Molecular Therapy
Review

Chimeric Antigen Receptors: A Cell and Gene Therapy Perspective

Isabelle Rivière[1] and Michel Sadelain[1]

[1]Center for Cell Engineering, Molecular Pharmacology and Immunology Programs, Memorial Sloan Kettering Cancer Center, New York, NY 10065, USA

Chimeric antigen receptors (CARs) are synthetic receptors that reprogram T lymphocytes to target chosen antigens. The targeting of CD19, a cell surface molecule expressed in the vast majority of leukemias and lymphomas, has been successfully translated in the clinic, earning CAR therapy a special distinction in the selection of "cancer immunotherapy" by *Science* as the breakthrough of the year in 2013. CD19 CAR therapy is predicated on advances in genetic engineering, T cell biology, tumor immunology, synthetic biology, target identification, cell manufacturing sciences, and regulatory compliance—the central tenets of CAR therapy. Here, we review two of these foundations: the genetic engineering approaches and cell types to engineer.

Chimeric antigen receptors (CARs) are synthetic receptors that target T cells to a chosen antigen and reprogram T cell function, metabolism, and persistence.[1,2] Through their extracellular domain, CARs bind cell surface molecules independently of major histocompatibility complex (MHC), in contrast to the physiological T cell receptor, which engages MHC/peptide complexes. CARs may thus target proteins, carbohydrates, or glycolipids and function irrespective of patient HLA haplotype. Binding to antigen triggers T cell activation, which is commonly mediated by the cytoplasmic domain of the CD3-ζ chain.[3–7] Merely providing T cell activation is, however, not sufficient to direct a productive immune response.[8–10] The CARs that have provided tangible clinical benefits incorporate a costimulatory domain,[11] which enables T cells to expand and retain their functionality upon repeated exposure to antigen.[12] These receptors have been dubbed second generation CARs[13] and are key to the design of persisting engineered T cells that constitute a "living drug" attacking tumors as long as they retain their functionality. Several recent reviews have addressed CAR design,[14–18] CAR prospects for solid tumors,[19,20] and T cell manufacturing.[21–23]

The targeting of CD19,[24] a cell surface molecule expressed in the vast majority of leukemias and lymphomas, has been successfully translated in the clinic, earning CAR therapy a special distinction in the selection of "cancer immunotherapy" by *Science* as the breakthrough of the year in 2013.[25] The genesis of CD19 CAR therapy is predicated on the convergence of scientific advances in genetic engineering, T cell biology, tumor immunology, synthetic biology, target identification, cell manufacturing sciences, and regulatory compliance, all of which were needed to enable innovative phase 1 clinical trials (Figure 1). We review here the vectors and cells that are the foundations of present and upcoming CAR therapies.

The Vectors

γ-Retroviral and Lentiviral Vectors

The implementation of T cell engineering begins with forging suitable tools to genetically modify primary lymphocytes. The first successful attempts made use of ecotropic γ-retroviral vectors to transduce mitogen-activated mouse T cells (M. Sadelain and R.C. Mulligan, 1992, Int. Congr. Immunol., abstract). This approach was subsequently adapted to human T cells, incorporating the gibbon ape leukemia virus (GALV) envelope to mediate retroviral vector entry.[26–30] These advances were pivotal for launching mouse and human T cell engineering, which had been hitherto limited to transfection of surrogate leukemia cell lines or hybridomas, which do not recapitulate several critical features of normal T cell proliferation, function, and survival. Receptors and signaling molecules could now be studied in authentic T cells easily harvested from peripheral blood. Retroviral vectors would eventually be the first to be evaluated in the context of T cell-based therapies[31–38] and continue to be relied upon in CAR therapy to this day.[39–42]

Retroviral vectors derived from murine leukemia virus require that the target cells divide to allow proviral integration.[43,44] This property was eventually exploited to preferentially transduce cycling T cells within mixed T cell populations.[45] In contrast, lentiviral vectors are able to successfully infect nondividing cells, owing to the nuclear translocation capability of the HIV-1 preintegration complex.[46–49] Lentiviruses, however, require that non-dividing cells proceed at least to the G_{1b} stage of the cell cycle to support reverse transcription and allow for completion of retroviral integration.[50] Although lentiviral vectors have been reported to transduce cytokine-activated T cells apparently without S phase progression,[51,52] lentiviral vectors are commonly used like γ-retroviral after in vitro activation of T cells.[53–55]

