In vivo engineered B cells secrete high titers of broadly neutralizing anti-HIV antibodies in mice

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Transplantation of B cells engineered ex vivo to secrete broadly neutralizing antibodies (bNAbs) has shown efficacy in disease models. However, clinical translation of this approach would require specialized medical centers, technically demanding protocols and major histocompatibility complex compatibility of donor cells and recipients. Here we report in vivo B cell engineering using two adeno-associated viral vectors, with one coding for Staphylococcus aureus Cas9 (saCas9) and the other for 3BNC117, an anti-HIV bNAb. After intravenously injecting the vectors into mice, we observe successful editing of B cells leading to memory retention and bNAb secretion at neutralizing titers of up to 6.8 µg ml⁻¹. We observed minimal clustered regularly interspaced palindromic repeats (CRISPR)–Cas9 off-target cleavage as detected by unbiased CHANGE-sequencing analysis, whereas on-target cleavage in undesired tissues is reduced by expressing saCas9 from a B cell-specific promoter. In vivo B cell engineering to express therapeutic antibodies is a safe, potent and scalable method, which may be applicable not only to infectious diseases but also in the treatment of noncommunicable conditions, such as cancer and autoimmune disease.

Broadly neutralizing antibodies (bNAbs) against HIV can suppress viremia. In particular, combination therapy with the bNAbs 3BNC117 and 10-1074 allowed long-term suppression on interruption of antiretroviral therapy in individuals with antibody-sensitive viral reservoirs. Similarly, viremic individuals with dual antibody-sensitive viruses experienced diminished viremia for 3 months following the first of up to three dual-bNAb infusions. However, the mean elimination half-life of the bNAbs is 16 and 23 days, respectively, allowing the virus to rebound. Moreover, individuals with previous resistance to one of the bNAbs have mounted resistance to the second antibody, and individuals with previous resistance to both antibodies were excluded from the trials. Limited bNAb persistence may be addressed by constitutive expression from muscle following viral vector transduction. However, anti-drug antibodies may develop, possibly because of improper glycosylation. Moreover, antibodies expressed from muscle do not undergo class switch recombination (CSR) or affinity maturation, which may be required for long-term suppression of a diverse and continuously evolving HIV infection. To overcome these challenges, we and others have developed B cell engineering for antibody expression. In particular, we previously combined Toll-like receptor (TLR)–mediated ex vivo activation of B cells with in vivo prime-boost immunizations, and demonstrated that engineered B cells allow immunological memory, CSR, somatic hypermutation (SHM) and clonal selection. However, cost and complexity of autologous B cell engineering ex vivo may be prohibitive. At the same time, use of engineered allogeneic B cells is challenging due to the requirement for human leukocyte antigen matching for receiving T cell help and avoiding graft rejection.

These challenges may be addressed using in vivo engineering. In vivo T cell engineering was previously demonstrated, using promiscuously integrating vectors, episomal adeno-associated viral (AAV) vectors or messenger RNA. However, in B cells, only the specific targeting of the IgH locus, using the endogenous constant exons with appropriate splicing signals, is expected to allow a well-regulated expression of the antibody, first as a membrane bound B cell receptor (BCR) and then, on antigen-induced activation, also as a soluble protein, released by progeny plasmablasts and plasma cells. IgH targeting is similarly required for memory retention, CSR, SHM and clonal selection. Therefore, we describe here an in vivo B cell engineering protocol based on a single system injection of AAV vectors coding for clustered regularly interspaced palindromic repeats (CRISPR)–Cas9 and for the desired bNAb cassette, which is targeted for integration into the IgH locus.
In vivo B cell engineering allows for high anti-HIV bNAb titers. AAV injections to mice were preceded by pre-immunizations, modeling a pre-existing infection. Indeed, B cell activation is required for efficient AAV transduction, and subsequent activation signals for the engineered B cells may benefit from previous priming of T helper cells and from presentation of appropriate immune complexes by follicular dendritic cells. In particular, C57BL/6 mice were immunized with 20 μg of the gp120 HIV antigen, which is the target of 3BNC117. On day 6 postimmunization, each mouse was injected with 5 × 10^11 viral genomes (vg) of a bNAb coding vector, 5 × 10^11 vg of the saCas9 coding vector or both (Fig. 2a). The mice then received additional immunizations on days 8, 23, 68, 98 and 128. Following the boosting regimen, mice receiving both a donor vector and an saCas9 vector had up to 5 μg ml^-1 of the 3BNC117 bNAb in their blood, being >50x the median virus neutralization IC₅₀ (half-maximum inhibitory concentration) for this bNAb (Fig. 2b). This is in concordance with previous reports, entailing transfer of low numbers of antigen-specific B cells from transgenic mice, demonstrating a potent immune response following immunizations. Here, 3BNC117 of multiple isotypes was found in the sera, and IgG 3BNC117 accounted for as much as 1% of the total response toward gp120 (Extended Data Fig. 1). IgG purified from treated mice can neutralize autologous YU2.DG and the heterologous tier-2 JRFL HIV pseudoviruses (Fig. 2c and Extended Data Fig. 2a). Mice injected with both a donor vector and an saCas9 vector had much higher 3BNC117 titers than mice receiving donor vector only. Nevertheless, 3BNC117 titers in mice receiving only the donor vector slightly exceeded the background levels measured in mice injected with PBS (Extended Data Fig. 2b). Indeed, integration of the antibody gene into the IgH locus was evident by PCR with reverse transcription (RT–PCR) of splenic B cell RNA from mice receiving dual vector injection but an additional, nested PCR was required to detect such integration in two of the three mice injected with the donor vector alone (Extended Data Fig. 2c–e). Very low editing frequencies without CRISPR genomic readout could similarly be detected by RT–PCR only in ex vivo edited lymphocytes (Extended Data Fig. 2f–i). Notably, when using dual vector injections, high titers could be obtained not only on immunizing the mice with the monomeric gp120 antigen of the clade B HIV strain YU2.DG, but also in independent experiments using either the clade A, BG505-based native trimer nanoparticle immuno-
nogen (MD39-ferritin)\(^7\) (Fig. 2d,e), or the stabilized soluble 2CC immunogen\(^4\), originating from the clade B HXBc2 strain (Fig. 2f,g), attesting for the breadth of the 3BNC117-expressing cells in vivo. The presence of 3BNC117-secreting cells in the bone marrow was established using enzyme-linked immunosorbent spot (ELISPOT) on the bone marrow of treated mice (Fig. 2h,i) and correlated well with splenic 3BNC117 expression in these mice (Extended Data Fig. 3a,b).

**In vivo engineered B cells undergo clonal expansion in germinal centers.** The frequency of 3BNC117-expressing cells reached 0.5% of total blood B cells following the later immunizations in all mice injected with both a bNAb vector and an saCas9 vector, but not in mice injected with PBS or with the bNAb vector alone (Fig. 2a and Extended Data Fig. 3c,d). On euthanizing the mice at day 136, 8 days after the last immunization, up to 23% of the plasmablasts in the spleen (Fig. 3a,b) and 5–10% of germinal center lymphocytes expressed 3BNC117 (Fig. 3c,d and Extended Data Fig. 3f).

Here, 0.6% of bone marrow cells expressed CD19 and 3BNC117 (Extended Data Fig. 3g,h). However, an additional 1.5% of bone marrow cells were CD19\(^+\) 3BNC117\(^+\) (Extended Data Fig. 3i).

To study SHM and clonal selection, we extracted DNA from the liver and the spleen of one of the treated mice at day 136 and performed Illumina sequencing of amplified 3BNC117 VH segments. Much of the mutation repertoire was shared between the liver and the spleen and may thus reflect heterogeneity in AAV production that is subjected to little or no selection\(^7,9\). In particular, all the 3BNC117 VH\(_R\) variants found to be over-represented in the liver are also over-represented in the spleen. However, the inverse is not true. The complementarity-determining region-1 substitution R30K is the most prevalent substitution in the spleen. It accounts for more than 20% of all mutants in the spleen but is found at very low abundance in the liver or in a representative AAV batch.
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The gp120. Including R30K, a total of four different positions along in 3BNC117 relieves some steric clash on binding to monomeric complementarity-determining region loops. The R30 position is indicated.

