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CAR-T cells and combination therapies: what's next in the immunotherapy revolution?

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Abstract

Cancer immunotherapies are dramatically reshaping the clinical management of oncologic patients. For many of these therapies, the guidelines for administration, monitoring, and management of associated toxicities are still being established. This is especially relevant for adoptively transferred, genetically-modified T cells, which have unique pharmacokinetic properties, due to their ability to replicate and persist long-term, following a single administration. Furthermore, in the case of CAR-T cells, the use of synthetic immune receptors may impact signaling pathways involved in T cell function and survival in unexpected ways. We hereby comment on the most salient aspects of CAR-T cell design and clinical experience in the treatment of solid tumors. In addition, we discuss different possible scenarios for combinations of CAR-T cells and other treatment modalities with a special emphasis on kinase inhibitors, elaborating on the strategies to maximize synergism. Finally, we discuss some of the technologies that are available to explore the molecular event governing the success of these therapies. The young fields of synthetic and systems biology are likely to be major players in the advancement of CAR-T cell therapies, providing the tools and the knowledge to engineer patients' T lymphocyte into intelligent cancerfighting micromachines.

Graphical abstract

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Keywords

CAR-T cells; solid tumors; targeted-therapies; combination therapies; synthetic biology; systems biology

1. Introduction

The presence of naturally occurring tumor-reactive T cells has been thoroughly documented in the peripheral blood, and within the tumors, of cancer patients (1–5). However, although T cells are capable of recognizing neoplastic cells, their presence is often insufficient to mediate clinical tumor regressions. Tumors employ a myriad of mechanisms to neutralize immune attack, particularly T cell-mediated responses (6–8). These mechanisms include downregulation of MHC molecule expression, or disruption of the antigen processing and presentation machineries (9–11), among others. Adoptive transfer of autologous T cells, modified *ex vivo* to express chimeric antigen receptors (CARs), has emerged as a new therapeutic tool to circumvent some of these obstacles. The resounding success of anti-CD19 CAR-T cells in the treatment of B-cell leukemias and lymphomas has raised the expectations of the scientific community and the private sector, bringing renewed hope to cancer patients and their families. As these therapies transition from experimental phase to widespread clinical implementation, a detailed understanding of their mechanism of action (alone or combined with other therapies) will be crucial to maximize their therapeutic potential, while preserving patient safety. In this article, we discuss the main challenges that lie ahead of us pertaining to the use of CAR-T cells beyond B-cell malignancies, and we present our view on how basic mechanistic studies can help facilitate the advancement of immunotherapy.

2. CAR-T cells 101

CARs are fusion proteins that can be structurally defined by three major domains: an *ectodomain*, commonly derived from a single chain variable fragment (scFv), which confers

antigen specificity, fused to a spacer that links the ectodomain with the *transmembrane domain*. Finally, an *endodomain* consisting of different combinations of cytoplasmic proteins that provide T cell activation signals (12). CARs have been classified according to the modules that conform the endodomain. First generation CARs display an endodomain with one signaling module derived from the ζ -chain of the TCR/CD3 complex. Second and third generation are designed to include one or more than one costimulatory regions accompanying the CD3 ζ chain, respectively (Figure 1). Although various costimulatory molecules including OX40, CD27 and ICOS, have been assessed in preclinical studies (13–16), CD28 and 4-1BB are the most commonly used in clinical trials. First generation CAR-T cells exhibited cytotoxic activity *in vitro*, but they showed suboptimal persistence *in vivo*, limiting their therapeutic potential. In fact, second generation CARs have shown better T cell persistence after infusion compared to first-generation CARs (17, 18). However, it has been reported that 4-1BB containing CARs exhibit a major persistence *in vivo*, in xenograft models as well as in patients, compared to those carrying CD28 domains (19–21). It was recently reported that CD28-based CAR-T cells have shown constitutive proliferation, effector memory differentiation and to be prone to exhaustion, while 4-1BB-based CAR T cells exhibit central memory features with enhanced survival and exhaustion inhibition (22–24). These characteristics could explain why CAR-T cells with different costimulatory domains have varied *in vivo* behavior. Since costimulatory domains improved CAR-T cells function, survival and persistence, third generation CARs combining two co-stimulatory domains were generated. However, the functional and therapeutic advantage of this approach remains a matter of discussion (25–27).

