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Applications of Molecular Engineering in T-cell-based Immunotherapies

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Abstract

Harnessing an individual's immune cells to mediate antitumor and antiviral responses is a life-saving option for some patients with otherwise intractable forms of cancer and infectious disease. In particular, T-cell based engineered immune cells are a powerful new class of therapeutics with remarkable efficacy. Clinical experience has helped to define some of the major challenges for reliable, safe, and effective deployment of T-cells against a broad range of diseases. While poised to revolutionize immunotherapy, scalable manufacturing, safety, specificity, and the development of resistance are potential roadblocks in their widespread usage. The development of molecular engineering tools to allow for the direct or indirect engineering of T-cells to enable one to troubleshoot delivery issues, amplify immunomodulatory effects, integrate the synergistic effects of different molecules, and home to the target cells in vivo. In this review, we will analyze thus-far developed cell- and material-based tools for enhancing T-cell therapies, including methods to improve safety and specificity, enhancing efficacy, and overcoming limitations in scalable manufacturing. We summarize the potential of T-cells as immune modulating therapies and the potential future directions for enabling their adoption for a broad range of diseases.

Graphical Abstract

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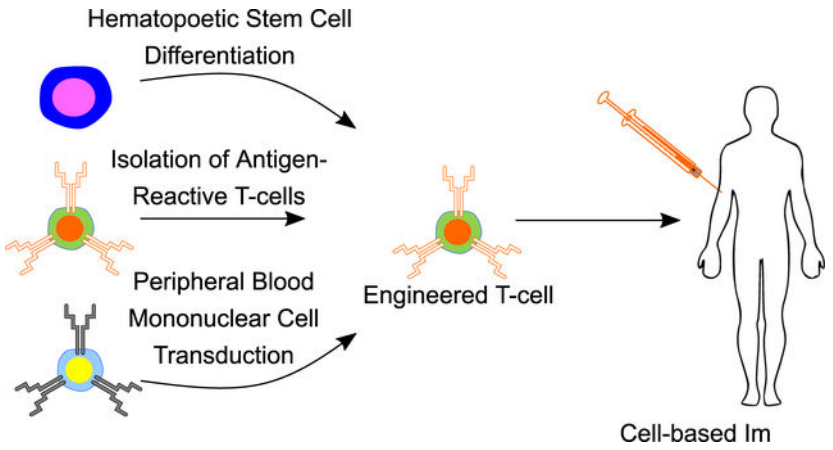

1 | INTRODUCTION

T-cells are essential mediators of adaptive immune defense against infectious pathogens and cancer. Deficiency in T-cell function is the basis of numerous immunological disorders, including immunodeficiency, autoimmunity and immunosurveillance disorders. T-cells develop from the subsets of hematopoietic stem and progenitor cells (HSPCs) that migrate to the thymus and differentiate into T-cells with clonotypic T-cell receptors (TCRs) that subsequently undergo thymic selection. The resulting T-cells are self-restricted and tolerant of self-tissues and initially circulate throughout the body at low frequency. T-cell priming and expansion requires TCR engagement by major histocompatibility (MHC)-antigen complexes to cognate antigens on the surface of antigen presenting cells (APCs) alongside concomitant ligation of co-stimulatory receptors by APCs within lymph nodes.

Since the first evidence of tumor treatment by T-cells, the principles of their using in cancer therapy have been systematically validated (Steven A Rosenberg, Restifo, Yang, Morgan, & Dudley, 2008; Steven A Rosenberg, Yang, & Restifo, 2004), (Billingham, Brent, & Medawar, 1954). Infusion of ex-vivo-expanded unmodified tumor infiltrating lymphocytes (TILs) was first used in melanoma patients(S. A. Rosenberg et al., 1988) and this approach is now used clinically for multiple types of cancers. It is established that tumor-specific T-cells recognize MHC-antigen complexes expressed on the surface of cancer cells to initiate the killing process and the effector T-cells, including $CD4^+$ T helper cells, $CD8^+$ cytotoxic T-cells, memory T-cells, and natural killer T-cells, release cytokines to facilitate tumor-killing(Schumacher & Schreiber, 2015). Therefore, engineered T-cell therapies have been a major focus in immuno-oncology based therapies(Chen & Mellman, 2013; Farkona, Diamandis, & Blasutig, 2016; Mellman, Coukos, & Dranoff, 2011). T-cell-based immunotherapy is defined by its character as a ‘living’ therapeutic platform(Lim & June, 2017) as the cells exhibit adaptive behaviors, and are unlike small molecules or antibodies(Chang et al., 2007).

Most current T-cell based adoptive cell transfer (ACT) therapies are isolated from peripheral blood via leukapheresis, genetically modified or sorted to enrich antigen specific T-cells, expanded, and subsequently reinfused into the patient. The efficacy and persistence of T-

cells in vivo may be further enhanced via the co-administration of immunomodulatory factors, of which immune checkpoint blockade is exemplary. However, difficuty with appropriate target identification, off-target effects, inefficient genetic modification techniques, lack of readily available histocompatible T-cells, and patient response variability still limit the widespread adoption of T-cell ACT therapies. To address these issues, a combination of molecular-scale technologies have been harnessed to engineer cellular signaling pathways to optimize the steps of ACT therapies and provide enhanced control and efficacy to T-cells in vivo.

Among the several forms of T-cells that have been developed for immunotherapy, engineered TCRs and chimeric antigen receptors (CARs) are the leading ACT therapies, with nearly 1500 ongoing clinical trials for multiple types of cancers. Both engineered CARs and TCRs are inserted ex-vivo into patient T-cells, which are then returned to the patient, and thereby deploy T-cells to target tumors. In some patients, CAR T-cells have generated durable therapeutic benefit after administration of a single dose(Porter et al., 2015) and most patients with acute lymphoid leukemia responded to CAR T-cell therapy(Davila et al., 2014; Shannon L Maude et al., 2014). While clinically approved CARs target cell surface receptors (e.g. CD19), genetically engineered T-cells can express highly active TCRs that target intracellular neoantigens expressed on peptide-MHC complexes to treat a variety of tumor antigens expressed in cancer patients(Park, Rosenberg, & Morgan, 2011).

The promising clinical results obtained with these forms of engineered T-cells has prompted the development of strategies to facilitate and broaden their applicability, which involve the use of T-cell sources that reduce the need for autologous cells and thereby enable cell transfer across histocompatibility barriers. These include virus-specific or allogeneic T-cells without native TCRs, expanded lymphoid progenitors, and induced pluripotent stem cell (iPSC)-derived T lymphocytes as off-the-shelf, genetically enhanced, histocompatible cell therapy products. The advances of T-cell engineering in oncology have also spurred T-cells engineering for treating infectious diseases, such as HIV, and autoimmunity such as graft-versus-host disease and induction of transplant tolerance(Ellebrecht et al., 2016).

In this review, we examine ongoing efforts in enhancing T-cell-based immunotherapies using cell-engineering approaches (Figure 1). We first discuss the engineering of peripheral T-cells in vitro and in vivo. We next discuss how progenitor T-cells are engineered to generate potent immunotherapies. We then discuss common transfection, transduction and expansion techniques applied to engineered T-cells. For each approach, we give a brief overview of recent advancement in that field of study. Finally, we conclude with some thoughts on important future directions for the development of future cell-based immunotherapies.

2 | CHIMERIC ANTIGEN RECEPTOR T-CELLS

Chimeric antigen receptors to enhance T-cell targeting are a widely-studied technique in T-cell engineering. The high clinical response rate of this approach in the treatment of patients with lymphoma and leukemia have resulted in the recent clinical approval of CD19-targeted CAR T-cell therapies and spurred ongoing efforts into engineering CARs to target other

types of tumors. Clinical experience with CAR T-cells has identified some of the current limitations of this approach including off-target neurotoxicity, resistance and limited therapuetic efficacy in solid tumors. CAR T-cell therapies have been extensively reviewed elsewhere(Bagley, Desai, Linette, June, & O'Rourke, 2018; June, O'Connor, Kawalekar, Ghassemi, & Milone, 2018) and here, we review recent advancements in molecular engineering to enhance the efficacy and safety of this T-cell based therapy.

All CARs consist of an ectodomain that determines the antigen specificity, a transmembrane region, and an endodomain that controls the intracellular signaling (Figure 2). The ectodomain consists of a single chain variable fragment (scFv) that is the light and heavy chain of an immunoglobulin bound with a linker and a spacer region, where the choice of immunoglobulin components primarily determines the specificity of the antigen recognized by the CAR T-cell. The linker and spacer regions can affect the efficacy, stability and targeting capabilities of CAR T-cells (Alabanza et al., 2017; Jonnalagadda et al., 2015). Variations in the transmembrane domain and hinge coding regions can influence CAR binding affinity and specificity.

1.1 – ENHANCING THE EFFICACY OF CAR T-CELL THERAPY

Most patients with relapsed B-cell lymphoma achieve remission after CD19-specific CAR T-cell treatment(S. L. Maude et al., 2018). However, the loss of the antigenic epitope on CD19 that is targeted by CAR T-cells has been identified as a dominant mechanism of tumor escape and can result in relapse of the cancer. Furthermore, the success of CAR T-cells in treating leukemic and lymphoblastic cancers has yet to be realized in treating solid tumors, in which the immunosuppressive microenvironment, lack of cancer-specific targets, and heterogeneity of the cancer cell population hinder CAR T-cell function(Harlin et al., 2009). To improve response rates, therapies that target alternative receptors and increase the persistence and function of CAR T-cells in tumor microenvironments are the subject of ongoing research efforts.

To overcome tumor antigen escape, alternative targeting motifs may allow for targeting the tumor through more than one receptor, such as CD22, which is also highly expressed in lymphomas. ACT with CD22 CAR T-cells cells has demonstrated remission in CD19 low pre-B cell acute lymphoblastic leukemia(Fry et al., 2018) that were not responsive to CD19 CAR T-cell therapy. As another alternative, B-cell activating factor receptor (BAFF-R) is being targeted in pre-clinical studies. CAR T-cells targeting BAFF-R conferred long term survival and tumor resistance to xenogeneic $CD19^{-}$ leukemia models(Qin et al., 2017).

Another strategy to prevent antigen escape in the use of CAR T-cells that target multiple antigens using Boolean OR logic gating. These constructs are termed either tandem CAR T-cells (TanCAR T-cells) if the targeting immunoglobulin domains are part of a single ectodomain, or dual CAR T-cells if each T-cell is engineered with two separate CARs. A TanCAR T-cell designed to target both CD19 and CD20 was designed and tested *in vivo* and compared to standard CD19 CAR T-cells. The use of both types of CAR T-cells results in comparable survival rates in a $CD19^{+}$ lymphoma model, but the TanCAR T-cells showed significantly better survival rates in a $CD19^{+}/CD19^{-}$ mixed lymphoma model when compared to the standard CD19 CAR T-cells. Subsequent work confirmed the efficacy of

CD19/CD20 TanCAR T-cells against a variety of leukemia models. A dual CAR T-cell targeting CD19 and CD123 has been developed and tested in in a mixed $CD19^+/CD19^-$ xenograft leukemia model and compared to treatments with CD19, CD123, and pooled CD19 + CD123 CAR T-cells (Ruella et al., 2016; Schneider et al., 2017). It is noteworthy that the dual CAR T-cell had a significantly higher long-term efficacy when compared to the pooled CAR T-cells. Additionally, dual CAR expression at a 1:1 ratio could be achieved using a single lentiviral vector, which suggested the feasibility of clinical manufacturing of this cell type.