For dots colored with lighter shades, (orange) of a single mouse or AAV (purple). The dotted line represents values >, dn/dS samples. From purified AAV. Orange shading indicates the R30K variant. Numbers in the middle of the pies indicate the total frequency of mutant reads in these samples.

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sharply by day 136, perhaps due to multiple cell divisions. The copy number was also found in the blood at day 37, but levels dropped

4a) and the levels were reduced by only tenfold at day 136 (Fig. 4b),

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To assess the possible off-target activity of saCas9, we performed circularization for high-throughput analysis of nuclease genome-wide effects by sequencing (CHANGE-seq)40 on genomic DNA from C57BL/6 mice. Of the reads, 95% corresponded to the on-target site (Fig. 4d). We then performed targeted sequencing on four potential off-target sites, as well as on the on-target site, using gDNA from liver and spleen of treated mice and of a negative control mouse. Relative to control DNA from the spleen of an untreated mouse, a trend for a higher mutation rate, indicating error-prone repair of CRISPR–Cas9-induced double-stranded DNA breaks, was evident in the liver and not in the spleen, and only at the IgH on-target site rather than in any of the tested off-target sites (Fig. 4e).

To better characterize the different populations of engineered cells, we next used a donor vector coding for green fluorescent protein (GFP) in addition to the 3BNC117 cassette (Fig. 5a). Recipient mice were immunized twice before analysis of GFP and/or 3BNC117 expression in the bone marrow and the spleen (Fig. 5b). As expected, GFP+, 3BNC117+ cells were enriched in the spleen

CRISPR–Cas9 cleavage is highly sequence specific but takes place also in undesired tissues. To assess the possible off-target effects of our in vivo engineering approach, we first quantified the copy number of the bNAb cassette in various tissues. The bNAb cassette was found at a high copy number in the liver at day 37 (Fig. 4a) and the levels were reduced by only tenfold at day 136 (Fig. 4b), reflecting high retention of AAV episomes in the liver. High copy number was also found in the blood at day 37, but levels dropped sharply by day 136, perhaps due to multiple cell divisions. The AAV copy number in the bone marrow was significantly increased from day 37 to day 136 and a nonsignificant similar trend was also
detected in the lymph nodes, indicating the possible accumulation of 3BNC117-expressing cells in these tissues (Fig. 4b). The copy number in the liver was similar whether or not the saCas9 coding AAV was coinjected to the mice. In contrast, the copy number of the bNAb cassette in the lymph nodes and in the bone marrow was found to be log higher with saCas9 AAV coinjection, signifying the selection of 3BNC117-expressing B cells (Fig. 4c).

Fig. 3 | In vivo engineered B cells are found in lymphatic tissues 130 days following AAV injection. a. Flow cytometry plots demonstrating 3BNC117 expression among plasmablasts (CD38+, CD138+, CD19+) in the spleen at day 136. Pregated on live, singlets. b. Quantification of a for engineered plasmablasts (CD38+ CD138+ 3BNC117+). Mean is indicated by the bars. NS, P = 0.9892; ****P < 0.0001, one-way ANOVA with Tukey’s multiple comparison. c. Flow cytometry plots demonstrating 3BNC117 expression of cells with a germlinal center phenotype (GL7+, CD95/Fas+) in the spleen. Pregated on live, singlets. FSC, Fourier shell correlation. d. Quantification of c. Mean is indicated by the bars. GC, germlinal center. NS, P = 0.8916; **P = 0.0054 one-way ANOVA with Tukey’s multiple comparison. e. Pie charts of 3BNC117 VH variants amplified from spleen and liver DNA at day 136 and from purified AAV. Orange shading indicates the R30K variant. Numbers in the middle of the pies indicate the total frequency of mutant reads in these samples. From dn/dS values for the positions along the VH segment, on the basis of illumina sequencing of DNA amplified from the spleen (blue) or liver (orange) of a single mouse or AAV (purple). The dotted line represents values >1, indicative of positive selection. For dots colored with lighter shades, the assignment of a dn/dS value >1 is not statistically significant. No position in the AAV sample reached statistical significance. Gray shading indicates complementarity-determining region loops. The R30 position is indicated.
Fig. 4 | AAV biodistribution and saCas9 off-target cleavage analysis reveal a high safety profile. a, Donor AAV copy number quantification by qPCR in indicated tissues at day 136 from mice injected with two AAVs as in Fig. 2a. b, Relative copy number of donor AAV between days 37 and 136 in selected tissues. c, Relative copy number of donor AAV between mice injected with two AAVs, as in Fig. 2a, and mice injected with donor AAV only, at day 136. For b and c, indicated are the mean of relative expression and error bars corresponding to lower and upper boundaries derived from two-sided unpaired t-test. For b, from left to right: *P = 0.0496, P = 0.0139, P = 0.0389, P = 0.0243; **P = 0.0046; comparison between the two time points. For c, from left to right: *P = 0.0128, P = 0.0147 for comparison between the two mouse groups. n = 3 biologically independent animals. The y axes in a–c use a log scale. LN, lymph nodes and BM, bone marrow. d, Unbiased CHANGE-seq analysis of potential saCas9 off-target cleavage with the sgRNA used in this study. Localization, annotation in the genome, number of mismatches and percentage read counts are indicated for each on- or off-target site. Sequence of the sgRNA with the protospacer adjacent motif (PAM) is indicated on the top. Black arrows indicate target sites used for analysis of mouse samples. Mismatches between off-target sites and intended sgRNA target are color-coded. e, On- and off-target saCas9 cleavage, of target sites indicated in d by black arrows, in the spleen (mauve) and liver (beige) of mice injected with two AAVs, as in Fig. 2a, at day 136, as compared to uncut, naïve splenic lymphocytes DNA. For spleen and liver tissues, n = 3 biologically independent animals. For the control uncut, naïve splenic lymphocyte DNA, n = 1. Mean values ± s.d. are indicated.

(Fig. 5c and Extended Data Fig. 4a) and the bone marrow (Fig. 5d and Extended Data Fig. 4a) of mice receiving both the donor and the saCas9 vectors. Coinjection with donor and saCas9 vectors has increased the rate of B cells expressing GFP (Fig. 5e and Extended Data Fig. 4a) and, in particular, the rates of B cells expressing both GFP and 3BNC117 (Fig. 5c and Extended Data Fig. 4a) in the spleen. We estimate that, for a typical spleen of 50 M cells, assuming an expansion factor of 25-fold for antigen-specific B cells following a single immunization⁴¹, as low as 140 cells may have been initially engineered. The rates of GFP+, 3BNC117+ cells among B220+ cells remained low (Fig. 5c and Extended Data Fig. 4a). Notably, within the GFP expressing B cells, coinjecting the saCas9 vector led to a marked increase in 3BNC117+, CD138+ plasmablasts, in both the spleen (Fig. 5f and Extended Data Fig. 4a) and the bone marrow (Fig. 5g and Extended Data Fig. 4a). In the bone marrow, we found a larger fraction of cells, stained by the anti-3BNC117 anti-idiotyp antibody, to be B220− (Extended Data Fig. 4a,b). The B220− cells, stained by the anti-idiotyp, were almost exclusively CD3− and most of them were CD11b+ cells (Extended Data Fig. 4a,c), indicative of possible FcR binding of secreted 3BNC117. Indeed, most of the CD11b+ cells in the bone marrow, stained with the anti-idiotyp, were GFP+ (Extended Data Fig. 4d,e). In addition, the same anti-idiotyp staining detected the ex vivo binding of soluble 3BNC117 by nonengineered CD11b+ cells at a much higher rate than by nonengineered B220+ cells (Extended Data Fig. 4f–i). An increase in the rate of cells, stained by the anti-idiotyp, among GFP expressing cells was seen even within this B220− cell population in the spleen (Extended Data Fig. 4a,j) and marrow (Extended Data Fig. 4a,k). This is in line with recent publications showing expression of membrane antibodies by cells of the myeloid lineage⁴¹–⁴³. Concordantly, we were able to ex vivo engineer CD11b+ cells to express 3BNC117 from the IgH locus (Extended Data Fig. 4l–p). Cumulatively, our data indicate that, pending CRISPR–Cas9 mediated on-target integration, both B and non-B cells can express the antibody on the membrane, but only B cells proliferate subsequent to antigen engagement.
Coding the sgRNA and Cas9 on different vectors prevents cleavage in the absence of donor DNA. The coding of the single guide RNA (sgRNA) together with the saCas9 on the same AAV is predicted to allow DNA cleavage in many cells that are not cotransduced with the donor AAV. The resulting, nonproductive, cleavage may be avoided if the sgRNA cassette is instead separated from the saCas9 gene and coded on the donor AAV (Extended Data Fig. 5a).

