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Engineering challenges and opportunities in autologous cellular cancer immunotherapy

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Abstract

The use of a patient's own immune or tumor cells, manipulated ex vivo, enables antigen- or patient-specific immunotherapy. Despite some clinical successes, there remain significant barriers to efficacy, broad patient population applicability, and safety. Immunotherapies that target specific tumor antigens, such as CAR T cells and some dendritic cell (DC) vaccines, can mount robust immune responses against immunodominant antigens, but evolving tumor heterogeneity and antigenic downregulation can drive resistance. In contrast, whole tumor cell vaccines and tumor lysate-loaded DC vaccines target the patient's unique tumor antigenic repertoire without prior neoantigen selection; however, efficacy can be weak when lower affinity clones dominate the T cell pool. CAR T and tumor-infiltrating lymphocyte (TIL) therapies additionally face challenges related to genetic modification, T cell exhaustion, and immunotoxicity. Here, we highlight some engineering approaches and opportunities to these challenges among four classes of autologous cell therapies.

Introduction

During tumor initiation and progression, mutated neoantigens can activate anti-tumor immune responses, but successful tumors evolve to escape immunity through numerous ways, including evasion (e.g., by downregulating immunogenic antigens) and suppression (e.g., by upregulating coinhibitory ligands like PD-L1, secreting suppressive factors, and recruiting suppressive immune cell subsets to the tumor microenvironment (TME) that can cause T cell inactivation or exhaustion) (1). Because different mechanisms may dominate resistance in different cancers, numerous immunotherapies have been developed over the past few decades. As an antibody drug, checkpoint inhibition is the most widely used, but as

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this lacks antigen specificity and boosts effector T cell function broadly, it carries significant risks.

Autologous cell therapies activate only tumor antigen-specific T cells, and thus have the potential for stronger tumor-targeted responses while posing less risk for autoimmune complications. Many are FDA-approved, and they fall into four general classes. Vaccines from irradiated whole tumor cells (WTCs) or lysed tumor cells provide all tumor-associated antigens (TAAs) in an inflammatory context, usually along with adjuvants or cytokines, to stimulate dendritic cells (DCs) in vivo to uptake antigen and activate tumor-specific T cells. DC vaccines take this first step ex vivo, pulsing isolated DCs with tumor lysate or specific tumor peptides with adjuvant to ensure potent activation of sufficient numbers of DCs before reintroduction, where they prime T cells in vivo. Autologous T cell therapies, which include chimeric antigen receptor (CAR) T cells and tumor infiltrating lymphocyte (TIL) therapy, bypass both steps by expanding cytotoxic T cells in vitro. While CAR T cells target a defined tumor antigen, TIL therapy expands all T cells from the tumor and targets the undefined pool of TAAs.

Defining promising target antigens – which require HLA restriction, expression by a substantial fraction of tumor cells, and sufficient immunogenicity – is a major challenge for defined antigen cell therapy, such as CAR T and some DC vaccinations. When immunodominant antigens can be identified, these therapies offer promising tumor-specific therapies with little off-tumor toxicity; however, these can lead to antigen loss-driven immune escape. In contrast, cell therapies that exploit the repertoire of each tumor's antigen pool, such as WTC vaccines or TIL therapy, can drive broader and more diverse T cell responses. However, not all antigens are created equal; dominant antigens exhibit stronger T cell receptor (TCR) signaling to antigen presenting cells generating CD8 T cell responders to immune checkpoint blockade, while subdominant antigens can result in a dysfunctional CD8 T cell subset inhibiting response to immune checkpoint blockade (2). Therefore, WTC vaccines and TIL therapy risk weaker overall efficacy compared to those targeting pre-defined antigens due to dilution by the expansion of lower affinity T cell clones.

As our understanding of tumor immunology continues to evolve, new challenges continue to be revealed in harnessing its full potential to cure cancer. In this review, we will focus on engineering challenges and opportunities facing these four classes of autologous cell therapies.

CAR T cells

Autologous chimeric antigen receptor (CAR) T cells are genetically modified patient T cells reprogrammed to express a synthetic receptor that targets a tumor antigen, such as CD19 on lymphoma cells, and signals cytotoxic effector functions upon binding. The CAR structure is composed of an extracellular binding domain, hinge region, transmembrane domain, intracellular signaling domain for co-stimulation and activation, and optional "armor" including cytokines, receptors or peptides to enhance functionality (3,4). CAR T cell therapy is FDA approved for the treatment of certain blood cancers such as lymphomas, leukemias, and multiple myelomas with a varying response rate of 40% to 98% (5). Still, CAR T cell

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therapy remains limited in efficacy and broader applicability, especially for solid tumors, due to a variety of challenges including trafficking, persistence, antigen heterogeneity, immunosuppression, toxicity, and facile manufacturing (6–10).

Improving gene editing efficiency

Genetic engineering of autologous T cells has been challenging to achieve at clinical scales with high efficiencies and low off-target effects. Both viral and non-viral delivery strategies have been used to genetically engineer primary human T cells with varying levels of success. In general, viral approaches have yielded higher transduction efficiencies and more stable gene expression, but are limited in cargo size, carry risks of random gene integration, and are costly to manufacture (11). Non-viral gene delivery has shown promise to increase cargo size and reduce costs, but typically yields lower transfection efficiencies (11–14). The most common non-viral gene delivery method is electroporation, but this can alter T cell phenotype and cause toxicity from high concentrations of large double-stranded DNA sequences (15–17). Alternative approaches to mitigate these issues include nanoparticle delivery and microfluidics-based cellular squeezing (17,18).

