C h a p t e r 1

LIVING CELLS AS A NEXT-GENERATION THERAPEUTIC MODALITY

1.1: The Necessity of Novel Therapeutic Platforms

The difficulty of treating diseases at the molecular scale has driven a great deal of innovation, and a corresponding increase in complexity, in the structure of therapeutic agents[1]. Initially, drugs consisted of small molecules which were isolated from natural sources[2]. Industrialization brought about a revolution in the capabilities of organic synthesis, leading to the creation of natural products from non-natural precursors, the derivatization of these molecules into novel chemical species, and the discovery of new medicinal chemicals[3]. The advent of high throughput screening brought about yet another method of developing small molecules into therapeutic entities, enabling diverse sets of synthetic chemicals to be rapidly examined for their ability to display potentially beneficial activities[4]. As the field of molecular biology developed, rational drug design became a viable proposition. Overall, the pharmaceutical industry has been largely dominated by small molecule drugs to date[5].

Despite the enormous success of small molecules at treating disease, a stubborn set of diseases remains refractory to treatment. Illnesses such as multi-drug resistant infections[6], many viral infections[7–9], neurodegenerative disorders such as dementias and motor impairments[10–13], and chemotherapeutic-unresponsive cancers[14] remain incurable and only partially treatable with conventional small molecule methods. These failings often stem from the fundamental structural properties of small organic molecules: their lack of size inherently

results in a relatively small contact area with their therapeutic targets, and their simplicity precludes them from executing logical computations such as sensing their environments and converting signals from their surroundings into structural rearrangements and altered modes of action[15,16]. The size of small molecule drugs limits their targets to those containing "druggable pockets," or molecular crevices which are able to maximize topological interactions with the contact surface of the drug molecule, and not all disease-driving molecules contain such sites. Furthermore, the lack of environmental sensing results in biological activity both "on target" (at the site of disease) and "off target" (in healthy tissue), resulting in side effects which are often dose-limiting[17,18].

To address the limitations in the specificity of small molecule drugs, new targeting systems are under constant development. Most notably, lipid-based drug carriers such as micelles and liposomes can overcome some of the targeting challenges associated with bare small molecules. In the context of cancer, the larger size of liposomes renders them susceptible to the Enhanced Permeability and Retention (EPR) effect, resulting in preferential deposition in the tumor via escape from the local abnormal and leaky vasculature[19]. This effect can be augmented by decorating the lipid coat with molecular targeting moieties, thereby enhancing drug deposition within specific environments[20]. Using environmentally-labile chemical linkages such as pH-cleavable bonds, these targeting agents can even induce transcytosis into physiological compartments that are traditionally difficult for therapeutic agents to access[21]. While nanoparticle-based drug delivery shows great promise, the method is not a panacea. Ultimately the small molecule payload cannot, upon delivery, discriminate between diseased vs. healthy tissue, and the ultimate therapeutic potency depends on the biological activity of the drug rather than of the carrier, thereby limiting molecular targets to those within the

druggable repertoire. Additionally, EPR-directed accumulation in tumors, while significant, leads to on-target delivery slightly in excess of off-target deposition in vital organs[22]. Furthermore, single-target guidance of the nanoparticles is often insufficient for disease discrimination, although more complex targeting strategies are in development[23,24]. Finally, the chemical simplicity of lipid membranes limits the variety of behaviors that these nanoparticles and drug carriers can display relative to more complicated, dynamic, and energy-driven systems, and these behaviors are often responsive to broad biological effects such as pH rather than the subtle molecular differences that can differentiate diseased tissue from an off-target, healthy environment.

