CHAPTER 4

# T Cell Engineering and the Rise of CAR-T Cell Therapies

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## INTRODUCTION

Immunotherapy is transforming the treatment of cancer. Although many approaches are currently under development to harness the immense cytotoxic potential of the immune system for therapeutic purposes, genetic engineering of T cells with a chimeric antigen receptor (CAR) to direct this potential towards cancer represents one of the most exciting advances in the field of immunotherapy. In this chapter, we review the history of this nascent field that culminated with the first FDA approval of a genetically engineered T cell therapy in 2017 and discuss some of the early lessons learned from the development of these therapies.

Like most innovation, the development of CAR-T cell therapy is a chimera of sorts, blending three initially separate fields: adoptive cell therapy, immunology and gene transfer technology (Fig. 4.1). Only when these three fields matured sufficiently were scientists able to create a successful CAR-T cell therapy. In turn, CAR-T cell therapies have also become useful tools in furthering research which are contributing the design and development of cellular therapies even beyond T cells.

## ADOPTIVE CELL THERAPY

The motivating idea behind CAR-T cell therapy, that the human immune system fights cancer, seems obvious now, but was controversial for much of the 20th century. The observation in 1917 by J.C. Mottram and Sidney Russ that rats are capable of rejecting rat sarcoma tumours provided some of the earliest evidence of immune-mediated rejection of a tumour (Mottram and Russ, 1917), and attempts to immunise patients to their own tumour, removed, irradiated and reinjected, occurred as early as 1922 (Kellock et al., 1922). Nevertheless, a review was published 7 years later, now quoted repeatedly, stating 'It would be as difficult to reject the right ear and leave the left ear intact as it is to immunize against cancer' (Rosenberg, 1999; Woglom, 1929). Similar conflicting

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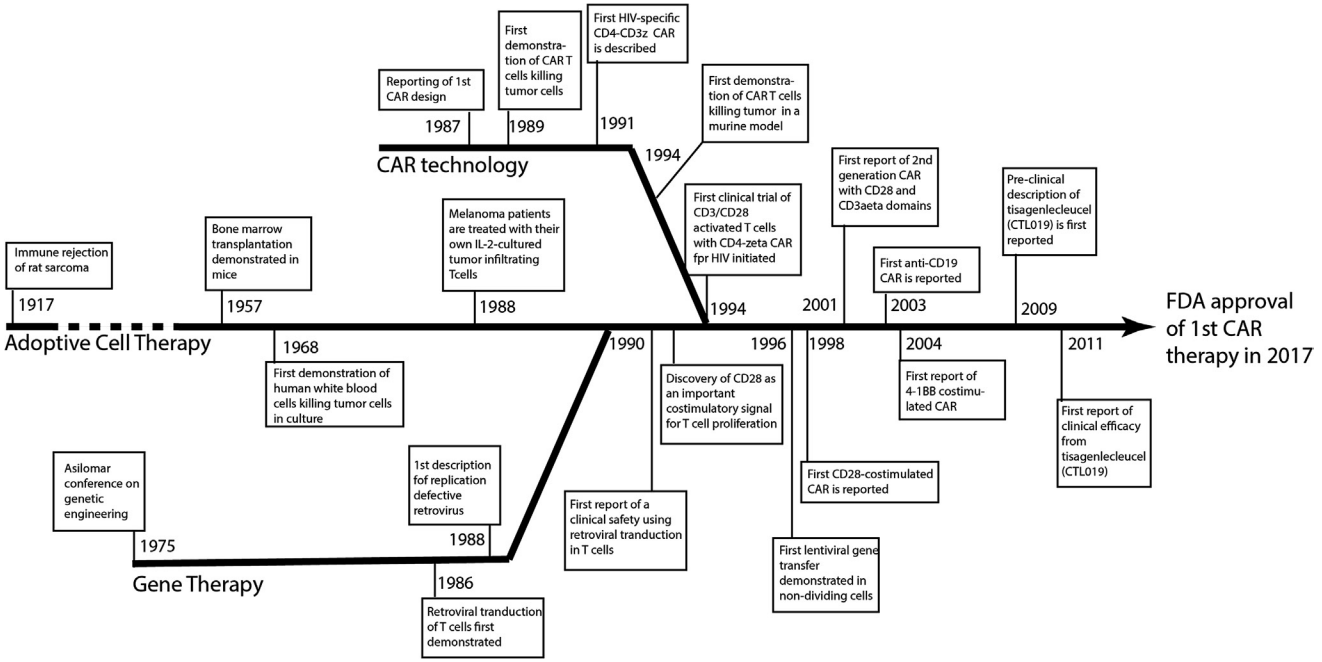


**Figure 4.1** T cell Engineering Timeline.

conclusions were drawn, and the field shifted from infusing patients with irradiated tumours to bone marrow in support of chemotherapies and radiation.

