6 Gene and Cell Therapy: How to Build a BioDrug

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6.1 Introduction

BioDrugs, or advanced therapeutic medicinal products (ATMP), are novel medicines involving genes, tissues, or cells for use in the treatment of a variety of diseases. The European Medicines Agency (EMA) classifies ATMP into three general categories: (1) gene therapies, (2) somatic-cell therapies, and (3) tissue-engineered medicines (https://www.ema.europa.eu/en/human-regulatory/overview/advanced-therapy-medicinal-products-overview), although there is overlap in the types of technologies used to create BioDrugs in these categories. For example, techniques used to edit or insert genes may be used to create a BioDrug used for gene therapy or for somatic-cell therapy. Development of a successful BioDrug requires in-depth knowledge of cellular biology and molecular genetics, complex manufacturing procedures, and completion of rigorous clinical trials in patients with serious medical illness. This chapter provides a resource for pediatric hematologist/oncologists to learn about the fundamental technologies involved in BioDrug development and will focus on the development of BioDrugs in the first two categories: (1) *gene therapies*, defined as a BioDrug that contain genes for insertion into the human genome or that contain gene-editing machinery for intracellular correction of genetic diseases, and (2) *cell therapies*, defined as cell products or tissues that have been manipulated to change their biologic characteristics with the aim to cure human disease.

As ATMP become more available, it is important to understand the principles involved in their development and the components required to produce a BioDrug. The genes, gene-editing tools, delivery systems, tissues, and cells used to generate ATMP can be thought of as existing in a BioDrug ToolKit (Fig. 6.1), which contains various components or tools for use in development of a BioDrug. The main categories of "tools" used in production of a BioDrug include cells, genetic materials and editing systems, and delivery systems. A vast choice of tools exists within each category, which can be combined to generate novel therapeutic agents that can be applied ex vivo in laboratory-based cell cultures or in vivo by direct administration to a patient for treatment of life-threatening malignancies or hematologic disorders.

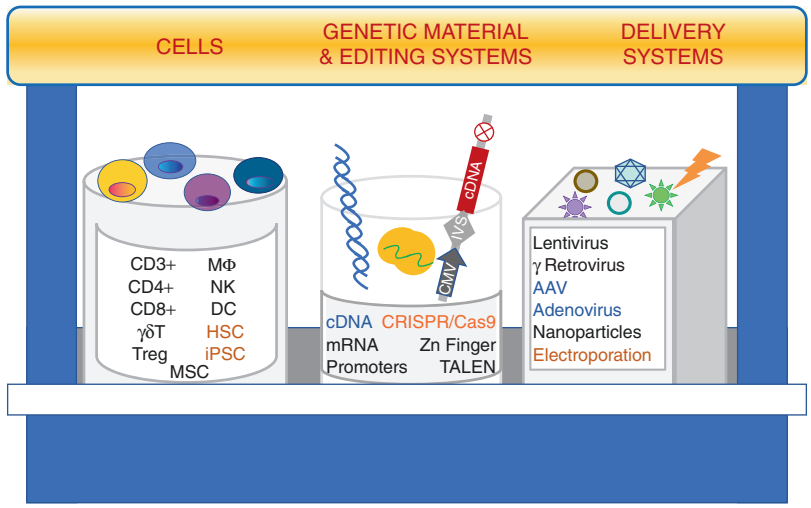

Fig. 6.1 The BioDrug ToolKit contains groups of tools and materials used to engineer cell and gene therapy. Combinations of cells, genetic material, and transgene delivery systems are used to engineer BioDrugs. Cells are selected for their biologic properties. The selection of the genetic material or editing systems is based on the desired genetic engineering approach. The selection of the delivery system is based on its carrying capacity and the efficiency of gene transduction

6.2 BioDrug ToolKit: Cells

The first important group of tools in developing a BioDrug are the cells or tissues that will be used either as targets of the BioDrug or as a component of the BioDrug itself. A cell may be used by itself as a BioDrug, or it may be genetically engineered to perform a specific function. BioDrug cells which are intended to be returned to the individual from which they were collected are termed "autologous" cells, whereas cells that are collected from one individual for administration to another individual are termed "allogeneic" cells. Allogeneic cells will differ genetically from the recipient and may express different major histocompatibility complex (MHC) antigens or minor histocompatibility antigens. The need to match the human leukocyte antigens (HLA) of the recipient with allogeneic cells depends upon the end use of the cells and whether long-term engraftment is desired. Depending on the type of immune cell, unmatched, unmanipulated allogeneic cells can mediate tissue damage in the recipient (i.e., graft-versus-host disease (GVHD)).

Cell products may be administered to the patient immediately after collection of the cells, such as in bone marrow transplantation procedures, or may also be cryopreserved for future use (Hornberger et al. 2019). Cryopreservation involves placing the cells in a solution with dimethyl sulfoxide (DMSO) which allows the cells to survive extremely low temperatures. The cell solution is cooled at a controlled rate until it can be stored in liquid nitrogen (approximately −195 °C). The shelf-life of cryopreserved cells depends on the cell type and cryopreservation methods.

The BioDrug ToolKit contains a variety of cells that can be developed for therapeutic use. The most commonly used cells are described here.

6.2.1 Hematopoietic Stem Cells (HSCs)

HSCs are capable of either self-renewal or differentiation into the various mature cells that comprise the hematopoietic system, including red cells, platelets, myeloid cells, and lymphocytes. HSCs reside in the bone marrow and can be obtained by direct aspiration of bone marrow or through mobilization of HSC into the bloodstream and removal via apheresis. HSCs are anchored in the marrow by adhesion to stromal cells; therefore, release of HSC into the peripheral blood requires interfering with these cellular bonds. The most efficient way to release HSC is through agents that disrupt adhesion bonds such as CXCR4-CXCL12. The most commonly used agents include granulocyte colony-stimulating factor (G-CSF) and more recently plerixafor (Giralt et al. 2014). While both bone marrow and mobilized peripheral blood stem cell (PBSC) products contain HSC, there are differences in composition that might affect the end use. Compared to mobilized PBSC, marrow products contain relatively more red cells and a lower proportion of T cells and may contain other bone marrow-derived cells, such as mesenchymal stromal cells. Bone marrow or mobilized PBSC may be administered to patients up to several days following collection, after which the viability is significantly reduced (Lazarus et al. 2009). When the intention is not for immediate use, both products may be cryopreserved and then thawed before administration to a patient or for use in generating an engineered cell product (Hornberger et al. 2019). HSCs also reside in the placenta and may be obtained by collection of postpartum umbilical cord blood and cryopreserved for future use.

In the setting of hematopoietic cell transplantation (HCT), HSCs can be viewed as a cell therapy product used to restore hematopoiesis after marrow-ablative therapy. HSCs also are used as the cellular component for the development of gene therapy products aimed at correcting genetic defects resulting in hematopoietic or immunologic diseases.

6.2.2 T Lymphocytes

T lymphocytes are the main effector cells of the adaptive immune system. Mature T cells recognize antigen via the T cell receptor (TCR), which is expressed early in T cell development (Davis and Bjorkman 1988). TCRs are heterodimers

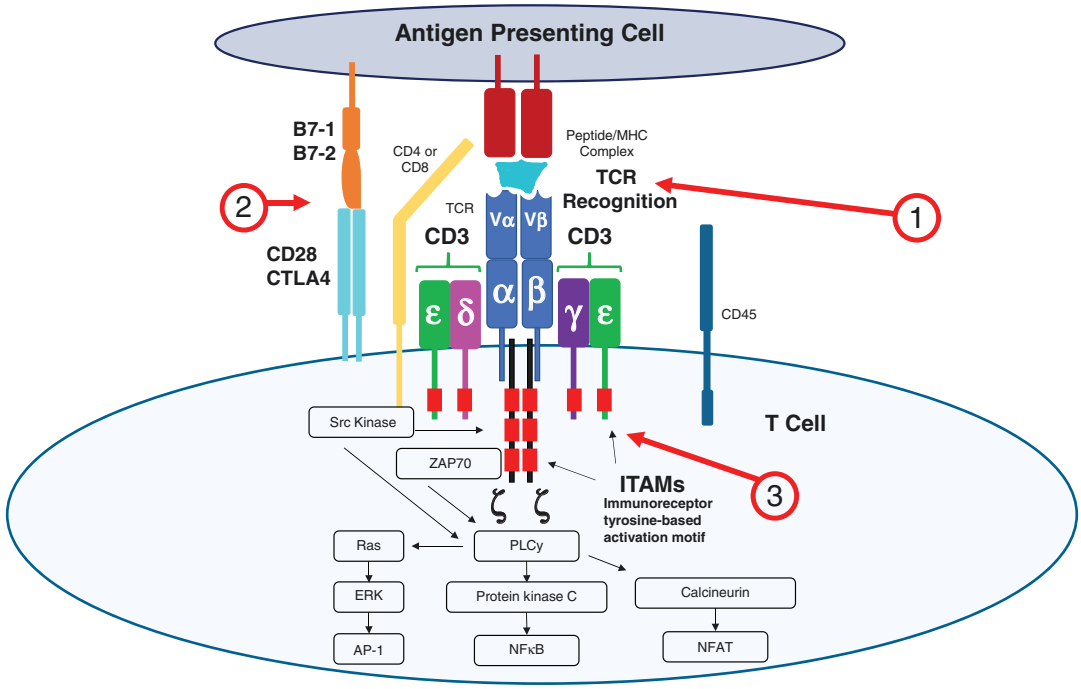

Fig. 6.2 The T cell recognition complex is comprised of (1) the T cell receptor (TCR); (2) the CD3 complex; and (3) the zeta (ζ) chain signaling molecules. The TCR is a heterodimer composed of alpha (α) and beta (β) chains. Each chain has a variable (V) and a constant region. When the TCR recognizes a peptide presented by a major histocompatibility antigen, signaling through the ζ chains results in phosphorylation of intracellular immunoreceptor tyrosine-based activation motifs (ITAMs). T cell activation involves three basic steps. Step 1: The T cell receptor recognizes the peptide in the context of the major histocompatibility complex (MHC) antigen of the antigen-presenting cell (APC). Step 2: A second signal or co-stimulatory signal is received by the T cell from the APC. Step 3: The TCR signal in concert with the co-stimulatory stimulates intracellular signaling leading to recruitment of ZAP-70 and Src kinase activation and subsequent generation of cytokines that promote T cell proliferation

comprised of α- and β-polypeptides linked by disulfide bonds. Each polypeptide has a variable region (Vα and Vβ, respectively) and a constant region (Fig. 6.2). The variable regions of the heterodimers are translated from a series of randomly juxtaposed sections of the V, D, and J genetic regions. When expressed on the cell surface, the TCR heterodimer associates with the CD3 heterodimeric complex externally, and internally associates with the ζ chain.

The entire complex, including the TCR, CD3, and ζ chain, is required for T cell antigen recognition and signaling (Alcover et al. 2018). In the simplest sense, activation and proliferation of antigen-specific T cells requires three main steps (Fig. 6.2): the first is TCR recognition of an antigen presented in the groove of a MHC molecule, which in humans are the human leukocyte antigens (HLA) (La Gruta et al. 2018; Smith-Garvin et al. 2009). Next a second signal or co-stimulatory signal must be received by the T cell from the antigen-presenting cell (APC). Importantly, if the second signal is not received, the T is rendered impotent or anergic (Sharpe and Freeman 2002; Azuma 2019). Co-signal receptors on the T cell include CD28, which interacts with B7-1 and B7-2 molecules, and the inducible T cell co-stimulator (ICOS), which interacts with the ICOS ligand. When stimulated, CD28 transmits a signal that in concert with the TCR signal results in the third step, phosphorylation of intracellular immunoreceptor tyrosine-based activation motifs (ITAMs) leading to recruitment of ZAP-70 and Src kinase activation and subsequent generation of cytokines such as IL-2 that promote clonal expansion.

To acquire sufficient numbers of T cells for cell therapy, large numbers of peripheral blood mononuclear cells (PBMC) are obtained by non-mobilized apheresis and then placed in culture conditions that support expansion of the desired T cell subset(s). Various T cell subsets have been used as a source for cell therapy products and can be selected from the apheresis product by their cell surface receptors. Most T cells within the product have heterodimeric TCRs composed of α- and β-polypeptides. αβT cells that express CD8 recognize peptides presented by class I HLA, whereas T cells that express CD4 recognize peptides presented by class II HLA. These subsets can be functionally defined further into naïve and memory subsets, which can be distinguished from each other by expression of other surface markers, e.g., CD62L, CCR7, and CD45RA expression on naïve T cells and CD45RO on memory T cell subsets (De Rosa et al. 2001). αβT cell subsets have been the predominant cell type used in the development of BioDrugs that target malignancies.

A small fraction of T cells have TCRs composed of γ- and δ-polypeptides, which play a role in both the adaptive and innate immune responses (Paul et al. 2014). γδT cells are found primarily in mucosal tissue and are capable of HLA-unrestricted cytotoxic activity, secrete cytokines that facilitate T and B cell activity, and are capable of antigen presentation. The development of BioDrugs using γδT cells is being explored as an alternative to αβT cells, based on their ability to infiltrate a wide variety of tumors and to recognize small phosphorylated non-peptide molecules emanated by tumor cells (Brandes et al. 2005; Gertner-Dardenne et al. 2012; Groh et al. 1999).

T regulatory (Treg) cells are another small population of CD4+ T cells that play a role in maintaining peripheral tolerance and preventing autoimmune disease. Tregs are characterized by surface expression of CD25 and intracellular FOXP3 (Owen et al. 2019). BioDrugs based on Treg cells currently are being explored for inducing tolerance in organ transplant recipients or mitigating GVHD after HCT and in patients with autoimmune diseases.

