Vector Strategies to Actualize B Cell-Based Gene Therapies

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Abstract

Recent developments in genome editing and delivery systems have opened new possibilities for B cell gene therapy. CRISPR/Cas9 nucleases have been used to introduce transgenes into B cell genomes for subsequent secretion of exogenous therapeutic proteins from plasma cells, and to program novel B cell antigen-receptor specificities, allowing for the generation of desirable antibody responses that cannot normally be elicited in animal models. Genome modification of B cells or their progenitor, hematopoietic stem cells (HSCs), could potentially substitute antibody or protein replacement therapies that often require multiple lifelong injections. To date, B cell editing utilizing CRISPR/Cas9 has been solely employed in preclinical studies wherein cells are edited *ex vivo*. In this review, we discuss current B cell engineering efforts and strategies for the eventual safe and economical adoption of modified B cells into the clinic, including *in vivo* viral delivery of editing reagents to B cells.

B Cells

B cells, or B lymphocytes, are key effectors of the adaptive immune response. One of their main roles is to produce antibodies (Ab), also called immunoglobulins (Igs). These are secreted proteins made up of covalently linked heavy (H) and kappa (κ) or lambda (λ) light chains with functionally distinct antigen-specific (variable), and immune effector (constant) regions. B cell ontogeny begins in the bone marrow and involves somatic recombination of Ig heavy (*IgH*) and light (*Igκ/Igλ*) chain loci. These events result in the cell-surface expression of immunoglobulins in the form of B cell antigen-receptors (BCRs). This process, also called V(D)J-recombination, generates unique BCR's in each B cell, with antigen specificity encoded by the complementarity-determining regions (CDRs) of the variable heavy (VH) and variable light (VL) regions. Cells with self-reactive BCR's are counter-selected in the marrow before they egress into the periphery to become circulating

naïve B cells (1). When naïve B cells receive activation signals from specific T helper (Th) cells and foreign antigen binding to the BCR, they undergo clonal expansion and affinity maturation in the germinal centers (GCs) of secondary lymphoid organs. In a process called class or isotype-switching, genomic recombination occurs in the *IgH* constant region, allowing for immunoglobulins to be expressed with alternative (IgG, IgA, IgE) effector, heavy chain constant genes. Mutations are also generated in *VH* and *VL* regions that alter BCR antigen affinity (2). Variants with improved BCR-affinities are preferentially selected for survival while those with lower affinity or recognizing self-antigens are deleted or maintained in an anergic state by peripheral tolerance (3). Affinity-matured cells differentiate into long-lived memory or plasma cells. Plasma cells, secrete large quantities of antibodies and can survive for decades in the bone marrow and gut-associated lymphoid tissues (GALT). Memory B cells also exhibit extended lifespans and can generate a rapid, high-affinity antibody response upon subsequent antigen-exposure (4). In addition to antibody production, B cells also express high levels of MHC class-II and act as antigen presenting cells (APC's), presenting antigens to $CD4^+$ T cells. As professional APC's B cells can either activate or tolerize T cell responses to antigen (5). This brief review describes recent efforts towards harnessing these various B cell effector functions in genetic medicine.

Gene Therapy

Broadly, gene therapy is the introduction of genetic material or gene-editing nucleases into a patient's cells for the treatment of disease. Gene therapy encompasses gene replacement, gene alteration, increasing or decreasing the transcribed levels of a gene product, and the introduction of completely novel transgenes. Decades of research have begun to pay off clinically, with 22 gene therapy drugs approved globally in 2019 (6). The main challenge for gene therapy is to achieve safe, specific, and long-term therapeutic changes in the targeted cells or tissues while minimizing off-target modifications. As highlighted with the tragic death of Jesse Gelsinger in 1999, immune tolerance of genetic medicines is also of paramount importance (7). Ideally, a patient would be dosed once with the genetic drug, supplanting the need for multiple injections in the case of enzyme replacement therapies.

Precision Genome Engineering Using DNA Nucleases

Prior to CRISPR/cas, integration-competent lentiviral (LV) transduction was commonly used to deliver transgenes to cultured B cells or HSCs for random insertion into the genome. For example, Lu et al transduced human $CD34^+$ cord blood cells with LVs containing HIV broadly neutralizing antibody (bnAb) IgG expression cassettes. These cells were then subjected to a B cell differentiation culture system leading to maturation of plasma-like cells that could secrete high levels of the bnAb *ex vivo* (8). In Fusil et al., similar expression cassettes were developed that could be alternatively spliced to generate either cell-surface BCRs in non-secretory B cell lines, or soluble antibody secreted from plasma cells after delivery using integration-competent lentiviral vectors (9). With the adoption of CRISPR/cas, more recent efforts employ precision genome editing strategies to knock-out or deliver transgenes to specific locations in the B cell genome by homology directed repair (HDR).