Bone marrow transplantation initially seemed to solve a problem created by radiation therapy: how to deliver enough chemotherapy to destroy a cancer without killing the patient. Early studies of bone marrow transplantation in mice demonstrated that bone marrow from a genetically identical mouse could be used to repopulate the full repertoire of cells of the blood (Barnes et al., 1956). Intriguingly, marrow from donor mice exposed to a tumour given to recipient mice was more successful in maintaining the recipient mice cancer-free than marrow from an unexposed donor (Barnes and Loutit, 1957). Yet, nearly 20 years later, an analysis of allogeneic bone marrow transplantation in 1975 concluded that 'the data on allogeneic marrow transplantation for hematologic neoplasia permit no conclusion about the existence or absence of an antileukemic effect of graft-versus-host disease…' (Thomas et al., 1975). Efforts to ameliorate graft-versus-host disease by depleting T cells found in the donor bone marrow ultimately highlighted the role of immune cells in treating cancer and foreshadowed future approaches focused on T cells (Goldman et al., 1988).

A patient's own immune cells became the next therapy, beginning an approach most resembling that used for CAR-T cell therapies available today. Patient-derived leukocytes were first observed to kill human tumours in the laboratory setting as early as 1968 (Hellström et al., 1968a,b). These reports and others successfully demonstrated cellular immunity to tumour cells, but without mechanisms for target recognition and the means to generate enough T cells in the laboratory to repeat experiments, and ultimately infuse therapeutic doses into patients, progress was hampered.

The first technique for sustaining leukocyte growth in the laboratory came from the cells themselves. In 1978, Steven Rosenberg at the Surgery Branch of the National Institutes of Health reported that his group successfully grew human killer T lymphocytes in the laboratory for over 90 days using media from cultures of activated white blood cells (Strausser and Rosenberg, 1978). Rosenberg and his team then applied the culture technique to lymphocytes derived from patients with melanoma, observing that these cells grown in the purified factor from the previous experiments, 'T cell growth factor', now known as IL-2, killed tumour cells from those patients in vitro (Lotze et al., 1981). Applying this approach of combining lymphocytes with IL-2, either grown in the laboratory or coadministered to patients, in a multitude of clinical scenarios, began in 1988 with the treatment of melanoma (Rosenberg et al., 1988). Patients in this study had tumour infiltrating lymphocytes (TILs) extracted from their tumours that were expanded ex vivo and then reinfused along with IL-2. Tumours regressed in 9 of 15 patients in this study, but in only one of those patients tumours did not recur in less than a year (Rosenberg et al., 1988). Although Rosenberg's group correctly postulated that a multitude of variables impact the efficacy of TIL therapy (Griffith et al., 1989), they identified a key challenge in achieving successful tumour clearance a year later: T cell persistence

(Rosenberg et al., 1990). Unlike the initial solution to expanding T cells in the laboratory, the approaches to enhancing T cell persistence in patients came from interactions between T cells and their targets.

T cells require at least two signals to drive a sustained response. The first one, through the receptor that defines the T cell itself, the T cell receptor (TCR), alone causes antigen inexperienced (i.e., naïve) T cells to become nonresponsive, a process termed anergy. To achieve optimal activation and proliferation of these T cells as well as promote their differentiation towards functional effector cells or long-lived memory cells, T cells require a second signal that is delivered through a plethora of proteins known as costimulatory receptors. These receptors are termed as such because, unlike the primary signal from the TCR, activating them alone has little effect on T cells (Gmünder and Lesslauer, 1984). Together, these signals drive T cells to replicate 20 to 30 times or more as well as make large quantities of growth factors such as IL-2 (Gmünder and Lesslauer, 1984; June et al., 1987, 1990). Much of the research in T cell biology during the 1970s and 1980s was focused on understanding these receptors, largely, away from the world of cancer. Some of the earliest efforts to apply the new understanding of TCR and costimulation were directed towards HIV infection. Carl June at the University of Pennsylvania and his team had investigated the role of the second signal mediated by CD28 for its activity against HIV (Levine et al., 1996). June and his team not only found that activating human T cells from HIV positive patients using antibodies to both the TCR and CD28 rendered CD4+ T cells resistant to HIV infection (Levine et al., 1996) but also found that this combinatorial technique could be used to grow huge numbers of human T cells in the lab (Levine et al., 1998). These observations led to adoptive cell therapy clinical trials using T cells expanded using either natural antigen-presenting cells with ligands for T cell costimulatory receptors or artificial scaffolds (e.g., magnetic polymer microbeads) coated with agonist antibodies to the TCR/CD3 complex (signal 1) and CD28 (signal 2).

Treatments consisting of polyclonal T cells activated using agonist antibodies to both the TCR/CD3 and CD28 signals and then infused back into patients had mixed results. The first trial reported using peripheral blood-derived, polyclonal T cells expanded with both TCR/CD3 and CD28 stimulation. Despite the ability of these T cells to kill tumour cells and produce cytokines in vitro, infusion of these cells had no measurable impact on tumours in vivo (Lum et al., 2001). Although activated autologous T cell therapy suffers from a paucity of cancer cell–specific T cells, this approach could enhance immune responses in cancer patients especially to vaccines in the postautologous haematopoietic stem cell (HSC) transplant setting (Rapoport et al., 2005). A phase I clinical trial using allogeneic, donor T cells in patients having received bone marrow transplants for various cancers of haematopoietic origin demonstrated that clinical responses could be achieved with this approach (Porter et al., 2006) However, toxicity from graft-versus-host disease produced by alloreactive T cells present in the activated T cell product created significant

challenges to applying this therapeutic strategy, thus highlighting the importance of antigen specificity.