By linking the epitope specificity of a monoclonal antibody with the killing ability of a specific T cell, CARs bypass the requirement of antigen presentation by MHC molecules. Moreover, CAR-T cells can also recognize non-classical TCR targets such as lipids and carbohydrates, conferring CAR-T cells with the ability of recognize a broader range of target antigens (28–30). These features have made CAR-T cell therapy more versatile than other T cell-based therapies such as TCR-engineered T cells or tumor-infiltrating lymphocytes (TIL) for certain indications. Unlike TCR-transgenic T cells, CAR-T cells do not require antigen processing and are not HLA-restricted, which allows this therapy to reach a larger subset of patients. In addition, CAR-T cell infusion product is obtained from peripheral blood, so patients with no resectable tumors or with low T cell infiltration into the tumor (non-eligible for TIL therapy) can benefit from CAR therapy (31). However, CAR-T cell-based therapies are still facing up three major limitations. First, the identification of truly tumor-specific target antigens. Second, the suboptimal performance in the treatment of solid tumors, which we discuss below, and third the clinical management of CAR-induced off tumor toxicities and cytokine release syndrome. These aspects have been thoroughly discussed in a recent review by Neelapu *et al.* (32).

3. CAR-T cells in the clinic

Adoptive transfer of CAR-T cells has shown spectacular results in the treatment of B-cell derived malignancies (18, 20, 21, 33, 34). As a result, the U.S. Food and Drug Administration (FDA) has recently approved the first two gene therapies, based on CAR-T cells, for B-cell malignancies. Kymriah (tisagenlecleucel) is a CD19-targeting second-

generation CAR, containing a 4-1BB-derived costimulation domain, approved for the treatment of pediatric acute B-cell lymphoblastic leukemia (<https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm574058.htm>). Yescarta (axicabtagene ciloleucel), also targeting CD19 antigen, is indicated for patients with diffuse large B-cell lymphoma. Unlike Kymriah, Yescarta contains a CD28-derived costimulatory module (<https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm581216.htm>). Treatment of solid tumors appears to be more challenging. CAR-T cells targeting solid tumors face a complex scenario: they need to traffic into the tumor and maintain their functionality within the immunosuppressive tumor-associated environment. These stumbling blocks may be part of the reason why CAR-T cell therapy against solid tumors has not yet shown its full potential. However, promising results have been reported in the treatment of brain tumors. Pule *et al.* (35) reported that the infusion of anti-GD2 CAR-T cells - generated from virus-specific cytotoxic T lymphocytes (CTLs) - induced tumor regression or tumor necrosis in neuroblastoma patients. Moreover, they next reported that this treatment induced a complete remission in three out of eleven patients, with no CAR-related toxicities (36). In an independent study, a patient with recurrent multifocal glioblastoma, treated with intraventricular infusions of CAR-T cells (targeting IL13R α 2), experienced a complete response lasting 7.5 months (37). These results indicate that CAR-T cell therapy has the potential to induce effective anti-tumor responses in solid malignant neoplasia.

To date, there are 207 registered clinical trials with CAR-T cells as main treatment. Among these, 33% are focused on solid tumors (clinicaltrials.gov). In fact, according to Beatty and O'Hara's report in 2016 (38), the number of CAR-T cell-based clinical trials against solid tumors has shown a 35% increase in one year (Figure 2).

4. Optimizing the function of CAR-T cells

Several strategies are currently in development with the goal of improving CAR-T cell function against solid tumors. Efforts are focused, for example, on improving the expansion, persistence, and activation of CAR-T cells by adding different costimulatory domains into the modular structure of the CAR (14, 17, 22–24, 39). Optimization of the structural components of the CAR molecule, including the hinge and transmembrane domains, is also an active area of research (40–42); as well as improving CAR-T cell migration and tumor-infiltration, by co-expressing a relevant chemokine receptor (43–45).

Once in the tumor microenvironment, CAR-T cells face a plethora of inhibitory signals that can impact their function, including: (i) immunosuppressive factors, like TGF- β , VEGF, adenosine, IDO, galectin-1 (46–49); (ii) immunosuppressive cell populations, like regulatory T cells, MDSCs, tumor-associated macrophages (50, 51); (iii) hypoxic, acidic, nutritionally-depleted or oxidizing environment (52–54); and (iv) immunosuppressive molecules, like PD-L1/PD-L2 (55, 56). All these factors may contribute to the fate of tumor-infiltrating CAR-T cells and the outcome of CAR-T cell-based therapies.