The advent of CRISPR/Cas genome editing has profoundly impacted emerging cell therapies and has been demonstrated in B cells for a variety of applications including the reprogramming of B cell antigen-receptor specificities and the secretion of therapeutic proteins from plasma cells, as further discussed in this review. CRISPR/Cas has largely displaced traditional DNA-nucleases used for genome modification, such as zinc fingers or TALENs, because this enzyme can be more easily adapted for cutting of different target sites in the genome (10). CRISPR/Cas machinery utilizes protein nucleases known as <u>C</u>RISPR <u>as</u>sociated proteins, or Cas. These ancient nucleases derived from archaeal and bacterial adaptive immune systems typically use a single guide RNA (gRNA) to locate a DNA target and produce a double stranded break (DSB) (11). The DSBs are then resolved by one of two mechanisms in diploid cells: (i) Nonhomologous end-joining (NHEJ) or (ii) homology-directed repair (HDR). The majority of DSBs will be repaired by NHEJ - a highly efficient, error-prone process that tends to introduce nucleotide insertions or deletions known as indels at the DNA break-site. Indels in a coding region can cause frameshift mutations that will result in gene knockout. In HDR-mediated DNA break-repair, template DNA (usually an exogenous "donor") is integrated at the site of the DSB using donor homology to sequences on either side of the break-site. This mechanism allows for seamless incorporation of exogenous genes or nucleotides in a controlled manner that does not generate random indels (12). Importantly, HDR occurs primarily during S/G2 phase, and is therefore restricted to cells that are actively dividing. The introduction of a DSB into the genome is inherently a dangerous process, leading to concerns about CRISPR/Cas editing causing undesirable genomic alterations (13). To improve safety, a variety of gRNA design algorithms have been developed to generate gRNAs with high on-target and minimal off-target DNA cutting activity (14). Another strategy utilizes Cas9 nickases that initiate HDR from a nick (single strand DNA break), instead of a DSB, in order to minimize indels (15). *Ex vivo* editing ensures modifications are restricted to the target cell population, which can be selected post editing for re-engraftment. Technologies being developed for specific *in vivo* delivery will be described in further sections.

B Cell Genome Editing Strategies

Editing at Safe Harbor Loci

Because transgenes inserted into random genomic locations can interact with the genome in unpredictable ways, recent gene delivery approaches target genomic safe harbor loci that have been evaluated for predictable transgene expression while minimizing unwanted interactions (16). As a proof-of-concept, Johnson et al inserted a splice acceptor and fluorescent reporter gene into a CRISPR/cas cut site in the AAVS1 for expression using cell endogenous promoters (17). Hung et al inserted transgenes of therapeutic proteins under control of the MND (myeloproliferative sarcoma virus enhancer, negative control region deleted, *d*l587rev primer-binding site substituted) promoter into the CCR5 safe harbor locus as it is not transcriptionally active in human B cells, it is not required for plasma cell differentiation, and null mutations are innocuous to humans (18). In a unique BCR editing approach, Pesch et al modified murine B cells for the expression of a novel chimeric B cell receptor (CBCR), at the Rosa26 safe harbor locus. A single-chain variable fragment (scFv) acting as the extracellular antigen-binding domain was joined to the CD28

transmembrane domain and the cytoplasmic domain of the endogenous murine BCR fused to the CD79β intracellular signaling domain via a spacer encoding a Strep tag. CBCRs could potentially offer a way to activate engineered B cells in an antigen-controllable manner (by vaccination), independent of the endogenous BCR (19). Luo et al. targeted an anti-PD-1 antibody cassette to the GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) locus in mouse primary B cells for co-expression of the transgene along with the GAPDH enzyme (20).

Editing the Immunoglobulin Loci

Engineering antibody transgenes into the immunoglobulin loci where they can be expressed using B cell endogenous heavy chain constant gene exons, allows them to function as both B cell antigen-receptors and subsequently as secreted therapeutic antibodies from long-lived plasma cells (Figure 1). Voss et al replaced the entire *VH* locus between distant (2MB) CRISPR/cas9 cut-sites with a homology repair template containing the VDJ sequence from a heavy chain-encoded HIV broadly neutralizing antibody in human B cells. The engineered heavy chain could be expressed using cell endogenous V-gene promoters, HC constant gene exons, and light chains. These antibodies could broadly recognize HIV and engineered cell lines could be affinity matured *in vitro*, however low engineering efficiencies in primary cells are limiting for clinical applications (21). Greiner et al. demonstrated that homology repair templates could replace or be inserted into commonly recombined VDJ and VJ regions in mature primary human B cells. Transgenes were expressed in primary human B cells using these endogenous V-gene promoters (22). Several groups have converged on a high efficiency engineering solution that introduces an antibody homology repair template encoding an *IgH* V-gene promoter, the light chain (including constant gene), heavy chain variable (VDJ) region and a splice donor site, into the heavy chain locus between the 3'-most J-gene and the downstream V-gene enhancer and constant gene region. In this way the antibody light and heavy chains are expressed from a single transcript spliced to cell endogenous HC-constant genes. A flexible linker or P2A self-cleaving peptide are placed between light and heavy chains to allow them to pair together for secretion as functional BCR or antibody (Figure 1). Hartweger et al. included a splice acceptor, stop codon and terminator sequence in front of the donor DNA V-gene promoter to stop transcription of upstream endogenous rearranged VDJ sequences, and a P2A self-cleaving peptide between light and heavy chains. They used CRISPR/cas9 protein complexed with guide RNA (ribonucleoprotein or RNPs) to target indels into the endogenous light chain kappa constant gene to minimize mispairing with endogenous light chains. They showed that *ex vivo* engineered mouse B cells could secrete class-switched 3BNC60 HIV bnAb for several days after adoptive transfer into congenic immunocompetent mice (23). Moffett et al used a 54-amino acid linker to join light and heavy chains in order to prevent mispairing with endogenous light chains. They could achieve high efficiency insertion with up to 60% or 24% of targeted cells expressing the engineered BCR in *ex vivo* activated primary human and mouse B cells respectively. Two antibody donor DNAs could be targeted to both HC alleles in up to 6% of double targeted B cells. They also showed that protective levels of pathogen specific RSV (Respiratory Syncytial Virus) antibody could be secreted from engineered cells for several days post adoptive-transfer into congenic immunocompetent mice, or for several weeks as bone marrow engrafted plasma cells in immunocompromised