In the 1980s, antibody-based therapeutics were introduced to the clinic[25]. Unlike traditional drugs, antibodies are large, complicated, and multifunctional molecules which can overcome some of the key disadvantages of small compounds. Antibodies contain a relatively large and genetically designable binding surface suitable for targeting non-cleft-like molecular surfaces, thereby greatly expanding the array of druggable protein targets[26]. Furthermore, in addition to simple binding and inhibition of molecular machinery, antibodies can interact with the host immune system by way of phenomena such as antibody-dependent cytotoxicity (ADCC), thereby increasing therapeutic potency beyond that conferred intrinsically by binding of the molecule to its target[27]. Due in large part to these expanded capabilities, antibodies have become a prominent portion of the modern therapeutic arsenal[28]. However, antibody-based therapies have their own limitations. Despite their favorable pharmacokinetics relative to small molecule drugs, antibody half-life is still measured on the order of days[29], which typically necessitates multiple expensive and uncomfortable infusion procedures[30]. Additionally, the large size of antibodies prohibits passive diffusion across the

cell membrane and endocytosis into the target cell typically depends on prior binding to an extracellular receptor[31], which precludes targeting of cytoplasmic antigens. The large size of antibodies also inhibits their transport from the blood to the diseased tissue[29]. Furthermore, the specificity of antibodies for a single molecular target can preclude them from being able to sufficiently distinguish healthy cells from diseased tissue, which often requires multiple markers[32–34]. Finally, the difficulty in controlling the therapeutic potency of antibodies *in situ* often leads to adverse effects related to runaway effects, particularly in biologics targeting the immune system[35].

The high complexity of extant therapeutic challenges has led to a concomitant increase in the complexity of the therapeutic agents engineered to treat them. Viruses represent a new class of such drugs, with a complexity exceeding even that of antibodies and nanoparticles[36]. Viruses consist of genetic material encapsulated by a protein, and sometimes lipid, shell. These biological particles are decorated with surface receptors that enable the complicated and coordinated molecular behavior required for efficient entry into the target cell. In the intracellular environment, the virus can act on targets in the cytoplasm or in the nucleus, and can alter cell function via transcriptional programs. Some types of viruses are even capable of integrating into the cell's genome, thereby enabling permanent modification of the cell's functionality. While viral therapy remains a novel research area, some clinical successes have already been achieved, culminating in the 2012 EMA approval of Glybera[37] and the 2017 FDA endorsement of Luxturna[38]. The relatively high complexity of viruses enables them to display some of the most desirable characteristics in therapeutic agents, such as precision targeting based on the molecular environment[39] and a broad and controllable range of biological activities[40]. However, as a natural environmental agent, viruses must combat the

intrinsic immunological defenses of the human body[41]. Not only does immunity severely limit the efficacy of viral therapy, it can also lead to significant and sometimes fatal toxicity by generating massive immune reactions at therapeutically-relevant doses[42]. While viruses remain an exciting therapeutic platform for treating some types of diseases, the fact that viral administration intrinsically renders the therapy at odds with the host immune system fundamentally limits the efficacy and persistence of this approach[43].

The past two decades have seen enormous progress in the development of technologies to engineer and control living cells. Many cell types, both microbial and animal, can be genetically altered and reprogrammed to execute arbitrary biological functions. Such engineered cells have found a variety of applications, ranging from the efficient production of biofuels to the manufacture of precursors for clothing[44] and other materials[45]. Such cells can also be used to produce both small molecule[46] and large molecule[47] therapeutic agents. Bioengineering and biotechnology have emerged as major industries within the United States and other developed nations, with one report estimating conservatively that in 2012 these sectors represented over 2% of the US GDP[48]. A particularly exciting area of application for engineered cells, whose leading edge is just now entering the clinic, is the engineering of live cells for direct therapeutic applications[49].

Cells offer a host of advantages over other therapeutic modalities. Their enormous complexity provides a plethora of biological “knobs” to tune – cells can be engineered to preferentially replicate under specific and multiparametric environmental stimuli, enabling the sensing of complex heuristics to differentiate between diseased and healthy tissue[50]. Cells can also persist for much longer than non-biological entities[51,52], and autologous cell grafts

do not compete with the host immune system for survival as is the case for viral therapeutics. Additionally, cells can proliferate at the site of disease[53–55], which can amplify therapeutics effects while also decreasing the required dosage for administration, thus reducing cost and potential off-target spread. Finally, cells can employ a suite of biological programs to interface with the local environment, interacting with the local immune system and augmenting[56] or ameliorating[57] inflammation as required by the given therapeutic context. The tremendous range of possibilities afforded by engineering cells for therapeutic applications has led to a great deal of pre-clinical and translational investigation, and the first wave of cell-based therapies has entered the clinic[58].

