Review

Bioengineering solutions for manufacturing challenges in CAR T cells

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Abbreviations: CAR, chimeric antigen receptor; **HSCT**, hematopoietic stem cell transplantation; **scFv**, small chain variable fragment; **TCR,** T cell receptor; **MHC,** major histocompatibility complex; **PBMC**, peripheral blood mononuclear cell; **DMSO**, dimethyl sulfoxide; **aAPC**, artificial antigen-presenting cell; **HLA**, human leukocyte antigen; **iPSC**, induced pluripotent stem cell; **TALEN**, transcription activator like effector nuclease; **ZFN**, zinc finger nuclease; **CRISPR**, clustered regularly interspaced short palindromic repeats; **TRAC**, T cell receptor alpha constant; **RNP**, ribonucleoprotein; **HDR**, homology directed repair; **GMP**, good manufacturing practice; **PAT**, process analytical techniques; **MPC**, model predictive control.

Abstract

The next generation of therapeutic products to be approved for the clinic is anticipated to be cell therapies, termed "living drugs" for their capacity to dynamically and temporally respond to changes during their production *ex vivo* and after their administration *in vivo*. Genetically engineered chimeric antigen receptor (CAR) T cells have rapidly developed into powerful tools to harness the power of immune system manipulation against cancer. Regulatory agencies anticipate approving CAR T cell therapies in the near future, due to their striking efficacy in treating some hematological malignancies. However, the engineering and manufacturing of such cells remains a challenge for widespread adoption of this technology. Bioengineering approaches including biomaterials, synthetic biology, metabolic engineering, process control and automation, and *in vitro* disease modeling could offer promising methods to overcome some of these challenges. Here we describe the manufacturing process of CAR T cells, highlighting potential roles for bioengineers to partner with biologists and clinicians to advance the manufacture of these complex cellular products under rigorous regulatory and quality control.

1 Introduction

The main pillars of cancer treatment are surgery, radiation, chemotherapy, and hematopoietic stem cell transplantation (HSCT). In the last two decades, immunotherapy has rapidly developed into a promising alternative, initially consisting mainly of monoclonal antibody and cytokine therapies [1], [2]. In the last five years, chimeric antigen receptor (CAR) T cell therapy has emerged at the forefront of the cancer immunotherapy field [3], [4]. In the CAR T approach, genetically modified lymphocytes are engineered to express a synthetic receptor comprised of an extracellularly expressed single chain variable fragment (scFv) of a monoclonal antibody, which is connected via a transmembrane linker to the intracellular signaling domains of common T cell co-receptors such as CD3 and CD28 [5]–[7] (**Fig.1**). The CAR can be used to target antigens expressed on the surface of cancer cells [8]–[10]. The scFv portion of the CAR is specific for a surface antigen (e.g., CD19, a B cell lineage surface marker used to target acute lymphoblastic and chronic lymphocytic leukemias [11]. This allows the CAR to bypass conventional interactions between the TCR and major histocompatibility complex (MHC), thus activating the cell upon recognition of the target antigen [12].

The typical CAR T cell manufacturing process (**Fig. 2A**) begins with harvesting the patient's peripheral blood mononuclear cells (PBMCs) through leukapheresis. These apheresed cells are virally transduced with the CAR transgene [13], activated, and expanded outside of the body (*ex vivo)* undergoing quality control (QC) testing before administration [14]. The entire manufacturing process requires a minimum of 22 days, beginning with T cell harvest and ending with intravenous delivery of the engineered CAR T cells back to the patient [15].

Amongst published trials targeting hematological malignancies, the therapy has resulted in complete or partial remissions across CAR designs and targets in approximately 70~94% of patients [16], [17]. The adoption of CAR T cell therapy into clinical practice shows similarities to the early stages of adoption of bone marrow transplantation (BMT). BMT was initially viewed with

skepticism and offered at few academic centers [18]. The therapy gained traction as its efficacy became apparent, and it is now available at a much wider number of centers across the globe, having been performed over one million times worldwide [19], [20]. Similarly, the full CAR T cell manufacture and therapy workflow including gene delivery, culture, and clinical care is limited to a handful of academic centers often in partnerships with industry (University of Pennsylvania with Novartis, Seattle Children's Hospital and Memorial Sloan Kettering Cancer Center with Juno Therapeutics, Baylor College of Medicine with Cell Medica, MD Anderson Cancer Center with Ziopharm Oncology and Intrexon Corporation, and the National Cancer Institute with Kite Pharma) with advanced manufacturing and clinical capabilities [14]. However, the geographical reach of CAR T cell therapy has increased with the advent of multicenter clinical trials supported by several pharmaceutical companies (e.g. fully recruited clinical trial NCT02435849 with 26 study locations).

A variety of biological challenges have limited the broad clinical applicability of CAR T cell therapy. First, CAR T cell therapy to date has only shown efficacy for certain hematological malignancies, and there are still problems present. The therapeutic process could be complicated by severe adverse events including cytokine release syndrome, neurotoxicities, and in the case of targeting CD19, B-cell aplasia [11]. These pose significant concerns, although standard treatment options such as chemotherapy and stem cell transplantation have equally severe side effects, including acute toxicity and the risk of graft-versus-host disease (GVHD), respectively [21], [22]. Additionally, recent attempts to treat solid tumors with CAR T cells have yielded lackluster results, due in part to heterogeneous CAR T cell populations that have performed inconsistently and in some cases failed to persist within the body [23], [24]. It has proved challenging to find proper target antigens for solid tumors, and strategies to improve T cell penetration into the tumor microenvironment are needed [23]. Furthermore, T cell exhaustion and differentiation are concerns for the lack of persistence *in vivo* [23]. While problems arising primarily from T cell

biology are currently being addressed [5], there is still a need to address manufacturing paradigms and processes to ensure that CAR T cell therapy can be translated widely.

