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TECHNICAL ADVANCES AND RESOURCES

HIV-specific humoral immune responses by CRISPR/Cas9-edited B cells

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

Introduction

Although a vaccine for HIV remains elusive, anti–HIV-1 broadly neutralizing antibodies (bNAbs) have been identified, and their protective activity has been demonstrated in animal models (Escolano et al., 2017; Nishimura and Martin, 2017; Kwong and Mascola, 2018; Sok and Burton, 2018). These antibodies are effective in suppressing viremia in humans, and large-scale clinical trials to test their efficacy in prevention are currently underway (Caskey et al., 2015, 2017; Ledgerwood et al., 2015; Lynch et al., 2015; Bar et al., 2016; Scheid et al., 2016; Schoofs et al., 2016; Nishimura and Martin, 2017; Mendoza et al., 2018). However, these antibodies typically have one or more unusual characteristics, including high levels of somatic hypermutation, long or very short complementarity-determining regions, and self-reactivity, that interfere with their elicitation by traditional immunization.

Consistent with their atypical structural features, antibodies that broadly neutralize HIV-1 have been elicited in camelids, cows, and transgenic mice with unusual preexisting antibody repertoires (McCoy et al., 2012; Dosenovic et al., 2015; Briney et al., 2016; Escolano et al., 2016; Tian et al., 2016; Sok et al., 2017). However, even in transgenic mice that carry super-physiological frequencies of bNAb precursors, antibody maturation required multiple immunizations with a number of different sequential immunogens. Moreover, bNAbs only developed for one of the epitopes targeted (Briney et al., 2016; Escolano et al., 2016; Tian et al., 2016). Consequently, elicitation of bNAbs in primates or humans remains a significant challenge.

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

Results

Expressing antibodies in primary mature, murine B cells

To edit mature B cells efficiently, they need to be activated and cultured in vitro. To determine whether such cells can participate in humoral immune responses in vivo, we used Igh^a CD45.1 B cells carrying the $B1\text{-}8^{hi}$ heavy chain that are specific for the hapten 4-hydroxy-3-nitro-phenylacetyl (NP; Shih et al., 2002). B1-8^{hi} B cells were activated in vitro with anti-RP105 antibody for 1–2 d and subsequently transferred into congenically marked (Igh^b CD45.2) C57BL/6J mice. Recipients immunized with NP conjugated to OVA developed GCs containing large numbers of the antigen-specific, transferred B cells (Fig. S1, A and B) and produced high levels of antigen-specific IgG1 (Fig. S1 C). In addition, transfection by electroporation did not affect the ability of transferred cells to enter GCs (Fig. S1, D and E).

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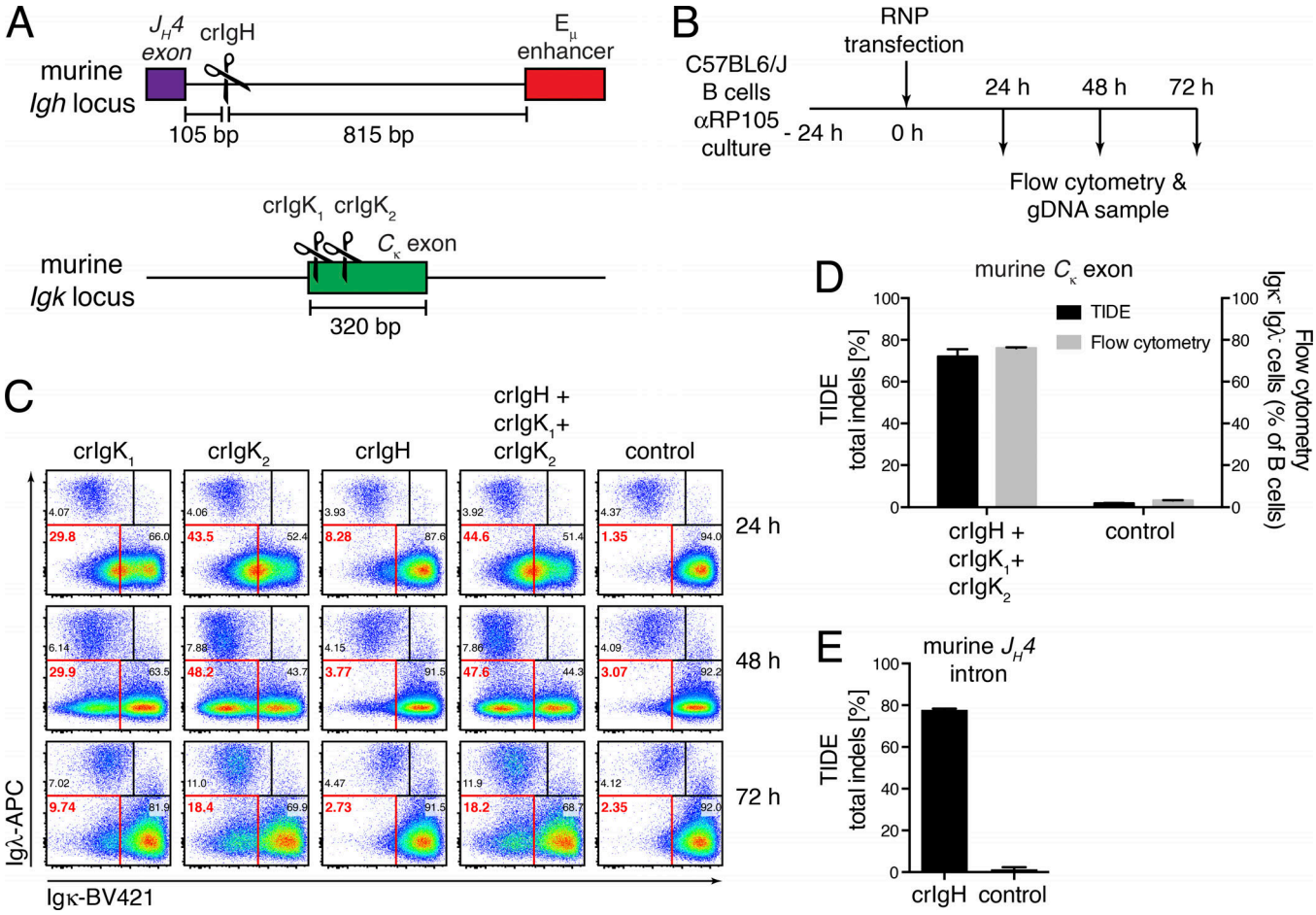