To enhance the likelihood of enriching for tumour-specific T cells, similar techniques to those pioneered by Rosenberg and his team at the National Cancer Institute (NCI) were used to expand T cells in the laboratory for cancer therapies (Curti et al., 1993; Rosenberg et al., 1988, 1985). These techniques involved isolating T cells derived either from resected tumours or peripheral blood, activating them through the TCR and adding IL-2, then reinfusing them into the patient with subsequent injections of IL-2 (Curti et al., 1993, 1998; Rosenberg et al., 1988). Deep and durable clinical results can be achieved in a range of cancers by these methods, most notably in melanoma (Radvanyi, 2015). Unfortunately, isolating or obtaining T cells with specificity towards cancer remains a major challenge. An appreciable fraction of patients treated with TILs experience targeting of noncancer tissues, most notably melanocytes in melanoma trials leading to the autoimmune syndrome of skin depigmentation termed vitiligo. Similar off-tumour toxicity in healthy tissues has presented significant challenges even when using cloned TCRs that are introduced into T cells (Johnson et al., 2009; Linette et al., 2013; Morgan et al., 2013). Strategies to isolate tumour-specific T cells using next generation sequencing to identify neoantigens arising from tumour-specific mutations offer the potential of further selection to focus on T cells with tumour specificity, and these approaches have yielded remarkable results in patients (Bethune and Joglekar, 2017); however, these bespoke approaches are complex, labour-intensive and difficult to implement in a timely fashion. Maintaining cancer specificity while avoiding normal tissue targeting therefore remains a major hurdle for the field of T cell immunotherapy. Nevertheless, the impressive results from TIL therapy encouraged a great deal of interest and research into ways to control T cell specificity for therapeutic purposes.

## CHIMERIC ANTIGEN RECEPTORS

CARs were first built to better understand the TCR. The first such effort was made in 1987 in Japan by Kuwana et al., who created a CAR by combining the DNA encoding heavy and light chains of an antibody targeting phosphorylcholine with that encoding the intracellular domains of the alpha and beta chains of the TCR (Kuwana et al., 1987). Using a CD4+ T cell line transfected with various combinations of the antibody and TCR components, T cell activation could be demonstrated on mixing with bacteria coated in phosphorylcholine, but not with bacteria lacking phosphorylcholine (Kuwana et al., 1987). A similar design was used by an American collaboration led by Stephen Hedrick at the University of California at San Diego (Becker et al., 1989). In these studies, a transgenic mouse line expressing a chimera of the heavy chain of an antibody targeting digoxin and the cytoplasmic domain of the TCR alpha chain demonstrated that splenocytes from transgenic mice proliferated and produced IL-2 in response to digoxin

coupled to BSA, confirming that this chimeric receptor associated with the rest of the components of the TCR to produce a functional TCR/CD3 complex signal to T cells (Becker et al., 1989).

The first group to demonstrate that a CAR could direct T cells to kill a cell coated in the target of the antibody component of the CAR was that of Zelig Eshhar at the Weizmann Institute of Science in Rehovot, Israel, in 1989 (Gross et al., 1989). Transfected mouse T cell hybridomas expressing a CAR composed of the variable fragments of an antibody targeting the hapten 2,4,6-trinitrophenyl (TNP) linked to the alpha or beta chain of the TCR killed cells from a mouse B-cell lymphoma line only when coated in TNP, but also produced IL-2 in response to TNP coupled to proteins (Gross et al., 1989). Importantly, this work demonstrated that chimeric immunoreceptors could direct a T cell to kill an antigen-coated target cell independent of antigen presentation.

Although altering the specificity of the TCR/CD3 complex is a viable approach to engineering T cell antigen specificity, the complexity of engineering this multisubunit receptor complex led to efforts to produce simpler receptors capable of achieving the same end result. In 1991, Bryan Irving and Art Weiss at the University of California at San Francisco created a chimera of the extracellular and transmembrane domains of CD8, a coreceptor normally expressed as a dimer, and the intracellular domain of the zeta chain, thereby dissociating it from the rest of the TCR but maintaining its dimeric form (Irving and Weiss, 1991). Expressing this CAR in the Jurkat human T cell line, these investigators demonstrated that anti-CD8 antibodies could activate TCR-like signalling suggesting that the zeta chain of the TCR/CD3 complex was sufficient even in the absence of the rest of the TCR (Irving and Weiss, 1991). Published in the same issue of the journal *Cell*, Charles Romeo and Brian Seed at Harvard and Massachusetts General Hospital used a similar CAR construction approach to direct T cells to kill their first disease-causing target: HIV (Romeo and Seed, 1991). In addition to the zeta chain, Romeo and Seed used a splice variant of the zeta chain, called the eta chain, and the gamma chain of the $F_c$ receptor each in combination with the extracellular domain of CD4, the coreceptor used by HIV to initiate target cell binding and subsequent viral entry. A human CD8+ T cell line, transduced using a vaccinia viral vector to express one of the three CARs described above, successfully killed HeLa cells transfected to express the HIV envelope protein gp120/41 (Romeo and Seed, 1991). This work formed the basis for the first clinical trial of a CAR in HIV that combined ex vivo expanded, CD28-costimulated T cells, which are transiently resistant to HIV infection, with a CD4-zeta-chain CAR that was introduced into the T cells by a retroviral vector (Mitsuyasu et al., 2000). This study not only demonstrated the safety of the approach but also established that adoptively transferred, gene-marked T cells could persist for over a decade offering the tantalising possibility of generating long-lived T cell 'memory' through adoptive T cell therapy (Scholler et al., 2012). Soon after the CD4-zeta and CD8-zeta CARs were described, Eshhar and his team published the first combination