This review will cover issues associated with the administration and scale up of CAR T cell therapy, as well as bioengineering solutions to address them. We will identify these challenges in the chronological order in which they arise during CAR T cell manufacture, including cell harvesting, shipment of the leukapheresis product, T cell activation and expansion, gene delivery, and QC [14]. We will also address regulatory requirements for medical centers that aim to offer these treatments. Ultimately, we seek to describe the significant role for bioengineering in the broader dissemination of CAR T cell therapy.

2 Cell Harvesting

CAR T cell therapies can use either autologous or allogeneic T cells, although allogeneic therapies may run a greater risk of immunogenic reactions [25]. Most current CAR T cell clinical trials use T cells collected from patients, although some of these patients had previous alloHSCT. CAR T cell therapy begins with the leukapheresis procedure to isolate PBMCs [13]. Leukapheresis typically occurs over several hours, during which the patient's blood is treated with anticoagulants and centrifuged to remove excess red blood cells and platelets. The patient's PBMCs are then either shipped to a manufacturing facility as a fresh product or cryopreserved for shipment in the future. Leukapheresis may be complicated for patients that have already been treated for their malignancies, as the resulting lymphopenia from chemotherapy can make it difficult to collect sufficient numbers of T cells [26]. Leukapheresis is also more challenging for infants and small children due to their lower total blood volume [27]. Prolonged treatment with anticoagulants during leukapheresis can pose problems due to the length of time that patients are connected to an external device [28].

Bioengineering solutions can be used to improve leukapheresis from an extended outpatient procedure to a process that substitutes implantable devices for traditional blood filtration. For instance, subcutaneous **biomaterial scaffolds** have been developed to recruit specific T cell subsets *in vivo* [29]. This approach was used to harvest diabetogenic T cells for *ex vivo* expansion and analysis using polylactide-co-glycolide scaffolds loaded with disease-specific antigens [30]. Methods have also been developed for analyzing rare T cell subtypes using novel peptide-MHC chemistries [31]. Additionally, functionalized carbon nanotubes have been shown to successfully recruit and activate T cells *in vitro* [32], and similar approaches could potentially be used *in vivo*. These technologies could be applied to the CAR T cell manufacturing workflow to produce collection devices coated with antigens specific to desired T cell populations, such as naïve and/or stem cell memory T cells. Within this model, the device would be implanted into the patient under a sterile field to reduce the probability of infection, and harvested a few days later with an enriched population of cytotoxic T cells suitable for transfection. By tailoring the avidity of the interaction between immobilized ligands and their target receptors, T cells could be harvested while potentially decreasing blood coagulation, inflammation, and fibrous encapsulation. This process could reduce stresses associated with large volume fluid shifts that occur during leukapheresis, and could further allow for selective isolation of highly cytotoxic T cell populations, thereby decreasing the total number of T cells required.

3 Transport of Harvested Cells

Once PBMCs have been isolated, some centers *cryopreserve* the cells and ship them to centralized manufacturing facilities for activation, viral transduction, and expansion [13]. The cells are cryopreserved in blood bags and shipped frozen, then thawed and activated after arrival at the manufacturing facility. However, transport of the T cells is an important consideration, as it is critical to ensure that desired cytotoxic populations are well preserved. Some studies have

indicated broad changes in PBMC transcriptomes after freezing and thawing [33], while others have shown that re-stimulation can rescue freeze/thaw-induced changes observed in regulatory T cells [34]. Aberrations in cell functionality due to cryopreservation proved prohibitive for Provenge®, the first FDA-approved autologous cell therapy product [35]. Provenge® was only viable for four hours post-thaw and could not be used after being frozen for 18+ hours [36]. As a result of strict delivery conditions and timelines, Provenge® was deemed financially unviable [37], although it remains an instructive case study for CAR T cell therapy. Thus, although some current clinical trials have successfully used freezing and thawing to transport T cells, there may be room for improvement.

As a starting point, better QC mechanisms will be required to confirm cell viability and immune profile changes [38] in the form of *in vitro* tumor cell killing assays and cell profiling techniques, which will be discussed in Section 6 of this review. Additionally, progress has been made to minimize the impact of cryopreservation reagents such as dimethyl sulfoxide (DMSO) to generate clinically safe products. **Microfluidic devices** to remove DMSO by diffusion have been described which allow over 95% of cells to be retained post-wash, thus improving yields by ~25% [39]. Furthermore, cell recovery outcomes may be improved through the use of hypothermic preservation solutions (e.g. HypoThermosol®), which allow cells to be transported without the need for freezing [40]. Such approaches not yet been implemented in CAR T cell manufacturing, but may one day improve production efficiency and safety.

4 Activation and Expansion of T cells

In order to trigger T cell killing mechanisms, CAR T cells must be stimulated via antigen recognition [41]. The most commonly used *activation* process is independent of antigen presentation, and involves culturing T cells with beads coated with CD3/CD28 antibody fragments, along with IL-2 supplementation [14]. While T cells are naturally activated in response to short-term antigen

presentation, sustained signaling can cause exhaustion, leading to a loss of proliferative capacity and cytotoxicity [42]. Therefore, it would be beneficial to ensure activation but limit exhaustion through the use of custom biomaterials. This has been achieved through **artificial antigen presenting cell (aAPC) technology** [43], which can include beads coated with a CD28-specific antibody, a specific antigen epitope, and soluble human leukocyte antigen immunoglobulin (HLA-Ig) [44]. More recently, cells expressing HLA-Ig that are engineered with an antigen epitope have been used as aAPCs [45].