1.2: Microbial Therapeutic Agents

Bacteria were the first living cells to be utilized in a clinical setting. The idea of cell-based therapy is often attributed to Dr. William Coley, an American surgeon who, in the late nineteenth century, recognized that cancer patients suffering from infection occasionally displayed spontaneous tumor regression[59]. Coley began injecting his patients with Erisypelas (now formally known as *Streptococcus pyogenes*) and observed some degree of therapeutic efficacy[60], although insufficient recordkeeping has rendered his results difficult to reproduce or justify as a modern therapeutic intervention. Additionally, Coley's use of non-engineered pathogenic bacteria was associated with the expected adverse effects of microbial infections such as sepsis and upon the development of X-ray radiation therapy and improvement of surgical techniques in the early twentieth century, Coley's method of treatment fell out of favor in the medical community[61,62].

An increased understanding of bacteriology and bioengineering has led to a resurgence in microbial therapeutics. Discovery of bacterial strains with restricted pathogenicity has enabled safe administration in the clinical setting, with some forms of microbes often purchased and consumed over the counter[63]. These naturally occurring bacteria have applications in the treatment of some disorders, particularly those of the gastrointestinal tract[64–66]. Some probiotic strains of bacteria, particularly *E. coli* Nissle 1917[67] and *L. lactis* NK34[68], have demonstrated anti-cancer activity in addition their conventional role in the GI tract.

Microbes are a versatile biological platform for modification and engineering. Decades of experience in bioengineering has resulted in the robust ability to engineer many strains of bacteria by the introduction of foreign nucleic acids, either by way of extrachromosomal DNA molecules (e.g. plasmids) or via chromosomal editing (such as via Lambda Red recombination)[69–71]. The expanded ability to manipulate and program microbial cells with novel functions has resulted in the design of strains that can sense disease states and either report their presence[72,73] or treat them *in situ*[63,74]. Such facile control over bacterial genetics has generated a great deal of interest in the scope of potential applications for microbial therapies[75,76].

One of the main limitations of conventional therapeutic molecules is the challenge of targeted delivery. After injection or ingestion, the drug molecule forms a concentration gradient away from the site of administration, often resulting in relatively low dosage to the disease site while maintaining an elevated level in circulation and at potential off-target tissues. Microbes can be utilized as *in situ* "micropharmacies" to directly synthesize therapeutic agents within

a diseased tissue[67,77–79]. By injecting microbes that directly home to the site of disease, the drug of interest can be locally produced, thereby maintaining the highest concentration at the target site and diluting at distal, healthy organs[80]. This method of drug delivery can mitigate the toxicity observed in the systemic administration of highly bioactive molecules, as has been limiting for IL-2 therapy for cancer[81].

Microbes can also be utilized to directly destroy diseased tissue, a behavior which has been of particular interest in cancer therapy. The majority of research into this targeting behavior has focused on the ability of microbes such as *Salmonella*[82,83] and *Clostridium*[84] to traffic to tumors and colonize them. Obligate anaerobic bacteria such as *Clostridium*[85] and *Bifidobacterium*[86] suffer toxicity from oxygen exposure and are thus restricted to surviving in the hypoxic cores of poorly vascularized tumors. In contrast, facultative anaerobes such as *E. coli*[87], *Salmonella*[88], and *Listeria*[89] are able to tolerate the presence of oxygen. These microbes can display preferential tumor accumulation by active chemotaxis to necrotic and nutrient-rich regions[90,91], engineered auxotrophy for necrosis-associated molecules[92] and by growth restriction to immune-privileged regions such as the tumor microenvironment[93]. While some destruction of diseased tissue is inherently caused by the colonization and replication of these microbes, this activity can be enhanced by augmenting bacteria with non-natural payloads[94]. Microbes have been engineered to deliver bacterial toxins[95], pro-apoptotic factors[96–98], cytokines[99,100], chemokines[101], anti-antiogenesis agents[102], tumor-specific siRNA[103,104], plasmid DNA bearing tumor-suppressive genes[105], pro-drug converting enzymes[106,107], and antibodies[108]. Additionally, the foreign surface markers of microbes can render them potent immune adjuvants[109,110], and delivering novel immunogenic proteins can further enhance the inflammatory response stimulated by these

