

1 **Antigen-specific humoral immune responses by CRISPR/Cas9-edited B cells**

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19 **Short title:** CRISPR/Cas9-edited anti-HIV B cells

20

21 **One-sentence summary:** B cells edited by CRISPR/Cas9 to produce antibodies

22 participate in humoral immune reactions and secrete neutralizing serum titers of anti-HIV

23 bNAbs.

24 **Abstract**

25 A small number of HIV-1 infected individuals develop broadly neutralizing-antibodies to
26 the virus (bNAbs). These antibodies are protective against infection in animal models.
27 However, they only emerge 1 - 3 years after infection, and show a number of highly
28 unusual features including exceedingly high levels of somatic mutations. It is therefore
29 not surprising that elicitation of protective immunity to HIV-1 has not yet been possible.
30 Here we show that mature, primary mouse and human B cells can be edited *in vitro*
31 using CRISPR/Cas9 to express mature bNAbs from the endogenous *Igh* locus.
32 Moreover, edited B cells retain the ability to participate in humoral immune responses.
33 Immunization with cognate antigen in wild type mouse recipients of edited B cells elicits
34 bNAb titers that neutralize HIV-1 at levels associated with protection against infection.
35 This approach enables humoral immune responses that may be difficult to elicit by
36 traditional immunization.

37 **Main text**

38 Although a vaccine for HIV remains elusive, anti-HIV-1 bNAbs have been identified and
39 their protective activity has been demonstrated in animal models¹⁻⁴. These antibodies
40 are effective in suppressing viremia in humans and large-scale clinical trials to test their
41 efficacy in prevention are currently under way^{2, 5-12}. However, these antibodies typically
42 have one or more unusual characteristics including high levels of somatic hypermutation
43 (SHM), long or very short complementarity determining regions (CDRs) and self-
44 reactivity that interfere with their elicitation by traditional immunization.

45
46 Consistent with their atypical structural features, antibodies that broadly neutralize HIV-
47 1 have been elicited in camelids, cows and transgenic mice with unusual pre-existing
48 antibody repertoires¹³⁻¹⁸. However, even in transgenic mice that carry super-physiologic
49 frequencies of bNAb precursors, antibody maturation required multiple immunizations
50 with a number of different sequential immunogens. Moreover, bNAbs only developed for
51 one of the epitopes targeted^{15, 17, 18}. Consequently, elicitation of bNAbs in primates or
52 humans remains a significant challenge.

53
54 To bypass this issue, we developed a method to reprogram mature B cells to express
55 an anti-HIV-1 bNAb. Adoptive transfer of the engineered B cells and immunization with
56 a single cognate antigen led to germinal center formation and antibody production at
57 levels consistent with protection.

58

59 **Results**

60 **Expressing antibodies in primary mature, murine B cells.**

61 To edit mature B cells efficiently, they need to be activated and cultured *in vitro*. To
62 determine whether such cells can participate in humoral immune responses *in vivo* we
63 used *Igh^a* CD45.1 B cells carrying the *B1-8^{hi}* heavy chain that are specific for the hapten
64 4-hydroxy-3-nitro-phenylacetyl (NP)¹⁹. B1-8^{hi} B cells were activated *in vitro* with anti-
65 RP105 antibody for 1 - 2 days and subsequently transferred into congenically marked
66 (*Igh^b* CD45.2) C57BL/6J mice. Recipients immunized with NP conjugated to ovalbumin
67 (NP-OVA) developed GCs containing large numbers of the antigen-specific, transferred
68 B cells (Supplementary Fig. 1a,b) and produced high levels of antigen-specific IgG1
69 (Supplementary Fig. 1c). In addition, transfection by electroporation did not affect the
70 ability of transferred cells to enter GCs (Supplementary Fig. 1d,e).

71

72 Despite having two alleles for each of the antibody chains, B cells express only one
73 heavy and one light chain gene, a phenomenon referred to as allelic exclusion²⁰⁻²².
74 Introducing additional antibody genes would risk random combinations of heavy and
75 light chains some of which could be self-reactive or incompatible. Thus, deletion of the
76 endogenous chains would be desirable to prevent expression of chimeric B cell
77 receptors (BCRs) composed of the transgene and the endogenous antibody genes. To
78 do so, we combined endogenous Ig disruption with insertion of a transcription unit that
79 directs expression of the heavy and light chain into the endogenous heavy chain locus.

80

81 CRISPR-RNAs (crRNAs) were designed to ablate the κ -light chain because 95% of all
82 mouse B cells express *Igk* (Fig1.a). The efficiency of κ light chain deletion was
83 measured by flow cytometry using the ratio of κ/λ cells to normalize for cell death due to
84 BCR loss. The selected crRNAs consistently ablated $Ig\kappa$ expression by 70 - 80 % of B
85 cells as measured by flow cytometry or TIDE (Tracking the Indels by DEcomposition²³)
86 analysis (Fig.1 b-d).

87

88 To insert a transgene into the heavy chain locus we designed crRNAs specific for the
89 first *Igh* intron immediately 3' of the endogenous VDJ_H gene segment, and 5' of the $E\mu$
90 enhancer. This position was selected to favor transgene expression and allow
91 simultaneous disruption the endogenous heavy chain (see below and ²⁴). We tested 7
92 crRNAs and selected a high-efficiency crRNA located 110 bp downstream of the J_{H4}
93 intron producing 77 % indels by the TIDE assay (Fig1e, Supplementary Fig.2a,b). This
94 location also allowed for sufficient homology to introduce a transgene, irrespective of
95 the upstream VDJ rearrangement.

96

97 The homology-directed repair template is composed of a splice acceptor (SA) stop
98 cassette to terminate transcription of upstream rearranged VDJ_H , and a V_H -gene
99 promoter followed by cDNAs encoding *Igk*, a P2A self-cleaving sequence, and IgV_H with
100 a J_{H1} splice donor (SD) site (Fig.2a). This design disrupts expression of the
101 endogenous locus, while encoding a transcription unit directing expression of the
102 introduced heavy and light chain under control of endogenous *Igh* gene regulatory

103 elements. In addition, it preserves splicing of the transgenic IgV_H into the endogenous
104 constant regions allowing for expression of membrane and secreted forms of the
105 antibody as wells as different isotypes by class switch recombination. Finally, correctly
106 targeted cells are readily identified and enumerated by flow cytometry because they
107 bind to cognate antigen.

108

109 A number of methods for producing ssDNA homology directed repair templates
110 (HDRTs) were compared. The most reproducible and least cytotoxic involved digestion
111 of plasmids with sequence-specific nickases, and ssDNA purification by agarose gel
112 electrophoresis (Supplementary Fig. 3a-c,^{25, 26}).