Significant effort has been made to shield CAR-T cells from this suppressive environment generated by tumors. Many of these approaches involve additional genetic modifications in T cells. For instance, to protect CAR-T cells from a cytokine like IL-4 with

immunosuppressive effect in pancreatic tumors, Mohammed *et al.* (57) generated an inverted cytokine receptor in which the IL-4 receptor exodomain was fused to the IL-7 receptor endodomain. As a result, they link an inhibitory input (IL-4) to a stimulatory output signal (enhanced proliferation). Similarly, the transduction of tumor-reactive T cells with a dominant-negative TGF- β receptor conferred a selective functional and survival advantage over untransduced T cells and improved antitumor activity (58–60). Cherkassky *et al.* (61) used an analogous approach involving a PD-1 dominant negative receptor to neutralize inhibition by PD-L1, resulting in enhanced CAR-T cell function and persistence.

More sophisticated synthetic biology approaches have been described, that involved modified Notch receptors (synNotch). As synNotch receptors contain the core regulatory domain of Notch, coupled to a synthetic extracellular recognition domain, and a synthetic intracellular transcriptional module, they can be generated to drive the fate of T cells to a particular differentiation or functional states. Consequently, they can be used to sculpt custom response programs in T cells. The authors demonstrate that synNotch T cells can sense tumor antigens and locally deliver cytokines and other therapeutic agents (62). The synNotch receptors are just an example of the tools available for the creation of customized sensor->effector circuits to empower therapeutic T cells. A system-level understanding of the complex signaling networks that govern T cell functions will be needed to facilitate the development synthetic biology interventions.

5. Combination therapies for solid tumors: the pursuit of synergy

In addition to genetic modifications built into CAR-T cells, other treatment modalities may synergize with cellular immunotherapies (Figure 3). Chemotherapy and radiotherapy can modify the tumor microenvironment enhancing immunotherapy (63, 64), affecting the persistence of adoptively transferred T cells, eliminating immunosuppressive cells, or facilitating T cell migration into the tumor (65–69). More recently, targeted therapies, such as monoclonal antibodies and small molecule inhibitors, have gained momentum. Monoclonal antibodies target specific antigens found on the cell surface meanwhile small molecule drugs can penetrate the cell membrane, and are designed to interfere with the enzymatic activity of specific proteins.

5.1 Monoclonal antibodies: pulling off the brakes

Tumor-targeting monoclonal antibodies (mAbs) can be directed towards receptors overexpressed by tumor cells (such as HER2, EGFR, VEGFR, etc.) or can target ligands for these receptors in order to neutralize them (such as Bevacizumab for VEGF) (70, 71). More recently, a different approach involving, mAbs has entered the scene. These mAbs are designed to improve T cell responses against tumors by targeting immune-inhibitory molecules.

Following activation, T cells express inhibitory receptors (iRs), like CTLA-4 and PD-1, as a physiological mechanism of self-regulation (72). In pathological conditions including cancer and chronic viral infections, T lymphocytes exhibit high levels of multiple inhibitory receptors, due to sustained TCR stimulation (73–75). This “exhaustion” phenotype has been associated with a progressive loss of function (1, 5, 76–80) and a specific gene expression

program (81). In order to neutralize this process, mAbs have been developed to prevent the binding of inhibitory ligands (expressed by tumors and/or stroma cells) to inhibitory receptors present on the surface of T cells. Immune checkpoint inhibition has shown remarkable clinical success in the treatment of melanoma, lung, bladder, ovarian, and renal carcinoma; and to lesser extent in triple negative breast cancer (82, 83). The most frequently targeted inhibitory axes are CTLA-4/B7 and PD-1/PD-L1.

Gene-engineered T cells can also express multiple iRs after adoptive transfer (84–87). Interestingly, adoptively transferred CAR-T cells located in the tumor exhibit higher levels of inhibitory receptors like PD-1 and Tim-3, and reduced functionality, than those found in the spleen (61). T cell hypofunction was reversed when the cells were isolated from the tumor, or after treatment with a blocking PD-1 antibody (61, 86, 87). These results provide evidence that blocking iRs with monoclonal antibody-based therapy can significantly restore the functionality of adoptively-transferred CAR-T cells in preclinical models of cancer. Interestingly, Chong et al. (88) report a successful combination of CD19-CAR-T cells and PD-1 blocking antibody after treating a patient with refractory diffuse large B-cell lymphoma. In light of these results, combination therapies with CAR-T cells and inhibitory receptors blockade emerge as a new strategy to overcome the tumor escape, and to further strengthen CAR-T cells. As of August 2017, there were 6 out of 69 active clinical trials for solid tumors registered using CAR-T cells able to produce antibodies against PD-1 or CTLA-4 (NCT03030001, NCT03179007, NCT02873390, NCT03182803, NCT02862028, NCT01454596, clinicaltrials.gov).

