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Emerging Cellular Therapies for Cancer

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Abstract

Genetically engineered T cells are powerful new medicines, offering hope for curative responses in patients with cancer. Chimeric antigen receptor (CAR) T cells were recently approved by the US Food and Drug Administration and are poised to enter the practice of medicine for leukemia and lymphoma, demonstrating that engineered immune cells can serve as a powerful new class of cancer therapeutics. The emergence of synthetic biology approaches for cellular engineering provides a broadly expanded set of tools for programming immune cells for enhanced function. Advances in T cell engineering, genetic editing, the selection of optimal lymphocytes, and cell manufacturing have the potential to broaden T cell-based therapies and foster new applications beyond oncology, in infectious diseases, organ transplantation, and autoimmunity.

Keywords

chimeric antigen receptor; CAR-T cell; adoptive cell transfer; immune-oncology; leukemia; synthetic biology

INTRODUCTION

Immunotherapy is the latest breakthrough in cancer therapy, thanks to the remarkable clinical results of checkpoint inhibitors (1) and chimeric antigen receptor (CAR) T cells (2). The US Food and Drug Administration (FDA) approval in 2017 of two CAR-T cell therapies for the treatment of B cell malignancies in pediatric and adult patients is a landmark for

cancer immunotherapies. In 2018, these therapies were approved in the European Union, the United Kingdom, and Canada.

These novel, effective immunotherapies rely on the ability of T cells to destroy cancer cells. Indeed, T cells, when adequately rewire, are proving to be the most powerful anticancer immune cell. Immunotherapy not only leads to unprecedented clinical responses in patients with otherwise refractory tumors but is also able to ensure long-lasting clinical remission in patients that were historically considered incurable due to their disseminated disease.

Among the different T cell immunotherapies, adoptive cell therapy (ACT) has attracted substantial attention and interest lately. ACT is a personalized therapy in which a patient's own immune cells are removed from, expanded in vitro to large numbers, and reinfused back into the patient to eradicate tumors. In the first attempts to apply this therapeutic strategy, tumor-infiltrating lymphocytes (TILs) were isolated from melanoma tumors. The infusion of TILs resulted in complete and durable responses in patients with metastatic melanoma (3). Rapid advances in the field of gene therapy, gene editing, T cell biology, and target identification make it now possible to genetically modify T cells to render them tumor specific. By using T cells easily collected from patients' blood and genetically modifying them with synthetic antigen-recognizing receptors, including T cell receptors (TCRs) or CARs, it is now potentially possible to target any type of cancer. The best clinical results with ACT have been obtained mostly with CAR-T cells for CD19⁺ leukemia and lymphoma (4–9). CAR-T cells targeting CD22 and B cell maturation antigen (BCMA) have also led to promising responses in B cell acute lymphoblastic leukemia (B-ALL) (10) and multiple myeloma (11), respectively. Of note, T cells engineered to express a TCR against NY-ESO-1 induced tumor regression in patients with synovial cell sarcoma and melanoma (12).

Despite exciting results in certain human malignancies, only a few trials have shown responses in solid tumors. Therefore, both academia and the pharmaceutical industry are intensively investigating how to improve ACT and develop next-generation T cell immunotherapies. Thanks to the novel gene-editing technologies and new combinatorial strategies, ACT has the potential to target virtually every cancer type. Moreover, T cells can be modified to express transgenes that could further enhance their effector functions and persistence and/or reduce toxicity, opening a myriad of possibilities in the emerging field of synthetic biology. Here, we highlight the most recent results and lessons learned in clinical trials with ACT, and we discuss the principles of effective treatment that are guiding the design of next-generation T cell therapies (Figure 1).

