B cells engineered to express pathogen-specific antibodies protect against infection

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Effective vaccines inducing lifelong protection against many important infections such as respiratory syncytial virus (RSV), HIV, influenza virus, and Epstein-Barr virus (EBV) are not yet available despite decades of research. As an alternative to a protective vaccine, we developed a genetic engineering strategy in which CRISPR-Cas9 was used to replace endogenously encoded antibodies with antibodies targeting RSV, HIV, influenza virus, or EBV in primary human B cells. The engineered antibodies were expressed efficiently in primary B cells under the control of endogenous regulatory elements, which maintained normal antibody expression and secretion. Using engineered mouse B cells, we demonstrated that a single transfer of B cells engineered to express an antibody against RSV resulted in potent and durable protection against RSV infection in RAG1-deficient mice. This approach offers the opportunity to achieve sterilizing immunity against pathogens for which traditional vaccination has failed to induce or maintain protective antibody responses.

INTRODUCTION

Protective vaccines have reduced morbidity and mortality from several infectious diseases, in large part, by activating the humoral immune response and subsequent production of high-affinity pathogen-specific antibodies produced by B cells. Unfortunately, vaccines for many common diseases are not yet available despite considerable research efforts. One example is respiratory syncytial virus (RSV), a common pathogen that infects the upper and lower respiratory tracts. RSV is a serious threat to infants, the elderly, those with cardiopulmonary disease, and those undergoing hematopoietic stem cell transplant, where it is a substantial cause of morbidity and mortality (1–3). The first RSV vaccine trial occurred in 1966 but proved harmful when subsequent natural infection caused severe lower respiratory disease and two deaths (4). The second RSV vaccine trial occurred in 2016 and also failed to protect against infection (5). Although several other candidate RSV vaccines are being developed, it is unclear whether any will elicit a protective response.

Despite the failure of vaccine trials, evidence exists for antibody-mediated protection against RSV. Several monoclonal antibodies including the RSV-specific monoclonal palivizumab have been shown to protect against RSV infection in vitro or in vivo (6, 7). Similarly, monoclonal antibodies protective against HIV, influenza virus, Epstein-Barr virus (EBV), human metapneumovirus virus, dengue, Zika virus, Ebola virus, and many other pathogens are also being developed (8). However, the infusion of monoclonal antibodies such as palivizumab is limited to high-risk populations because monthly reinfusion is required to maintain protection. Although new approaches to increase the antibody half-life after injection have been developed (9), even the most promising of these strategies would require lifelong reinfusion to maintain protection.

To overcome the need for reinfusion, alternative strategies to generate long-term immunity have been explored. One approach involves viral transduction of muscle cells with an adenoviral vector encoding a protective antibody (10, 11). Another approach is transduction of hematopoietic stem cells with a lentivirus-encoded secreted antibody, which are differentiated into antibody-secreting plasma cells in vitro before infusion or allowed to differentiate in vivo after infusion (12, 13). A shared limitation of the adenoviral/muscle cell and lentiviral/stem cell approaches is that the level of antibody produced is fixed and unresponsive to infection. In contrast, protective vaccines elicit both long-lived memory B cells and antibody-secreting plasma cells. Memory B cells express a membrane-bound form of antibody that allows these cells to rapidly respond and differentiate into additional antibody-secreting cells upon infection.

In an effort to mimic the protective B cell response, we developed a genetic engineering strategy that allowed for the expression of protective antibodies against RSV, HIV, influenza, or EBV in mouse or human B cells under endogenous regulatory elements. This was challenging because fully functional B cells require alternative splicing and polyadenylation to produce membrane-bound and secreted antibodies, a process that is difficult to recapitulate in a viral transgene (14, 15). Adding an additional level of difficulty, antibodies are produced as the product of two genes, the heavy chain gene (IgH) and either the kappa (Igk) or lambda (Igl) light chain gene. Targeting the IgH locus is complicated by the large size and extreme genetic heterogeneity of this area in antibody-expressing B cells. Each developing B cell undergoes recombination of V, D, and J segments over more than a megabase of DNA within the IgH locus, and this results in variable regions that are essentially unique to each cell (16). This sequence variability makes directly targeting antibody coding regions challenging. One group recently bypassed this limitation by replacing the entire IgH variable region with the heavy chain VDJ of their choosing (17). This approach is promising but limited to antibodies that bind antigens without light chain involvement (17). Another recent study inserted the full light chain into the light chain V region and a secreted version of the IgH into the IgH V region (18). This work is limited in that only a secreted antibody was expressed, and it was unclear from this work whether expression of the endogenous antibody was eliminated (18). To build on this previous work, we developed a single-cut approach

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RESULTS
Targeting strategy and emAb cassette design

To circumvent the complexity of the antibody heavy chain gene, we focused on a small 2600-nucleotide (nt) region of DNA present in all B cells between the last J gene segment and the region involved in class switching. This region was further limited because of the presence of a critical intronic Eμ enhancer, one of several strong enhancer elements that cooperate to drive high-level expression of recombinant VDJ genes despite the weak promoters of V gene segments (19, 20). Activity of these enhancers is regulated in part by the proximity of promoters relative to the Eμ enhancer, and insertion of a transgene between the recombinant VDJ segments and the Eμ enhancer can completely block transcription of the upstream VDJ segment (21). We therefore inserted a synthetic VDJ under the control of a heavy chain promoter upstream of the Eμ enhancer that would allow for physiological expression of the inserted engineered monoclonal antibody (emAb).