γ-retroviral vectors and lentiviral vectors both integrate semi-randomly in the human genome, with similar preference for transcribed genic regions but with some differences (near transcriptional

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Correspondence: Michel Sadelain, Center for Cell Engineering, Memorial Sloan Kettering Cancer Center, New York, NY 10065, USA.
E-mail: m-sadelain@ski.mskcc.org

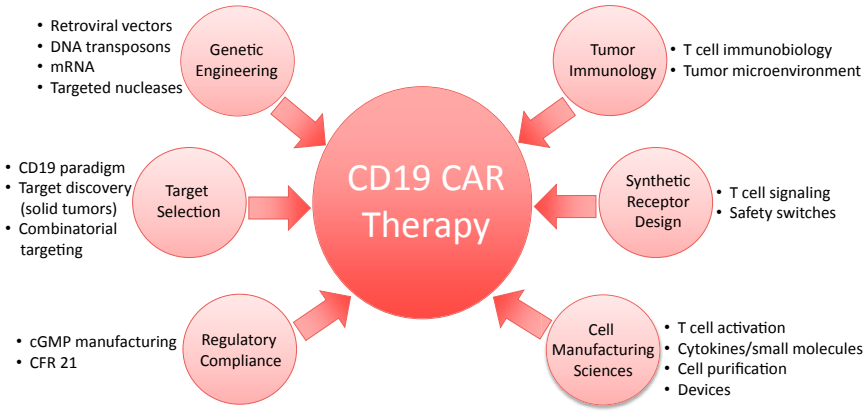

Figure 1. Assembling a CAR
CAR therapy is predicated on cell engineering and the convergence of multiple disciplines and technologies.

start sites for the former, more evenly distributed intra-genically for the latter).[56–58] This subtle difference is thought to contribute to the lesser genotoxicity of lentiviral vectors in hematopoietic progenitors.[59] The relevance of this divergence between retroviral vector types to T cell therapy is, however, uncertain, given the rarity of oncogenic T cell transformation encountered with γ-retroviral vectors.[60–63] Another feature of retroviral vectors is their mutation rate incurred during reverse transcription, which is about 5-fold higher for HIV-1 reverse transcriptase compared with that of Moloney murine leukemia virus (Mo-MLV).[64–69]

DNA Transposons

γ-retroviral and lentiviral vectors are complex biological reagents that require expensive biosafety testing and storage. Non-viral approaches would be advantageous, provided that they were as effective. The *Sleeping Beauty* transposon/transposase system[70] has been used to introduce CARs into T cells by electroporation.[71] The advantages of this system are its simple manufacturing procedure, relatively low cost, and straightforward release testing. Integration is random, posing a potential oncogenic risk secondary to mutagenesis.[72] Ongoing CD19 CAR T cell trials using the *Sleeping Beauty* transposon/transposase show low T cell toxicity.[73] However, the efficacy of CAR T cells generated by this approach remains to be demonstrated.

RNA Transfection

In contrast to the stable and permanent transgene expression afforded by retroviral infection or plasmid DNA transfection, transient expression can be obtained following electroporation or endocytosis of in vitro transcribed messenger RNA (mRNA). This approach eliminates the concerns of genotoxicity and potential generation of a replication-competent retrovirus. RNA transfection allows the expression of the transgene for up to 1 week and has been used to deliver mRNA for physiological T cell receptor (TCR)/CAR, chemokine receptors, and cytokines.[74–76] This approach may be advantageous to screen potentially toxic CAR molecules that could cross-react with normal tissues. Beatty and colleagues[77] found that repetitive infusions of mRNA-transduced CAR T cells could elicit an anti-tumor effect in some patients.

Genome Editing

Genome editing technologies are further expanding the landscape of human cell engineering. Four technologies based on the use of targeted nucleases, including meganucleases, zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and CRISPR/Cas9, enable gene disruption in human cells.[78–83] ZFNs and CRISPR/Cas9 are presently the most developed of these tools and have been used, for example, to efficiently target the HIV co-receptor CCR5.[84–86] Gene editing techniques have also been used to disrupt TCR genes and others (e.g., PD-1) in primary T cells,[86–92] further expanding the possibilities of T cell engineering. A first proof-of-concept study reported that the electroporation of TALENs specific for disrupting TCRα and CD52 molecules allows the generation of off-the-shelf CAR T cells from third-party healthy donors.[93] We recently demonstrated that knocking in a CAR cDNA to the TCR locus improved CAR expression and signaling, resulting in superior anti-tumor activity.[94] T cell genome editing is poised to significantly advance the therapeutic potential of engineered T cells.