ADOPTIVE T CELL THERAPY: LESSONS LEARNED

Tumor-Infiltrating Lymphocytes

Adoptive transfer of naturally occurring tumor-specific T cells has been used in melanoma patients for over 20 years. The rationale behind this approach is that melanoma tumors are often infiltrated by tumor-specific T cells, but they appear to be unresponsive and fail to control the tumor. Removing these T cells from the immunosuppressive tumor microenvironment and culturing them in vitro with IL-2 enables their activation and large-scale expansion before they are infused back into the patient. Current efforts are focused on

enhancing the quality and characterization of the final T cell product used for the treatments and simplifying the methods to obtain these tumor-specific T cells (Figure 2). Accumulating evidence suggests that tumor regression following TIL transfer is mediated by T cells targeting tumor-specific antigens, also known as neoantigens (13, 14). TILs are a heterogeneous population, and thus enrichment of the final product in neoantigen-reactive T cells may enhance clinical efficacy. In this regard, PD-1 and 4-1BB have been proposed as markers for the tumor-reactive repertoire in the tumor (13, 15, 16). Whether isolation and infusion of these neoantigen-specific T cells will be more effective than bulk TIL transfer requires clinical investigation. A more recent report showed that expression of PD-1 identifies neoantigen-reactive lymphocytes in the peripheral blood of melanoma patients (17), providing a novel noninvasive strategy to develop T cell-based personalized therapies. Alternatively, tumor-specific T cells could potentially be isolated following vaccination or treatment with checkpoint blockade therapy. In this regard, personalized vaccines targeting neoantigens have been shown to promote preexisting, as well as de novo, neoantigen-specific T cell immunity (18).

TCR-Engineered Lymphocytes

Despite the exciting results in melanoma patients treated with TILs, and recent progress in the field, the translation of TIL therapy into a setting of routine personalized medicine is still challenging and limited by the logistics of obtaining tumor biopsies. An alternative to the process of isolating TILs is to genetically modify T cells isolated from the blood to express transgenic TCRs able to recognize tumor antigens and induce cancer cell killing. In early approaches, T cells were modified to express TCRs targeting shared antigens, which are expressed at high levels on different cancers but can also be expressed in normal tissues. Treatment with TCR-T cells targeting MART-1, gp100, CEA, and MAGE-A3 resulted in unfavorable safety profiles, including poor antitumor effects and/or severe toxicity (19–24). By contrast, adoptive transfer of high-avidity TCR-T cells targeting NY-ESO-1, a cancer-testis antigen, induced objective clinical responses in patients with synovial cell sarcoma, melanoma, or myeloma, without severe toxicity (12, 25), and this is likely to be the first transgenic TCR to be approved by the FDA.

The observation that T cells recognizing neoantigens have a critical role in tumor regressions after TIL therapy or checkpoint blockade has shifted the focus of TCR-T cell therapy from self-antigens to neoantigens. T cells targeting neoepitopes are not subjected to negative selection and may therefore express high-avidity TCRs with the potential of inducing strong antitumor effects in the absence of toxicity. Recent technological advances in tumor exome sequencing and epitope prediction algorithms permit rapid identification of the immunogenic neoepitopes resulting from somatic gene mutations in tumor cells. Isolation of T cells with reactivity against these targets, and identification of mutation-reactive TCRs that can be genetically introduced into autologous T cells, is being actively explored for the development of personalized therapies.

Although the infusion of neoantigen-specific T cells holds great potential, the current protocols to generate good manufacturing practice (GMP)-grade, neoantigen-specific, personalized autologous T cells are costly, labor intensive, and time consuming. Moreover,

limitations of transgenic TCRs (and TIL therapy) are that they are dependent on external costimulation and presentation of the targeted neopeptide via the MHC-I complex, which is often downregulated in cancer cells (26–29). An alternative approach that may address some of these challenges is to genetically modify T cells to express CARs that are MHC independent.

Chimeric Antigen Receptors

CARs are synthetic hybrid receptors created by genetic engineering that combine an extracellular domain, typically derived from an antibody single-chain variable fragment (scFv), with intracellular signaling domains providing activation signals that are derived from endogenous TCRs and costimulatory signals (2). Since antigen recognition by CAR-T cells is based on scFv binding to intact surface antigens, the targeting of tumor cells is neither MHC restricted nor dependent on processing and effective presentation of target epitopes. However, CAR recognition requires surface expression of the tumor antigen.

