgA2 combination was
270 the sum of mass concentrations of individual mAbs. Dimeric IgA2 has a molecular
271 weight of ~315 kDa, compared to ~150 kDa for IgG1; thus, the molar concentration of
272 dIgA2 taken at the same mass concentration as IgG1 is twofold lower than for IgG1.
273 However, dIgA2 bears four Fab regions, and IgG1 has only two. Therefore, the dIgA2
274 solution with a twofold lower molar concentration than the IgG1 solution contained the
275 same molar concentration of antigen combining sites as the IgG1 solution. These
276 considerations explain the similar neutralization curves for HGN194 IgG1, dIgA2, and
277 combination of both.

278

279 **The combination of IgG1+dIgA2 does not inhibit virus transcytosis *in vitro***

280 As we previously reported [20], only HGN194 dIgA1, but not dIgA2 or IgG1 as single
281 agent, was able to inhibit virus transcytosis *in vitro*. We evaluated whether the
282 combination of HGN194 IgG1+dIgA2 could inhibit the transcytosis of SHIV-1157ipEL-p
283 at pH 6. A low pH has been reported to enhance antibody-mediated virus transcytosis
284 [26]. Additionally, the pH of colonic rectal fluid for *Macaca* species was reported to
285 range between 5.1 and 7.8 [27, 28]. As shown, the mean concentration of dIgA2
286 administered i.r. was 231.6 µg/ml 30 min after topical application [20]. To reflect the *in*
287 *vivo* observed concentration of dIgA2, we performed the transcytosis assay at 200
288 µg/ml of dIgA2 or dIgA1 used as a positive control mixed with different concentrations of

289 HGN194 IgG1 or the isotype control IgG1 Fm-6 (Figure 6D). At pH 6, HGN194 IgG1
290 alone or in combination with dIgA2 enhanced transcytosis of SHIV-1157ipEL-p across
291 HEC-1A cells. As expected, dIgA1 used as a positive control completely reversed the
292 effect of specific IgG1 and inhibited transcytosis almost completely at any IgG1
293 concentration tested. The fact that the IgG1+dIgA2 combination was unable to prevent
294 enhanced virus transcytosis *in vitro* suggests that this combination is not likely to exert
295 its protective effect through inhibition of transcytosis.

296

297 **Discussion**

298 We have shown that HIV-1 Env-specific IgG1 plasma Abs in combination with mucosal
299 dIgA2 of the same epitope specificity completely protected RMs against high-dose
300 mucosal challenge with SHIV-1157ipEL-p. Initially, we demonstrated that RMs that had
301 remained aviremic in previous passive immunization experiments had developed low-
302 level, virus-specific cell-mediated immune responses and thus represent a relevant
303 model to assess immunization efficacy among HIV-1-exposed, uninfected individuals.
304 The striking 100% protection we observed here in RMs with the combination of i.v. IgG1
305 plus i.r. dIgA2 mAbs was unexpected – given that the group treated with the IgG1
306 version alone had 0% protection and that i.r. dIgA2 had protected only 17% of RMs
307 challenged with the same clade C SHIV earlier. Lastly, we demonstrated that inhibition
308 of virus transcytosis, as suggested for dIgA1 [20], was unlikely to be the mechanism of
309 protection by the combination of IgG1+dIgA2. Thus, our findings suggest that mucosal
310 dimeric IgA2, if generated by active immunization, will complement HIV-1-specific

311 plasma IgG1 in preventing virus acquisition rather than diminishing the protective role of
312 plasma IgG1.

313 The gastrointestinal mucosa is the largest mucosal surface in the human body,
314 and it represents the major portal of HIV-1 entry during mother-to-child transmission via
315 breastfeeding, sexual transmission in men who have sex with men, as well as during
316 heterosexual anal intercourse [reviewed [29]]. The risk of HIV-1 transmission through
317 receptive anal sex was estimated by meta-analysis at 1.4% (an average of one
318 transmission event occurred for every 71 exposures), which is at least 10 times higher
319 than for unprotected vaginal intercourse [reviewed [30]]. On another note, the
320 distribution of IgG and IgA varies considerably between different body compartments
321 [31]. While serum contains 3.5 – 14 times more IgG than IgA, IgAs are more prevalent
322 in gastrointestinal tract secretions. Within the intestine, secretions of the digestive part
323 contain more IgA1 than IgA2, whereas secretions of the colon generally possess slightly
324 more IgA2 than IgA1 [31]. In the current study, IgG1 was administered i.v. and thus
325 was distributed systemically as well as into some mucosal fluids. After topical
326 administration, dIgA2 remained localized at the rectal mucosa, because there is no IgA
327 back-transfer from the intestinal lumen across the epithelial barrier. In contrast, the Fc
328 neonatal receptor (FcRn) can shuttle IgG in both directions; it unloads IgG or IgG-
329 immune complex cargo in a pH-dependent manner [reviewed in [32]]. Thus, our
330 passive immunization study reflected the compartmentalization of Ab responses and
331 addressed the vulnerability of the rectal mucosa for HIV-1 infection.