Apart from the delivery method, T cell gene editing strategies have included the use of transposons like *Sleeping Beauty* (SB), designer nucleases like zinc finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN), and clustered regularly interspaced short palindromic repeats (CRISPR)/ CRISPR-associated protein 9 (Cas9) (19). While the SB transposon is capable of introducing larger transgenes into the cell compared to viral gene delivery methods, ZFN, TALEN, and CRISPR/Cas9 approaches can additionally replace a gene or disrupt gene function (20,21). Unlike ZFN and TALENs, which require protein engineering approaches to design the DNA targeting sequence, CRISPR relies on RNA targeting, resulting in a more facile design approach (22).

The CRISPR/Cas system has advanced CAR T cell genetic engineering capabilities by enabling high-throughput site-specific editing using both viral and non-viral delivery (23). CRISPR/Cas9 contains a guide RNA sequence that targets the DNA sequence of interest and Cas9 to induce a double stranded break (DSB). DNA is then repaired through either homology-directed repair, which uses a DNA template resulting in precise genetic alterations, or non-homologous end joining (NHEJ), which occurs during all cell cycle phases but can result in random insertions or deletions (23). CRISPR/Cas has improved the uniformity of CAR expression and reduced CAR tonic signaling by placing the CAR construct under transcriptional control of the endogenous T-cell receptor α constant locus (24). However, depending on the cargo type and design, delivery strategy, cell phenotype, method of DNA repair, and culture conditions, careful consideration must observed to sufficiently edit T cells with CRISPR/Cas (25). Challenges using CRISPR/Cas for T cell engineering include off-target effects, suboptimal editing efficiency, limited editing at motif specific sites, and large base pair deletions or chromosomal aberrations (26). Multiple efforts are addressing these obstacles, such as reducing DSBs using base or prime editors to mitigate unintended genomic alterations or leveraging NHEJ mediated repair to improve CAR T cell yields (27–29). Additionally, CRISPR/Cas strategies employing co-transfection with CRISPR/Cas9 complexes or single stranded DNA templates have reduced

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electroporation associated toxicity (30,31). While innovative genetic engineering strategies further CAR T cell capabilities, gene editing efficiency and cost-effectiveness must be considered for large-scale manufacturing.

Identifying promising genome perturbations

While the efficiency of T cell gene editing improves, identifying key genetic modifications that translate to enhanced CAR T cell efficacy remains challenging. Designing novel CAR T cells has generally been a low-throughput and laborious process, hindering rapid identification of novel genetic manipulations. Recent approaches using CRISPR-based pooled screens allow for high-throughput discovery of different CAR or T cell products; for example, a pooled library of 40 co-stimulatory domains rapidly identified domains with clinical potential within a single donor (32). To evaluate multiple combinations of CAR T cell signaling domains, a restriction enzyme based cloning method shuffled and assembled domains from two pools to generate 180 unique combinations of CAR T cells (33). Functional screening using paired single cell RNA and CAR sequencing enabled high-throughput validation of domain combinations with superior tumor killing abilities and T cell phenotype. However, this approach is limited in its modular nature as well as pre-identifying genes of interest.

CRISPR pooled screens have also enabled thousands of gene perturbations to identify novel genes that enhance CAR T cell efficacy (34). CRISPR gene activation and interference screens have been optimized in human T cells to uncover novel genes impacting T cell function (35,36). Unfortunately, there are inherent challenges with T cell CRISPR screens including inefficient genetic editing, off-target effects, and minimal activity (25). A deeper understanding of screening methodologies, such as transcriptional phenotype, cytokine secretion, or surface marker expression, that translate to CAR T cell anti-tumor effectiveness will improve clinical translation (37).

Performing simultaneous genome edits

It is becoming increasingly evident that multiple genetic alterations may be necessary to improve CAR T cell functionality. CAR T cells co-expressing multiple genes including IL-7, CCL21, CCL19, and Flt3L have improved intratumoral persistence and dendritic cell infiltration in non- or suboptimal lymphodepleting conditions (38–40). While these approaches expressed multiple proteins in one plasmid, creating gain and loss of function edits in a single construct is challenging. Various strategies have used a combination of SB and ZFN, TALEN, and CRISPR/Cas9 approaches for multiplex CAR T cell engineering (41–43). However, introducing multiple DSBs at one time increases the risk off-target effects, and sequential gene editing can be a time-consuming process. To reduce DSBs, CRISPR/Cas9 splice site disruption via base editors achieved greater than 80% efficiency of multiplex knockout of three DNA targets (28). However, this strategy required optimization of each guide RNA, and potential off-target edits can still occur. Instead of reprogramming individual genes, genetic manipulation of the epigenome can impact expression of gene networks. Epigenetic editing of CAR T cells was shown to affect their differentiation, phenotype, persistence, and metabolism (44). Recent work combined overexpression of a single transcription factor to promote T cell memory with a transcription factor knock-out

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CRISPR screen to identify novel gene regulators of T cell memory in human donors (35). Increasing the throughput of strategies that enable simultaneous genome edits will promote rapid CAR T cell innovation, uncovering novel mechanisms and functionalities.