The extracellular glycoprotein CD19 is the most common B cell target for CAR-T therapies. Within the past few years, clinical trials using CD19-targeting T cells (CD19-CAR-T cells) have shown high rates of sustained complete responses that are unprecedented in relapsed and refractory B-ALL (7, 30, 31, 32). Significant responses have been observed also in chronic lymphocytic leukemia (CLL) (33, 34) and non-Hodgkin lymphoma (NHL) (9, 35, 36). Some key lessons were learned in these trials: (a) An effective proliferative response is the best predictor of clinical efficacy—T cells have to expand and survive (33); (b) CD19-CAR-T cells show differential rates of complete responses among different B cell neoplasms, with ~80% complete-response rate in pediatric and young adult patients with B-ALL and 30–50% complete-response rate in adult patients with diffuse large B cell lymphoma; (c) CAR-T cell dysfunction (37) and loss of CD19 in leukemia cells (38) are the main relapse mechanism in patients treated with CD19-CAR-T cells; (d) CAR-T treatment can cause significant toxicities, such as cytokine-release syndrome (CRS) and neurotoxicity (35); and (e) the use of anti-IL-6R antibody (tocilizumab) and steroids can successfully control these adverse events in most patients (39).

Importantly, CAR-T cells are also being evaluated in other hematologic malignancies that do not express CD19 (40). For acute myeloid leukemia (AML) several targets are under investigation, such as CD33 (41), CD123 (42–47), and CD44v6 (48); however, off-tumor expression of these antigens suggests potential toxicities. Clinical data have shown some efficacy of anti-CD30 CAR-T therapy for Hodgkin lymphoma (49). In multiple myeloma anti-BCMA CAR-T cell therapies (50) are leading to excellent clinical responses in multiple clinical trials (51, 52).

The success shown by CAR-T cells in hematologic cancers supports the translation of this technology to the challenging setting of solid tumors that are responsible for more than three-quarters of cancer-related deaths in humans and therefore represent a large unmet medical need. Several clinical trials testing CAR-T cells for the treatment of solid tumors are ongoing. Early clinical trials reported poor CAR-T cell efficacy with different levels of toxicity (53–55). However, recent reports of patients with glioblastoma, pancreatic cancer, mesotheliomas, and sarcomas have provided evidence supporting the feasibility of CAR-T

cells, with transient activity and absence of serious adverse events (56–60). Of note, in a recent report CAR-T cells directed to IL-13R α 2 induced a complete regression of metastatic glioblastoma in one patient (61). Some of the valuable lessons learned by initial clinical trials with solid tumors that will drive the design of future CAR-T cell therapies include the following: (a) Despite CAR-T cells trafficking to the tumor, initially proliferating in situ and exerting some direct antitarget activity, substantial antitumor responses are rarely observed (57, 59); (b) antitumor efficacy is often limited by a lack of significant expansion and/or survival of CAR-T cells in the tumor (55, 58, 62); (c) significant decrease or loss of targeted-antigen expression has been observed following CAR-T cell administration, suggesting some level of transient CAR-T cell activity and pointing at antigen heterogeneity and antigen loss as major barriers to overcome (57, 59); and (d) severe on-target off-tumor toxicity has been observed in some trials, even when antigens are expressed at very low levels. As CAR-T cells can dramatically proliferate in vivo, great caution should be taken when designing and implementing novel CAR-T cell therapies to mitigate toxicities (53, 54, 62).

CURRENT ROADBLOCKS FOR T CELL IMMUNOTHERAPIES

As discussed, the efficacy of ACT needs to be improved in solid tumors, but in CD19⁺ neoplasms additional optimization of the treatment could improve the long-term success and safety of CD19-CAR-T cells. Finally, when these new exciting products are commercialized, fair access to them should be ensured to all patients. To better understand how to improve ACT, a deep knowledge of the current roadblocks is required, and optimal strategies need to be developed.