Figure 1. **Efficient generation of indels in primary mouse B cells by CRISPR/Cas9. (A)** Targeting scheme for *Igh* (crIgH) and *Igk* crRNA guides (crIgK$_1$, crIgK$_2$). **(B)** Experimental setup for C–E. Primary mouse B cells were cultured for 24 h in the presence of anti-RP105 antibody and then transfected with Cas9 RNPs and analyzed at the indicated time points. gDNA, genomic DNA. **(C)** Flow-cytometric plots of cultured B cells at the indicated time points after transfection. Control uses an irrelevant crRNA targeting the HPRT gene. **(D)** Quantification of C, percentage of Igκ$^-$ Igλ$^-$ B cells by flow cytometry (right y axis), and percentage of cells containing indels in the *Igkc* exon by TIDE analysis (left y axis). Control bars include irrelevant HPRT-targeting crRNAs or a scramble crRNA without known targets in the mouse genome. **(E)** Percentage of cells containing indels in the J_H4 intron by TIDE analysis after targeting with crIgH or control. Bars indicate mean ± SEM in two (TIDE) or four (flow cytometry) independent experiments.

Despite having two alleles for each of the antibody chains, B cells express only one heavy and one light chain gene, a phenomenon referred to as allelic exclusion (Pernis et al., 1965; Cebra et al., 1966; Nussenzweig et al., 1987). Introducing additional antibody genes would risk random combinations of heavy and light chains, some of which could be self-reactive or incompatible. Thus, deletion of the endogenous chains would be desirable to prevent expression of chimeric B cell receptors (BCRs) composed of the transgene and the endogenous antibody genes. To do so, we combined endogenous Ig disruption with insertion of a transcription unit that directs expression of the heavy and light chain into the endogenous heavy chain locus.

CRISPR-RNAs (crRNAs) were designed to ablate the κ light chain because 95% of all mouse B cells express *Igk* (Fig. 1 A). The efficiency of κ light chain deletion was measured by flow cytometry using the ratio of κ/λ cells to normalize for cell death due to BCR loss. The selected crRNAs consistently ablated Igκ expression by 70–80% of B cells as measured by flow cytometry or tracking of indels by decomposition (TIDE; Brinkman et al., 2014) analysis (Fig. 1, B–D).

To insert a transgene into the heavy chain locus, we designed crRNAs specific for the first *Igh* intron immediately 3′ of the endogenous variable, diversity, and joining region (VDJ)$_H$ gene segment, and 5′ of the *Eμ* enhancer. This position was selected to favor transgene expression and allow simultaneous disruption the endogenous heavy chain (see below and Jacobsen et al., 2018). We tested seven crRNAs and selected a high-efficiency crRNA located 110 bp downstream of the J_H4 intron producing 77% indels by the TIDE assay (Fig. 1 E and Fig. S2, A and B). This location also allowed for sufficient homology to introduce a transgene, irrespective of the upstream VDJ rearrangement.

The homology-directed repair template (HDRT) is composed of a splice acceptor stop cassette to terminate transcription of upstream rearranged VDJ$_H$, and a V$_H$-gene promoter followed by cDNAs encoding *Igk*, a self-cleaving porcine teschovirus-1 2A peptide (P2A) sequence, and IgV$_H$ with a J$_H$1 splice donor site