The Cells

Autologous T Cells

Autologous T cells are the logical starting point for T cell engineering because they will not attack the recipient or be rejected by the recipient because of their self-origin. Unlike the isolation of tumor-infiltrating T cells, they do not require a surgical intervention and can be simply collected from blood. The engineered T cells have to be able to migrate to tumor sites, expand, and persist in a functional state long enough to eradicate the tumor. While the CAR itself retargets and reprograms the genetically modified lymphocyte, the native properties of the engineered T cell can affect therapeutic potency.[95,96] After leaving the thymus, naive T cells (T_N) differentiate into distinct subsets that play specific roles in protective immunity, including memory stem (T_{SCM}), central memory (T_{CM}), effector memory (T_{EM}), tissue resident memory (T_{RM}), and effector (T_E) cells, which are endowed with different functional and expansion capabilities.[97–102] At this time, genetic modification of T_N, T_{SCM}, and T_{CM} subsets appears to be better suited for enhanced therapeutic efficacy.[95,103–109]

Allogeneic T Cells

Autologous approaches now have a proven track record in the clinic, but personalized manufacture imposes significant logistical constraints and may be challenging in some instances, for example, in patients with chemotherapy or HIV-induced immune deficiency, or in small infants. The promising clinical results of autologous engineered T cell therapy could be further broadened if potent and histocompatible T cells were

Table 1. CD19 CAR Therapy for Acute Lymphoblastic Leukemia

References	Disease	CAR	V	N	T Cells	CR Rate (%)
[147]	ALL (Ad)	CD28	gRV	16	auto	88
[154]	ALL (Ped)	4-1BB	LV	2	auto	100
[148]	ALL (Ped)	4-1BB	LV	25	auto	90
[150]	ALL (Ped)	CD28	gRV	21	auto	68
J.H. Park, et al., 2016, J. Clin. Oncol., abstract	ALL (Ad)	CD28	gRV	46	auto	91
C.J. Turtle, et al., 2016, J Clin Invest, abstract	ALL (Ad)	4-1BB	LV	29	1:14/8	93
[93]	ALL (Ped)	4-1BB	LV	2	allo[a]	100

Ad, adult; allo, allogeneic T cells; auto, autologous T cells; CR, complete remission; gRV, γ-retroviral vector; LV, lentiviral vector; Ped, pediatric; V, vector type; 1:1 CD4/CD8 ratio.
[a]With TALEN-mediated TCR deletion.

readily available. Although T cells can be easily harvested from donors, their use is restricted by their alloreactive potential.[110] This property underlies the high risk of graft rejection in transplant recipients and of graft-versus-host disease (GVHD) in recipients of donor-derived T cells. To provide an acceptable risk-benefit ratio, donor T cells must be devoid of alloreactive potential. Two strategies are currently in use, based either on the selection of virus-specific T cells or the ablation of TCR expression. Virus-specific T cells lacking alloreactive potential are one potential cellular vehicle for CAR-mediated tumor targeting. Although alloreactivity and unanticipated TCR cross-reactivity cannot be prospectively eliminated with full certainty,[111,112] recent studies suggest that virus-specific T cells can be administered to multiple recipients with limited risk of GVHD.[113,114] Virus-specific T cells (VSTs) may thus serve as cellular vehicles for TCR or CAR therapy. A first trial evaluating VSTs to express CARs found that such T cells expanded in response to viral reactivation, although anti-tumor activity was modest.[115] Murine studies have recently revealed the challenges of having both a TCR and a CAR functioning in the same T cell.[116] Another approach thus consists in abrogating TCR expression, which is now feasible with targeted nucleases. TCR-deleted lymphocytes cannot mediate GVHD reactivity, but their long-term persistence could potentially be compromised, because homeostatic proliferation is in part dependent on TCR-MHC interactions.[117,118] Furthermore, gene disruption technologies are still in early stages of development and require optimization to afford efficient targeting without genotoxicity at a reasonable cost. Finally, one should be reminded that preventing GVHD potential does not address the opposite response—donor T cell rejection by the recipient—and is thus limited to the treatment of immunocompromised recipients. Donor T cell approaches are thus still labor intensive and constrained by the limited replicative potential of mature T cells.[96]