113

114 Co-transfection of the ssDNA template with pre-assembled Cas9 ribonucleoproteins
115 (RNPs) containing the crRNAs resulted in expression of the encoded anti-HIV antibody
116 in 0.1 - 0.4 % of mouse B cells by antigen-specific flow cytometry (TM4 core^{27, 28} or
117 10mut²⁹) (Fig.2c,d). Transgene expression was stable over the entire culture period of 3
118 days on feeder cells³⁰, during which the overall number of B cells expanded by 6 to 20-
119 fold (Fig.2e-h). However, expression of transgenic antibodies differed depending on the
120 antibody and were generally reflective of their expression in knock-in mouse models
121 (Fig.2c, f)^{16, 18, 28, 29, 31}.

122

123 To determine whether edited cells are allelically excluded we transfected *Igh*^{a/b} B cells
124 with 3BNC60^{SI}, a chimeric antibody composed of the mature heavy chain and germline

125 light chain of the anti-HIV bNAb 3BNC60 (Supplementary Fig.4b, c). The majority of
126 edited cells expressing the 3BNC60^{SI} transgene, expressed it using either *Igm^a* or *Igm^b*
127 allele as determined by flow cytometry. Only 5.21 % of 3BNC60^{SI}-expressing B cells
128 showed co-expression of both IgM^a and IgM^b indicative of allelic inclusion of the
129 endogenous allele or successful integration of the transgene into both alleles. Thus, the
130 majority of edited B cells express only the transgene.

131

132 We conclude that mature mouse B cells can be edited *in vitro* to produce anti-HIV-1
133 bNAbs from the *Igh* locus.

134

135 **Antibody gene editing in human B cells**

136 To determine whether this method could be adapted to edit human B cells we isolated
137 them from peripheral blood of healthy volunteers and activated them using an anti-
138 human RP105 antibody³². Analogous crRNAs were selected for targeting the human
139 *IGKC* and the first intron 3' of *IGHJ6* (Fig.3a-d, Supplementary Fig.5a-b). The best
140 *IGKC*-targeting crRNA caused 85 % of κ -bearing B cells to lose BCR expression,
141 whereas λ -bearing cells increased proportionally indicating that they were unaffected.
142 TIDE analysis of the J_H6 intron sequences showed that the most efficient crRNA
143 induced 64% indels. In conclusion, activation of human, primary B cells with anti-RP105
144 allows efficient generation of indels using Cas9 RNPs.

145

146 To target bNAbs into the human J_H6 intron we adapted the ssDNA HDRT and replaced
147 mouse with human homology arms, the human C_μ splice acceptor, the human *IGHV1-*
148 *69* promoter, a codon-modified human *IGKC* constant region to avoid targeting by
149 crRNAs and the human *J_H4* splice donor (Fig 3a). In contrast to mouse cells, 2.9 – 4 %
150 of λ⁻ B cells expressed 3BNC60^{SI} or 10-1074 antibodies respectively as determined by
151 flow cytometry using the cognate antigen (Fig 3e,f). Thus, the efficiency of transgene
152 integration is at least 10-times higher in human B cells. Furthermore, viability was also
153 higher in human B cells, ranging from 60 to 85 % of live cells after transfection
154 (Supplementary Fig.5c).

155

156 We conclude that primary human B cells can be edited by CRISPR/Cas9 to express
157 anti-HIV bNAbs, and that this is significantly more efficient than in mouse B cells.

158

159 **Adoptive transfer of antibody-edited B cells**

160 To determine whether edited B cells can participate in immune responses, we
161 adoptively transferred mouse 3BNC60^{SI}-edited *Igh^b* B cells, into congenically-marked
162 *Igh^a* wild type mice and immunized with the high-affinity, cognate antigen TM4 core in
163 Ribi adjuvant (Fig.4a). Transgene-specific responses were detected using anti-idiotypic
164 antibodies as an initial capture reagent in ELISA. Similar to endogenous humoral
165 immune responses, transgenic antibodies were detected on day 7 after immunization,
166 they peaked at day 14 and started to decrease by day 21 (Fig.4b, c). Importantly, the
167 transgenic immune response included secondary isotypes indicating that the re-

168 engineered locus supports class switch recombination (Fig.4c). Finally, the magnitude
169 of the response was directly correlated to the number of transferred cells. However,
170 prolonged *in vitro* culture under the conditions tested decreased the efficiency of
171 antibody production *in vivo* (Fig.4d).

172

173 To determine whether the transferred cells retained the ability to produce neutralizing
174 antibodies we used B cells that were edited to produce 10-1074, a potent bNAbs, or
175 3BNC60^{SI} a chimeric antibody with limited neutralizing activity^{31, 33}. 4×10^7 transfected
176 B cells were transferred into wild type Igh^a mice that were subsequently immunized with
177 the appropriate cognate antigen 10mut²⁹ or TM4 core^{16, 27, 28, 31}. Serum IgG was purified
178 from 3 mice each that received an estimated $\sim 10^3$ edited B cells expressing 10-1074 or
179 3BNC60^{SI}. The serum antibodies were tested for neutralizing activity in the TZM-bl
180 assay³⁴. Two of the 3 mice that received 10-1074 edited cells showed IC₅₀s of 21.59
181 $\mu\text{g/mL}$ and a third reached 49 % neutralization at 118 $\mu\text{g/mL}$ (corresponding to
182 approximately 1:500 and 1:100 dilution of serum, Fig.4e, Supplementary Fig.6a, b). As
183 expected, neutralizing activity was not detected in mice receiving 3BNC60^{SI} because
184 this antibody is 2 - 3 orders of magnitude less potent against the tested viral strains than
185 10-1074 (Supplementary Fig.6e).

186

187 We conclude that edited B cells can be recruited into immune responses and produce
188 sufficient antibody to confer potentially protective levels of humoral immunity³⁵.

189

190 Discussion

191 T cells can be reprogrammed to express specific receptors using retrogenic methods³⁶⁻
192 ³⁸ or non-viral CRISPR/Cas9 genome targeting²⁶. In contrast, B cell receptor
193 reprogramming in primary cells using retroviruses has not been successful³⁹. Moreover,
194 although antibody heavy chains have been targeted into human B cells using
195 CRISPR/Cas9⁴⁰, little is known about how CRISPR/Cas9 genome targeting might be
196 used to introduce complete antibody genes into mature B cells that retain the ability to
197 participate in immune responses *in vivo*.