Reducing pre-infusion product variability

Traditionally, manufacturing CAR T cells includes T cell activation, genetic manipulation, and expansion. Improving CAR T cell manufacturing has involved promoting a memory T cell phenotype, more rapid cell expansion, minimizing production time, increasing automation, and reducing costs (45–48). Significant efforts have decreased production time from around 14 days to a few days (49). Recent work produced functional CAR T cells in one day in non-activated T cells, eliminating the need for T cell activation and expansion (50). While innovative, this research, as well as many other preclinical studies, used T cells derived from healthy donors. Patient-derived T cells can exhibit a more differentiated phenotype than healthy donors with reduced levels of naïve and memory phenotypes (51,52). Additionally, T cells can be difficult to harvest from patients with lymphopenia or patients treated with chemotherapy or radiotherapy (53). Thus, patient T cell variability presents a challenge for manufacturing a consistent CAR T cell product. For example, an optimized manufacturing protocol using IL-7 and IL-15 could rescue dysfunctional T cells in B-cell acute lymphoblastic leukemia patients, but failed to expand early memory T cell phenotypes in patients with pancreatic ductal adenocarcinoma, possibly due to increased average patient age or the solid tumor indication (51). Instead of transducing bulk patient T cells, T cells can be sorted based on phenotype prior to genetic manipulation (54). However, cell sorting can affect T cell functionality, reduce product quantity, and increase manufacturing complexity. New innovations in microfluidic platforms can sort based on secreted factors for function specific separation (55).

Predicting Efficacy and Toxicity

Since patient CAR T cell variability can influence therapeutic efficacy and toxicity (56,57), efforts have been made towards developing computational models that can accurately predict CAR T cell responses from patient pre-infusion products and thus improve quality control and patient safety (58–60). A recent study in CD19 CAR T cell indications used machine learning based models to compare pre-infusion single-cell transcriptomics with patient responses and identify a beneficial transcriptional signature more predictive of clinical response than cellular phenotyping (61). In terms of how CAR T cell transcriptional signatures may evolve over treatment time and disease progression, post-infusion CAR T cell monitoring has enhanced our knowledge of clonal kinetics and revealed phenotypes associated with progressive disease, reduced neurotoxicity, long-term persistence and decreased exhaustion (62–65). However, since most of this work has been performed in hematological malignancies, there is a need for more data on CAR T cell kinetics in other tumor types in order to improve the translatability of predictive modeling to solid tumors (66).

CAR T cell therapy is associated with toxicities such as cytokine release syndrome and neurotoxicity (67). Predicting CAR T cell toxicity involves complex interactions between the pre-infusion product and patient biology. Innovative strategies to mitigate CAR T

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cell toxicity implement suicide switches, logic gates, or spatial localization (68). If a patient experiences adverse events during treatment, CAR T cells can be pharmacologically depleted by engineering suicide switches into the CAR T cells (69,70). Logic gated CAR T cells respond to multiple input signals; for example, NOT gates prohibit CAR T cell activation upon recognition of a healthy tissue antigen by incorporating an inhibitory signaling domain while allowing activation upon neoantigen identification (71). Although these new approaches enhance CAR T cell safety, they are not 100% effective (70,72), and thus more complex logic gates are being engineered to tune CAR T cell tumor specificity (73). Furthermore, identifying robust antigens allowing for T cell activation from cancer cells and T cell inhibition from healthy cells remains challenging. In-depth computational analysis of single-cell data can identify both neoantigens for CAR T cell targeting and potential off-target toxicity in healthy cells (74–76). Opportunities to develop more comprehensive toxicity models include improving access to quality data and furthering our understanding of the immunological complexity of TCR binding promiscuity (77).

Tumor Infiltrating Lymphocytes

Tumor infiltrating lymphocyte (TIL) therapy uses T cells from the patient's own tumor microenvironment, which are assumed to recognize TAAs. This heterogeneous population of T cells, which mostly targets tumor neoantigens, is beneficial for treating solid tumors like melanoma that have few shared clonal antigens (78). In TIL therapy, T cells are first expanded from patient tumor fragments *ex vivo* in a 'pre-rapid expansion protocol' (pre-REP), then activated and expanded further over the course of several weeks with IL-2 or other cytokines in a 'rapid expansion protocol' (REP). Then, TILs with high doses of IL-2 are reinfused back into the patient who has been pre-conditioned with a lymphodepletion regimen. TIL therapy has achieved complete responses in some metastatic melanoma patients, but the response in other cancer types has been limited (79–81).

The first TIL therapy trial against metastatic melanoma in 1988 showed an improved patient response compared to IL-2 administration alone (82,83). These original TILs were expanded from patient biopsies using high doses of IL-2 over the course of six to eight weeks, with no additional ex vivo genetic modification. Although these first TIL trials showed some efficacy, the lengthy in vitro expansion period resulted in pre-infusion T cell exhaustion. Additionally, during manufacturing time, patients' condition worsened to the point that some became ineligible for therapy (84). Since the early trials, there have been improvements in manufacturing and administration of TILs that have made them more accessible to patients. In contrast to CAR T cell therapy, currently there are no FDA-approved TIL therapies, although many are currently seeking approval (85).

Reducing complexity in manufacturing

TIL manufacturing involves two major steps: (i) the resection of a biopsy that must be cut into small pieces and expanded with IL-2 during the pre-REP phase, and (ii) the activation and further expansion of these cells with anti-CD3 antibodies, IL-2, and feeder cells (typically irradiated PBMCs) during REP phase (86). This labor-intensive and complex manufacturing process requires specialized staff and facilities equipped to produce good

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manufacturing practice (GMP) TILs (87). Several approaches have attempted to reduce this complexity. For example, a simplified REP protocol was developed that uses a closed-system bioreactor to send “waves” into the media for improved oxygenation and allow a significant workload reduction during the manufacturing process (88). Another technology, called G-Rex flasks developed by the company Wilson Wolf, allows for large expansion of suspension cells in a closed system without need to expand into larger flasks or change media, due to the silicone gas-permeable membrane at the base of the flask (89). A semi-automatic and GMP-compliant process using G-Rex flasks was recently approved by the Swiss regulatory agency, Swissmedic (87).