mice (24). Nahmad et al showed that primary mouse B cells engineered to express the 3BNC117 HIV bnAb as antigen receptor could be matured in germinal centers after adoptive transfer and vaccination in congenic immunocompetent mice, resulting in a class-switched and somatically mutated memory and plasma cell response (25). Huang et al showed similar results in mice using HIV bnAb VRC01 engineered B cells, and that such responses could be durable and boosted multiple times. They also showed that *ex vivo* activated mouse primary cells go on to express memory markers and remain subject to peripheral tolerance mechanisms that delete autoreactive cells after adoptive transfer into immunocompetent animals. In addition, they developed an alternative engineering strategy where heavy and kappa chain variable regions in separate donor DNAs were targeted to their endogenous loci for expression using cell native constant genes. Cells engineered in this way however did not respond to immunization after adoptive transfer in to immunocompetent mice (26).

Applications of Engineered B Cells

B cells have several immune effector functions that make them intriguing targets for genetic modification, mainly: (i) their ability to clonally expand and differentiate into long-lived memory and plasma cells capable of secreting large quantities of highly specific antibodies, (ii) their ability to function as antigen presenting cells, sustaining effector T cell and T follicular helper responses and iii) their production of inflammatory or immune suppressive cytokines (27). These characteristics make them promising cells for the treatment of genetic diseases (18, 19, 28), infectious diseases (20–24, 26, 29, 30), cancers (20, 22, 31, 32), and autoimmune disorders (22).

Secretion of therapeutic proteins from plasma cells

B cells are the natural secretory cells for antibodies and cytokines, making them an ideal gene therapy target cell choice for secreted therapeutic proteins with clinical relevance for monogenic disorders (18, 24, 33, 34). A major challenge for protein replacement therapy is the ability to sustain effective serum concentrations of the desired exogenous protein long-term without eliciting anti-drug immune responses that could reduce or neutralize the therapeutic effect (35). As plasma cells can persist for an entire life span, one injection of engineered plasma cells secreting high doses of therapeutic proteins might act as a life-long cure. Alternatively, engineered B cells expressing both a secreted therapeutic protein and an antigen-specific BCR could be regulated *in vivo* to express the transgene through vaccination as demonstrated in Takacs et al., using adoptive transfer and immunization of *Ig* knock-in mouse B cells expressing therapeutic transgenes (33). Furthermore, antigen-presentation by B cells often leads to T cell tolerance (36–38), raising the possibility that transgene expression from B cells may generate reduced neutralizing anti-drug immune responses as compared with injected protein or gene therapies that result in exogenous protein secretion from other tissues such as the liver or muscle (39). Most immunocompetent mouse models used to study *in vivo* engineered B cells to date have made use of the black 6 (C57BL/6 or B6) strain because this inbred model is most reliable for congenic adoptive cell transfer experiments where donor cells are tolerated by the recipient animals immune system. This strain has also been shown to tolerate foreign transgene-encoded proteins however, such as human factor IX (FIX), α−1 trypsin and human Erythropoietin

(hEPO) (40–42) and therefore may not be the ideal choice for experiments designed to study the tolerogenic properties of B cells. Humanized mouse models have often been used to assess cell-based therapies, wherein immunodeficient mice are injected with human hematopoietic stem cells capable of recapitulating the human immune system. While stem cells are capable of differentiating into mostly transitional or immature B cells in these models, antigen-driven B cell maturation with memory and plasma cell differentiation remains difficult (43). Adoptive transfer of CD19^{+} human B cells, modified *ex vivo* for transgene expression, along with autologous CD4^{+} human T cells into immune-deficient NSG (NOD *scid* gamma) mice, could allow engineered B cells to home to the spleen and bone marrow, generating plasma cells in 4–5 weeks. The homing was believed to be due to expression of the crucial homing receptors CD62L, CXCR4 and LFA1 on select transduced plasma cells. Lévy et al, used this model to transduce B cells to secrete human factor IX (FIX), a clotting factor that is deficient in Hemophilia B patients, at therapeutic levels (28). Hung et al showed that B cell activating factor (BAFF) could be delivered to human primary B cells and these cells showed improved engraftment and secretion of antibody in NSG mice after *ex vivo* differentiation to plasma cells (18).