6.2.3 Natural Killer (NK) Cells

NK cells are large granular lymphocytes that play a pivotal role in the innate immune response to viral pathogens and tumors as well as have an adjunctive role in the adaptive immune response (Campbell and Hasegawa 2013; Caligiuri 2008; Sun et al. 2009). NK cells reside in lymphoid tissue as well as circulate in the blood and are characterized by surface expression of CD56 and lack of CD3 expression. NK cells interact with their environment through multiple inhibitory and activating receptors, including killer-cell immunoglobulin-like receptors (KIRs), CD16, or NKG2D, which engage MHC class I as well as non-MHC molecules. Activation of NK cells can occur either through engagement of a ligand with an activating receptor or by lack of engagement of an inhibitory KIR with its MHC class I ligand. Once activated, NK cells can directly kill target cells through perforin/granzyme production or through death receptor pathways (Smyth et al. 2001; Bryceson et al. 2006). Activated NK cells also produce gamma interferon (IFNγ), thus stimulating components of the adaptive immune response. NK are generated for cell therapy by apheresis and subsequent selection of CD56-positive cells, which are then placed in culture or cryopreserved (Kottaridis et al. 2015). NK cells currently are being developed as autologous or allogeneic cell therapies, either as unmanipulated cell products or as engineered tumor-directed cells.

6.2.4 Macrophages (MΦs)

MΦs reside in a variety of tissues and function to maintain homeostasis through cell-to-cell contact and elaboration of cytokines. Depending upon the microenvironment, MΦs become reversibly polarized toward a pro-inflammatory (M1) or an anti-inflammatory (M2) phenotype. M1 polarization occurs after stimulation of MΦs by pro-inflammatory agents such as IFNγ or lipopolysaccharide (LPS) which activate the NFκB pathway (Lee et al. 2016a; Mills et al. 2000). Pro-inflammatory MΦs play a role in the

innate and adaptive immune systems through phagocytosis, antigen presentation, and cellular cytotoxicity. M2 polarization occurs in response to IL-4 signaling, as well as M-CSF, IL-10, IL-13, and TGF-β. These anti-inflammatory cytokines are prominent within the tumor microenvironment and result in polarization of tumor-associated MΦs (TAMs) (Italiani and Boraschi 2014). TAMs facilitate tumor persistence by contributing to the immunosuppressive environment and promoting angiogenesis and tumor invasion.

MΦs are obtained for cell therapy by apheresis or through lavage of alveolar or peritoneal tissues. Once collected, MΦs are placed into culture conditions that stimulate the M1 phenotype (Lee et al. 2016a). However, unlike T lymphocytes which can be expanded to large numbers in appropriate culture conditions, there is limited ability for MΦs to proliferate ex vivo. As an alternative approach, MΦs have been generated from conditional progenitor cell lines that allow differentiation to MΦs under specific culture conditions (Wang et al. 2006). M1 MΦs currently are currently being studied as the platform for solid tumor-directed engineered cell therapies (Klichinsky et al. 2020).

6.2.5 Dendritic Cells (DCs)

DCs are potent APCs involved in both the innate and adaptive immune responses. DCs arise from bone marrow CD34+ stem cells and reside in various tissues (Liu and Nussenzweig 2010). Mature DC subsets include myeloid/conventional DC1 (cDC1), myeloid/conventional DC2 (cDC2), and plasmacytoid DC (pDC) (Collin and Bigley 2018). DCs interact with their environment through multiple signaling receptors and produce various cytokines in response to stimulation. pDCs produce IFNα, TNF, IL-6, and granzyme B in response to receptor signaling by viral nucleic acids. cDC1 express MHC class I and present antigen to CD8+ T cells, as well as produce IFNα and IL-12. cDC2 also present antigen and secrete high levels of IL-12.

Dendritic cell therapy has been explored as a mechanism to increase anti-tumor immune responses through "vaccination" with cells that present tumor antigen to native CD8+ T cells (reviewed in depth in Sabado et al. 2017). DCs can be generated ex vivo from monocyte precursors or CD34+ HSC. Antigen loading of DCs is accomplished by incubation with proteins, RNA, or tumor cells along with GM-CSF to produce activated APCs. Sipuleucel-T is an example of an ex vivo cultured cell product that includes activated DCs, which is now approved for treatment of prostate cancer.

6.2.6 Mesenchymal Stem Cells (MSCs)

MSCs are defined by expression of CD73, CD90, and CD105, lack of HLA-DR expression, as well as the ability to adhere to plastic and to differentiate into mature mesenchymal tissues including adipocytes, chondrocytes, and osteoblasts (Wagner et al. 2005). MSCs can be derived from bone marrow, umbilical cord blood, as well as other adult and fetal tissues (Ullah et al. 2015). Tissue or blood MSCs are isolated by seeding onto plastic culture plates in specific culture conditions that can generate mesodermal, ectodermal, or endodermal lineages. MSCs are being studied for use in cancer immunotherapy as well as immunomodulating therapies for degenerative or autoimmune diseases. Currently there is considerable interest in using MSCs as either treatment for or prevention of graft-vs-host disease after allogeneic HCT (reviewed in Zhao et al. 2019).

6.2.7 Human-Induced Pluripotent Stem Cells (iPSCs)

Human iPSCs are not natural human cells but are mentioned here as these have been used to engineer BioDrugs. Human iPSCs are created by reprogramming a differentiated cell, such as a fibroblast, by insertion of genetic instructions (reviewed in Hockemeyer and Jaenisch 2016). This results in a personalized pluripotent cell that can then undergo differentiation by manipulating culture conditions to regenerate mature tissues, such as cardiac or neurologic tissues.

6.2.8 Target Tissues

Normal body tissues may be the target of genetic engineering in order to correct a genetic mutation within that specific cell. Examples include pulmonary epithelial cells in patients with cystic fibrosis, or retinal cells in patients with biallelic *RPE65* mutation-associated retinal dystrophy. Alternatively, an organ may play the role of host to new genetic machinery that produces a protein which functions elsewhere. The liver is a common target of in vivo gene therapy, because many viral vectors and nonviral particles that transport genetic material are hepatotropic. For example, the liver has been the target organ for expression of factor VIII in patients with hemophilia A (Pasi et al. 2020).

Abnormal tissues, such as solid tumors, are another type of target for gene or cell therapies. Tumors present challenges to effective drug delivery due to the heterogenous nature of the tumor and stromal cells. The tumor "ecosystem" results from interaction between tumor clones, stromal cells such as endothelial cells and fibroblasts, and host immune cells such as TAMs (Petty and Yang 2017). This symbiotic environment promotes tumorigenesis and creates an immunosuppressive tumor microenvironment (TME). Tumor heterogeneity poses a challenge to identify uniformly expressed tumor-specific antigens. Mechanisms to evade anti-tumor immune responses also vary within and between tumor types, resulting in a tumor-specific microenvironment that may be unique to the host (Hinshaw and Shevde 2019; Wu and Dai 2017).

6.3 BioDrug ToolKit: Genetic Material and Gene-Editing Machinery

The second important group of tools employed in the creation of a BioDrug are the genetic materials and machinery used for giving target cells new instructions or for correction of dysfunctional genes. The choice of genetic material depends upon whether gene replacement or gene editing is desired, whether long-term gene expression is desired, and the nature of the target tissue. Genetic information may be delivered to a cell as either mRNA or cDNA. cDNA must be transcribed into mRNA and therefore must enter the cell nucleus which contains the transcriptional machinery. Once in the nucleus, the genetic material of cDNA may become integrated into the host cell genome or can remain episomal, depending upon the approach used to deliver the cDNA into the cells. Integration of cDNA into the genome usually occurs randomly, although some vectors preferentially deliver the genetic material into specific genomic locations. The advantage of integrated cDNA is that the new genetic material will be replicated and carried into daughter cells during mitosis. Nonintegrated cDNA will be lost over time as its host cell undergoes mitosis. Accordingly, most cellular targets for nonintegrated cDNA are long-lived postmitotic cells in organs with low cell turnover, such as the liver, heart, or nervous system.

Protein expression from mRNA sequences requires just the intracellular translational machinery which can be found in the cytoplasm. However, protein expression is transient, persisting intracellularly for less than a month as mRNA will be degraded by intracellular RNase. Several techniques may increase the stability and durability of intracellular mRNA, for example, optimizing the non-translated genetic material at either end of the mRNA, such as 3′ untranslated regions (UTR) or the 5′ cap analogs (Orlandini von Niessen et al. 2019; Stepinski et al. 2001). The severe acute respiratory syndrome coronavirus 2 (SARS-CoV2) vaccine is an outstanding example of successful mRNA therapy that easily adapts to new viral mutations due to the relative ease of reprogramming the mRNA cassettes.

Transcription from either integrated or nonintegrated transgenes cannot occur without additional regulatory elements. Viral vectors used for episomal gene transfer may contain DNA replication and activation motifs sufficient to express the inserted mammalian genetic material (Van Craenenbroeck et al. 2000). Most vectors used in gene therapy con-

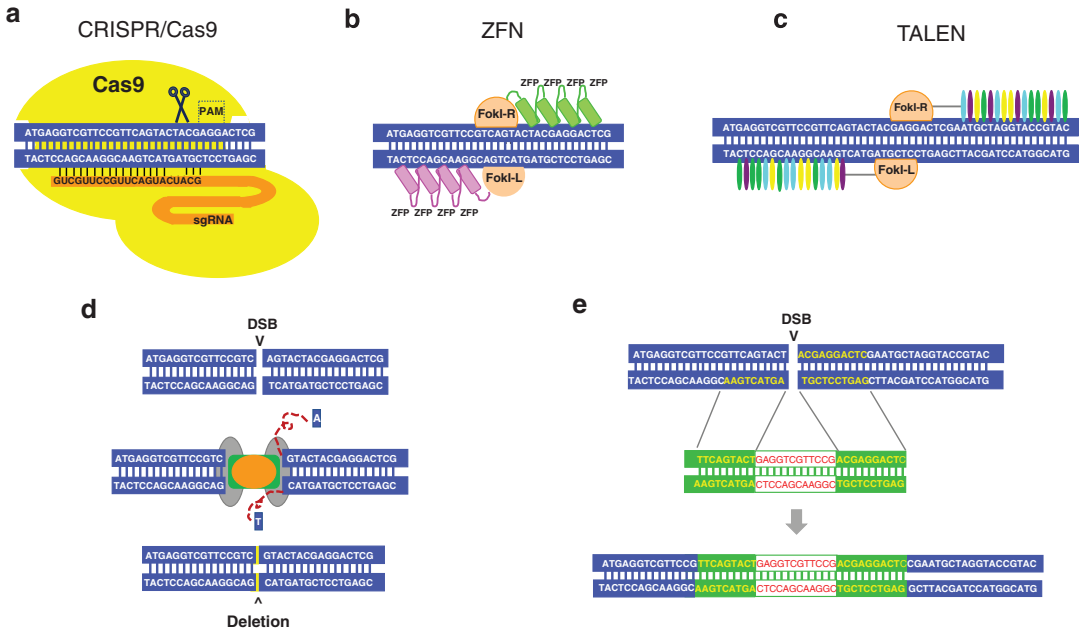

Fig. 6.3 Systems for gene editing in use most commonly include the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) protein 9 or CRISPR/Cas9 system (panel **a**); zinc-finger nucleases (panel **b**); and transcription activator-like effector nucleases or TALENs (panel **c**). Each of these editing systems results in a double-strand DNA break. The broken strands are reunited by one of two natural repair pathways. The most common repair mechanism is nonhomologous end joining, in which the broken ends are directly ligated (panel **d**). Mistakes in the end ligation, such as a loss of a nucleotide, result in a deletion that can knock out the gene. Homology-directed repair (panel **e**) requires the presence of a homologous piece of DNA for religation. Insertion of new genetic sequence is accomplished by providing a length of DNA that has the new sequence, flanked by sections that are homologous the regions on either side of the double-strand break

tain an expression cassette, which consists of a promoter and a polyadenylation signal in addition to the therapeutic gene. DNA transcription is activated by a promoter within the cassette. The level of DNA transcription depends on promoter strength and tissue-specific activity. Elongation factor 1 alpha or cytomegalovirus (CMV) promoters are constitutively expressed and transactivate high levels of transgene expression (Kim et al. 2002; Teschendorf et al. 2002). In some situations, tissue-specific gene expression is desired, which requires knowledge of the natural promoter region and its location relative to the gene (Saukkonen and Hemminki 2004; Zheng and Baum 2008; Boulaire et al. 2009).

When gene editing is the desired objective, the BioDrug may be used to remove or inactivate a gene involved in the pathogenesis of a disease or to "fix" a gene by replacing a mutation with the correct genetic sequence. In either case, gene-editing "machinery" is delivered to the nucleus along with instructions for targeting the correct gene. The machinery includes a cutting device that causes a double-strand break followed by DNA repair by the endogenous repair mechanisms. The commonly used gene-editing machinery is described below (Fig. 6.3).

The clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) protein 9 system was first recognized as a bacterial defense against virus and phage infection (reviewed in Zhang et al. 2014). The CRISPR part of the system acts as a template to produce a sequence of RNA that is complementary to parts of the viral DNA. The Cas9 part of the system is an enzyme that cuts DNA producing double-strand breaks. Together the CRISPR RNA (crRNA) and a transactivating crRNA (tracrRNA) bind to Cas9 and guide it to sections of the DNA

that contain a short protospacer adjacent motif (PAM). Once the PAM sequence is recognized, the CAS:RNA complex unwinds the DNA from the first 10–12 nucleotides after the PAM sequence. If that section of DNA is complementary to the crRNA sequence, the Cas9 produces a double-strand break (Jinek et al. 2012; Gasiunas et al. 2012).