In addition to manufacturing complexity, the length of time from biopsy to infusion is a major challenge with TIL therapy. Although the manufacturing time has been significantly reduced since the first trials, long expansion time is necessary because sufficient numbers of TILs remains a limiting factor for their clinical efficacy. During the 6-8 week manufacturing time during the first TIL trials, patients’ conditions worsened, resulting in a 50% patient dropout rate (84); this process has now been reduced to 2-3 weeks. Later work showed that “young TILs,” which were expanded for less time and had no prior $CD8^+$ T cell selection, maintained less exhausted phenotypes and led to similar therapeutic efficacy in terms of patient survival compared to the original, longer TIL protocol (90,91). A US-based company is clinically testing a TIL manufacturing process using G-Rex flasks in their latest design that decreases the time to infusion from 22 to 16 days (92).

While TIL therapy has worked well in metastatic melanoma, it has had limited success in other tumor types. To expand TIL therapy to unresectable tumors, multiple groups have developed protocols to isolate and expand TIL-like tumor-reactive T cells from the patient’s blood. Co-cultures of patient peripheral blood lymphocytes with either autologous tumor organoids or tumor neoantigen peptide pools can enrich tumor-reactive T cells (93,94) identified by surface markers such as PD1, CD39, and CD103 (95,96). Furthermore, a novel microfluidics approach has recently been developed as an alternative to expanding TILs from tumor fragments during pre-REP; instead, $CD8^+CD103^+$ tumor-reactive lymphocytes are isolated from circulating blood and then expanded through a REP-like process (96). T cells isolated this way showed similar tumor reactivity in murine models compared to TILs isolated in the traditional method (96).

Improving TIL phenotype through genetic engineering

While standard methods of TIL production can expand large quantities of T cells, the desired phenotype and heterogeneity is not always maintained. Therefore, TILs can be genetically engineered to improve survival of multiple clones, prevent exhaustion, induce memory, and increase tumor cell killing. As a first proof of concept, TILs were transduced retrovirally with a neomycin-resistance gene to track their persistence and trafficking in vivo (97). These gene-modified cells could be recovered from patients weeks to months later, demonstrating that stable transduction of TILs over this time period is achievable (97). Many of the same innovative genetic modification methods used for CAR-T cell engineering can be applied to TILs.

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One major challenge with TIL therapy is limiting T cell exhaustion, which results in loss of effector function and tumor recurrence. In an effort to decrease T cell exhaustion, melanoma TILs were genetically edited using ZFN and CRISPR/Cas9 at the PD-1 locus (98–100). These engineered TILs showed improved efficacy in mouse xenograft models, but as they have not yet undergone clinical trials, the safety and efficacy of these PD-1-edited TILs in patients remain unknown. Interestingly, a clinical trial using similar CAR T cell with editing at the PD-1 locus did not meet the pre-set median survival criteria (101). As an alternative to genetically preventing or reversing exhaustion, skewing TILs toward a memory phenotype may also increase the long-term efficacy of TIL therapy.

Stem-like T cells, characterized by their ability to self-renew and generate all effector and memory T cell subsets, are associated with better clinical efficacy in adoptive cell transfer (102,103). Pre-clinical efforts suggest modulating cell metabolism and signaling during expansion may skew T cell phenotype toward more stem-like states (103). Additionally, cytokines such as IL-7, IL-15, and IL-21, which are associated with T cell memory development, may be incorporated during REP expansion (104,105). TILs have been engineered to express membrane-bound IL-15 on their cell surface to integrate this memory-related cytokine in both in vitro and in vivo expansion (106,107).

TIL heterogeneity is an important indicator of patient survival across several immunotherapies in order to target multiple TAAs in heterogeneous solid tumors (108,109). Although T cell clones in the biopsy sample may be highly diverse in both phenotype and function, this TCR repertoire can change drastically during the traditional in vitro expansion (110,111). Unfortunately, markers related to antigen experience, indicating more differentiated T cells, are correlated with poor proliferation of a clone in vitro, due to increased sensitivity to cell stress during expansion (103,111). Improved REP protocols incorporating agonistic antibodies against CD3 and 4-1BB in addition to IL-2 are better able to maintain the original tumor clonal T cell diversity compared to the traditional TIL REP expansion (112).

To reduce cytotoxicity, TILs have also been transduced to secrete cytokines such as IL-2, TNFa, and IL-12 (113–118) . However, because these potent cytokines can be associated with increased toxicity, regulation of these genes may be necessary. One group engineered a TIL product to transiently express IL-12 using mRNA electroporation (118). TILs have also been engineered to express chemokine receptors to improve their trafficking to the tumor (79,119).