T Cell Persistence and Expansion

Significant expansion and persistence of adoptively transferred T cells are considered critical predictors of clinical efficacy. Several clinical trials testing the infusion of TILs or genetically modified T cells have confirmed the correlation between the persistence of the infused T cells in the circulation and the likelihood of tumor regression (37, 63). By contrast, most ACT clinical trials where lack of overall efficacy was observed have reported poor T cell persistence, especially in the context of solid tumors. Likewise, lack of response in some patients and relapse in patients responding to ACT are often associated with loss of tumor-reactive T cells. Several factors can influence the persistence of adoptively transferred T cells, including patient preconditioning, ex vivo culture conditions, development of T cell exhaustion, and lack of costimulation or host immune responses against the cellular infusion product.

Early trials with melanoma patients demonstrated that sustained persistence of TILs is only achieved after preparative lymphodepletion of the patient before TIL transfer (64). Preclinical models suggest that lymphodepletion creates a homeostatic space for T cell expansion, depletion of regulatory T cells, and activation of the innate immune cells that leads to a better engraftment and stimulation of infused antitumor CD8⁺ T cells (65, 66). Clinical studies indicate that the intensity of lymphodepletion is an important variable governing the expansion and persistence of CAR-T cells (7).

Some of the protocols currently used to culture T cells for ACT result in extensive in vitro proliferation and T cell differentiation. Moreover, TILs may contain exhausted or terminally differentiated T cell populations. T cell differentiation is associated with telomere shortening, phenotypic changes (including loss of expression of CD27, CD28, and CCR7), and loss of replicative capacity. In this regard, studies in melanoma patients using biological correlates indicated that short TIL culture duration, longer telomeres of the infused cells, and the number of CD8⁺CD27⁺ cells infused are associated with objective responses (67). Similarly, one study conducted at the University of Pennsylvania evaluating the biomarkers predictive of clinical response to CD19-CAR-T cells in patients with CLL found that the preinfusion product of responding patients was enriched in less-differentiated T cells (CD27⁺CD45RO⁻) and Th17 cells (37). In this same study, long-term remissions were associated with infusions containing transcriptomic signatures of early memory CAR-T cells, while T cells from nonresponding patients were enriched in genes related with terminal differentiation and exhaustion. In line with this, preclinical studies in animal models have shown that adoptive transfer of less-differentiated T cell subsets, including naive and memory stem cells, results in superior in vivo expansion, persistence, and antitumor response compared to the more-differentiated T cell subsets. The rationale behind this is that less-differentiated T cells persist longer and are a continuous source of effector cytotoxic cells. Other studies suggest that Th17 cells may have enhanced antitumor effects when used in ACT due to their higher in vivo survival, self-renewal capacity, and resistance to senescence compared to Th1 or nonpolarized T cells (68, 69).

T Cell Dysfunction

Some patients fail ACT despite tumor-specific T cells accumulating within tumors that express the target antigen. A growing body of evidence suggests that this inability of T cells to eliminate tumors may be due to a progressive loss of T cell effector functions that can be caused by both intrinsic (T cell fitness) or extrinsic (tumor microenvironment) factors (70).

T cell dysfunction, including T cell anergy, exhaustion, and senescence, arises during many chronic infections and cancer and is driven in part by chronic persistence of antigen (71). Dysfunctional T cells are characterized by impaired proliferation and effector function and expression in different degrees of various inhibitory receptors, including PD-1, TIM-3, LAG-3, TIGIT, and KLGR-1 (71, 72). In melanoma patients, the majority of tumor-reactive cells express PD-1 when isolated from tumors (16). Clinical results with adoptive transfer of TILs or immune checkpoint blockade demonstrate that T cell dysfunction can be partially reverted and that reinvigorated T cells can mediate complete responses in patients with solid tumors, underscoring the therapeutic potential of counteracting immune inhibition.

T cell exhaustion has been mainly studied in virus-specific or tumor-specific, naturally occurring T cells. Exhausted T cells have distinct genetic and epigenetic reprogramming states that appear to be stable and not reversible (73). CAR-T cells have been modified with chimeric receptors that do not exist in nature, and thus the dynamics and features of T cell exhaustion may be different compared to the signaling through the TCR. The presence of exhaustion markers on CD19-CAR-T cells at the time of infusion predicts poor response to therapy in CLL patients. Whether chronic antigen exposure can induce T cell exhaustion in

patients treated with CAR-T cells is currently unknown, but it is likely that preventing T cell exhaustion will be important to improve treatment.