Infectious Diseases

Vaccines are designed to provide protection from infectious diseases, often through the production of pathogen-specific neutralizing antibodies. However, traditional vaccines have failed to generate broadly neutralizing antibodies (bnAbs) capable of generating universal protection against pathogens with diverse and rapidly evolving immunodominant regions, such as Human Immunodeficiency Virus (HIV), Influenza and Hepatitis C (44, 45). The importance of this problem is emphasized by the frequent spillover of antigenically distinct pathogenic coronaviruses into humans leading to the current COVID-19 pandemic (46), and by antigenic drift of the SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus-2) pandemic strain that has threatened the efficacy of neutralizing antibody responses generated by approved vaccines (47). The ability to elicit broadly-neutralizing antibodies from engineered B cells could revolutionize the development of vaccines against such variable pathogens when traditional approaches fail. Programming engineered Ab isotypes could also be useful. Cheong et al., developed a method to induce isotype switching by mimicking AID cleavages with CRISPR/cas (48). For example IgA was found to contribute to neutralization of SARS-CoV-2 to a greater extent compared to other isotypes and could be programmed into bnAb-engineered cells (49). Infusion of recombinant monoclonal antibodies (mAbs) has been explored for several diseases including COVID-19 (50), but this approach is not ideal for therapy in the case of chronic viral infections, or as a prevention strategy, because the short half-life of mAbs (1–3 weeks) necessitates repeated administration, driving practical and financial challenges (51). Immunogenicity of the therapeutic mAb for some patients (~7%), is also an issue (52, 53). Traditional gene therapy approaches have been utilized for *in vivo* antibody generation from non-B cell populations. Vectored delivery of antibody genes to muscle cells has been developed as a strategy to generate long-term expression of protective antibodies (39, 54, 55). A drawback to this approach is the fixed quantity of antibody produced, which is unresponsive to a dynamic infection. Additionally, such an approach is unable to stimulate the natural immune response to develop the cellular and humoral memory necessary for successful, long-term vaccination. As clearly demonstrated

in one study using rhesus monkeys given a vector encoding anti-HIV bnAbs, there is also the danger of developing antibodies that neutralize the transgenic bnAbs (39). Furthermore, pre-existing immunity to viral vectors can cause low levels of transgene antibody expression (56, 57).

Self-renewing HSCs have also been targeted for anti-pathogen antibody production, as stem cells are capable of B cell and plasma differentiation. This approach has the potential advantage of short-circuiting V(D)J-recombination to prevent generation of endogenous Igs during B cell development and continuous generation of transgene expressing B cells *in vivo*. Several groups have used lentiviral vectors encoding secreted bnAbs against HIV to edit HSCs (8, 29). This HSC-based delivery of bnAbs was capable of persistent secretion, penetrating relevant viral reservoir tissues, and stimulating a protective immune response in humanized mice (30). However, current HSC gene therapy involves harvesting HSCs from the patient, *ex vivo* genetic engineering, and re-infusion following myeloablative conditioning. This procedure is largely unsuitable for vaccination purposes due to the associated toxicities and risks of low engraftment.

Until recently, genetic engineering of B cells has been limited by technical challenges including delivery of genome editing reagents, the complexity of native antibody development, and the culture and differentiation of HSCs and primary B cells. CRISPR/cas has recently enabled methods to reprogram novel antigen receptor specificities into B cells, an approach that could help surmount difficulties eliciting protective responses against antigenically variable pathogens (58). BCR editing has been used to introduce antibodies specific for HIV (21, 23–26) and respiratory syncytial virus (RSV) (24), with promising results, indicating that engineered B cells are capable of initiating a protective immune response in animal models. In sum, potential advantages of expressing antibody transgenes from BCR-engineered B cells for infectious disease applications are that: 1) Titers could be increased by vaccination or in response to infection. 2) The engineered antibody response would be able to mature in affinity against rapidly evolving pathogens. 3) Engineered antibody could be expressed as all effector isotypes. 4) The engineered antibody should be tolerated by the immune system and eliminated if self-reactive.

Cancer

While not requiring B cell genome editing, the antigen-presentation function of B cells can potentially be harnessed to initiate T cell responses targeting cancer neoantigens. Indeed, there have been a few early-stage clinical trials for B cell-based cancer vaccines, many utilizing the CD40 pathway to activate B cells for presentation of cancer antigens (59). B cells have been loaded with antigens through BCR-mediated uptake (60), CD19 receptor-targeting (61), or through microfluidic-based loading (62). The isolation and enrichment of B cells with defined anti-tumor specificity from the tumor site or surrounding lymph nodes is another approach that has shown some success (63).