332 While a negative correlation was established between circulating anti-HIV-1 Env
333 IgA in the RV144 trial, our data strongly suggest that a successful HIV-1 vaccine must

334 generate both mucosal IgA and systemic IgG responses. Such a defensive strategy is
335 best described by the military term “defense-in-depth” – an approach to defend a vital
336 core by pre-planned, well-armed, multiple lines of defense that can provide backup in
337 case the frontline is breached. Defense-in-depth against mucosal HIV-1 transmission
338 can be described as follows. When HIV-1 virions first encounter mucus in the mucosal
339 cavity, they are exposed to secretory IgAs (SIgAs), which may either crosslink the
340 virions, prevent virus transcytosis across the epithelial barrier, or simply neutralize virus.
341 In case some virions remain free, they can penetrate the mucus and the epithelium by
342 transcytosis or other mechanism. Such virions then face ubiquitous IgG and may be
343 neutralized by it. The neutralization capacity of antiviral IgG is limited by its affinity and
344 by the accessibility of HIV-1 Env epitopes. In other words, the tissue and/or plasma
345 concentration of neutralizing IgG must be high enough to neutralize incoming virus as it
346 penetrates the mucosa.

347 In our current study, the concentration of HGN194 IgG1 used for i.v.
348 administration (1.45 mg/kg) was expected to protect approximately half of the RMs from
349 SHIV challenge based on data from our previous study, where 50% of RMs were
350 protected with 1 mg/kg of HGN194 IgG1 [23]. However, the RMs used for the current
351 study had developed low-level cellular immune responses as a consequence of their
352 earlier experience to live virus; in fact, proliferative responses among CD4⁺ lymphocytes
353 tended to predominate. Thus, the HGN194 IgG1 dose of 1.45 mg/kg was not protective
354 and all the animals of Group B became infected. When applied mucosally (i.r.) as
355 single mAbs in an earlier study [20], IgG1 protected only 33% and dIgA2 17% of RMs,
356 respectively against the same challenge virus. However in the current experiment, the

357 combination of i.v. IgG1 plus i.r. dIgA2, in which both mAbs were administered at sub-
358 protective doses, completely prevented virus acquisition. It is reasonable to suggest
359 that in this case, part of the challenge virus was neutralized in the rectal lumen by dIgA2
360 and to a lesser extent by transudated IgG1; the residual virus, which was not
361 neutralized in the lumen and crossed the epithelium, was met and neutralized by IgG1
362 in tissues or in the circulation. Thus, our RM model of passive immunization with the
363 combination of topically applied dIgA2 and systemically administered IgG1 followed by
364 mucosal SHIV challenge reflects the outcome of a successful HIV-1 vaccine that
365 induces both systemic IgG and mucosal IgA responses. Our current study provides
366 proof-of-concept for the defense-in-depth strategy against mucosal transmission of HIV-
367 1.

368 The results of the current study, taken together with our previous findings
369 regarding the protective role of dIgA1 [20], provide a rational explanation of a possible
370 way of preventing HIV-1 acquisition if active vaccination were to generate both mucosal
371 IgA and systemic antibody responses. In this regard, to achieve the complete
372 prevention of HIV-1 infection, a successful vaccine must induce different immune
373 effectors, including HIV-1 Env-specific Abs in mucosal secretions as frontline defense,
374 with back-up provided by neutralizing Abs in tissues and in the circulation and cytotoxic
375 T cells, thus generating defense-in-depth.

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378

379

380 **Conclusions**

381 Passive immunization with mAb HGN194 IgG1 given systemically together with
382 HGN194 dIgA2 given mucosally completely protected all RMs from high-dose mucosal
383 SHIV challenge, while no monkey given the IgG1 mAb i.v. alone was protected.
384 These results together with our previous findings regarding the protective role of
385 mucosal dIgA1 [20] provide proof-of-concept for defense-in-depth against mucosal
386 transmission of HIV-1.

387

388 **Materials and Methods**

389 **Cell lines, reagents, and virus**

390 The following reagent was obtained through the NIH AIDS Reagent Program, Division
391 of AIDS, NIAID, NIH: TZM-bl from Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme
392 Inc.. A3R5 cells were kindly provided by Dr. David Montefiori. MAb Fm-6 and VRC01
393 were kindly provided by Drs. Wayne Marasco (Dana-Farber Cancer Institute) and John
394 Mascola (Vaccine Research Center, NIH), respectively. The SHIV-1157ipEL-p stock
395 (grown in RM PBMC) had a p27 concentration of 792 ng/ml and 7.8×10^5 50% tissue
396 culture infectious doses (TCID₅₀)/ml as measured in TZM-bl cells. Recombinant
397 HGN194 mAb forms were prepared as described previously [20].

398

399 **Lymphocyte proliferation assay**

400 PBMC were stained with CFSE (CellTrace™ CFSE Cell Proliferation Kit, Invitrogen)
401 and incubated with or without SIVmac239 Gag peptides (2 µg/ml for each peptide). The
402 peptides (obtained through ARRRP) were 15-mers with an 11-amino acid overlap

403 between sequential peptides and represented the complete protein sequence. Cells
404 without any stimuli were used to determine background proliferation. After incubation
405 for 5 days at 37°C, cells were stained with anti-CD3-Alexa Fluor 700 (clone SP34-2),
406 anti-CD4-PerCP (clone L200), and anti-CD8-PE (clone RPA-T8) Abs (all from BD
407 Pharmingen). After fixation, at least 10,000 CD3⁺ cells were acquired by flow
408 cytometry, and data were analyzed using FACSDiva (BD Biosciences) software. The
409 percentages of proliferating CD3⁺CD4⁺ and CD3⁺CD8⁺ cells were determined by CFSE
410 dilution; background proliferation (without stimulation) was subtracted.