In addition to achieving tumor-specific targeting, modifications to the endodomain can improve CAR T-cell activation. The endodomain controls the intracellular signaling by the incorporation of an immunoreceptor tyrosine-based activation motif (ITAM) to activate the T-cell. First generation CAR T-cells use an ITAM signaling domain and transmembrane region conserved from the CD3-ζ subunit that flanks TCRs. The FcεRI-γ domain is also used in some first generation CAR T-cells, but cells with this domain show lower persistence in vivo than those designed with the CD3-ζ domain(Haynes et al., 2001; Heuser, Hombach, Losch, Manista, & Abken, 2003) and undergo rapid anergy after initial activation(Kershaw et al., 2006; Pule et al., 2008). To improve the proliferation of CAR T-cells in vivo, second generation CARs use an additional signaling domain, generally CD28, 4-1BB, DAP10, OX40 or ICOS(Finney, Akbar, & Lawson, 2004). These domains provide a costimulatory signal necessary to maintain robust T-cell functionality and has resulted in an improvement in CAR T-cell persistence and expansion and thus the potency of the therapy(Savoldo et al., 2011). Third generation CAR T-cells combine multiple costimulatory domains and display improved persistence and expansion after tumor challenge as compared to the second generation(Carpenito et al., 2009; J. Wang et al., 2007). Whether this strategy provides a clinical benefit over second generation CAR T-cells is the subject of ongoing investigation.

Some types of cancer cells may not have a specific targetable cell-surface receptor (motif-naïve). For enhancing the persistence and function of ACT in solid tumors, fourth generation CARs T-cells, termed “T-cells redirected for universal cytokine killing” (TRUCKs), have been developed. Generally, TRUCKs locally recruit immune cells via a cytokine gradient for potentiating the killing of cancer cells that would otherwise escape normal T-cell targeting[91]. TRUCKs recruited immune cells via IL-12 production upon activation in the tumor microenvironment. In this approach, inducible transgenes containing a promoter region that bound to nuclear factor of activated T-cells (NFAT) enabled transgenic IL-12 expression only upon T-cell activation. In a murine melanoma model, treatment with inducible IL-12 T-cells resulted in significant improvement in tumor eradication and reduced toxicity when compared to standard TCR-specific T-cell treatment(Chmielewski & Abken, 2017; Ling Zhang et al., 2011).

IL-18 promotes T-bet expression, and has been demonstrated to enhance cytolytic activity in $CD8^+$ T-cells and reduce FoxO1 expression, a transcription factor linked with exhaustion, in both $CD4^+$ and $CD8^+$ T-cells. IL-18 TRUCKs increased the number of M1 macrophages and NK cells, whereas regulatory T-cells, suppressive dendritic cells (DCs), and M2 macrophages decreased, compared to CAR T-cell controls. Treatment of pancreatic

carcinomas and xenogeneic disseminated lung adenocarcinoma-bearing mice with IL-18 TRUCK enhanced survival rate compared to CAR T-cell treatment[93].

1.2 – MITIGATING OFF-TARGET REACTIVITY OF CAR T-CELLS

In contrast to diminished function, hyperactivation and off-target reactivity of CAR T-cells have resulted in treatment related adverse events in patients. As both lymphomas and normal B cells express the CD19 receptor, CD19 targeting CAR T-cells deplete normal B-cells even after remission is achieved, leading to B cell aplasia and necessitating intravenous immunoglobulin transfusions in patients(Nazimuddin et al., 2013). Additionally, off target effects such as cytokine release syndrome (CRS) and neurological toxicities are also linked with CAR T-cell therapies(Brudno & Kochenderfer, 2016). To improve the safety of these treatments, ongoing research efforts seek to develop methods to induce death of CAR T-cells after therapy and increase the specificity of CAR T-cell activation.

One strategy to limit the period of B-cell aplasia is to delete the CAR T-cells after remission is achieved. In this approach, the incorporation of the inducible caspase 9 (iCasp9) gene produces the intracellular portion of human caspase 9 fused to a mutated version of the FKBP12 drug binding domain(Gargett & Brown, 2014). The mutated FKBP12 binds with a pharmacologically inert chemical inducer of dimerization (CID) inducing dimerization of the iCasp9 products and results in cell apoptosis. Alternatively, incorporation of a caspase 9 suicide gene can be induced via administration of clinically approved rapamycin (rapaCasp9)(Stavrou et al., 2018). In this approach, the rapamycin binding FRB domain from the mammalian target of rapamycin (mTOR) was included at the end of the standard iCasp9 sequence, and the linking domain length between the FRB, FKBP, and Caspase 9 segments was optimized for responsiveness to rapamycin. *In vivo*, significant rapamycin-specific ablation of the rapaCasp9 T-cells was achieved, which was not observed in T-cells without rapaCasp9. rapaCasp9 and iCasp9 systems achieved similar efficiency in CAR T-cell ablation.

Bi-specific Boolean gating strategies have been used to integrate multiple signals to increase the specificity of CAR T-cells. Separation of the stimulatory and costimulatory domains between two receptors with different ectodomains creates an AND gate that requires the recognition of two signals before the CAR T-cell will activate. CAR T-cells engineered to express mesothelin targeting CARs with the CD3-ζ endodomain and a-folate targeting CARs with the CD28 endodomain were tested in a murine model of epithelial ovarian cancer(Lanitis et al., 2013). The AND gated CAR T-cells and conventional second-generation CAR T-cells for tumors expressing both antigens were comparable in tumor clearance. However, when tumors expressed mesothelin only, AND gated CAR T-cells were comparable to first generation CAR T-cells, displaying initial lysis of target cells but minimal subsequent expansion. Tuning receptor affinity may provide further control over dual signal response(Kloss, Condomines, Cartellieri, Bachmann, & Sadelain, 2013; Wilkie et al., 2012).

An alternative bi-specific mechanism uses a NOT gate, in which a CAR T-cell will not activate upon signal recognition. These inhibitory CARs (iCARs) are created with an ectodomain attached to the intracellular domain from either CTLA-4 or PD1. In vitro,

conventional CAR T-cells eliminated the both $CD19^+$ and $CD19^+$ human prostate specific membrane antigen$^+$ ($PMSA^+$) artifiicial antigen presenting cells (aAPCs), whereas CD19 iCARs targeting PMSA selectively eliminated single positive $CD19^+$ aAPCs(Fedorov, Themeli, & Sadelain, 2013).

CAR T-cells that activate only upon simultaneous small molecule and target motif recognition may be a means for additional mitigation of off-target reactivity. One example is “ON-switch” CAR T-cells that require both the recognition of a rapamycin analog (rapalog) and CD19(C.-Y. Wu, Roybal, Puchner, Onuffer, & Lim, 2015). $CD8^+$ ON CAR T-cells exhibited cytotoxic function that was dependent on rapalog concentration. In this apporach, the cells had no toxicity in the absence of rapalog, but could achieve the same level of cell lysis as conventional CAR T-cells when switched on (Figure 3). Additionally, because the concentration of rapalog influences the level of cytotoxic function, the activation of T-cells may be tuned.

2 | ENGINEERING ANTIGEN SPECIFIC T-CELLS

In general, CARs target surface markers, while TCRs target MHC molecules containing peptide fragments from intracellular proteins. Human leukocyte antigen (HLA) peptide targeted CARs lose specificity above a threshold affinity, and high antigen binding affinity TCR-like CARs exhibit impaired activation when compared to lower affinity native TCRs(Oren et al., 2014),(Harris et al., 2018). The low affinity may limit the applicability of CARs to new targets for immunotherapy. Therefore, to harness the full potential of TCR targeting capabilities, antigen specific T-cells have been harnessed for cancer immunotherapy.

The development of antigen specific T-cells is achieved by the isolation of T-cells with TCRs that have high binding affinities with the target peptide-MHC complex. Therefore, methods to isolate and sequence T-cells are crucial for subsequent engineering. Additionally, T-cells may be genetically modified to express TCRs of interest, though off-target effects due to endogenous and transgenic TCR mispairing remain a concern. Here we review advances in techniques for isolating and engineering TCRs to generate antigen-specific T-cells.

2.1 – ISOLATION OF NEOANTIGEN REACTIVE TUMOR INFILTRATING LYMPHOCYTES

ACT methods for treating metastatic cancer have used tumor infiltrating lymphocytes (TILs) that were isolated, expanded and subsequnetly re-infused into patients(S. A. Rosenberg et al., 1985; Steven A Rosenberg et al., 1988). While effective in some patients, only ~10% of $CD8^+$ TILs recognize autologous tumor antigens(Scheper et al., 2018) in the highly heterogenous tumor environment. As a result, the therapeutic outcomes of TIL-based therapy is highly variable. Ongoing research efforts seek to better identify and isolate TILs with neoantigen receptors for effective targeting in cancer immunotherapy.

One approach to better identify tumor-reactive TILs used cataloging TCR reactivity of TILs. Here, TILs were harvested and expanded from the tumor, and whole exome sequencing of tumor samples and healthy tissue was used to identify protein mutations (Figure 4). Multiple

cultures of expanded TILs were then co-cultured with tandem minigene transfected or peptide pulsed autologous APCs. The T-cells were then analyzed for interferon-γ (IFN-γ) and IL-2 expression levels using single cell sequencing to determine TCRs associated with neoantigen-induced activation(Y. C. Lu et al., 2018). In a clinical trial for patients with metastatic breast cancer(Zacharakis et al., 2018), T-cells transduced with TCR sequences that were identified using the above described method induced complete and durable regression. It is noteworthy that presorting of TILs for activation markers or PD-1 has also improved isolation of neoantigen reactive TCRs(Gros et al., 2016; Parkhurst et al., 2017). TILs sorted for CD137 and PD-1 expression prior to in vitro culture and screening resulted in the identification of 19 neoantigens as compared to 8 found with no presorting(Seliktar-Ofir et al., 2017; Yossef et al., 2018).

In addition to genomic and cell-surface marker based sorting techniques, nanoparticle based antigen-specific T-cell separation techniques are used for sorting T-cells based on antigen specificity(Hickey et al., 2018). Optimization of nanoparticle-based magnetic beads has allowed for the recovery of T-cells highly enriched for a chosen cognate antigen from a starting population comprised of less than 0.01% of T-cells specific for the target antigen. This platform may allow for the development of an effective system that can simultaneously isolate and expand a subset of antigen specific T-cells, though further optimization may be required for selection of T-cells from an initially small starting population.

2.2 – TCR ENGINEERING IN MATURE T-CELLS

While the isolation of antigen specific T-cells is an important step in creating effective immunotherapies, a small initial starting population of neoantigen specific T-cells imposes a significant limitation on the feasibility of treatments that rely on expansion of isolated T-cells. The genetic engineering of T-cells with antigen-specific TCRs offers a method for rapidly generating a large population of tumor-reactive T-cells. The focus of TCR-engineered T-cells has been transducing $CD8^+$ T-cells to express transgenic TCRs against cancer-specific neo-antigens[72–74]. Ongoing research efforts have focused on addressing off-target reactivity from endogenous-transferred TCR-mispairing encountered when transducing mature T-cells, augmenting TCR-engineered $CD8^+$ T-cell therapies by co-administration of $CD4^+$ T-cells, and developing TCR-engineered $CD4^+$ T-cell therapies for tumor-associated antigens in cancers that lack expression of MHC molecules.

TCR engineering in PBMC-derived T-cells may result in mispairing of introduced and endogenous TCRs leading to lethal off-target toxicity(Bendle et al., 2010). The use of TCR single chain signaling complexes has been shown to limit mispairing while maintaining the ability of TCRs to target pMHC complexes(Stone et al., 2014). The use of TCR single chain signaling (TCR-SCS) complexes was examined to limit mispairing while maintaining the ability of TCRs to target pMHC complexes(Stone et al., 2014). The efficacy of these constructs was demonstrated in a murine melanoma model using polyclonal T-cells, T-cells engineered with m33 TCRs, and T-cells expressing m33 TCR-SCS. Both $CD4^+$ and $CD8^+$ T-cells were transduced and tested. $CD4^+$ and $CD8^+$ TCR-SCS T-cells showed improved inhibition of tumor growth compared to $CD8^+$ T-cells engineered with standard m33 TCRs but worse suppression of tumor growth relative to $CD4^+$ T-cells engineered with m33 TCRs.