As current methods for activation are time consuming [14] and can lead to exhaustion, there is significant room for improvement in this stage of CAR T cell manufacturing. **Tissue engineering** approaches may improve the activation process via customizable ligand-presenting scaffolds in the place of aAPCs. These could potentially feature controlled spatial or temporal patterns of ligand presentation. For example, spatially patterned ligands have been used to study and control cell adhesion [46], and degradable materials may be useful to slowly release ligands, thus modulating the activation response [47]. It has also been shown that micropattered T cell costimulatory ligands can enhance secretion of IL-2 by CD4+ T cells via a CD3/CD28 costimulation array. These same technologies could be utilized to potentially ameliorate activation-associated problems such as exhaustion [48]–[50].

Another vital process in the CAR T cell manufacturing pipeline is *expansion*. Expansion is required to increase the population of T cells available for transduction or infusion to the patient and can occur either before or after gene transduction, depending on the manufacturer [51], [52]. Currently, this process can be accomplished via several platforms. Wave-mixed bioreactors (e.g., GE, Sartorius bioreactors) feature a bioprocessing bag (e.g., Cellbag®, Flexsafe®) on a rocking base for efficient gas exchange and media perfusion, and are widely used across academic and industrial labs to support clinical trials [53]. Fully automated closed systems such as CliniMACS® are also being developed to allow for GMP-compliant production without the need for clean room facilities

[54]. The cell expansion process takes approximately ten days, upon which cells are harvested and cryopreserved for distribution [14].

Current expansion platforms use CD3 and CD28 antibody-functionalized beads to expand general T cell populations (e.g., Dynabeads™ [55]). These beads are prone to aggregation, particularly when used in agitated systems such as GE WAVE [56], [57]. Additionally, ligand presentation needs to be optimized, to ensure that sufficient quantities of cells are activated. Beads have the advantage of a high surface area to volume ratio, which allows for a greater density of ligand presentation. However, the process of removing the beads can cause a loss of product if T cells fail to dissociate or are damaged by shear forces due to binding [58], [59]. To address this issue, bead-free T cell expansion systems utilizing tetrameric CD3/CD28 antibody complexes have been developed by Juno Therapeutics and others, including Expamer™ technology [14]. **Ligand-functionalized surfaces** could potentially be utilized to circumvent some of these difficulties, enabling the use of other bioreactor architectures. These surfaces could be within hollow fiber membrane bioreactors [60], packed bed bioreactors [61], and potentially, stainless steel stirred tank bioreactors, as antibody functionalization of stainless steel surfaces has been demonstrated [62]. Once expanded, T cells could then be detached using controlled chemistries that release the bound cell from the surface [63], [64]. Such an approach could reduce aggregation and shear stress on the cells.

Cellular metabolic profiles provide an additional phenotypic measurement that can be used to affect cell fate decisions to preferentially expand cells in a mixed culture [65], [66]. In cardiac differentiation of induced pluripotent stem cells (iPSCs), cardiomyocytes metabolize lactate better than non-cardiomyocyte populations generated during differentiation: bioengineers dosed mixed cultures with lactate to increase cardiomyocyte purity in culture [67]. Similar **metabolic engineering** approaches may prove advantageous for preferentially expanding T cell subsets. Activation of mammalian Target of Rapamycin (mTOR), a regulator of cellular metabolism [68], can

influence T cell differentiation fates by altering responses to metabolite changes [69]. Positive and negative mTOR signaling modulators could be used to control *ex vivo* expansion. Levels of amino acids, including tryptophan, arginine, and glutamine, have been indicated in T cell proliferation; hence, amino acid titration is another tool that could be utilized to improve T cell proliferation [70]–[73]. Fatty acid titration could also be employed, as fatty acids have been implicated in CD8+ T cell proliferation, survival and activation [74]. Metabolites can be assayed using many techniques, including fluorescence-based methods to monitor intracellular metabolism in real time [75], and microfluidics [76].

5 CAR Gene Transfer and Editing

CAR T cell manufacturing for clinical trials currently uses *viral vectors* (mostly lentiviruses and retroviruses) to transfer the CAR transgene [14], [17], all of which have high transduction efficiencies (approximately 68% for retroviruses, depending on the multiplicity of infection) [77]. However, viral approaches have several major drawbacks, both in terms of patient safety and manufacturing practicality (**Table 1**). Since viral vectors insert transgenes randomly into the genome, there is a risk of gene silencing or insertional oncogenesis [78]. Additionally, heterogeneous copy numbers may result in T cell populations with highly variable cytotoxic abilities due to altered levels of surface expression [79]. There are additional manufacturing issues associated with viral vectors, which are expensive to produce and require costly QC [80]. While the scale of viral manufacturing has been adequate for phase I/II clinical trials, this will be a significant barrier to entry for centers that wish to implement CAR T cell therapy for larger patient populations [81].

Recent advances in **non-viral transfection techniques** have shown promise in ameliorating some of the issues associated with viral vectors. One approach utilizes transposons, including the Sleeping Beauty [82] and Piggybac transposon systems [83]. Both transposons have

been used to successfully generate CAR T cells [43], [84]–[86]. However, they utilize random transgene insertion, which carries risks for clinical safety and efficacy. Additionally, transposons by nature allow the transferred gene to repeatedly change genomic location [87], which further complicates QC efforts. To address these concerns, many researchers are turning to genome editing methods that allow for site-directed mutagenesis to improve CAR T cell manufacture.