To enable one-hit insertion, we designed an emAb cassette that contained a IgH promoter followed by a complete light chain gene linked to a recombinant IgH VDJ containing a splice junction to allow for splicing to downstream endogenous heavy chain constant regions (Fig. 1A). Using the endogenous heavy chain constant region reduced the insert size and allowed emAbs to be expressed in membrane-bound and secreted forms of all isotype classes under the control of endogenous regulatory elements. When expressed, the emAb light chain is physically linked to the IgH with a 57–amino acid glycine-serine linker (Fig. 1B), which has been used previously in single-chain Fab fragments (22). The linker also contained three tandem Strep-Tag II motifs to facilitate the detection and enrichment of engineered cells (23). Physically linking the heavy and light chains also minimized the possibility of mispairing between an inserted emAb IgH and the endogenous light chain.

CRISPR-Cas9–mediated emAb expression in RAMOS B cells

Insertion and expression of emAb cassettes were first tested in the Burkitt-lymphoma–derived RAMOS B cell line that natively expresses membrane-bound and secreted antibodies. Analyzing the region between the terminal J segment and Eμ using the CrispRGold algorithm (24), several potential Cas9 guide RNA (gRNA) binding sites were identified. We focused on hulgH296 gRNA, which targeted a region 296 nt downstream of IgHβ6 where single-nucleotide polymorphisms with a frequency above 1% have not been reported (25). Electroporation of RAMOS cells with hulgH296 gRNA precomplexed with Cas9 protein resulted in efficient DNA cutting, with insertions or deletions (indels) at this site being detected in ~72% of genomic DNA (Fig. 2A). After electroporation, we incubated cells with adenovirus (AAV) encoding an engineered RSV-emAb cassette derived from palivizumab flanked by 450-nt homology arms on either side of the hulgH296 target site. Because the AAV does not include the IgH constant regions that are essential for antibody expression, RAMOS cells would only gain the ability to bind RSV F antigen if the RSV-emAb cassette was successfully inserted into the IgH locus. Flow cytometry was used to assess RSV-emAb expression on the cell surface by measuring binding to fluorescent RSV F antigen and Strep-Tactin, a modified streptavidin with high affinity for the Strep-Tag II motifs in the linker (26). Using this approach, ~30% of RSV-emAb–engineered RAMOS cells bound RSV F antigen and Strep-Tactin compared with less than 0.3% of control RAMOS cells (Fig. 2B). To determine whether the endogenous IgH was silenced in emAb-expressing B cells, we examined the surface expression of the endogenous Igλ, which would only be present if it were able to pair with the endogenous IgH. For this, we focused on RAMOS cells expressing high levels of surface B cell receptor (BCR) by gating on B cells expressing high levels of CD79b (Fig. 2C). As expected, surface expression of endogenous Igλ expression was eliminated in RSV-emAb–engineered RAMOS cells that bound to RSV F antigen (Fig. 2C), although the expression of Igλ gene was not inactivated by our strategy. These results indicate that emAb engineering replaces the endogenous antibody expressed by RAMOS B cells.

To assess the functionality of the RSV-emAb, we FACS (fluorescence-activated cell sorting)–purified RAMOS cells that bound RSV F antigen and Strep-Tactin to create an RSV-emAb cell line. To confirm functional interaction between the RSV-emAb and the BCR signaling complex, we stimulated control and RSV-emAb RAMOS cells with tetramerized RSV F antigen or with polyclonal αlg F(ab′)2. Only the RSV-emAb cell line fluxed calcium in response to RSV F antigen, whereas the RSV-emAb and control RAMOS cell lines had similar responses to αlg (Fig. 2, D and E). These results indicate that emAb engineering programs B cells with a functional monoclonal antibody.

CRISPR-Cas9–mediated emAb expression in primary human B cells

We next engineered human primary B cells using a multistep process of expansion and differentiation (Fig. 3A). Human CD19+ B cells were magnetic-activated cell sorting–purified from peripheral blood mononuclear cells (PBMCs) and stimulated with a cocktail of cytokines,
The emAb expression efficiency ranged from 5 to 59% for the three additional antiviral emAbs (Fig. 3D). All four emAb B cell populations also secreted engineered antibodies when induced to expand and differentiate into CD38^+CD27^+ antibody-secreting cells through additional culture (Fig. 3, E to G). These data demonstrate the flexible nature of the emAb platform for engineering primary B cells to produce and secrete protective monoclonal antibodies.

Engineered B cells can coexpress emAbs off of both heavy chain loci

Work with transgenic mice demonstrated that productive VDJ sequences on both IgH loci result in simultaneous transcription and translation of both IgH (30). These results indicate that if the emAb cassette was only inserted into the unproductive IgH, then cells simultaneously expressing their endogenous antibodies and emAbs could be produced (Fig. 4A). This would be problematic if the endogenous antibody caused autoimmune tissue destruction due to binding self-antigens. This is a concern given that up to 20% of the naive B cell repertoire has been shown to express antibodies that can bind self-antigens (31). To determine whether B cells coexpressing both the emAb and endogenous antibodies were produced, we FACs-purified CD19^+ B cells that expressed antibodies using Igλ (Fig. 4B) before engineering with Flu-emAb, which used Igκ. Because many cells in the culture down-regulated surface BCR expression as a result of the culture conditions, we gated on cells expressing high levels of CD79b to focus on cells retaining high surface BCR expression (Fig. 4B). Within the Flu-emAb–engineered CD79b^+ B cells, most cells that gained the ability to bind Flu HA lost surface expression of Igλ (Fig. 4B). The loss of Igλ expression indicated that the inserted emAb cassette blocked expression of the endogenous antibody in these cells. However, nearly half of Flu HA–binding B cells retained Igλ expression on the cell surface (Fig. 4B). These results suggested that, in many of the cells, emAb insertion occurred on the nonproductive IgH locus, resulting in coexpression of the emAb and the endogenous antibody. This was not detected in RAMOS experiments because RAMOS cells have a c-Myc translocation in one IgH locus between the Eμ and constant regions (32, 33).