Monoclonal antibodies have also been used to enhance immune responses, such as checkpoint blockade therapy, which uses antibodies targeting PD-1, PD-L1, or CTLA-4 to activate T cell anti-cancer effector functions (64). In addition, chimeric antigen receptor (CAR) T cells edited to target a tumor-associated antigen have achieved great clinical

results for the treatment of multiple myeloma (65). CAR-T cells have even been modified to constitutively secrete inhibitory checkpoint antibodies, such as anti-PD-1 (66) and anti-PD-L1 (67). Luo et al edited B cells to express an anti-PD-1 antibody at the GAPDH locus using integrase-defective lentiviral vectors containing either CRISPR/cas9 and gRNA or homology repair template. The engineered B cells demonstrated antitumor activity comparable to existing anti-PD-1 monoclonal antibodies in a tumor xenograft mouse model (20). A drawback to this approach, is that engineered cells expressing antibodies targeting self-antigens will have to bypass deactivation by peripheral tolerance (26) and also persistent secretion of a checkpoint antibody might induce an overly activated immune system and increase T cell autoimmunity against the patient (68). One potential solution would be to edit these cells to express a stable selection marker or suicide gene that could be used to eliminate the engineered cells and avoid adverse side effects. An additional applicati84on of B cell editing is the induction of chromosomal translocations for the development of new models to study B cell malignancies. Johnson et al modeled the t(8;14) translocation characteristic of Burkitt Lymphoma using RNPs targeting c-Myc and the IgH locus (17).

Autoimmune Disorders

Autoimmune disorders develop when self-tolerance mechanisms fail, resulting in the immune system attacking the body's own cells. B cells are known to play a mediating role in autoimmunity through the production of autoantibodies, presentation of "self" proteins to T cells, and cytokine secretion (69) (70). There have been a few studies editing activated primary B cells *ex vivo* for the induction of tolerance to self-antigens via retroviral delivery of antigen fusions to the tolerogenic Ig backbone (71). Adoptive transfer of such genetically modified B cells has been shown to induce tolerance in animal models for diseases including diabetes (72), multiple sclerosis (73), rheumatoid arthritis (74), posterior uveitis (75), hemophilia (76), and experimental allergic encephalomyelitis (77). Another potential application for B cell editing to treat autoimmune disorders lies in the secretion of antibodies targeting inflammatory cytokines (e.g., TNF-α and IL6) (22, 77). However, *in vivo* use of engineered B cells that continuously secrete antibodies specific to self-antigens may be problematic as previously described. It remains challenging to intervene in autoimmune disease without overly compromising the normal immune response.

Delivery Methods

Ex vivo

Ex vivo gene delivery to HSCs and primary T cells is now routine in cell therapies used to treat several diseases (78, 79). *Ex vivo* editing allows for the selection of targeted cell types prior to editing and subsequent enrichment of successfully engineered cells. Almost all published B cell editing studies utilizing CRISPR/Cas found at the time of this review's writing have described *ex vivo* engineering methods (Table 1). In these studies, CRISPR/cas9 and corresponding gRNAs are generally delivered as RNPs by electroporation. Electroporation of nuclease in this format is extremely efficient, non-toxic, and ensures minimal off-target cutting compared with mRNA or DNA formats. (17, 18) (19, 22–26). Plasmid DNA is also toxic (dsDNA), and is not as efficiently electroporated into B cells (26). Nonviral methods for delivery of homology repair templates to primary B cells has

included electroporation of single stranded DNA (ssDNA) or endotoxin-free plasmid donor DNA (17, 18, 21, 23, 26). Viral vector transduction of donor DNA has also been used with great success. Adeno-associated virus (AAV) vectors delivering homology repair templates in combination with electroporated RNPs is the most widely used method for delivery of HDR genome-editing reagents at this time, and has shown some of the highest reported efficiencies in mouse and human primary B cells, ranging from 10–84% (Table 1). AAVs are commonly used to deliver donor DNA templates because of their stability and low toxicity, however AAV has several limitations, including a small packaging capacity (~5 kb), modest transgene expression levels, and production of low titers (80). AAV serotype-6 is most commonly reported for transduction of human primary B cells while AAV.2 or AAV.DJ has been successfully used in mouse B cells (Table 1). Lentiviral vector transduction of donor DNA templates has not been widely used for CRISPR-based genome editing of B cells however they are becoming increasingly popular because they are capable of transducing both replicating and non-replicating cells, and because they can be psuedotyped with receptor binding proteins that allow for retargeting to different cells types. They are however limited by a small packaging capacity (9 kb), issues with large-scale production (81–83), and risk of insertional mutagenesis (84). LV's pseudotyped with alternative envelopes such as BaEV, measles virus (H/F glycoproteins), feline endogenous retrovirus (RD114), or gibbon-ape leukemia virus (GaLV) have shown naïve B cell transduction rates up to 20–50% (9, 20, 85). Luo et al. could achieve 20% HDR efficiency in activated human B cells by transduction of two BaEV-pseudotyped integration-deficient LVs that delivered both HDR template and CRISPR/cas9 by transduction (14). Adenovirus (Ad) vectors are another common choice for gene delivery. They are stable, have a large packaging capacity (36 kb), can be grown to high titer, efficiently transduce dividing and non-dividing cells, and pose a low risk for insertional mutagenesis (86). The main limitation for Ad-based *ex vivo* editing is poor transduction of primary B cells (87). This problem could be addressed with strategies to incorporate novel B-cell targeting moieties into the Ad fiber protein, which mediates cell infection: (i) a B-cell specific natural ligand (88), (ii) a B-cell specific single domain antibody (89), (iii) peptides (90), or (iv) alternative Ad serotype or xenotype (91) (Figure 2). One group showed that an Ad5 vector with a polylysine-containing fiber was able to transduce lipopolysaccharide (LPS)-activated murine B cells and B cell lines (92). Another group found up to 6.5-fold enhancement binding of B cell lines upon insertion of a CD21 binding sequence into the Ad5 fiber (93).