Repeating the above mouse experiments (Fig. 2a) with this new pair of AAVs allowed high 3BNC117 titers capable of neutralizing autologous YU2.DG and heterologous JRFL HIV pseudoviruses following repeated immunizations (Extended Data Fig. 5b–d), and the frequency of 3BNC117-expressing cells reached 0.5% of total blood B cells (Extended Data Fig. 5e,f). On killing the mice at day 136, up to 10% of splenic plasmablasts expressed 3BNC117 (Extended Data Fig. 5g,h). In addition, up to 7% of splenic B cells with a germinal center phenotype expressed 3BNC117 (Extended Data Fig. 5i,j), while 1 and 3% of the bone marrow cells expressed 3BNC117, with or without coexpressing CD19, respectively (Extended Data Fig. 5k–m). These results are of the same range as those obtained when the sgRNA was coded together with the saCas9, although direct side-by-side comparison is hindered by the use of different ubiquitously active promoters. The overall numbers of splenic plasmablasts, germinal center B cells and bone marrow plasma cells were similar to those in the control groups (Extended Data Fig. 5n,o), mitigating concerns of B cell neoplasm (Extended Data Fig. 3e,f).

Driving Cas9 expression by a B cell-specific promoter prevents cleavage in undesired tissues. To further increase the safety of our approach, we next coded the saCas9 under the control of the CD19, B cell-specific, promoter44 (Fig. 6a).

Fig. 5 | Assessing expression of the transgene in different subsets of cells. a, Vector design. The donor cassette expresses a GFP, separated from the 3BNC117 cassette by a 2A peptide. b, Experimental design. c,d, Quantification of GFP+ 3BNC117+ in the spleen (c) or bone marrow (d) of recipient mice. Mean values and standard deviation are indicated. For each group, n = 3 biologically independent mice. *P = 0.0284, ***P = 0.0004 for two-way ANOVA. e, Quantification of GFP+ cells in spleen. ****P < 0.0001 for two-way ANOVA and ****P < 0.0001 for two-way ANOVA with Tukey’s multiple comparison. f,g, Quantification of the 3BNC117+ CD138+ population from B220+, GFP+ cells in the spleen (f) *P = 0.0147 for unpaired two-tailed t-test, or bone marrow (g) *P = 0.0471 for unpaired two-tailed t-test. Mean values are indicated.
were immunized with 20 µg of HIV gp120, and 6 days later each mouse was coinjected with one vector coding for saCas9, regulated by the CD19 promoter, and a second vector coding both the bNAb and the sgRNA (Fig. 6a,b). The mice then received up to six additional immunizations. Already, after four immunizations, treated mice had up to 2 µg ml⁻¹ of the 3BNC117 bNAb in their blood (Fig. 6c). 3BNC117 blood titers did not go down by 30 days later, irrespective of whether additional immunizations were administered (Extended Data Fig. 6a). Regardless of the immunization regimen, similar titers were obtained using promiscuous or B cell-specific regulation over saCas9 expression. Concordantly, 45 days after the fourth immunization, similar rates of 3BNC117-secreting cells
could be detected in the bone marrow using ELISPOT (Fig. 6d,e), irrespective of whether additional immunizations were administered and irrespective of the saCas9 promoter (Extended Data Fig. 6b–d). Therefore, replacing the saCas9 promoter does not preclude the therapeutic effect, which is stable after four immunizations. In addition, using CD19 rather than CMV promoter, to drive saCas9 expression, reduces the engineering rate of B cell progenitors as assessed following in vitro differentiation of IL7R enriched bone marrow cells (Extended Data Fig. 7). Even when the CMV promoter is used to drive saCas9 expression, bone marrow hematopoietic stem and progenitor cells may not be engineered, as similarly low 3BNCl17 staining and enzyme-linked immunosorbent assay (ELISA) levels are obtained following syngeneic transplantation of Lin− enriched cells from mice injected with the donor vector with or without the saCas9 coding vector (Extended Data Fig. 8).

To assess possible effects on biodistribution and safety, different groups of mice were euthanized for tissue analysis, 3 days after having been coinjected with the donor + sgRNA vector and with a second vector coding for saCas9 under the control of either a ubiquitous promoter or the B cell-specific CD19 promoter. Similar transduction rates were obtained for vectors coding the saCas9 under the regulation of the CD19 or spleen focus forming virus (SFFV) promoters (Fig. 6f,g). However, the CD19 promoter significantly reduced saCas9 expression in the liver, while not reducing expression in peripheral blood mononuclear cells (Fig. 6h). The rates of on-target cleavage in the liver or the spleen, as measured by TIDE analysis, were significantly above background only when using the CMV or SFFV promoters, rather than the CD19 promoter, to drive saCas9 expression (Fig. 6i). Therefore, separating the coding of saCas9 and the sgRNA between the two AAVs and expressing saCas9 under a B cell-specific promoter reduce undesired cleavage to below our limit of detection while allowing high 3BNCl17 titers following immunizations.

**Discussion**

Eliciting a specific, neutralizing antibody response to hypervariable viruses is a long-standing challenge in medicine. B cell engineering provides an opportunity to express desired therapeutic antibodies for adaptive immunity. Here, we uniquely demonstrate that B cells can be safely and robustly engineered in vivo. A single, systemic dose of dual AAV-DJ coding for CRISPR–Cas9 and donor cassettes in mice allowed for site-specific integration, with limited off-target Cas9 expression and DNA double-strand breaks. On immunizations, the engineered B cells underwent antigen-induced activation leading to memory retention, clonal selection and differentiation into plasma cells that secrete the bNAb at neutralizing levels.

The monoclonal bNAb titers obtained by in vivo engineering in this work are similar or higher than those obtained by ex vivo engineering followed by adoptive transfer to immunocompetent mice17,18,19,20, with the exception of Huang et al17. The response of the in vivo engineered B cells to the antigen is not hindered by the endogenous polyclonal response to immunization, which can be highly potent21. In contrast to ex vivo engineering, in vivo B cell engineering is simple, fast and cost effective. It can and will be provided at the point of care, requiring no specialized facilities. Our approach further allows for CSR and clonal expansion, but the full functional consequences of these attributes will have to be tested in the prevention or treatment of infection models. Yet additional experiments may determine whether in vivo B cell engineering allows proper antibody glycosylation and expression patterns to avoid the formation of antidrug antibodies, as seen following muscle transduction for antibody expression5. The effects of anti-AAV antibodies and T cell responses would similarly have to be assessed. In engineered B cells, autoreactivity may occur due to heterogeneity in AAV production or due to pairing of the engineered heavy chain with the endogenous light chain. Future clinical applications must aim to reduce these sources of heterogeneity, if not sufficiently eliminated by natural tolerance mechanisms22,23. Still, future modifications may include coding the bNAb as a single chain to reduce mispairing of the bNAb heavy chain with the endogenous light chain, potentially improving both safety and efficacy. Such single chain coding can further allow the expression of bispecific bNAbs, which may be required to provide long-term protection from HIV resurgence. Safety may be further improved by using more specific nucleases24,25 and by having the bNAb gene preceded by a splice acceptor rather than by a promoter, to reduce expression from off-target integration26,27. Both safety and efficacy may benefit from embedding B cell-specific targeting moieties in the AAV vector28 or in a nonviral alternative29. The therapeutic impact of our approach may best be evaluated in nonhuman primates with HIV-like infections. In the nonhuman primates as in HIV infected individuals, undergoing controlled treatment interruption, we expect a continuous and much more potent antigen-induced activation, which can either replace or complement an immunization regimen to achieve higher antibody titers and do so in a shorter time frame. Finally, in vivo B cell engineering may have diverse future applications as it may be used to address other persistent infections as well as to treat autoimmune diseases, genetic disorders and cancer.

**Online content**

Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41587-022-01328-9.