The expression of emAb from both the productive and nonproductive IgH offered the possibility of producing dual-emAb B cells by insertion of a different cassette into each locus. To test this possibility, we assessed B cells engineered simultaneously with AAVs encoding RSV-emAb and Flu-emAb cassettes. Simultaneous engineering of cells with RSV-emAb and Flu-emAb resulted in ~6% of cells binding both RSV F and Flu HA, a population that is not detected in RAMOS experiments because RAMOS cells have a c-Myc translocation in one IgH locus between the Eμ and constant regions (32, 33).

Murine emAb B cells protect against RSV infection

Having demonstrated the ability to engineer primary B cells, we next assessed the protective capability of these cells in a murine model of infection. Murine emAb-expressing B cells were produced using a process of priming, electroporation, and emAb cassette delivery similar to that used in human primary B cells (Fig. 5A). Electroporation in combination with precomplexed muIgH^367 gRNA and Cas9 resulted in indels in ~80% of target alleles (Fig. 5B). Delivery of a murine RSV-emAb cassette encoded by AAV resulted in 8 to 24% of murine B cells binding RSV F antigen 2 days later (Fig. 5, C and D). RSV F peptide

a multimerized CD40 ligand, and CpG for 48 hours before electroporation with huIgH^367 gRNA/Cas9. Sequencing of genomic DNA revealed the presence of indels in ~67% of IgH sequences from six independent donors (Fig. 3B). To test the performance of the emAb backbone across multiple independently derived antibodies, we designed three additional emAb cassettes encoding for the HIV-1 broadly neutralizing antibody VRC01, the influenza broadly neutralizing antibody MEDI8852, and the EBV neutralizing antibody AMM01 (27–29). All emAb cassettes were efficiently introduced into primary human B cells and resulted in the generation of populations that bound RSV F antigen, influenza hemagglutinin (Flu HA) stem, EBV gh/gL, or HIV-1 Envelope (Env) (Fig. 3C). Tests of RSV-emAb engineering in eight independent donors resulted in RSV antigen being bound by 16 to 44% of engineered B cells, compared with under 1% of mock-engineered control B cells. The emAb expression efficiency
antigen binding of 1 to 7% of B cells could be detected when an RSV-emAb cassette was delivered during the electroporation as a double-stranded DNA (dsDNA) containing short 36-nt homology regions (Fig. 5, C and D), offering a potential to produce emAb B cells without the use of an AAV. Similar to human cells, murine B cells secreting engineered antibodies when induced to expand and differentiate into CD19^{CD138-}CD138+ antibody-secreting cells through additional culture (Fig. 5, E to H). Most of the cells in this culture also lost the expression of IgM and IgD between days 3 and 7 (Fig. 5, I and J), indicating the ability of these cells to undergo isotype switching.

To assess the protective ability of RSV-emAb B cells, we transferred 0.5 × 10^7 to 1.5 × 10^7 Balb/c mouse B cells binding RSV antigen into wild-type Balb/c recipients (Fig. 6A). Six days after transfer, RSV-specific antibodies (3 to 29 µg/ml) were present in the serum from mice that received RSV-emAb B cells but absent in those that received control B cells (Fig. 6B). These titers were not maintained, however, as antibody levels returned to baseline levels 25 days after transfer (Fig. 6C). We next assessed whether RSV-emAb B cells provided protection against infection while antibody levels were still high. For this, animals were challenged intranasally with 10^6 plaque-forming units (PFU) of RSV 7 days after cell transfer, and viral titers were measured in the lungs 5 days later. About 5000 PFU of RSV was detected in lungs from control mice that did not receive cells and recipients of control B cells (Fig. 6D). In contrast, RSV was nearly undetectable in mice that received RSV-emAb B cells (Fig. 6D). This protection was comparable with the protection afforded by the injection of a clinical dose of palivizumab (15 mg/kg) 2 days before infection.

Because hematopoietic stem cell transplant recipients are one of the most susceptible groups for RSV infection, we next assessed whether protective antibody levels would be sustained in immunodeficient hosts. Because of the profound immunodeficiency resulting from transplant, RSV infection in the 3-month period after transplant carries a significant risk of lower respiratory tract infection, pneumonia, and death (34). For this reason, we tested the capacity of RSV-emAb B cells to provide long-term protection in immunodeficient RAG1−/− mice, which lack B and T cells (Fig. 7A). Transfer of 1.5 × 10^7 CD45.1+ C57Bl/6 RSV-emAb B cells into RAG1−/− mice led to a rapid accumulation of more than 40 µg/ml of RSV-specific antibodies in serum, which was maintained at this level for 40 days (Fig. 7B). Beginning at day 40, antibody levels declined, reaching ~3 µg/ml by 72 days.


Fig. 3. CRISPR-Cas9–mediated replacement of endogenous antibodies with engineered emAbs targeting RSV, HIV-1, influenza, or EBV in primary human B cells. (A) Schematic representation of the human B cell engineering protocol. (B) Frequency of indels detected in IgH sequences from B cells 2 days after electroporation with hulgH_{516} gRNA/ Cas9 (n = 8 individuals). (C) Representative flow cytometric analysis and (D) quantitation of antigen binding to human B cells from different individuals (n = 3 to 7) engineered to express emAbs based on the HIV-1 broadly neutralizing antibody VRC01 (HIV-emAb), the influenza broadly neutralizing antibody MEDI8852 (Flu-emAb), the EBV neutralizing antibody AMM01 (EBV-emAb), or RSV-emAb. Cells were analyzed at day 4 shown in (A) and compared with control B cells that were mock-electroporated and cultured similarly. (E) ELISA-mediated quantitation of antigen-specific antibodies in day 10 supernatants from two to three independent B cell cultures per specificity. (F) Combined data from two experiments displaying the number of emAb B cells in cultures at days 7, 11, and 14 displayed as a fold expansion over the number of cells sorted at day 4 (n = 2 to 6). (G) Representative flow cytometric analysis of CD38 and CD27 expression by B cells at the start of culture and days 4 and 10. The mean ± SD% in each quadrant was generated from three individuals. Data are representative of three similar experiments.
after cell transfer (Fig. 7B and C). Despite this decline in titers, intranasal challenge of mice with RSV 82 days after RSV-emAb B cell transfer revealed near-complete protection similar to mice challenged 7 days after transfer when RSV PFU in the lungs were assessed at days 87 and 12, respectively (Fig. 7D). Similar to wild-type recipients, protection did not appear to be mediated by a boosting effect because serum from infected mice did not contain increased levels of serum antibody compared with their uninfected counterparts (Fig. 7E).