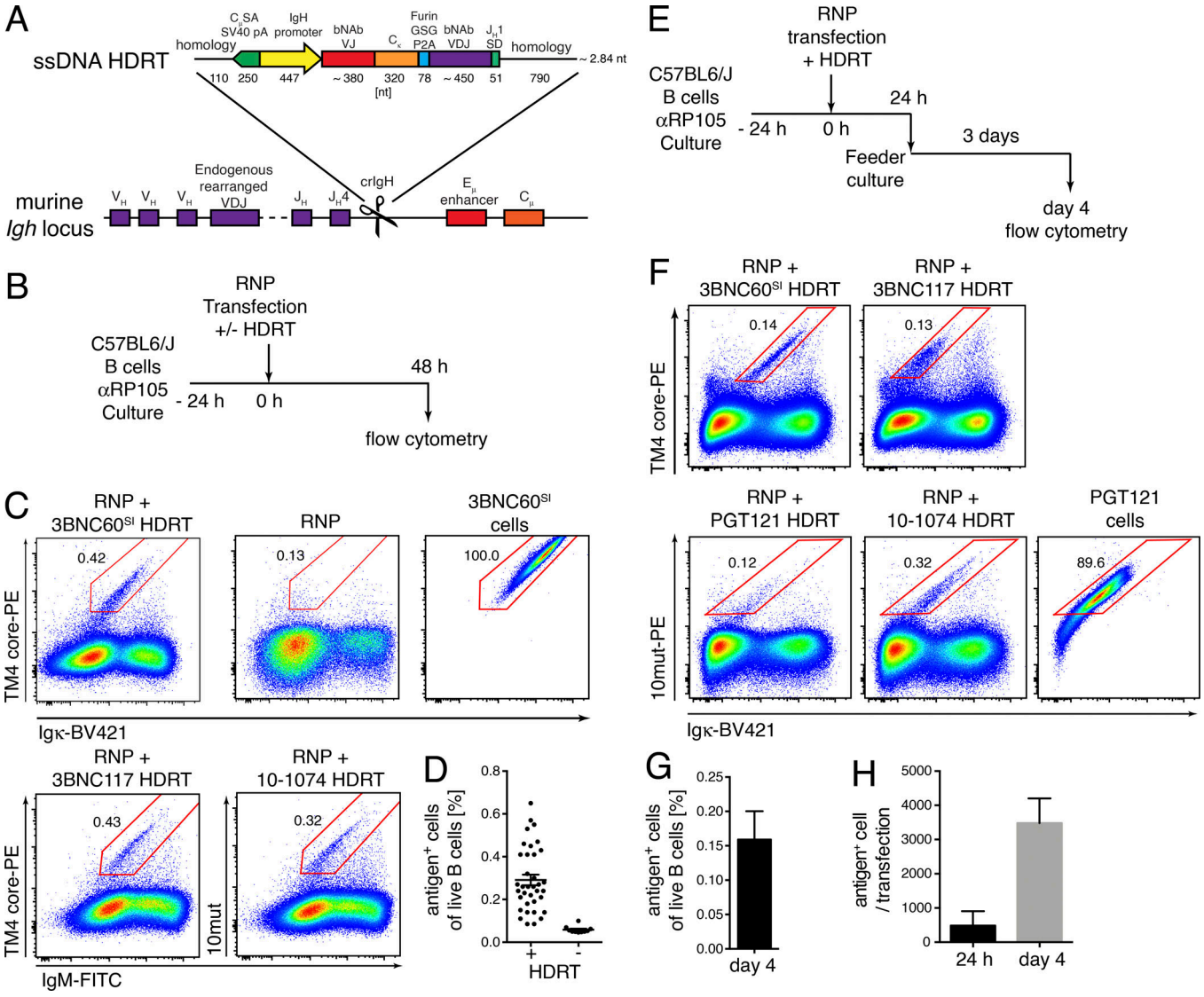

Figure 2. **Engineering bNAb-expressing primary mouse B cells. (A)** Schematic representation of the targeting strategy to create bNAb-expressing primary mouse B cells. ssDNA HDRT contained 110 nt 5′ and 790 nt 3′ homology arms flanking an expression cassette. The 5′ homology arm is followed by the 111 nt long splice acceptor site and the first two codons of Cμ exon 1, a stop codon, and a SV40 polyadenylation signal (CμSA SV40 pA). Then the mouse *Ighv4-9* gene promoter, the leader, and variable and joining regions (VJ) of the respective antibody light chain and mouse κ constant region ($C_κ$) are followed by a furin-cleavage site, a glycine-serine-glycine (GSG)–linker, and a P2A self-cleaving oligopeptide sequence, the leader, VDJ of the respective antibody heavy chain, and 45 nt of the mouse J_H1 intron splice donor site to splice into downstream constant regions. **(B)** Experimental setup for C. **(C)** Flow-cytometric plots of primary, mouse B cells, activated and transfected with RNPs targeting the *Ighj4* intron and *Igkc* exon with or without ssDNA HDRTs encoding the 3BNC60^{SI}, 3BNC117, or 10-1074 antibody. Non-transfected, antigen-binding B cells from 3BNC60^{SI} knock-in mice cultured the same way are used as control for gating. **(D)** Quantification of C. Each dot represents one transfection. Data from seven independent experiments (B–D). **(E)** Experimental setup for F–H. **(F)** Flow-cytometric plots of primary, mouse B cells, activated and transfected using ssDNA HDRT encoding the antibodies 3BNC60^{SI}, 3BNC117, PGT121, or 10-1074. B cells were expanded on feeder cells for 3 d. Cultured, nontransfected, antigen-binding B cells from PGT121 knock-in mice are shown for gating. **(G)** Quantification of F. **(H)** Total number of antigen-binding B cells before (24 h) or after 3 d (day 4) of feeder culture. Bars indicate mean ± SEM. Combined data from two independent experiments for E–H.

(Fig. 2 A). This design disrupts expression of the endogenous locus, while encoding a transcription unit directing expression of the introduced heavy and light chains under control of endogenous *Igh* gene regulatory elements. In addition, it preserves splicing of the transgenic IgV_H into the endogenous constant regions, allowing for expression of membrane and secreted forms of the antibody as wells as different isotypes by class switch recombination. Finally, correctly targeted cells are readily identified and enumerated by flow cytometry because they bind to cognate antigen.

A number of methods for producing single strand DNA (ssDNA) HDRTs were compared. The most reproducible and least cytotoxic involved digestion of plasmids with sequence-specific nickases, and ssDNA purification by agarose gel electrophoresis (Fig. S2, C–E; Yoshimi et al., 2016; Roth et al., 2018).