A related strategy to generate a durable anti-tumoral response involves adoptive immune transfer of antigen-specific $CD4^+$ cells(Hunder et al., 2008; Tran et al., 2014). The adoptive immune transfer of autologous TCR-transduced $CD4^+$ T-cells recognizing melanoma-associated antigen-A3 (MAGE-A3) was co-administered with high dose IL-2 to patients with MAGE-A3 expressing tumors. Before treatment, $CD4^+$ peripheral blood lymphocytes were isolated from patients and transduced with a γ-retroviral vector that encodes for HLA-DPB*0401 -restricted MAGE-A3 TCR. Patients were then treated with a nonmyeloablative chemotherapy followed by single infusion of autologous TCR-transduced $CD4^+$ T-cells and intravenous IL-2 administration. Objective responses were observed in patients during a preliminary cell dose escalation study(Y.-C. Lu et al., 2017).

3 | ENHANCEMENT OF IN VIVO FUNCTION OF T-CELLS

Modifying the cell machinery and biomaterials-based strategies provides a means to enhance the native cytotoxic function of effector T-cells to lyse tumor cells. In this section we will discuss methods to enhance T-cell function to overcome these suppression and escape mechanisms, as well as regulatory switches for controlling in vivo activation of transferred cells.

3.1 – ENGINEERING THE INTRACELLULAR T-CELL MACHINERY

By modulating the T-cell response upon antigen recognition, one may engineer enhanced anti-tumor efficacy. To this effect, it has been demonstrated that engineered synthetic Notch (synNotch) receptors can be used to drive prespecified transcriptional pathways(Roybal, Williams, et al., 2016). Like a CAR, the native Notch receptor has three main components – the extracellular binding region comprised of epidermal growth factor repeats, a core region that regulates cleavage of the receptor, and a Notch intracellular domain (NICD) that is released to regulate transcriptional pathways. The synNotch receptors replace the EGF repeats with a synthetic scFv region for either $CD19^+$ or Her2 and the NICD is replaced with a DNA binding domain fused to a transcriptional activator domain. Upon activation, synNotch receptors release transcriptional factors to upregulate pre-specified genetic pathways to release cytokines. In addition, synNotch receptors influence cell fate to induce preferential differentiation into the Th1 phenotype in the presence of a user-specified antigen via release of T-bet. SynNotch receptors have also been used to engineer T-cells to require the recognition of two antigens before they exhibit cytotoxic properties, thereby minimizing off-target effects in bystander tissue(Roybal, Rupp, et al., 2016).

The development of synthetic receptors may also be used to overcome naturally exhaustive pathways. In one approach, a chimeric PD-1:CD28 receptor was designed to couple the extracellular domain of PD1 with the transmembrane and intracellular machinery of CD28 and incorporated into $CD8^+$ T-cells. A lentiviral vector was used to incorporate the chimeric receptors into human $CD8^+$ T-cells that lack endogenous expression of both PD1 and CD28. The cell transformation converted an exhaustion signal into a costimulatory signal, resulting in significantly higher amounts of IL-2, TNF-α, and IFN-γ production from the transformed cells in the presence of PD-L1 when compared to non-transformed cells(Prosser, Brown, Shami, Forman, & Jensen, 2012).

T-cells may be engineered to express non-functional versions of receptors involved in T-cell suppression pathways to decrease the signaling of functional inhibitory pathways. In one study, T-cells were transduced with retroviral vectors to express TGF-β-dominant-negative receptor II (DNRII) or secrete either soluble TGF-β-RII (sRII) or a soluble immunoglobulin-receptor hybrid (sRIIFc) to prevent TGF-β mediated inhibition of T-cell activation. The *In vitro* results showed all three methods prevented phosphorylation of smad-2, with the DNRII cells exhibiting the best TGF-β blockade. Melanoma bearing mice treated with DNRII expressing cells had a reduced tumor burden and prolonged survival time when compared with control treatment groups(L Zhang et al., 2013).

Cas9-mediated gene editing of T-cells may be use to reduce immune checkpoint PD-1 expression. Cas9 mediated disruption of the Pdcd1 gene resulted in CAR T-cells that exhibited improved cell lysis of $CD19^{+}PDL1^{+}$ tumor cells compared to control Cas9 nucleofected cells. In contrast, conventional CAR T-cells co-cultured with PD-L1 expressing cells exhibited a decrease in lytic function when compared to Cas9 edited CAR T-cells treated in the same manner. CAR T-cells with the disrupted Pdcd1 gene eliminated all tumors in mice whereas about the tumors were cleared on only 17% of mice by the control cells at the same dose(Rupp et al., 2017).

Gene silencing microRNA (miRNA) switches triggered by small molecule recognition have been used to control T-cell proliferation via mediation of cytokine receptor expression(R. S. Wong, Chen, & Smolke, 2017). MicroRNAs (miRNA) are short, non-coding RNA fragments that can direct post-transcriptional gene silencing via the RNA interference (RNAi) pathway. MicroRNA switches are designed by incorporating the miRNA with an RNA sequence known as an aptamer that can be designed to bind to a user-specified molecule. Incorporation of the aptamer into the miRNA segment prevents maturation of the miRNA and inhibits its ability to influence gene expression. After the aptamer binds to its target molecule, conformational changes allow for the miRNA to be processed and function normally. This method has been used to control T-cell proliferation in vitro using an aptamer that binds to biologically inactive (6R)-folinic acid, and decreases IL-2Rβ expression via RNAi.

3.2 – BIOMATERIAL-BASED DELIVERY FOR DIRECTING T-CELL FUNCTION

In addition to synthetic biology approaches to engineer T-cell function, the recent success of immune checkpoint blockade therapies to enhance ACT demonstrates the importance of modulating the signals encountered by the immune cells. However, these small molecule and protein-based immunotherapies are still limited by off-target side-effects and non-responsive patients. To effectively synergize immune checkpoint therapy with engineered T-cells, biomaterial-based delivery can improve their safety profiles and anti-cancer benefits. The use of modular nanoparticles capable of targeting T-cells and $PD\text{-}1^{+}$ cells has been shown to deliver therapeutic payloads to inhibit TGF-β signaling. The nanoparticles are created by conjugating antibody binding domains to maleimide functionalized polyethylene glycol coated poly(lactic-co-glycolic acid) (PLGA) beads. PD-1 targeted nanoparticle delivery of a small molecule (SD-208) to inhibit TGF-β signaling led to extended life in murine colorectal cancer models using doses of anti-PD-1 and SD-208 that showed no physiological effect

when administered free(Schmid et al., 2017). The same results were shown for the delivery of a Toll-like receptor 7/8 agonist.

An alternate biomaterial-based delivery approach used ECM binding peptide to localize checkpoint blockade antibodies to tumor sites(Ishihara et al., 2017). In this study, checkpoint blockade antibodies for PD-L1 and CTLA4 were bound to placenta growth factor-2 (PlGF) which binds strongly to multiple ECM proteins and was attributed to an increase in infiltrating T-cells into the tumor tissue. In vivo administration of PIGF-conjugated antibodies resulted in a reduced tumor burden when compared with peritumorally administered unconjugated antibodies. Additionally, the conjugation of immune checkpoint blockade antibodies to PIGF decreased off-target systemic side effects such as the risk of autoimmune diabetes. This technique both show the capabilities of cancer-targeted drug delivery techniques to work synergistically with T-cells to prevent exhaustion and enhance the potency of the therapy.

In addition to blocking inhibitory pathways, T-cells may be delivered with stimulatory molecules to improve intratumoral persistence using drug–loaded nanoparticles conjugated to the cell surface(Stephan, Moon, Um, Bershteyn, & Irvine, 2010; Tang et al., 2018). The nanoparticles provided sustained activation signaling to the conjugated cells, mimicking autocrine stimulation. This method of cell delivery resulted in marked enhancements in cell persistence and tumor elimination in a B16F10 melanoma model. Similarly, a nanogel system capable of attaching to T-cells and releasing protein payloads in response to T-cell activation has also been developed[47]. This method was used to deliver IL-15 super-agonist (IL-15sa) complex along with therapeutic cells and the nanogel carrying T-cells were shown to improve T-cell proliferation in tumors relative to free administration of the cytokine. Up to 80% of tumors were eradicated in mice treated with IL-15sa nanogel backpacks, while no eradication was observed for mice that received a comparable dose of free IL-15sa in a murine model for human glioblastoma.

As an alternative to isolating antigen-specific T-cells, nanoparticle-based vaccines which concentrate in draining lymph nodes and activate immune cells after subcutaneous or intraperitoneal injection have been widely studied(Liu et al., 2014; Reddy, Rehor, Schmoekel, Hubbell, & Swartz, 2006; Thomas, Vokali, Lund, Hubbell, & Swartz, 2014). A recent report describes a synthetic glyco-adjuvant called p(Man-TLR7) which targets DCs via mannose-binding receptors or activates DCs via Toll-like receptor 7 (TLR7)(Wilson et al., 2019). Antigens attached to p(MAN-TLR7) monomers via a self-immolative linker are released upon internalization by DCs, which are also activated by endosomal TLR7. When used as a vaccination against a malaria derived protein plasmodium falciparum-derived circumsporozoite protein (CSP) p(MAN-TLR7)-based vaccination conferred greater protection than the clinical standard in mice. Nanoparticles have also been decorated with antigen-specific and tumor cell binding moieties, termed antigen-specific T-cell redirectors (ATRs), to redirect T-cells to tumor cells(Schutz et al., 2016). In this approach, anti-mouse IgG microbeads were conjugated with CD19 antibodies and either a pMHC-Ig dimer or anti-TCR antibodies specific for human influenza. The particles were then cultured with $CD19^{+}$ Raji cells and FluM1 specific cytotoxic lymphocytes. Ratios above 2:1 of T-cell:tumor cell binding moieties increased cell lysis more than two fold over control nanoparticles that

contained only tumor cell binding moieties. ATRs also demonstrated the ability to inhibit tumor growth in vivo, inducing a significant reduction in tumor growth in a murine B cell lymphoma model compared to the same controls.

4 | T-CELLS ENGINEERED FROM HEMATOPOIETIC STEM AND PROGENITOR CELLS (HSPCs)

Hematopoietic stem and progenitor cells offer a potential source to generate large quantities of engineered T-cells for ACT. Generating T-cells from HSPCs offers certain advantages over engineering mature T-cells such as the elimination of endogenous and transferred TCR mispairing[39,40], prolific *ex vivo* expansion of HSPCs as compared to mature T-cells[18,19], and sustained *in vivo* generation of antigen specific T-cells[24–29]. Notch signaling is key in directing HSPCs towards the T-cell lineage, and the induction of Notch signaling in HSPCs by delta-like ligand (DL) expressing murine stromal feeder-layers, such as OP9-DL1 or MS5-DL1, or synthetic systems are often used for developing and expanding T-cells engineered with antigen-specific T-cell receptors (TCRs) *ex vivo*(Holmes & Zuniga-Pflucker, 2009; Schmitt & Zuniga-Pflucker, 2002).