198
199 We have developed a method to produce transgenic antibodies in primary mouse and
200 human B cells using CRISPR/Cas9. The new method involves short term culture *in*
201 *vitro*, silencing of the endogenous *Ig* genes, and insertion of a bi-cistronic cDNA into the
202 *Igh* locus. Mouse B cells edited to express an anti-HIV-1 bNAbs by this method can
203 produce transgenic antibody levels that are protective in animal models^{35, 41-43}.

204
205 Mouse and human B lymphocytes typically express a single antibody despite having the
206 potential to express 2 different heavy chains and 4 different light chains. Theoretically
207 the combination could produce 8 different antibodies and a series of additional chimeras
208 that could interfere with the efficiency of humoral immunity and lead to unwanted
209 autoimmunity. Allelic exclusion prevents this from happening and would need to be
210 maintained by any gene replacement strategy used to edit B lymphocytes. In addition,

211 genetic editing is accompanied by safety concerns due to off-target double strand
212 breaks and integrations.

213

214 The approach reported maintains allelic exclusion in part by ablating the *Igkc* gene. In
215 the mouse, 95% of B cells express *Igkc*. In the absence of *Igkc* expression these cells
216 will die by apoptosis because they cannot survive unless they continue to express a B
217 cell receptor^{44, 45}. Since the introduction of the transgene into the heavy chain locus
218 disrupts endogenous *Igh* expression, editing maintains allelic exclusion because only
219 cells expressing the introduced antibody can survive. Our strategy also interferes with
220 the survival of cells that suffer off-target integration events, because such cells would be
221 unable to express the B cell receptor and they too would die by apoptosis.

222

223 A potential issue is that there are two heavy chain alleles in every B cell and allelic
224 exclusion would be disrupted if the transgene were only integrated in the non-productive
225 *Igh* allele allowing for expression of the original productive *Igh*. However, our flow
226 cytometry data indicates that this is a very rare event. Thus, either both alleles are
227 targeted or the occasional remaining endogenous *Igh* gene is unable to pair with the
228 transgenic *Igk*.

229

230 In contrast to the mouse, *IGL* is expressed by 45 % of all B cells in humans. Therefore,
231 this locus would either need to be ablated, or alternatively, cells expressing *IGL* could

232 be removed from the transferred population by any one of a number of methods of
233 negative selection.

234

235 Similar to antibody transgenes in mice, expression of the edited BCR varied between
236 different antibodies. Some combinations of heavy and light chains were refractory to
237 expression in mature B cells. In addition, although the level of B cell receptor expression
238 was within the normal range, it was generally in the low end compared to polyclonal B
239 cells. This is consistent with generally lower level expression of a similar transgene in
240 knock-in mice²⁴. Low BCR expression could also be due to the bi-cistronic design since
241 expression was higher in knock-in mice that expressed the identical Ig from the native
242 *Igk* and *Igh* loci³¹. Nevertheless, expression levels were adequate to drive antigen-
243 induced antibody production *in vivo*.

244

245 bNAb mediated protection against infection with simian-human immunodeficiency
246 viruses in macaques requires IC₅₀ neutralizing titers of 1:100^{35, 41-43}. Thus the titers
247 achieved by CRISPR/Cas9 edited B cells in mice would be protective if they could be
248 translated to macaques and by inference humans. Moreover, our neutralization
249 measurements may be an underestimate since we excluded bNAbs produced as IgM or
250 isotypes other than IgG.

251

252 CAR T cell therapy typically involves transfer of millions of edited cells to achieve a
253 therapeutic effect. Whether similar numbers of edited B cells would also be required to

254 achieve protective levels of humoral immunity can only be determined by further
255 experimentation in primate models. In addition, the longevity of the antibody response
256 produced by edited B cells, and its optimization by boosting or adjuvant choice will
257 require further experimentation.

258

259 Most protective vaccine responses depend on humoral immunity. Neutralizing antibody
260 responses are readily elicited for most human pathogens, but in some cases, including
261 HIV-1, it has not yet been possible to do so. The alternatives include passive antibody
262 infusion, which has been an effective means of protection since it was discovered at the
263 turn of the last century. We have shown that passive transfer of mouse B cells edited by
264 CRISPR/Cas9 can also produce protective antibody levels *in vivo*. This proof of concept
265 study demonstrates that humoral immune responses can be engineered by
266 CRISPR/Cas9. The approach is not limited to HIV-1 and can be applied to any disease
267 requiring a specific antibody response.

268 **Methods**

269 **crRNA design**

270 crRNAs were designed with the MIT guide design tool (crispr.mit.edu), CHOPCHOP
271 (chopchop.cbu.uib.no) and the IDT crRNA design tool (www.idtdna.com). Designs were
272 synthesized by IDT as Alt-R CRISPR-Cas9 crRNAs. crRNA sequences are listed in
273 Supplementary Table 1.

274

275 **ssDNA HDRT preparation**

276 HDRT sequences, listed in Supplementary Table 2, were synthesized as gBlocks (IDT)
277 and cloned using *NheI* and *XhoI* (NEB) into vector pLSODN-4D from the long ssDNA
278 preparation kit (BioDynamics Laboratories, Cat.# DS620). ssDNA was prepared
279 following the manufacturer's instructions with the following modifications: In brief, 2.4 mg
280 sequence verified vector was digested at 2 $\mu\text{g}/\mu\text{L}$ in NEB 3.1 buffer with 1200 U
281 *Nt.BspQI* for 1 h at 50 °C followed by addition of 2400 U *XhoI* (NEB) and incubation for
282 1 h at 37 °C. Digests were desalted by ethanol precipitation and resuspended in water
283 at < 1 $\mu\text{g}/\mu\text{L}$. An equal volume of formamide gel-loading buffer (95 % de-ionized
284 formamide, 0.025 % bromophenol blue, 0.025 % xylene cyanol, 0.025 % SDS, 18 mM
285 EDTA) was added and heated to 70 °C for 5 min to denature dsDNA. Denatured
286 samples were immediately loaded into dye-free 1 % agarose gels in TAE and run at 100
287 V for 3 h. Correctly sized bands were identified by partial post-stain with GelRed
288 (Biotium), then excised and column purified (Machery Nagel Cat.# 740610.20 or

289 740609.250) according to the manufacturer's instructions. Eluate was ethanol
290 precipitated, resuspended in water, adjusted to 2.5 $\mu\text{g}/\mu\text{L}$ and stored at $-20\text{ }^{\circ}\text{C}$.