Site-specific editing tools appeared in the early 2000s with the development of zinc finger nucleases (ZFNs) [88] and transcription activator-like effector nucleases (TALENs) [89]. ZFNs and TALENs are chimeric, customizable restriction enzymes that are engineered to target specific loci in the genome, including validated safe-harbor loci [90]. The cost to manufacture ZFNs and TALENs is significant, as individual proteins must be designed for each editing locus [91], [92]. ZFN technology has yet to advance to clinical trials for CAR T cell therapy, although it has been used for other clinical targets, including Hemophilia B and HIV [93],[94]. TALENs have been used preclinically to successfully treat two infant patients ahead of planned phase I clinical trials [95]. In these cases, TALENs were used to knock out the endogenous TCR in allogeneic T cells, although the CAR itself was delivered virally. This technology is actively being developed by Cellectis for their UCART19 product, which is scheduled to begin clinical trials this year [95].

In recent years, the development of CRISPR/Cas9 technology has revolutionized genome editing in laboratory settings [96]. CRISPR/Cas9 involves the use of a nuclease coupled to a short guide RNA, which can be designed to target nearly any locus in the genome [97], [98]. The nuclease can be delivered in the form of a ribonucleoprotein (RNP), or as a plasmid that is expressed by the target T cell [96]. A donor template, typically in the form of a plasmid, is then used to incorporate the desired transgene via homology-directed repair (HDR) [97]. CRISPR is currently an efficient and flexible genome-editing technology, and a recent preclinical study [79] has demonstrated its use to produce CAR T cells with a high degree of homogeneity and superior survival outcomes in a murine model. Specifically, this study inserted the CAR at the endogenous T cell receptor alpha constant

(*TRAC*) locus, which improved CAR T cell cytotoxicity. This finding suggests that strategic and precise CAR integration may be important for developing reliable and effective therapies.

While all three non-viral gene modification tools for directed mutagenesis can achieve targeted edits, editing efficiencies for CAR knockin remain low, with successful editing rates up to 20% [99]. As this is a limiting factor in the overall efficacy of CAR T cell therapies, bioengineering strategies to improve gene transfer are in high demand. New **nanomaterials** based on biotin-streptavidin conjugation have been used to deliver and link donor templates to Cas9 in human cells, improving rates of gene transfer by 5-fold relative to conventional methods [100]. Other labs have directly modified Cas9 protein to achieve high-fidelity edits without off-target effects, as well as recognize a wider range of potential editing sites, thus improving both the safety and versatility of the system [101]. Additionally, new **high-content analysis platforms** have been developed to nondestructively measure editing efficiencies *in vitro*, which can be used to assay new methods and materials for genome editing [102], [103].

6 Quality Control and Assurance

The complete CAR T cell therapeutic process requires extensive equipment and technical expertise to manufacture cellular products of high quality in a relatively short period of time [14]. Facilities must be capable of handling clinical-grade vectors, conducting gene transfection, and performing their own QC before reinfusing cells to the patient. Additionally, they require the infrastructure to care for CAR T cell recipients both prior to infusion, when they have active disease, and post-infusion, upon which they may experience severe side effects. Few places can currently offer all of these components; as such, the current CAR T cell manufacturing approach is moving towards a centralized format, in which academic clinical centers ship patient's cells to a facility for genome editing and expansion under ISO5 GMP conditions [52]. This centralized model has led to the

development of rapidly expanding CAR T cell companies including Juno Therapeutics, Kite Pharma, Novartis, Cellectis, Bluebird Bio, Bellicum, and others [104].

Bioengineers can assist in quality control and assurance for CAR T cell products through the use of **process analytical techniques** (PAT) and **model predictive control** (MPC). MPC is a tool in which workflows are managed through mathematical predictions of outcomes based on the current measured state of the process, enabling significant gains in efficiency and automation [105] (**Fig. 3**). However, these techniques are rarely used for mammalian cell culture-based processes [106], primarily due to a lack of monitoring tools [107]. Studies on the metabolic requirements of T cell subsets could yield useful monitoring targets, as advanced process control techniques for mammalian cell culture rely on metabolic flux analysis [108], [109]. PAT for T cell culture could include immune biosensors [110] and spectroscopic techniques [111]. Soft sensors could be used to integrate measurements of secreted cytokines and metabolite concentrations with software modeling to estimate other components [112]. In CAR T cell expansion, multiphoton redox-based imaging could be used to measure intracellular respiration [75] in combination with biosensors to detect secreted cytokines [113], thus potentially identifying T cell phenotype distributions *in situ*. As with biopharmaceuticals [114], it is expected that regulatory agencies will request quality-by-design-based improvements in cell manufacturing, PAT, and automation to be integrated into current CAR T cell production paradigms.

7 Outlook

Despite the various challenges outlined, CAR T cell therapy remains poised to revolutionize cancer treatment. As research progresses, there is significant space for bioengineers to improve safety, efficacy, and access to such therapies for patients with diverse malignancies (**Fig. 2B**).