In parallel to a growing number of immune checkpoint pathways described in literature (89–91), the number of clinical trials testing combinations of checkpoint-blocking antibodies is on the rise. In many cases, antagonistic antibodies targeting 2 or 3 pathways are administered in combination, aiming for an additive effect (92, 93). Although appealing, anti-checkpoint antibodies may reach a plateau of clinical benefit due to the redundancy of some inhibitory circuits. In the setting of combinatorial approaches involving CAR-T cells, it remains to be determined which pathway is the most relevant for gene-modified T cells. A molecular understanding of the immunobiology of CAR-T cells will be needed to boost their clinical efficacy through combination with other treatment modalities. This may mean that we need to revisit our knowledge on T cell function, and study patient-derived CAR-T cells as a separate entity. To that end, a joint effort involving basic, translational, and clinical scientists will be required. Most importantly, only a strong cooperation between industries and research centers will make this possible.

5.2 Small molecule inhibitors: mighty sidekicks

Small molecule drugs are compounds, usually of less than 500Da, designed to target a specific portion of a molecule. Because of their small size, they have the ability to translocate through the plasma membrane, thus inhibiting not only cell-surface proteins but also intracellular molecules. Most of these drugs antagonize signaling pathways associated with tumor growth, survival, angiogenesis and metastasis. They can target protein kinases, matrix metalloproteinases, heat shock proteins, proteasome and other proteins involved in apoptosis (94). However, the majority of FDA-approved small molecules drugs target

tyrosine and/or serine/threonine kinases, which constitute the main signal transduction mechanisms for proliferation and survival in many tumors. In many cases, their off-target effect on kinases that control physiological processes, in non-neoplastic cells, remains underexplored (95). This constitutes a promising area of research, which may lead to the repurposing of FDA-approved agents for new indications (96).

In the context of T-cell based immunotherapies, the optimal scenario would be the use of a drug capable of targeting a driver mutation in tumor cells, while stimulating T lymphocytes. Constitutive activation of all the mitogen-activated protein kinase (MAPK) signaling cascade, including RAS, RAF, MEK and ERK proteins has been described in many tumors, including melanoma. Selective BRAF and MEK small molecule inhibitors like vemurafenib and dabrafenib (BRAF inhibitors) and trametinib (MEK inhibitor) have shown impressive success in controlling up to 50% of metastatic disease in melanoma patients (97–99). Several preclinical studies, suggested that inhibitors of BRAFV_{600E} had also immunostimulatory properties. Koya *et al.* (100) showed superior anti-tumor responses in BRAFV_{600E} melanoma-bearing mice after receiving antigen-specific T cells combined with vemurafenib, compared to adoptive cell transfer treatment alone. More recently, a clinical trial showed that vemurafenib increased T cell infiltration of melanoma tumors, but had an inhibitory effect on TIL proliferation and viability *ex vivo* (101), highlighting the complexity of the underlying biology. While, Hong *et al.* (102) demonstrated that BRAF inhibition in patients with metastatic tumors exhibiting BRAFV₆₀₀ mutations had minimal inhibitory effects on peripheral blood immune cells. These findings open new questions such as whether BRAF inhibition has different effects on T cells depending on their anatomical location or natural history (peripheral blood T cells, tumor-infiltrating T cells, *ex vivo* expanded T cells, etc.). These works suggest that adoptive cell transfer and BRAF inhibition could play well together. Since BRAF inhibition therapy have shown resistance, combination therapies with BRAF and MEK inhibitors exhibited an improvement in the progression-free survival in patients with metastatic melanoma (99). Recently, Gargett *et al.* (103) analyzed the effect of the three approved drugs, at usual concentrations found in serum of treated-cancer patients, in melanoma-specific CAR-T cells. They reported that vemurafenib alone or the combination of dabrafenib and trametinib result in the inhibition of CAR-T cells effector function, while dabrafenib alone is innocuous. In the same direction, Liu *et al.* (104) reported that dabrafenib did not suppress human CD4+ or CD8+ T cell function *in vitro* while trametinib induced a partial/transient inhibition of T cell proliferation and function. However, when analyzed the effect of trametinib *in vivo*, in a mice model of CT26 tumors, they did not observe inhibitory effects on circulating immune cells and more importantly, trametinib enhance the immune response against the tumor. This result, suggest that the effect of these drugs on CAR-T cells biology should be also addressed in models *in vivo*.