411

412 **ELISAs**

413 To evaluate HGN194 IgG1 pharmacokinetics, ELISA plates (Nunc) were coated with 1
414 µg/ml of SHIV-1157ip gp120 in PBS. After washing, plates were blocked with 4% Non-
415 fat dry milk (Bio-Rad), 0.05% Tween-PBS (blocking buffer). Plates were then incubated
416 with serial dilutions of RM plasma samples in triplicates. HGN194 IgG1 was included as
417 a standard ranging from 0.1 to 31 ng/ml. After washing, plates were developed by
418 incubation for 1 h with goat anti-human IgG HRP-conjugated Ab (Jackson
419 Immunoresearch) or goat anti-human HRP-conjugated Ab that had been adsorbed with
420 monkey IgG (Southern Biotech) to avoid cross-reactivity with monkey anti-gp120 Abs.
421 Color reaction was performed with TMB solution (Life Technologies).

422 Analysis of RM plasma binding to SHIV-1157ip gp120 was done essentially as
423 described above. Plates were coated with gp120, blocked and incubated with RM
424 plasma samples at different dilutions. To detect binding, plates were incubated with

425 mouse monoclonal anti-monkey IgG HRP-conjugated Ab with no cross-reactivity to
426 human IgG (Southern Biotech) and developed with TMB solution.

427 To evaluate RM anti-human IgG responses, plates were coated with 1 µg/ml of
428 HGN194 IgG1 in carbonate buffer, pH 9.6. After blocking and incubation with RM
429 plasma samples, plates were probed mouse monoclonal anti-monkey IgG HRP-
430 conjugated Ab with no cross-reactivity to human IgG (Southern Biotech) and developed
431 with TMB solution.

432

433 **Animals**

434 RMs were housed at the Yerkes National Primate Research Center (YNPRC, Atlanta,
435 GA) in accordance with standards of the National Institutes of Health Guide for the Care
436 and Use of Laboratory Animals. Animal experiments were approved by the Institutional
437 Animal Care and Use Committees at Emory University and the Dana-Farber Cancer
438 Institute (DFCI) via a Collaborating Institution Animal Use Agreement. Blood was
439 collected under ketamine or Telazol anesthesia.

440

441 **Passive immunization and mucosal SHIV-1157ipEL-p challenge**

442 All RMs were Mamu B*008 and B*017 negative and aged between 12 to 16 months at
443 the time of challenge. Mamu A*001-positive animals were evenly distributed in each
444 group, as were RMs with different FcγRIIIa genotypes (Table 1). As depicted on Figure
445 3, Group A RMs (n=6) were treated i.v. with 1.45 mg/kg of HGN194 IgG1 at -24 h, and
446 i.r. with 1.25 mg (in 2.1 ml of PBS) of HGN194 dIgA2 30 min before challenge. The six
447 macaques of Group B were treated i.v. with 1.45 mg/kg of HGN194 IgG1 only at -24 h.

448 The control Group C consisted of two untreated animals. All monkeys were challenged
449 i.r. with 31.5 50% AID₅₀ of SHIV-1157ipEL-p [24].

450

451 **Plasma viral RNA levels**

452 Plasma viral RNA levels were measured as described [25, 33].

453

454 **In vitro neutralization assays**

455 For all the assays, mAbs were incubated with virus for 1 h at 37°C and then the cells
456 were added to the mixture. The TZM-bl assay was performed as described [34]. In
457 brief, virus was added to cells in the presence of DEAE-dextran (Sigma), washed 1x on
458 day 1 and luminescence was measured on day 2 using luciferase substrate Bright-Glo
459 (Promega). The A3R5 cell-based assay was performed as described [35] with
460 NL.LucR-1157ipEL virus encoding the *env* gene of SHIV-1157ip-EL envelope [36] and
461 *Renilla* luciferase [37]. Human PBMC-based assays were performed as described [23].

462

463 **Inhibition of transcytosis**

464 HEC-1A cell (ATCC) monolayers were created on 0.4 µm polyethylene terephthalate
465 (PET) membrane hanging transwell inserts (Millipore). Electrical resistance of >400
466 mOhms/cm² across the membrane confirmed monolayer integrity. Cell-free SHIV-
467 1157ipEL-p (2 ng/ml of p27) was preincubated for 1 h at 37°C alone or with various
468 concentrations of HGN194 dIgA1, HGN194 dIgA2, or IgG1, or control IgG1 Fm-6. Next,
469 virus or virus/mAb mixtures were added to the apical surface of the cell monolayer in the

470 upper chamber. After 12 h, fluid in the lower chamber (“subnatant fluid”) was collected
471 and used to measure viral RNA copy numbers by RT-PCR [25, 33].

472

473 **Statistical Analysis**

474 Statistical analyses were performed using Graph Pad Prism for Windows, version 6
475 (Graph Pad Software Inc., San Diego, CA).