7.1 Safety

The predominant safety concerns for therapies currently in the clinic are cytokine release syndrome, neurotoxicities, and off-target CAR T cell activity, all of which have resulted in severe adverse events, and in some cases, patient deaths [115]. Efforts to mitigate these issues are of utmost importance. One approach has been to employ small molecule modulation of CAR T cells *in vivo*. For instance, apoptotic switches have been engineered into CAR T cells that allow them to be quickly destroyed if a patient experiences an adverse event [116] (completed clinical trial NCT02107963). Others have explored the use of transient mRNA-mediated CAR expression, in contrast to conventional workflows in which the CAR is genomically integrated (NCT01355965). While this approach may require multiple infusions of mRNA to sustain a therapeutic effect, transient expression may help protect against off-target activity. Alternatively, some studies have focused on tuning functionality of the CAR itself. For instance, CARs have been designed with split signaling and recognition domains, which can be linked to form a single functionally active CAR following drug administration. This small molecule serves as an "ON-switch" for CAR activity, thus allowing it to be controlled or inactivated as necessary [117]. CAR affinities can also be manipulated to preferentially bind cancer cells over healthy tissue, thus preventing off-target effects and diversifying the range of antigens that can be safely targeted [118]. These designs exemplify the growing role for **synthetic biology** in allowing precise control over CAR T cells after infusion to safeguard the patient.

Bioengineers are also actively developing **tissue engineered *in vitro* toxicity models**, which may prove useful in the CAR T cell space. For instance, human embryonic stem cells have been used to generate brain organoids as a screening platform for chemical toxicity [119]. These models could be adapted using iPSCs to study neural toxicities on a patient-by-patient basis, thus providing personalized safety checks and quality control. Ultimately, bioengineers may combine *in vitro* modeling tools with *in vivo* synthetic biology approaches to both predict and rapidly reverse adverse events, thereby improving the safety of CAR T cell therapy.

7.2 Efficacy

In addition to increasing the overall safety of CAR T cell therapies, bioengineering solutions may also improve their efficacy. For instance, **multiplexed gene edits** may be combined with CAR transgene insertion to boost CAR T cell performance. CRISPR-Cas9 technologies have been demonstrated to improve the performance of CAR T cells primarily through the knockout of PD-1 to limit *in vivo* exhaustion, and are now in clinical trials [120] (NCT02793856). Cellectis has developed CD52/DCK knockout strategies to generate T cells that are chemo-resistant to lymphodepletion agents, thus allowing such drugs to be deployed as combinatorial therapies [121]. Gene editing tools can also enable nuanced recognition of tumor antigens by implementing Boolean logic gates on CAR T cells using **synthetic biology** approaches [122]. Multiplexed CAR designs implementing AND [123], NOT [124], and OR [125] gates have been demonstrated. The synthetic biology approach to CAR T cell design has also led to tuning CAR affinity to discriminate between healthy and cancerous tissue [118].

Recent modifications to the CRISPR system have been used to tune genomic transcription *in vivo* for directed reprogramming [126]. This approach could potentially be used to tune expression of genes involved in T cell activation or exhaustion, or even to control CAR T cell differentiation post-infusion. Catalytically dead Cas9 proteins, which lack the ability to induce double strand breaks, have been coupled with **transcriptional modulators** to selectively activate gene expression in various tissue types [127]–[129], enabling tunable implementation of biological circuits.

Finally, *in vitro* **organ/disease-on-a-chip** approaches are actively being developed to probe CAR T cell functionality, with the aim of assessing heterogeneity in T cell populations and selecting for therapeutically effective cells [130]. Like the aforementioned neural organoid models for safety testing, these could be used to recapitulate the patient's cancer microenvironment, thus

informing a personalized treatment approach. Ultimately, the field is moving towards building smarter and more efficient CAR T cells, and new modeling technologies may go a long way toward improving the therapy's reliability [51], [122], [131].

7.3 Accessibility

As a final consideration, there is a pressing need to increase accessibility to CAR T cell therapies. Current estimates suggest that autologous therapy may cost over $500,000 per patient; thus, new cell sources are highly desirable. While allogeneic therapies have been limited in scope due to the risk of immune rejection, new engineering approaches may allow for the production of non-immunogenic T cells. Work to date has focused on knocking out HLA and the endogenous TCR locus to **eliminate alloreactivity**, thus creating potent "universal" CAR T cells, which could potentially be produced en masse for large patient populations [132]. Human iPSCs could also be used as a cell source to generate large quantities of T cells for patients for whom sufficient T cells cannot be acquired. Furthermore, elimination of viral vectors through the use of novel **non-viral transfection techniques** could increase accessibility by simplifying manufacturing workflows.

Ultimately, *ex vivo* culture may become irrelevant with the advent of *in situ* transgenesis, which could eliminate significant costs and be easily scaled as an off-the-shelf therapy [133]. In one recent study, **nanomaterials** were used to perform CAR gene transfer *in situ* to create CAR nanocarriers. These were directly injected into a murine model, resulting in successful regression of leukemia with no obvious toxicity. While still quite recent, this technique has the potential to produce off-the-shelf gene editing products that eliminate the need for *ex vivo* culture altogether.

8 Conclusions

In summary, we anticipate that advances in biomaterials, genome engineering, tissue engineering, metabolic engineering, process control, and synthetic biology will lead to the next generation of

CAR T cell therapies, which could be manufactured readily and implemented at a wide array of medical centers. As the field progresses, it is hoped that CAR T cells may prove to be a safe and viable treatment for patients with diverse malignancies, and perhaps finally offer cures for conditions that were once a death sentence.

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Conflict of Interest

The authors declare no commercial or financial conflicts of interest.

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Table 1. Comparison of Gene Delivery Approaches for CAR T Cell Manufacture. Plus signs indicate positive characteristics associated with each approach while minus signs indicate negative characteristics.