The CRISPR/Cas9 system subsequently has been modified such that it can now be used to specifically target and cut precise areas in the human genome (reviewed in Thurtle-Schmidt and Lo 2018). The original bacterial crRNA and tracrRNA have been fused to create a single-guide RNA (Jinek et al. 2012; Gasiunas et al. 2012). Since PAM sequences occur approximately every 8 base pairs, CRISPR/Cas9 can interrogate the entire genome for a genetic sequence of interest. To target a specific gene, the crRNA is engineered to an RNA sequence of about 20 nucleotides complementary to the target gene. This engineered complex is termed the CRISPR/Cas9 site-specific endonuclease or sgRNA:Cas9. Once the sgRNA:Cas9 complex has bound at the target gene sequence, the Cas9 cuts both DNA strands. From that point, the native DNA machinery will repair the break by nonhomologous end joining. Since there are no overlapping homologous ends to form a template for repair, nonhomologous end joining frequently results in addition or subtraction of base pairs that effectively causes a disruption of the native gene. More sophisticated gene editing can be performed when two guide RNAs are used to target sites on either side of the mutation, resulting in loss of a segment of dsDNA. Nonhomologous repair can be used to replace the lost segment with an "inert" segment of dsDNA that essentially rejoins the cut ends. Alternatively, homology-directed repair can be used to substitute a section of dsDNA that contains the corrected genetic sequence, thus repairing the gene mutation and resulting in a functional gene.

Several challenges must be addressed for efficient and safe CRISPR/Cas9 gene editing. The design of the sgRNA must allow for the relatively short complementary RNA sequence to identify the correct part of the target gene for editing and at the same time avoid any chance of cutting at similar genetic sequences in nontarget genes (off-target cutting). Correction of genetic mutations remains more difficult than simply introducing a double-strand break that results in a deletion or mutation, because nonhomologous end joining is far more common than homology-directed repair (Maruyama et al. 2015). Optimization of Cas9 activity also must be achieved, for example, by using a Cas9 protein that recognizes a unique PAM sequence or other means to increase its enzymatic activity (Jinek et al. 2012).

Several other systems exist as alternative platforms for engineering customized DNA-binding nucleases. Meganucleases are homing endonucleases (enzymes that cut DNA) that recognize up to 40 base pairs of DNA sequence as binding sites for cleavage. In order to customize the meganuclease for gene editing, the DNA-binding sites of naturally occurring meganucleases are reengineered to target the desired DNA sequence (Ashworth et al. 2010). Meganucleases can be put together using selected protein units that have been created for this purpose (Smith et al. 2006; Arnould et al. 2006). Meganucleases potentially have less risk for off-target cleavage due to their very high specificity; however, other methods for creating double-strand DNA breaks are more easily customized.

Zinc-finger nucleases (ZFNs) are an engineered hybrid system that combines the DNA-cutting activity of the restriction endonuclease Fok1 with the DNA-binding specificity of zinc-finger proteins. A zinc-finger protein is a compact unit of approximately 30 amino acids arranged with as a double β-sheet linked to an α-helix that binds to DNA via surface amino acid side chains (Pavletich and Pabo 1991). An individual zinc finger will make contact with three to four base pairs in the major groove of DNA. Several zinc-finger proteins can be linked together in tandem to form domains that can bind to longer DNA sequences providing more specificity (Pabo et al. 2001). By combining zinc-finger proteins with unique DNA-binding specificity, ZFNs can be engineered to recognize specific genomic sequences for gene editing (reviewed in Gersbach

et al. 2014). The second component of ZFNs, the restriction endonuclease Fok1, must be dimerized in order to cut DNA. To accomplish this, two ZFNs are delivered, each of which recognizes a sequence on opposite DNA strands that is 5–7 bp from the target cutting. This allows the Fok1 endonucleases to align, forming a dimer that allows cleavage of each strand resulting in a double-strand DNA break. ZFN-mediated double-strand breaks allow for homology-directed repair as well as nonhomologous end joining.

Transcription activator-like effector nucleases (TALENs) work in a similar manner by combining the endonuclease activity of the Fok1 restriction enzyme with a DNA-binding TALE proteins (Li et al. 2011a). TALE proteins have 33–35 amino acids that bind a single base pair in the major groove and wrap around the DNA in a superhelical structure. DNA-binding specificity can be engineered by assembling TALE repeats in a modular fashion to target almost any DNA sequence (Christian et al. 2010; Morbitzer et al. 2010). The TALE bonding domain is fused to the Fok1 endonuclease, which functions when dimerized in the same manner as in ZFNs. The two TALENs recognize sequences on opposite DNA strands 12–20 base pairs from the target cleavage site, inducing double-strand breaks for nonhomologous end joining or homology-directed repair (Li et al. 2011b). Advantages of TALEN-mediated gene editing include its limitless targeting capability and straightforward engineering; the disadvantage is the large size of TALE arrays which pose a significant barrier to using some in vivo delivery systems.

6.4 BioDrug ToolKit: Delivery Systems

The third important group of tools for creating a BioDrug are delivery systems to ensure the genetic materials or editing machinery are transferred into the target cells. Delivery systems fall into two broad categories: viral-based and nonviral-based delivery systems. Selection of a delivery system is dictated by the nature of the target tissue and whether the gene is to be transferred ex vivo or in vivo. The cellular targets for ex vivo gene transfer include HSC, T cells, and other hematopoietic cells that can be removed and kept healthy in culture before reintroduction into the body. In this case the delivery system should allow for transgene stability in dividing cells. Selection of a delivery system for in vivo gene therapy depends primarily on its cell tropism. This section describes the characteristics of the various delivery systems that can be used for building a BioDrug.

6.4.1 Viral-Based Delivery Systems

Viral vectors are used to infect target cells and then deliver genetic material into the nucleus or cytoplasm. Viral vectors used clinically are based on naturally occurring viruses known to infect human cells but rendered replication incompetent by removal of most of the native viral genes. Selection of a specific viral vector depends upon the target cell and whether the transferred genetic material is intended to be integrated into the genome. Integration of genetic material is important when the genetic information must not be lost during mitosis, such as when the target cells are HSC, other progenitor cells, or cells that expand in vivo such as T cells.

Factors that affect transgene expression within the target cell include the specific transgene being delivered and the *cis*-elements incorporated within the vector, such as the type of promoters and regulatory motifs and the orientation of the transgene within the vector. These elements influence the degree to which transgene expression may be repressed by the target cell silencing machinery or eliminated by host immune responses.

The optimal vector for gene therapy will result in stable and high-level transgene expression, high transfection efficiency, high carrying capacity of genetic material, no insertional mutagenesis, no host immune response, and no ability to transform and incite secondary malignancy. As yet, no viral delivery system has met all criteria, and each has specific advantages as well as car-

ries specific risks for gene delivery into human cells. The most commonly employed viral vectors are described below.

Retroviral (RV) vectors are RNA viruses which require reverse transcription to generate cDNA for integration into the host genome (reviewed in Biasco et al. 2017). Although RV vectors are derived from wild-type retroviruses, substantial portions of the original viral genomes have been deleted or altered to render them acceptable for use in human gene therapy. RV vectors retain the genetic elements that encode for the reverse transcription machinery as well as the viral proteins required for integration into the host cell genome. These characteristics allow RV vectors to transfect dividing and sometimes nondividing cells, resulting in stable long-term expression of the integrated transgene. RV-based gene transduction is primarily used in ex vivo gene delivery, such as gene transfer into HSC or lymphocytes.

Production of retroviral vectors occurs by transfecting a packaging cell line with the various components of the retrovirus required for host cell infection, delivered in separate cassettes that mitigate the possibility of generating replication-competent virus (reviewed in Cockrell and Kafri 2007). These components include (1) the envelope cassette that contains the viral genes required to form the envelope and which dictates the cell tropism and vector entry via endocytosis; (2) the packaging cassette that contains constitutive promoters that drive expression of packaging elements; (3) the vector cassette that contains the viral elements required for reverse transcription; and (4) the transgene expression cassette that includes the transgene sequence and promoter elements. Together these cassettes within the packaging cell line result in production of viral particles that contain the transgene. The viral particles can be harvested from the supernatant of the producer cells and purified. Target cells are then incubated with the virus at an optimal ratio of virus particles to cell, termed multiplicity of infection (MOI). The viral particle enters the cell through direct membrane fusion or attachment via a surface receptor. Once in the cell, the viral particle is uncoated to release the reverse transcription complex (RTC) which is transported to the chromosomal DNA where integration occurs (reviewed in Milone and O'Doherty 2018). The viral RNA is converted within the RTC into proviral DNA. The RV viral proteins deliver the proviral DNA into the nucleus where the RV integrase enzyme catalyzes the integration of the transgene DNA into the host genome. Each class of retrovirus has preferential DNA sequences for insertion. Following integration, the transgene is expressed by the host cellular transcription machinery.

The goal for most gene therapy using RV vectors is a single transduction event in the target cell genome that does not interfere with normal gene function and that results in stable high-level transgene expression. Producing a high-potency RV vector must take into consideration the incorporation of the specific envelope protein elements that dictate the appropriate RV pseudotype and the constitutive and tissue-specific promoters that dictate transgene expression. Additional considerations include the potential for transgene silencing by the host cell, which depends upon the *cis*-elements within the vector and the specific transgene being delivered (reviewed in Ellis 2005). Potential risks of all RV vectors include insertional mutagenesis, generation of replication-competent vectors, and germ-line transmission of vector sequences.

The first successful RV-based gene transduction was developed using the gamma retrovirus (γRV) murine leukemia virus (MLV). γRV vectors have a large capacity for transgenes, however, are restricted by the requirement for target cell mitosis for uptake. Thus, clinical use of γRV-based gene transfer has been limited to target cells that undergo cell division. A second limitation of γRV vectors is that genome integration is nonrandom with a preference for integration into actively transcribed loci near the initiation of transcription (Biasco et al. 2012, 2017). In clinical trials using γRV-based gene delivery, nonrandom integration has led to insertional mutagenesis resulting in leukemia (Hacein-Bey-Abina et al. 2008).

Lentiviral (LV) vectors have emerged as a potentially safer and more broadly applicable

approach to delivering transgenes (reviewed in Cockrell and Kafri 2007; Kafri 2004; Escors and Breckpot 2010). LV vectors were initially derived from the human immunodeficiency virus-1 and are capable of infecting both dividing and nondividing cells (Lewis et al. 1992; Yamashita and Emerman 2006). Safety of LV vectors is enhanced by deletion of specific viral sequences that result in self-inactivating (SIN) vectors (Miyoshi et al. 1998; Zufferey et al. 1998). LV vectors have demonstrated high efficiency of infection and long-term stable expression in many tissues (Naldini et al. 1996; Kafri et al. 1997). Compared to γRV vectors, LV vectors appear to have a more favorable integration profile with less risk for insertional mutagenesis. Furthermore, LV vectors are less immunogenetic, which may decrease the risk for host cell silencing.

Foamy virus (FV) vectors have broad tropism and can carry large transgene cassettes (Trobridge 2009). The virus itself is not pathogenic in humans. FV vectors require cell division for efficient transduction and integration into the host genome. However, FV vectors can infect a quiescent cell and form a stable transduction intermediate that can then integrate into the host genome once the cell undergoes mitosis. Safety of FV vectors has been enhanced by deletion of sequences involved in viral replication. FV vectors have been used in both ex vivo and in vivo gene delivery (Liu et al. 2008; Simantirakis et al. 2020).

Other viral vector delivery systems have been developed that more effectively allow transgene delivery to nondividing cells and avoid the risks of insertional mutagenesis. These viral vectors may be used to deliver transgenes in vivo, without the requirement for ex vivo incubation with the target cell which offers an advantage over RV-based vectors.

Adenoviral vectors used for gene therapy can transfer large amounts of genetic material into both dividing and nondividing cells (Quantin et al. 1992; Athanasopoulos et al. 2017). Adenoviral vectors are nonintegrating dsDNA virus vectors capable of carrying payloads exceeding 30 kb (Youil et al. 2003). High transfection efficiency is achievable with adenoviral vectors, although transgene expression typically is transient. Because transgene expression may be lost when target cells undergo mitosis, the most appropriate target cells are stable nondividing cells such as hepatic or muscle cells. Since there is no need for target cells to undergo mitosis, adenoviral vectors can be administered directly in vivo by intravenous or other routes. Much of the native adenovirus genome has been deleted to render adenovirus vector replication incompetent; however, adenoviral vectors remain highly immunogenic since most humans have been exposed to wild-type adenoviruses (Nwanegbo et al. 2004). The immunogenicity of adenoviral vectors can result in target tissue inflammation and inhibition of transgene expression (Raper et al. 2003). Several engineering strategies have been developed to reduce immunogenicity such as the inclusion of adenoviral E3 genes that downregulate host cell MHC expression (Youil et al. 2003).

Adeno-associated virus (AAV) is a small nonenveloped virus that cannot self-replicate unless aided by an adenovirus (Lukashev and Zamyatnin 2016). AAV-based vectors do not integrate into the target cell genome, even though integration into a specific location on chromosome 19 has been observed for a very small proportion of wild-type AAV. Most AAV vectors are hepatotropic; moreover, depending on the serotype of the wild-type AAV, other tissue types can be targeted (Athanasopoulos et al. 2017; Balakrishnan and Jayandharan 2014). One constraint to AAV-based gene delivery is the limit to transgene capacity of approximately 4.5 kb. To overcome this capacity limitation, the genetic material may be divided into expression cassettes with complementary sequences that can anneal to form full-length dsDNA in the nuclei (Pasi et al. 2020). Compared to adenovirus, there is a lower likelihood for eliciting an immune response, as the proportion of humans previously exposed to AAV ranges from 10 to 50%, depending upon the serotype of AAV and the prevalence of AAV in the population (Louis Jeune et al. 2013).