Alternative T Cell Sources: Lymphoid Progenitors and Pluripotent Stem Cells

Lymphocyte engineering is not limited to mature T cells and may be performed in lymphoid precursors. T cell progenitors can be generated in vitro[119] and be transplanted across MHC barriers, generating T cells that are restricted to host MHC.[120] When transduced with a CAR, allogeneic lymphoid progenitors yield tumor-targeted T cells without causing GVHD.[121] T cells may also be generated from human embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSC) in vitro.[122–126] iPSC-derived T cells expressing a CAR can eradicate tumors in vivo,[126] providing a foundation for further exploiting the formidable potential of self-renewing stem cells to engineer therapeutic T cells. The combination of iPSC technology and immune engineering may thus provide an opportunity to generate T cells that uniquely combine favorable attributes including antigen specificity, lack of alloreactivity, enhanced functional properties, and histocompatibility.[127]

Regulatory T Cells

The potential of engineered T cells may extend beyond cancer to autoimmunity. CAR T cells can eliminate autoreactive B cells as demonstrated in a mouse model of pemphigus vulgaris.[128] Engineered T regulatory cells may also be harnessed to dampen immune responses,[129,130] which may be useful in the context of autoimmunity[129,131,132] and transplantation tolerance.[133] This field of application of CAR technology is poised for further development.

Clinical Results: The CD19 Paradigm

To successfully translate CAR therapy in the clinic, one not only needs a powerful CAR but also a suitable target, which ideally is expressed in all tumor cells and absent from all normal cells, or at least vital cells. We identified CD19 as a promising target[24] based on its cell-surface expression in most leukemia and lymphomas, and its role in signaling.[134–136] As expected, targeting CD19 induces a B cell aplasia,[137–140] which is clinically manageable especially if it is limited in time. Of interest to us was the added possibility that B cell elimination would prevent the emergence of putative anti-CAR antibodies, which were eventually observed in other CAR T cell trials.[141,142]

Our studies on CD19 CARs were the first to demonstrate complete tumor eradication of established, systemic lymphoma following a single infusion of CAR T cells,[24] providing the foundation and rationale for subsequent clinical studies. Three groups reported promising early results in three different B cell malignancies: diffuse large B cell lymphoma,[143] chronic lymphocytic leukemia (CLL),[144] and acute lymphoblastic leukemia (ALL).[145] These studies focused on relapsed, chemotherapy refractory patients, and made use of either CD28- or 4-1BB-based CARs.[16] Strikingly, despite differences in disease histology, single-chain variable fragment (scFv), vector utilization, and manufacturing process, all groups have reported remarkably high rates of overall and complete response, especially in ALL[109,146–150] (J.H. Park, et al., 2016, J. Clin. Oncol., abstract) (Table 1). These results have placed T cell engineering and CAR T cells at the center of the cancer immunotherapy revolution that is unfolding today in oncology.[25] These clinical results have been extensively reviewed elsewhere.[16,151–153] They arguably rank among the

most compelling clinical achievements obtained through cell and gene therapy to date.

CAR Therapy in the Next Decade

Cell and gene engineering technologies have propelled CAR therapy to an effective and tractable therapy, based on the CD19 paradigm.[16] CAR therapy has, however, not yet reached its full potential. As reviewed here, there still are numerous questions as how to best harness genetic engineering and cell biology to generate the best medicines. Resolving the toxicities sometimes encountered with engineered T cells and devising how to tackle the vast realm of solid tumors are the next tasks to address. CARs also have the potential to impact on autoimmunity and transplantation tolerance. CD19 CAR therapy has sparked pharmaceutical industry interest more than any other cell therapy before it. The flourishing intellectual and financial capitalization of CAR therapy will likely benefit all other cell engineering therapies. T cell engineering was not easily accepted, but it will now, hopefully, inspire a new generation of scientists and physicians who seek curative medicines through engineered immunity.

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