Decreasing lymphodepletion and IL-2 related toxicity

Lymphodepletion prior to ACT improves proliferation of transferred T cells in vivo, increasing efficacy of the treatment (120,121). Multiple mechanisms have been defined for improved efficacy with ACT after lymphodepletion, including: removal of immunosuppressive Tregs, increased availability of homeostatic cytokines IL-7 and IL-15 due to elimination of “cellular sinks,” and damage to the gut epithelium that leads to activation of an inflammatory immune response (engagement of APCs and their toll-like receptors) (122). Commonly, this lymphodepletion regimen will be non-myeloablative cyclophosphamide and fludarabine, which leaves the patient lymphopenic and neutropenic

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for approximately 7 days (123). Despite the increased efficacy, lymphodepletion leads to toxicity risks such as risk of neutropenic sepsis, anemia, and coagulopathy (123). Potential engineering solutions that engage the innate immune system, directly deplete Tregs, or increase cytokine availability are needed to reduce this toxicity.

In addition to lymphodepletion, another toxicity risk associated with TIL therapy is the high dose of IL-2 administered in vivo. Although cytokine release syndrome-related events are not common with TIL therapy, high dose IL-2 requires strict patient criteria for trial admittance to prevent toxicity. Alternative cytokine approaches have been engineered to remove IL-2 both during REP and in vivo. TILs transduced to express membrane-bound IL-15 under the control of ligand acetazolamide can be expanded through REP without IL-2 and show similar tumor reactivity to TILs expanded traditionally with IL-2 (124).

Although there are many advantages of heterogeneous targets, there is also a concern about toxicity if the target is undefined. In TIL therapy for melanoma, on-target, off-tumor effects have been reported. T cells in a melanoma biopsy can potentially target antigens present on normal epithelial cells. Clinicians have reported cases of vitiligo, loss of skin pigmentation, and hearing loss and imbalance issues (125,126). These on-target, off-tumor effects are a challenge for progressing TIL therapy because they cannot be predicted where the target antigens are unknown. A potential engineering solution would be to transduce TILs with a kill switch to stop their action in the case of toxicity.

Dendritic Cell Vaccines

DCs are the major activators of both naïve and memory T cells, but a variety of suppressive features in the TME can hinder their uptake of tumor associated antigens (TAAs), migration to the LNs, maturation, and expression of coreceptors and cytokines necessary to stimulate a potent antitumor T cell response (127). However, ex vivo activation of patient-derived DCs can bypass these issues, allowing control of activation strength as well as antigen specification. The DCs can be pulsed with either a tumor-specific peptide or protein or with whole tumor lysate. When such TAA-pulsed DCs are re-injected as a vaccine, they can readily migrate to the lymph nodes and both activate naive T cells and boost memory T cell response (128). Therefore, in contrast to traditional vaccines, DC vaccines are less sensitive to the suppressive features of the tumor microenvironment and not dependent on antigen uptake.

In the first DC vaccine demonstration, patient-derived DCs were pulsed with a synthetic MAGE-1 peptide for treatment of melanoma (129). These DCs induced peptide-reactive T cells that were found in both the primary tumor as well as in distant metastases, and further studies demonstrated tumor regression after DC vaccination (129,130). Since 1995, more than 300 clinical trials using DC vaccines have been performed (131,132). The first DC vaccine to be FDA approved was sipuleucel-T (Provenge) in 2010, which showed a 4.1-month increase in median survival for prostate cancer compared to placebo (133). DCs have been activated with a wide range of antigen sources: tumor lysate, a synthetic protein, or single or multiple synthetic peptides (129,133,134). While these vaccines are generally safe and well tolerated by patients, their overall efficacy has been disappointing (132,135).

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Here, we highlight some of the engineering challenges facing efforts to improve DC vaccine efficacy.

Improving DC source and phenotype

Because sorting DCs from PBMCs yields low numbers, most clinical DC vaccines have used monocyte-derived DCs (moDC) expanded from the abundant $CD14^+$ peripheral blood monocytes over 5-7 days using a combination of granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 (88,132). Another strategy involves differentiating DCs from bone marrow-derived $CD34^+$ hematopoietic stem cells, which results in a mixed population of APCs, the majority of which are moDCs (132). Although large numbers of moDCs can be differentiated from PBMCs, these ex vivo-derived cells are less migratory and have decreased T cell stimulatory capacity compared to other DC subtypes, and therefore further differentiation into other DC subtypes may be preferred (136–138).

Conventional type 1 DCs (cDC1s) have been identified to have a major role in anti-tumor immunity, with cDC1 gene signature expression in the tumor found to correlate with improved prognosis across multiple cancer types (139). In addition to their ability to cross-present antigen on MHC I to activate $CD8^+$ T cells, cDC1 release of chemokines and cytokines shape the TME in an immune response against the tumor (140). Using a moDC-based vaccine, amplified T cell responses were found to be dependent on cross-presentation by endogenous host cDCs (141). In another study, cDC1-based vaccines were able to cross-present and effectively respond to the tumor independent of host cDC1s (142). Pre-clinical work has shown that cDC1s can be differentiated and used in DC vaccines, and cDC1-based vaccines are currently in clinical trials (143). While inducing cDC1s at a large scale for delivery to patients has been difficult due to longer manufacturing, several engineering solutions exist to improve this differentiation and expansion process. As described in the TIL section, a microfluidics approach may be appropriate for isolating and expanding circulating DCs from peripheral blood (88). For an alternative approach to achieving a desired DC phenotype, monocytes can be transduced with lentiviral vectors to induce a specific DC phenotype. For example, functional moDCs have been produced from monocytes transduced with GM-CSF, IL-4, and the TAA at the same time (144). Another proof of concept study showed that mouse and human embryonic fibroblasts can be reprogrammed to become functional induced cDC1s by lentiviral transduction with PU.1, IRF8, and Batf3 (145).