Analysis of transferred CD45.1+ cells revealed thousands of RSV-emAb B cells in the spleen and bone marrow of recipient mice (Fig. 8, A and B). Many of the RSV-emAb B cells in the bone marrow expressed CD138 and low levels of CD19, a phenotype consistent with long-lived antibody-secreting plasma cells (Fig. 8, C and D). In contrast, most of the RSV-emAb B cells in the spleen expressed CD19 and CD38 but not IgM, IgD, or CD138 (Fig. 8, C to H), which would be consistent with the phenotype of an isotype-switched memory B cell. The expression of these markers was indistinguishable in infected mice compared with their uninfected counterparts (Fig. 8, F and H). CD38 expression was low on the CD19+CD138− emAb B cells at the time of transfer (Fig. 8, E and F), suggesting that cells reexpressed this molecule after transfer. Together, these results demonstrate that B cells can be efficiently engineered to provide robust and durable protection against infection.

**DISCUSSION**

The isolation of monoclonal antibodies has transformed medicine as therapeutics (35–37). However, although the use of antibody-producing
primary B cells in adoptive cellular therapy has lagged behind that of other cell types, there has recently been a wave of innovation in this area that paves the way for future clinical trials. There has been some recent genetic engineering work focused on taking advantage of the potent protein secretion capabilities of B cells to produce nonantibody therapeutic proteins (38, 39). Strategies to reprogram B cells to produce therapeutic antibodies have also been developed (17, 18), and several more will likely be published in the coming year. Our approach targets an intronic region, allowing for universal B cell engineering without a priori knowledge of the endogenous VDJ sequences. Engineered emAb receptors are not limited by the gene segments and recombination events that generate the endogenous B cell repertoire. This could be important in situations where current vaccines have failed. For example, many of the broadly neutralizing antibodies that have been identified for HIV-1 contain features that are rare in the naive B cell repertoire (40). As an alternative to isolation of rare antibodies, targeting domains could be designed in silico based on either the backbone of antibody variable domains or alternative high-affinity binding domains (41, 42). Combining these approaches with the emAb platform could allow targeting of pathogens for which no protective antibody has been isolated.

Insertion of emAb cassettes into this region was complicated by the ability of B cells to simultaneously express productive IgH from both loci, which allowed the emAb to be expressed from one IgH allele and an endogenous IgH from the other. Endogenous antibody/emAb coexpression can be circumvented by selecting against emAb B cells that express both Igκ and Igλ when the endogenous antibody and emAb antibodies express one or the other. Alternatively, B cells can be engineered and selected based on the simultaneous expression of two emAbs. The latter strategy is intriguing because complimentary epitopes on the same pathogen or escape mutations could be simultaneously targeted by the same engineered cells.

Although we favor coexpression of two antibodies, there may be downsides to this approach. Normal B cells do not express multiple heavy and light chains because this would result in numerous antibodies expressed one antibody. For example, if one antibody is expressed to higher levels due to increased stability or a more active promoter, then the expression of the second antibody could be greatly reduced compared with B cells only expressing a single antibody. This is particularly true of surface-expressed antibodies, where the level of CD79a and CD79b expressed by B cells is limiting. Another confounding factor is that the two antibodies in the cell could use different IgH constant regions unless class switching was carefully controlled. Future work is necessary to probe these issues.
Our results indicate that serum antibody levels produced by emAb B cells were not stable long-term in wild-type recipients. One explanation is that cell fitness is decreased because of in vitro culture, and we are exploring alternative culture methodologies to increase emAb B cell persistence in wild-type recipients. Another explanation is that emAb B cells are rejected by T or B cell responses targeting the emAb protein and/or decreased cell fitness due to off-target cutting by Cas9. Future work is necessary to assess these mechanisms, and the strategy may need to be altered if cell rejection or off-target cutting is problematic.

Off-target cutting by Cas9 is a major concern of any engineering strategy using gene cutting approaches. We have attempted to minimize the potential for off-target mutations by confining our strategy to a single gRNA and cut site for expression of both heavy and light chains and by using precomplexed gRNA and Cas9 (43). Nevertheless, before emAb B cells could be used in the clinic, a thorough and deep analysis of off-target mutations must be conducted. If mutations are detected, then other gene cutting approaches or Cas9 variants with higher fidelity could be used (44).

Serum antibody levels were maintained for 40 days when emAb B cells were transferred into immunocompromised RAG1−/− mice that lack endogenous T and B cells. Although titers declined after this time point, mice were protected for at least another 82 days because of the persistence of emAb B cells. In the vast majority of the murine cells we used at the start of culture, it may not be appropriate to describe in vitro–differentiated B cells that only encountered antigen at the time of cell sorting as “memory” B cells although many of these cells are isotype-switched. In the future, preselection of B cell subsets with reduced dependence on T cell help may improve the response to infection.

In situations where T cell help is present, emAb B cells may be able to enter the germinal center reaction and undergo affinity maturation. This could be beneficial in that the affinity of the engineered protective antibody can be enhanced or mutated to allow for control of escape variants. We have not assessed whether activation-induced deaminase (AID) will effectively mutate inserted emAb genes. Using a different approach to replace the heavy chain VDJ, Voss et al. (17) demonstrated successful AID targeting and somatic hypermutation within the insert. This suggests that the emAb insert would be effectively targeted for somatic hypermutation, but it is possible that the inclusion of the light chain and insertion into the intronic region eliminates this targeting.