In Vivo

Despite the success of *ex vivo* cell engineering, there are several drawbacks that make it an impractical method for several major applications of genetically altered B cells, particularly vaccines. Briefly, *ex vivo* editing of primary B cells for adoptive transfer requires (i) cell isolation and sorting (ii) incubation with immune activating agents or differentiation media (HCSs/plasma cells) (iii) transduction with a gene-delivery vehicle, and (iv) characterization of cells before reinfusion into the patient. Furthermore, quiescent B cells require stimulation with cytokines, activating ligands, or other factors to transition from the G0 to G1 phase of the cell cycle to be transduced/modified by HDR. This process is complex, costly and requires significant infrastructure. It would be highly advantageous to simply inject an appropriate vector into a patient and introduce desired changes to the cells *in vivo*.

To date, there is a single report of successful *in vivo* HDR-based editing of B cells using CRISPR/cas and homology repair templates in the mouse model. Here, saCas9/ gRNA (transcriptionally targeted for expression in only B cells), and HIV bnAb donor DNA, were packaged in separate AAV.DJ vectors (due to packaging size restrictions). Systemic co-delivery of the vectors in mice could edit enough B cells to allow subsequent elicitation of transgene derived bnAb serum responses through vaccination with HIV envelope immunogens. (94). This success highlights that despite significant reduction in engineering efficiency that occurs when nuclease and donor DNA must be packaged separately, relatively few engineered cells are required for generation of a therapeutic effect. Vaccination of low numbers of engineered cells can drive 100x clonal expansion and affinity maturation *in vivo* after boosting (26). While B cell targeting *in vivo* can be fairly efficient, it is so far non-specific. One group used high doses of an exosome-associated AAV8 vector *in vivo* that transduced 3–9.5% of the primary B cells in the blood, spleen, and lymph nodes and 40% of the B cells in the liver, with significant off-target cell expression (95). Several strategies exist for improving *in vivo* targeting of B cells using AAVs however. 'Designed ankyrin repeat proteins' (DARPins) (96) or nanobodies (97) can be embedded into the viral capsid to enhance B cell specific targeting for example. Recent work engineering B cells *ex vivo* and new vector developments highlight other possibilities for achieving safe and effective *in vivo* B cell therapies. LV vectors are commonly used for *in vivo* editing and exhibit low levels of immunogenicity (98) and as mentioned, pseudotyped LVs can specifically target B cells. The packaging capacity is again problematic for LVs when both nuclease and HDR template DNA must be delivered to the same cell *in vivo*. Some recent reports have described novel IDLVs capable of packaging RNP into the same vector as donor DNA, which should dramatically improve efficiencies for *in vivo* applications (99, 100). Ad vectors also have a well-established safety profile and have recently been able to deliver CRISPR/Cas machinery for long-term gene expression at therapeutic levels (101). Ads are extremely immunogenic (102). Approaches for dealing with Ad immunogenicity for *in vivo* gene delivery include: vector genome modifications to dampen immune responses; chemical shielding to reduce unwanted surface interactions (i.e. polyethylene glycol) (103); capsid modifications and the use of non-human serotypes; and the use of immunosuppressive drugs during vector administration for transient dampening of anti-vector responses (104). The hepatotoxicity of first-generation Ad vectors has also been addressed using a chimeric capsid strategy for liver de-targeting (105). One study showed *in vivo* delivery of antigen to B cells in mice using an Ad5 vector modified with an Ad serotype 35 fiber. The treatment was capable of suppressing tumor growth and eliciting antigen-specific T cell responses (106). Although it will likely require significant development before clinical adaptation as compared with *ex vivo* methods, *in vivo* delivery to B cells holds more potential as feasible one-shot, universal B cell gene therapy.

Conclusions

Genetic engineering of B cells is a novel but rapidly growing field, driven by recent technological advances in genome editing and delivery technologies, and by the numerous potential therapeutic applications in disease treatment and prevention. B cells have several important immune effector functions that could be harnessed for genetic medicine. For

example, they could be used to durably express therapeutic proteins or antibodies from long-lived plasma cell transgenes. Desirable antibodies can be elicited from B cell receptor engineered cells using vaccine immunogens, and these cells have been shown to be capable of affinity maturation in germinal centers in immunocompetent animal models. However promising, further characterization of *in vivo* function, safety, and therapeutic efficacy of engineered B cells in relevant animal models is required before adapting the most promising applications for clinical testing. Current B cell engineering methods target *ex vivo* activated cells which would require autologous re-engraftment into the patient. *Ex vivo* engineering offers many important advantages including the ability to specifically target and select correctly engineered B cells. However, if genome modified B cells are to become a cost-effective and widely available therapeutic option, methods for safe, specific, and efficient *in vivo* editing should be developed. Adenoviral vectors (Ads), integration-deficient lentiviral vectors (IDLVs) and adeno-associated viral (AAV) vectors are all promising options for *in vivo* delivery of genome editing payloads to B cells. Each of these options have unique advantages and disadvantages that should be explored as this fledging cell therapy matures towards the clinic.