An important mechanism of T cell exhaustion in CAR-T cells that has not been described in naturally occurring T cells is antigen-independent tonic signaling due to spontaneous clustering of CAR molecules. Emerging evidence suggests that the optimal configuration of all the elements within a CAR, including the scFv, hinge, transmembrane (TM) domain, and intracellular domains, is essential for optimal CAR-T cell function and persistence. Depending on the selection and configuration of CAR modules, and the levels of CAR expression on the cell surface, some CAR-T cells can have tonic signaling during ex vivo expansion (74–78). Tonic signaling is associated with augmented T cell apoptosis, early T cell differentiation and exhaustion, and impaired antitumor effects. CAR-T cells with tonic signaling have been used in preclinical and clinical trials, leading to poor antitumor activity and results that have been difficult to interpret.

Tumor Heterogeneity and Antigen Loss

In the presence of fully functional tumor-specific T cells, tumor cells can develop adaptive resistance to avoid being eliminated by the immune system. Tumor evasion due to antigen loss, heterogeneity of antigen expression, or impaired antigen presentation has been widely reported after ACTs in both preclinical and clinical studies, and these are considered main causes of treatment failure.

In the case of engineered T cells targeting a single antigen, to avoid tumor escape, the target antigen should be expressed in all tumor cells; alternatively, its expression should be essential for tumor growth. However, even in this ideal scenario, tumor escape can potentially occur. For example, in the setting of CAR-T cells targeting CD19, a cell surface antigen that is uniformly and highly expressed on B lineage cells, emergence of antigen-negative leukemic cells is observed in a significant subset of treated patients (30). It is unclear if these cells are present in the initial cancer and overgrow thanks to a selective advantage under pressure from the T cell therapy, or if they develop de novo mutations to escape the selective pressure. Lack of surface expression of CD19 may be due to mutations (79, 80), alternative splicing in CD19 (exon 2 skipping) (80), or mutations in the B cell receptor protein CD81 (81, 82). New evidence suggests that loss of antigen expression might contribute to treatment failure when targeting not only CD19 but also other antigens like CD22. Similar to CD19, CD22 is uniformly expressed in B lineage cells and has been proposed as an alternative antigen to target CD19-negative relapsed ALL patients (83, 84). Diminished CD22 surface expression on ALL B cells without detectable CD22 mutations has been recently identified as a mechanism of relapse following CD22-CAR-T therapy (10). Sequential loss of both CD19 and CD22 following sequential CAR-T cell treatment has also been observed in one patient with NHL (85). A novel mechanism of CD19-negative immune escape includes lineage switch from lymphoid to myeloid leukemia with consequent loss of expression of B lymphoid lineage antigens (86, 87).

Solid tumors are characterized by heterogeneous antigen expression with regard to intensity and distribution, which increases the possibility of tumor escape when compared to hematologic malignancies. CAR-T cell therapies preferentially kill tumor cells with high

targeted-antigen expression but may fail to eliminate cells with lower antigen densities (88–90). Diminished expression of the targeted antigen after T cell therapy in patients with solid tumors has been reported in several trials with both CAR-T cells and TCR-T cells (12, 57, 59). Targeting multiple tumor antigens, using combination therapies, or implementing strategies to promote epitope spreading could mitigate tumor escape.

TIL therapy or infusion of patient-specific engineered TCR-T cells targeting different neoantigens could provide a solution to the difficulties of finding single tumor antigens to serve as universal targets. However, the mutation or decreased expression of genes involved in antigen-presentation pathways, including specific deletion of HLA class I alleles or loss of β_2 microglobulin (β_2m), may also lead to tumor escape (26, 27, 29, 91). An interesting example of this was reported by Tran et al. (28) after treatment of one patient with metastatic colorectal cancer with TILs targeting mutant KRAS G12D. Despite an evident clinical benefit, one metastatic lesion relapsed by acquiring uniparental disomy, which maintains the surface expression of irrelevant MHC molecules (preventing tumor cell elimination by natural killer cells) but precludes the presentation of the mutant KRAS peptide.