Other DC subsets, including conventional type 2 DCs and plasmacytoid DCs, have demonstrated anti-tumor capacity, although these subtypes have been less explored than cDC1s. It is possible that a vaccine packaging multiple DC subsets could lead to enhanced anti-tumor response due to the different functions of each DC subset (140).

Identifying antigens and loading DCs

As mentioned, a wide range of antigen sources can be used to for DC vaccines. One issue with manufacturing is determining a robust, immunogenic TAA. While many DC vaccines have targeted shared TAAs, such as MART-1, MAGE-1, and gp100, these antigens are not present on all tumors and can only be used for patients matching the identified peptide's HLA restriction (146). As an alternative to shared TAAs, DC vaccines can be loaded

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with patient-specific neoantigens, but identifying these neoantigens requires next-generation sequencing and bioinformatics approaches (147). Either neoantigen or shared TAAs can traditionally be loaded into DCs for vaccination through co-culture of DCs with synthetic peptide or protein. However, this traditional method relies on DC uptake and processing of the peptide or protein in vitro. To address this problem, DCs have been engineered to uptake TAA using mRNA nanoparticles or retroviral transduction (148,149).

Due to antigen loss resulting in tumor immune escape, targeting a single TAA may be insufficient. Therefore, DCs have been loaded using a variety of strategies that introduce them to multiple or all TAAs, which can induce polyclonal antigen-specific cytotoxic T cells (150,151). Whole cell lysate or killed tumor cells as a source for DC vaccine antigens allow for decreased manufacturing time and increased specificity compared to other alternatives (130,152). Because the whole cell lysate is not inherently immunogenic, methods to increase immunogenicity are used to increase DC uptake of TAA (147,153). Some methods used include repeated freeze-thaw cycles or hypocholorous acid-oxidization of the tumor lysate, which both induce necrosis, ultraviolet B ray-irradiation, which induces apoptosis, or heating of tumor cells, which increases MHC I expression and upregulates heat shock proteins (153,154).

As an alternative strategy that exposes DCs to all TAAs, DC-tumor fusion cells have been developed, using combinations of autologous and allogeneic DCs and tumor cells (155,156). DCs and tumor cells are mixed together at a defined ratio, fused using polyethylene glycol (PEG), and cultured with GM-CSF and IL-4 (155). DC-tumor fusion cells have demonstrated variable success in clinical trials (156,157). Across each of these innovations for loading DCs with TAAs, there have been improvements in ability to present multiple TAAs, but there are still major problems with efficacy of DC vaccines.

Enhancing immunogenicity to improve post-infusion efficacy

While changes to the source and loading of DC vaccines may increase their efficacy, other advances have been made to increase DC trafficking. Functional DCs should be present in both the lymph node and the tumor site for optimal activation of T cells (158). DC vaccines are usually injected intradermally, but direct injection into the tumor-draining lymph node, (intranodal) or into the tumor (intratumoral) have also been explored (153). Although a significantly decreased number of DCs reach the lymph node with intradermal injection, both intradermal and intranodal injection result in similar antigen-specific T cell activation (159,160). While the most effective route of administration is not clear, improving DC trafficking is important for DC vaccine efficacy. To increase DC trafficking to the lymph node, researchers have induced expression of the lymph node homing chemokine receptor CCR7 by viral transduction, micelle delivery, or miRNA-induced epigenetic regulation (161,162). A separate study delivered a DC vaccine intratumorally that was transduced to secrete chemokine CCL21, the ligand for CCR7, which resulted in increased T cell and endogenous DC recruitment to the tumor site (163,164).

Because DC vaccine performance relies on efficient activation of T cells in vivo, the immune system of the cancer patient can influence the immune response. Skewing toward an inflammatory rather than immunosuppressive phenotype is necessary to prevent

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immunosuppression-related inefficacy. One potential solution is combination with antibody therapies to induce immunogenic cell death. A recent phase I/II clinical trial treated breast cancer patients with an autologous DC vaccine targeting two HER2 epitopes (E90 and E75) combined anti-HER2 antibody (134). Combination with antibody therapy was hypothesized to synergize with DC vaccine to skew the DCs toward an inflammatory phenotype because the antibody can engage Fc receptors on DCs and activate immunogenic cell death; however, this trial had only moderate results in extending survival and no complete responses (134). Overall, more work must be done to increase the immune response induced by DC vaccines and the trafficking of DCs and T cells between the tumor and its draining lymph node.

Whole Tumor Cell Vaccines

Excised tumor tissue contains a plethora of tumor-specific antigens that serve as starting material for personalized therapeutic cancer vaccines. Autologous tumor cell-derived vaccines include whole tumor cells, lysate, extracellular vesicles or cellular membrane components that can mount polyclonal anti-tumor immune responses (165–167). While tumor lysate vaccines have less manufacturing challenges, whole tumor cell (WTC) vaccines can be genetically engineered to deliver immune modulating factors such as GM-CSF or vascular endothelial growth factor C (VEGF-C) (168,169). Autologous WTC vaccines are typically sourced from resected patient tumors that are digested, lethally irradiated, and cryopreserved until administration (170). Irradiation prevents tumor growth, induces immunogenic cell death, and releases tumor antigens as tumor cells die (171). Herein, we examine the engineering challenges and opportunities of WTC vaccines to generate anti-tumor immune responses.