291

292 **Murine cell culture**

293 Mature, resting B cells were obtained from mouse spleens by forcing tissue through a 70
294 μm mesh into PBS containing 2 % heat-inactivated fetal bovine serum (FBS). After ACK
295 lysis for 3 min, untouched B cells were enriched using anti-CD43 magnetic beads (MACS)
296 according to manufacturer's protocol (Miltenyi Biotec) obtaining > 95 % purity. 3.2×10^7
297 cells/10 cm dish (Gibco) were cultured at $37\text{ }^{\circ}\text{C}$ 5 % CO_2 in 10 mL mouse B cell medium
298 consisting of RPMI-1640, supplemented with 10 % heat-inactivated FBS, 10 mM HEPES,
299 antibiotic-antimycotic (1X), 1 mM sodium pyruvate, 2 mM L-glutamine and 53 μM 2-
300 mercaptoethanol (all from Gibco) and activated with 2 $\mu\text{g}/\text{mL}$ anti-mouse RP105 clone
301 RP/14 (produced in house or BD Pharmingen Cat.# 562191).

302

303 NB-21 feeder cells³⁰ were maintained in DMEM supplemented with 10 % heat-inactivated
304 FBS and antibiotic-antimycotic (1X). For co-culture, feeder cells were irradiated with 80
305 Gy and seeded simultaneously with B cells, 24 h after transfection, into B cell culture
306 medium supplemented with 1 ng/mL recombinant mouse IL-4 (PeproTech Ca.# 214-14)
307 and 2 $\mu\text{g}/\text{mL}$ anti-mouse RP105 clone RP/14.

308 **Human cell culture**

309 Frozen human leukapheresis samples were collected after signed informed consent in
310 accordance with Institutional Review Board (IRB) approved protocol TSC-0910. Cells
311 were thawed in a 37°C water bath and resuspended in human B cell medium composed
312 of RPMI-1640, supplemented with 10 % heat-inactivated FBS or human serum, 10 mM
313 HEPES, antibiotic-antimycotic (1X), 1 mM sodium pyruvate, 2 mM L-glutamine and 53
314 µM 2-mercaptoethanol (all from Gibco). B cells were isolated using EasySep human
315 naïve B cell Enrichment Kit (Stemcell Cat.# 19254) according to the manufacturer's
316 instructions and cultured in the above medium supplemented with 2 µg/mL anti-human
317 RP105 antibody clone MHR73-11 (BioLegend Cat.# 312907).

318

319 **RNP preparation and transfection**

320 Per 100 µL transfection, 1 µL of 200 µM crRNA and 1 µL 200 µM tracrRNA in duplex
321 buffer (all IDT) were mixed, denatured at 95 °C for 5 min, re-natured for 5 min at room
322 temperature. 5.6 µL PBS and 2.4 µL 61 µM Cas9 V3 (IDT, Cat.# 1081059) were added
323 and incubated for 15 - 30 min. If required RNPs were mixed at the following ratios: 50 %
324 crlgH, 25 % crlgK₁ and 25 % crlgK₂ (mouse) or 50% crlHgH₃ and 50 % crlHgK₃ (human).
325 4 µL 100 µM electroporation enhancer in duplex buffer or 4 µL HDRT at 2.5 µg/µL were
326 added to 10 µL mixed RNP and incubated for a further 1-2 min.

327

328 24 h after stimulation, activated mouse or human B cells were harvested, washed once
329 in PBS and resuspended in Mouse B cell Nucleofector Solution with Supplement

330 (murine B cells) or Primary Cell Nucleofector Solution 3 with Supplement (human B
331 cells) prepared to the manufacturer's instructions (Lonza) at a concentration of 4 - 5 ×
332 10⁶ cells / 86 μL. 86 μL cells were added to the RNP/HPRT mix, gently mixed by
333 pipetting and transferred into nucleofection cuvettes and electroporated using an Amaxa
334 Ilb machine setting Z-001 (murine B cells) or Amaxa 4D machine setting EH-140
335 (human B cells). Cells were immediately transferred into 6-well dishes containing 5 mL
336 prewarmed mouse or human B cell medium supplemented with the relevant anti-RP105
337 antibody at 2 μg/mL and incubated at 37°C 5 % CO₂ for 24 h before further processing.

338

339 **TIDE assay**

340 Genomic DNA was extracted from 0.5 - 5 × 10⁵ cells by standard phenol/chloroform
341 extraction 24 - 42 h after transfection. PCRs to amplify human or mouse Ig loci targeted
342 by CRISPR-Cas9 were performed using Phusion Green Hot Start II High-Fidelity
343 polymerase (Thermo Fisher Cat.# F-537L) and primers listed in Supplementary Table 3.
344 Thermocycler was set to 40 cycles, annealing at 65°C for 30 s and extending at 72°C
345 for 30s. PCR product size was verified by gel electrophoresis, bands gel-extracted and
346 sent for Sanger sequencing (Genewiz) using the relevant PCR primers. ab1 files were
347 analyzed using the TIDE web tool (tide.nki.nl) using samples receiving scramble or
348 irrelevant HPRT-targeting crRNA as reference²³.

349 **Flow cytometry**

350 Mouse spleens were forced through a 70 μ m mesh into FACS buffer (PBS containing 2
351 % heat-inactivated FBS and 2 mM EDTA) and red blood cells were lysed in ACK lysing
352 buffer (Gibco) for 3 min. Cultured cells were harvested by centrifugation. Then cells
353 were washed and Fc-receptors blocked for 15 min on ice. Cells were stained for 20 min
354 on ice with antibodies or reagents listed in Supplementary table 4 and depending on the
355 stain, washed again and secondary stained for another 20 min on ice before acquisition
356 on a BD LSRFortessa. GC B cells were gated as single/live, B220⁺, CD38⁻ FAS⁺, GL7⁺,
357 IgD⁻. Allotypic markers CD45.1 and CD45.2 were used to track adoptively transferred B
358 cells.

359

360 **Mice**

361 C57BL/6J and B6.Igh^a (B6.Cg-Gpi1^a Thy1^a Igh^a/J) and B6.SJL were obtained from the
362 Jackson Laboratory. Igh^{a/b} mice were obtained by intercrossing B6.Igh^a and B6.SJL mice.
363 B1-8^{hi} ¹⁹, 3BNC60^{SI} ³¹ and PGT121^{18, 29} strains were generated and maintained in our
364 laboratory. Experiments used age and sex-matched animals. All experiments were
365 performed with authorization from the Institutional Review Board and the Rockefeller
366 University IACUC.