Tumor Contamination Leading to Resistance

Tumor contamination in the cellular therapy product is known to correlate with relapse in the setting of autologous stem cell transplantation (92). CD19-CAR-T cells are generated from the peripheral blood lymphocytes and, for patients with acute leukemia, leukemic blasts can often be present in the peripheral blood at the time of collection. Usually by the end of the CD19-CAR-T cell expansion, no residual B cell blasts are detected by flow cytometry, as CD19-CAR-T cells effectively purged them *ex vivo*. However, we recently reported a patient treated with CD19-CAR-T cells (CTL019) who relapsed nine months after infusion with CD19-negative leukemia that aberrantly expressed the anti-CD19 CAR protein (93). The CD19-CAR-T gene was unintentionally introduced into a single leukemic B cell by lentiviral transduction during CD19-CAR-T cell manufacturing. Interestingly, the CAR19⁺ leukemic cells were able to survive the CD19-CAR-T cell purging during expansion as the CAR protein expressed on the surface of leukemic cells bound in *cis* to the CD19 epitope, masking it from recognition by CD19-CAR-T cells. Although very rare, this case warrants careful evaluation of the minimal residual disease in the ACT products (93).

Altogether, these results highlight the strong immune selective pressure that targeted T cells can impose, as well as the great capacity of tumor cells to find new mechanisms to evade the immune system. Several lessons from the infectious diseases field are instructive in strategies that will be required to overcome resistance to ACT (94).

Toxicity

Together with the remarkable clinical activity, ACT has demonstrated significant and unique toxicities (95). Potent immune activation and general inflammation are triggered by the infused T cells recognizing tumor cells. In the setting of CD19-CAR-T cell therapy, CRS is observed in the majority of B-ALL patients and in subsets of B-CLL and B-NHL patients (6, 8, 9, 33, 96–98). This syndrome is characterized by increased levels of cytokines (IL-6, TNF- α , and others) and other inflammatory markers (ferritin, C-reactive protein), alongside

with fever, hypotension, myalgia, and additional systemic symptoms. In some patients treated with CD19-CAR-T cells, CRS is followed within days by neurotoxicity of unknown etiology. While this syndrome is generally self-limiting and resolves within days, in rare instances it can result in fatal cerebral edema (99, 100). The prompt use of an anti-IL-6R monoclonal antibody, tocilizumab, can control CRS in the majority of patients (101, 102), but it does not prevent or treat neurotoxicity. New murine models that recapitulate key features of CRS and neurotoxicity suggest that blocking IL-1 may abolish both CRS and neurotoxicity, enabling new therapeutic interventions (103–105).

Another important toxicity is on-target, off-tumor toxicity that occurs when healthy tissues bearing the same antigen being targeted by the cellular therapy are also damaged. An example of this adverse event is B cell aplasia, caused by the presence of CD19 on normal B cells and subsequent killing by CAR-T cells (106). This event can be predicted in most cases but can be an unexpected clinical adverse event. Treatment with CAR-T cells targeting Her2/neu antigen, which is expressed at low levels in the lungs, resulted in rapid and fatal toxicity in one early trial (54). Off-target, off-tumor aberrant reactivity, also known as cross-reactive toxicity, describes unexpected targeting of healthy tissues by T cells that had not been anticipated, in particular with TCR-T cells. In this regard, two different trials have reported severe toxicity, including lethal events, after treatment with T cells redirected to a MAGE-A3 peptide (23, 24). In one trial, T cells induced neurotoxicity due to TCR cross-reaction with MAGE-A12 (107), while in the other, T cells recognized an unrelated muscle-specific protein, Titin, leading to cardiac toxicity (108, 109).