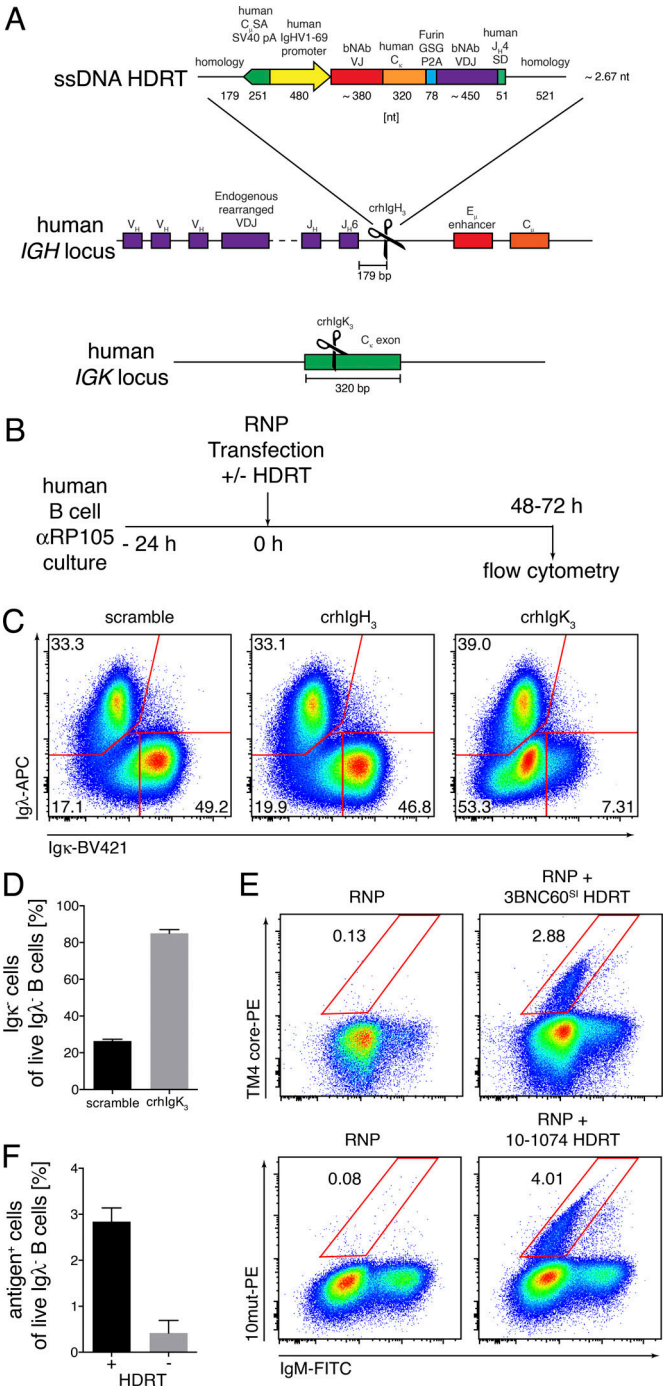

Figure 3. **Engineering bNAb-expressing primary human B cells. (A)** Schematic representation of the targeting strategy to create bNAb-expressing primary human B cells. The ssDNA HDRT is flanked by 179 nt and 521 nt homology arms. The central expression cassette contains 112 nt of the human splice acceptor site and the first two codons of Cμ exon 1, a stop codon and a SV40 polyadenylation signal (CμSA SV40 pA). Then the human *IGHV1-69* gene promoter, the leader, variable and joining regions (VJ) of the respective antibody light chain, and human $C_κ$ are followed by a furin-cleavage site, a GSG-linker, and a P2A self-cleaving oligopeptide sequence, the leader, VDJ of the respective antibody heavy chain, and 50 nt of the human J_H4 intron splice donor site to splice into downstream constant regions. **(B)** Experimental setup for C and D. Primary human B cells were cultured for 24 h in the presence of anti-RP105 antibody and then transfected with RNPs ± HDRT. **(C)** Flow-cytometric plots of primary human B cells 48 h after transfection with RNPs containing crRNAs without target (scramble) or targeting the *IGHJ6* intron or the *IGKC* exon. **(D)** Quantification of C. Bars indicate mean ± SEM. Combined data from three independent experiments are shown (B–D). **(E)** Flow-cytometric plots of antigen binding by Igλ⁻ primary human B cells 72 h after transfection of RNPs targeting both the *IGHJ6* intron and the *IGKC* exon with or without HDRTs encoding 3BNC60^{SI} or 10-1074. **(F)** Quantification of E. Bars indicate mean ± SEM. Combined data from two independent experiments with two to four replicates each (E and F).

Co-transfection of the ssDNA template with preassembled Cas9 ribonucleoproteins (RNPs) containing the crRNAs resulted in expression of the encoded anti-HIV antibody in 0.1–0.4% of mouse B cells by antigen-specific flow cytometry using antigens TM4 core (McGuire et al., 2014, 2016) or 10mut (Steichen et al., 2016; Fig. 2, C and D; and Fig. S3 A). Transgene expression was stable over the entire culture period of 3 d on feeder cells (Kuraoka et al., 2016), during which the overall number of B cells expanded by 6–20-fold (Fig. 2, E–H). However, expression of transgenic antibodies differed depending on the antibody and were generally reflective of their expression in knock-in mouse models (Fig. 2, C and F; Dosenovic et al., 2015, 2018; Escolano et al., 2016; McGuire et al., 2016; Steichen et al., 2016).

To determine whether edited cells are allelically excluded at the heavy chain locus, we transfected $Igh^{a/b}$ B cells with 3BNC60^{SI}, a chimeric antibody composed of the mature heavy chain and germline light chain of the anti-HIV bNAb 3BNC60 (Fig. S3, B and C). The majority of edited cells expressing the 3BNC60^{SI} transgene expressed it using either Igm^a or Igm^b allele as determined by flow cytometry. Only 5.21% of 3BNC60^{SI}-expressing B cells showed coexpression of both IgM^a and IgM^b, indicative of allelic inclusion of the endogenous allele or successful integration of the transgene into both alleles. Thus, the majority of edited B cells express only the transgene.

Promoter-containing expression cassettes have the potential to cause unwanted ectopic gene expression or allelic inclusion since they can be expressed from either the rearranged or germline IgH locus. To address these potential problems, we designed a smaller, promoterless antibody expression cassette that depends on integration into a rearranged IgH allele for expression (Fig. S3 D). Cell surface expression of the 3BNC60^{SI} from the promoterless construct was higher than the promoter-driven version (Fig. S3, E and F). Thus, the smaller promoterless and potentially safer construct efficiently directs knock-in antibody expression.

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

Antibody gene editing in human B cells

To determine whether this method could be adapted to edit human B cells, we isolated them from peripheral blood of healthy volunteers and activated them using an anti-human RP105 antibody (Miura et al., 1998). Analogous crRNAs were selected for targeting the human *IGKC* and the first intron 3′ of *IGHJ6* (Fig. 3, A–D; and Fig. S4, A and B). The best *IGKC*-targeting crRNA caused 85% of κ-bearing B cells to lose BCR expression, whereas λ-bearing cells increased proportionally, indicating that they were unaffected. TIDE analysis of the J_H6 intron

sequences showed that the most efficient crRNA induced 64% indels. In conclusion, activation of human primary B cells with anti-RP105 allows efficient generation of indels using Cas9 RNPs.

To target bNAbs into the human J_H6 intron, we adapted the ssDNA HDRT and replaced mouse with human homology arms, the human Cμ splice acceptor, the human *IGHV1-69* promoter, a codon-modified human *IGKC* constant region to avoid targeting by crRNAs, and the human J_H4 splice donor (Fig. 3 A). In contrast to mouse cells, 2.9–4% of λ^- B cells expressed 3BNC60^{SI} or 10-1074 antibodies, respectively, as determined by flow cytometry using the cognate antigen (Fig. 3, E and F). Thus, the efficiency of transgene integration is ≥10 times higher in human B cells. Furthermore, viability was also higher in human B cells, ranging from 60 to 85% of live cells after transfection (Fig. S4 C).

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

Adoptive transfer of antibody-edited B cells

To determine whether edited B cells can participate in immune responses, we adoptively transferred mouse 3BNC60^{SI}-edited Igh^b B cells into congenically marked Igh^a wild-type mice and then immunized the mice with the high-affinity, cognate antigen TM4 core in Ribi adjuvant (Fig. 4 A). Transgene-specific responses were detected using anti-idiotypic antibodies as an initial capture reagent in ELISA. Similar to endogenous humoral immune responses, transgenic antibodies were detected on day 7 after immunization, peaked at day 14, and started to decrease by day 21 (Fig. 4, B and C). Importantly, the transgenic immune response included secondary isotypes, indicating that the re-engineered locus supports class-switch recombination (Fig. 4 C). Finally, the magnitude of the response was directly correlated to the number of transferred cells. However, prolonged in vitro culture under the conditions tested decreased the efficiency of antibody production in vivo (Fig. 4 D).