4–1 | INDUCTION OF ANTIGEN SPECIFIC TCRs IN VITRO

The generation of T-cells from HSPCs is generally achieved by (i) differentiation and (ii) TCR selection of HSPCs. First, extended Notch signaling of HSPCs on a feeder layer, such as an OP9-DL1 stromal cells, induces TCR rearrangement and differentiation into $CD4^+CD8^+$ double positive (DP) T-cells with heterogeneous TCR clonotypes[14,15]. Next, APCs, such as DCs or fibroblasts, are genetically modified to express pathogen-derived antigens are used to induce antigen specific positive selection of DP T-cells into CD8 single positive (SP) T-cells in a manner analogous to *in vivo* thymic positive selection(Oelke et al., 2003; Suhoski et al., 2007). Examples of antigens include TRP-2 and LCMV-associated antigen gp33(Dervović, Ciofani, Kianizad, & Zúñiga-Pflücker, 2012). Phenotypic, molecular, and functional analyses of *in vitro* generated T-cells have demonstrated consistency with thymic derived T-cells, and are activated by TCR and CD28 co-stimulation, as determined by effector phenotype and INF-γ secretion. Furthermore, activated TRP-2 or gp-33 antigen specific T-cells can undergo peripheral expansion in mice following adoptive cell transfer (Dervovic, Ciofani, Kianizad, & Zuniga-Pflucker, 2012).

An alternative method of generating antigen specific T-cells is the genetic modification of HSPCs with antigen specific TCR sequences. Unlike non-transduced HSPCs, the presence of the transgenic TCR precludes TCR rearrangement in these cells during T-cell lineage differentiation. As a result, TCR-transduced HSPCs differentiate into DP T-cells with a homogenous TCR sequence after seeding onto OP9-DL1 cell culture (Fernandez, Ooi, & Roy, 2014; Giannoni et al., 2013; Snauwaert et al., 2014; Yang & Baltimore, 2005; Yang, Qin, Baltimore, & Van Parijs, 2002; Zhao et al., 2007). Human HSPCs transduced with one of a variety of TCRs and seeded onto OP9-DL1 co-cultures have been demonstrated to undergo positive selection using relevant agonist peptide added to culture. Successful positive selection has been verified by the measurement of SP-specific genes. SP TCR-

transduced T-cells generated in this manner exhibit effector function and cytotoxic killing of T2 cells pulsed with agonist peptide[20].

A common strategy for developing T-cell based cancer immunotherapy is isolating TILs that recognize known tumor antigens(Stromnes et al., 2012). However, the affinity of the TCRs in these cells is still limited by negative selection in the endogenous thymus, which may delete cells with higher affinity TCRs. A strategy to generate higher affinity TCRs is the transduction of an α-chain coding region of a TIL-derived parental TCR into HSPC progenitors seeded on OP9-DL1 co-culture before TCR rearrangement(Schmitt et al., 2017). This method still permits TCR rearrangement of the β-chain coding region and thereby results in a TCR with transgenic α-chain and endogenous β-chain. Subsequently, cognate peptide fragments are added to cell cultures to induce positive selection and cells are subsequently isolated and screened to determine relative TCR binding affinity and specificity. The resulting binding affinity of the engineered TCRs to their cognate antigen was demonstrated to be significantly higher than the parental TCR (Figure 5).

4–2 | ENGINEERING 3D AND FEEDER-FREE DIFFERENTIATION SYSTEMS

Notch ligand-expressing stromal cell feeder layers together with exogenous cytokines are widely used for many engineered HSPC-based T-cell therapies. Co-cultured HSPCs exhibit phenotypic markers of all thymocyte lineages, where the generation of DP T-cells reaches a maximum at day 20–25 and by day 40 SP $CD8^+$ T-cells represent only 2 – 4% of cultured cells[16,(De Smedt, Hoebeke, & Plum, 2004)]. The requirements for complex infrastructure, the extended period of co-culture, the requirement for serum-containing medium and the xenogeneic nature of feeder layers limits the translational potential of this system. The development of new technologies addresses some of the shortcomings of these feeder layer systems.

Artificial thymic organoids (ATOs) support more efficient *in vitro* differentiation of human T-cells(De Smedt et al., 2011; La Motte-Mohs, Herer, & Zuniga-Pflucker, 2005). ATOs are formed by a compaction re-aggregation technique in which MS5-DL1 stromal cells and HSPCs are aggregated by centrifugation and seeded on the air-fluid interface of a cell culture insert(Chung et al., 2014; Sheridan, Taoudi, Medvinsky, & Blackburn, 2009). Cell culture medium supplemented with differentiation cytokines was identified as a serum-free medium that supported robust T-cell differentiation. The ATO expanded functional NY-ESO-$1_{157–165}$ peptide specific TCR-transduced HSPCs. When compared to OP9-DL1 monolayer system, the ATO system sustained higher number of DP T-cell generation up to 6 weeks in vitro (Seet et al., 2017). Improved positive selection was determined by presence of functional SP CD8 T-cells in the ATOs, but were not detected in the OP9-DL1 monolayer cultures, that were reactive in a humanized mouse tumor model(Seet et al., 2017).

Serum-free cell culture in plate-bound DL4 vascular cell adhesion molecule 1 (VCAM-1) can enhance NOTCH signaling and direct progenitor T-cell (proT-cell) differentiation. Plate-bound DL4 + VCAM-1 cell culture yielded proT-cell expansion that was comparable to OP9-DL4 cultures after 14 days. To test the in vivo engraftment potential of cells generated in this manner, proT-cells from bound DL4 + VCAM-1 cell culture were injected intra-hepatically into neonatal humanized mice. Subsequently harvested thymi contained high

levels of human $CD45^+$ cells at 4 weeks and mature functional SP $CD8^+$ T-cells were observed after 10–12 weeks *in vivo*(Shukla et al., 2017).

In the context of hematopoietic stem cell transplantation (HSCT), the in vivo generation of progenitor T-cells might be a means to generate large quantities of T-cell competent immune cells without the requirement for extensive infrastructure needed for ex-vivo T-cell manufacture using an off-the-shelf biomaterial-based scaffold that mimics features of T-cell lymphopoiesis in the bone marrow(Shah et al., 2019). The scaffold, termed a bone marrow cryogel (BMC), releases bone morphogenetic protein-2 to recruit stromal cells, and presents the Notch ligand Delta-like ligand-4 to facilitate T-cell lineage specification of mouse and human hematopoietic progenitor cells. BMCs subcutaneously injected in mice at the time of HSCT enhanced T-cell progenitor seeding of the thymus, T-cell neogenesis and diversification of the T-cell receptor repertoire. Compared with adoptive transfer of T-cell progenitors, BMCs increased donor chimerism, T-cell generation and antigen-specific T-cell responses to vaccination.

4–3 | IN VIVO EXPANSION OF TCR-ENGINEERED HSPCs

The adoptive immune transfer of in vitro expanded TCR-engineered PBMC-derived T-cells has demonstrated remarkable antitumor efficacy in clinical trials(Johnson et al., 2009; Y.-C. Lu et al., 2017; Morgan et al., 2006; Robbins et al., 2011). However, the *in vivo* expansion of TCR-engineered HSPCs has certain advantages over adoptive immune transfer of TCR-engineered mature T-cells. First, the presence of a transgenic TCR precludes endogenous TCR expression and mitigates off-target TCR reactivity stemming from mispairing of transferred and endogenous TCR dimers. TCR mispairing occurs when there is an incorrect pairing between introduced TCR α or β- chains and endogenous TCR α or β- chains in TCR-transduced mature T-cells(Reuss et al., 2014; Sommermeyer et al., 2006) and remains a barrier in widespread clinical adoption of engineered mature T-cells(Bethune et al., 2016; Bunse et al., 2014). Second, engraftment and long-term in vivo expansion of TCR-engineered HSPCs into TCR-engineered T-cells provides a robust method for long-term persistence of TCR-engineered T-cells.

A head-to-head comparison of therapeutic efficiency of TCR-transduced mature T-cells and TCR-transduced HSPC-derived T-cells was conducted in mice using the lymphocytic choriomeningitis virus specific P14 TCR which recognizes gp33 antigen of LCMV. P14 TCR-transduced HSPCs transplanted into mice resulted in P14 TCR^+ T-cells in the peripheral blood 6 weeks after transplant. Isolation and co-culture of P14 TCR^+ T-cells derived from transduced HSPCs on gp33 peptide-loaded splenocytes activated T-cells, and was equivalent to that of P14 TCR-transduced mature T-cells that were similarly stimulated. Both types of P14 TCR^+ T-cells mediated equivalent tumor suppression in a B15-gp33 melanoma model, demonstrating that TCR-transduced HSPCs differentiate into mature T-cells with TCR specificity similar to that of TCR-transduced T-cells (Starck, Popp, Pircher, & Uckert, 2014).

While long-term persistence of TCR-engineered T-cells has been associated with better antitumor responses in patients(Chodon et al., 2014; Rapoport et al., 2015), it has been difficult to achieve long-term clinical responses with adoptive transfer of terminally

differentiated T-cells(Robbins et al., 2015). As a result, co-administration of both TCR-engineered $CD8^+$ T-cells and HSPCs to improve the persistence of TCR-engineered T-cells is a key research focus. Persistence may be improved by the engraftment of TCR-transduced HSPCs and their subsequent differentiation into antigen specific T-cells. As an example, HSPCs transduced with a lentiviral vector encoding a cancer/testes antigen NY-ESO-1 and PET/suicide gene sr39TK. In vivo safety was demonstrated by ablation of PET signal in long-bones, spleen, and thymus of humanized mice after receiving bone marrow transplants with NY-ESO-1/sr39-TK transduced HSPCs. The transduced HSPCs differentiated into all blood lineages and isolation of *in vivo* generated T-cells demonstrated antigen- and HLA-restricted effector function against NY-ESO-1 expressing melanoma cells *ex vivo*(Gschweng et al., 2014). An upcoming phase I clinical trial aims to determine the efficacy of co-administration of the NY-ESO-1 TCR transduced HSPCs together with NY-ESO-1 TCR transduced T-cells(Puig-Saus et al., 2018).

4–4 | DEVELOPMENT OF CHIMERIC ANTIGEN RECEPTOR – T-CELLS FROM HSPCs

As with TCR-engineered T-cells, the efficacy of CAR-T-cell therapy also depends on persistence and effector function(Kalos et al., 2011; G. Li et al., 2018; Porter et al., 2015; Porter, Levine, Kalos, Bagg, & June, 2011). The clinical experience of recently approved CAR-T therapies for CD19 expressing B-ALL, has spurred interest in developing therapies over which better control can be exerted as well as broaden the applicability of CAR to different subsets of immune cells and to other immunological diseases(Morrissey et al., 2018; Rezvani, Rouce, Liu, & Shpall, 2017). The use of HSPCs for the CAR cell source may result in greater control over CAR T-cell function, and the ability to engineer other immune cells for CAR expression, including natural killer (NK) cells and myeloid cells(De Oliveira et al., 2013; S. Larson & De Oliveira, 2014).

In one approach for the treatment of CD19-expressing B-ALL, the modification of HPSCs with a second-generation anti-CD19 CAR and herpes simplex virus thymidine kinase (HSVsr39TK) suicide gene allowed for ablation of the gene-modified cells. Similar engraftment of non-transduced, CAR-T only, and CAR-T + HSVsr39TK HSPCs was quantified in NSG mice. The efficacy of anti-CD19 CAR was also unaffected by the presence of the suicide gene as determined by anti-tumor response to a subcutaneous tumor inoculation with $CD19^+$ human lymphoblast-like cells and subsequent long-term survival. Furthermore, treatment with ganciclovir ablated CAR-T + HSVsr39TK populations(S. M. Larson et al., 2017).

5 | T-CELLS ENGINEERED FROM INDUCED PLURIPOTENT STEM CELLS

For both allogeneic and autologous HSPC sources, the process of mobilizing and collecting HSPCs is burdensome, invasive and time consuming and therefore may not be suitable for some patients. Induced pluripotent stem cells (iPSCs) from PBMCs may overcome some of the practical hurdles to the obtaining suitable numbers of HSPCs (Loh et al., 2010; Staerk et al., 2010). (Seki et al., 2010). Furthermore, the use of autologous T-iPSC antigen specific T-cells mitigates concerns about graft-versus-host diseas(Vizcardo et al., 2013).