of a single chain variable fragment (scFv) with the zeta, eta or gamma chains to produce a functional CAR with controlled antigen specificity. Although these studies utilised TNP, which is not a target normally found on cells, this CAR became the basis for what is currently termed a 'first-generation' CAR design (Eshhar et al., 1993). Eshhar and his team would go on to publish not one, but two functional 'first-generation' CARs directed towards two different tumour-associated antigens, the alpha-folate receptor and Her2 [ERBB2] (Hwu et al., 1993; Stancovski et al., 1993). Together, these groups established a parallel approach to constructing a chimeric immunoreceptor with a far simpler design that forms the basis for the signalling domain used in nearly all CARs at present.

Engineering T cells to kill tumour cells in vitro progressed to attempts at using these 'first-generation' CAR-T cells to eradicate tumours in mice. The first group to publish such an attempt was that of Bernd Groner in 1994 (Moritz et al., 1994). In these studies, a mouse CD8+ T cell line was retrovirally transduced with a zeta chain-based CAR targeting the ERRB2 antigen. Although these CAR-T cells were able to kill ERBB2-expressing cells specifically and produce interferon gamma when mixed with them in vitro, they were only marginally effective at reducing tumour size in mice (Moritz et al., 1994). Similar attempts were made by Rosenberg and his colleagues using the same $F_c$-gamma chain CAR targeting the folate receptor described by Hwu et al. (1993), but transduced into mouse T cells extracted from colon adenocarcinoma tumours in mice instead of T cell lines. In spite of these T cells being able to reduce the number of lung metastases formed by the injection of mouse fibrosarcoma cells overexpressing the folate receptor, they were only able to marginally lengthen the survival of immunocompromised mice injected with human ovarian cancer cells that overexpress the folate receptor (Hwu et al., 1995). These early failures in mouse models with 'first-generation' CARs would ultimately prove predictive of human clinical trials (Deeks et al., 2002; Kershaw et al., 2006; Park et al., 2007; Till et al., 2008) despite contradictory evidence from a report using a zeta-chain CAR targeting ERBB2 in a mouse breast cancer model (Altenschmidt et al., 1997), and another demonstrating that a zeta-chain CAR was superior to a gamma chain CAR in a colon cancer model (Haynes et al., 2001). The solutions to this difference in efficacy of 'first-generation' CAR-T cells in experimental models and human trials would be found by those studying the fundamental biology of T cells.

The first report of a fundamental challenge to the 'first-generation' CAR design came as focus increased on the nature of the cell into which the CAR was being introduced. Thomas Broker and Klaus Karjalainen of the Basel Institute for Immunology used a transgenic mouse model in which they were able to express a zeta-chain CAR targeting a component of the human TCR in primary mouse T cells. This model allowed them to test the function of the CAR without previously activating the T cells as done for viral vector-based transduction or having to use an immortalised T cell line that can be transfected for gene delivery. They demonstrated that cross-linking the CAR with

antibodies, as done to activate the CAR in prior studies, produced no T cell activation as measured by proliferation or calcium flux in otherwise quiescent primary naive T cells (Brocker and Karjalainen, 1995). Consistent with previous work, however, these T cells were observed to become activated in response to CAR cross-linking when previously stimulated through their endogenous TCRs (Brocker and Karjalainen, 1995). The observation that previously unactivated or antigen 'naïve' T cells cannot be activated by signals from the zeta chain alone would be critical to the development of the first successful design of a CAR for human therapies. These studies implicated second signals through costimulatory receptors as being crucial to promoting the activation and proliferation of primary T cells.

CD28 costimulation promotes efficient T cell activation (Gmünder and Lesslauer, 1984), even in the presence of immunosuppressive agents (June et al., 1987), enhances tumour killing by T cells (Townsend and Allison, 1993) and prolongs T cell survival (Boise et al., 1995), all of which would help address the inadequacies of the first-generation zeta-chain CARs. The first CD28 CAR-like construct was generated by Luis Alvarez-Vallina and Robert Hawkins at the Centre for Protein Engineering in Cambridge, England (Alvarez-Vallina and Hawkins, 1996). Created by fusing the intracellular and transmembrane domains of CD28 with an scFv targeted to either the NIP or phOx haptens, these CD28 CAR signals combined with those from a zeta-chain CAR with the same scFv consistently drove Jurkat T cells transfected with these constructs to produce more IL-2 compared with signals delivered by the first-generation CAR alone and similar amounts as of IL-2 secreted on costimulation through the natural CD28 receptor (Alvarez-Vallina and Hawkins, 1996). Focused once again on the cell into which the CAR is transduced, Michel Sadelain and his team at the Memorial Sloan Kettering Cancer Center showed similarly enhanced IL-2 production as well as enhancement of cell viability and growth by an scFv-CD28 fusion transduced into primary human T cells (Krause et al., 1998). These first fusions of CD28 with an scFv laid the groundwork for improvements in CAR design that addressed the shortcomings of first-generation CARs.