To determine whether the transferred cells retained the ability to produce neutralizing antibodies, we used B cells that were edited to produce 10-1074, a potent bNAb, or 3BNC60^{SI}, a chimeric antibody with limited neutralizing activity (Mouquet et al., 2012; Dosenovic et al., 2018). 4×10^7 transfected B cells were transferred into wild-type Igh^a mice that were subsequently immunized with the appropriate cognate antigen 10mut (Steichen et al., 2016) or TM4 core (McGuire et al., 2014, 2016; Dosenovic et al., 2015, 2018). IgG was purified from the serum of three mice that received an estimated ∼10^3 edited B cells expressing 10-1074 or 3BNC60^{SI}. The purified serum antibodies were tested for neutralizing activity in the TZM-bl assay (Montefiori, 2005). Two of the three mice that received 10-1074 edited cells showed half-maximal inhibitory concentrations (IC_{50})s of 21.59 μg/ml, and a third reached 49% neutralization at 118 μg/ml (corresponding to ∼1:500 and 1:100 dilution of serum; Fig. 4 E and Fig. S5, A and B). As expected, neutralizing activity was not detected in mice receiving 3BNC60^{SI} because this antibody is two to three orders of magnitude less potent against the tested viral strains than 10-1074 (Fig. S5 C).

We conclude that edited B cells can be recruited into immune responses and produce sufficient antibody to confer potentially protective levels of humoral immunity (Shingai et al., 2014).

Discussion

T cells can be reprogrammed to express specific receptors using retrogenic methods (Eyquem et al., 2017; Lim and June, 2017; Sadelain et al., 2017) or nonviral CRISPR/Cas9 genome targeting (Roth et al., 2018). In contrast, BCR reprogramming in primary cells using retroviruses has not been successful (Freitag et al., 2014). Moreover, although antibody heavy chains have been targeted into human B cells using CRISPR/Cas9 (Voss et al., 2019), little is known about how CRISPR/Cas9 genome targeting might be used to introduce complete antibody genes into mature B cells that retain the ability to participate in immune responses in vivo.

We have developed a method to produce transgenic antibodies in primary mouse and human B cells using CRISPR/Cas9. The new method involves short-term culture in vitro, silencing of the endogenous *Ig* genes, and insertion of a bi-cistronic cDNA into the *Igh* locus. Mouse B cells edited to express an anti–HIV-1 bNAb by this method can produce transgenic antibody levels that are protective in animal models (Mascola et al., 1999; Shibata et al., 1999; Parren et al., 2001; Shingai et al., 2014).

Mouse and human B lymphocytes typically express a single antibody despite having the potential to express two different heavy chains and four different light chains. Theoretically the combination could produce eight different antibodies and a series of additional chimeras that could interfere with the efficiency of humoral immunity and lead to unwanted autoimmunity. Allelic exclusion prevents this from happening and would need to be maintained by any gene replacement strategy used to edit B lymphocytes. In addition, genetic editing is accompanied by safety concerns due to off-target double strand breaks and integrations. This approach lowers these risks by using nonviral gene editing with ssDNA templates, which limits random integrations and by keeping culture time short to prevent expansion of any such cell.

The approach reported maintains allelic exclusion in part by ablating the *Igkc* gene. In the mouse, 95% of B cells express *Igkc*. In the absence of *Igkc* expression, these cells will die by apoptosis because they cannot survive unless they continue to express a BCR (Lam et al., 1997; Kraus et al., 2004). Since the introduction of the transgene into the heavy chain locus disrupts endogenous *Igh* expression, editing maintains allelic exclusion in the majority of cells because only cells expressing the introduced antibody can survive.

Our strategy also interferes with the survival of cells that suffer off-target integration events, because the majority of such cells would be unable to express the BCR, and they too would die by apoptosis.

A potential issue is that there are two heavy chain alleles in every B cell, and allelic exclusion would be disrupted if the transgene were only integrated in the nonproductive *Igh* allele, allowing for expression of the original productive *Igh*. However, our flow cytometry data indicate that this is a very rare event.