476

477 **List of abbreviations**

478 Ab, antibody; mAb, monoclonal antibody; nmAb, neutralizing monoclonal antibody;
479 ADCC, antibody-dependent cellular cytotoxic activity; AID₅₀, 50% animal infectious
480 doses; C1, first conserved region; CTL, cytotoxic T-lymphocyte; dIgA, dimeric IgA; Env,
481 envelope; FcγR, Fcγ receptor; FcRn, Fc neonatal receptor; PBS, phosphate buffered
482 saline; HIV-C, HIV-1 clade C; IC₅₀, 50% inhibitory concentration; IC₉₀, 90% inhibitory
483 concentration; Ig, immunoglobulin; i.r., intrarectal; i.v., intravenous; RM, rhesus monkey;
484 SHIV, simian-human immunodeficiency virus; TCID₅₀, 50% tissue culture infectious
485 dose; V1V2, variable loops 1 and 2; vRNA, viral RNA.

486

487 **Competing interests**

488 A. Lanzavecchia is the scientific founder of Humabs LLC, a company that develops
489 human Abs for treatment of infectious diseases. D. Corti and G. Agatic are currently
490 employees of Humabs. A. Lanzavecchia hold shares in Humabs.

491

492

493 **Authors' contributions**

494 Contribution: A.M.S., J.D.W., Q.S., R.A.W., D.C., A.L., J.L.H. and R.M.R conceived the
495 study and designed the experiments; A.M.S., J.D.W., H.K.V., S.K.L., S.T., M.Z., G.H.,
496 and B.C.B. performed the experiments; F.V. managed primate experiments; S.G. and
497 D.N.F. performed transcytosis analysis; G.A., D.C. and A.L. prepared mAbs; A.M.S. and
498 R.M.R analyzed the data and wrote the manuscript.

499

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512

513

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656 **heterologous R5 clade C SHIV: prevention of infection and correlates of**
657 **protection. *PLoS One* 2011, 6:e22010.**
- 658

Table 1 Group reassignment of virus-experienced uninfected RMs

Groups	Animal	HGN194 version	Type of previous mAb treatment	MHC allele			TRIM5 α genotype restriction	Reference
				A*001	B*008	B*017		
Group A	RBk-14	IgG1 _{wt}	i.v.	-	-	-	moderate	-
IgG1+dIgA2	Rlr-14	IgG1 _{kif}	i.v.	-	-	-	high	-
	RKv-14	IgG1 _{wt}	i.r.	-	-	-	high	[20]
	RLz-14	dIgA1	i.r.	-	-	-	high	[20]
	RNv-14	dIgA1	i.r.	+	-	-	moderate	[20]
	RWw-14	dIgA1	i.r.	-	-	-	high	[20]
Group B	RAy-14	dIgA1	i.r.	-	-	-	moderate	[20]
IgG1	RCw-14	IgG1 _{wt}	i.r.	-	-	-	moderate	[20]
	RIk-14	IgG1 _{wt}	i.v.	+	-	-	moderate	-
	ROw-14	dIgA1	i.r.	-	-	-	high	[20]
	RUq-14	IgG1 _{kif}	i.v.	-	-	-	high	-
	RYv-14	dIgA2	i.r.	-	-	-	high	[20]
Group C	REo-14	IgG1 _{wt}	i.v.	+	-	-	moderate	-
controls	RIm-14	IgG1 _{kif}	i.v.	-	-	-	moderate	-

660 All RMs had no anti-HIV Env Ab responses at the time of the 2nd virus challenge. IgG1_{wt},

661 wild type of HGN194 IgG1; IgG1_{kif}, afucosylated version of HGN194 IgG1.

662

663 **Table 2 Concentration and IC₅₀ of HGN194 IgG1 in RM plasma on the day of virus**
 664 **challenge**

Groups	Animal #	IgG1 concentration, µg/ml	Plasma IC₅₀, µg/ml
Group A IgG1+dIgA2	RBk-14	4.0	0.54
	Rlr-14	2.5	ND
	RKv-14	2.8	ND
	RLz-14	2.5	0.32
	RNv-14	3.2	0.86
	RWw-14	4.9	0.30
Group B IgG1	RAy-14	3.5	0.53
	RCw-14	2.4	0.41
	Rlk-14	3.9	ND
	ROw-14	3.6	0.56
	RUq-14	3.2	0.40
	RYv-14	2.6	0.32

665
 666 Plasma IC₅₀ concentrations were determined using the concentration of mAb in RM
 667 plasma on the day of challenge and the dilution of this plasma sample showing 50% of
 668 neutralization in TZM-bl cell assay. Calculations were performed in respect of
 669 neutralization obtained with the plasma sample from the same RM taken before the
 670 mAb administration at the same dilution. ND, not determined. Experiment performed in
 671 triplicate.