Shortly after reports of successful T cell activation by scFv-based fusion proteins separately containing the signalling domains of the zeta chain and the costimulatory molecule CD28, the creation of the first fusion protein containing both CD28 and the zeta-chain signalling domains in a single chimeric protein was reported (Finney et al., 1998). The first of these 'second-generation' CARs were created by Neil Weir and his colleagues at Celltech Therapeutics in Slough, United Kingdom. This group empirically evaluated a range of different CAR designs that combined an scFv targeting CD33, a haematopoietic marker targeted in acute myeloblastic leukaemia, with a linker from either the IgG1 antibody or a piece of the extracellular domain of CD28, the transmembrane and intracellular domain of CD28 and the zeta chain (Finney et al., 1998). Only CARs bearing an intracellular domain of CD28 closest to the membrane enhanced IL-2

secretion by Jurkat cells relative to CARs with the zeta chain alone. Albeit limited in scope and conducted in a cell line instead of in primary human T cells, these first studies opened the floodgates to developing and evaluating CARs with a multitude of costimulatory domains (van der Stegen et al., 2015).

## VIRAL VECTOR-BASED GENE TRANSFER

Although several CAR designs had been developed that are capable of activating and driving T cell proliferation, much of the research evaluating these CAR architectures utilised either murine T cells or immortalised T cell lines, most notably Jurkat cells. Methods for gene transfer into primary human peripheral blood-derived T cells lymphocytes were fairly inefficient, and vectors continued to retain large portions of the original viral genome raising important safety concerns. It was in the arena of viral gene transfer that critical bridges were crossed to enable the efficient and safe engineering of primary human cells, a prerequisite for generating therapeutic CAR-T cells.

The origins of gene transfer predate 1975, but this is the year in which the rules governing all future gene transfer research and development were set (Berg et al., 1975). Berg et al. summarise the conclusions reached by those gathered at the Asilomar Conference on DNA molecules. They conclude that the temporary ban in existence at the time on research involving the production of new DNA be lifted. The conference participants felt that there was too much to be learned not to continue with such research, but that significant and evolving safeguards would need to be put in place to prevent potential catastrophes resulting from such work. Although they state that they could not see any application for recombinant DNA at the time, they aptly conclude that 'there is every reason to believe that they [new techniques in recombinant DNA methodology] will have significant practical utility in the future' (Berg et al., 1975). So began the 40-year effort to develop the first FDA-approved gene therapy.

Although there were attempts to achieve gene delivery to T cells by using many different viral vectors, retroviruses have become the mainstay for stable insertion of genes into T cells. Retroviruses, which are RNA-based viruses, are capable of efficiently integrating their proviral DNA genomes into the host cell through a process that uses both viral and host cell factors (Bishop, 1981). The first successful demonstration of retroviral gene transfer used a Rous sarcoma virus-derived vector to deliver the thymidine kinase gene of herpes simplex virus into rat and chicken cells (Shimotohno and Temin, 1981). Similar viruses were quickly found to be able to deliver genes to immortalised human fibroblast cells (Miller et al., 1983), mouse HSCs (Joyner et al., 1983) and most importantly for the purpose of making CAR-T cells, human HSCs (Hock and Miller, 1986) and immortalised T cells (Kantoff et al., 1986). Yet all of these efforts used vectors that encoded most or all of the virus from which they were derived, making them capable of continuous infection, replication and possible oncogene activation.

The construction of replication-deficient viral vectors, a major safety feature required for these vectors, was a multistep process. David Baltimore's group at the Whitehead Institute for Biomedical Research in Cambridge, Massachusetts, discovered that deleting part of the genome of the Moloney murine leukaemia virus, an early retroviral vector and the precursor to the murine stem cell virus vector used in one of the commercial CAR-T cell therapies today, rendered it incapable of producing viral particles that contained its own genome (Mann et al., 1983). Unfortunately, replication-competent virus would sometimes be spontaneously generated by vectors of this design through recombination events (Miller and Buttimore, 1986). Although an effort was made to correct this defect using the same overall design (Miller et al., 1986), the most effective method found to ensure the absence of replication-competent virus production was to split the viral genes responsible for reverse transcription and integration from that responsible for envelope production (Markowitz et al., 1988). This split genome approach became the standard method for nearly all retroviral-mediated gene transfer.

In parallel to innovations in safety, multiple changes were made to retroviral vectors to broaden the range of cell types these vectors could efficiently transduce, which was key to extending these vectors to T cells. The first combination of a replication-incompetent retrovirus with a broadened range of targets was created in 1984 by Roger Cone and Richard Mulligan at the Massachusetts Institute of Technology. Cone and Mulligan exchanged the Moloney virus gene coding for native envelope protein with a similar gene from a virus capable of infecting cells from a multitude of species including humans (Cone and Mulligan, 1984). This vector was then combined with the replication-incompetent retrovirus created a year prior (Mann et al., 1983), thereby creating amphotropic viral vectors.