Acknowledgments

Abbreviations used in this article:

AAV	adeno-associated virus
Ab	antibody
Ad	adenovirus
APC	antigen presenting cells
BaEV	baboon retrovirus envelope
BAFF	b cell activating factor
BCR	b cell receptor
bnAb	broadly neutralizing antibody
C	constant
CAR	chimeric antigen receptor
Cas	crispr associated proteins
CBCR	chimeric b cell receptor
CDR	complementarity-determining region
CPG	5'-cytosine-phosphodiester bond-guanine-3'

CRISPR	clustered regularly interspaced short palindromic repeats
D	diversity
DSB	double stranded break
EBV	epstein-barr virus
FIX	factor IX
GALT	gut-associated lymphoid tissues
GaLV	gibbon-ape leukemia virus
GC	germinal center
gRNA	guide ribonucleic acid
H	heavy
HC	heavy chain
HDR	homology-directed repair
HEL	hen egg lysozyme
HIV	human immunodeficiency virus
HSC	hematopoietic stem cell
Ig	immunoglobulin
IgA	immunoglobulin isotype A
IgE	immunoglobulin isotype E
IgG	immunoglobulin isotype G
IgH	immunoglobulin heavy
Ig κ	immunoglobulin kappa light
Ig λ	immunoglobulin lambda light
Indel	insertion or deletion
IRES	internal ribosome entry site
J	joining
LC	light chain
LPS	lipopolysaccharide
LV	lentivirus
mAb	monoclonal antibody

NHEJ	nonhomologous end-joining
NSG	non-obese diabetic severe combined immunodeficiency gamma
RNP	ribonucleoprotein
RSV	respiratory syncytial virus
scFv	single-chain variable fragment
SLO	secondary lymphoid organ
TALEN	transcription activator-like effector nuclease
Th	t helper
TNF-α	tumor necrosis factor alpha
V	variable
VH	variable heavy
VL	variable light
κ	kappa
λ	lambda

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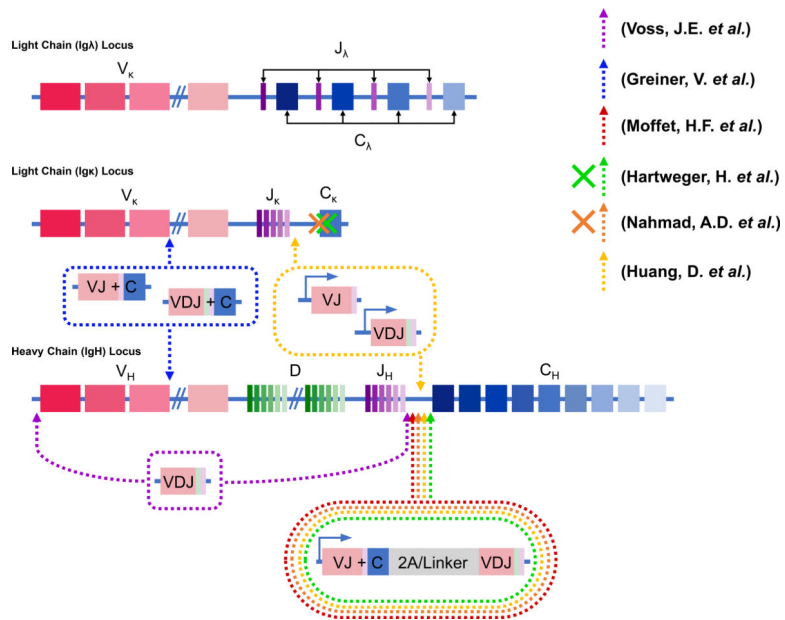
Figure 1: CRISPR/Cas9 BCR Editing