Our RAG1−/− experiments model immunodeficiencies where common viral infections frequently lead to hospitalization, disability, and death. Hematopoietic stem cell recipients are a particularly relevant group because they are vulnerable to infection after transplant
and are already receiving a cellular product as part of treatment for an underlying disease. If donor B cells were engineered and infused as emAb B cells targeting RSV, HMPV, EBV, and CMV, then thousands of hospital visits, disabilities, and deaths could be prevented each year. For lower-risk populations, in vitro culture and infusion of engineered cells could pose a barrier to the clinical translation of emAb B cells. However, new technology is being developed to bypass patient-specific in vitro preparation of adoptive cellular therapies such as the production of universal donor cells (50), as well as nanocarrier-driven in vivo transduction of primary cells (51).

In summary, we have demonstrated specific and efficient engineering of primary mouse and human cells to produce multiple potent antiviral antibodies. Modified IgH loci in these engineered B cells retain the ability to undergo alternative splicing to generate both cell surface BCR and secreted antibodies at protective levels after adoptive transfer. This technique offers the possibility of engineering humoral immunity to produce sterilizing immunity to diseases for which no current therapy exists.

**MATERIALS AND METHODS**

**Study design**

The aim of this study was to use CRISPR-Cas9 to replace the endogenous antibody expressed by human and murine B cells with antibodies known to be protective against RSV, influenza, HIV-1, or EBV. In the murine system, we also aimed to demonstrate that CRISPR-Cas9–engineered B cells could protect mice from infection. The size of the experimental groups is specified in the figure legends. For RSV infection experiments, mice were randomly selected into infected versus uninfected groups. For most experiments, the analysis was conducted unblinded, with the exception of quantitation of RSV PFU in the lung 5 days after infection.

**Cell lines**

3T3-msCD40L cells were obtained from M. Connors at the National Institutes of Health (NIH) AIDS Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, NIH (catalog no. 12535) and cultured in Dulbecco’s modified eagle medium (DMEM) with 10% fetal bovine serum (Gibco), penicillin (100 U/ml) and streptomycin (100 μg/ml; Gibco). Human embryonic kidney (HEK) 293 cells were obtained from ATCC (CRL-1596) and cultured in RPMI 1640 medium with 10% fetal bovine serum (Gibco) and penicillin (100 U/ml) and streptomycin (100 μg/ml; Gibco). Human embryonic kidney (HEK) 293 cells were obtained from ATCC (CRL-1596) and cultured in RPMI 1640 medium with 10% fetal bovine serum (Gibco) and penicillin (100 U/ml) and streptomycin (100 μg/ml; Gibco). Vero cells were obtained from ATCC (CCL-8) and cultured in DMEM with 10% fetal bovine serum (Gibco) and penicillin (100 U/ml) and streptomycin (100 μg/ml; Gibco).

**Design of single-chain antibody template sequences**

Human emAb cassettes consisted of a 450–base pair homology arm, the IgVH1–69 heavy chain promoter region, the full-length antibody light chain gene, a segment encoding a 57–amino acid glycine-serine linker containing three tandem copies of the Strep-Tag II motif, the variable region of the heavy chain, and a splice junction with 60 base pairs of flanking sequence derived from matching IgH variable regions followed by a 450–base pair homology arm. Antibody variable domain sequences were derived from the humanized monoclonal antibody MEDI-493/palivizumab (6) and the human monoclonal antibodies AMM01, VRC01, and MEDI8852 (27–29).

Murine emAb cassettes consisted of an upstream homology arm, the J555H10 heavy chain promoter region (20), a full-length codon-optimized antibody light chain, a segment encoding a 57–amino acid glycine-serine linker containing three tandem copies of the Strep-Tag II sequence, a codon-optimized IgH VDJ, and a splice junction with 60 base pairs of flanking sequence derived from the mouse IgH J3 gene segment followed by a downstream homology arm. Antibody variable domains were derived from mouse monoclonal antibody 1129 (6). The AAV emAb cassette included a 503–base pair upstream homology arm and a 968–base pair downstream homology arm, whereas the dsDNA emAb cassette included a 36–base pair upstream and downstream homology arm.

**Production of recombinant emAb-AAV**

To generate AAV plasmids for homologous recombination, we isolated linearized AAV backbone from pAAV–green fluorescent protein (GFP; Addgene plasmid no. 32395) by digestion with SnaBI (New England Biolabs), and homology arms for mouse and human heavy chain flanking an EcoRV restriction site were inserted using an NEBuilder HiFi DNA assembly master mix (New England Biolabs). emAb constructs were then synthesized as gene fragments (Integrated DNA Technologies) and cloned into the EcoRV site using Gibson HiFi master mix.

AAVs were generated by triple transfection of AAV emAb plasmid, serotype 6 capsid, and adenoviral helper plasmids into HEK293 cells using polyethylenimine (Polysciences). Eighteen hours after transfection, the medium was changed to serum-free DMEM, and the cells were incubated for 48 hours before being lysed by freeze thaw, treated with 20 U of benzonase (Thermo Fisher Scientific) per 1 ml of viral lysate for 30 min at 37°C, and then purified over iodixanol gradient. Purified AAV was concentrated into 1× Dulbecco’s phosphate-buffered saline (DBPS) using an Amicon Ultra-15 column (EMD Millipore) (52) before viral titer determination by quantitative polymerase chain reaction (PCR) of AAV genomes (53), which ranged from 1 × 10^{10} to 7 × 10^{10} per microliter.