Changing the tropism, or the targeting of the viral vector, enabled transduction of the wide range of cell types previously mentioned and made possible the first human clinical trial of retrovirally transduced T cells. Armed with retroviral vectors capable of transducing human T cells, Rosenberg and his group at the NCI performed the first safety (Cornetta et al., 1990) and feasibility (Rosenberg et al., 1990) trials treating patients with retrovirally gene-modified TILs. TILs of five patients diagnosed with metastatic cancers were transduced with a gene that conferred the TILs with resistance to the antibiotic neomycin as a marker of successful gene transduction. Even though none of the patients were cured, the observation that transduced TILs were detected in tumours and peripheral blood for months after administration constituted an important proof of concept (Rosenberg et al., 1990). From this trial, retroviral gene transduction of human T cells for therapeutic use was established as safe and feasible, but transduction efficiency remained low. Efficient production of genetically engineered T cells would require additional enhancements to these vector systems.

Enhanced efficiency of T cell transduction was achieved by combining several strategies. Virus production was substantially enhanced through the development of optimised

viral production systems developed by Margo Roberts and her group at Cell Genesys in San Francisco, California (Finer et al., 1994; Roberts et al., 1994; Tran et al., 1995). These efforts led to high efficiency protocols for producing large quantities of viral vector. Altering the tropism of the vector to that of the vesicular stomatitis virus (VSV) by use of the VSV-g envelope glycoprotein broadened the tropism of these vectors to allow infection of nearly all mammalian cell types including T cells (Gallardo et al., 1997). T cells, an unusually difficult cell type to transduce, were also found to be substantially more susceptible to transduction when activated and proliferating (Hagani et al., 1999; Koehne et al., 2000; Pollok et al., 1999). Long-term gene expression due to methylation and subsequent silencing of the retrovirally transferred gene was also noted (Challita and Kohn, 1994). Numerous approaches have been described to improve gene expression and safety through modification of the promoters, insulator and enhancer sequences within the vector (Cooray et al., 2012).

In parallel, HIV researchers had developed replication-deficient HIV-based vectors, known as lentiviral vectors. These vectors belong to the same family of viruses as the retroviruses discussed previously, in that they both carry their genomes as RNA, and utilise reverse transcription followed by stable integration of a proviral DNA genome into the host cell as part of their natural life cycle. However, a distinguishing feature of lentiviral vectors is their ability to infect and transduce nondividing cells (Lewis et al., 1992; Naldini et al., 1996). Didier Trono and his collaborators at the Salk Institute in La Jolla, California, in collaboration with Dr. Mulligan at the Whitehead Institute, demonstrated the ability of a lentiviral vector to transduce cell lines arrested in the first phase of the cell cycle with either the gene encoding beta-galactosidase, the enzyme responsible for breakdown of lactose, or luciferase, an enzyme capable of causing light to be emitted on breakdown of its substrate, luciferin (Naldini et al., 1996). They found their lentiviral vector to be nearly 20-fold more efficient at transducing nondividing cells compared with retroviruses. This finding seemed to be confirmed by Nobuku Uchida and his collaborators at SyStemix in Palo Alto, California, who found that lentiviral vectors could transduce nondividing human haematopoietic cells (Uchida et al., 1998). As T cells in circulation are not dividing, with the exception of those responding to an infection, lentiviral transduction of T cells seemed to be a promising method to transduce peripheral blood T cells for therapeutic use.

Although target cells do not require the dissolution of the nuclear envelop for lentiviral gene integration, lentiviral vectors still appeared to require T cell activation to achieve efficient and stable transduction of primary human T cells (Korin and Zack, 1999; Sonza et al., 1996; Zack et al., 1990, 1992). As discussed previously, retroviral vector transduction also requires T cells to be activated and proliferating. This lesson enabled groups working with lentiviral vectors to use previously developed technologies to successfully apply lentiviral technology to stably transduce primary human T cells (Barry et al., 2000; Unutmaz et al., 1999). By the end of the 1990s and the start of the 21st

century, stable gene transfer into human primary T cells seems to have been achieved, but two key safety events involving gene therapy forced all researchers in the field to proceed with caution.

The first event involved the death of a patient in a Phase I trial of an adenoviral vector for the gene correction of ornithine transcarbamylase deficiency. The patient, Jesse Gelsinger, had an unanticipated and ultimately fatal immune reaction to the viral vector itself, illustrating the importance of considering vector immunogenicity in gene therapy trials as well as selection of appropriate subjects for first-in-human studies (Stolberg, 1999). The second event was the development of T cell leukaemia in a patient with X-linked severe combined immunodeficiency disease who received HSC transduced with a retroviral vector encoding a functional form of the common gamma chain. The leukaemia in this patient, and eventually several more patients in this trial and others using retroviral vectors, appears to have arisen through the selective integration of the retroviral vector into the genome in proximity to the LMO-2 oncogene, the expression of which became dysregulated by the powerful enhancers used in the retroviral vector (Howe et al., 2008). Although neither of these events occurred in the context of direct gene transfer into T cells, the latter case being one in which HSCs were transduced, further efforts were taken to ensure the safety of gene therapy products, and these events arguably slowed the development of gene therapies.