672

673 **Figure legends**

674 **Figure 1 Antiviral T-cell responses after previous SHIV-1157ipEL-p challenge [20].**

675 PBMC were stimulated with overlapping peptides representing SIVmac239 Gag and
676 proliferation of CD4⁺ and CD8⁺ cells was measured using the CFSE dilution method as
677 described in Materials and Methods. The y-axis indicates % proliferating cells. PBMC
678 isolated from two naïve macaques (RCy-5 and RSf-12) were used as a negative control
679 and PBMC from a previously vaccinated, aviremic animal RAT-9 [38] served as a
680 positive control, respectively. **Panel A.** Positive (RAT-9) [38] and negative (RCy-5 and
681 RSf-12) controls. **Panel B.** T-cell responses of RMs that had received wild-type
682 (IgG1_{wt}) or afucosylated (IgG1_{kif}) versions of HGN194 IgG1 systemically (i.v.)
683 (unpublished data). **Panel C.** T-cell responses of animals that had previously received
684 HGN194 IgG1, dimeric IgA1 or dimeric IgA2 topically (i.r.) [20].

685

686 **Figure 2 Antibody responses in RMs previously given passive immunization with**

687 **different forms of HGN194 [20]. Panels A and B,** Only animals that had received
688 HGN194 systemically were analyzed. Mucosally treated RMs had been tested earlier
689 and HGN194 had not been detected in the plasma (data not shown). Red bars, RMs
690 assigned to Group A of the current study; blue bars, RMs assigned to Group B; black
691 bars, RMs assigned to Group C. Solid bars show recipients of HGN194 IgG1_{wt} and
692 striped bars depict recipients of IgG_{kif}. **Panel A.** Residual concentration of HGN194
693 IgG1 at different time points after administration. HGN194 IgG1 was used as a
694 standard. Secondary goat anti-monkey HRP-conjugated Ab was RM IgG adsorbed.
695 **Panel B.** RM anti-human IgG responses at different time points after HGN194 IgG1 i.v.

696 administration. Grey bars, goat anti-human Ab served as a positive control; +C, positive
697 control (goat anti-human Ab HRP-conjugated). **Panels C and D.** HIV Env binding
698 ELISA analysis of RM plasma samples collected at different time points after virus
699 challenge. **Panel D.** Blue, Group B RM plasma samples. SHIV-1157ip gp120 served
700 as antigen. **Panel C.** Red, Group A RM plasma samples. Open bars, pooled naïve RM
701 plasma was used as a negative control; grey bars, plasma of RRI-11 [39] was used as
702 positive control. The secondary Ab was mouse anti-monkey HRP-conjugated
703 secondary Ab with minimal cross-reactivity to human IgG.

704

705 **Figure 3 Study timeline and design.** Three groups of RMs were enrolled. Group A (n
706 = 6) received the combination of i.v. HGN194 IgG1 (1.45 mg/kg); and i.r. HGN194 dIgA2
707 (1.25 mg). Group B RMs (n = 6) received i.v. HGN194 IgG1 (1.45 mg/kg) only. Group
708 C (n = 2) RMs served as virus-only controls. Black arrow, mAb administrations; yellow
709 arrow, 24 h after IgG1 administration and 30 min after dIgA2 topical application, if any,
710 animals were challenged i.r. with 31.5 AID₅₀ of SHIV-1157ipEL-p.

711

712 **Figure 4 The combination of HGN194 IgG1+dIgA2 completely protects RMs from**
713 **high-dose mucosal virus challenge.** **A.** Red, viral RNA loads for individual RMs for
714 Group A (IgG1+dIgA2); blue, vRNA loads for Group B (IgG1) RMs; black, vRNA loads
715 for Group C (controls) RMs. **B.** Kaplan-Meier analysis of time until vRNA load
716 exceeded 50 copies/ml. Log rank test significance P value is indicated. Red, Group A;
717 blue, Group B.

718

719 **Figure 5 Analysis of HGN194 IgG1 levels in plasma. Panel A.** HGN194 IgG1
720 pharmacokinetics in RM groups. Black arrow indicates SHIV-1157ipEL-p challenge; red,
721 RM of Group A; blue, RMs of Group B. **Panel B.** Analysis of HGN194 IgG1 half-life in
722 RMs. Red, RMs of Group A; blue, RMs of Group B. Statistical analysis was performed
723 by Mann-Whitney test ($P < 0.05$).

724

725 **Figure 6 The combination of HGN194 IgG1+dlgA2 neutralizes virus similarly to the**
726 **individual mAbs and does not inhibit virus transcytosis. A-C.** Neutralization of
727 SHIV-1157ipEL-p by HGN194 IgG1, dlgA2 and combination of both. The concentration
728 of IgG1+dlgA2 combination is the sum of concentrations of individual mAbs. MAbs
729 VRC01 and Fm-6 were used as positive and negative controls, respectively (not
730 shown). **A.** Human PBMC-based assay; **B.** TZM-bl cell assay; and **C.** A3R5 cell assay.
731 **D.** Inhibition of transcytosis. Solid lines, HGN194 IgG1; dashed lines, isotype control
732 mAb Fm-6. Both IgG1 Abs ranging from 1 to 100 $\mu\text{g/ml}$ were incubated with the virus
733 alone (blue, HGN194; light blue, Fm-6), or together with 200 $\mu\text{g/ml}$ of dlgA2 (red,
734 HGN194; light red, Fm-6), positive control dlgA1 (grey, HGN194; light grey, Fm-6).
735 Next, virus or virus/mAb mixtures were added to the HEC-1A cell monolayer. Twelve h
736 later, vRNA copy numbers were measured. Percent of transcytosis inhibition was
737 calculated in comparison with the number of vRNA copies determined for wells with
738 virus alone. Negative values on Y axis show percent transcytosis enhancement. All
739 experiments were repeated at least twice.