367

368 **Cell transfers and immunizations**

369 After culture, mouse B cells were harvested at the indicated time points and
370 resuspended in mouse B cell medium without anti-RP105 antibody and rested for 2 - 3

371 h at 37°C, 5 % CO₂. Then cells were washed once in PBS and resuspended in 200 µL
372 PBS/mouse containing the indicated number of initially transfected cells. 200 µL cell
373 suspension/mouse were injected intravenously via the retroorbital sinus. Number of
374 transferred, edited B cells was estimated as follows: Number of cells transfected × 20 %
375 survival × 0.15 - 0.4 % transfection efficiency × 50 % handling/proliferation × 5 %
376 transfer efficiency³¹. Mice were immunized intraperitoneally within 24 h after cell transfer
377 with 200 µL containing 10 µg TM4 core²⁷ or 10mut²⁹ in PBS with 50 % Ribi (Sigma
378 Adjuvant system, Sigma Aldrich) prepared to the manufacturer's instructions. Mice were
379 bled at the indicated time points from the submandibular vein. Blood was allowed to clot
380 and then serum was separated by centrifugation for 10 min at 20817 g. Serum was
381 stored at -20°C.

382

383 **Anti-idiotypic antibody**

384 IgG producing hybridomas were isolated from mice immunized with iGL-VRC01 at the
385 Frederick Hutchinson Cancer Research Center Antibody Technology Resource.
386 Hybridoma supernatants were screened against a matrix of inferred germline (iGL)
387 VRC01 class antibodies as well as irrelevant iGL-antibodies using a high throughput
388 bead-based assay. One anti-idiotypic antibody, clone iv8, bound to additional VRC01
389 class antibodies, but it also bound to a chimeric antibody with an iGL-VRC01 class light
390 chain paired with the 8ANC131 heavy chain (which is derived from VH1-46), and to
391 3BNC60^{SI}.

392

393 **ELISAs**

394 For determination of 3BNC60^{SI} levels, Corning 3690 half-well 96-well plates were
395 coated overnight at 4°C with 25 µL/well of 2 µg/mL human anti-3BNC60^{SI} (clone iv8)
396 IgG in PBS, then blocked with 150 µL/well PBS 5% skimmed milk for 2 h at room
397 temperature (RT). Sera were diluted to 1:50 with PBS and 7 subsequent 3-fold dilutions.
398 Recombinant 3BNC60^{SI} (produced in house as mouse IgG1, κ) was diluted to 10 µg/mL
399 in PBS followed by six 5-fold dilutions. Blocked plates were washed 4-times with PBS
400 0.05 % Tween 20 and incubated with 25 µL diluted sera or antibody for 2 h at RT.
401 Binding was revealed by either anti-mouse IgG-horseradish peroxidase (HRP) (Jackson
402 ImmunoResearch, Cat.# 115-035-071) or anti-mouse IgG1a-biotin (BD Pharmingen
403 Cat.# 553500) or anti-mouse IgG1b-biotin (BD Pharmingen Cat.# 553533), all diluted
404 1:5000 in PBS, 25 µL/well and incubation for 1 h at RT. Biotinylated antibodies were
405 subsequently incubated with Streptavidin-HRP (BD Pharmingen Cat.# 554066), diluted
406 1:1000 in PBS, 25 µL/well for 30 min at RT. Plates were washed 4-times with PBS 0.05
407 % Tween 20 in between steps and 6 times before addition of substrate using a Tecan
408 Hydrospeed microplate washer. HRP activity was determined using TMB as substrate
409 (Thermo Scientific Cat.# 34021), adding 50 µL/well. Reactions were stopped with 50
410 µL/well 2 M H₂SO₄ and read at 450 and 570 nm on a FLUOstar Omega microplate
411 reader (BMG Labtech). Data were analyzed with Microsoft Excel and GraphPad Prism
412 6.0. Absolute 3BNC60^{SI} titers were interpolated from sigmoidal fits of recombinant
413 3BNC60^{SI} standard curves.

414

415 For determination of NP-binding antibodies the following modifications applied. Plates
416 were coated with 10 μ g/mL NP₃₁-bovine serum albumin (BSA, Biosearch Technologies)
417 and blocked with PBS 3 % BSA. Sera, antibodies and secondary reagents were diluted
418 in PBS 1% BSA 0.05 % Tween20.

419

420 **Neutralization assays**

421 Collected mouse serum was pooled and IgG purified using protein G Ab SpinTraps (GE
422 Healthcare Cat.# 28-4083-47) then concentrated and buffer-exchanged into PBS using
423 Amicon Ultra 30K centrifugal filter units (Merck Millipore Cat.# UFC503024) according to
424 the manufacturers' instructions.

425 TZM-bl assays were performed as previously described³⁴. Neutralizing activity was
426 calculated as a function of the reduction in Tat-inducible luciferase expression in the
427 TZM-bl reporter cell line in a single round of virus infection.

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439

440 **Author contributions**

441 H.H. and M.J. conceived, planned and performed experiments, analyzed data, and wrote
442 the manuscript. M.H. performed experiments and prepared ssDNA HDRT. P.D. assisted
443 with experimental design and ELISAs. D.Y. and A.G. expressed antibodies. A.T.G., J.J.T.
444 and L.S. designed and provided iv8 antibody. M.S.S. performed neutralization assays.
445 M.C.N. planned and supervised experiments, analyzed data, and wrote the manuscript.

446

447 **Competing Interests statement**

448 There are patents on 3BNC117 and 10-1074 on which M.C.N. is an inventor. M.C.N. is
449 a member of the Scientific Advisory Boards of Celldex and Frontier Biotechnologies.