Schematic representation of the targeting strategies used to engineer the endogenous BCR of primary B cells. The human κ and λ light chain loci lie on chromosomes 2 and 22 respectively, while both mouse light chain loci lie on chromosome 6. The light chain loci are comprised of variable (V), joining (J) and constant (C) regions. The heavy chain locus lies on the human chromosome 14 and the mouse chromosome 12. The heavy chain locus is comprised of variable (V), diversity (D), joining (J), and constant (C) regions. Cutting sites used by different groups for transgene insertion are indicated by dashed arrows and cutting sites for light chain ablation are indicated by x's. Depictions of the expression cassettes for each group are depicted in circles of dashed lines (purple (25), blue (29), red (26), green (27), orange (28), and yellow (30)).
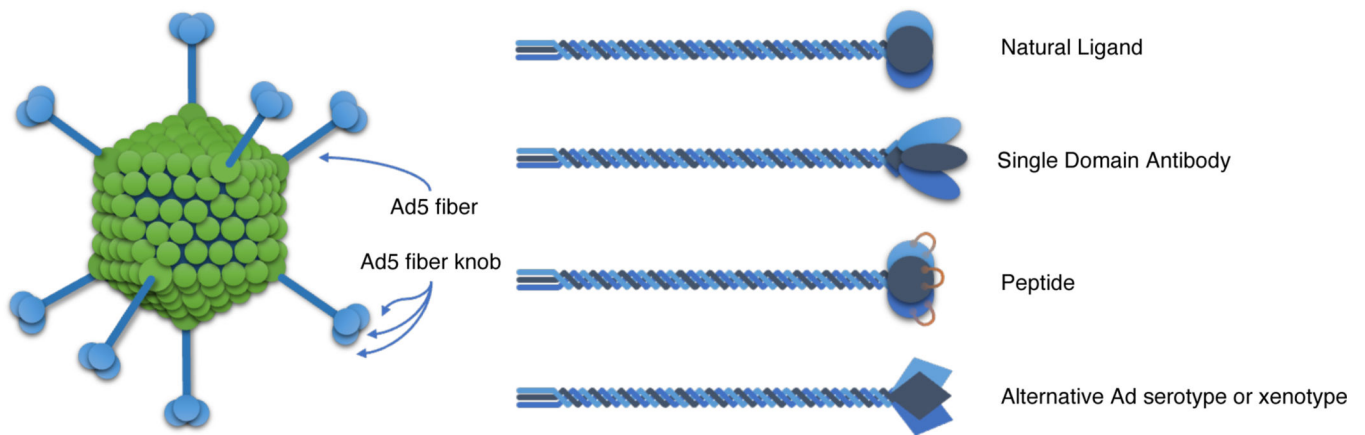
Figure 2: Strategies for Ad Transductional Targeting of B Cells

Adenoviral cell entry involves interactions of the Ad fiber knob with cell surface receptors. B cell targeting using an Ad vector requires ablation of tropism for its native receptor and incorporation of a targeting moiety into the knob that can redirect binding to a B cell surface receptor. This can be accomplished using a fiber-fibritin chimera fused to a novel knob epitope. Strategies to target B cells include modifying the knob to incorporate: (i) a B cell specific natural ligand, (ii) a B cell specific single domain antibody, (iii) a peptide with B cell affinity, and (iv) an alternative Ad serotype or xenotype knob.

Table 1:

CRISPR/Cas9-mediated HDR in Primary B cells

	Johnson (17)	Hung (18)	Pesch (19)	Luo (20)	Voss (21)	Greiner (22)	Hartweger (23)	Moffett (24)	Nahmad (25)	Huang (26)
Modified Cell Type	Human B cells	Human B cells	Murine B cells	Human B cells	Human B cells	Human B cells	Human and murine B cells	Human and murine B cells	Human and murine B cells	Murine B cells
Pre-transduction activation	CD40L and IL4	CD40L, CpG, IL2/10/15	IL4, BAFF, CD40L feeder cells	CD40L, IL2/10/15, and CpG	CD40L and IL4	CellXVivo Human B Cell Expansion Kit	Anti-TLR4 Ab (RP105)	hCD40L, IL2/10/15, and CpG (h) mCD40L + IL4 (m)	Anti-TLR4 Ab (RP105), IL7 (h) LPS (m)	LPS, CpG
Delivery Vehicle	AAV6 + RNPs	AAV6 + RNPs	Linear dsDNA/ AAVDJ +RNPs	BaEV-pseudotyped Integrase-deficient LV	plasmid	ssDNA + RNPs	ssDNA + RNPS	AAV6 + RNPs	AAV6 + RNPs (h) AAVDJ + RNPs (m)	Plasmid / AAV2 RNPs
Integration Site	AAVS1 locus	CCR5 locus	Rosa26 locus	GAPDH locus	IgH locus	IgH and IgK loci	IgH locus	IgH locus	IgH locus	IgH and IgK loci
Cutting efficiency	Not evaluated for AAVS1 locus	43–84%	Not evaluated	48.3%	Not evaluated	not quantified for Ig loci	64% (h) 80% (m)	72% (h) 71% (m)	29% (h) 30% (m)	55–80%
Inserted sequences	EGFP	FIX and BAFF	CBCR	αPD1-CD90	VH	HC/nanobody (into IgH) KC (into IgK)	KC-P2A-VH	KC-linker-VH	KC-linker/ P2A-VH	KC-P2A-VH or VH (into IgH) VL (into IgK)
HDR efficiency	25%	10–40%	0.3%	21.6%	0.21%	Approx. 15–35%	0.4% (h) 4% (m)	10–60% (h) 10–24% (m)	11% (h) 10–84% (m)	10% (HC)
Promoter	endogenous PPP1R12C	donor derived MND	donor derived CMV	endogenous GAPDH	endogenous HC V-gene	endogenous HC V-gene	donor derived HC V-gene	donor derived HC V-gene	HC V-gene promoter or splice acceptor	donor derived HC V-gene
Target	N/A	Hemophilia/ B cell engraftment	HEL	PD-1	HIV	TNF-α	HIV	RSV, HIV, Influenza, and EBV	HIV	HIV
Secretion in immunocompetent mice	N/A	N/A	N/A	N/A	N/A	N/A	7 days	7–12 days	33 days	252 days
Secretion in NSG mice	N/A	21 days	N/A	150 days	N/A	N/A	N/A	72 days	N/A	N/A