No direct evidence of Ras/Erk inhibition with small molecule drugs in T cells has been reported. However, since diacylglycerol kinases (DGK) are enzymes that regulate Ras/Erk activation pathway, essential for T cell activation, Moon *et al.* (86) explored their role in CAR-T cells by knocking them down. They found higher Erk activation, in DGK-knocked down CAR-T cells, in response to antigenic stimulation *in vitro* and higher anti-tumor ability

in vivo. This suggests that inhibition of Ras/Erk pathway could also benefit CAR-T cell-mediated anti-tumor responses.

The PI3K/Akt/mTOR pathway plays a pivotal role in many physiological processes, and in pathological conditions like cancer. Several PI3K inhibitors are currently in early-phase clinical trials (105), and certain Akt and mTOR inhibitors have been approved for clinical use (94). For instance, the approved Akt inhibitor perifosine has shown antitumor effects against several cell lines *in vitro*, as well as induction of apoptosis in leukemia cells and rhabdomyosarcoma (106, 107). Independently, it has been demonstrated that defective Akt signaling in T cells can induce cellular senescence (108), while sustained activity of Akt increases T cell effector function and drives them to terminal differentiation (109). Taken together, these results suggest that perifosine might act as a double-edged sword, by inhibiting tumor cells but also inhibiting T cells. However, a recent report by Urak *et al.* (110) showed that Akt inhibition during *ex vivo* expansion of CD19-CAR-T cells conferred them a less differentiated phenotype, and enhanced antitumor capacity *in vivo*, without affecting their ability to proliferate. In contrast, Sun *et al.* (111) reported that GD2-specific CAR-T cells modified to express a constitutively active Akt (caAkt-CAR-T cells) showed enhanced proliferation and survival, and exhibited reduced apoptosis after being co-cultured with a GD2-expressing tumor cell line. caAkt-CAR-T cells also exhibited increased cytokine production and were resistant to TFG- β -mediated regulatory T cells conversion. These results suggest that manipulation of Akt activity could potentiate the therapeutic efficacy of cellular therapies. At the same time, they highlight the fact that an integral understanding of the effects of Akt inhibition on both tumor cells and T lymphocytes is necessary to design the most effective intervention, to control tumor growth while empowering CAR-T cells. mTOR signaling is crucial for both tumor and T cells. Beyond the inhibitory effects of rapamycin on tumor cells and effector T cells, mTOR inhibition may potentially reverse tumor immune suppression by downregulating IL-10, VEGF and PD-L1 expression (112, 113). In order to exploit these anti-tumor effects of rapamycin in combination with adoptive T cell therapy, Huye *et al.* (114) designed a rapamycin-resistant CD19-second generation CAR-T cell. They demonstrated that those CAR-T cells maintain mTOR signaling, proliferate and show effector function even in the presence of rapamycin. Moreover, they displayed greater antitumor activity against Burkitt's lymphoma and pre-B ALL cell lines *in vitro*, suggesting that mTOR could potentiate the functionality of CAR-T cells.

Several growth factor receptors have shown aberrant activation or deregulation in tumors. For instance, members of the family of epidermal growth factor receptor (EGFR: like HER1, HER2, HER3), vascular endothelial growth factor receptors (VEGFR), insulin-like growth factor receptor (IGFR) and fibroblast growth factor receptor (FGFR) can be targeted with kinase inhibitors (KIs) currently approved by FDA (94). In fact, different KIs targeting VEGFR are also in use for several types of cancer like renal cell carcinoma (RCC), hepatocellular carcinoma (HCC), metastatic colorectal cancer, between others. While the expression of VEGF-R2 has been described in human CD8⁺ T cells (115), its role on CAR-T cells is still unknown. VEGF signaling induces suppression of CD8⁺ T cell proliferation and also reduces their cytotoxic activity (116). Moreover, VEGF-A can induce the expression of PD-1 on tumor-infiltrating CD8⁺ T cells (115), suggesting that CAR-T cells might benefit from VEGF-VEGFR pathway blockade.