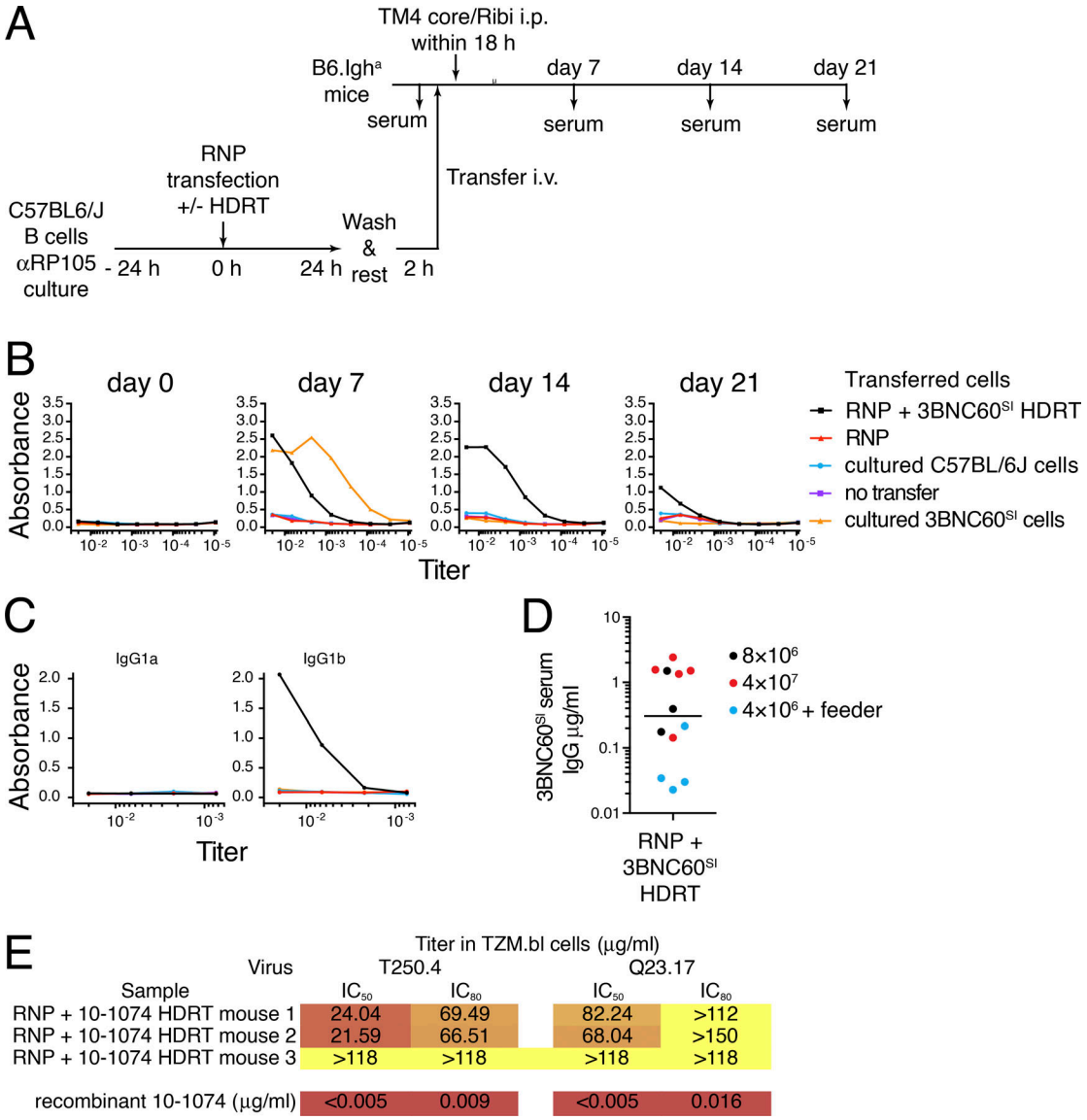

Figure 4. **Engineered bNAb-expressing primary mouse B cells participate in humoral immune responses in vivo. (A)** Experimental setup for B–E. **(B)** Anti-3BNC60^{SI} idiotype-coated, mouse IgG ELISA of sera from mice adoptively transferred with the indicated B cells and immunized with the cognate antigen TM4 core at the indicated time points. Representative plots of seven independent experiments. **(C)** Anti-3BNC60^{SI} idiotype-coated mouse IgG1^{a} or IgG1^{b} ELISA of day 14 sera, as above. Representative plots of two independent experiments. **(D)** 3BNC60^{SI} serum IgG levels 14 d after immunization in mice transferred with 3BNC60^{SI}-edited cells. Numbers of total B cells/mouse at transfection are indicated. Cells were transferred either 24 h after transfection or after additional culture on feeder cells as in Fig. 2 D. Determined by anti-3BNC60^{SI} idiotype-coated mouse IgG ELISA over seven independent experiments. Each dot represents one mouse, and the line indicates the arithmetic mean. **(E)** TZM.bl neutralization data of protein G–purified serum immunoglobulin days 14–21 after immunization from mice treated as in A but transfected with 10-1074 HDRT and immunized with cognate antigen 10mut. Combined data from two independent experiments are shown.

Thus, either both alleles are targeted or the occasional remaining endogenous *Igh* gene is unable to pair with the transgenic *Igk*. A small number of B cells that have not deleted endogenous *Igk* might also integrate the transgene into the *Igh* locus. This could decrease the efficiency of knock-in antibody expression if the endogenous kappa pairs with the transgenic heavy chain.

The use of a promoterless construct increases surface BCR expression and improves safety. This construct relies on integration into an allele with in-frame VDJ rearrangement. Furthermore, the absence of a promoter makes off-target gene activation less likely, thereby increasing the safety of this approach.

In contrast to the mouse, *IGL* is expressed by 45% of all B cells in humans. Therefore, this locus would need to be ablated, or alternatively, cells expressing *IGL* could be removed from the transferred population by any one of a number of methods of negative selection.

Similar to antibody transgenes in mice, expression of the edited BCR varied between different antibodies. Some combinations of heavy and light chains were refractory to expression in mature B cells. In addition, although the level of BCR expression was within the normal range, it was generally on the low end compared with polyclonal B cells. This is consistent with generally

lower-level expression of a similar transgene in knock-in mice (Jacobsen et al., 2018). Low BCR expression could also be due to the bi-cistronic design since expression was higher in knock-in mice that expressed the identical Ig from the native *Igk* and *Igh* loci (Dosenovic et al., 2018). Nevertheless, expression levels were adequate to drive antigen-induced antibody production in vivo.

bNAb-mediated protection against infection with simian–human immunodeficiency viruses in macaques requires IC_{50} neutralizing titers of 1:100 (Mascola et al., 1999; Shibata et al., 1999; Parren et al., 2001; Shingai et al., 2014). Thus, the titers achieved by CRISPR/Cas9-edited B cells in mice would be protective if they could be translated to macaques and, by inference, humans. Moreover, our neutralization measurements may be an underestimate since we excluded bNAbs produced as IgM or isotypes other than IgG.

Chimeric antigen receptor T cell therapy typically involves transfer of millions of edited cells to achieve a therapeutic effect. Whether similar numbers of edited B cells would also be required to achieve protective levels of humoral immunity can only be determined by further experimentation in primate models. In addition, the longevity of the antibody response produced by edited B cells, and its optimization by boosting or adjuvant choice, will require further experimentation. Finally, adoptive cell therapies are currently prohibitively expensive, but this is likely to change with future scientific developments.