5.1 | INDUCED PLURIPOTENT STEM CELLS DERVIED FROM ANTIGEN SPECIFIC T-CELLS

iPSCs derived from T-cells (T-iPSCs) inherit rearranged TCR genes upon T-cell re-differentiation(Kaneko, 2016; Maeda et al., 2016; Vizcardo et al., 2018) and may be sourced from from neo-antigen specific tumor infiltrating lymphocytes (TILs)(Fernandez-Poma et al., 2017; Forget et al., 2014; Forget et al., 2017; Hall et al., 2016). It has been demonstrated that T-iPSCs can be generated from human T-cells specific for melanoma epitope MART-1. These T-iSPCs were co-cultured on OP9/DL1 cells to generate $TCR\beta^{+}CD4^{+}CD8^{+}$ DP cells expressing TCR specific for the MART-1 epitope. Instead of using MHC-TCR ligation to induce positive selection, TCR stimulation of DP T-cells was achieved with anti-CD3 antibody. The resulting T-cells $CD8^{+}$ and were 95% were specific for MART-1 epitope and activated in an antigen-specific manner.

Using the OP9/DL1 co-culture method, Maeda et al. generated iPSCs from latent membrane protein 2 (LMP2) and WT1-specific human T-cells, an Epstein-Barr virus (EBV)-encoded antigen and an acute myeloid leukemia (AML) antigen respectively. The regenerated WT1-specific T-cells were functional and induced in vitro cytotoxic cell death of HLA-matched AML cells expressing WT1 protein and extended survival in mice inoculated with human leukemia cells. The cytotoxic response of these cells was comparable to the original LMP2-specific T-cells and did not exhibit have long-term tissue toxicity in mice[62].

5.2 | CHIMERIC ANTIGEN RECEPTOR T-CELLS FROM INDUCED PLURIPOTENT STEM CELLS

CAR-T-cell generation from mature T-cells currently involves labor-intensive engineering of autologous T-cells from each individual patent and limits their proliferative capacity *in vitro*(Thistlethwaite et al., 2017). To overcome this manufacturing roadblock, an alternative strategy is the generation of CD19 CAR-T from iPSCs. The T-iPSCs are expanded and transduced with a bicistronic lentiviral vector encoding 19–28z (1928z-T-iPSC), a second generation CD19 CAR. The 1928-T-iPSCs can then be cultured on an OP9-DL1 feeder layer to induce T-cell lymphoid differentiation. $CD3^{+}TCR\alpha\beta^{+}$ cells expressing the CD19 CAR are harvested and co- cultured on NIH-3T3-based artificial antigen-presenting cells (AAPCs) expressing CD19 antigen. The activated 1928z-T-IPSCs exhibited cytotoxic function in vitro, and in a humanized mouse model (Figure 6). By removal of alpha or beta chain coding regions, these cell lines may be modified to eliminate the endogenous TCR, thereby reducing the risk of development of graft-versus-host disease in an off the shelf product(Themeli et al., 2013).

6 | TRANSFECTION AND TRANSDUCTION METHODS FOR ENGINEERED T-CELLS

A major limitation to the widespread adoption of T-cell therapies that involve genetic modification, including CAR T-cells and engineered TCRs, is cell transfection or transduction(Varela-Rohena et al., 2008). Gamma-retroviral vectors, lentiviral vectors, and mRNA transposon/transposase systems, are used in the majority of edited T-cells using a

viral vector platform. In this section we will briefly review the established techniques and highlight advances in targeted gene editing methods for T-cell engineering.

6.1 – GAMMA RETROVIRAL TRANSFECTION

Gamma-retroviral vectors consists of a viral protein envelope with a virion core scaffold and transduce reverse transcriptase and mRNA encoding the gene in target cells. The Moloney murine leukemia virus (MLV), effectively reverse transcribes mRNA into DNA and subsequently incorporate it into the cell genome of T-cells in both mice and humans with high levels of transgene expression(Engels et al., 2003). Traditional gamma-retroviral machinery requires that cells be undergoing mitosis for successful transduction, therefore cells must be stimulated for transduction to occur and is unsuitable for transduction of HSPCs and other cells that must be maintained in a quiescent state. MLVs used in the treatment of severe combined immunodeficiency-X1 (SCID) has been linked to the development of leukemia due to insertional mutagenesis(Howe et al., 2008). Subsequent findings have suggested that MLV preferentially incorporates transgenes near promoter regions, potentially causing a higher incidence of oncogenic activation due to proto-oncogene mutagenesis than random insertions(Lewinski & Bushman, 2005; X. Wu, Li, Crise, & Burgess, 2003). Ongoing work seeks direct introduced genes away from promoter regions(Aiyer et al., 2014). As mature T-cells are capable of resisting oncogenic transformation, gamma-retroviral transduction remains a viable method for engineering mature T-cells.

6.2 – LENTIVIRAL TRANSFECTION

Lentiviral vectors have emerged as a common technique for cell transfection, and relative to gamma retroviruses, incorporates two additional components, the Rev protein and the cPPT sequence. These additional components improve nuclear export and import allowing lentiviral vectors to efficiently transduce non-dividing cells(Follenzi, Ailles, Bakovic, Geuna, & Naldini, 2000). This provides a significant advantage over gamma retroviral techniques as it allows for the incorporation of genes into intermitotic mature cells as well as in HSPCs without the need to potentially induce lineage commitment. Furthermore, incorporation into these slow cycling cells may improve the persistence of the edited cells(Milone & O'Doherty, 2018). Like with gamma-retroviral vectors there is a risk of oncogenic activation with lentiviral vectors. However, the incorporation of the reverse transcribed DNA with this technique does not exhibit a preference for promoter regions, but rather proximity to the edge of the nuclear envelope, theoretically reducing the possibility of cancer-inducing mutagenesis(R. W. Wong, Mamede, & Hope, 2015).

The separation of the genes encoding env and gag along with pol, during the expansion of gamma retroviral vectors in packaging cells mitigates some of the concerns about the production of self-replicating viruses. This process significantly reduces the probability of producing viruses containing all genes, thus limiting the production of self-replicating viruses. A similar method is used to produce replication inert lentiviral vectors, with the rev gene also being introduced separately(Dull et al., 1998).

6.3 NON-VIRAL TRANSDUCTION

While most T-cell engineering methods employ viral vectors, the length of development and approval process associated with creating new viral vectors in substantial. Therefore, the development of other non-viral techniques is an important research focus in T-cell engineering. One example is the Sleeping Beauty system, which incorporates predefined DNA sequences into the host genome(Ivics, Hackett, Plasterk, & Izsvák, 1997). Here, transposase, an enzyme cuts and "pastes" a gene flanked by a specific repeating pattern known as a terminally inverted repeat (TIR) allowing the introduction of plasmids encoding for the desired gene, surrounded by TIRs, into the cell without the need for a viral vector. The transposase can either be introduced as a protein, mRNA for translation, or a plasmid for transcription and translation. This method alleviates the potential risk of mutations that may be introduced during reverse transcription of viraus-based transfection systems. However the Sleeping Beauty system has a much lower rate of transfection compared to viral vectors as it relies on electroporation for DNA uptake (Singh et al., 2008; Yant et al., 2000). Additionally, by requiring multiple extra expansions the T-cells may lose some functionality.

A common limitation of all the above methods is the lack of site specificity in DNA incorporation, and therefore the potential risk of oncogenic mutagenesis. The use of methods that allow more site specific genome editing, such as with the use of clusters of regularly interspaced short palindromic repeats (CRISPR) and Transcription activator-like effector nucleases (TALENs) are a focus of ongoing research efforts(C.-A. M. Wu et al., 2018). Optimization of electroporation techniques has been shown to enable non-viral CRISPR-Cas9 genome editing(Roth et al., 2018). Both single and double stranded DNA segments of significant length may be incorporated in both $CD4^+$ and $CD8^+$ T-cells, and can result in enhanced function (Figure 7). TALEN has also been shown to allow for site specific gene editing, and may provide a pathway to "off the shelf" T-cells for ACT by removal of endogenous TCRs to mitigate the graft-versus-host effects that limit the use of allogeneic CAR T-cell therapies(Poirot et al., 2015).

7 – ENGINEERED PLATFORMS FOR ROBUST EXPANSION OF T-CELL THERAPIES

A key step in the widespread use of ACT T-cell therapies is the expansion of T-cells *ex vivo*. T-cell expansion is dependent upon presentation of a stimulatory and costimulatory signal, termed signal 1 and signal 2. *In vivo*, this is achieved via antigen presenting cells (APCs), such as DCs. In early clinical trials, T-cell expansion was achieved using autologous DCs isolated from patients to expand TILs. However, this method was found to be highly variable between patients, and DCs in cancer patients were found to be diminished in both number and function(Almand et al., 2000). In this section, we will review artificial APCs (aAPCs) and expansion platforms which seek to overcome these deficiencies by optimizing cell-culture conditions and presentation methods of the molecular activation signals for the expansion of T-cells.

7.1 – PARTICLE AND CULTURE-BASED EXPANSION OF T-CELLS

The most widespread method of T-cell expansion in vitro relies on stimulation of the TCR via anti-CD3 (αCD3) and costimulation with anti-CD28 (αCD28) along with culture in IL-2. The αCD3 and αCD28 may be plate-bound or presented to cells using alternative methods. The most common alternative method of presentation uses immobilized forms of the antibodies on beads and shows marked advantages over plate-bound methods. The majority of clinical trials use paramagnetic polystyrene microparticles that display both αCD28 and αCD3. Dynabeads (Life Technologies) are an example of this, and can be separated prior to transfer. Bead-bound systems more efficiently expanded $CD4^+$ T-cells when compared to plate bound systems (Y. Li & Kurlander, 2010), while expansion rates of $CD8^+$ T-cells did not differ significantly. These results also suggest that optimization of $CD4^+$ versus $CD8^+$ T-cell expansion may require different systems or that expansion may occur on different time scales. MACS GMP TransAct CD3/28 beads (Miltenyi) and MACS GMP ExpAct Treg beads for T-cell expansion (Miltenyi) are alternative industry standards for bead-based presentation. Of these, The ExpAct and Dynabeads require magnetic separation after culturing, while the TransAct system presents a biodegradable alternative, an attractive idea for expanding T-cells on a clinically relevant scale. Comparisons of these methods show similar levels of expansion(X. Wang et al., 2016).

Similarly, the expansion of antigen specific T-cells has been achieved using αCD28 and pMHC conjugated to paramagnetic beads and co-cultured with T-cells for 1 hour. The population is then sorted using a magnetic column, allowing for antigen-specific cell selection. This method may provide an advantage over antigen-specific TCR transduction or transfection as there is no risk of potential undesired immunogenic effects. Additionally, it minimizes dilution of the desired population via polyclonal expansion. Antigen specific populations expanded with this platform showed 1000-fold increase in the target population over the course of one week(Perica et al., 2015).

It has been demonstrated that the size and geometry of aAPCs also determine functionality. Larger beads (>300nm) loaded with pHMC and αCD28 were shown to induce over twice the expansion of smaller beads (500nm) expansion in CD8 T-cells over one week (Hickey, Vicente, Howard, Mao, & Schneck, 2017), though after a critical bead size the expansion rate stayed constant. However, magnetic induced clustering of smaller beads resulted in expansion comparable to larger beads, suggesting that multi-receptor ligation is necessary for inducing expansion. Based on this finding, a system using a combination of small beads expressing single ligands was developed to provide a modular platform for studying and optimizing T-cell activation(Kosmides, Necochea, Hickey, & Schneck, 2018).