Other viruses have been studied for gene delivery as episomal virus-derived vectors (reviewed in Van Craenenbroeck et al. 2000). These include vectors derived from BK virus, SV40 virus, bovine papilloma virus, and

EBV. Episomal vectors contain a viral origin of DNA replication and activation motifs, which allow replication of the inserted genetic material without the need for integration into cellular DNA. Persistence of episomal vectors in multiple copies in the nucleus allows for high transgene expression; however, long-term stability of transgene expression has not been established.

6.4.2 Nonviral Delivery Systems

Effective delivery of transgenes may also be accomplished through physical- or chemical-based systems. These systems tend to be less immunogenic than viral-based systems and have no limits to the transgene size. However, nonviral delivery systems are relatively inefficient compared to viral vectors.

6.4.2.1 Chemical Methods of Delivery

Nucleic acids are negatively charged, which allows genetic material to be packaged in cationic lipids or polymers forming a nanoparticle (Zhang et al. 2004). Nucleic acids within the complex are protected from degradation. Nanoparticle complexes are taken up by cells through endocytosis; subsequently the genetic material is released from the endosomes and translocated to the nucleus (Khalil et al. 2006). Nanoparticles are engineered to target specific cellular receptors by incorporating ligands in the lipid or polymer layer (Chiu et al. 2004; Hood et al. 2002). Advantages of chemical delivery systems include low risks for toxicity, immunogenicity, and insertional mutagenesis. The main disadvantage is the low efficiency of gene transduction.

Cationic liposomes form nanoparticles with DNA and can be used for ex vivo and in vivo gene delivery. The efficiency of delivery depends on the size, structure, charge ratio between transgenic DNA and cationic liposome, the cellular target, and whether a "helper lipid" is added (Birchall et al. 1999). Cationic liposome nanoparticles have been studied for delivery of the CRTR gene in patients with cystic fibrosis and shown to be well tolerated (Caplen et al. 1995; Alton et al. 1999). Cationic polymer nanoparticles also can be used for both in vitro and in vivo gene delivery. Particle engineering is critical to optimize gene delivery efficiency as well as toxicity (Tang and Szoka 1997; Tang et al. 2010).

6.4.2.2 Physical Methods of Delivery

There are a number of physical methods to deliver genetic material into target tissues; however, in most cases transduction efficiency is much lower than viral- or chemical-based delivery systems. It is possible for naked DNA to be transferred into cells via direct injection into cells or tissue (Herweijer and Wolff 2003). Electroporation is a procedure in which cells are placed in solution that contains the transgene and are subjected briefly to an electrical current. This allows the transgene to penetrate both cell and nuclear membranes (Heller et al. 2011). Magnetic fields also have been employed in combination with viral vectors to increase gene delivery to cells for which the virus has low tropism (Scherer et al. 2002). Other methods to penetrate the cell membrane include ultrasonic waves and mechanical forces, such as bombarding the tissue with DNA-coated metallic particles shot from a gene gun (Mahvi et al. 1997).

6.5 Building a BioDrug

Now that we understand the tools required for building a BioDrug, we can start putting them together. The following sections will focus on building BioDrugs for pediatric hematology/oncology patients.

6.5.1 Building Gene Therapies: Putting Together Genetic Material, Gene-Editing Machinery, Delivery Systems, and Target Cells

Gene therapy broadly covers a number of genetic engineering approaches aimed toward ameliorating human disease (Fig. 6.4). A gene therapy medicinal product contains a recombinant nucleic acid, the product of which is intended to regulate, repair, replace, delete, or augment an existing

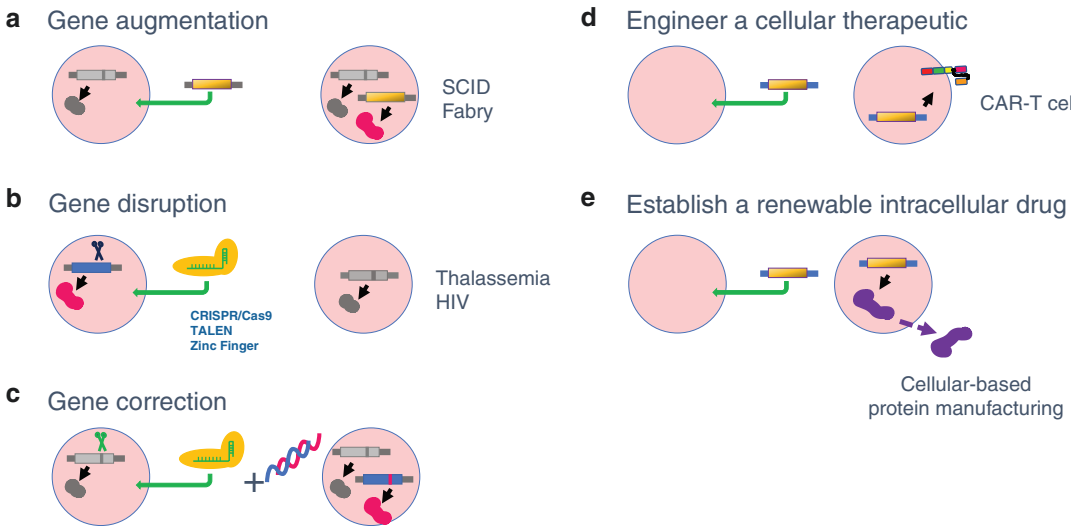

Fig. 6.4 Various genetic engineering strategies can be used to produce a BioDrug. In panels **a**–**c**, diseases are corrected using genetic engineering strategies that alter a protein product involved in the disease. In panel **a**, a new gene (yellow bar) that produces a normal protein (red globule) is transferred into the cell to replace a mutated gene (gray bar) and its abnormal protein product (gray globule). Examples of potentially treatable diseases using gene augmentation include inherited immunodeficiency disorders, which might be corrected using ex vivo transduction of hematopoietic stem cells (HSCs), or metabolic disorders, in which infusion of the gene therapy product for in vivo transduction might be effective. In panel **b**, a normal protein that may contribute to the disease is rendered dysfunctional using gene-editing machinery to delete part of its gene (gray bar). Examples of potential uses include disrupting the co-receptor required for HIV1 entry into CD4+ cells or disrupting the regulatory genes that silence HbF transcription in beta-thalassemia. In panel **c**, gene deletion is followed by homology-directed repair to replace the incorrect gene sequence (in gray) with the correct gene sequence (in red) to produce a normal protein. Sickle cell disease is an example of a disorder that might be amenable to this gene correction strategy. Genetic engineering strategies can also be used to turn cells into BioDrug products. In panel **d**, a T cell is transduced with a transgene construct that generates a chimeric antigen receptor (CAR). The CAR T cell is then used as a BioDrug to attack and destroy cancer cells. In panel **e**, a transgene construct that encodes a therapeutic protein, such as a cytokine, is introduced into a cell. The cell becomes an in vivo "manufacturing site" for the cytokine

human gene or genes. Gene therapies have been developed to correct inherited genetic defects, to interfere with acquired genetic mutations as might occur in a malignancy, or to artificially increase the amount of a gene product produced within a cell, such as a cytokine or functional protein, for therapeutic purposes.

The BioDrug ToolKit can be used to correct inherited genetic defects by delivering new genetic information into cells (gene augmentation) or by disabling genes that contribute to disease. The basic steps in building a gene therapy using the toolkit include:

1. Selection of the appropriate strategy for correcting the genetic disease, which can be gene replacement, gene editing, or a combination approach.
2. Engineering the transgene cassettes that includes the gene and regulatory elements or engineering the components of the gene-editing machinery.
3. Determination of the target cell, which dictates whether the gene transduction will be performed in vivo or ex vivo.
4. Selection of the optimal delivery system capable of carrying the genetic information to the targeted cell.

Building a gene therapy for correction of an inherited disorder begins with understanding the underlying pathophysiology of the genetic mutation. The design of the system must be based on knowledge of how the genetic mutation affects the resulting protein product as well as the factors important for the function of the protein. The

following considerations will determine the type of genetic manipulation (e.g., gene replacement and/or gene editing), the type of vector, and the target tissue required for successful gene therapies.

How many mutations are involved in the disease? A disorder caused by a single nucleotide substitution, such as sickle cell anemia, might be treated by a gene-editing approach since the same gene-editing machinery could be used for every patient. However, for a disorder such as Wiskott-Aldrich syndrome in which there are over 100 known mutations, gene editing would be prohibitive, and a gene replacement strategy would be substantially more practical.

What is the consequence of the mutation? Premature termination codons or other nonsense mutations may abrogate production of the protein altogether, in which case a simple replacement therapy that provides genetic instructions for the normal protein may suffice. However, in many situations the mutation results in an aberrant protein that directly causes the disorder, such as in transthyretin amyloidosis, or that might interfere with the function of a normal protein. For example, the presence of β^S chains in red cells might lead to sickling even if a transgene were generating normalized β-chains, depending on the intracellular concentrations of each protein product (Mansilla-Soto et al. 2011). For these situations, the engineering approach might also include knocking out the function of the pathogenic gene.

What is the required level of gene expression for correction of symptoms? For any given disorder, there will be a level of protein expression that is required for amelioration of symptoms. Furthermore, there may be requirements for a given amount of protein expressed within a cell or for the overall number of cells that express any amount of protein. Lessons learned from treating nonmalignant diseases with allogeneic HCT illustrate disease-specific differences in requirements for the level of intracellular protein expression or for the proportion of cells with normal protein expression. For example, improvement in sickle cell vaso-occlusive symptoms and normalization of hemoglobin are feasible without achieving full donor chimerism and can also be achieved by transplantation of cells from a donor with sickle cell trait, i.e., donor cells with half normal levels of HbA per cell, provided full chimerism is achieved (Abraham et al. 2017; Eapen et al. 2019). In contrast, allogeneic HCT for correction of mucopolysaccharidosis type I must result in a normal level of α-l-iduronidase for disease response, which cannot be achieved with cells from a carrier donor because the intracellular protein expression is low, nor can normal donor cells produce sufficient enzyme levels if full donor chimerism is not attained (Peters et al. 1998).

Optimization of protein expression requires selection of the appropriate regulatory elements to include in the transgene cassette. The number of copies of the gene established within the cell also affects protein expression. The vector copy number (VCN) is a measurement of the average number of transgenes integrated into the genome. Too few integrated copies will result in low expression, whereas too many may increase the risk for insertional mutagenesis. Sensitive polymerase chain reaction (PCR) techniques are used to quantify VCN in preclinical studies as well clinically as a correlation with disease response (Lin et al. 2016; Thompson et al. 2018). Assessment of VCN in a clinical setting after gene therapy requires easily accessible tissue; thus, practically speaking it has been limited to monitoring results of gene-modified hematopoietic or lymphoid cells.

What is the requirement for tissue specificity of gene expression? The cell that expresses the transgene may not matter for normal function of some proteins. For example, hepatocytes or myocytes may express transgenes that encode for proteins normally made by other organs and which function systemically. The liver is the most easily targeted organ for in vivo delivery because hepatocytes take up nanoparticles through endocytosis and many of the viral vectors are hepatotropic. The size of the gene and regulatory elements in the transgene construct will dictate the options for in vivo delivery to hepatocytes, as transfer of large amounts of genetic material may not be feasible with rAAV vectors.

There are circumstances in which tissue-specific expression might be desired, such as expression of beta globin in erythrocytes; there-

fore, building a tissue-specific gene therapy will involve additional considerations. Design of the transgene cassette must include the appropriate regulatory elements, such as tissue-specific promoters. A delivery system can be selected to further optimize tissue targeting, based on tropism of viral vectors or the capability of transducing dividing or quiescent cells.

6.5.2 Building Gene Therapies: Progress and Challenges

Many of the early challenges to developing gene therapies have been overcome by progress made in improvement of gene delivery systems and gene-editing technologies, resulting in a plethora of clinical trials in patients with genetic disorders. While few gene therapy products have been approved to date, it is expected that many more will be approved within the next decade. This section summarizes the progress to date in gene therapy technologies that have led to gene therapies for pediatric patients with inherited hematologic or immunologic disorders.

Until recently, the mainstay for treatment of life-threatening inherited hematologic disorders has been allogeneic HCT, which can be viewed as a very crude form of gene therapy. In its simplest sense, replacement of the entire hematologic and immunologic system is done in order to correct a single mutation that may affect function of only one cell compartment. While often effective at correcting symptoms caused by the genetic defect, the immunologically mediated graft-versus-host and host-versus-graft reactions, and consequent risk for graft-versus-host disease or graft rejection, form major barriers to successful allogeneic HCT. Ex vivo gene therapy permits a more focused correction of the specific mutation within the affected autologous cells of an individual (Fig. 6.5).