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Methods

Plasmid cloning. For the CMV−Cas9gRNA vector, pX601 (ref. 7) (Addgene) was cleaved with Bsal and pre-annexed, phosphorylated (PNK, NEB), sgRNA coding oligo-deoxynucleotides were ligated using T4 DNA Ligase (NEB). For the CD3Δ Cas9 vector, pBAR270 (ref. 7) was cleaved using NotI and SpeI (NEB) and an scCas9 coding fragment, amplified from pX601, as well as the murine CD19 promoter, amplified from wild type C57BL/6OlaHsd gDNA, were assembled using Hi-Fr DNA Assembly Mix (NEB). For the SFFV−Cas9 vector, pBAR270 was cleaved with NotI and SpeI (NEB). The fragment coding the SFFV promoter was amplified from GW17 (Cay Laboratory, Stanford) and the scCas9 was amplified from pX601. The fragments were assembled using Hi-Fi DNA Assembly Mix (NEB). For the donor gRNA vector, the U6−gRNA fragment was amplified from ligated pX601 with the murine Igf1 sgRNA used in this study, and the fragment was assembled using Hi-Fr DNA Assembly Mix (NEB) into the donor vector pADN171XS (ref. 7).

IgH Cas9 vector, pAB270 (ref. 51) was cleaved using NotI and SpeI (NEB) and the saCas9 oligo-deoxynucleotides were ligated using T4 DNA Ligase (NEB). For the CD19−flash)52. The default parameters were changed to allow for lower maximum TapeStation. Combined libraries were loaded at 5 pM with 25% PhiX control were quantified using Qubit (Invitrogen) and analyzed using an Agilent 4200

sites was performed using the proofreading PrimeStarMAX Polymerase (Takara) to nucleotides to the donor vector from Nahmad et al.7 and named ‘donor’ in this paper), a fragment was amplified from pADN171CF2 (GFP expressing vector from Nahmad et al.) and a homology arms-bearing vector cleaved with XhoI7. A list of primers used for cloning were amplified with 0.1 and 0.001 mg g−1 of antigen in Alum (Invitrogen)7,8. For AAV injections, mice were anesthetized or MD39-ferritin were performed as previously described, using 20 μg ml−1 of an anti idiotypic antibody against 3BC117 in PBS overnight at 4 °C. Plates were washed with PBS with Tween (PBST), blocked for 1 h at 37 °C and 5% CO2. Viability at the time of an anti-idiotypic antibody against 3BC117 in PBS overnight at 4 °C. Plates were washed with PBS with Tween (PBST), blocked for 1 h at 37 °C and 5% CO2. Viability at the time
Neutralization assays. Under sterile BSL2/3 conditions, the P3G3 plasmid was cotransfected into HEK293T cells along with JRF1 or YU2 HIV envelope plasmids and Lipofectamine 2000 transfection reagent (ThermoFisher Scientific) to produce a single round of infection of AAV9. For comparison among multiple clades of HIV, HEK293T cells were plated in advance overnight with DMEM medium + 10% FBS + 1% Pen/Strep + 1% l-glutamine. Transfection was done with Opti-MEM transfection medium (Gibco) using Lipofectamine 2000. Fresh medium was added 12 h after transfection. Supernatants containing the viruses were harvested 72 h later. In sterile 96-well plates, 25 µl of virus was immediately mixed with 25 µl of serially diluted (2x) bead protein A/G purified IgG (ThermoFisher) from mouse sera (starting at 500 ng µl⁻¹) and incubated for 1 h at 37 °C to allow for antibody neutralization of the pseudoviruses. 10,000 TZM-bl cells per well (in 50 µl of media containing 20 µg ml⁻¹ Dextran) were directly added to the antibody virus mixture. Plates were incubated at 37 °C for 48 h. Following the incubation, TZM-bl cells were lysed using 1x luciferase lysis buffer (25 mM G-6-P, pH 7.8, 15 mM MgSO4, 4 mM EGTA, 1% Triton X-100). Neutralizing antibody titers and qPCR System (Applied Biosystems) using SYBRGreen (PCR Biosystems). For fold change of AAV titers and qPCR was performed using RevertAid (ThermoFisher) and random treatment on-column and postpurification using RQ1 DNAse (Promega). Reverse transcription was performed using RevertAid (ThermoFisher) and random hexamer primers. Data collection and analysis were performed on a StepOnePlus qPCR System (Applied Biosystems) using SYBRGreen (PCR Biosystems). For fold change of AAV titers and Cas9 relative expression, we used the relative quantity method.[58]

Flow cytometry. Collected cells from spleen, bone marrow or blood were resuspended in cell staining buffer (Biolegend) and incubated with 2 µg per 100 µl of human anti-3BNC117 and, where applicable as indicated in the legend, with 1 µg per 100 µl of TruStain FcX (Biolegend) for 10 min, washed and resuspended again in cell staining buffer containing conjugated primary antibodies. A list of antibodies and respective dilutions used in these experiments can be found in Supplementary Table 1. Secondary staining was performed in the dark, for 15 min, with anti-human IgG1 Alexa488 (Abcam) or anti-human IgG BV421 (Biolegend) or anti-human IgGl FITC (Biolegend). For primary IgG1 staining, cells were incubated with 2 µg per 100 µl of gp120. Then, cells were washed and data acquisition was performed on a CytoFLEX (Beckman Coulter) or Attune NxT (Life Technologies) or FACS Aria III (BD Biosciences) for experiments involving cell sorting. Flow data collection was performed with CytExpert. Data were compiled and analyzed using Kaluza Analysis v2.1 (Beckman Coulter). Gating strategies can be found in Extended Data Fig. 9.

In vitro B progenitor differentiation. For enrichment of IL7R+ cells, bone marrow from the tibia and femur bones of each mouse was collected at day 77 by flushing (Extended Data Fig. 7a). BRC lysis was performed for 10 min using RBC Lysis Buffer (Biorad) at room temperature. After washing, cells were resuspended at 2 × 10⁶ cells per ml in PBS supplemented with 10% FCS (Sigma). PE-conjugated antihuman IL7R (120-048-801, Milteny) was added 1/100 and binding was performed on ice for 30 min. Cells were subsequently washed and resuspended at 100,000 cells per ml in PBS/1% FCS. Anti-CD19 and anti-human IgG1 FITC (Biolegend) were used as conjugates and for a total of 30 min. CD19+ B cells were detected with 100,000 cells per ml and separated on liquid separation or magnetic separation magnetic columns (Milteny). Following separation, eluate was analyzed for IL7R and CD19 expression (Extended Data Fig. 7c) and in vitro differentiation was performed as follows. Cells were seeded in RPMI 1640 (Biological Industries) supplemented with P/S (Biological Industries), 55 µM β-mercaptoethanol, 10% FCS [Sigma] and 10 ng ml⁻¹ mouse IL7 (Peprotech) at approx. 2 × 10⁵ cells per ml. Three days following seeding (Extended Data Fig. 7b) cells were washed and reseeded in RPMI 1640 (Biological Industries) supplemented with P/S (Biological Industries), 55 µM β-mercaptoethanol, 10% FCS [Sigma] and 10 ng ml⁻¹ mouse IL4 (Peprotech), 10 µM lipopolysaccharide (LPS) (Peprotech). Two days following seeding, cells were washed and seeded on 60 mm irradated CD40L/BL feeder cells in RPMI 1640 (Biological Industries) supplemented with P/S (Biological Industries), 55 µM β-mercaptoethanol, 10% FCS [Sigma] and 10 ng ml⁻¹ mouse IL4 (Peprotech) and 10 ng ml⁻¹ mouse IL21 (Peprotech) at 5 × 10⁵ cells per ml. Two days following seeding, supernatant and cell aliquots were collected for ELISA and flow cytometry, respectively. The rest were washed and reseeded on 60 gray irradiated CD40L/BL feeder cells in RPMI 1640 (Biological Industries) supplemented with P/S (Biological Industries), 55 µM β-mercaptoethanol, 10% FCS [Sigma] and 10 ng ml⁻¹ mouse IL21 (Peprotech) at 5 × 10⁵ cells per ml. Finally, 2 d following seeding, cells and supernatant were collected for flow cytometry and ELISA, respectively.