Expanding patient eligibility

Currently, autologous WTC vaccines are limited to patients with resectable tumors of an established size (170). Procuring sufficient tumor tissue for WTC vaccine manufacturing varies depending on the tumor type, resection mass, and disease progression. Overall, vaccine production from solid tumors have had relatively high success rates of >80% in eligible patients (168,172,173). Factors that impeded successful manufacturing included insufficient viable tumor cells and contamination (168,174). For hematological malignancies vaccine production can have slightly less favorable outcomes than in solid tumors, potentially, due to the lack of sufficient excisable tumor tissue or circulating tumor cells (173,175). However, despite successful vaccine generation, patient withdrawal, inability to complete the vaccine schedule, and disease progression impede treatment completion (170,173,174). Although manufacturing methods for WTC vaccines can take less than two days, quality control may take up to three weeks (174,176). Rapid sterility testing could reduce time-to-treatment along with improved protocols to reduce contamination; nonetheless, personalized cellular products need specialized manufacturing facilities to ensure quality product (177,178).

Mounting a robust immune response

Following the manufacturing process, patients commonly undergo a series of intradermal or subcutaneous vaccine injections, administered either weekly, bi-weekly, or monthly

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(170,174). Thus, vaccination schedules may span several months to reach completion. And while increasing the vaccine dosage frequency can enhance patient survival, CD8 T cell vaccine responses have been reported to diminish during the vaccination schedule (174). Strategies improving vaccine efficacy include targeting the tumor microenvironment during vaccination, enhancing immune cell infiltration and activation, reducing immunosuppression, and enhancing antigen presentation (166,179).

To enhance immune cell infiltration, tumor cells have been modified to secrete GM-CSF (GVAX) to promote macrophage, granulocyte, and DC infiltration as well as myeloid maturation, DC antigen presentation, and an inflammatory milieu at the vaccine site (178,180). GVAX has been extensively studied in hematological cancers and solid tumors and showed early promise in prostate cancer, however, demonstrating significant clinical response in later phase trials has been challenging (168). Tumor cells have also been modified to secrete Flt3L to enhance CD8 T cell and plasmacytoid DC infiltration or VEGF-C to increase naïve T cell infiltration and lymphatic endothelial cell formation at the vaccine site (169,181). Preclinical studies demonstrated that VEGF-C WTC vaccines upregulated CCL21, resulting in naïve T cell recruitment, and enhanced lymphatic transport of antigens by DCs in the draining lymph node (169).

In addition to promoting the infiltration of antigen presenting cells and T cells, strong activation of immune cells is required for a robust anti-tumor response. WTC vaccines have been combined with a variety of adjuvants as well as engineered to express immune stimulatory proteins, such as CD40L and CD80 (182–184). Enhancing TAA presentation through upregulation of MHCI or MHCII has also been used to increase tumor-specific CD8 and CD4 T cells responses (182,185,186). Alternatively, blocking suppressive mechanisms can also improve T cell activation and function. For example, gene knock-down of a don't-eat-me signal CD47 or expression of anti-PD-1 antibodies in tumor cells, led to increased DC expansion and ratios of effector to regulatory T cells, respectively (187,188).

Coordinating vaccine elements to enable precise spatial and temporal control of immune responses holds the potential to amplify vaccine effectiveness. Near-infrared (NIR) laser irradiation is a useful tool to trigger cancer immunotherapies due to deep tissue penetration and engagement with agents that lead to the generation of heat or reactive oxygen species. Tumor cells loaded with nanoparticles or coated with polymers sensitive to NIR laser irradiation have the potential to control endogenous adjuvant release, improve DC infiltration and maturation, and suppress regulatory T cells (189,190). Although these tumor cells were either completely or partially non-viable, there is opportunity to combine protein secreting tumor cells with NIR laser responsive materials to improve immune infiltration while temporally controlling activation.

Employing stable and consistent genetic engineering

Genetic modification of tumor cells has shown impressive ability to improve immune cell infiltration and activation. However, both viral and non-viral genetic engineering approaches have resulted in variable vaccine products. Retroviral vectors were initially employed in clinical trials for GVAX, however, the application of adenoviral vectors was found to be associated with low toxicity and improved transduction in resting cells without the

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need to establish primary cultures (178). Adenoviral vectors still have challenges with consistent protein production, for example, GM-CSF secretion ranged from less than 1 nanogram to 6 micrograms per million cells in 24 hours in transduced tumor cells (170). Non-viral gene delivery is an attractive approach due to reduced manufacturing labor and costs; however, variable protein expression is still a challenge. Electroporated cells from various tumor tissues programmed to secrete GM-CSF and an anti-sense TGF-B2 nucleotide sequence demonstrated protein expression from 7 picogram to 7 nanograms per million cells, and TGF-B2 knockdown from 9% to 100% (191). This WTC vaccine functions by reducing immunosuppression associated with TGF-B and subsequentially increased IFN-γ secretion in peripheral blood mononuclear cells of patients with solid tumors. While the electroporation protocol was optimized for cellular viability instead of efficiency, adjusting gene delivery methods for each patient can become costly and time-consuming. Variable gene expression represents a challenge to develop a consistent product, especially since high levels GM-CSF secretion is known to be immunosuppressive (192). Codon optimization can improve poorly expressing genes, however, more robust genetic engineering tools are necessary for consistent protein expression across various patient tumors (193).