**Production of murine dsDNA emAb templates**

dsDNA templates containing short homology regions were generated from RSV-emAb AAV plasmids through PCR amplification using Platinum PCR SuperMix High Fidelity (Thermo Fisher Scientific) and modified DNA oligos. PCR product was purified and concentrated using MinElute PCR cleanup columns (Qiagen). The following primers were used for amplification, with mouse genomic homology region in bold and phosphorothioate-stabilized DNA bonds denoted by an asterisk (*):

5’/3’:

Phosphate/ACACCATTCTGTGACAGCATTTACAGTATCCGATTGGAACTGTGACTCAGGTTAGGTCTCTTATGTTAGATCTCAGCTATTTACTGCTCTATTTAGTCTATTTTCTCCTACTCCTTGTTCCTATGTTGACCCAAATGATATCTCCACC

(Forward) and TAAAGAAAATGCCCACATGCACTTCTTGTCTTCCTATGTTGACCCAAATGATATCTCCACC (reverse).

**Mouse B cell culture and electroporation**

Mouse B cell medium consisted of RPMI supplemented with 10% fetal bovine serum (Gemini Biosciences), 10 mM Hepes (Gibco), 55 μM β-mercaptoethanol (Sigma-Aldrich), and penicillin (100 U/ml) and streptomycin (100 μg/ml; Gibco) except in antibiotic free steps as noted. B cells were isolated from spleen and lymph nodes via negative isolation and are already receiving a cellular product as part of treatment for an underlying disease. If donor B cells were engineered and infused as emAb B cells targeting RSV, HMPV, EBV, and CMV, then thousands of hospital visits, disabilities, and deaths could be prevented each year. For lower-risk populations, in vitro culture and infusion of engineered cells could pose a barrier to the clinical translation of emAb B cells. However, new technology is being developed to bypass patient-specific in vitro preparation of adoptive cellular therapies such as the production of universal donor cells (50), as well as nanocarrier-driven in vivo transduction of primary cells (51).

In summary, we have demonstrated specific and efficient engineering of primary mouse and human cells to produce multiple potent antiviral antibodies. Modified IgH loci in these engineered B cells retain the ability to undergo alternative splicing to generate both cell surface BCR and secreted antibodies at protective levels after adoptive transfer. This technique offers the possibility of engineering humoral immunity to produce sterilizing immunity to diseases for which no current therapy exists.
selection with magnetic beads (Miltenyi Biotec), and 2 × 10^6 cells/ml were cultured for 24 hours at 37°C in a tissue culture incubator in B cell medium supplemented with recombinant carrier free HA-tagged mouse CD40L (100 ng/ml; R&D Systems), αHA antibody (100 ng/ml; clone 543851, R&D Systems), and mouse interleukin-4 (IL-4) (4 ng/ml; R&D systems). Next, the B cells were electroporated using the Neon Transfection System: Cas9 protein (Invitrogen) and synthetic gRNA (Synthego) were precomplexed at a 1:3 molar ratio in Neon Buffer T at room temperature for 20 min. The mulgH_{367} gRNA sequence with the protospacer adjacent motif (PAM) site in bold is TTATACAGTATCGATGCATAGG. B cells were washed with 1× DPBS and suspended in Neon Buffer T at a final density of 2.5 × 10^5 cells/ml with 12 μg of Cas9 per 10^6 cells. When dsDNA emAb cassettes were used, 7.5 μg of dsDNA template per 10^6 cells was included in the electroporation. Cells were electroporated with three 10-ms pulses at 1675 V and immediately dispensed into pre-warmed antibiotic-free mouse B cell medium. For AAV experiments, after electroporation, concentrated AAV in 1× DPBS was added at an up to 20% of final culture volume at a final multiplicity of infection (MOI) of 10^5 to 10^6 genome copies per cell and incubated for 1 hour. After AAV infection, B cells were expanded for an additional 48 hours with B cell medium supplemented with recombinant carrier-free HA-tagged mouse CD40L (100 ng/ml), αHA antibody (100 ng/ml), mouse IL-4 (4 ng/ml; R&D systems), and mouse IL-21 (20 ng/ml; BioLegend). For additional expansion, B cells were cocultured with irradiated (80 grays [gy]) NIH 3T3-CD40L feeder cells in the presence of mouse IL-21 (20 ng/ml) for 6 to 8 days, with passage onto fresh irradiated 3T3-CD40L feeder cells every 4 days.