740

Figure 1

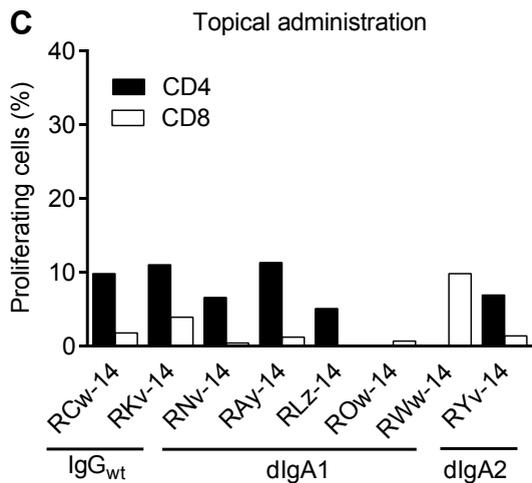
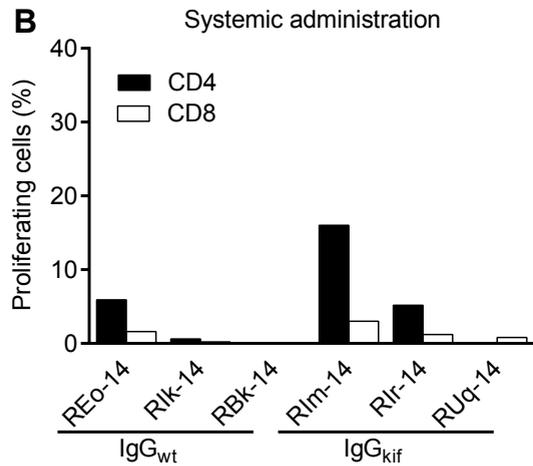
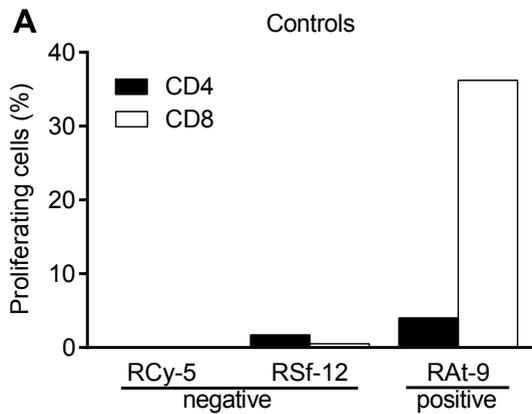


Figure 2

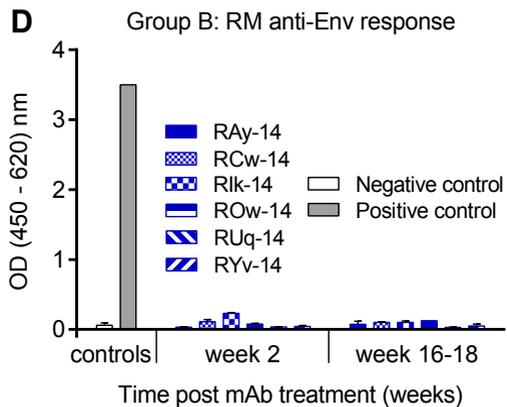
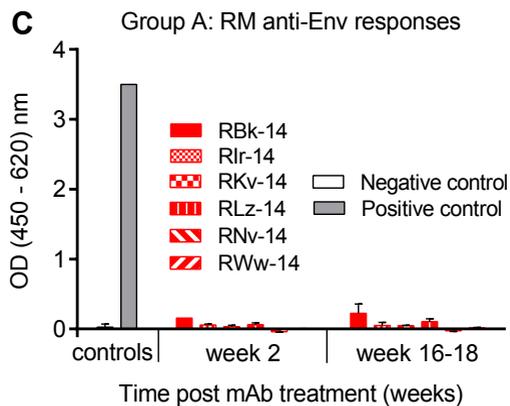
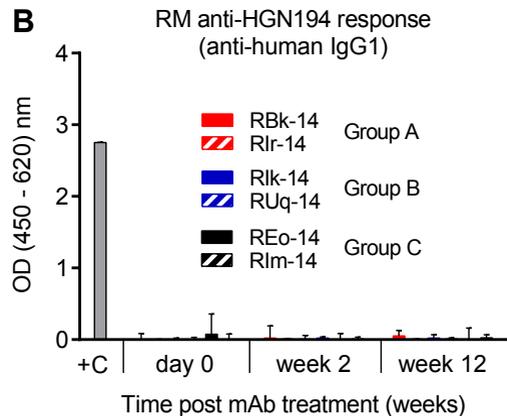
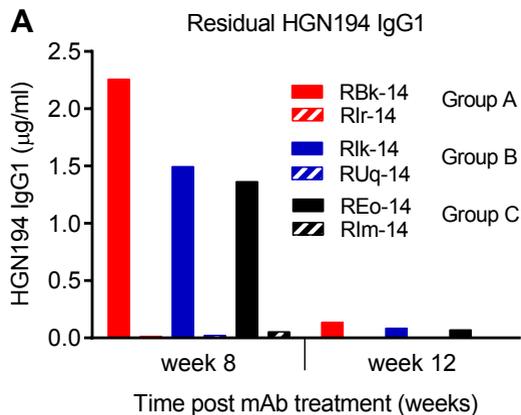
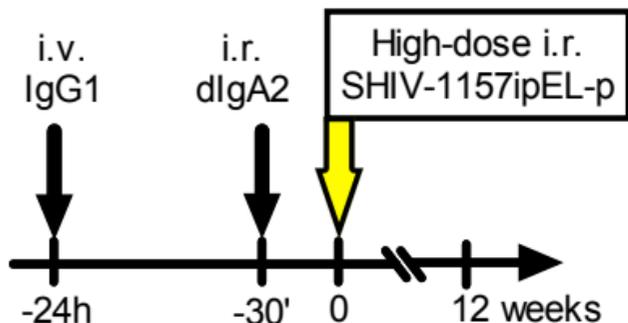