Conclusions

Regardless of cell type for each therapy, there are innate manufacturing challenges in deriving a personalized therapeutic from patient cells. These challenges include long manufacturing times, complex and specialized expansion processes, stable cell engineering, and quality control. Efficacy challenges include trafficking to the tumor and draining lymph node, priming the therapeutic and the patient's immune system into an inflammatory state to avoid suppression, and avoiding T cell exhaustion. T cell therapies have greater risk for patient toxicity than cell-based vaccines, due to risk of cytokine storm, lymphodepletion, and intensive cytokine regimens. Overall, we have highlighted innovative engineering solutions and opportunities to address these challenges for the improvement in cell therapy for cancer.

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Abbreviations

APC	antigen presenting cell
CAR	chimeric antigen receptor
Cas9	CRISPR-associated protein 9
cDC1	conventional type 1 dendritic cell
CRISPR	clustered regularly interspaced short palindromic repeats
DC	dendritic cell

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DSB	double stranded break
GM-CSF	granulocyte-macrophage colony stimulating factor
GMP	good manufacturing practice
NHEJ	non homologous end joining
NIR	near-infrared
REP	rapid expansion protocol
TAA	tumor associated antigen
TALEN	transcription activator-like effector nucleases
TCR	T cell receptor
TIL	tumor infiltrating lymphocyte
TME	tumor microenvironment
VEGF-C	vascular endothelial growth factor-C
WTC	whole tumor cell
ZFN	zinc finger nuclease

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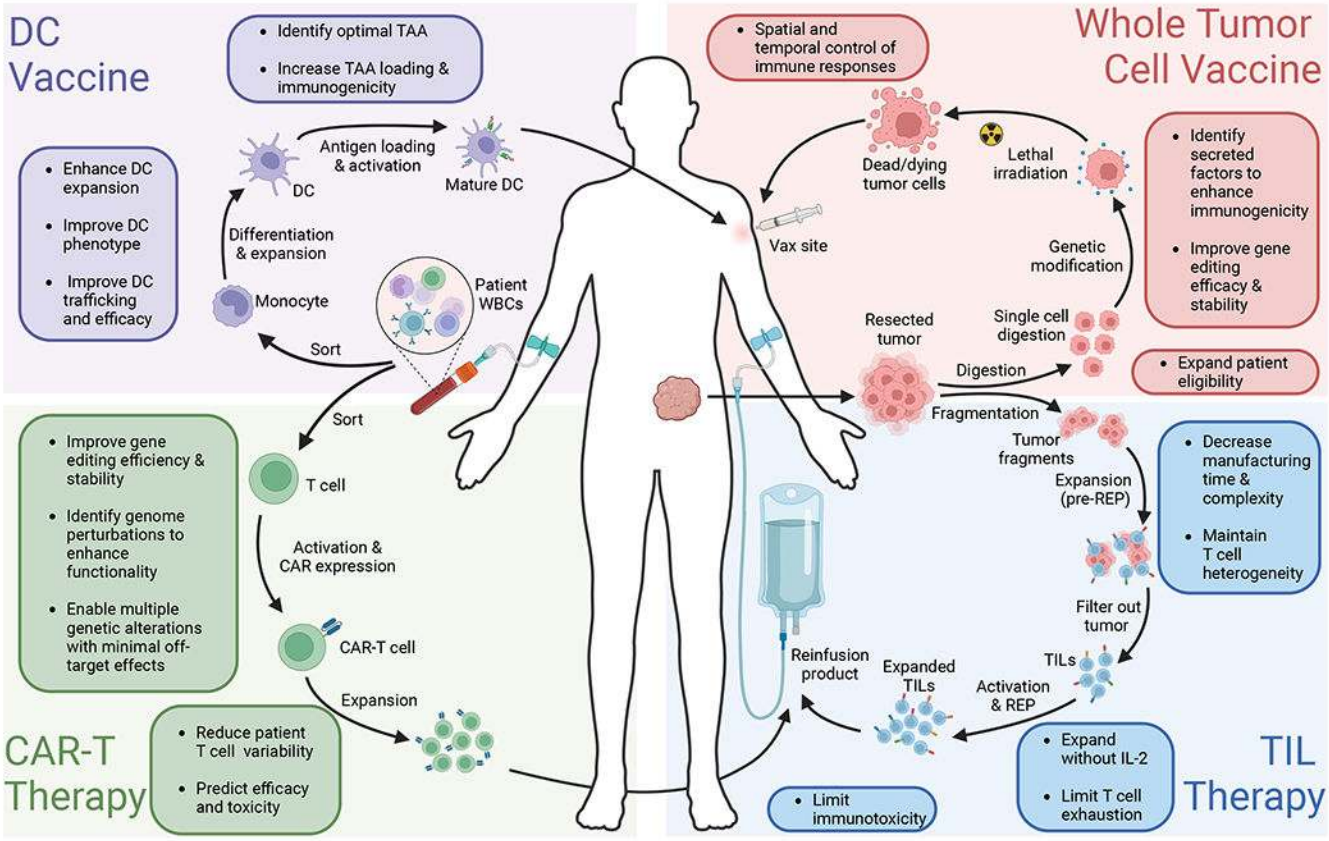


Figure 1.

Engineering challenges in cancer cell therapy. Both DC vaccine and CAR-T cells use cell sources from patient blood. Tumor cell vaccine and TIL therapy use cell sources from tumor biopsy samples. DC vaccine and tumor cell vaccine are typically injected intradermally as a vaccination, and these therapies activate T cells in vitro. CAR-T cells and TIL therapy are activated ex vivo and reinfused into the patient intravenously. Boxes highlight engineering challenges being explored in manufacturing, efficacy, and toxicity of each therapy.