agents[111]. Arming microbes with disease-related peptides, proteins, or nucleic acids can prime the host immune system to recognize antigens which it would otherwise tolerate, an application of interest for inducing recognition of tumor-associated self-antigens that are frequently protected by immunological tolerance[112–116].

Attenuation of bacterial strains can also result in growth restriction to diseased tissue which can be harnessed in a clinical setting[117]. In the early 1900s, researchers at the Pasteur Institute searched for avirulent strains of Bacillus for use as vaccination strains against *Mycobacterium tuberculosis*, the causative agent of tuberculosis infection. By passaging a slightly attenuated strain on glycerine potato medium, the scientists generated a sufficiently avirulent line of *Bacillus* for use as a vaccine, and this became the strain utilized in the well-accepted BCG vaccination for tuberculosis[118]. In the 1970s this strain was found to promote tumor regression in non-muscle-invasive bladder cancer (NMIBC) patients, and intravesical administration of live BCG is now a standard treatment for this disease[119]. The mechanism of BCG homing and therapeutic activity against bladder tumors is not fully determined, although recent research has suggested a model in which the microbe binds to the carcinoma surface[120] and becomes internalized via micropinocytosis[121] stimulating a host immune response[122], a process which may be accelerated in cells mutated in the PTEN tumor suppressor[123]. Biosimilar strains have subsequently been developed, and expanded therapeutic applications such as treatment of colorectal cancer are under investigation. The strategy of virulence attenuation has since resulted in the development of the therapeutic bacterial strains *Salmonella* VNP20009[124], AR-1[92], and ΔppGpp[125], *Clostridium Novyi* NT[126], and others promising variants including a derivative of *S. pyogenes*, the bacterium that launched the field of microbial therapeutics[127].

Progress in bacterial therapy has been hindered by several factors. First, the fact that microbes are immunologically foreign objects in a patient's body renders them subject to immune reaction and as such, the dosages are restricted to minimize the severity of potential immune reactions[128]. Even BCG therapy, despite its clinical approval, must be prematurely terminated in a small number of patients due to toxicity[129]. Additionally, while some specific disorders such as NMIBC possess cognate bacteria which display strong replicative preference for the diseased tissue, many bacterial therapies are reliant on more general environmental cues for targeting. Such signals include hypoxia and the metabolic profile within the tumor core. Some other compartments within the body such as the bone marrow can share some of these molecular heuristics[130] and induce off-target colonization and therapeutic activity. Even in experiments demonstrating successful targeting of a disease site, a substantial concentration of bacteria can accumulate in healthy organs such as the spleen and liver[131]. Finally, there have been notable failures of pre-clinically successful therapeutic strategies failing to translate from animal models into human trials[132], and most positive effects observed in humans have been non-curative[133–137]. In the most notable failed trial, a patient injected with an engineered *Listeria* strain to vaccinate against HPV-positive oropharyngeal cancer suffered systemic listeriosis, resulting in the halt of the trial[138]. The modest success of translating efficacious microbial interventions from animal models to the clinic suggests that a significant amount of research and development must be undertaken to augment the potency of microbial therapeutics while restricting their action to sites of disease.

1.3: Mammalian Therapeutic Agents

The first implementation of mammalian cells as therapeutic agents in human patients was arguably in 1818, when the British physician James Blundell performed a blood transfusion to treat hemorrhage[139]. In the early 1900s, preliminary molecular research enabled physicians to segregate patients according to blood type, thereby establishing the paradigm of antigen matching in cell transplantation[140,141]. The 1950s saw another type of transplantation enter medical acceptance: that of stem cells[142]. Using this newly discovered, non-terminally differentiated cell type, physicians could rebuild the hematopoietic system in cancer patients who received high doses of chemotherapy. Both RBC transfusions and HSC transplantations have revolutionized aspects of healthcare and are enduring components of the modern medical industry.