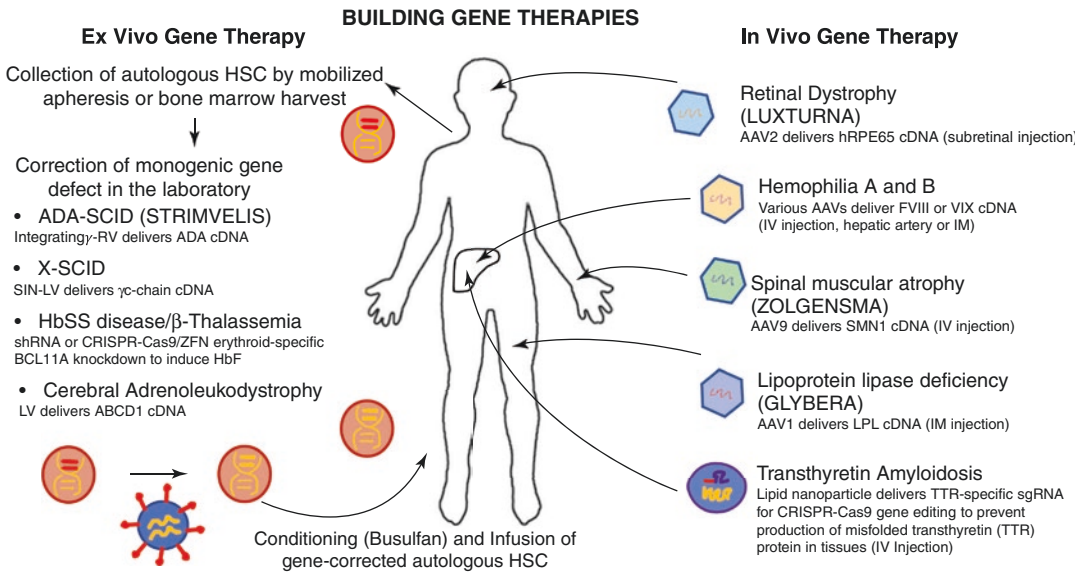

Fig. 6.5 Gene therapies can be distinguished based on "ex vivo" and "in vivo" approaches. "Ex vivo" gene therapy is utilized to correct monogenic gene defects in hematopoietic stem cells (HSCs). The patient's autologous HSCs are collected by apheresis following mobilization with G-CSF or Plerixafor (the latter being used in HbSS) or by bone marrow harvest. Cells are then placed in culture and gene-modified using the various approaches from the BioDrug ToolKit. After HSCs have been successfully corrected, the patient undergoes conditioning (typically busulfan-based), and gene-corrected autologous HSCs are reinfused intravenously with the goal of engraftment by the gene-corrected HSCs. In contrast, gene correction occurs in the patient's body rather than the laboratory when "in vivo" gene therapy approaches are utilized. Using elements of the BioDrug ToolKit such as adeno-associated virus and nanoparticles, cDNA or gene-editing tools are delivered via intravenous, intramuscular, or direct injection into the target organ

Introduction of a normal transgene into autologous HSC is especially relevant when a large number of possible mutations have been identified each of which can result in a nonfunctional gene product, such as occurs in many inherited immunodeficiency disorders (Bradford et al. 2017; Imai et al. 2003). Strimvelis is the first gene therapy product approved by the FDA for ex vivo gene augmentation of an inherited disorder, specifically to supersede mutations in the adenosine deaminase (ADA) gene that result in severe combined immunodeficiency (SCID) (https://www.ema.europa.eu/en/documents/product-information/strimvelis-epar-product-information_en.pdf). Components from the BioDrug ToolKit used to build Strimvelis include HSC as the target cells, adenosine deaminase cDNA as the genetic material, and a gamma retrovirus as the delivery system. Drug approval was based on safety and efficacy data from three trials with a combined total of 18 children. At 1 and 3 years following the procedure, genetically modified cells comprised a median of ~30% of CD19+ cells and ~70% of CD3+ cells, and by 8 years close to 100% of each subset were genetically modified. The 3-year overall survival was 100% and the rate of severe infection was reduced by 50% from baseline. There was a significant improvement in both the median number of T cells and the percent of dAXP in red blood cells. Adverse events related to Strimvelis included autoimmune reactions that were observed in 1–10% of patients, including autoimmune-mediated anemia, thrombocytopenia, thyroiditis, hepatitis, Guillain-Barré syndrome, and antineutrophil cytoplasmic antibodies. As would be expected, the most commonly observed adverse events in the clinical trials, such as anemia, neutropenia, and elevation of hepatic enzymes, were considered to be related to busulfan given to the children as conditioning before Strimvelis infusion.

Table 6.1 lists the ex vivo gene replacement trials currently in progress in patients with life-threatening hematologic or immunodeficiency disorders. A number of challenges remain for successful development of each of these BioDrugs, posed by complexities of disease indication as well as the limitations of current technologies available from the ToolKit. In addition to the issues that must be considered in designing a gene replacement therapy, such as strategies to avoid gene silencing or off-target cell expression, there remains an incomplete understanding of the variables involved in the cell engineering procedure that correlate with therapeutic efficacy, such as the optimal number of transduced HSC or the optimal VCN in the target cells. Furthermore, clinical toxicities have been observed in recipients of ex vivo genetically manipulated HSC, which may have relevance to BioDrug design.

The most serious toxicity observed to date has been the development of leukemia as a consequence of insertional mutagenesis. Initial trials that explored ex vivo gene replacement for X-SCID used a design strategy similar to

Table 6.1 Gene replacement and gene-editing trials for correction of inherited hematologic or immunodeficiency disorders (listed as open or recruiting on ClinicalTrials.gov as of April 2021)

Disorder	Trial
ADA-SCID	NCT03645460 NCT03765632
Artemis-SCID	NCT03538899
Beta-thalassemia	NCT03276455
Chronic granulomatous disease	NCT03645486
Fanconi anemia	NCT04248439 NCT03351868 NCT04069533
Hemophilia A	NCT04418414 NCT03818763 NCT03217032
Hemophilia B	NCT03961243
Infantile osteopetrosis	NCT04525352
Leukocyte adhesion deficiency	NCT03825783 NCT03812263
RAG1-SCID	NCT04797260
Sickle cell disease	NCT03964792 NCT04293185 NCT04443907 NCT04819841
X-linked SCID	NCT03311503 NCT01512888 NCT03601286 NCT04286815 NCT01306019 NCT03217617

ADA adenosine deaminase, *RAG* recombinant activating gene, *SCID* severe combined immunodeficiency

Strimvelis. Retroviral vectors based on the MLV were used to insert a copy of the common gamma chain (γ_c) cDNA into autologous CD34+ cells; expression of the γ_c cDNA was under control of the MLV promoter and enhancer within the retroviral long terminal repeat (LTR). The transduced CD34+ cells were reinfused into the patients without myeloablative conditioning. Of the 20 patients reported, reconstitution of normal T cell numbers and function were observed in 19, and normal B cell function was achieved in 8 (NCT01410019 and NCT01175239) (Hacein-Bey-Abina et al. 2010, 2014; Gaspar et al. 2004, 2011). However, five of the patients developed an acute T cell leukemia caused by insertion of the transgene near the LMO2 proto-oncogene. Oncogene activation was attributed to the activity of the strong T cell-tropic enhancer within the U3 region of the viral LTR. A similar experience occurred in patients with Wiskott-Aldrich syndrome after infusion of ex vivo transduced HSC using a γRV vector (Braun et al. 2014). Sustained engraftment and partial or full amelioration of immunodeficiency and thrombocytopenia were achieved in nine of ten patients; however, seven patients developed acute leukemia involving myeloid or T lymphocyte lineages.

To address this problem, LV vectors have supplanted γRV vectors as the preferred vector delivery system for ex vivo transduction of HSC. LV vectors have been further engineered to reduce the likelihood for replication-competent RV, termed self-inactivating (SIN), by removing viral transcriptional elements and including an enhancer-blocking element (Zhou et al. 2010; Morris et al. 2017). In 2019 the EMA approved betibeglogene autotemcel (Zynteglo) for treatment of non-β0/β0 beta-thalassemia (https://www.ema.europa.eu/en/documents/product-information/zynteglo-epar-product-information_en.pdf). Components from the BioDrug ToolKit used to build Zynteglo include mobilized HSC as the target cells and a LV vector delivery system (Thompson et al. 2018). The genetic material included an extended β-globin gene with regulatory segments of the locus control region. Drug approval was based on safety and efficacy data from 4 trials with a combined total of 32 adolescents and adults. Transfusion independence was demonstrated for 78–90% of patients at 24 months following infusion, and transfusion independence was maintained for at least 1 year following. Similar to the Strimvelis experience, the most commonly observed adverse events were related to the busulfan conditioning.

Transduction of autologous HSC using LV vectors also has shown early promise for delivering cDNA to replace the mutated *ABCD1* gene in patients with adrenal leukodystrophy and for delivering microRNA-adapted short hairpin RNA to interfere with expression of the HBB^s gene in patients with sickle cell disease (Eichler et al. 2017; Esrick et al. 2021). The clinical trials listed in Table 6.1 also employ LV vectors as the delivery system.

The degree of risk for development of leukemia after LV-mediated transduction of HSC remains unknown. In the Lentiglobin trial for sickle cell disease, in which a LV vector is used to transduce HSC with an anti-sickling β-globin, one patient has developed myelodysplastic syndrome approximately 3 years after gene therapy (Hsieh et al. 2020). Extensive analysis of the marrow found no clonal dominance of the insertion site in gene-modified cells, and there was no enrichment of the VCN in the MDS blasts compared to peripheral blood cells. In this case, leukemogenesis was considered to be caused by busulfan conditioning effects. However, long-term monitoring for insertional mutagenesis in all recipients of LV-transduced HSC will be essential, and the FDA has provided guidance for long-term follow-up of patients enrolled in trials of ex vivo transduced cell products (https://www.fda.gov/media/113768/download). In addition to insertional mutagenesis, gene therapy products based on retroviral vectors, including LV vectors, have the potential to transmit replication-competent retrovirus (RCR). While technologies for creating optimal vector designs and vector producing cells have markedly reduced the chance for transmission of RCR, the FDA has provided guidelines for RCR testing of both the product and the recipient of the gene-modified cells (https://www.fda.gov/media/113790/download).

Selection of the optimal target cell for ex vivo gene transduction also may pose a challenge. The initial trials of gene therapy for SCID used T lymphocytes as the target cell; however, long-term persistence of the gene-modified T cells was not achieved (Bordignon et al. 1995). The optimal target cell for ex vivo gene modification, whether for correction of disorders of hematopoiesis or immunodeficiency, is the HSC, which can provide a continual renewable source of lymphoid or myeloid lineage precursors. Because acquisition of high numbers of HSC may be critical to ensure a sufficient number of genetically modified cells for reinfusion, most studies utilize PBSC mobilized with G-CSF with or without plerixafor. However, G-CSF has been associated with severe adverse events in patients with sickle cell disease, and for these and other patient populations, alternative mobilization regimens such as plerixafor alone are being explored (Adler et al. 2001; Grigg 2001; Lagresle-Peyrou et al. 2018). It also may not be feasible to collect PBSC in very young infants due to the lack of vascular access for apheresis. Novel strategies to increase the total number of or to enrich the population of pluripotent HSC from harvested BM are being investigated (Radtke et al. 2020; Adair et al. 2018; Frangoul et al. 2007).

The early clinical trials of Strimvelis also showed that, despite optimal engineering and selection of appropriate vectors, engraftment of gene-modified HSC was impeded by competition from endogenous cells (Bordignon et al. 1995; Muul et al. 2003). Subsequent gene therapy trials included strategies to reduce in vivo completion by addition of conditioning with busulfan (BU) to create space for engraftment (Aiuti et al. 2002, 2009). Currently most trials include either sub-myeloablative or myeloablative Bu-based conditioning. Selection of dose intensity depends on the level of engraftment required for correction of the disease and comfort with the higher risk for toxicity associated with more intense conditioning. While myeloablative BU conditioning has been used for decades in conditioning for allogeneic HCT, it carries the risks of prolonged pancytopenia and liver toxicity. Sub-myeloablative BU dosing once daily for 1–2 days is preferable in most conditions (Mamcarz et al. 2019; Bradford et al. 2020). An additional concern associated with myelotoxic regimens is the potential for genotoxic effects on the host hematopoietic cells, which has been suggested by the development of myelodysplastic syndrome without evidence for insertional mutagenesis in recipients of LV-transduced HSC given BU conditioning (Hsieh et al. 2020). Improved conditioning regimens, such as antibodies that target CD34 or c-kit, are being explored as a method to decrease competition for marrow space while avoiding systemic toxicities and the risk for genotoxicity (Chandrasekaran et al. 2014; Srikanthan et al. 2020).

Gene-editing technologies, such as the CRISPR/Cas9 system, have the potential to overcome some of the limitations of gene replacement therapy. The ability to edit a mutation within the genome allows for the native transcriptional regulatory elements to control gene transcription, thus circumventing the need to engineer a transgene cassette with additional promoter elements. Gene-editing technology also can be used to knock out mutated genes that could interfere with gene replacement strategies or to knock out regulatory elements to reduce or enhance endogenous gene expression. For example, one strategy to improve hematopoiesis in patients with beta-thalassemia has been to "reawaken" fetal hemoglobin production by disrupting the regulatory genes that silence HbF transcription (Bauer et al. 2012). Some inherited disorders, such as transthyretin amyloidosis, are caused by gain-of-function mutations, in which case gene disruption has the potential to directly treat the disorder by knocking out production of the dysfunctional protein (Sekijima 2015; Gillmore et al. 2021). Currently there are multiple trials investigating gene-editing technology for correction of hematologic and immunodeficiency disorders (reviewed in Daniel-Moreno et al. 2019). Components from the BioDrug ToolKit used to build these products include HSC as the target cells, a selection of gene-editing machinery of which the CRISPR/Cas9 system is emerging as the most adaptable, and a selection of delivery systems that have included viral and nonviral

methodologies. Given the rapid advances in gene-editing technologies, it is expected that approval of a gene-edited HSC BioDrug will occur in the near future.