Hematopoietic stem and progenitor enrichment and adoptive transfer. For bone marrow Lin- hematopoietic stem and progenitor cells enrichment, cells were collected from the tibia and femur bones of female CD45.2 C57BL/6OlaHsd mice at day 97 by flushing (Extended Data Fig. 8a). RBC lysis was performed for 10 min using RBC Lysis Buffer (Biorad) at room temperature. Following washing, cells were CD19+Lin- positively enriched using Mouse Lineage CD19-CD45-CD3- Cell Depletion Kit (Milteny) using liquid separation or magnetic separation magnetic columns (Milteny). According to the manufacturer’s instructions.

Following separation, flow through aliquots were analyzed for Lin and B220 expression. The rest of the cells were adoptively transferred into mice as with an adaptation of a previously described protocol.[56] In short, recipient female, 8 weeks old, CD45.1 C57BL/6OlaHsd mice were sublethally irradiated at 150 cGy and, the following day, received 6 × 10⁶ cells per 100 µl per mouse in PBS by retro-orbital injections.

In vitro FcR loading. Primary bone marrow CD11b+ were collected 5 days following activation and 2 x 10⁵ cells were further cultured in FBS/ granulocyte-macrophage colony-stimulating factor (GMCSF) supplemented DMEM with 2 µg ml⁻¹ of purified mouse Igk/IgG2a 3BNC117. Cells were collected after 20 min and then FcRx blocked before analysis by flow cytometry using the anti-3BNC117 anti-idiotype antibody.

In vitro engineering of B cells. CRISPR-Cas9 RNP electroporations and AAV transductions of B cells was performed as described previously.[61] In short, total splenocyte lymphocytes were collected from spleens of naïve mice and were cultured in FBS supplemented RPMI with 2 µg ml⁻¹ of purified mouse Igk/IgG2a 3BNC117. Cells were collected after 20 min and then FcRx blocked before analysis by flow cytometry using the anti-3BNC117 anti-idiotype antibody.

In vitro engineering of CD11b+ cells. Total bone marrow cells were extracted by flushing tibia and femur. Cells were cultured in DMEM (Biological Industries) supplemented with 15% FBS (Biological Industries) and 50 ng ml⁻¹ of GMCSF (Peprotech) to activate and proliferate CD11b+ cells. Five days following activation, 1 x 10⁶ cells were electroporated with pregenenerated complexes of 20 µmol spCas9 (IDT) and 25 pmol sgRNA (IDT) in buffer T at 1,600 V, 20 ms, 1 pulse. Immediate AAAD-J transduction was performed at 200,000 MOI. Cells were subsequently cultured for 1 day at 1 x 10⁶ cells per ml in GMCSF/FBS supplemented DMEM and for an additional 5 d without GMCSF before analysis flow cytometry.

Nucleic acid manipulations. For RT-PCR demonstrating 3BNC117 gene integration into the Igk locus, RNA was extracted from sorted engineered B cells (3BNC117+, C64+, CD19+) on a FACs BD Aria III (BD Biosciences). As a positive control, we used in vitro engineered mouse splenic lymphocytes, as described previously. In short, mouse splenic lymphocytes were activated with 10 µg ml⁻¹ LPS (Santa-Cruz Biotechnology) and 10 ng ml⁻¹ IL-4 (Peprotech) for 24h, electroporated by CRISPR–Cas9 RNP using a Neon Electroporation System (Invitrogen) and transduced at 50,000 MOI of the donor AAAD-J vector. For RNA extraction, we used RNAesy Mini Kit (Qagen) with DNase treatment on-column and postpurification using RQ1 DNase (Promega). Reverse transcription was performed using RevertAid (ThermoFisher) and random hexamer primers. Data collection was performed with CytExpert. Data were compiled and analyzed using Kaluza Analysis v2.1 (Beckman Coulter). Gating strategies can be found in Extended Data Fig. 9.
beads (Beckman Coulter) at a 1:1 ratio. Purified amplicons were Sanger sequenced at the DNA Sequencing Unit, Tel Aviv University. For each sample, multiple sequencing reactions were performed using either primers. Then, samples were compared using TIDE (https://tide.nki.nl/). For control samples, we performed reciprocal sample comparisons from the independent initial PCR reactions. For all samples, along with the CMV expressed Cas9, mice received the donor vector. Along with the SFFV or CD19 expressed Cas9, mice received the donor vector. Control samples come from naïve splenic lymphocytes (Fig. 6i).

Anti-idiotype to 3BNCl17 scFv was generated by phage display. Candidates were cloned into pcDNA3.1 vectors with the human kappa and IgG1 heavy chain. In short, antibodies were produced by transfection of both antibody chains into Expi293F cells (Gibco) by ExpiFectamine (Gibco) and purified using MabSelect (GE Healthcare) as previously described. Specificity and sensitivity of the antibody were verified in vitro using primary B cells engineered, as described previously, to express one of the three bNabs, 3BNCl17, VRC01 or 10-1074. Both 3BNCl17 and VRC01 bind to the CD4 binding site. However, only 3BNCl17 binds to its anti-idiotype (Extended Data Fig. 10).

For reverse transcription PCR of ex vivo engineered cells, RNA was extracted, two days following treatment, from 5 × 10⁷ splenic lymphocytes or 6 d following treatment from 5 × 10⁶ GMCSF activated bone marrow cells. RNA extraction was performed using Quick-RNA micro prep (Zymo Research) without DNAse treatment. Reverse transcription was performed using RevertAid (ThermoFisher) and Oligo dT primers. PCR of exon–exon junctions on the resulting cDNA was performed for 30 cycles using HS Taq Mix (PCRBio), a list of primers used for these reactions can be found in Supplementary Table 1.

Statistics. Statistical analyses were performed on distinct samples using Prism (GraphPad). For the area under the curve (AUC), in each group, the mean AUC and s.d. were calculated and these values were compared by t-test. All t-tests were performed as two-tailed. For the TIDE analysis, each comparison between -test. All statistics. Statistical analyses were performed on distinct samples using Prism (GraphPad). For the area under the curve (AUC), in each group, the mean AUC and s.d. were calculated and these values were compared by t-test. All t-tests were performed as two-tailed. For the TIDE analysis, each comparison between -test. All comparisons were performed using TIDE (https://tide.nki.nl/). For control samples, we performed reciprocal sample comparisons from the independent initial PCR reactions. For all samples, along with the CMV expressed Cas9, mice received the donor vector. Along with the SFFV or CD19 expressed Cas9, mice received the donor vector. Control samples come from naïve splenic lymphocytes (Fig. 6i).

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Author contributions
A.D.N. designed, performed and analyzed the study. C.R.I. performed CHANGE-seq; S.Q.T. supervised CHANGE-seq experiments. N.Z. and T.K. performed bioinformatical analyses. A.S. and R.R.-A. supervised the bioinformatical analyses. N.Z., M.H.-F. and I.R. helped with sample processing. Y.R. helped with vector design and cloning. D. Nataf and L.D. designed the B cell progenitor enrichment. M.T. and D.H. performed neutralization assays. D. Nemazee and I.E.V. supervised neutralization assays. I.D. contributed to supervising the study. Y.C. helped with experimental design. A.D.N. and A.B. drafted and revised the manuscript. A.B. conceptualized and supervised the study.

Competing interests
A.D.N., D. Nataf, M.H.-F. and D.H. are listed as inventors on patent applications covering B cell engineering. A.D.N. and A.B. have an equity stake in and receive monetary compensation from Tabby Therapeutics Ltd, a B cell engineering company. S.Q.T. is a coinventor on patents covering the CHANGE-seq method. S.Q.T. is a member of the scientific advisory boards of Kromatid, Inc. and Twelve Bio. The other authors declare no competing interests.