In the mid-1990s, Steven Rosenberg's group at the NIH demonstrated that immune cells could also be used in a therapeutic context[143]. Reasoning that T-cells activated within the tumor environment could be expected to attack the surrounding malignancy, Rosenberg and colleagues isolated T-cells from the tumors of melanoma patients, expanded them *ex vivo*, and then reinfused them in large numbers back into the patient of origin. These artificially selected T-cells resulted in regression of the melanoma, thereby establishing cell-mediated adoptive immunotherapy as a viable therapeutic strategy. Autologous immunotherapy was subsequently utilized for dendritic cells as well. In 2010, the US FDA approved Sipuleucel-T, a blood product generated by treating peripheral blood mononuclear cells with a fusion protein consisting of GM-CSF to enrich dendritic cells and Prostatic Acid Phosphatase, a common prostate cancer antigen, to generate cognate antigens[144]. Other artificially stimulated dendritic cells generated via exposure to tumor-derived peptides, nucleic acids, or raw lysates are currently in late stage clinical trials[145].

The advent of reliable genetic modification of mammalian cells revolutionized the field by expanding the scope of accessible biological functions and providing novel strategies to control their activation[146]. Gene delivery into mammalian cells via chemical (cationic and polycationic complexes)[147,148], physical (electroporation or mechanoporation)[149,150], and biological (viral)[36] mechanisms, as well as precision gene editing via customized proteins and biomolecular complexes such as Zinc Finger Nucleases, TALENs, and CRISPR-Cas9[151], enables unprecedented control over cell function and fate. Aided by the 1970s revolution in recombinant DNA technology, which enabled facile manipulation of DNA sequences, gene delivery technologies offered a host of potential therapeutic modalities. A landmark experiment in 1980 demonstrated safe *ex vivo* transfer of recombinant plasmid DNA into human bone marrow cells followed by reinfusion into the patients of origin[152]. Although this study failed to demonstrate clinical efficacy and was roundly criticized as premature within the scientific and medical communities, it also opened the door for later studies of gene therapies.

One of the first technological beneficiaries of recombinant DNA technology was viral gene therapy, which can repair defective patient cells or introduce therapeutic functionality into host cells *de novo*[40]. Viruses had been explored (largely unsuccessfully) as therapeutic agents in their wild type context, but the ability to edit their genomes and utilize them to deliver arbitrary genes of interest garnered a great deal of scientific and public attention[152]. This ambition led to the use of recombinant viruses in clinical trials, with the first attempt aiming to correct X-SCID, a deficiency in immune cell maturation, in a batch of pediatric patients at the turn of the millennium[153,154]. While the field of gene therapy encountered significant turbulence during its inception[36], more recent research has yielded promising results and

several clinical approvals. The first viral gene therapy was approved in China to treat head- and neck squamous cell cancer in 2003[155] and another therapeutic adenovirus was brought to this market in 2005[156]. 2012 saw the landmark EMA approval of Glybera[157], an adeno-associated virus carrying lipoprotein lipase to correct a hereditary deficiency in this enzyme[158]. The first viral gene therapy approved in the U.S. was Luxturna, an AAV carrying a transgene to restore retinoid cycle function in a subset of retinal dystrophy patients, in 2017[38]. A large array of clinical trials for *in vivo* and *ex vivo* gene therapies for a variety of diseases is currently ongoing (reviewed by Dunbar et al[40]). Genetically engineered viruses have also garnered significant attention for oncolytic therapy via the wide array of alterations that bias viral replication to preferentially occur in tumor cells[159], with Amgen's Imlygic being the first to receive FDA approval in 2015[158].