Collectively, these results suggest that a systematic and integrated understanding of the molecular consequences of KIs on T cells, tumor cells, and the microenvironment will be needed for the optimal design of combination therapies.

6. CAR-T cells v2.0

Although a great deal of knowledge exists on the signaling mechanisms and downstream of the T cell receptor, a systems level perspective may be necessary to better understand the signaling properties of CAR-T cells. This information will allow for the design of more predictable and controllable systems. For example, newly engineered versions of CAR-T cells may have unique interactions, from the signaling point of view, with immune checkpoint molecules being activated or inactivated by the tumor stroma. How can we begin to decipher all the ensuing complexity this is likely to bring? One answer could be to apply modern proteome-wide technologies such as liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). This technology platform has in recent years been applied to cancer cell models to better understand the complexity of cancer signaling. In simple terms, this approach starts with a protein sample from a cell, digests the protein into peptides (typically with trypsin enzyme digestion), uses various enrichment strategies to capture subpopulations of the proteome, separates the peptides using LC, and then uses the increasingly powerful mass spectrometers to not only identify the peptide (and any post-translational modifications) but also quantify peptide abundance. Through various enrichment strategies, various aspects of the proteome can be captured and analyzed. This includes capturing post-translational modifications through antibody based reagents, such as tyrosine phosphorylated peptides using anti-phosphotyrosine antibodies (117–119). This can be extended to other post-translational modifications, including acetylation and ubiquitination (120). These experiments then allow in depth characterization of signaling cascades in biological samples, for example tyrosine kinase signaling, acetylation networks, and ubiquitination signaling systems.

Other approaches can use chemical biology tools to label active sites of enzymes and then enrich for these activated enzymes. Activity based protein profiling (ABPP) has been used to enrich various enzyme classes such as kinases and serine hydrolases through such technology (121, 122). Last, another approach is to use proteins as “baits” to capture protein-protein interactions. In this way, knowledge on protein complexes can be experimentally determined in the context of various signaling proteins. For example, our group has used affinity purification coupled with LC-MS/MS to experimentally determine protein complexes and larger networks of protein complexes termed “interactomes” in EGFR and ALK driven lung cancer cell lines (123, 124).

We envision applying these now mature tools that have been road tested largely in cancer models to better understand CAR-induced signaling events, and their dynamic nature in the context of the tumor stroma and drug therapy. Our group has begun studies using different variants of CAR-T cells to begin pulldown studies to determine how alterations in cytoplasmic domains affect not only protein complexes around the CAR but also change downstream kinase signaling pathways. An important area for future research is extending these types of studies beyond simple cell culture models to more complex models that

simulate the CAR-T cells interactions with tumor stromal cells and activation of various immune checkpoints. Ideally, one would like to co-culture CAR-T cells with tumor cells to model these interactions, but this brings about more complexity with determining the compartment source (CAR-T cell or tumor cell) of signaling proteins identified by mass spectrometry. One particular way to overcome this hurdle is through the use of a novel cell labeling strategy termed CTAP (125, 126). This is a variant of SILAC-based labeling where heavy isotopes are used to metabolically label cell proteomes, which can then be identified through the mass shift in the resulting mass spectrometry data. While still an emerging technology, creating CAR-T cell/tumor cell co-culture models and employing CTAP-based technology may enable fine tune mapping of signaling events driven by CARs.

Last, one major area of research that could be translated using this approach is developing CAR-T cell-based therapies in conjunction with small molecule drugs that perturb signaling events. Signaling downstream of CARs will require various enzymes, including kinases, acetylases, and deubiquitinases, all of which have chemical compounds that perturb their activity. Through a better understanding of these networks, we envision the pairing of small molecule inhibitors (for example, kinase inhibitors) that tune outputs from CAR-T cells in therapeutically important ways. For example, applying kinase inhibitors at particular targets may boost CAR-T cell activity in a temporal fashion. Similarly, small molecule inhibitors could be used to tune down the activity of CAR-T cells (or even eliminate them) in the face of severe toxicity. An overlay of drug targets elucidated by chemical proteomics, such as through drug immobilization, capture of targets, and identification of such targets via mass spectrometry, could be a powerful approach to repurpose existing compounds or compounds with interesting but mechanistically not understood phenotypes (117, 127–131) (Figure 4).