Solid Tumors

With rare exceptions, the outcomes of ACT in patients with nonmelanoma, solid tumors remain poor. Clinical trials conducted with CAR-T cells targeting solid tumors suggest that CAR-T cells can engraft in the peripheral blood, traffic to tumors, and respond to antigen but fail to expand, persist, and mediate objective responses (56, 58, 59, 61). Compared to hematologic malignancies, solid tumors pose extra barriers of complexity (110, 111). First, T cells must traffic and infiltrate into the solid mass. On arrival at the tumor, T cells encounter an immunosuppressive environment that includes immunosuppressive cells (regulatory T cells, tumor-associated macrophages, myeloid-derived suppressor cells), hypoxia, necrosis, nutrient shortage, and an array of immunosuppressive molecules (PD-L1, IL-10, TGF- β). Moreover, many enzymes with immune regulatory activity are up-regulated in cancer patients, either in the tumor environment or in draining lymph nodes, such as arginase 1, inducible nitric oxide synthase, and IDO, and production of nitric oxide and reactive oxygen and nitrogen species is increased (112, 113). These conditions can lead to inadequate T cell activation and/or T cell inhibition, restricting the persistence of tumor-specific T cells within the tumor and limiting the benefits of ACTs (114). Furthermore, if the tumor persists, continuous antigen exposure could lead to the T cell-intrinsic program of exhaustion. Finally, in the case that T cells are able to survive in the immunosuppressive tumor environment and maintain their effector functions, loss of antigen or the components in the antigen-presentation pathway may result in overgrowth of antigen-negative populations and tumor escape.

Financial Toxicity

In developed economies, there is an emerging debate about rising prices for cancer drugs (115, 116). In the United States the price tag for all new cancer drugs approved in 2017 is more than \$100,000 per year. ACT is a powerful approach that provides durable responses and the potential for cure in patients with previously refractory cancers. Two CD19-CAR-T cell products were approved for clinical use in 2017 in the United States at a price of \$373,000 to \$475,000. Due to their personalized nature, the bespoke GMPs of TILs, CAR-T cells, and TCR-T cells are associated with high development and manufacturing costs, stringent regulatory requirements pertaining to gene transfer, and reimbursement challenges. The current high cost of CAR-T cell manufacturing might be mitigated by an approach such as annuity payments with risk sharing, also known as pay-for-performance (117). Novartis has implemented this approach in the United States and charges for tisagenlecleucel CAR-T cells only upon clinical benefit in patients with ALL (118). There is an urgent need to control the escalating costs of cancer therapy, and in the case of ACT, improved cell manufacturing is the most direct strategy to lower the cost and improve access to this emerging technology.

STRATEGIES TO IMPROVE ADOPTIVE CELL THERAPY

Enhancing Persistence

Most efforts directed at enhancing T cell persistence have been focused on optimizing CAR constructs to contain intracellular domains that support T cell persistence and/or identifying strategies to enrich the final T cell product in T cell subsets with enhanced regenerative capacity.

The molecular design of CARs can strongly influence T cell expansion and persistence. While first-generation CARs containing only the CD3 ζ chain persisted long-term in HIV patients (119), they failed to persist in cancer patients (55). The addition of a costimulatory domain in second-generation CARs greatly enhanced T cell persistence and antitumor effects. CARs containing either CD28 or 4-1BB costimulatory domains have been the most widely used in the clinic, and both CAR designs yielded significant tumor regressions in patients with hematologic malignancies. Several studies indicate that CD28-based CARs have greater initial antitumor activity, while 4-1BB signaling enhances CAR-T cell persistence and mitigates exhaustion. The enhanced persistence of CAR-T cells containing the 4-1BB signaling domain may be explained, at least in part, by the increased mitochondrial biogenesis and oxidative metabolism induced by 4-1BB signaling (120). Other signaling domains, including CD27, OX40, and ICOS, are also being investigated in preclinical trials. CD27, which like 4-1BB is a member of the tumor necrosis factor receptor (TNFR) family, has been shown to promote T cell survival similarly to 4-1BB (121). Signaling through ICOS, a member of the CD28 family, has a key role in enhancing the persistence of CD4⁺ T cells (78, 122). Third-generation CARs combining two costimulatory domains, usually one from the CD28 family (CD28 or ICOS) and one from the TNFR family (4-1BB or OX40), can activate different downstream signals, combining potency with long-term persistence, and are promising candidates for clinical evaluation (78).