Ex vivo gene modification of HSC is not suitable for treatment of genetic disorders that affect other tissue compartments, such as the nervous or musculoskeletal systems. For these disorders, delivery of the transgene must be targeted to the appropriate tissue via an in vivo delivery system (Fig. 6.5). Currently there are no approved in vivo gene therapy products for treatment of hematologic or immunodeficiency diseases; however, several have been approved for treatment of other inherited disorders. The first product for treatment of an inherited disorder was Glybera (alipogene tiparvovec) was approved by the EMA in 2012. Components from the BioDrug ToolKit used to build Glybera include human lipoprotein lipase (LPL) cDNA as the genetic material and the AAV1 viral vector as the delivery system, which has tropism for skeletal muscle and neurologic tissue (Scott 2015; Naso et al. 2017). Clinical trials demonstrated significant reductions in plasma triglyceride levels after a onetime series of intramuscular injections in patients with lipoprotein lipase deficiency, a rare autosomal recessive disorder which can cause severe pancreatitis. Luxturna (voretigene neparvovec-rzyl) was the first in vivo gene therapy approved in the USA for treatment of an inherited disorder, specifically to treat children and adults with an inherited retinal dystrophy resulting in vision loss. Components from the BioDrug ToolKit used to build Luxturna include *hRPE65* cDNA driven by a CMV enhancer and chicken beta actin (CβA) promoter as the genetic material and the AAV2 viral vector as the delivery system, which has broad tropism including retinal cells (Naso et al. 2017). In clinical trials, patients with biallelic *RPE65* mutation-associated retinal dystrophy who received subretinal injections of Luxturna showed a statistically significant clinical improvement compared to control patients over a period of 1–5 years, and adverse reactions were limited to ocular events (described in the FDA Summary Basis for Regulatory Action https://www.fda.gov/media/110141/download). The second US approval for in vivo gene therapy was for Zolgensma (onasemnogene abeparvovec-xioi), indicated for treatment of pediatric patients with spinal muscular atrophy caused by biallelic mutations in the *SMN1* gene, encoding for the SMN protein which is critical to the function and survival of motor neurons. Components from the BioDrug ToolKit used to build Zolgensma include *SMN1* cDNA under control of a CMV enhancer and CβA hybrid promoter as the genetic material and the AAV9 viral vector as the delivery system, which has broad tropism including for neurons (Foust et al. 2009). In clinical trials, a statistically significant improvement in survival and motor milestone achievement was observed for infants with SMA1 given a single intravenous infusion compared to natural history controls (described in the FDA Summary Basis for Regulatory Action https://www.fda.gov/media/127961/download) (Mendell et al. 2017; Al-Zaidy and Mendell 2019). In contrast to the experience with locally administered AAV-based gene therapy in the Glybera (i.e., intramuscular) and Luxturna (i.e., intraocular) trials, serious adverse reactions were observed, including severe liver toxicity in 6.8% of patients.

The studies supporting approval of these drugs provide several lessons for development of in vivo gene therapy for treatment of hematologic disorders. The target cells for each product were postmitotic, thus allowing for sustained gene expression without genomic integration of the transgene. Selection of the delivery system was based on tropism of the AAV vector to achieve sufficient levels of gene product within the target tissue. Clinical studies of AAV-based gene replacement for hemophilia A and B have been underway over the last decade and show promising results (reviewed in Perrin et al. 2019). In these trials, the components from the BioDrug ToolKit include factor VIII or factor IX cDNA as the genetic material, AAV vectors as the delivery system, and the liver as the target organ for a systemically administered product (Pasi et al. 2020). Barriers to broader application of these therapies mainly are related to the immunogenicity of AAV vectors, which trigger both cytotoxic T lymphocyte (CTL) and humoral immune responses

(reviewed in Mingozzi and High 2013). The presence of neutralizing antibodies (NAb) to the AAV capsid, even at low titers, can impede transduction of target tissues (Manno et al. 2006). The prevalence of NAb to AAV depends on the serotype and likely increases with age (Louis Jeune et al. 2013; Fu et al. 2017). Most clinical trials of AAV-based gene therapy require assessment of pre-existing NAb prior to enrollment of patients and take one of two general approaches in the management of NAb-positive patients. Exclusion of NAb-positive patients may improve interpretation of the dose-response data in early phase trials, as done in recent trials in patients with hemophilia B (Ertl and High 2017; Miesbach et al. 2018; Nathwani et al. 2011, 2014). However, depending on the serotype, this approach may exclude up to 50% of patients and seriously affect enrollment of the trial, particularly if focused on a rare disease. Several studies enrolled patients with detectable NAb, for example, the ZOLGENSMA clinical trials allowed anti-AAV9 titers ≤1:150 (Mendell et al. 2017; Al-Zaidy and Mendell 2019). Current trials that allow NAb-positive patients include a course of prophylactic immune suppression to block CTL responses, an approach taken in the clinical trials of Luxturna (Mingozzi and High 2013; Mingozzi et al. 2007; Jiang et al. 2006).

In NAb-negative patients, development of NAb also has been observed to occur weeks to months after receipt of AAV-based gene therapy, particularly when systemically administered. Therefore, AAV-based gene therapy protocols incorporate post-infusion monitoring for NAb and institution of immune suppression when detected (Nathwani et al. 2014). This phenomenon of post-infusion development of AAV-specific NAb also has implications for the design of early phase dose-escalation trials. Because any exposure to an AAV vector can elicit NAb, and because presence of NAb will exclude the patient from receiving AAV-based gene therapy in the future, it is important that the study minimize the number of patients exposed to a subtherapeutic dose (measured in vector genomes (vg) per kilogram recipient weight). For this reason, regulatory agencies have allowed dose escalation after demonstration of safety in a single patient, as reported in the initial hemophilia A trial (Rangarajan et al. 2017).

For systemic delivery of AAV-based gene therapy, the liver has become an ideal target tissue because it is a biosynthetic organ for which many AAV vectors have tropism and in which stable long-term transgene expression can be achieved (Mak et al. 2017). However, liver inflammation has emerged as a potential toxicity thought to be a consequence of the immune response to AAV (Miesbach et al. 2018; Nathwani et al. 2014). Clinical trials in patients with hemophilia observed elevations in liver transaminase levels that generally occurred between 6 and 16 weeks after infusion of AAV vectors. Current clinical trials require close monitoring of liver transaminase levels and prompt institution of a course of prednisolone given once levels exceed 1.5 times the upper limit of normal. Several recent trials have also incorporated a course of prophylactic corticosteroids during the first month or so after infusion, which could reduce the burden of monitoring transaminase levels.

Viral vector-based gene therapy products also raise the concern for vector shedding and the risk of transmission to untreated individuals. Vector viral shedding was observed in studies of AAV-based gene therapy for hemophilia B, with vector detected in nasal secretions, saliva, feces, urine, and semen for up to 48 weeks after systemic administration (Miesbach et al. 2018). The FDA has produced guidance for incorporating studies of vector shedding in clinical trials (https://www.fda.gov/regulatory-information/search-fda-guidance-documents/design-and-analysis-shedding-studies-virus-or-bacteria-based-gene-therapy-and-oncolytic-products).

The development of nanoparticles as delivery systems may help overcome the challenges of viral shedding and immune-mediated interference with transduction and transgene expression. Currently there are no approved nanoparticle-based gene therapies, but several clinical trials have commenced for study of local or systemic nanoparticle-based delivery of cDNA or mRNA in patients with solid tumors. Nanoparticles have been studied for delivery of the CFTR gene to the nasal epithelium in patients with cystic fibrosis

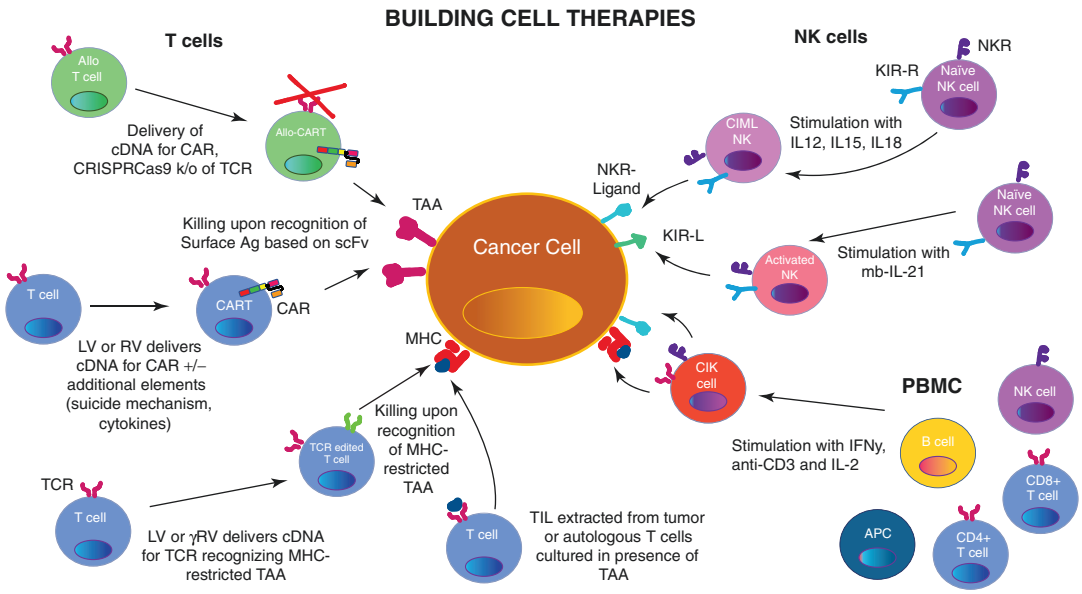

Fig. 6.6 The BioDrug ToolKit is also used to develop novel cellular immunotherapies to treat cancer. These strategies include non-engineered cells or gene-modified immune cells. Non-engineered approaches include extraction and ex vivo expansion of tumor-infiltrating lymphocytes (TILs) or peripheral blood T cells cultured in the presence of tumor-associated antigen (TAA), and ex vivo expansion and stimulation of NK cells with various approaches (IL-12, IL-15, IL-18 to generate cytokine-induced memory-like NK cells) or stimulation with membrane-bound IL-21, to generate NK cells with enhanced anti-tumor cytotoxicity. The stimulation of peripheral blood mononuclear cells with IFN-γ, anti-CD3, and IL-2 generates cytokine-induced killer (CIK) cells equipped with TCR and NK-cell receptor recognition to eliminate cancer cells. Conversely, immune cells may be genetically modified in the laboratory, utilizing viral vectors from the BioDrug ToolKit to generate T cells expressing a chimeric antigen receptor (CAR). The CAR-binding domain (typically derived from a single-chain fragment variable region of an antibody) recognizes the cognate surface antigen in an MHC-independent fashion and can kill cancer cells in highly efficient fashion. While currently approved CART cell therapies are individualized to collect and gene-modify autologous T cells, CART cells derived from allogeneic donors for an off-the-shelf approach are increasingly explored in clinical trials. To prevent GVHD, the endogenous allogeneic T cell receptor has to be knocked out in this approach and is generally combined with additional strategies to minimize rejection of allogeneic T cells by the patient's immune system via MHC recognition on the allogeneic T cells. For recognition of intracellular tumor-associated proteins, introduction of a foreign high-affinity TCR recognizing an HLA-restricted peptide can be utilized

and have been studied for delivery of mRNA in preclinical models of inherited hematologic disorders for delivery of mRNA (Caplen et al. 1995; Russick et al. 2020).

6.5.3 Building Cell Therapies: Using the BioDrug ToolKit for Treatment of Malignancy

The BioDrug ToolKit provides a variety of cells that have been given for therapeutic purposes and additional tools that can be used to create highly engineered cells for treatment of advanced malignancies. This section provides examples of how the BioDrug ToolKit has been used to generate cell-based products for clinical trials in patients with malignancies (Fig. 6.6).

Non-engineered cells have been used as a "living drug product" for decades in treatment of hematologic malignancies, the classic example being transplantation of allogeneic HSC. Adoptive cell therapies (ACT) are good examples of more recent non-engineered BioDrugs that utilize the innate capabilities of T cells to provide the therapeutic effect. The goal of ACT is to exploit the capacity of endogenous T cells to generate an ongoing immune response to a tumor-associated

antigen (TAA). TAA targeted by ACT can be neoantigens that arise from somatic mutations in cancer cells or may be normal tissue antigens that are overexpressed by malignant cells. Identification of targetable TAAs poses an enormous challenge that has been a significant barrier to the development of ACT.

The earliest studies of ACT avoided the problem of TAA identification by collecting and expanding lymphocytes found within the parenchyma of solid tumors, known as tumor-infiltrating lymphocytes (TILs) (Topalian et al. 1988). The presence of lymphocytes within tumor tissue has been shown to be a favorable prognostic biomarker for many tumors, and suggests the presence of an endogenous population of lymphocytes that recognize TAAs (Zhang et al. 2003; Djenidi et al. 2015). To generate the TIL product, small tumor sections are placed in culture medium with IL-2. The proliferating lymphocyte populations are harvested and placed in a second culture for rapid expansion in the presence of feeder cells, anti-CD3 antibody and IL-2 (Klapper et al. 2009; Dudley et al. 2003). The resulting product contains up to 1×10^{11} lymphocytes that have the potential to recognize a variety of TAAs. TIL therapy has been explored as an ACT for several tumor types (Dafni et al. 2019; Rohaan et al. 2018; Andersen et al. 2016). Infusion of TILs typically follows a lymphodepleting regimen, based on the hypothesis that reduction of the endogenous lymphocyte compartment decreases competition for homeostatic cytokines that support T cell function, such as IL-7 and IL-15. Post-infusion support with IL-2 also has shown to improve response in studies of melanoma-specific TIL therapy (Dafni et al. 2019). The FDA recently granted breakthrough status of a TIL product for advanced cervical cancer (https://ccr.cancer.gov/news/article/fda-grants-breakthrough-therapy-designation-of-new-til-therapy-for-advanced-cervical-cancer).