Along with methods of signal presentation, culture conditions play a role in T-cell expansion and in promoting T-cell function. It has been demonstrated that the VIP antagonist VIPhyb and PI3K-delta inhibitor idelalisib prevent terminal differentiation of T-cells during expansion. The cells cultured with these factors had lower cell exhaustion markers and increased T-cell function(Petersen et al., 2018). Development of serum free media for T-cell expansion also shows promise for improving the scalability of ACT therapies(Medvec et al., 2018).

7.2 – BIOMIMETIC AND CELL-BASED EXPANSION OF T-CELLS

The rigidity of the presentation platform also affects T-cell expansion rate(Lambert et al., 2017). Supporting this finding is an approach that examined improving T-cell expansion by creating biomimetic scaffolds that presented both survival and activation signals in a way that more closely resembled in vivo interactions. To achieve this, microscale mesoporous silica rods were electrostatically coated sequentially with IL-2 followed by a lipid membrane, which was then embedded with anti-CD3 and anti-CD28. The electrostatic adsorption of the IL-2 provided a prolonged release of IL-2, and the lipid membrane mimicked the physical conditions of membrane-bound signal presentation of found on DCs. These constructs displayed a significant improvement relative to Dynabead-induced expansion, with a formulation-dependent $CD4^+$ to $CD8^+$ ratio, to selectively expand T-cell subpopulations (Figure 8) (Cheung, Zhang, Koshy, & Mooney, 2018)

Another platform for expansion are irradiated K562 human erythroleukemic cell line derived from a patient with chronic myelogenous leukemia. K562 cells do not exhibit endogenous expression of HLA molecules, or CD1d and thereby prevent the expansion of allospecific T-cells that may cause GvHD upon transfusion and establish an effective immunological synapse(Klein et al., 1976). (Butler et al., 2007). These cells may be genetically modified to express desired HLA molecules as well as the immunostimulatory motifs to promote antigen specific T-cell expansion. K562 cells may also be modified via lentiviral transfection to stably express multiple costimulatory ligands to facilitate T-cell expansion(Suhoski et al., 2007).

8 | CONCLUSIONS

The advances in engineering molecular pathways to drive the development and function of T-cells have greatly enhanced the repertoire of cell-based therapies for with improved clinical outcomes in oncology, which in turn have driven the rapid evolution of engineering strategies. For all engineered T-cells, the genetic engineering, differentiation, selection and expansion of the clinical product have common complexities associated with manufacturing. Therefore, the development of technologies to generate optimal cellular components will increase safety, efficacy and reproducibility while decreasing costs associated with manufacturing. The rise of T-cell therapies has incentivized cell-based manufacturing platforms and the establishment of standards driven by the formation of consortia such as CCRM (http://ccrm.ca), NIIMBL (http://www.niimbl.us), CMaT (http://cellmanufacturingusa.org) and the involvement of industry(Kaiser et al., 2015). These and other initiatives have identified the significant challenges to achieve better clinical efficacy with T-cell engineering strategies and these will be briefly reviewed below.

The early clinical successes of genetically engineered T-cells have been accomplished by obtaining PBMC-derived T-cells from patients without further fractionation, which is likely a significant source of heterogeneity in clinical products. Studies in preclinical models have shown that engineering T-cells selected from naïve and central memory T-cell subsets, or expanding naïve T-cells while inhibiting T-cell differentiation, generate T-cells with superior engraftment, proliferation and anti-tumor effects after adoptive transfer(Gattinoni, Klebanoff, & Restifo, 2012). These observations suggest that the potency of engineered T-

cells may be improved if therapeutic products were prepared from purified subsets with superior activity in preclinical models and formulated uniformly for infusion to the patient. Here, strategies to manipulate T-cell differentiation in favor of specific T-cell types that might better counteract tumor cells have been considered. By exposing T-cells to cytokines such as IL-7, IL-15 and IL-21 prior to adoptive T-cell transfer to drive T-cell differentiation, gene-engineered T-cells that have a central memory phenotype, prolonged peripheral persistence and potent antigen reactivity have been generated(Kaneko et al., 2009; Pouw et al., 2010). An alternative approach is focused on the direct selection, isolation and transfer of specific genetically modified $CD8^+$ T-cell populations(Hinrichs et al., 2009). However, a combined $CD4^+$ and $CD8^+$ T-cell response might provide a therapeutic advantage and that selecting single-cell populations might risk limiting therapeutic efficacy as discussed above.

Although less effort has been put into learning how to control the amplitude and timing of T-cell activity, such regulatory capabilities are now appreciated as increasingly important in light of the observed clinical toxicities. Toxicity induced by TCR-engineered is difficult to predict due to the large size of the human proteome expressed on the genetically heterogeneous MHC complex in humans makes this a daunting task. Better methods to screen such T-cells for reactivity against self antigens are needed to test for off-target recognition of engineered TCRs(Tannock & Hickman, 2016). In addition, engineered heterodimeric TCRs may potentially pair with the endogenous TCR chains, creating novel specificity for unknown targets and remain a serious concern. One approach to obviate this risk is the use of various gene-editing technologies to ablate the endogenous TCR(Provasi et al., 2012).

As a key component of TCR-engineered T-cells, the identification of patient specific tumor antigens will determine the applicability of TCR-engineered T-cells for any patients. Better algorithms to identify potential neoantigens have expanded potential targets for some types of cancers. However, the available TAAs for poorly immunogenic cancers are still very limited. Clinical experience suggests that targeting multiple antigens can potentially elicit stronger antitumor immune responses. Therefore methods to identify as well as induce the generation of neoantigens to potentiate cell-based immunotherapy will be an important focus area for future work. For example, the induction of immunogenic cell death is widely recognized to potentiate other forms of immunotherapy and may be a means to broaden the potential targets for engineered T-cells.

The functional activation and proliferation of T-cells is determined not only by interactions between the T-cell and its target but also by T-cell co-stimulatory signals. Solid tumors often present antigens in the absence of co-stimulatory ligands, which can result in exhausted T-cells with reduced proliferative capacity and effector function(Capece, Verzella, Fischietti, Zazzeroni, & Alesse, 2012). Alternatively, the tumor environment might induce an upregulation of T-cell co-inhibitory molecules, which compromise tumor-specific T-cell responses. Both TCRs and CARs are being developed with a signaling cassette that harbors a co-stimulatory molecule that should provide a stimulatory trigger to the T-cell even when one is not provided by tumor cells. Alternatively, prior to transfer into patients, T-cells can be stimulated *ex vivo* with human artificial antigen-presenting cells that express co-stimulatory ligands; this process has the potential to improve function *in vivo*.

T-cells are a potent form of immunotherapy for treating the complex pathologies that are found in cancer, autoimmunity and infectious disease. The continued advancement of molecular engineering tools to manufacture safer and more potent engineered cells is anticipated to generate new life-saving drugs for multiple diseases with a substantial unmet therapeutic need.

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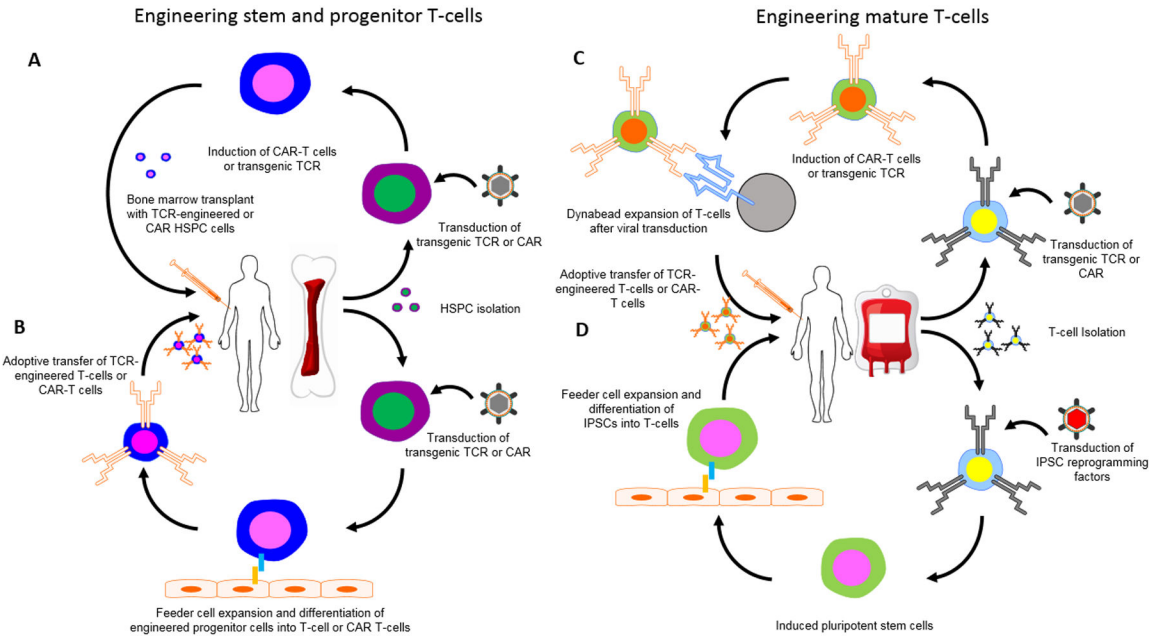

Fig. 1. Methodologies for T-cell Engineering.

Examples of T-cell manufacturing techniques for generating immunotherapies derived from hematopoietic stem and progenitor cells (HSPCs) or mature T-cells. (A) HSPCs are transduced with viral vectors coding for transgenic T-cell receptor (TCR) or chimeric antigen receptor (CAR). Transduced HSPCs are expanded *ex vivo* and intravenously administered to a conditioned patent resulting in sustained *in vivo* differentiation into TCR-transduced T-cells or CAR T-cells. (B) Isolated HSPCs are transduced with viral vectors coding for transgenic TCR or CAR-T and differentiated on feeder cell layer allowing for *ex vivo* differentiation of TCR-transduced T-cells or CAR-T cells for adoptive immune transfer. (C) Isolated mature T-cells are transduced with viral vectors coding for transgenic TCR or CAR. TCR transduced T-cells or CAR-T cells are subsequently expanded for adoptive immune transfer. (D) Isolated mature T-cells are transduced with viral vectors coding for pluripotent stem cell reprogramming factors. Induced pluripotent stem cells (ISPCs) are expanded and seeded onto feeder cell layer to induce T-cell differentiation for adoptive immune transfer.

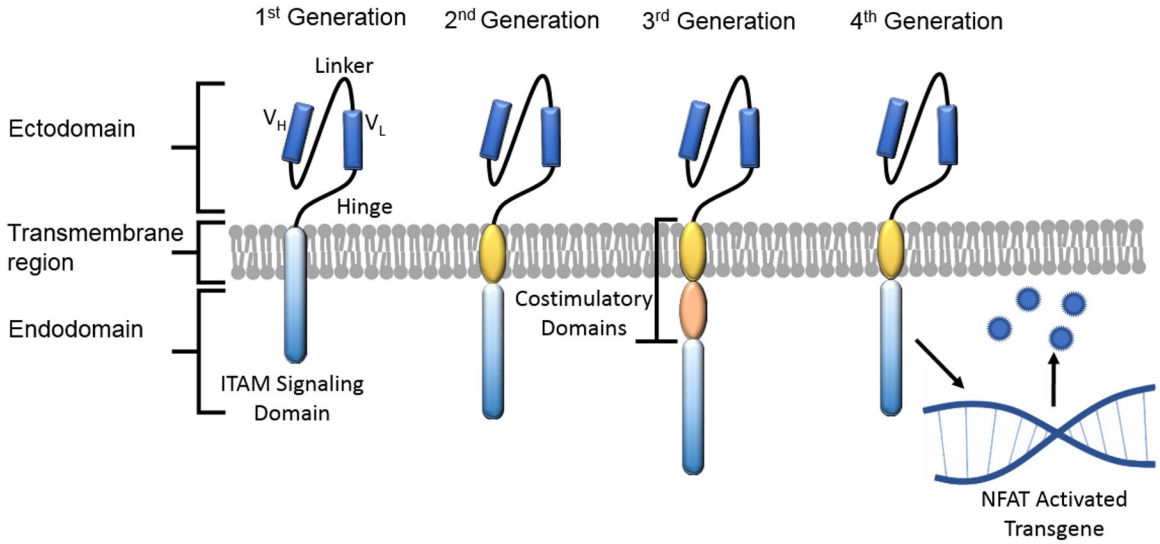

Fig. 2. Design of CAR T cells.