## IT ALL COMES TOGETHER

By the early 2000s, all three fields, adoptive cell therapy, CARs and viral gene transfer, had developed to a point that allowed researchers to efficiently construct successful CAR-T cells for cancer therapy. Most groups were focused on CARs that employed the CD28 costimulatory domain in a second-generation design (Hombach et al., 2001; Maher et al., 2002; van der Stegen et al., 2015). Many of these designs targeted antigens on solid tumours. Identifying suitable antigens for targeting remains one of the most challenging problems faced by researchers in the field. Michael Jensen's group at the City of Hope Medical Center and the Fred Hutchinson Cancer Research Center postulated that directing T cells specifically to leukaemia cells would be a potent technique based on the success of bone marrow therapy, but would spare the patient the toxicities of graft-versus-host disease (Cooper et al., 2003). Cooper et al. created a 'first-generation' CAR targeted to CD19, a universal B-cell marker and one present on nearly all leukaemia cells of B-cell origin (Cooper et al., 2003). These human anti-CD19 CAR-T cells efficiently killed CD19-expressing leukaemia cells in culture. As was the case with all previous 'first-generation' CARs, this CAR would be replaced with more effective 'second-generation' CARs (Brentjens et al., 2007; Kochenderfer et al., 2009; Kowolik et al., 2006; Milone et al., 2009).

In somewhat of a twist, the first 'second-generation' anti-CD19 CAR to achieve regulatory approval did not contain CD28 costimulation. Although CD28 is an important

costimulatory receptor for T cells, 4-1BB (also known as TNFSFR9 or CD137) can also provide costimulation to T cells and, unlike CD28, belongs to the family of type II, non-death domain containing tumour necrosis receptors. 4-1BB can substitute for CD28 costimulation in T cells, especially CD8+ T cells (DeBenedette et al., 1997; Saoulli et al., 1998), limit T cell death from activation (Hernandez-Chacon et al., 2011) and was found to be critical for CD8+ T cell memory (Wang et al., 2009). Dario Campana and his team at the St. Jude Children's Research Hospital in Memphis, Tennessee, demonstrated that a CD19-specific CAR constructed with the 4-1BB cytoplasmic domain were better killers of leukaemia cell blasts (Imai et al., 2004). Our group at the University of Pennsylvania incorporated this CAR into an optimised lentiviral vector and combined this construct with a well-established microbead-based T cell culture platform developed in the early CD4-zeta CAR trials in HIV. We demonstrated that CD19-specific CAR-T cells that employ a 4-1BB costimulatory domain are more effective than a CD28-costimulated, second-generation CARs in humanised mouse models of primary acute lymphoblastic leukaemia (Milone et al., 2009). Based on these results, we chose to pursue a 4-1BB-costimulated second-generation CAR for clinical development, forming the foundation for the regulatory approval of tisagenlecleucel (CTL019, Kymriah).

## TRANSFORMING T CELL THERAPIES INTO A COMMERCIAL REALITY

For a long time, treating cancer with T cell therapies was relegated to primarily the academic settings where this therapeutic approach was viewed as an interesting boutique therapy that was unlikely to achieve widespread success or commercialisation. This view of the T cell immunotherapy world changed dramatically in 2011 with the publication of data from the first three patients treated with what is now tisagenlecleucel (Kalos et al., 2011; Porter et al., 2011). The demonstration that CAR-T cell therapies could produce deep and durable remission of otherwise refractory chronic lymphocytic leukaemia sparked a great deal of excitement around engineered T cell therapy. This impressive clinical activity was replicated by several groups using similar, but distinct CAR-T cell designs culminating with the FDA approval of two of these therapies, tisagenlecleucel and axicabtagene ciloleucel in 2017. Since the entry of Novartis Pharmaceuticals (Basel, Switzerland) into the first commercial alliance with the University of Pennsylvania centred around CAR-T cell immunotherapy in 2012 (2012), the field of T cell immunotherapy has undergone a major transformation. More than 350 CAR-T cell therapies are now under development from preclinical to late state clinical development, driven by actors well beyond academia including highly capitalised pharmaceutical companies (e.g., Gilead, Celgene, Janssen) and biotechnology companies (e.g., Juno Therapeutics, Bluebird Bio) (Yip and Webster, 2018). The interest is also global with more than half of CAR-T cell trials occurring outside of the United States including more than 200 listed on clinicaltrials.org in China as of September 2018.

Despite the success in achieving regulatory approval, many barriers to commercial success still remain for the CAR-T cell technology to overcome. The manufacture of autologous T cell therapies is currently complex and labour-intensive, thereby significantly raising costs. Improvement in the manufacturing of these therapies is bound to occur over time as is typically observed in economies of scale and economies of learning. There are now commercial incentives to develop dedicated manufacturing equipment and systems to retrofit what was primarily repurposed from other fields for use in T cell manufacturing (Levine et al., 2017). Reducing ex vivo culture is one approach that is actively being pursued to reduce cost as it increases capacity of manufacturing facilities and reduces growth medium needs. Data from our group suggest that it may also improve the quality of T cells in these therapies (Ghassemi et al., 2018). Changing the methods used for genetic engineering, such as transposon-based gene delivery (Monjezi et al., 2016; Singh et al., 2008) or gene-editing with homology-directed repair (Eyquem et al., 2017), offers the potential to eliminate the need for viral vectors, which have their own challenges to manufacturing (Chen, 2015).