Human B cell culture and electroporation

Human B cell medium was Iscove’s modified Dulbecco’s medium supplemented with 10% fetal bovine serum (Gemini Biosciences) and penicillin (100 U/ml; Gibco) and streptomycin (100 μg/ml; Gibco), except in antibiotic-free steps as noted. Blood was obtained from healthy, HIV-seronegative adult volunteers as a part of the General Quality Control study in Seattle, WA by venipuncture and was approved by the Fred Hutchinson Institutional Review Board. Informed consent was obtained before enrollment. PBMCs were isolated from whole blood using ACCUSPIN System-Histopaque-1077 (Sigma-Aldrich) resuspended in 10% dimethylsulfoxide in heat-inactivated FBS and cryopreserved in liquid nitrogen before use. PBMCs were thawed, and B cells were isolated using negative selection using the Human B Cell Isolation Kit II (Miltenyi Biotec) according to the manufacturer’s recommendations. Isolated B cells were resuspended at 0.5 × 10^6 to 1.0 × 10^6 cells/ml in stimulation medium, which consisted of human B cell medium supplemented with MEGACD40L (100 ng/ml; Enzo Life Sciences), recombinant IL-2 (50 ng/ml; BioLegend), IL-10 (50 ng/ml; Shenandoah Biotechnology), IL-15 (10 ng/ml; Shenandoah Biotechnology), and CpG oligodeoxynucleotide 2006 (1 μg/ml; Integrated DNA Technologies). After 48 hours, cells were electroporated using the Neon Transfection System. Cas9 protein (Invitrogen) and gRNA (Synthego) were precomplexed at a 1:2 molar ratio in Neon Buffer T for 20 min at room temperature. Cells were washed with 1× DPBS and resuspended to 2.5 × 10^6 cells/ml in Neon Buffer T containing 12 μg of precomplexed gRNA/Cas9 per 10^6 cells. The hulgH_{367} gRNA sequence with the PAM site in bold is GTCT-CAGGAGCGGTGTCTCTGTAGG. The cell/gRNA/Cas9 mixture was electroporated with one 20-ms pulse at 1750 V and immediately plated into stimulation medium as described above, without antibiotics. After 30 min, AAV was added to a final concentration of up to 20% culture volume amounting to a MOI of 10^5 to 10^6 genome copies per cell and incubated for 2 to 4 hours. Cells were next transferred to a larger culture dish to allow for further expansion. Two days after electroporation, we labeled cells with fluorochrome-labeled antigen and/or Strep-Tactin, and engineered cells were FACS-purified. For secondary expansion, B cells were cocultured for 4 to 8 days with irradiated (80 gy) NIH 3T3-CD40L feeder cells in human B cell medium containing human recombinant insulin (5 μg/ml;Sigma), transferrin (50 μg/ml; Sigma), human IL-2 (50 ng/ml; BioLegend), human IL-21 (20 ng/ml; BioLegend), and human IL-15 (10 ng/ml; Shenandoah Biotechnology). Cells were passaged onto fresh 3T3-CD40L feeder cells every 4 days. To promote differentiation to plasma cells, cells were washed and transferred from expansion conditions into fresh feeder-free culture conditions containing human B cell medium supplemented with human recombinant insulin (5 μg/ml;Sigma), transferrin (50 μg/ml; Sigma), Universal Type I Interferon Protein (500 U/ml; R&D Systems), IL-6 (50 ng/ml; Shenandoah Biotechnology), and IL-15 (10 ng/ml; Shenandoah Biotechnology).

Assessment of gRNA activity by Sanger sequencing

Total genomic DNA was isolated from 0.5 × 10^6 to 2 × 10^6 mock and gRNA/Cas9-treated cells 2 to 5 days after electroporation using the DNeasy Kit (Qiagen). The genomic DNA region flanking the gRNA target site was amplified by PCR using the following primers: mouse IgH, GGCTCCACCAAGCTCTCTCTA (forward) and AACCTGATCAGCTGCTCTCCT (reverse); and human IgH, ACAGTAAGCATGCTGCTCCTAA (forward) and GCCACTCTAGGCCCTTTGTT (reverse).

The resulting PCR product was purified using a MinElute reaction cleanup kit (Qiagen) and Sanger-sequenced (Genewiz). The frequency of indels in gRNA/Cas9-electroporated cells relative to control cells was determined using the Inference of CRISPR Editing (ICE) algorithm (54).

Protein antigens

RSV prefusion F antigen trimers, EBV gH/gL complexes, and modified HIV-1 Env GP140 trimers (426c TM44AV-1) were produced as described (29, 55, 56). Stabilized Flu HA stem was produced from VRC clone 3925, derived from strain H1 1999 NC as described (57). All antigens were conjugated to biotin–N-hydroxysuccinimide (NHS) ester (Thermo Fisher Scientific) followed by tetramerization with streptavidin–R-Phyceroerythrin (PE), streptavidin–allophycocyanin (APC), or streptavidin (all from ProZyme) as described previously (58). RSV F antigen was conjugated to Alexa Fluor 488 NHS ester (Thermo Fisher Scientific) according to the manufacturer’s recommendations and used for flow cytometry.

Flow cytometry

Cells were incubated in 50 μl of FACS buffer containing a cocktail of antibodies for 30 min on ice before washing and analysis on a FACSSymphony (BD Bioscience) or sorted on FACS Aria II (BD Bioscience). FACS buffer consisted of 1× DPBS containing 5 mM EDTA and either 1% newborn bovine serum (Life Technologies) or 1% bovine serum albumin (BSA; Sigma) for experiments including Strept-Tactin staining. For murine experiments, cells were labeled with a cocktail including combinations of Strept-Tactin PE (IBA Lifesciences), anti-CD45.1 APC (A20, BioLegend), anti-IgM PerCP-eFluor710 (II/41, eBioscience), anti-IgD PE-Cy7 (11-26c, 58).
eBioscience), anti-CD138 BV421 (281-2, BioLegend), anti-CD38 AF700 (90, eBioscience), anti-CD19 BV395 (ID3, BD Biosciences), anti-CD38 BV510 (145-2C11, BD Biosciences), anti-Gr-1 BV510 (RB6-8C5, BioLegend), anti-F4/80 BV510 (BM8, BioLegend), and a fixable viability dye (eBioscience) before analysis. For human experiments, cells were labeled with a cocktail including combinations of Strep-Tactin PE, anti-IgG. PE (MHL-38, BioLegend), anti-IgM V450 (JDC-12, BD Biosciences), anti-CD19 APC (GS25C1, BD Biosciences), anti-CD20 BV395 (2H7, BD Biosciences), anti-CD27 PE-Cy7 (LG.7F9, eBioscience), anti-CD38 BV650 (HB-7, BioLegend), anti-CD45, (H130, BD Biosciences), anti-CD79b APC/Fire750 (3B3-1, BioLegend), and a fixable viability dye.

For ex vivo analysis of transferred cells after RSV infection in mice, single-cell suspensions of spleen and bone marrow from the femurs were generated by manual disassociation and filtration. Cells were stained with anti-CD45.1 APC (A20, BioLegend) and purified anti-CD16/32 (2.4G2, Bio X Cell) for 30 min on ice, washed with FACS buffer, and then incubated with 25 μl of anti-APC–conjugated magnetic microbeads (Miltenyi Biotec). After a 15- to 30-min incubation on ice, 3 ml of FACS buffer was added, and the sample was passed over a magnetized LS column (Miltenyi Biotec). The tube and column were washed once with 5 ml of FACS buffer and then removed from the magnetic field. FACS buffer (5 ml) was pushed through the column with a plunger twice to elute column-bound cells. Cells from the column-bound and 1/40 of the column flow through fractions were stained as described above. Twenty thousand AccuCheck counting beads (Invitrogen) were added to the samples to calculate total cell numbers. To account for cells in bones that were not harvested, we multiplied the number of cells detected in the pooled femurs by 10 (59).