Figure 3

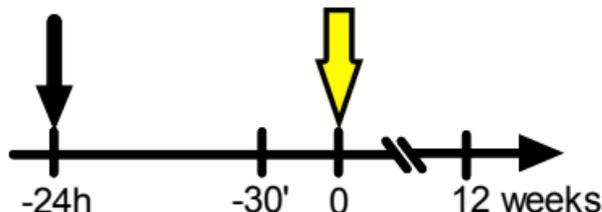
Group A: IgG1+dIgA2

RBk-14	RLz-14
RIr-14	RNv-14
RKv-14	RWw-14



Group B: IgG1

RAy-14	ROw-14
RCw-14	RUq-14
RIk-14	RYv-14



Group C: controls

REo-14	RIm-14
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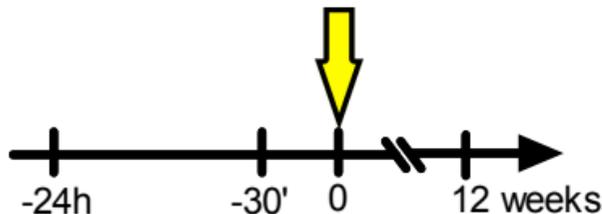


Figure 4

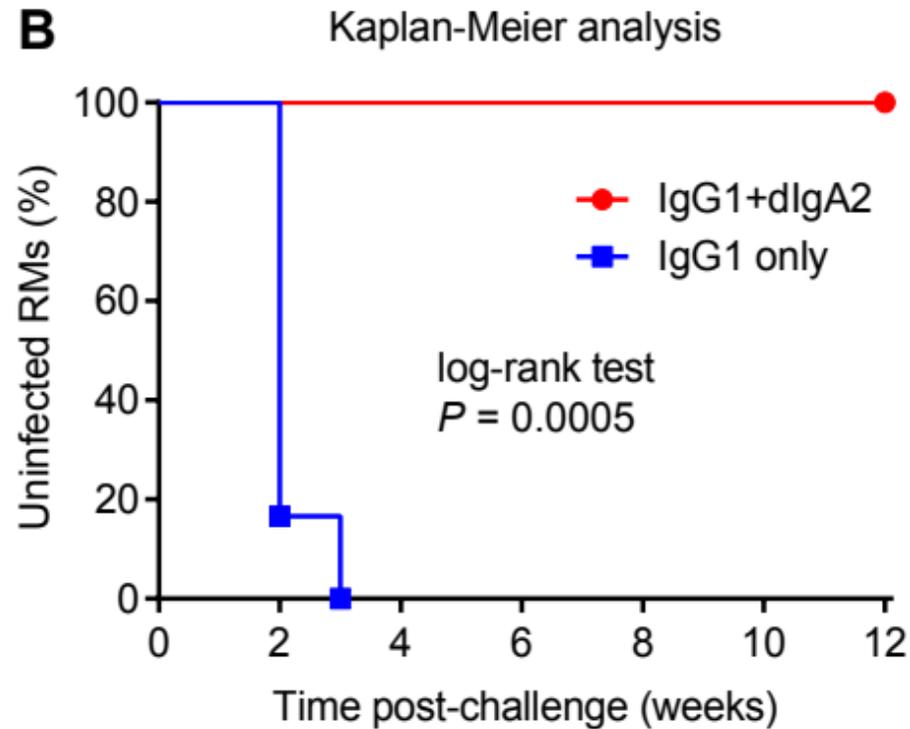
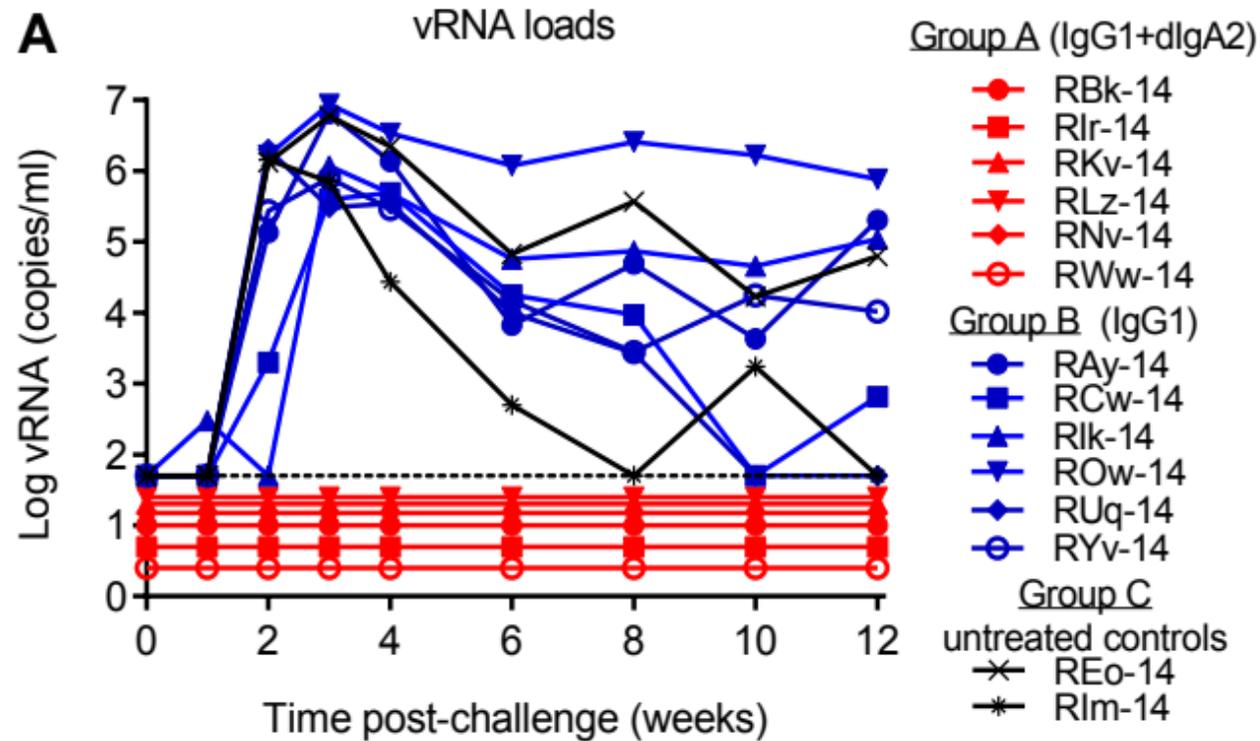