	Viruses			Transposons		Targeted Nucleases		
	Lenti	Retro	Adeno	Sleeping Beauty	Piggybac	ZFN	TALEN	CRISPR-Cas9
Site-Directed Integration						+++	+++	++
Transfection Efficiency	+++	+	+	++	++	+	+	+
Prevalence in Clinical Trials	+++	+	+	+			+	+
Used for Gene Knockout	+		+			+++	+	+
Insertional Oncogenesis Risk	- - -	- -	- -	- -	- -	-	-	-
Manufacture Costs	- - -	- -	- -	- -	-	-	-	
Random Transgene Integration	- - -	- -	- -	- -	- -	-	-	-

Figure 1. Schematic of a Chimeric Antigen Receptor (CAR). CARs feature a single chain variable fragment (scFv) specific to a particular antigen, a transmembrane domain, and intracellular signaling domains. The example shown is a third generation CAR containing OX40, CD28, and CD3ζ intracellular signaling domains. $V_{H,}$ variable heavy; $V_{L,}$ variable light; IgG Fc, immunoglobulin G crystallizable fraction.

Figure 2. Challenges and potential bioengineering solutions during CAR T manufacturing. A) An autologous CAR T cell Manufacturing Process. Autologous cell therapy involves cell harvesting

via apheresis, followed by T cell activation, CAR gene transfer, T cell expansion, and Quality Control and Assurance (QC/QA), upon which CAR T cells are infused into the patient. Each of these steps has multiple extant challenges that affect the safety, efficacy, and scale of CAR T cell production. **B)** Bioengineering approaches to improve CAR T cell manufacturing. PAT, process analytical techniques; MPC, model predictive control; aAPC, artificial antigen-presenting cell.

Figure 3. Process Analytical Techniques (PAT) and Model Predictive Control (MPC) implementation for CAR T cell populations during manufacturing. Culture medium from the bioreactor is sampled using in-line spectroscopy to determine amino acid composition and metabolite concentrations. Cells from the bioreactor are analyzed using fluorescent techniques to determine their respiratory characteristics. These outputs are combined using modeling to estimate the cellular composition within the bioreactor and modulate medium composition *in situ* to optimize cell yields.

Figure 1

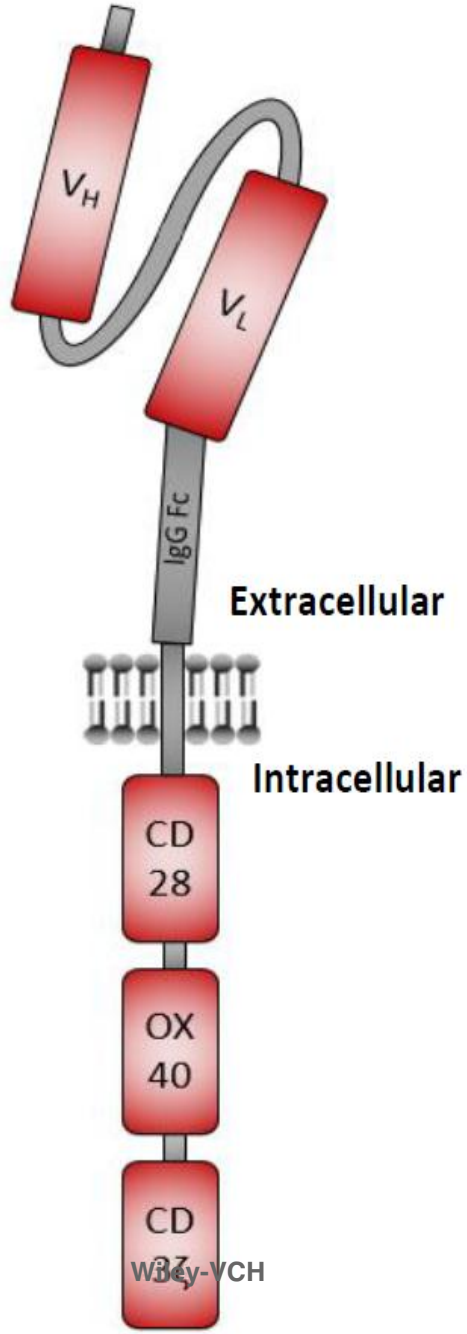
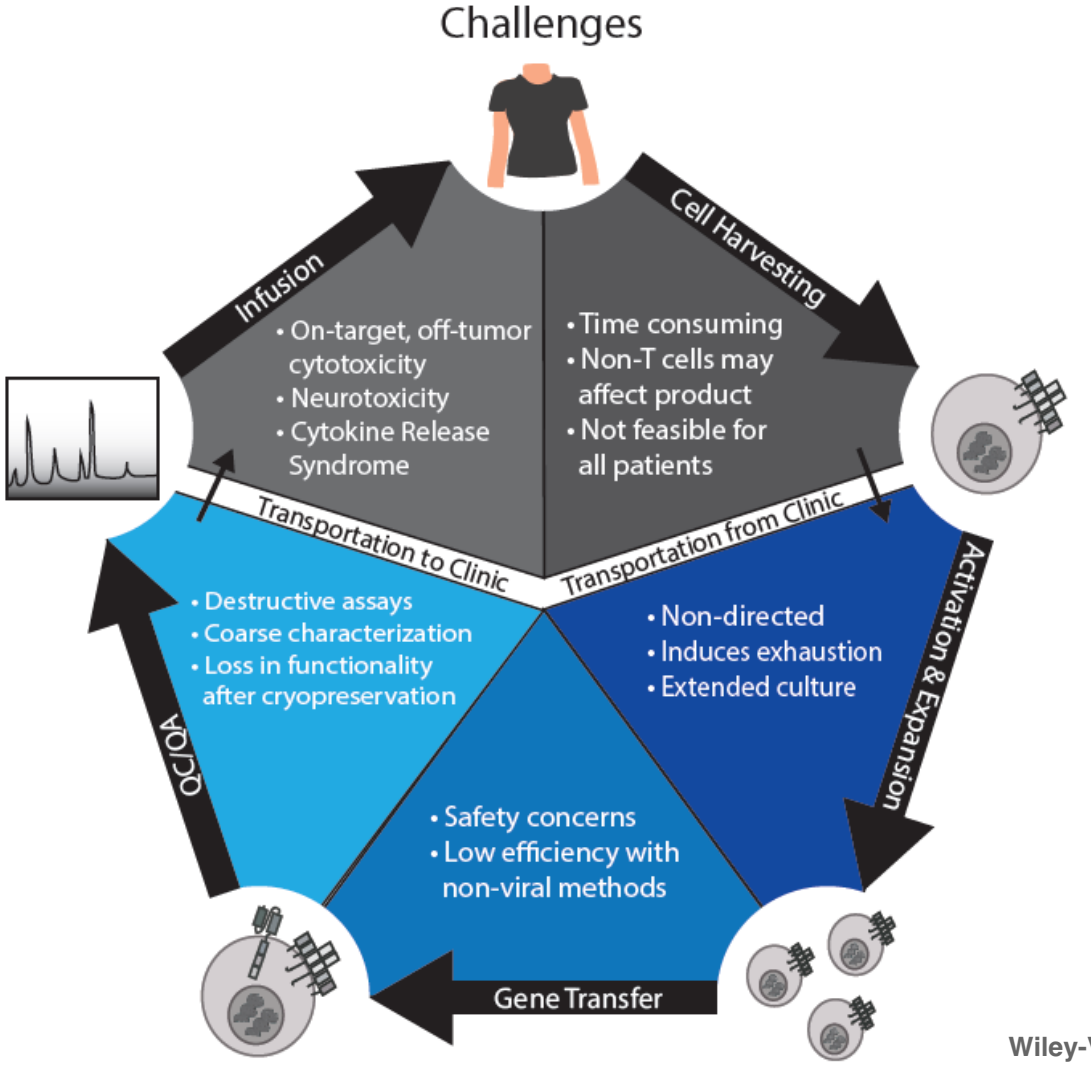