The development of 'universal' donor approaches to CAR-T cell therapies represents one of the most exciting areas of research in the field as such allogeneic CAR-T cell products hold the potential to replace the complex manufacturing of autologous therapies with an 'off-the-shelf' product. CAR-T cell products generated using allogenic donor T cells, which are gene-edited to eliminate the endogenous TCR and otherwise manufactured using similar approaches to autologous therapies, are the most clinically advanced (Ren et al., 2016). Although TCR disruption can reduce or eliminate the risk of potentially fatal graft-versus-host disease that arises from alloreactive T cells in the donor, immune recognition of the donor cells by the host still represents a major barrier to engraftment and persistence. Gene-editing approaches may solve this problem such as that of Cellectis to disrupt CD52 to make the CAR-T cells resistant to the highly immunosuppressive antibody, alemtuzumab(Poirot et al., 2015). Perhaps the most exciting manufacturing solution lies in the potential to generate a virtually endless supply of allogeneic donor T cells from induced pluripotent stem cells (Themeli et al., 2013).

A continuing challenge for the commercialisation of new CAR therapies is that of targeting. Although CD19 has proven to be a largely successful target for B-cell leukaemias, its benefits and drawbacks are illustrative of the barriers new CAR developers are working to overcome. A critical aspect to the success of CD19-targeted therapies is that CD19 is universally expressed in nature on all B cells, including malignant B cells (Nadler et al., 1983; Uckun et al., 1988). This universal expression constitutes a double-edged sword, allowing CAR-T cells to target all of the malignant cells in patients with B-cell leukaemias and lymphomas, yet also mediating one of the central toxicities of anti-CD19 CAR therapy: B-cell aplasia (Kalos et al., 2011). Both aspects of this paradigm become challenges for other potential CAR targets, especially on solid tumours. Potential CAR targets on solid tumours are rarely universally expressed across the entire tumour (Newick

et al., 2016). Equally worrisome is that the normal tissues that also express these targets cannot have their biological functions replaced as can that of B cells. A number of strategies are being pursued to address these issues of tumour targeting and potential on-target, off-tumour toxicities. To broaden CAR-T cell targeting, CAR-T cells that target multiple antigens are under development, allowing them to kill tumours that have a number of associated targets that are not universally expressed throughout the tumour (Wilkie et al., 2012). To improve CAR-T cell safety, new systems biology approaches are being applied to enhance the specificity of CAR-T cells. One such approach employs conditional 'AND gates' in which a synthetic notch receptor targeting one nonexclusive tumour-associated antigen induces the expression of a CAR targeting another tumour-associated target (Roybal et al., 2016). This two-step process allows the CAR-T cell to only kill cells that have both antigens, while potentially sparing normal tissues that might have one of the two targets. Finally, in spite of the ubiquity of CD19 expression on B-cell malignancies prior to CAR therapy, 60% of relapses post CAR therapy are CD19 negative, necessitating the identification of new targets for B-cell malignancies, such as CD20 (Martyniszyn et al., 2017; Till et al., 2012; Zah et al., 2016), and prompting the adoption of combinatorial approaches during initial CAR-T cell therapy, such as the addition of ibrutinib to CAR-T therapy for patients with chronic lymphocytic leukaemia (Fraietta et al., 2016). In spite of the challenge of targeting remaining for the field of CAR-T cell therapeutics, a multitude of new approaches are in development to produce safe and effective products.

## PERSPECTIVES

The theme throughout the history of CAR-T cells, like most areas of science, is that few of the key ideas that led to the first FDA-approved therapies were recently conceived, but rather have been long in the making. The idea that the immune system can attack cancer can be traced back to as early as ancient Egypt (Rosenberg, 1999). Cellular therapy in the form of bone marrow transplantation was practiced as early as the 1950s. Transferring genetic material into cells was already widespread enough in 1975 that an entire conference was convened on how to ethically and safely continue such work (Berg et al., 1975). This molecular biology allowed for the development of synthetic genes like CARs, which were themselves initially conceived in the 1980s. In addition, the massive investment in HIV research in the 1980s enabled the underlying advances in understanding of the immune system. The massive investment in HIV research in the 1980s also enabled significant advances in our understanding of the immune system as well as the initial testing ground for many of the technological advances used in CAR-T cell therapies like lentiviral vector-based gene delivery (Levine et al., 2006).

The success of CAR-T cell therapy in B-cell malignancies has clearly invigorated the wider field of cellular therapy by demonstrating that these complex treatments can reach regulatory approval and be commercialised. It has also paved the way for other

engineered cellular therapies with promise in treating diseases well beyond cancer, such as autoimmunity (Bluestone et al., 2015; Ellebrecht et al., 2016), transplantation (Hoeppli et al., 2016; Lu et al., 2016) and genetic diseases (Thompson et al., 2018). The major investments that were already made in the field of cell therapy, and continue to be made at a high level, are also having a major effect on the field by drawing in new investigators and technology, which may solve some of the many remaining challenges and create a new generation of cytotherapies. Only time will reveal the ultimate place of CAR-T cell therapy within history. However, it seems clear that CAR-T cell therapies have already helped make the future of cell therapy brighter and have brought hope to patients suffering from previously untreatable diseases.

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