In the past two decades, the use of genetic engineering to modify human cells for therapeutic efficacy has become a focus of significant research interest. Cells can also be extracted from a patient, genetically modified *ex vivo,* and subsequently reintroduced into the host[160]. The main advantage of this therapeutic approach is that the cells can be manipulated under controlled conditions, evaluated for quality, and then reinfused as a pre-modified product. Two cell types, hematopoeietic stem cells (HSCs) and leukocytes (particularly T-cells), have been of particular interest in this context[40]. HSCs are progenitor cells found in the bone marrow and are able to differentiate into adult blood cells of virtually any type. HSCs had previously found application in the context of allogenic transplantation of unmodified patient cells for treatment of chemotherapy-associated lymphodepletion[161]. T-cells are the main immune cell subtype involved in the adaptive cytotoxic response. By virtue of their unique T-Cell Receptor (TCR), which is randomized and selected during T-cell development, they

are able to recognize unique antigens presented on the Major Histocompatibility Complex (MHC) of potential target cells and selectively kill or otherwise direct an immune response against these specific targets[162]. The serial killing behavior of this cell type renders it ideal for applications such as cancer therapy in which cell ablation, rather than reprogramming, is required to treat the disease.

New advances in gene editing have enabled *ex vivo* modification of HSCs from patients with genetic defects in blood cell function such as β-Thalassemia, Sickle cell anemia, Adenosine deaminase deficiency (ADA), and several others[40]. After genetic reprogramming, these cells are reinfused into the patient and migrate back to the bone marrow, where they subsequently serve as progenitors to functionally repaired blood cell progeny[163]. Addition or replacement of mutant alleles in the HSCs has resulted in several successful clinical trials as well as the 2016 EU approval of Strimvelis for ADA.

Immunotherapies have also benefitted tremendously from advances in gene delivery. Following the pioneering work of the Rosenberg group in the 1990s on expanding tumor-specific lymphocytes, the past decade has seen a great deal of interest in the *ex vivo* genetic modification of T-cells and other lymphocytes for improved efficacy against cancer[164–166]. One of the first instances of *ex vivo* genetic engineering was the modification of T-cells with a genetic tracer for evaluation of their biodistribution following infusion into a patient[167]. A great deal of subsequent work has aimed to augment T-cell function for the treatment of cancers. A seminal paper by Eshhar and colleagues described genetic retargeting of T-cells toward native (non-MHC-presented) surface antigens by a hybrid protein consisting of an extracellular single-chain antibody domain for recognition of novel antigens and an

intracellular domain from the CD3ζ chain of the TCR for activation of the T-cell effector functions[168]. The extracellular domain in this design is modular and this fundamental architecture, now known as the Chimeric Antigen Receptor (CAR) has since been optimized and retargeted against the B-cell antigen CD19 for treatment of B-lineage blood cancers, culminating in the 2018 FDA approvals of Yescarta and Kymriah, the first CAR-T therapies[58].

Despite the immense progress which has been made in the genetic conversion of patient cells into therapeutic agents, significant challenges in the field remain. For direct viral gene delivery into patients, immunotoxicity and genotoxicity are the two key concerns. Viruses can elicit a strong immune response which can inhibit therapeutic efficacy by vector inactivation[41,169]. This immune reaction can also destroy virally modified cells by directing T-cell responses against viral capsid proteins or therapeutic transgenes presented by the MHC molecules of the genetically modified cells, thereby blunting the therapeutic effect[170]. Finally, the immune response may amplify from a local to a systemic phenomenon, driving signal transduction cascades that result in life-threatening systemic inflammation. Such a runaway immune response was famously responsible for the death of Jesse Gelsinger, a patient enrolled in an adenoviral gene therapy trial for Ornithine Transcarbamylase Deficiency[171]. This tragedy not only killed the patient, but also brought significant public and regulatory scrutiny toward the field, highlighting the safety concerns of these approaches. Another highly publicized mishap in the history of gene therapy occurred in one of the earliest clinical trials against SCID-X1. While the initial results of the intervention were highly promising, with immune functionality partially restored in the majority of subjects[172], several of the enrolled patients subsequently developed leukemia[173,174]. This result

underscores the potential for viral integration-induced genotoxicity, in which the process of viral insertion can disrupt the innate cellular machinery responsible for regulation of cell division, leading to malignant transformation. Similarly, a potential pitfall of stem cell-based therapy is the uncontrolled proliferation of undifferentiated cells, or teratocarcinoma[175]. While this phenomenon has not been observed in humans, several rodent studies have demonstrated it to be a concerning possibility in such therapeutic approaches[163,176].