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Extended Data Fig. 1 | Multiple isotypes of the 3BNC117 antibody are expressed by engineered B cells. a–d. ELISA for each isotype. a. IgM, b. IgG1, c. IgG2c and d. IgA. All samples come from the CMV-Cas9gRNA+ donor injected mice at different time points, as indicated in each legend. Mean and SD are indicated. $n = 3$ biologically independent animals. e,f. Area under the curve (AUC) for A–D. e. IgM, ns; $p_v = 0.1288, **; p_v = 0.0008, ***; p_v = 0.0062, f. IgG1, ns; $p_v = 0.131$ and from top to bottom as presented in the graph, **; $p_v = 0.0044$ and $p_v = 0.0013, **; p_v = 0.0195, **; p_v = 0.0098, h. IgA, ns; $p_v = 0.0587, **; p_v = 0.0013, *; p_v = 0.0403$, for two-sided unpaired t-test. $n = 3$ biologically independent animals. For A–H, sample collection day is indicated. Mean values are indicated. i. Fraction of 3BNC117 IgG titers as quantified by ELISA using purified gp120 binding sera from donor injected mice immunized with gp120, at day 37. ***; $p_v = 0.0007$ for two-sided unpaired t-test. $n = 3$ biologically independent samples. Mean values are indicated.
Extended Data Fig. 2 | bNAb genomic integration, sera titers and neutralization as a function of immunizations and co-injection of the CRISPR-Cas9 vector. **A.** Area under the Curve (AUC) of Fig. 2d for YU2.DG (left) and JRFL (right) **; pv = 0.0036 (YU2.DG) and pv = 0.005 (JRFL) for unpaired t-test for CMV-Cas9gRNA + donor to PBS comparison and #; pv = 0.0072 (YU2.DG) and pv = 0.0063 (JRFL) for one-sample t-test for Naive to PBS comparison. n = 3 for CMV-Cas9gRNA + donor and PBS. Naive sample is from a single, non-immunized, non-AAV-injected mouse. Mean values ± SD are indicated. **B.** Area under the curve (AUC) of Fig. 2c. From top to bottom, ***; pv = 0.0185 and pv = 0.0103, **; pv = 0.0036 for two-sided unpaired t-test. n = 3 biologically independent animals. Mean values ± SEM are indicated. **C.** RT-PCR on RNA from sorted, 3BNC117⁺, CD19⁺, CD4⁻ blood lymphocytes from day 37. Here, we used a reverse primer in a membranal exon of either IgHCμ or IgHCγ (all subtypes) and a forward primer on the VH of the coded 3BNC117. Numbers indicate different mice, injected with either a) PBS, b) the donor vector and the CMV-Cas9gRNA vector, or c) the donor vector only, as indicated above the gels. Control sample (C++) comes from in-vitro engineered primary mouse splenic lymphocytes, as described previously7. Ladder sizes are indicated on the left. Arrow indicates the expected amplicon size. For each group, experiment was reproduced 3 times with independent samples, as indicated by the numbers. Molecular weight markers (M) and their respective size in base pairs (MW) are indicated. **D.** Total DNA from the previous reaction as in (C) was purified and a semi-nested PCR with the same forward primer and a reverse primer on the CH1 of the respective constant domains. Ladder sizes are indicated on the left. Arrow indicates the expected amplicon size. For each group, experiments were reproduced 3 times with independent samples. Molecular weight markers (M) and their respective size in base pairs (MW) are indicated. **E.** Sanger sequencing alignment and chromatogram of the purified amplicon from the previous step. Reference sequences are indicated above the alignment. For the IgHCγ, each subtype reference is indicated. Sequencing of the IgHCμ amplicon of donor 3 has failed. **F.** Experimental design for (G-I). Splenic lymphocytes were activated with LPS and IL-4 and engineered, ex vivo, by AAV transduction and Cas9 electroporation with or without a gRNA. **G.** Flow cytometry of engineered splenic lymphocytes two days following treatment. Pregated on live, singlets. FcR block was used in the staining. Engineering parameters are indicated above each plot. **H.** EtBr gel electrophoresis showing products of an RT-PCR reaction with RNA from cells two days following treatment as in (F). For each sample, a control (C) reaction was performed amplifying the endogenous IgHGI cDNA. Ladder sizes are indicated on the left. Arrow indicates the expected amplicon size. The experiment was reproduced once, with similar results. Molecular weight markers (M) and their respective size in base pairs (MW) are indicated. **I.** Sanger sequencing of the previous amplicons, confirming the integration.
Extended Data Fig. 3 | Detection of engineered B cells in the spleen, the blood and the bone marrow. a. Flow cytometry plots demonstrating 3BNC117 expression among CD19^+ CD11b^- cells in the spleen at day 82 of 2CC immunized mice. Pregated on live, singlets. FcR block was used in the staining. b. Quantification of B. ***; \(p = 0.0006\) for two-sided unpaired t-test. c. Flow cytometry plots demonstrating 3BNC117 expression among blood B cells (CD19^+, CD4^-). d. Quantification of blood 3BNC117-expressing cells over time. The black arrows indicate immunizations and the blue arrow indicates AAV injection. ####; \(p < 0.0001\) for Two-Way ANOVA comparison between groups and *; \(p = 0.0133\), ****; \(p < 0.0001\) Two-Way ANOVA with Šidák's multiple comparison for time points comparison to PBS. For each group, each line represents the mean ± SD of \(n = 3\) biologically independent mice. e. Quantification of total CD38^+ CD138^+ in spleens of recipient mice as in Fig. 3a. From top to bottom: ns; \(p = 0.2380\) and \(p = 0.9907\) for One-Way ANOVA with Tukey's multiple comparisons test. f. Quantification of total GL7^+ CD95^+ cells as in Fig. 3e. From top to bottom: ns; \(p = 0.9857\) and \(p = 0.9985\) for One-Way ANOVA with Tukey's multiple comparison. g. Flow cytometry plots demonstrating the presence of 3BNC117-expressing B cells in the bone marrow. h-i. Quantification of G. ns; \(p = 0.9965\), ****; \(p < 0.0001\) (G) and from top to bottom: ns; \(p = 0.9857\) and \(p = 0.0372\) (H) for One-Way ANOVA with Tukey's multiple comparison.
Extended Data Fig. 4 | See next page for caption.
Extended Data Fig. 4 | Assessing expression of the transgene in different subsets of cells. 

**a.** Flow cytometry examples for Fig. 5c-g and Extended Data Fig. 3b-e, j-k. 
**b.** Quantification of 3BNC117+ cells in bone marrow. 

- #; pv = 0.0129 for Two-Way ANOVA and *; pv = 0.0255 for Two-Way ANOVA with Tukey’s multiple comparison.
- &; pv = 0.0335, **** = pv < 0.0001 for One-Way ANOVA with Tukey’s multiple comparison.

**c.** Quantification of the indicated populations from 3BNC117+ B220− cells in the bone marrow.

- *; pv = 0.0214, **** = pv < 0.0001 for unpaired two-tailed t-test. Mean values are indicated.

**f.** Experimental scheme for (G). Bone marrow cells were collected and activated in the presence of GMCSF. 5 days later, cells were cultured in the presence of purified mouse 3BNC117 for FcR presentation and then collected for analysis by flow cytometry.

**i.** Analysis by flow cytometry, using the anti-3BNC117 anti-idiotypic antibody, of bone marrow cells activated by GMCSF and subsequently cultured with 3BNC117. Experimental conditions are indicated above. Pre-gated on CD11b, live, singlets.

**l.** Experimental scheme of in-vitro engineering of primary GMCSF activated bone marrow cells for (M-N).

**m.** EtBr gel electrophoresis showing the product of an RT-PCR reaction of RNA from activated bone marrow cells, six days following treatment as in (L). For each sample, two reactions were performed (N or E). N amplifies endogenous IgHC µ mRNA. E amplifies the transgene mRNA joined by splicing to the IgHC µ exons following engineering. Indicated labeling of the amplicons (E1, E2, N1 and N2) performed for reference in (N). The experiment was performed once with expected results. Molecular weight markers (M) and their respective size in base pairs (MW) are indicated.

**n.** Sanger sequencing of the amplicons, confirming correct integration. Amplicons are annotated E1, E2, N1 and N2 as in (M).

**p.** Flow cytometry example of engineered GMCSF activated primary bone marrow cells. Pre-gated on B220−, live, singlets. 