7. Concluding remarks

The success of CAR-T cell therapies has made waves in the clinical and research arenas, attracting the attention of the scientific community, and the society as a whole. This opens a universe of possibilities for the development of more efficacious and less toxic cancer therapies. However, the path ahead of us is not devoid of challenges. Two major fronts will require special attention as we work towards the universalization of adoptive T cell therapies beyond a handful of cancer types: extending the clinical success to solid tumors; and increasing the tumor specificity, to prevent treatment-related toxicity. In both cases, a thorough and integrated understanding of the complex signaling networks triggered in the immune cells, in the tumor cells, and also in stromal cells present within the tumors, will be required. The ultimate frontier is the generation of T cells programmed with tailored biochemical algorithms, capable of trafficking efficiently to tumor lesions, recognizing malignant cells, and unleashing their cytotoxic potential in spite of the multiple inhibitory signals present in the tumor microenvironment. T cells should also be able to recognize normal, vital healthy tissues, and refrain from attacking them. The notion of truly tumor-specific antigens, such as those arising from somatic mutations; or tumor-associated antigens expressed in dispensable normal tissues, may not apply to the majority of patients. Therefore, the detection of one or two tumor-associated proteins on the surface of malignant cells may not be sufficient. Signals that can be used to discriminate malignant tissues/cells from normal include: aberrant expression of protein, lipids, carbohydrates; altered pH and/or

oxygen tension; metabolic traits; among others. Effector functions to be executed by therapeutic T cells include accumulation in tumor lesions; induction of apoptosis of tumor cells; recruitment of endogenous, unmodified immune effectors; polarization of the immune response; control of inflammation; tissue repair, etc. The current challenge is to bridge those input and output signals in a controlled, productive fashion, by hacking the T cell signaling machinery. The integration of constitutively active or dominant negative kinase domains; synthetic docking sites; or drugable signaling hubs, as part of the CAR structural domains will likely allow us to fine tune the aforementioned biological processes. The vast body of knowledge acquired during years of research and clinical testing of small molecule agents will be a solid stepping-stone for the design of complex control commands. In some cases, the immune-related effects of agents developed to target tumor cells will need to be assessed, and the development of fully human *ex vivo* systems will be required draw an integrated map of those biochemical algorithms. In any instance, the advancement of the field of cancer immunotherapy will require the concerted contributions of clinicians, immunologists, pharmacologists, and molecular biologists to maximize the impact of scientific developments on the quality of life of cancer patients.

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Abbreviations

CAR	<u>ch</u> imeric <u>a</u> ntigen <u>r</u> eceptor
iRs	<u>i</u> nhibitory <u>r</u> eceptors
mAbs	<u>m</u> onoclonal <u>a</u> ntib <u>o</u> dies
SMIs	<u>s</u> mall <u>m</u> olecule <u>i</u> nhibitors
KIs	<u>k</u> inase <u>i</u> nhibitors

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Figure 1. Basic structure of successive generations of chimeric antigen receptors
Schematic representation showing the ectodomain (scFv plus hinge and spacer modules), the transmembrane domain, and the intracellular signaling domain of CAR-expressing T cells. First generation CARs contain a CD3ζ-derived signaling module, while second and third generation CARs contain also one or two co-stimulatory domains, respectively.

CAR-T cells in Clinical trials

Figure 2. Clinical trials using CAR-T cells

Comparison between active clinical trials using CAR-T cells, reviewed by Beatty G.L. and O'Hara M. (Pharmacology & Therapeutics) by March 2016 and the active clinical trials registered to August 2017 (clinicaltrials.com). The pie charts show the proportion of clinical trials targeting B-cell malignancies, myeloid leukemia and solid tumors. The arcs represent the proportion of different tumor-associated antigens that are being targeted with CAR-T cells for the treatment of solid tumors. The bar graph shows the different solid tumors currently targeted with CAR-T cells.

Figure 3. Improvement options for CAR-T therapy against solid tumors
Genetic modifications that can be built into CAR-T cells (intrinsic), and possible combination therapies (extrinsic) to enhance CAR-T cell therapies.

Figure 4. Kinase inhibitors

Schematic representation of potential effects of kinase inhibitors (KI) on CAR-T cells, when used in combination.