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

Materials and methods

crRNA design

crRNAs were designed with the Massachusetts Institute of Technology guide design tool (Hsu et al., 2013), CHOPCHOP (Montague et al., 2014; Labun et al., 2016; http://chopchop.cbu.uib.no), and the Integrated DNA Technologies crRNA design tool (http://www.idtdna.com). Designs were synthesized by Integrated DNA Technologies as Alt-R CRISPR/Cas9 crRNAs. crRNA sequences are listed in Table S1.

ssDNA HDRT preparation

HDRT sequences, listed in Table S2, were synthesized as gBlocks (Integrated DNA Technologies) and cloned using *NheI* and *XhoI* (New England Biolabs) into vector pLSODN-4D from the long ssDNA preparation kit (DS620; BioDynamics Laboratories). ssDNA was prepared following the manufacturer's instructions with the following modifications. In brief, 2.4 mg sequence-verified vector was digested at 2 µg/µl in NEB 3.1 buffer with 1,200 U Nt.*BspQI* for 1 h at 50°C followed by addition of 2,400 U *XhoI* (New England Biolabs) and incubation for 1 h at 37°C. Digests were desalted by ethanol precipitation and resuspended in water at <1 µg/µl. An equal volume of formamide gel-loading buffer (95% de-ionized formamide, 0.025% bromophenol blue, 0.025% xylene cyanol, 0.025% SDS, and 18 mM EDTA) was added and heated to 70°C for 5 min to denature double-stranded DNA. Denatured samples were immediately loaded into dye-free 1% agarose gels in Tris base, acetic acid, and EDTA (TAE) buffer and run at 100 V for 3 h. Correctly sized bands were identified by partial post-stain with GelRed (Biotium), then excised and column-purified (740610.20 or 740609.250; Machery Nagel) according to the manufacturer's instructions. Eluate was ethanol-precipitated, resuspended in water, adjusted to 2.5 µg/µl, and stored at −20°C.

Murine cell culture

Mature, resting B cells were obtained from mouse spleens by forcing tissue through a 70-µm mesh into PBS containing 2% heat-inactivated fetal bovine serum (FBS). After ammonium-chloride-potassium buffer lysis for 3 min, untouched B cells were enriched using anti-CD43 magnetic beads according to the manufacturer's protocol (Miltenyi Biotec) obtaining >95% purity. 3.2×10^7 cells/10 cm dish (Gibco) were cultured at 37°C 5% CO_2 in 10 ml mouse B cell medium consisting of RPMI-1640, supplemented with 10% heat-inactivated FBS, 10 mM Hepes, antibiotic-antimycotic (1×), 1 mM sodium pyruvate, 2 mM L-glutamine, and 53 µM 2-mercaptoethanol (all from Gibco) and activated with 2 µg/ml anti-mouse RP105 clone RP/14 (produced in house or 562191; BD Pharmingen).

NB-21 feeder cells (Kuraoka et al., 2016) were maintained in DMEM supplemented with 10% heat-inactivated FBS and antibiotic-antimycotic (1×). For co-culture, feeder cells were irradiated with 80 Gy and seeded simultaneously with B cells, 24 h after transfection, into B cell culture medium supplemented with 1 ng/ml recombinant mouse IL-4 (214–14; PeproTech) and 2 µg/ml anti-mouse RP105 clone RP/14.

Human cell culture

Leukapheresis samples of healthy human individuals were collected after signed informed consent in accordance with protocol TSC-0910 approved by the Rockefeller University Institutional Review Board. Peripheral blood mononuclear cells were prepared, stored in liquid nitrogen, then thawed in a 37°C water bath and resuspended in human B cell medium composed of RPMI-1640, supplemented with 10% heat-inactivated FBS or human serum, 10 mM Hepes, antibiotic-antimycotic (1×), 1 mM sodium pyruvate, 2 mM L-glutamine, and 53 µM 2-mercaptoethanol (all from Gibco). B cells were isolated using the EasySep human naive B cell Enrichment Kit (19254; Stemcell) according to the manufacturer's instructions and cultured in the above medium supplemented with 2 µg/ml anti-human RP105 antibody clone MHR73-11 (312907; BioLegend).

RNP preparation and transfection

Per 100 µl transfection, 1 µl of 200 µM crRNA and 1 µl 200 µM trans-activating crRNA in duplex buffer (all from Integrated

DNA Technologies) were mixed, denatured at 95°C for 5 min, and renatured for 5 min at room temperature. 5.6 µl PBS and 2.4 µl 61 µM Cas9 V3 (1081059; Integrated DNA Technologies) were added and incubated for 15–30 min. If required, RNPs were mixed at the following ratios: 50% crIgH, 25% crIgK$_1$, and 25% crIgK$_2$ (mouse) or 50% crhIgH$_3$ and 50% crhIgK$_3$ (human). 4 µl 100 µM electroporation enhancer in duplex buffer or 4 µl HDRT at 2.5 µg/µl was added to 10 µl mixed RNP and incubated for a further 1–2 min.

24 h after stimulation, activated mouse or human B cells were harvested, washed once in PBS, and resuspended in Mouse B cell Nucleofector Solution with Supplement (murine B cells) or Primary Cell Nucleofector Solution 3 with Supplement (human B cells) prepared following to the manufacturer's instructions (Lonza) at a concentration of 4–5 × 10^6 cells/86 µl. 86 µl cells were added to the RNP/HPRT mix, gently mixed by pipetting, transferred into nucleofection cuvettes, and electroporated using an Amaxa IIb machine setting Z-001 (murine B cells) or Amaxa 4D machine setting EH-140 (human B cells). Cells were immediately transferred into 6-well dishes containing 5 ml prewarmed mouse or human B cell medium supplemented with the relevant anti-RP105 antibody at 2 µg/ml and incubated at 37°C 5% CO_2 for 24 h before further processing.

TIDE assay

Genomic DNA was extracted from 0.5–5 × 10^5 cells by standard phenol/chloroform extraction 24–42 h after transfection. PCRs to amplify human or mouse Ig loci targeted by CRISPR/Cas9 were performed using Phusion Green Hot Start II High-Fidelity polymerase (F-537L; Thermo Fisher Scientific) and primers listed in Table S3. A thermocycler was set to 40 cycles, annealing at 65°C for 30 s and extending at 72°C for 30 s. PCR product size was verified by gel electrophoresis, and bands were gel-extracted and sent for Sanger sequencing (Genewiz) using the relevant PCR primers. ab1 files were analyzed using the TIDE web tool (http://tide.nki.nl) using samples receiving scramble or irrelevant HPRT-targeting crRNA as the reference (Brinkman et al., 2014).