Control cells are cells transduced with the AAV and electroporated only with the spCas9, without the sgRNA (-gRNA). For A-P and Fig. 3, FcR block was used in the staining. For each group, n = 2 biologically independent samples.
Extended Data Fig. 5 | See next page for caption.
Extended Data Fig. 5 | Engineering B cells with the sgRNA coded on the donor AAV. a. Vector maps of the AAVs coding for the donorRNA and the SFFV-Cas9. b. 3BNC117 IgG titers as quantified by ELISA over time in the SFFV-Cas9 + donorRNA group. The black arrows indicate immunizations and the blue arrow indicates AAV injection. Each line represents a mouse. ***; pv = 0.0005 for Two-Way ANOVA comparing the SFFV-Cas9 + donorRNA group to the donor group. n = 3 biologically independent mice. In this panel, the PBS and donor control groups are the same as for Fig. 2B. c. Transduction neutralization of TZM.bl cells by the YU2.DG (top) and JRFL (bottom) HIV pseudoviruses in the presence of day 136 sera IgGs. Neutralization is calculated as percent reduction from maximal luminescence per sample. The PBS control received immunizations, while the naïve control represents serum IgG from an untreated mouse. ns = non-significant, *= pv = 0.0462 and from top to bottom: ***, ***, ***; pv < 0.0001, Two-Way ANOVA with Šidák’s multiple comparison for time points comparison to PBS. d. Area under the Curve (AUC) for C. for YU2.DG and JRFL. ns; pv = 0.0667 (YU2.DG) and *; pv = 0.0103 (JRFL) for two-sided unpaired t-test for CMV-Cas9gRNA + donor to PBS comparison and #; pv = 0.0097 (YU2.DG) and *; pv = 0.0078 (JRFL) for two-sided one-sample t-test for naïve to PBS comparison. n = 3 for CMV-Cas9gRNA + donor and PBS. Naive sample from a single, non-immunized, non-AAV-injected mouse. In C-D, the PBS and control group is the same as for Fig. 2c and Extended Data Fig. 2a. Mean values ± SD are indicated. e. Representative flow cytometry analysis of 3BNC117+, CD19+, CD4− blood lymphocytes over time in the SFFV-Cas9 + donorRNA group. f. Quantification of E. The black arrows indicate immunizations and the blue arrow indicates AAV injection. ###; pv = 0.0006 for Two-Way ANOVA comparison between groups and ****; pv < 0.0001 for Two-Way ANOVA with Šidák’s multiple comparison for time points comparison to PBS. For each group, each line represents the mean ±SD of n = 3 biologically independent mice. g. Representative flow cytometry analysis of 3BNC117+, CD19+, CD38+, CD138+ plasmablasts in the spleens of the SFFV-Cas9 + donorRNA group at day 136. h. Quantification of G. Mean is indicated by the bars. ns; pv = 9892, **; pv = 0.0005, one-way ANOVA with Tukey’s multiple comparison. i. Representative flow cytometry analysis of GL7+, Fas/CD95+ GC B cells in the spleens of the SFFV-Cas9 + donorRNA group at day 136. j. Quantification of I. Mean is indicated by the bars. ns; pv = 0.8916, **; pv = 0.0075, one-way ANOVA with Tukey’s multiple comparison. k. Representative flow cytometry analysis of 3BNC117+ cells in total bone marrow (BM) of the SFFV-Cas9 + donorRNA group at day 136. l. Quantification of k. Mean is indicated by the bars. One-way ANOVA with Tukey’s multiple comparison. n-o. Assessing overall immune homeostasis. Quantification by flow cytometry of total CD38+ CD138+ plasmablasts in spleen (N) ns; pv = 0.5622, total GL7+, Fas+ GC B cells in the spleen (O), at day 136 ns; pv = 0.9926. Mean is indicated by the bars. One-way ANOVA with Tukey’s multiple comparison. For E-O, the PBS and donor control groups are the same as for Fig. 3 and Extended Data Fig. 3.
Extended Data Fig. 6 | Long-term persistence of serum antibodies and antibody-secreting cells in the bone marrow. **a**. Experimental design. Here, all mice were immunized 4 times (days 8, 23, 38 and 53) and selected groups received 2 additional immunizations on days 68 and 83. **b**. 3BNC117 IgG titers as quantified by ELISA over time. For each group, each line represents the mean ± SD between day 22 and day 67 represents n = 6 for biologically independent mice and n = 3 for day 67 to 97. **c–d**. Representative ELISPOT experiments (C) and quantification (D) of 3BNC117-secreting cells from the bone marrow of mice of the indicated groups. Numbers in parentheses represent the number of immunizations. From top to bottom followed by left to right, ns; p = 0.9844 and p = 0.9963 and p = 0.0884, *; p = 0.0135, **; p = 0.008, ***; p = 0.0005 and p = 0.0003 for One-Way ANOVA with Tukey’s multiple comparison. Mean values are indicated. Part of the data presented in panels B–D is presented also in Fig. 6c–e.
Extended Data Fig. 7 | Using CD19 rather than CMV promoter, to drive saCas9 expression, reduces the engineering rate of B cell progenitors. a. Origin of cells used in this experiment. Bone marrows were extracted 100 days following AAV injection and enriched for IL7R⁺. b. Experimental scheme. Enriched IL7R⁺ cells were grown in the presence of multiple activation factors as indicated in the representative timeline, numbers indicate days. Horizontal bars below the timeline indicate the presence of a specific factor supplemented to the growth media. c. Representative flow cytometry analysis of in-vitro differentiation of the IL7R⁺ enriched cells over time. Days as in (B) are indicated above the plots. d. Quantification of C. For each group, each line represents n = 3 biologically independent samples. e. Representative flow cytometry analysis of IL7R enrichment from mice, as in (A). f. Quantification of E. g. Representative flow cytometry analysis of 3BNC117⁺ CD19⁺ expression by cells, following 9 days of in-vitro differentiation, as in (B). h. Quantification of G. Each dot represents cells collected from a single mouse as in (A). ns; p = 0.9, *; p = 0.0122 and **; p = 0.0077 for One-Way ANOVA with Tukey's multiple comparison. For C-G, FcR block was used in staining.
Extended Data Fig. 8 | Low 3BNC117 staining and ELISA levels are obtained following syngeneic transplantation of Lin-enriched cells from mice injected with the donor vector with or without the saCas9 coding vector. a. Experimental scheme. CD45.2 mice received either a donor AAV expressing the gRNA or both a donor AAV and an AAV expressing saCas9 and the gRNA. Immunization protocol is indicated by the bars in black. On day 97, bone marrow cells were collected, enriched for lineage negative cells (Lin-) and transplanted into, recipient CD45.1 mice. Recipient mice are sublethally irradiated before transplantation and immunized after transplantation. b–c. Representative flow cytometry analysis (B) and quantification (C) of the enriched Lin- population from the donor mice. ns; pv = 0.9627 for One-Way ANOVA with Tukey’s multiple comparison. d. Representative flow cytometry analysis (D) of spleens (top) or bone marrows (bottom) from recipient mice at day 140, as in (A) e. Quantification of D. for the CD45.2+ CD45.1- population. From top to bottom: ns; pv = 0.4595 and pv > 0.9999 for Two-Way ANOVA with Tukey’s multiple comparison. f–g. Quantification of D. for the rate of 3BNC117-expressing cells for the spleen (F) or the bone marrow (G). From top to bottom: ns; pv = 0.9994 and pv = 0.0668 (F) and pv = 0.2371 and pv = 0.0560 for Two-Way ANOVA with Tukey’s multiple comparison. h. Serum 3BNC117 IgG titers at the indicated time points. The scale of the Y-axis was chosen to correspond to the other 3BNC117 titer plots in this manuscript. ns; pv = 0.1726t for Two-Way ANOVA. i. Representative ELISPOT assay of a day 140 bone marrow from CD45.1 recipient mice. j. Quantification I. ns; pv = 0.1756 for two-sided unpaired t-test. For B–D, FcR block was used in staining.
Extended Data Fig. 9 | Gating strategy for each experiment in this study. Gating strategy for each experiment in this study.
Extended Data Fig. 10 | Assessment of the specificity and sensitivity of the anti-idiotype for 3BNC117. a. Experimental scheme. Cells are engineered a day following extraction from spleen and activation. We used three different donor AAVs, each expressing a different antibody: either 3BNC117, VRC01 or 10−1074. b. Flow cytometry of gp120 or anti-idiotype binding of engineered cells, two days following treatment. Staining procedure is indicated above the plots. FcR block was used in staining. Each row indicates a different AAV used. Untransduced cells serve as the negative control. c. Quantification of B. ****; pv < 0.0001 for Two-Way ANOVA with Tukey's multiple comparison.
Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a Confirmed