T cells also can be expanded ex vivo to generate cytotoxic T lymphocyte (CTL) lines to leverage the adaptive immune response to a specific antigen, such as viral protein or a TAA. The general steps in the production of an antigen-specific CTL product start with the establishment of a population of antigen-presenting cells derived from the patient, such as monocytes, dendritic cells, or an EBV-transformed B lymphocyte. Next the antigen-presenting cells are given the requisite antigen(s) for presentation, either by pulsing the cells with the peptide(s) or transfecting the cells with a vector that encodes the peptide sequence (Sili et al. 2012; Patel et al. 2018). Once the antigen-presenting stimulator cells have been established, peripheral blood mononuclear cells (PBMC) obtained from the patient are placed into the culture. T cells within the PBMC that recognize antigen become activated and expand in numbers. These T cells are collected and further expanded in culture to produce lines of CTLs that can be used for immunotherapy (Riddell and Greenberg 1990). Ex vivo expanded CTL lines have been studied for treatment of viral infections in immunocompromised patients and for malignancies, such as melanoma, for which TAAs have been defined (Sili et al. 2012; Hont et al. 2019; Weber et al. 2013). One advantage to this form of ACT is that T cell lines with a broader array of TCRs can be generated, which may increase the likelihood of antigen recognition. However, to date a limited number of TAA peptides have been identified. Furthermore, tumor cells may downregulate MHC, thus circumventing TCR recognition.

In contrast to T cell therapies, cells that comprise the innate immune system do not require antigen recognition in the context of MHC for activity. The potent anti-tumor activity of NK cells has prompted much interest in developing NK cell therapies for treatment of malignancy. One approach has been to exploit the "missing ligand" concept, which allows activation of NK cells when their inhibitory KIR fails to engage the cognate MHC class I inhibitory ligand. Much of this work has been done in the setting of HLA-haploidentical HCT, first brought to attention by Ruggeri and colleagues who reported a significantly lower risk for relapse among recipients who lacked the inhibitory HLA molecule for the donor NK cells (Ruggeri et al. 2002). Donor NK alloreactivity also has been utilized in HLA-matched HCT by selection of donors that have more favorable activating KIR phenotypes (Cooley et al. 2018; Hsu et al. 2006). Subsequently, alloreactive HLA-haploidentical

NK cells have been studied outside the setting of HCT for treatment of advanced myeloid malignancies (Kottaridis et al. 2015; Miller et al. 2005; Lee et al. 2016b; Curti et al. 2016). These studies obtained allogeneic NK cells from adult donors; however, allogeneic NK cell products have also been generated from umbilical cord blood (UBC) or established NK cell lines resulting in readily available "off-the-shelf" products (Spanholtz et al. 2011; Arai et al. 2008). Efforts also have been focused on enhancing NK cell activation, either by placing cells in culture with IL-12, IL-15, and IL-18, termed cytokine-induced memory-like (CIML) NK cells, or by in vivo activation of infused NK cells by administration of IL-2, IL-15, or membrane-bound IL-21 (Lee et al. 2016b; Uppendahl et al. 2019; Berrien-Elliott et al. 2015; Phillips et al. 1987; Romee et al. 2016). Tumor antigen-directed NK cells also have been engineered using genetic modification to generated chimeric antigen receptors, as described in the sections below (Liu et al. 2020).

Cells that have characteristics of both NK and T cells, including expression of both CD3 and CD56, capable of both MHC-restricted and non-restricted cytotoxicity, termed cytokine-induced killer (CIK) cells can be generated by incubating peripheral blood mononuclear cells with interferon gamma (IFNγ), IL-1, IL-2, and anti-CD3 (Lu and Negrin 1994; Pievani et al. 2011). The safety of CIK therapy has been shown in pilot studies, and efficacy is being assessed in patients with advanced malignancies (Rettinger et al. 2016; Introna et al. 2007; Schmeel et al. 2015).

6.5.4 Engineered Cell Therapy: Putting Together Cells, Genetic Material, Gene-Editing Machinery, and Delivery Systems to Target Malignant Cells

Cellular engineering strategies have evolved to overcome the biologic limits of the innate and adaptive immune systems by insertion of genetic instructions that direct the cells toward specific antigens and augment cellular immune responses. These technologies all rely on pre-identification of the tumor-associated antigen (TAA) for targeting tumor cells. This section describes the various approaches to genetic engineering of adaptive or innate immune responses to tumor antigens and the contributions of the various tools available from the BioDrug ToolKit.

The basic steps in building a tumor-directed BioDrug using the ToolKit include (1) selection of the appropriate TAA for targeting; (2) engineering the transgene cassettes to provide tumor-targeting genes and regulatory elements; (3) selection of the optimal delivery system capable of carrying the genetic information to the targeted cell; and (4) selection of the appropriate cell type as the best weapon to eradicate the tumor. To date, T cells have been the preferred cell for use in engineering a tumor-specific immune response. As described above, the endogenous adaptive immune response occurs when the TCR recognizes antigen in the context of MHC, which in concert with a co-stimulatory signal results in T cell activation. However, tumors that express self-antigens, even if overexpressed, are unlikely to be recognized by endogenous T cells, since these will have been deleted during thymic selection. Endogenous T cells may also fail to recognize tumor antigens due to inadequate presentation of TAA, downregulation of MHC, or lack of co-stimulatory signals within the tumor milieu. These limitations to endogenous TCR recognition of TAA have hampered the success of TIL and CTL therapies and led to the development of chimeric antigen receptor (CAR) T cells.

Building CAR T cells requires all the tool components in the BioDrug ToolKit, including cell culture systems, complex genetic material, and viral delivery systems. The genetic material is designed to express a long protein that links together a TAA-recognition domain expressed on the cell surface with intracellular signaling domains (Fig. 6.7). The TAA-recognition domain is most commonly a single-chain variable fragment (scFv) derived from a monoclonal antibody, linked to a "hinge" or transmembrane region that connects the surface antibody receptor to the intracellular signaling domains. The intracellular

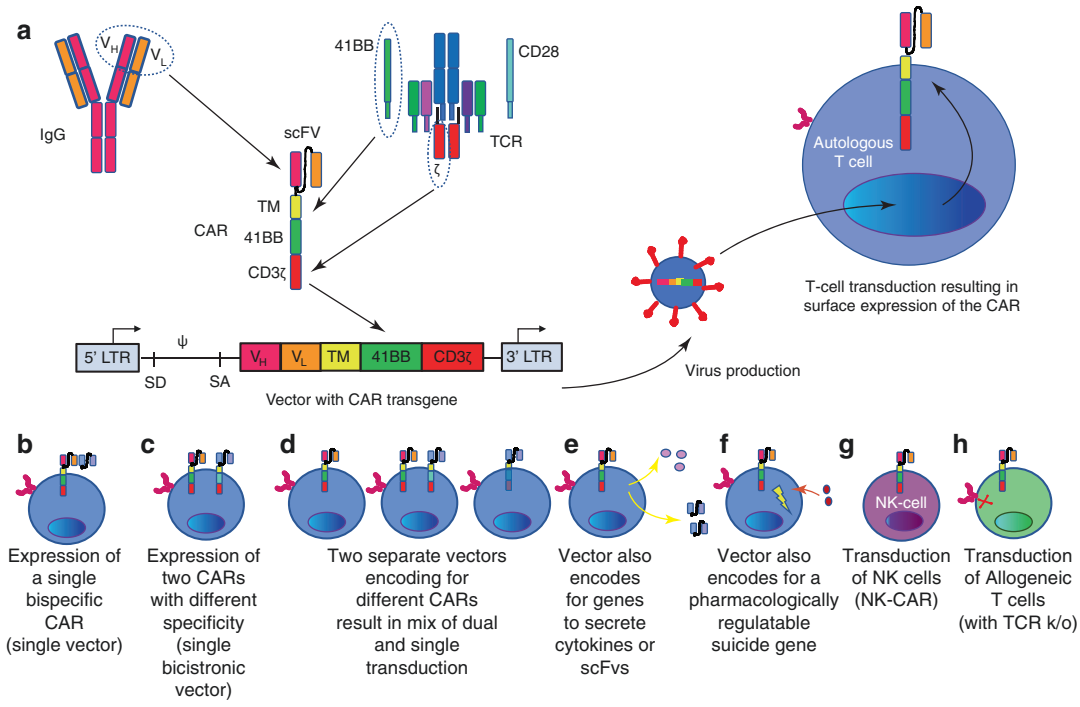

Fig. 6.7 (**a**) A typical CAR T cell configuration consists of a single-chain fragment variable region (scFV) derived from the variable chains of an IgG antibody as the antigen-recognition domain, a hinge/transmembrane (TM) region, a co-stimulatory moiety such as 41BB or CD28 and the CD3ζ chain derived from the T cell receptor (TCR). The CAR gene is cloned into a lentiviral or γ-retroviral vector. After production of replication-incompetent lentivirus or γ-retrovirus, these integrating viruses are used to transduce autologous T cells, leading to CAR expression on the cell surface. Several iterations of the classic CART approach are currently being explored. (**b**) To address antigen-escape mechanisms, CARs with dual antigen specificity have been developed. This may be achieved by inclusion of two separate scFVs in the transgene, resulting in a bi-specific CAR with a common signaling domain that is delivered by a single vector. (**c**) Alternatively, a single vector may encode for two separate CARs, each with their own signaling domain contained in the same transgene separated by a ribosomal skipping sequence such as T2A and delivered by a single bicistronic vector. (**d**) Dual specificity may also be achieved by utilizing two different vectors, each encoding for a different CAR to transduce T cells. This may result in a mixed T cell pool of cells expressing either one or both CARs. (**e**) In an effort to enhance CART efficacy, so-called "armored" CARs have been developed in which the transgene may include genes for cytokines or scFVs that can be secreted by the T cell. (**f**) In an effort to enhance the safety profile of CART cells, the transgene may include a suicide gene that can be activated by administering a drug to the patient. (**g**) CARs can also be introduced into autologous or allogeneic NK cells, which generally do not mediate graft-versus-host disease. (**h**) Approaches to develop universal off-the-shelf CAR T cells are underway, in which allogeneic T cells are transduced to express the CAR. However, this must be combined with a gene-editing approach to knock out the TCR to prevent GVHD. Additionally, it is frequently combined with strategies to minimize rejection of allogeneic CAR T cells based on HLA mismatch

signaling domains consist of the CD3ζ protein from the native TCR linked to one of the "second signal" proteins, either CD28 or 4-1BB. This entire construct artificially replicates the three important steps in the generation of an adaptive immune response. In step one the antibody domain engages the tumor antigen and replaces the need for TCR recognition of the antigen-MHC complex. Engagement of the antibody with antigen automatically stimulates both CD3ζ signaling and the second co-stimulatory signal, replacing both of these steps to initiate T cell activation. The engineered transgene construct may also include other linked domains for proteins that activate or modify cell migration, antigen recognition, or immune responses. In addition to

the multiple transgenes, the CAR construct must include promoter regions that can drive expression of long RNA encoding multiple gene products (Rad et al. 2020). The choice of promoters currently is limited to more well-characterized promoters such as EF-1 and CMV, and depends on the level of desired CAR transgene expression. To generate the target cells, PBMC are collected by apheresis for initiation of T cell cultures. PBMC can be placed directly into T cell culture systems as described above, or T cell subsets can be selected from apheresis product prior to manufacturing (Shah et al. 2020; Turtle et al. 2016). Once the target T cells have been obtained and the CAR construct has been built, retroviral or lentiviral vectors are used for delivery of the genetic material for integration into the genome. The CAR T cells are expanded in culture and then cryopreserved for future administration. Several excellent reviews discuss the development of CAR T cell technology, including the stepwise incorporation of co-stimulatory domains (termed second-generation CARs) that have improved CAR T activation and efficacy (June et al. 2018; Boyiadzis et al. 2018).

To date, four CAR T cell products manufactured from autologous PDMC have gained FDA approval for treatment of hematologic malignancies (Table 6.2). In order to commercialize products originally conceived in academic laboratories, biotech companies were required to demonstrate that the manufacturing process and controls were capable of yielding a product with consistent quality and that chain of identity and chain of custody could be maintained throughout the manufacturing process. Kymriah (tisagenlecleucel) and Yescarta (axicabtagene ciloleucel) were the first CAR T cell products to be approved, and while both are directed at the CD19 antigen, they differ in the genetic material incorporated in the CAR construct and the vector delivery systems. Kymriah is generated from autologous T cells using a LV vector to deliver the CAR construct which contains the 4-1BB co-stimulatory domain, whereas the Yescarta CAR construct contains the CD28 co-stimulatory domain and is delivered by a RV vector. The latest CD19-directed CAR T cell product to be approved, Breyanzi (lisocabtagene maraleucel), also contains the 4-1BB co-stimulatory domain in its CAR construct but differs in its end composition which includes a fixed ratio of CD4+/CD8+ cells. Abecma (idecabtagene vicleucel) is the first CAR T cell approved for treatment of multiple myeloma. The CAR construct includes a B cell maturation antigen (BCMA) recognition single-chain variable fragment domain and the 4-BB and CD3ζ intracellular signaling domains, transduced into autologous T cells by a LV vector.