Figure 2A



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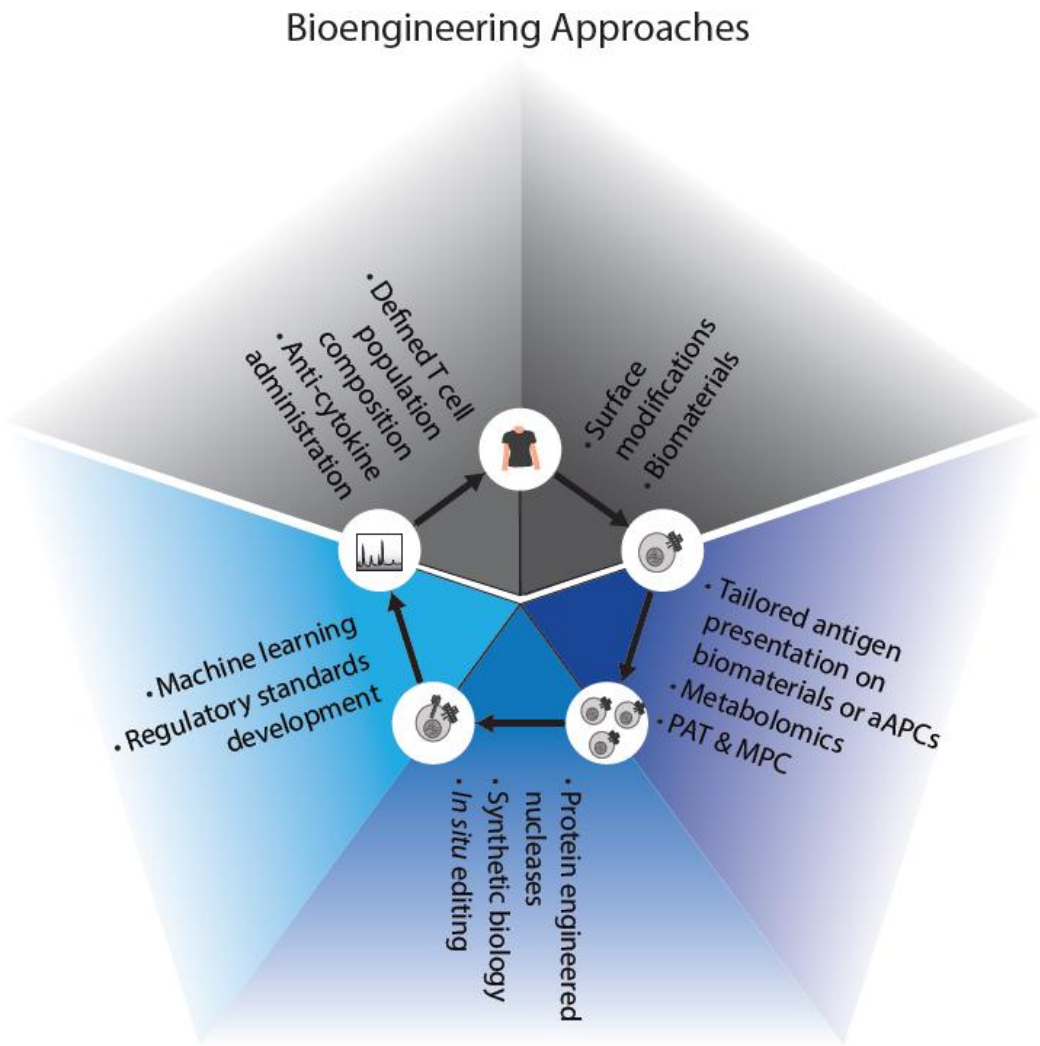
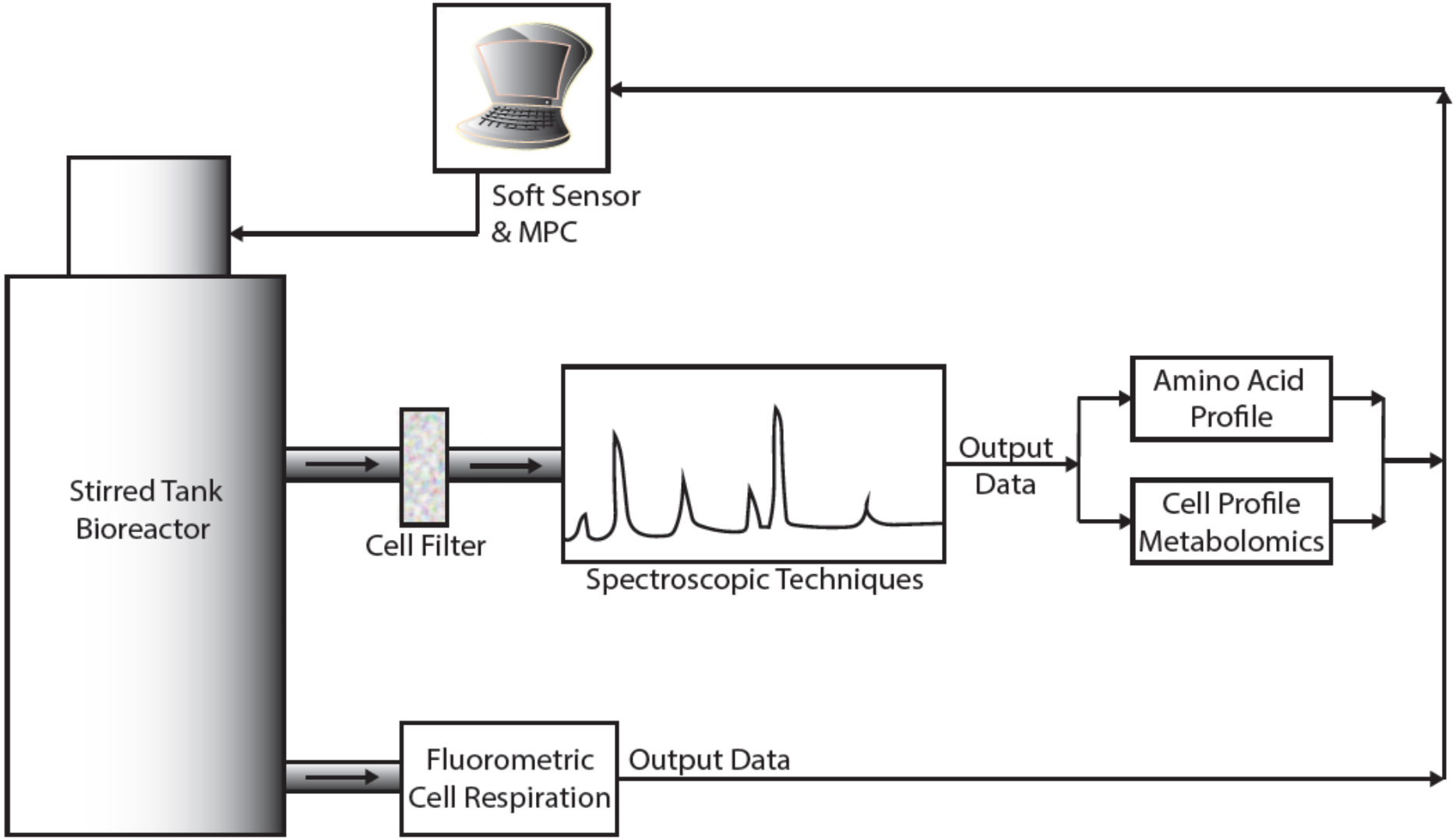


Figure 3



Short CV and Headshot

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Krishanu Saha is an Assistant Professor in the Department of Biomedical Engineering at the University of Wisconsin-Madison. He is also a member of the Wisconsin Institute for Discovery, Carbone Cancer Center, and Stem Cell and Regenerative Medicine Center. Prior to his arrival in Madison, Dr. Saha studied Chemical Engineering at Cornell University and at the University of California in Berkeley. He was a Society in Science: Branco-Weiss fellow at the Whitehead Institute for Biomedical Research at MIT and in the Science and Technology Studies program at Harvard University. Major thrusts of his lab involve gene editing and cell engineering of human cells found in the retina, central nervous system and blood.

Christian Capitini, MD

Bio:
Christian Capitini graduated with an MD with Distinction in Research at the University of Rochester School of Medicine and Dentistry in 2002. He then completed a residency in Pediatrics at the University of Minnesota in 2005. Dr. Capitini then completed a fellowship in Pediatric Hematology/Oncology through the joint program of Johns Hopkins University/National Cancer Institute in 2008. Dr. Capitini joined the faculty of the University of Wisconsin-Madison as an Assistant Professor in 2011. His research focuses on developing cell-based therapies, including NK cells and CAR T cells, for treating pediatric cancer and for complications associated with bone marrow transplant.