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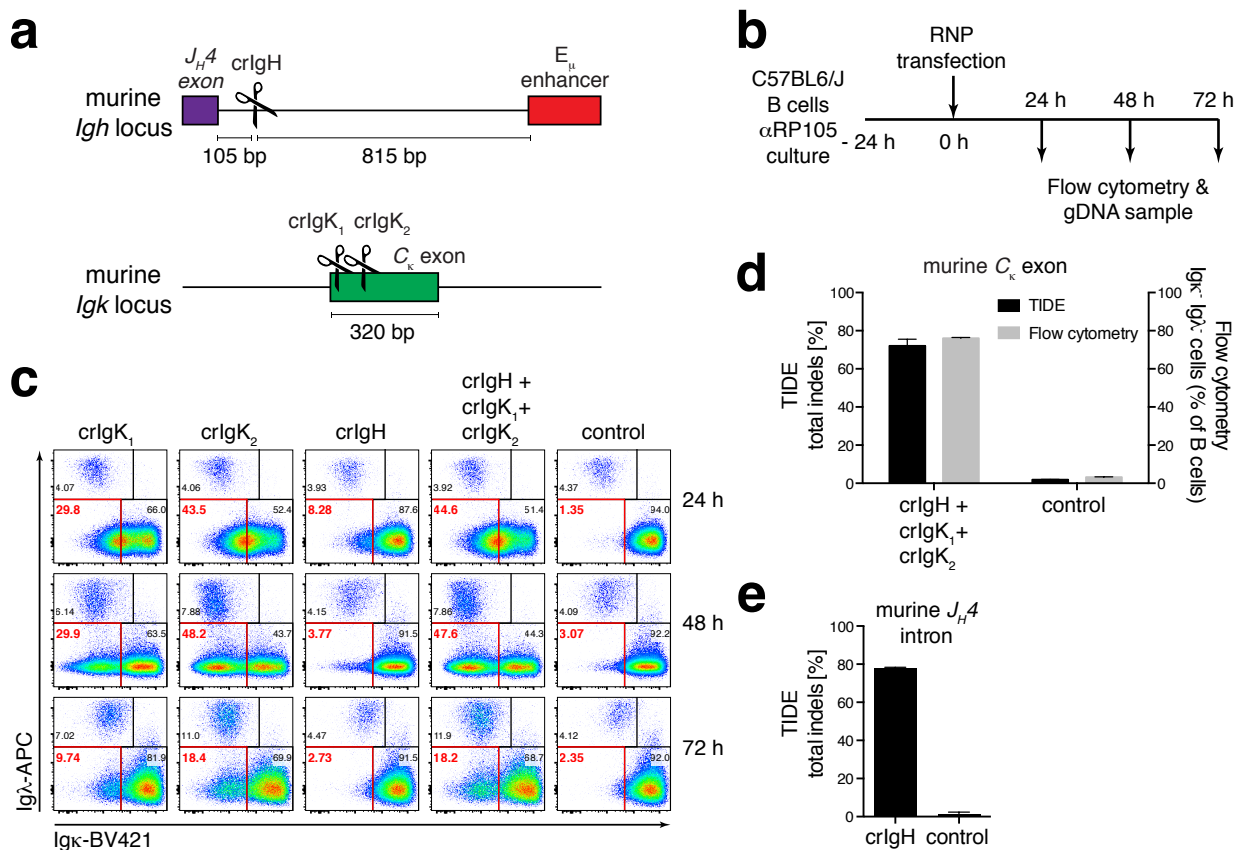
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- 556

557 **Figures**

Fig. 1



558

559 **Figure 1: Efficient generation of indels in primary mouse B cells by CRISPR/Cas9.**

560 **(a)** Targeting scheme for *Igh* (crlgH) and *Igk* crRNA guides (crlgK₁, crlgK₂). **(b)**

561 Experimental set up for (c-e). Primary mouse B cells were cultured for 24 h in the

562 presence of anti-RP105 antibody and then transfected with Cas9 ribonucleoproteins

563 (RNPs) and analyzed at the indicated time points. **(c)** Flow cytometric plots of cultured B

564 cells at the indicated time points after transfection. Control uses an irrelevant crRNA

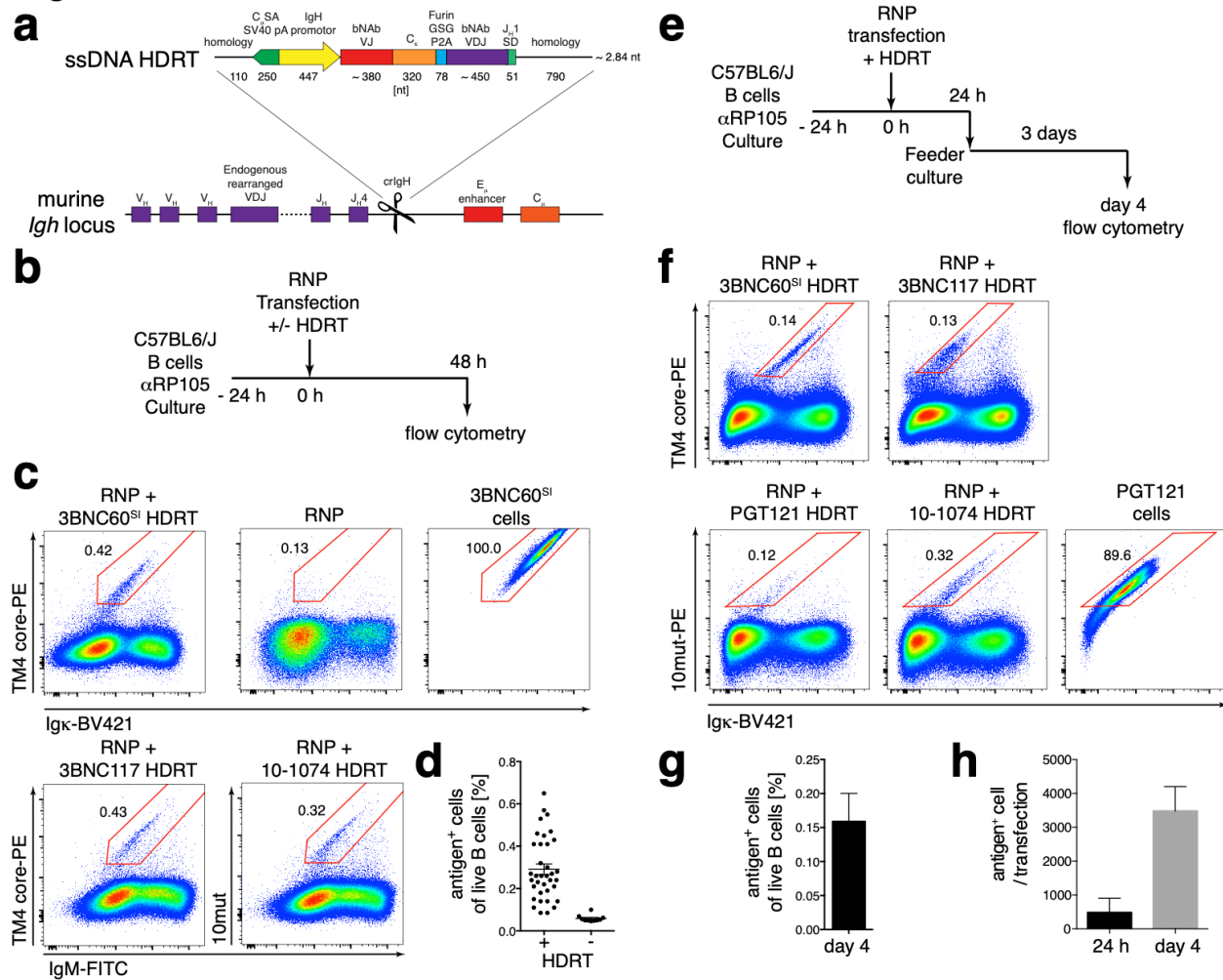
565 targeting the HPRT gene. **(d)** Quantification of (c), percentage of Igk⁻ Igλ⁻ B cells by flow

566 cytometry (right y-axis) and percentage of cells containing indels in the *Igkc* exon by

567 TIDE analysis (left y-axis). Control bars include irrelevant HPRT-targeting crRNAs or a

568 scramble crRNA without known targets in the mouse genome. **(e)** Percentage of cells
569 containing indels in the J_H4 intron by TIDE analysis after targeting with crIgH or control.
570 Bars indicate mean \pm SEM in two (TIDE) or four (flow cytometry) independent
571 experiments.