Approval of each of these products was based on results of multicenter, open-label, single-arm trials, and with respect to the CD19-directed CAR T cell products, there have not as yet been head-to-head comparisons (Grupp et al. 2013; Maude et al. 2014; Cappell et al. 2020; Locke et al. 2017, 2019; Wang et al. 2020; Neelapu et al. 2017; Schuster et al. 2019). However, these trials exhibited similar findings with lessons learned to

Table 6.2 Approved chimeric antigen receptor T cell products (of April 2021)

Cell therapy product	Indication	Target antigen	ORR CR
Abecma (idecabtagene vicleucel)	Multiple myeloma	BCMA	72% 28%
Breyanzi (lisocabtagene maraleucel)	Relapsed or refractory large B cell lymphoma	CD19	73% 54%
Kymriah (tisagenlecleucel)	Refractory B cell precursor acute lymphoblastic leukemia Relapsed or refractory large B cell lymphoma	CD19	50% 32%
Yescarta (axicabtagene ciloleucel)	Relapsed or refractory large B cell lymphoma	CD19	72% 51%

BCMA B cell maturation antigen, *CD* cluster of differentiation, *CR* complete remission, *ORR* overall response

guide future trials. First, the median time from leukapheresis to final manufactured product was approximately 2–4 weeks, during which time the patient may need to receive bridging chemotherapy to maintain control of the malignancy. Second, both efficacy and toxicity correlated with the degree of in vivo expansion of the CAR T cells. Expansion typically peaked between 7 and 14 days after infusion, and the area under the curve within the first month was significantly higher in responding compared to non-responding patients. However, responding patients also had higher levels of cytokines, such as IL-6, associated with toxicity. Third, delivery of a lymphodepleting regimen before infusion of the CAR T cell product facilitated CAR T expansion, presumably by reducing competition and immunogenicity from endogenous T cells (Hirayama et al. 2019).

The speed at which the CD19-directed CAR T cells have been shown to be effective illustrates the promise of this therapy when a tumor expresses an antigen on its surface that can be directly recognized by the CAR T cell receptor, and when elimination of cells that express the surface antigen does not result in serious off-tumor effects. CD19 expression is limited to malignant and nonmalignant B cells; thus, CD19-directed CAR T cell therapy typically causes profound B cell aplasia. The on-target off-tumor consequence of hypogammaglobulinemia is treatable by administration of gamma globulin. Development of CAR T cells for other tumor types is made more challenging by the fact that tumor antigens may be internally expressed, therefore only "visible" to a T cell when processed peptides are expressed in the context of MHC.

For this reason, investigators are exploring alternatives to CAR T cells that exploit the entire TCR complex for TAA recognition in the context of self-MHC (Fig. 6.6). Compared to CARs, TCRs can target virtually every tumor protein, independent of their cellular localization, and are reactive at lower antigen densities than CARs (Harris et al. 2018). This process starts by identification of the T cells that recognize the desired peptide in the context of MHC, which in and of itself is a challenge, since MHC genes are highly polymorphic. Most studies use peptides restricted to HLA-A*02, because it is the most common HLA allele, present in up to 50% of the population depending on the ethnic background. Several systems can be used to isolate T cells with the desired TCRs, such as affinity-enhanced phage display (Varela-Rohena et al. 2008). Once identified, the TCRα and TCRβ chains can be cloned and inserted into viral vectors for delivery into T cells. The engineering can become more sophisticated by using CRISPR/Cas9 or other gene-editing tools to knock out the native TCRα and TCRβ chains so as not to interfere with the transgenic TCRαβ complex. The engineered T cells can be expanded in culture similar to the process for CAR T cells. Theoretically, by infusing T cells with an intact, albeit engineered, TCR complex, intracellular signaling occurs through the six TCR subunits in contrast to the single CD3ζ signal from CAR T cells, which exploits the full potential of TCR-driven T cell activation, effector function, and regulation. A hybrid system has also been developed that combines the MHC-unrestricted antigen recognition properties of CAR T cells with the native TCR signaling. T cells are transduced with a construct containing an antigen recognition domain, such as an scFv, which is connected to a transmembrane spacer domain and then to the CD3ε chain. These hybrid constructs overcome the limitation of HLA-A*02 restriction, however, may be limited by the need for surface expression of the antigen.

6.5.4.1 Toxicities of Engineered T cell Therapy

Clinical trials have brought to attention unique toxicities related to the biologic activity of engineered T cells (Neelapu et al. 2018). It is not yet known whether the toxicities observed in trials of CD19- and BCMA-directed T cells will be observed with T cells directed toward other TAA, or with TCR-engineered T cell therapies, since these toxicities may be driven by the antigen in addition to the biology of T cell activation. Specific to the individual TAA will be the potential for on-target off-tumor effects, such as the B cell aplasia observed with CD19-directed ther-

apy. To the extent that TAA expression is unique to the tumor cells, off-tumor effects will be minimized.

Toxicities that result from T cell activation and proliferation become a potential concern for any engineered T cell. In the studies that supported approval of CAR T cell products, a dose-toxicity relationship was observed, such that a greater proportion of patients and a higher grade of toxicity was observed in patients given higher CAR T cell doses. The most important toxicities reported in these patients include cytokine release syndrome (CRS), immune effector cell-associated neurotoxicity (ICANS), macrophage activation syndrome (MAS), and prolonged cytopenias (Neelapu et al. 2018). CRS represents the double-edged sword of T cell therapies, because it is associated with T cell activity and is observed to some extent in most patients who have tumor response. Onset of the symptoms correlates with in vivo expansion and proliferation of activated T cells and usually occurs within a week after infusion of the T cell product. The hallmark of CRS is fever (>38.0 °C) which occurs in all patients. CRS can progress to a state of vasodilation and capillary leak, resulting in hypotension, and respiratory distress (Acharya et al. 2019). A standardized grading system is used to aid in diagnosis and management of patients (Lee et al. 2019). The symptoms are caused by release of inflammatory cytokines from activated T cells, including interleukin (IL)-6, IL-2, IL-10, IL-15, and IL-18 (Hay et al. 2017). Markers of inflammation are often elevated, including ferritin, C-reactive protein, lactic acid dehydrogenase, interferon gamma (IFNγ), and soluble IL-2 receptor. Management of CRS involves supportive care and judicious medical intervention guided by staging criteria and accepted algorithms (Lee et al. 2014, 2019). The primary treatment is tocilizumab, an IL-6 receptor antagonist that is approved by the FDA for treating CRS (Le et al. 2018; Gardner et al. 2019). Dexamethasone can be added for patients who do not respond to tocilizumab or other anti-IL-6 agents. MAS is another potentially life-threatening complication of CAR T cell therapy, observed in 1–5% of CAR T cell recipients, and may be difficult to differentiate from CRS, as a markedly elevated serum ferritin is associated in both disorders. Treatment of MAS typically includes etoposide; however, its role in treatment of CAR T cell recipients has not been established (reviewed in Sandler et al. 2020).

ICANS has been defined as a disorder characterized by a pathologic process involving the central nervous system following any immunotherapy that results in the activation or engagement of endogenous or infused T cells and/or other immune effector cells (Lee et al. 2019; Sheth and Gauthier 2021). ICANS was observed initially in trials of CD19 CAR T cells and later in the BCMA CAR T cell trials (Raje et al. 2019). ICANS is characterized by speech difficulties, tremor, dysgraphia, cognitive difficulties, and/or altered level of consciousness (reviewed in Rice et al. 2019). Symptoms typically occur within the first week after infusion of CAR T cells and range from mild to severe. Similar to CRS, a standardized grading system has been developed to aid in diagnosis and management of ICANS (Lee et al. 2019). While it is likely that inflammatory cytokines play an important role in the development of ICANs, it appears treatment aimed toward inhibiting IL-6 may not be sufficient for control, some evidence even suggests that tocilizumab paradoxically contributes to worsening ICANS; therefore, treatment relies upon supportive care, control of seizures, and corticosteroids (Rice et al. 2019; Gust et al. 2020).

Building a less toxic cell T cell therapy must take into consideration that almost all patients with tumor response also develop some degree of CRS; thus, strategies must not interfere with TAA recognition and T cell activation. Engineering strategies include cloning in suicide genes such as inducible caspase 9, which was shown capable of “turning off” alloimmune T cell activation in recipients of HLA-haploidentical HCT (Di Stasi et al. 2011), or genes that express cell surface molecules that can be targeted with monoclonal antibodies (Fig. 6.7). Others have proposed developing a “universal CAR” that recognizes one moiety on a bi-specific engager, which recruits the CAR T cell to the tumor via its TAA engager (Yu et al. 2019). Alternatively, non-

integrating vectors such as AAV might be used to deliver constructs that would be expressed for a limited timeframe (Rotolo et al. 2018).

6.5.4.2 Building a More Effective T cell BioDrug: Remaining Challenges

The recent approval of several CAR T cell BioDrugs and the proliferation of clinical trials for engineered immunotherapeutic cell products speak to the progress and promise of tumor-directed cell therapies. The studies that have supported development of the currently approved CAR T cell products unequivocally demonstrated the power of T cells not only for killing malignant cells but for maintaining tumor surveillance and preventing relapse. Currently approved T cell products achieve disease response in 50–75% of patients and complete remission in 28–54%. However, the success in targeting B cell malignancies has not yet translated to solid tumors. Building a T cell BioDrug for treatment of a solid tumor begins with identifying the barriers to success, and the design of the system must be based on knowledge of the interactions between T cells and the TME. The difficulties must be overcome along with potential approaches to build improved cellular therapy for solid tumors. Some of the obstacles to current cellular therapies and strategies for building improved tumor-targeting BioDrugs are outlined below and shown in Figs. 6.6 and 6.7.

How can we improve tumor cell targeting? In contrast to CD19 and BCMA, many tumor antigens are also expressed by a variety of normal cells, resulting in the potential for significant off-tumor toxicity, and expression is heterogenous within the tumor, resulting in inadequate recognition of tumor cells. Furthermore, overexpressed TAA may be intracellular antigens not normally found on the cell surface, such as NY-ESO-1 or WT1. As described above, engineered TCR approaches were developed to recognize TAA presented in the context of HLA. However, much of the improvement in solid tumor antigen recognition will come through complex genetic engineering to enable T cells to recognize patterns of gene or protein expression that differentiate malignant cells from normal cells (Springuel et al. 2019). Examples of proposed strategies include multi-specific CAR constructs that recognize different TAA, with a specific recognition pattern required to initiate T cell activation or that recognize TAA in the context of other signals expressed by tumor cells, such as stress-induced ligands in the TME.

How can we prevent antigen negative escape? Approximately 60% of patients with relapsed disease after treatment with CD19-directed CAR T cells had a recurrence with a CD19-negative malignancy. Antigen-negative relapse results from the pressure that CAR Ts place on leukemic cells that leads to natural selection of alternatively spliced variants of the CD19 molecule (Sotillo et al. 2015). Strategies proposed to reduce the risk for antigen-negative relapse include administration of CAR T cells with different specificity (CAR pools), for example, coadministration of CD19- and CD20-directed CAR T products, based on the idea that there is a lower probability of losing two different antigen targets (reviewed in Ruella et al. 2016). Others have proposed developing multi-antigen specific CAR constructs allowing each T cell to recognize multiple TAAs. An alternative approach is based on the success of bi-specific T cell engagers (BiTEs), wherein the CAR construct has a universal recognition site activated by antibodies or other molecules bound to the tumor surface. Various antibodies can be delivered independently, each capable of activating the universal CAR T cells (Ayyappan and Maddocks 2019; Darowski et al. 2019).

How can we build a stronger BioDrug? T cell exhaustion caused by continual antigen stimulation results in impaired in vivo proliferation and lack of persistence, both correlated with lower anti-tumor efficacy. One strategy to strengthen the overall BioDrug product is to consider the optimal cell to engineer. For example, investigators have proposed to start with T cells that are less prone to exhaustion by upfront selection of less differentiated naïve or central memory phenotypes and/or to modify culture conditions that support T cell persistence (Gattinoni et al. 2011; Ghassemi et al. 2018; Ceppi and Gardner 2019).

Alternatively, collection of T cells from healthy donors not previously exposed to cancer therapy might improve T cell fitness. Gene-editing machinery, such as CRISPR/Cas9, has been used to remove the endogenous TCR and/or HLA molecules that could lead to alloimmune responses. Alternatives to T cells, such as NK cells or MΦs, also are being explored as optimal cells for overcoming the immunosuppressive TME (Fig. 6.6). In addition to novel cell selection, genetic engineering may contribute to strengthening cellular products. One example is the advance made by the addition of co-stimulatory genes to the first-generation CAR constructs, now termed "second-generation" CAR T cells, which led to improved T cell activation and proliferation products. Genetic engineering strategies proposed to improve T cell persistence include the addition of genes for cytokines that support T cell proliferation, such as IL-2, or precisely target the insertion of the transgene next to endogenous T cell regulatory elements using gene-editing machinery by using gene editing such as CRISPR/Cas9 (Perales et al. 2018).

How can we overcome the immunosuppressive TME? The main obstacle to successful TIL therapy has been the immunosuppressive nature of the TME, which presumably will pose a challenge to even the most potent T cell products. The TME combines the interactions of stromal cells, secretory factors, tumor vasculature, and immune regulatory cells such as Tregs, myeloid-derived suppressor cells, and TAMs within a hostile hypoxic and nutritionally depleted environment, each part of which can form a barrier to T cell function (reviewed in Ye et al. 2018). Lessons from the development of immuno-oncology drugs, such as the checkpoint inhibitors that have radically changed the treatment of solid tumors, will need to be incorporated into cellular therapy strategies to ensure a potent anti-tumor response. The simplest approaches have proposed to administer checkpoint inhibitors, IL-12, or other immuno-oncology drugs alongside cell therapy products; however, these strategies may increase the risks for added systemic toxicities from the additional agents. More complex strategies seek to incorporate these genes within the transgene constructs, resulting T cells capable of locally secreting pro-inflammatory cytokines within the TME (Springuel et al. 2019).

6.6 Summary

The future holds bright promise for new curative therapies for life-threatening malignancies and inherited blood disorders in children based on BioDrug technology. Building better BioDrugs in the future will incorporate many of the strategies outlined in this chapter, and the components in the Toolkit will be utilized. Undoubtedly the ToolKit will expand to include technologies and components not yet imagined for BioDrug development. The resources provided here are meant to provide pediatric hematologist/oncologists with the knowledge to understand current and future developments so that they can better inform their patients and guide them through clinical trials and complex therapies aimed toward permanently correcting genetic disorders and eradicating childhood malignancies.

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