Cell-based T-cell therapy has also demonstrated significant dangers. Antigen-redirected T-cells can mount a significant immunological response which, similar to the effect induced by high doses of viral genetic vectors, can prompt runaway and potentially fatal inflammatory reactions, which are termed Cytokine Release Syndrome (CRS)[177]. While modest CRS is an expected corollary to CAR-T therapy and in fact can serve as a biomarker of therapeutic efficacy, severe CRS has proven fatal in multiple cases[178]. Engineered T-cells can also demonstrate toxicity by erroneously attacking off-target tissues. This has occurred by way of "off-target, off tumor" toxicity in which the cells mistakenly recognize a healthy tissue antigen with structural similarity to the tumor antigen[179], or via "on-target, off tumor" behavior in which the target antigen is found, contrary to prior expectation, to be expressed on healthy tissue as well as in the cancer cells[180,181]. Both modes of mis-targeting have resulted in fatal reactions during clinical trials. In a trial of an affinity-enhanced TCR against the MAGE-A3 melanoma antigen, a patient suffered fatal cardiac toxicity when the engineered T-cells recognized and attacked Titin, a protein expressed in striated muscle[182]. "On target, off tumor" attack has been more common, with several trials reporting deaths from this mode of toxicity[180,183]. Improving the safety of viral and mammalian cell-based

therapeutics without compromising therapeutic efficacy remains the primary challenge of the field.

1.4: Common Challenges in Gene and Cell-Based Therapy

While viral and nonviral gene therapy, engineered bacterial therapeutics, and cell-based therapies are disparate strategies with their own idiosyncratic challenges, several overarching themes unify the obstacles that must be overcome before these next-generation interventions become standard clinical practices. The tissue and organ specificity of next-generation therapeutics must be well controlled to ensure that only sites of disease are treated or modified. The complex, and in some cases self-renewing, nature of these therapies is correlated with a high degree of potency, the regulation of which is critical for ensuring patient health. In cases where the intervention proves deleterious, such as malignant expansion of engineered cells[184] or life-threatening activity-related toxicity[185], physicians must be able to robustly abort the therapy. Finally, the structural and manufacturing complexity of next generation therapies leads to high cost, and as such, the therapeutic designs should be controllable by *ex vivo* factors to avoid necessitating premature cessation of the treatment.

The targeting specificity of genetic modification therapies is largely dictated by the method of transduction. While *ex vivo* editing of mammalian cells pre-selects against off-target genetic modification by physically separating the desired cells from the patient prior to modification[186], control of *in vivo* gene delivery specificity largely relies on the vector for editing. In some cases, differential viral tropism for specific tissues can bias entry and delivery, although natural viral tropism is rarely exclusive to single tissue types[187,188].

Tropism can be modified via engineering of viral surface proteins, yielding vectors with altered infectivity profiles[189]. Utilization of tissue-specific promoters to drive expression of the genetic payload can further increase specificity of gene delivery[190]. As a whole, the cell-type specificity of systemically administered genetic interventions is virtually never as precise as that of *ex vivo* engineered approaches due to the inability to pre-sort the target cell population prior to editing. Introducing methods to control the timing and location of gene delivery within the patient has the potential to vastly improve the safety and efficacy of such methods.