☐ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement

☐ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly

☐ The statistical test(s) used AND whether they are one- or two-sided

*Only common tests should be described solely by name; describe more complex techniques in the Methods section.*

☐ A description of all covariates tested

☐ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons

☐ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)

☐ For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted

*Give P values as exact values whenever suitable.*

☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings

☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes

☐ Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection | Data collection for flow cytometry was performed using CytExpert software v3.2.1 | Data collection for MiSeq Illumina sequence was performed using MiSeq control software v3.1

Data analysis | For flow cytometry, data analysis was performed using Kaluza Analysis v2.1 [Beckman Coulter] | Statistical analysis and data visualization was performed using Prism 8 v8.4.3 or 9 v9.0.1 [GraphPad] | Pair-end alignment were performed using Fast Length Adjustment of Short Reads (FLASH) v1.2.11 [https://github.com/ewigler/flash] | On target CRISPR induced double stranded breaks were analyzed using CRISPRpic v1 [https://github.com/compbio/CRISPRpic] | Alignments were performed using Bowtie2 alignment analysis v2.4.2 [https://github.com/CaseLife/bowtie2] | Selection analysis was performed using SELECTION v2.4 [http://selecton.tau.ac.il/] | CHANGE-seq analysis was performed using CHANGE-seq analysis software v1.2.9.1 [https://github.com/tsalab/SJ/changeseq] | CRISPR induced double stranded breaks were compared using TIDE v3.3 [https://tide.nki.nl/] | Alignment of Sanger sequencing chromatograms was performed using Snapgene v5.3 and above (GSL Biotech)

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.
Data

Policy information about availability of data
All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

Data is available in the main text, in the Supplementary Data and Materials. Illumina sequencing data can be accessed in the SRA database under accession code PRJNA706552. The authors declare that all unique materials used are readily available from the authors upon MTA agreement.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- [x] Life sciences     - [ ] Behavioural & social sciences     - [ ] Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nure.com/documents/mp-reporting-summary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size
Due to ethical and financial consideration we used the minimal amount of animals that allowed the derivation of statistically significant conclusions. Thus, cohorts of 3 mice were used as the minimal amount of animals to allow the derivation of statistically significant conclusions. For other experiments, sample size was not predetermined and data was compiled as is.

Data exclusions
No data was excluded

Replication
All attempts for replication were successful. Experiments were replicated or performed independently at least once and up to four times, as described in the figures legends.

Randomization
Mice were allocated randomly into the different experimental groups. For experiments other than those involving mice, samples were allocated randomly.

Blinding
Experiments were not blinded. For off-target CRISPR induced double stranded breaks and somatic hypermutation / clonal selection, analysis was performed independently of experiments. For other experiments, analysis was performed by the same researchers that conducted the assays.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

| n/a | Involved in the study |
|-----|------------------------|
| [x] | Antibodies             |
| [x] | Eukaryotic cell lines  |
| [x] | Palaeontology and archaeology |
| [x] | Animals and other organisms |
| [x] | Human research-participants |
| [x] | Clinical data          |
| [x] | Dual use research of concern |

Methods

| n/a | Involved in the study |
|-----|------------------------|
| [x] | ChiP-seq               |
| [x] | Flow cytometry         |
| [x] | MRI-based neuroimaging |

Antibodies

| Antibodies used |
|-----------------|
| HRP conjugated anti-mouse IgM (Jackson ImmunoResearch, 715-035-140) |
| HRP conjugated anti-mouse IgG (Jackson ImmunoResearch, 715-035-151) |
| HRP conjugated anti-mouse IgG1 (Jackson ImmunoResearch, 715-035-205) |
| HRP conjugated anti-mouse IgA (SouthernBiotech 1040-06) |
| HRP conjugated anti-mouse IgG2c (BioRad, STAR-135P) |
| FITC labeled anti-mouse CD19 (Biolegend, 152403) |
| BV421 labeled anti-mouse CD19 (Biolegend, 115538) |
Validation

All BD Bioscience antibodies listed are tested by the manufacturer for flow cytometry on murine cells.

Murine targeting Biolegend antibodies listed are tested by the manufacturer for flow cytometry on murine cells.

Human targeting Biolegend antibodies listed are tested by the manufacturer for flow cytometry on human cells.

All invitrogen antibodies are tested by the manufacturer for flow cytometry on mouse splenocyte suspensions.

The Miltenyi Biotec antibody is tested by the manufacturer for flow cytometry on mouse splenocytes.

Southern Biotech antibodies listed are tested by the manufacturer for flow cytometry and ELISA with known reactivity to mouse.

All Jackson Immunoresearch antibodies were reported to work with ELISA on murine samples.

All BioRad antibodies listed are tested by the manufacturer for ELISA and flow cytometry on murine samples.

All abcam antibodies listed are tested by the manufacturer for ELISA and flow cytometry on murine samples.

In house produced antibodies were tested for flow cytometry and ELISA and have specificity for the idiotype of 38NC117 with data provided in this manuscript and in Nahmad et al. 2020, Nature Communications.

eBioscience antibodies are tested by the manufacturer for flow cytometry, on mouse splenocytes.

Eukaryotic cell lines

Policy information about <cell lines>

Cell line source(s) Exp293F cell line (ThermoFisher, A14635) was provided by the Wine lab, Tel Aviv University. 293t cells (ATCC CRL-3216) were provided commercially.

Authentication 293t cells were commonly identified by flow cytometry during study-unrelated experiments in the lab.

Expi293F cells were commonly identified by microscopy during routine checks.

Mycoplasma contamination Cell lines tested negative for mycoplasma contamination during routine checks.

Commonly misidentified lines (See ICLAC register) No commonly misidentified cell lines were used.

Animals and other organisms

Policy information about <studies involving animals, ARRIVE guidelines> recommended for reporting animal research.

Laboratory animals 6-9 weeks old female CD45.2 C57Bl/60lHsd mice (Envigo), housed in standard animal facility conditions as regulated by the Tel Aviv University Animal Facility, CD45.1 C57Bl/60lHsd mice were provided by the Lapidot lab, Weizmann Institute of Science and were also housed in standard animal facility conditions as regulated by the Tel Aviv University Animal Facility.

Wild animals The study did not involve wild animals.

Field-collected samples The study did not involve samples collected from the field.

Ethics oversight All mouse experiments were done with approval of Tel Aviv University ethical committees.

Note that full information on the approval of the study protocol must also be provided in the manuscript.
Flow Cytometry

Plots

_confirm that:

☒ The axis labels state the marker and fluorochrome used (e.g., CD4-FITC).
☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
☒ All plots are contour plots with outliers or pseudocolor plots.
☒ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

Blood samples from mice were collected in heparin. Cells and serum were separated by centrifugation. For spleens, whole spleens were extracted from mice and mechanically crushed in PBS to be filtered in a 70 μm cell strainer (Corning). For bone marrow, cells were flushed from the posterior femur and tibia. For blood, spleen and bone marrow, cells were processed with red blood cell lysis buffer (Biolegend) and plated in 1640 RPMI (Biological Industries) supplemented with 10% Hi FBS (Biological Industries) until processing, 30min-1hour following extraction.

Harvested cells from spleen, bone marrow or blood were resuspended in cell staining buffer (Biolegend) and incubated with 2μg/100μl of human anti-3BNC117 and, were relevant, 1μg/100μl of Anti-mouse CD16/32 (TruStain FcX, Biolegend) for 10 mins, washed and resuspended again in cell staining buffer containing secondary antibodies. Secondary staining was performed in the dark, for 25 mins. Cells were washed and resuspended in staining buffer before reading in an a CytoFLEX (Beckman Coulter), Attune Nxt (Life Technologies) flow cytometers or BD AriaII (BD Biosciences) FACS.

Instrument

CytoFLEX (Beckman Coulter) or Attune Nxt (Life Technologies) or BD AriaII (BD Biosciences)

Software

Kaluza (Beckman Coulter)

Cell population abundance

1000-5000 cells were sorted for engineered B cells [3BNC117+, CD4-, CD19+] phenotype on a FACS BD AriaII (BD Biosciences). RNA was extracted directly, in bulk, following sorting. No assessment for purity was performed.

Gating strategy

The gating strategy is detailed in the legend of each panel and depicted in figure S13

☒ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.