Flow cytometry

Mouse spleens were forced through a 70-µm mesh into FACS buffer (PBS containing 2% heat-inactivated FBS and 2 mM EDTA), and red blood cells were lysed in ammonium-chloride-potassium buffer lysing buffer (Gibco) for 3 min. Cultured cells were harvested by centrifugation. Then cells were washed and Fc-receptors blocked for 15 min on ice. Cells were stained for 20 min on ice with antibodies or reagents listed in Table S4 and, depending on the stain, washed again and secondary-stained for another 20 min on ice before acquisition on a BD LSRFortessa. Anti-idiotype 3BNC60^{SI} (iv8) produced as human IgG1/κ was detected with anti-human Igκ-BV421 on edited mouse B cells. GC B cells were gated as single/live, B220$^+$, CD38$^-$ FAS$^+$, GL7$^+$, and IgD$^-$. Allotypic markers CD45.1 and CD45.2 were used to track adoptively transferred B cells.

Mice

C57BL/6J and B6.Igh^a (B6.Cg-Gpi1^a Thy1^a Igh^a/J) and B6.SJL were obtained from the Jackson Laboratory. Igh$^{a/b}$ mice were obtained by intercrossing B6.Igh^a and B6.SJL mice. B1-8^{hi} (Shih et al., 2002), 3BNC60^{SI} (Dosenovic et al., 2018), and PGT121 (Escolano et al., 2016; Steichen et al., 2016) strains were generated and maintained in our laboratory on a C57BL/6J background. All experiments used age- and sex-matched animals, littermates when possible. All experiments were performed with authorization from the Institutional Review Board and the Rockefeller University Institutional Animal Care and Use Committee.

Cell transfers and immunizations

After culture, mouse B cells were harvested at the indicated time points and resuspended in mouse B cell medium without anti-RP105 antibody and rested for 2–3 h at 37°C, 5% CO_2. Then cells were washed once in PBS and resuspended in 200 µl PBS/mouse containing the indicated number of initially transfected cells. 200 µl cell suspension/mouse were injected intravenously via the retroorbital sinus. The number of transferred, edited B cells was estimated as follows: number of cells transfected × 20% survival × 0.15–0.4% transfection efficiency × 50% handling/proliferation × 5% transfer efficiency (Dosenovic et al., 2018). Mice were immunized intraperitoneally within 24 h after cell transfer with 200 µl containing 10 µg TM4 core (McGuire et al., 2014) or 10mut (Steichen et al., 2016) in PBS with 50% Ribi (Sigma Adjuvant system; Sigma-Aldrich) prepared according to the manufacturer's instructions. Mice were bled at the indicated time points from the submandibular vein. Blood was allowed to clot, and then serum was separated by centrifugation for 10 min at 20,817 *g*. Serum was stored at −20°C.

Anti-idiotypic antibody

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

ELISAs

For determination of 3BNC60^{SI} levels, Corning 3690 half-well 96-well plates were coated overnight at 4°C with 25 µl/well of 2 µg/ml human anti-3BNC60^{SI} (clone iv8) IgG in PBS, then blocked with 150 µl/well PBS 5% skimmed milk for 2 h at room temperature (RT). Sera were diluted to 1:50 with PBS and seven subsequent threefold dilutions. Recombinant 3BNC60^{SI} (produced in house as mouse IgG1,κ) was diluted to 10 µg/ml in PBS followed by six fivefold dilutions. Blocked plates were washed four times with PBS 0.05% Tween 20 and incubated with 25 µl diluted sera or antibody for 2 h at RT. Binding was revealed by anti-mouse IgG-HRP (115–035-071; Jackson ImmunoResearch), anti-mouse IgG1a-biotin (553500; BD Pharmingen), or anti-mouse IgG1b-biotin (553533; BD Pharmingen), all diluted 1:5,000 in PBS, 25 µl/well, and incubated for 1 h at RT. Biotinylated antibodies were subsequently incubated with streptavidin-HRP

(554066; BD Pharmingen) and diluted 1:1,000 in PBS, 25 µl/well, for 30 min at RT. Plates were washed four times with PBS 0.05% Tween 20 in between steps and six times before addition of substrate using a Tecan Hydrospeed microplate washer. HRP activity was determined using 3,3′,5,5′ tetramethylbenzidine as substrate (34021; Thermo Fisher Scientific), adding 50 µl/well. Reactions were stopped with 50 µl/well 2 M H_2SO_4 and read at 450 and 570 nm on a FLUOstar Omega microplate reader (BMG Labtech). Data were analyzed with Microsoft Excel and GraphPad Prism 6.0. Absolute 3BNC60^{SI} titers were interpolated from sigmoidal fits of recombinant 3BNC60^{SI} standard curves.

For determination of NP-binding antibodies, the following modifications applied. Plates were coated with 10 µg/ml NP_{31}-BSA (Biosearch Technologies) and blocked with PBS 3% BSA. Sera, antibodies, and secondary reagents were diluted in PBS 1% BSA 0.05% Tween 20.

Neutralization assays

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

TZM-bl assays were performed as previously described (Montefiori, 2005). Neutralizing activity was calculated as a function of the reduction in Tat-inducible luciferase expression in the TZM-bl reporter cell line in a single round of virus infection.

Online supplemental material

Fig. S1 shows that B cells cultured and stimulated as for RNP transfection are able to participate in GCs and produce antibodies. Fig. S2 relates to the choice of murine IgH crRNAs and production of HDRTs. Fig. S3 provides data on murine B cell viability after transfection, *Igh* allelic exclusion, and a promoterless HDRT to improve allelic exclusion. Fig. S4 relates to the choice of human crRNAs and viability of human B cells after transfection. Fig. S5 provides details and additional data of neutralization assays. Table S1 lists crRNA sequences. Table S2 contains annotated HDRT sequences. Table S3 contains primer sequences for the TIDE assay, and Table S4 details flow-cytometric reagents.

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There are patents on 3BNC117 and 10-1074, of which M.C. Nussenzweig is an inventor. M.C. Nussenzweig is a member of the Scientific Advisory Boards of Celldex and Frontier Biotechnologies. The authors declare no additional competing financial interests.

Author contributions: H. Hartweger and M. Jankovic conceived, planned, and performed experiments; analyzed data; and wrote the manuscript. M. Horning performed experiments and prepared ssDNA HDRT. P. Dosenovic assisted with experimental design and ELISAs. D. Yost and A. Gazumyan expressed antibodies. A.T. McGuire, J.J. Taylor, and L. Stamatatos designed and provided iv8 antibody. M.S. Seaman performed neutralization assays. M.C. Nussenzweig planned and supervised experiments, analyzed data, and wrote the manuscript.

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