Calcium flux was measured by flow cytometry with the Fluo-4 Direct kit (Thermo Fisher Scientific). Briefly, 1.5 × 10^6 cells were labeled with 1 ml of Fluo-4 according to manufacturer instructions for 30 min at 37°C. Fluo-4 baseline florescence was measured for 60 s, and then, cells were stimulated with 1 μg/ml of tetramerized RSV F antigen or 1 μg/ml of polyvalent eGFP (eBioscience), followed by 180 s of measurement, and last, cells were stimulated with 1 μg/ml of ionomycin in dimethylsulfoxide, and fluorescence was measured for an additional 60 s. Fluorescence intensity data were binned by time and displayed as fold change over baseline measurement. Flow cytometry data were analyzed using FlowJo X software (Tree Star).

**Animals**

Animal studies were approved and conducted in accordance with the Fred Hutchinson Cancer Center Institutional Animal Care and Use Committee. Six- to ten-week-old male and female BALB/cByJ, CByJ.SJL(B6)-Ptprca/J (BALB/C CD45.1), B6.129S7-Rag1tm1Mom/J (RAG1−/−), and B6.SJL-Ptpcrca Pecpb/BoyJ (CD45.1) mice were obtained from the Jackson Laboratory. For transfer of emAb B cells, age-matched BALB/cByJ mice or RAG1−/− mice received a single intraperitoneal injection of emAb or control B cells derived from CD45.1 congenic donor mice or a single intraperitoneal injection of palivizumab (15 mg/kg) at the indicated 2 days before RSV challenge.

**RSV infections and titer measurement**

In RSV challenge experiments, mice were inoculated intranasally with 10^6 PFU of sucrose-purified RSV expressing enhanced GFP (eGFP) (60) in 40 μl 1× DPBS. Lungs were harvested 5 days after infection and the titer was determined using a plaque assay (61).

Briefly, lungs were homogenized in 2 ml of DMEM using a gentleMACS M Dissociator using preset program lung_02 (Miltenyi Biotec) followed by centrifugation at 400g for 10 min. Supernatant was flash frozen and stored at −80°C. The supernatant was diluted 1:10 and 1:20 in DMEM and 100 μl of each dilution was added in duplicate to confluent Vero cells in 24 well flat-bottoms tissue-culture plates and incubated for 2 hours at 37°C. An overlay of 0.8% methylcellulose was then added and plates were incubated for 5 days before imaging on a Typhoon imager (GE Healthcare) with filter settings for eGFP. The titer in PFU per lung was calculated by counting the number of eGFP plaques with ImageJ software in the highest positive dilution and correcting for the dilution factor.

**Enzyme-linked immunosorbent assay**

Nunc MaxiSorp 96-well plates (Thermo Fisher Scientific) were coated with RSV F antigen, HIV Env, or Flu HA (all at 1 μg/ml) in 1× DPBS overnight at 4°C. Plates were washed three times with 1× DPBS containing 0.05% Tween-20 (PBST) and blocked with 150 μl per well PBST and 3% BSA (Sigma-Aldrich) for 1 hour at room temperature. Alternatively, EBV gH/gL (1 μg/ml) in 1× DPBS was coated on preblocked 96-well Ni-NTA plates (Qiagen) for 1 hour at room temperature and then washed three times with PBST. Antigen-coated plates were incubated with culture supernatant or mouse plasma samples in duplicate diluted in PBST and 3% BSA, and a standard curve was generated using the purified recombinant mouse RSV-specific antibody 1127, purified recombinant human RSV-specific palivizumab (Synagis clinical grade, MedImmune), Flu HA–specific MED18852, EBV gH/gL–specific AMM01, or HIV-1 Env–specific VRC01-positive control antibodies for 90 min at room temperature. Plates were washed five times with PBST before 1 hour of incubation with horseradish peroxidase–conjugated goat anti-mouse or anti-human total Ig (SouthernBiotech) diluted 1:4000 in PBST and 3% BSA. Plates were then washed three times with PBST before a 2- to 15-min incubation with enzyme-linked immunosorbent assay (ELISA; 100 μl per well) 1× trimethylboron substrate (Thermo Fisher Scientific), and absorbance was measured at 405 nm using a SoftMax Pro plate reader (Molecular Devices). The concentration of antigen-specific antibody in each sample was determined by reference to the standard curve and dilution factor.

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism 7. Pairwise statistical comparisons were performed using unpaired two-tailed t test with Welch’s correction. P < 0.05 was considered statistically significant. Data points from individual samples are typically displayed, and raw values can be found in data file S1.

**Supplementary materials**

immunology.sciencemag.org/cgi/content/full/4/35/eaax0644/DC1
Data file S1. Raw values for data displayed in figures.

**References and notes**

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B cells engineered to express pathogen-specific antibodies protect against infection
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B cells enter the cell therapy game
Antibodies are currently being used to treat a number of ailments from infectious diseases to cancers and autoimmunity. Like other drugs, patients often require multiple doses of antibodies. Because production and storage of antibodies are expensive, there has been considerable interest in finding alternative strategies to deliver antibodies. Here, Moffett et al. have engineered both human and murine B cells to express antibodies targeting a number of viruses, including respiratory syncytial virus (RSV), and report that a single injection of B cells expressing RSV-specific antibodies into mice lacking T and B to be protective. Their technology opens up the possibility of using engineered B cells as therapeutics.

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