Fig. 2



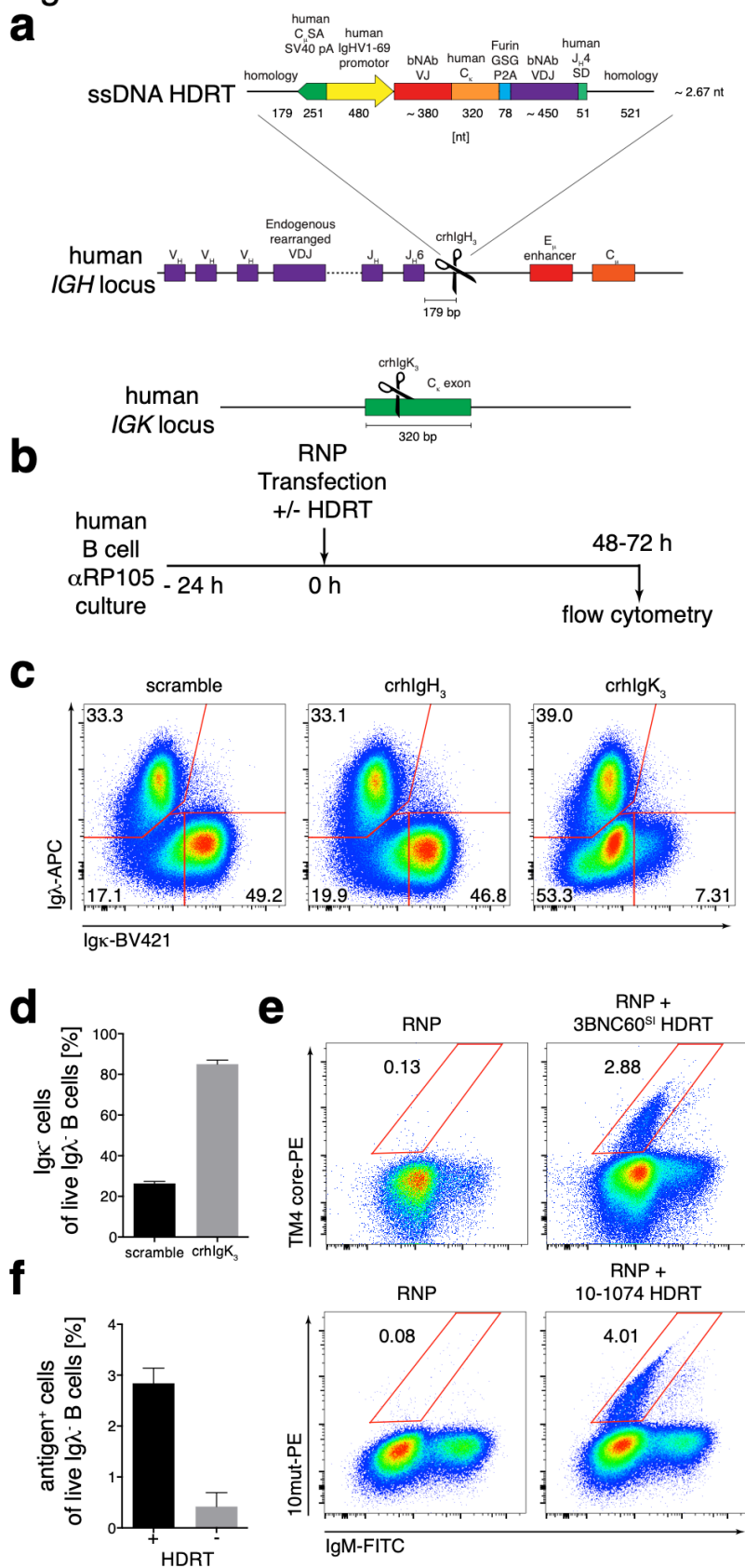
572

573 **Figure 2: Engineering bNAb-expressing, primary, mouse B cells.**

574 **(a)** Schematic representation of the targeting strategy to create bNAb-expressing,
 575 primary mouse B cells. ssDNA homology-directed repair template (HDRT) contained
 576 110 nt 5' and 790 nt 3' homology arms flanking an expression cassette. The 5'
 577 homology arm is followed by the 111 nt long splice acceptor site and the first 2 codons
 578 of C_μ exon 1, a stop codon and a SV40 polyadenylation signal (C_μSA SV40 pA). Then
 579 the mouse *Ighv4-9* gene promoter, the leader, variable and joining regions (VJ) of the
 580 respective antibody light chain and mouse κ constant region (C_κ) are followed by a furin-

581 cleavage site, a GSG-linker and a P2A self-cleaving oligopeptide sequence (P2A), the
582 leader, variable, diversity and joining regions (VDJ) of the respective antibody heavy
583 chain and 45 nt of the mouse J_H1 intron splice donor site to splice into downstream
584 constant regions. **(b)** Experimental setup for (c). **(c)** Flow cytometric plots of primary,
585 mouse B cells, activated and transfected with RNPs targeting the *Igh* J_H4 intron and
586 *Igkc* exon with or without ssDNA HDRTs encoding the 3BNC60^{SI}, 3BNC117 or 10-1074
587 antibody. Non-transfected, antigen-binding B cells from 3BNC60^{SI} knock-in mice
588 cultured the same way are used as control for gating. **(d)** Quantification of (c). Each dot
589 represents one transfection. Data from 7 independent experiments. **(e)** Experimental set
590 up for (f-h) **(f)** Flow cytometric plots of primary, mouse B cells, activated and transfected
591 using ssDNA HDRT encoding the antibodies 3BNC60^{SI}, 3BNC117, PGT121 or 10-1074.
592 B cells were expanded on feeder cells for 3 days. Cultured, non-transfected, antigen-
593 binding B cells from PGT121 knock-in mice are shown for gating. **(g)** Quantification of
594 (f). **(h)** Total number of antigen binding B cells before (24 h) or after 3 days (day 4) of
595 feeder culture. Bars indicate mean \pm SEM of combined data from 2 independent
596 experiments.
597