Figure 5

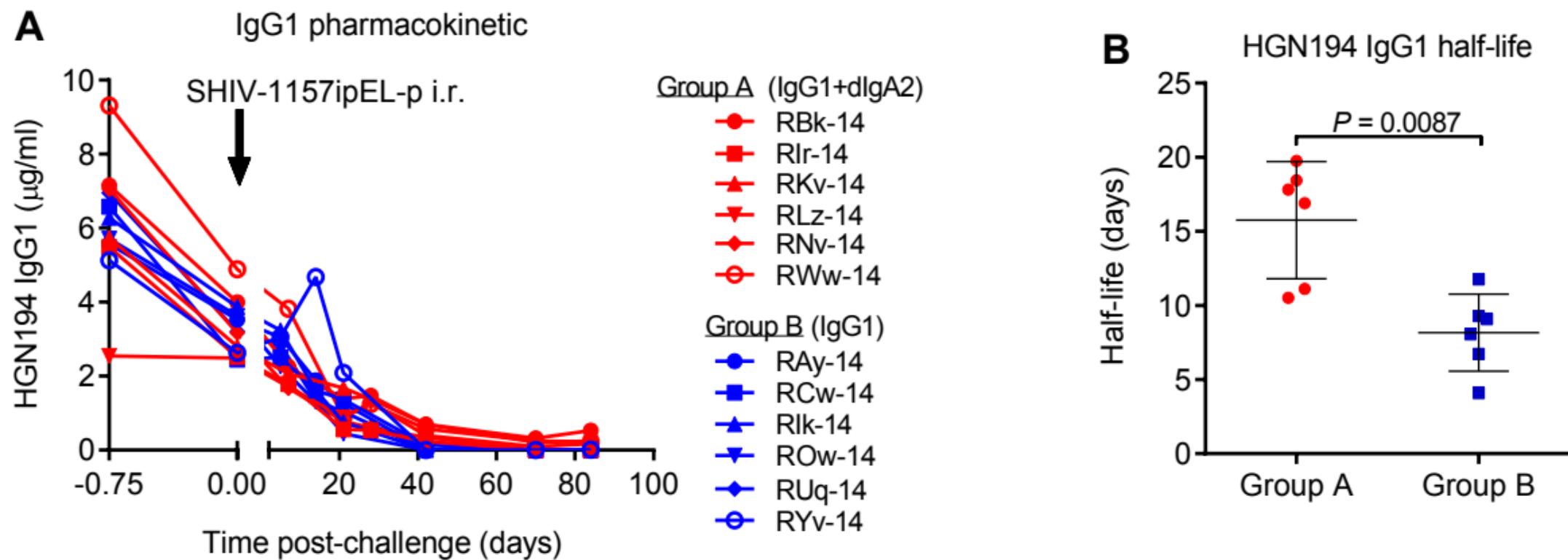


Figure 6