The spatial specificity of cell-based therapies is also of great interest, due largely in part to the aforementioned observations of off-tumor toxicity. With the exception of HSC engineering, where activity is usually restricted to its intended niche via the stem cells' useful property of intrinsic homing and engraftment into the patient's bone marrow after reinfusion[161], cell-based therapies are largely limited by off-target adverse effects. Bacterial homing and growth specificity are largely governed by the permissivity of the surrounding environment. Despite the wealth of available mechanisms to support bacterial proliferation in tumors, off-target colonization can occur in organs such as the spleen and liver, and in "tumor-like" niches such as the bone marrow. Additionally, some diseases for which cell-based therapies would be beneficial do not have obvious environmental markers that would promote bacterial growth. In contrast to bacterial therapies, the spatial localization of cell-based immunotherapies is mostly guided by the location of the target antigen[191]. Ligation of the antigen with the TCR or CAR results in strong pro-inflammatory signaling leading to local immune cell proliferation and recruitment. Unfortunately, this mechanism of spatial localization leaves therapies such as CAR-T vulnerable to off-target expression of antigens

or target antigen misrecognition, both of which can result in efficacy and toxicity in healthy tissues. As such, a great deal of work is being invested into improving molecular recognition using inhibitory receptors[192], logic-gated receptor systems[193], and even novel receptor platforms such as modular synthetic variants of the Notch receptor (SynNotch)[194] which can be utilized to orthogonally modulate specific biological functions including expression of the primary antigen receptor.

In some cases the administration of a therapy can go awry, leading to unpleasant or potentially fatal side effects that justify abortion of the therapy. The field of gene therapy has observed several such instances, such as the death of an adenoviral therapy patient due to severe and systemic inflammation[171] and the onset of leukemia in pediatric SCID patients[195]. CAR-T based therapies have also resulted in patient deaths during clinical trials due to off-target antigen recognition[196]. As such, the potency of next generation living therapeutics justifies engineered control strategies to enable abortion of the therapy at the whim of the administrating physician. Artificial control over the viability of the treatment can largely address this necessity. In bacterial therapy, auxotrophic strains which require continuous delivery of a nutrient for survival have been developed[197]. Additionally, kill switches which rely on detection of exogenous factors have been developed to inducibly halt bacterial growth or damage viability[198]. These methods can be utilized in combinatorial fashion for highly effective biocontainment[199,200]. For gene therapy and mammalian cell therapy, in which the modified cells are typically patient-derived, complex metabolic engineering is often not an option. Therefore, control of viability is typically enacted in kill switch fashion using inducible pro-drug-conversion enzymes such as HSV-tk[201] or intrinsically lethal proteins such as iCasp9[202]. A key obstacle to auxotrophy and kill switch-mediated control systems is

mutation[203]. Introduction of a genetic system that damages viability exerts a strong selection pressure on cells, and leaky selection strategies enable populations of escape variants to grow out, thereby continuing the undesired biological activity. This phenomenon is more prevalent in the bacterial setting, in which polymerase fidelity is not as robust as in mammalian cells and where proliferation can thus occur rapidly.

The relative expense of biological therapies in comparison to conventional drugs results in abortion of treatment being a last resort. Instead, exogenous modulation of therapeutic efficacy can be engineered to allow physicians to tune the potency of the intervention without necessitating complete elimination. A common strategy is the design of systems dependent on inducible gain of function. These include therapeutic genes which are natively transcribed at low levels but can be induced upon detection of an exogenous stimulus, such as engineered T-cells harboring a chimeric antigen receptor driven by a doxycyline-inducible promoter[204] and CAR-Ts in which receptor signaling is dependent on chemically-induced dimerization enabled by an infused drug[205]. While such systems are useful to prevent severe toxicity, the requirement for constant or repeated infusion of the inducer can render them unsuitable for long-term therapies, such as repair of genetic deficiencies. To address this issue, genetic state switches which can toggle in response to external stimuli are under investigation[206].

Gene therapies, bacterial cell-based therapies, and mammalian cell therapies are exciting and potentially revolutionary treatment modalities which may enable treatment of previously undruggable targets and diseases. The molecular complexity of these systems renders them highly engineerable and customizable, and also enables a high degree of therapeutic efficacy and specificity. Nevertheless, off-target activities and undesirable behaviors largely remain

to be addressed. The next generation of biological therapies will likely carry engineered therapeutic modules, possibly in multiplex, to enable precise control over biological behavior. The ability to exert novel biological programs, coupled with exogenous strategies to modulate of abort the intervention if needed, will render these “programmable” therapies a powerful tool in the armament of future physicians.

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