T cells can be redirected to have specificity for tumors by the introduction of chimeric antigen receptors (CAR) proteins. CARs targeting is controlled via an extracellular ectodomain that is comprised of light and heavy variable regions (VL and VH) from an antibody bound together with a peptide linker and attached to the transmembrane region with a hinge peptide. The endodomain controls intracellular signaling and activation and is comprised of conserved modules. First-generation CAR endodomains use the CD3-ζ ITAM, whereas second generation CARs include one costimulatory domain and third generation CARs contain multiple costimulatory domains. Fourth generation CARs termed “TRUCKs” that include an inducible pathway for the expression of a transgenic product.

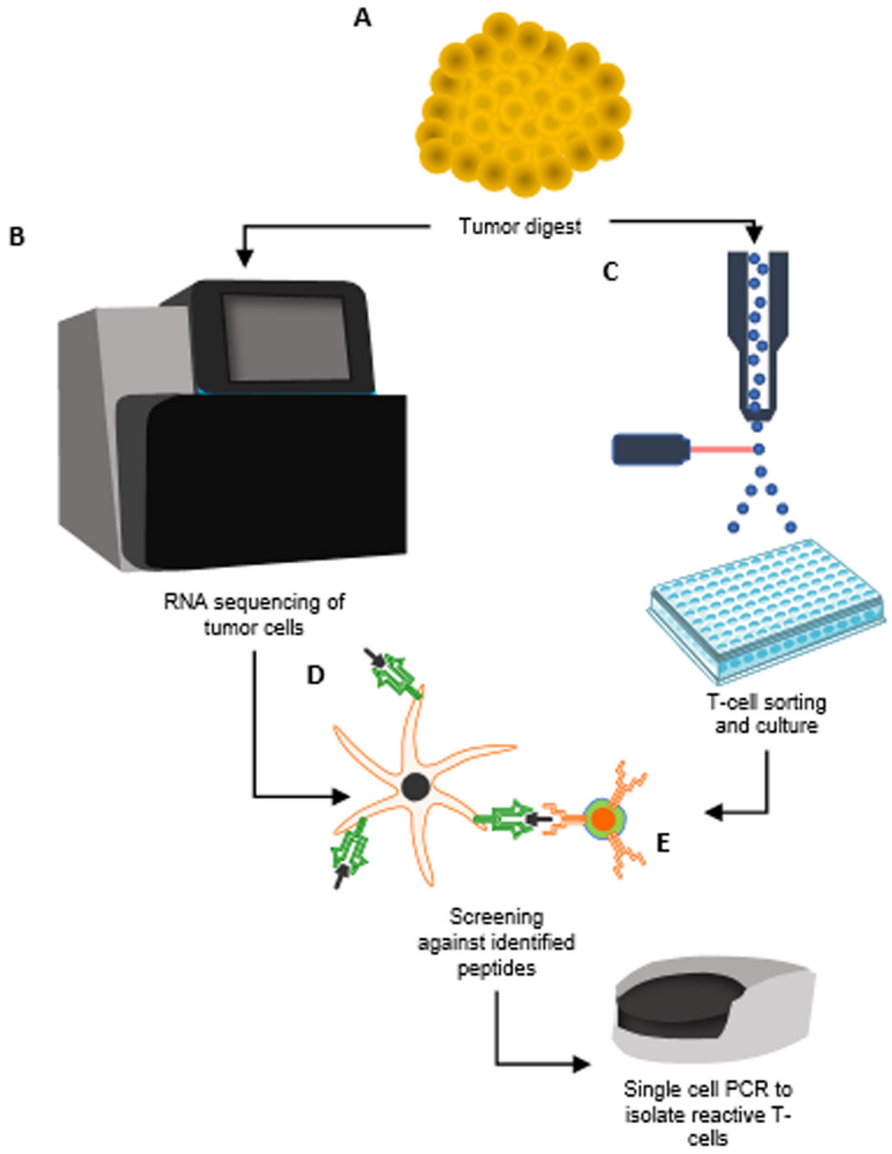

Fig. 3. Schematic illustration of novel high-throughput approach for enrichment, culturing, and screening strategy of TILs.

(A) Tumor cell digests were thawed and rested overnight in complete media in the absence of exogenous cytokines. (B) A piece of the tumor underwent whole-exome sequencing (WES) and RNA sequencing to identify nonsynonymous mutations. Based on mutation calls, 25mer peptides encompassing the mutations at position 13 were synthesized. (C) Cells were washed, labeled, and sorted based on PD-1 and/or activation markers (CD134 or CD137) expression. Sorted cells were cultured in 96-well plates at 3 cells/well in the presence of irradiated allogeneic feeder cells, 3,000 IU/ml IL-2, and anti-CD3ε (OKT3) for expansion. (D) Peptide pools were pulsed on autologous APCs that served as a target in a coculture with sorted cells that grow in the microwell cultures. To minimize the assays, cells from 2 or 3 cultures were combined in the assay wells. (E) Cells from coculture assay were labeled and reactive T cells were single-cell sorted into 96-well plates containing lysis buffer

and PCR primers for TCR sequencing. Adapted from Lu, Y.-C. et al. (2018). *Molecular Therapy*, *26*(2), 379–389.

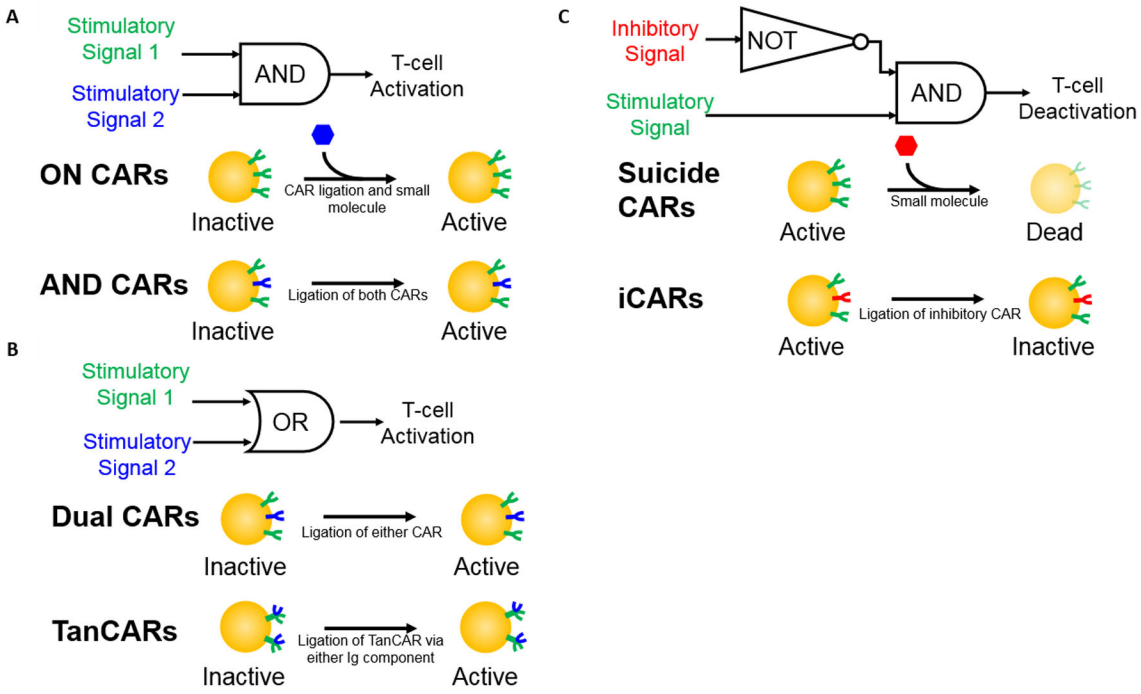

Fig. 4. Data showing function of ON-switch CAR demonstrating antigen-specific and titratable killing of target cell population by engineered primary cytotoxic ($CD8^+$) T cells.

Schematic illustrating the variety of control mechanisms for CAR T-cells as well as their respective mechanisms for activation or deactivation. (A) ON CARs and AND CARs replicate AND gated Boolean logic, requiring multiple signals to initiate T-cell function, thereby improving specificity. (B) Dual CARs and TanCARs replicate OR gated Boolean logic, requiring one or both signals for T-cell activation to prevent tumor escape. (C) Suicide CARs and iCARs replicate Boolean logic in which a there is NOT gate on the inhibitory signal followed by an AND gate. This means that the T-cells will only function in the condition where there is stimulatory signal with no inhibitory signal. iCARs may be used to prevent activation when encountering epitopes found on healthy tissue, while suicide CARs represent the possibility of eliminating transferred T-cells after therapy via the administration of a small molecule.

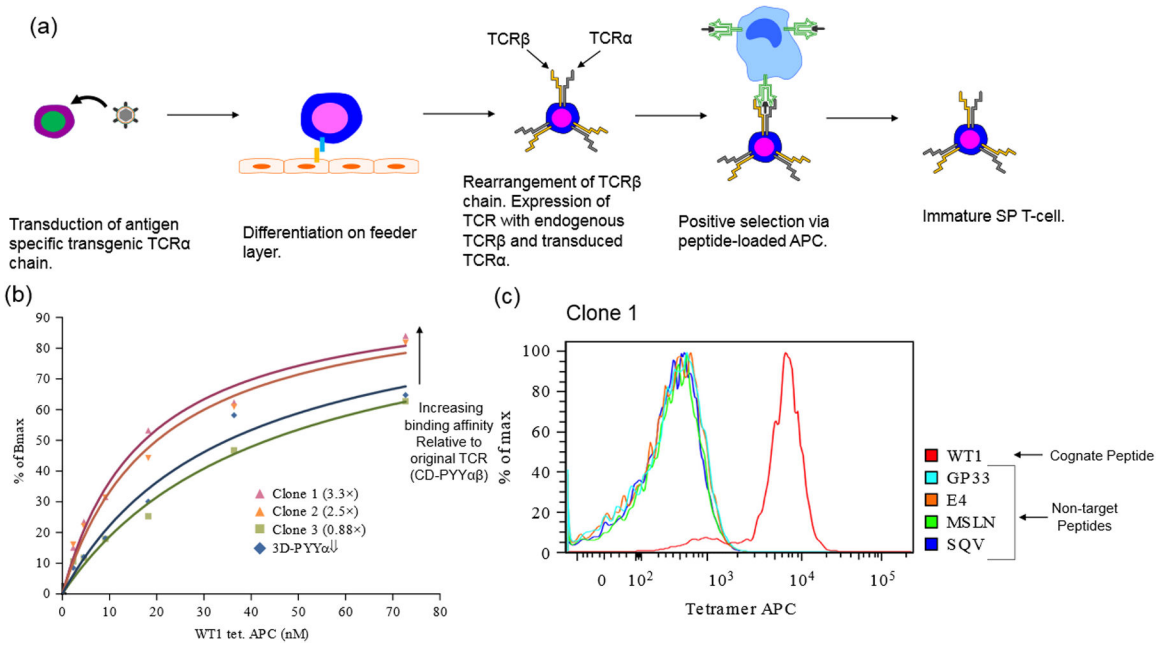

Fig. 5. Generation of Higher Affinity TCRs.