Fig. 3

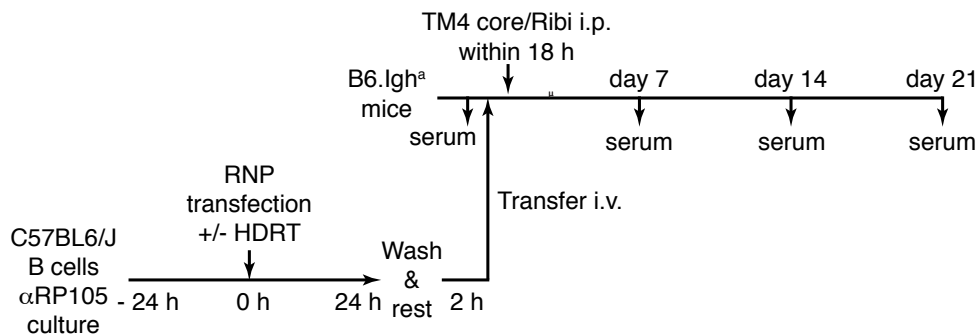


599 **Figure 3: Engineering bNAb-expressing, primary, human B cells.**

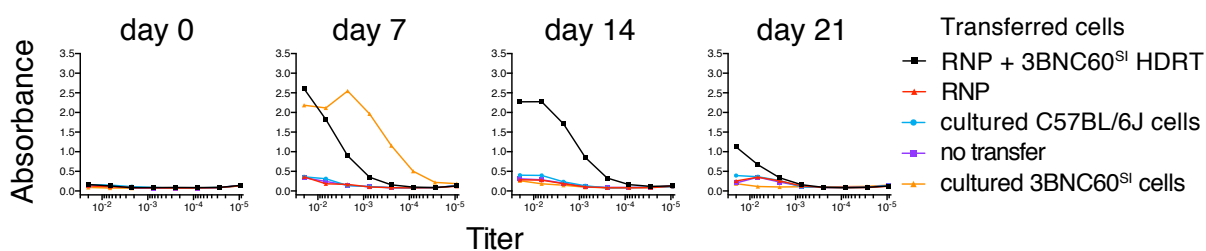
600 **(a)** Schematic representation of the targeting strategy to create bNAb-expressing,
601 primary human B cells. The ssDNA HDRT is flanked by 179 nt and a 521 nt homology
602 arms. The central expression cassette contains 112 nt of the human splice acceptor site
603 and the first 2 codons of C μ exon 1, a stop codon and a SV40 polyadenylation signal
604 (C μ SA SV40 pA). Then the human *IGHV1-69* gene promoter, the leader, variable and
605 joining regions (VJ) of the respective antibody light chain and human κ constant region
606 (C κ) are followed by a furin-cleavage site, a GSG-linker and a P2A self-cleaving
607 oligopeptide sequence (P2A), the leader, variable, diversity and joining regions (VDJ) of
608 the respective antibody heavy chain and 50 nt of the human J_{H4} intron splice donor site
609 to splice into downstream constant regions. **(b)** Experimental set up for c,d. Primary
610 human B cells were cultured for 24 h in the presence of anti-RP105 antibody and then
611 transfected with RNPs \pm HDRT. **(c)** Flow cytometric plots of primary human B cells 48 h
612 after transfection with RNPs containing crRNAs without target (scramble) or targeting
613 the *IGHJ6* intron or the *IGKC* exon. **(d)** Quantification of (c). Combined data from 3
614 independent experiments is shown. **(e)** Flow cytometric plots of antigen binding by Ig λ ⁻
615 primary human B cells 72 h after transfection of RNPs targeting both the *IGHJ6* intron
616 and the *IGKC* exon with or without HDRTs encoding 3BNC60^{SI} or 10-1074. **(f)**
617 Quantification of (e). Bars indicate mean \pm SEM of combined data from 2 independent
618 experiments with 2 - 4 replicates each.

Fig. 4

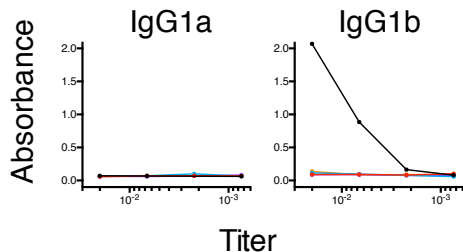
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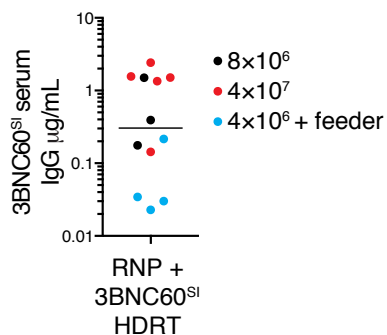
b



c



d



e

Sample	Virus	Titer in TZM.bl cells (μ g/mL)			
		T250.4		Q23.17	
		IC ₅₀	IC ₈₀	IC ₅₀	IC ₈₀
RNP + 10-1074 HDRT mouse 1		24.04	69.49	82.24	>112
RNP + 10-1074 HDRT mouse 2		21.59	66.51	68.04	>150
RNP + 10-1074 HDRT mouse 3		>118	>118	>118	>118
recombinant 10-1074 (μ g/mL)		<0.005	0.009	<0.005	0.016

619

620 **Figure 4: Engineered bNAbs-expressing, primary, mouse B cells participate in**

621 **humoral immune responses *in vivo*.**

622 **(a)** Experimental set up for (b-e). i.v. intravenous; i.p. intraperitoneal. **(b)** Anti-3BNC60^{SI}

623 idiotypic-coated, mouse IgG ELISA of sera from mice adoptively transferred with the

624 indicated B cells and immunized with the cognate antigen TM4 core at the indicated

625 time points. Representative plots of 7 independent experiments. **(c)** Anti-3BNC60^{SI}
626 idiotype-coated, mouse IgG1^a or IgG1^b ELISA of day 14 sera, as above. Representative
627 plots of 2 independent experiments. **(d)** 3BNC60^{SI} serum IgG levels 14 days after
628 immunization in mice transferred with 3BNC60^{SI}-edited cells, numbers of total B
629 cells/mouse at transfection are indicated. Cells were transferred either 24 h after
630 transfection or after additional culture on feeder cells as in Fig.2d. Determined by anti-
631 3BNC60^{SI} idiotype-coated, mouse IgG ELISA over 7 independent experiments. Each
632 dot represents one mouse. **(e)** TZM.bl neutralization data of protein G-purified serum
633 immunoglobulin days 14 – 21 after immunization from mice treated as in (a) but
634 transfected with 10-1074 HDRT and immunized with cognate antigen 10mut. Combined
635 data from 2 independent experiments are shown.