(A) Schematic depicting transduction of antigen specific TCRα chain into T-cell progenitor cells and differentiation into SP T-cells after interaction with peptide loaded APC. Only TCRα chain coding region is transduced into progenitor cells allowing for normal rearrangement of TCRβ coding region. (B) The newly generated TCRs with endogenous TCRβ and transduced TCRα are subsequently screened for relative affinity for the cognate peptide by titrating the amounts of peptide/MHC tetramer and analyzing by flow cytometry. The relative change in affinity compared to the parental TCR is listed in parenthesis. (C) To measure off-target reactivity to a subset of antigens, Clone#1 from (B) is stained with WT1 specific tetramer, as well as several non-specific H-2Db tetramers.

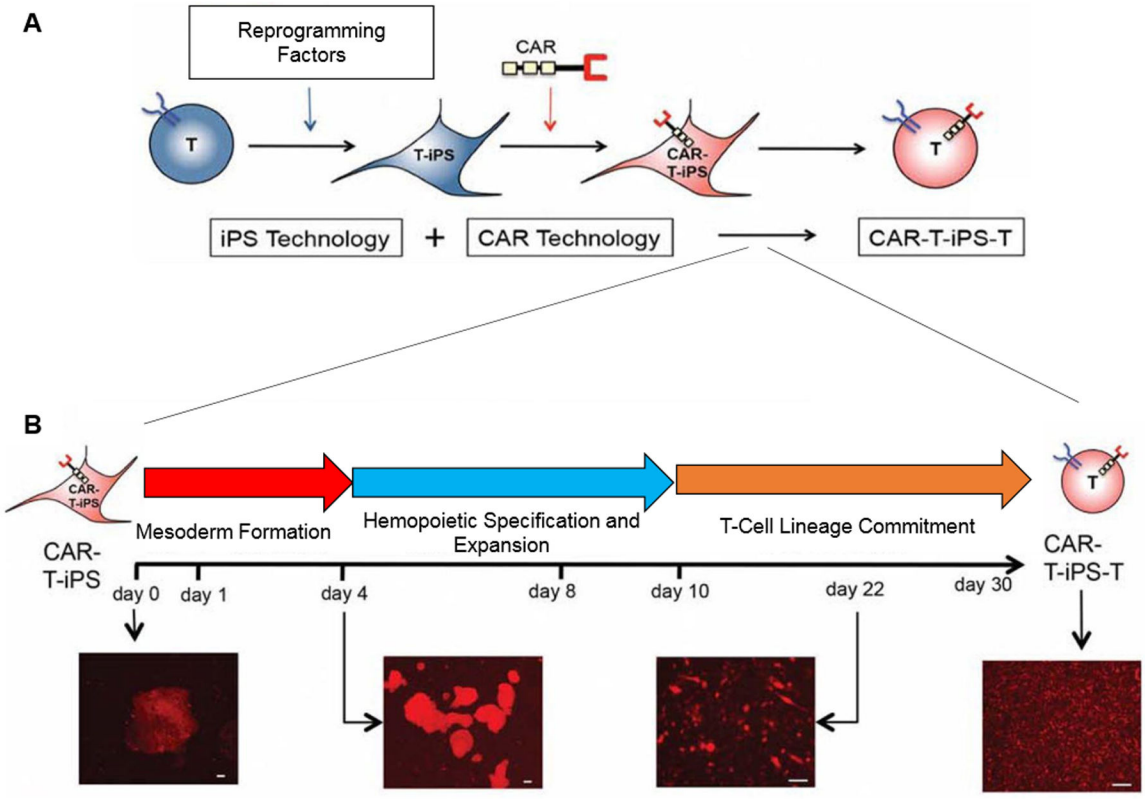

Fig. 6. Reprogramming of peripheral blood T-cells into CAR-T-iPSC-T Cells.

(A)Schematic of study. Peripheral blood lymphocytes are reprogrammed to pluripotency by transduction with retroviruses encoding reprogramming factors c-MYC, SOX2, KLF4 and OCT-4. The resulting T-iPSCs are genetically engineered to express a CAR and are then differentiated into T cells that express both the CAR and an endogenous TCR. (B) In vitro lymphoid differentiation protocol. T-iPSCs were stably transduced with a lentiviral vector encoding the 19–28z CAR and the fluorescent marker mCherry. Differentiation in three steps: (i) mesoderm formation (days 1–4), (ii) hematopoietic specification and expansion (days 5–10) and (iii) T-lymphoid commitment (days 10–30). Fluorescence microscopy images (below) show mCherry expression was maintained throughout the differentiation process. Scale bars=100 μM.

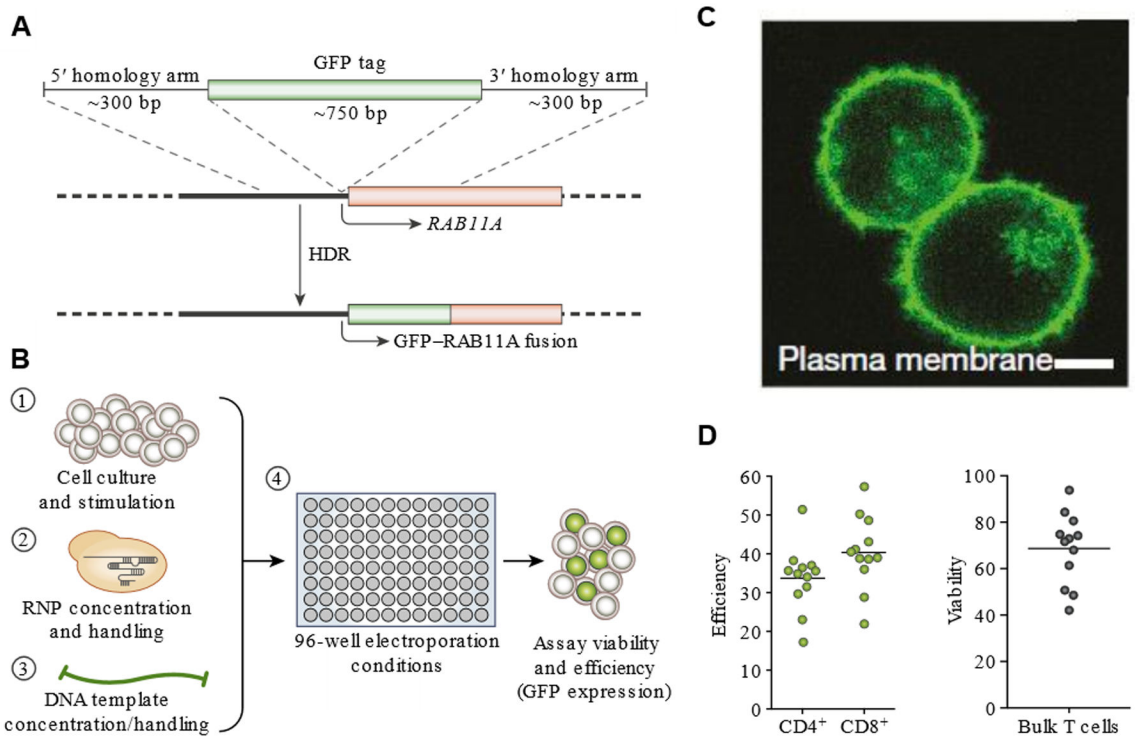

Fig. 7. Data and methodology for optimization of electroporation based incorporation of CRISPR/Cas9 and DNA for gene editing.

(A) Schematic illustrating CRISPR/Cas9 integration of a GFP fusion tag to the housekeeping gene *RAB11A*. (B) Conditions considered and strategy used for development and optimization of non-viral genome targeting for both cell viability and HDR efficiency. (C) The ability to target multiple sites was confirmed by inserting a GFP fusion tag into various endogenous genes using non-viral targeting in primary human gated $CD4^+$ and $CD8^+$ T cells using HDRT, HDR template. (D) Average efficiency with the RAB11A–GFP HDR template was 33.7% and 40.3% in $CD4^+$ and $CD8^+$ cells, respectively. (E) Viability (number of live cells relative to non-electroporated control) after non-viral genome targeting averaged 68.6%. Efficiency and viability were measured 4 days after electroporation. Mean values of $n = 12$ independent healthy donors are shown (horizontal bars, d, e). Adapted from Roth TL et al. (2018) *Nature* 2018, 559:405 with permission from Springer Nature.

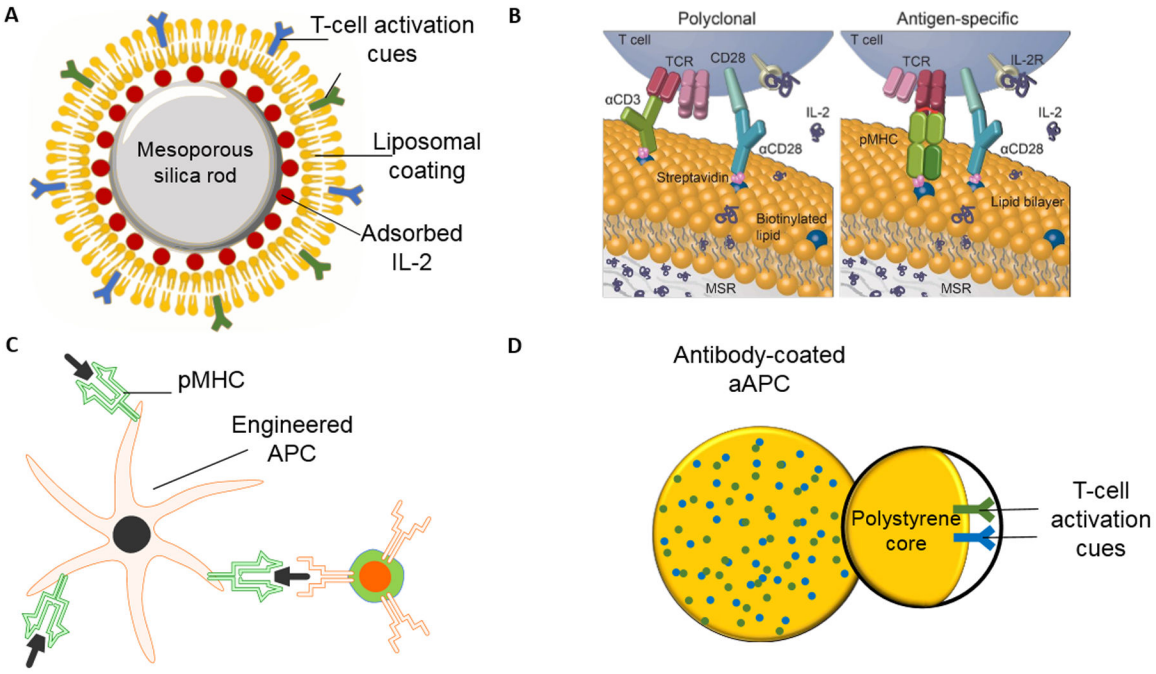

Fig. 8. Expansion of T-cells using antigen presenting cell mimic scaffolds.

(A) Schematic showing a cross-sectional view of the biomimetic scaffold components. Notably, a liposomal coating allows the APC-ms to better mimic natural antigen presentation. (B) T-cell activation cues used to prepare APC-ms were either anti-CD3 and anti-CD28 or pMHC and CD28 allowing for both polyclonal and antigen specific expansion. (C) Illustration of cell based antigen presentation for T-cell expansion. K562 may be engineered to express a variety of additional surface ligands to enhance expansion. (D) Illustration of a Dynabead aAPC. Beads are generally coated with anti-CD3 and anti-CD28 to promote T-cell expansion and require administration exogenous IL-2 to achieve optimal expansion. Adapted from Cheung et al. (2018) *Nature biotechnology* 36:160 with permission from Springer Nature