A different strategy to enhance persistence is to enrich the infusion product in T cells with stem cell properties. Current approaches focus on shortening the expansion protocols and optimizing them to include cytokines or agents able to delay T cell differentiation. Rapid-expansion protocols used to expand TILs induce rapid T cell differentiation, while TILs that undergo shorter expansion ex vivo persist longer (123, 124). A recent work proposes to shorten the expansion protocol for CAR-T cells from day 9 to day 3, which would limit T cell differentiation while simplifying production methods and increasing access (125). Exposure to IL-7, IL-15, or IL-21 during expansion protocols has been shown to preserve a less-differentiated state and improve in vivo persistence (126–128). However, whether enriching the final product in certain subsets, rather than using a sorted population, will be sufficient to increase ACT efficacy remains unknown. In this regard, it has been proposed that T cell subsets can synchronize their differentiation state, and therefore the presence of antigen-experienced subsets in the initial product can promote the differentiation of naive cells (129). This process is mediated by T cell-T cell interaction through a nonapoptotic Fas signal, resulting in Akt-driven cellular differentiation. Blocking the interaction between naive T cells and more-differentiated T cells, through sorting of naive T cells or Fas signaling blockade, may be used to prevent differentiation. Alternatively, activation of the wnt signaling (130) or pharmacologic inhibition of Akt can also enhance the expansion of T cells with memory cell characteristics (131, 132). Another strategy to enhance persistence is to enrich the infusion product in Th17 cells by culturing T cells with Th17 polarizing cytokines and optimized costimulation. ICOS signaling plays a key role in the function of Th17 cells (133), and CARs containing the ICOS ICD have been shown to polarize T cells toward a Th1/Th17 phenotype, which results in enhanced persistence of CD4⁺ T cells and superior antitumor effects (78, 122).

Recent work shows that in exceptional conditions, the progeny of a single CAR-T cell is sufficient to mediate potent antitumor effects in advanced leukemia, suggesting that quality of the infused T cells may be more critical than quantity (134). However, the majority of ongoing current trials with ACT use bulk T cells. The advantage of using selected populations with less-differentiated phenotypes awaits confirmation in clinical trials.

Manipulating Host Hematopoiesis

The absence of cancer-restricted surface markers is a major impediment to antigen-specific immunotherapy for solid tumors and several hematologic malignancies. As an example, anti-CD33 CAR-T cells can effectively kill CD33⁺ AML cells, but as CD33 is also expressed in the hematopoietic stem cell, they result in severe myelotoxicity. Kim et al. (135) preclinically demonstrated that CD33 can be safely targeted by deleting CD33 from normal hematopoietic stem and progenitor cells, generating a hematopoietic progeny resistant to CART33 (Figure 3). Therefore, infusion of CD33-deleted hematopoietic stem cells could allow efficient elimination of CD33⁺ leukemia cells by CART33 without myelotoxicity.

Overcoming T Cell Dysfunction

One possible strategy to overcome T cell dysfunction is to shield adoptively transferred T cells from inhibitory signals. Infused tumor-specific T cells undergo activation-induced upregulation of coinhibitory receptors, including PD-1, TIM-3, LAG-3, and CD200,

although the role of these markers is not fully understood yet. On the other hand, tumor cells can augment the expression of coinhibitory ligands, such as PD-L1, upon T cell activation-mediated release of Th1 cytokines. Strategies that combine CAR-T cells with checkpoint blockade, most of them focusing on the PD-1/PD-L1 interaction, are being actively investigated. PD-1/PD-L1 pathway interference through short hairpin RNA blockade, PD-1 dominant negative receptor, or chimeric switch-receptors have been shown to augment the efficacy of CAR-T cells in both solid tumors and hematologic malignancies (136). Using genome editing, it is now possible to delete the PD-1 gene locus on T cells. Preclinical studies indicated that PD-1 editing enhanced anticancer activity of engineered T cells, with promising evidence of efficacy in solid tumors (137, 138). A first-in-human phase 1 CRISPR gene-editing clinical trial is now testing the safety and efficacy of NY-ESO-specific TCR-T cells lacking both the endogenous TCR and PD-1 in patients with melanoma, synovial sarcoma, and multiple myeloma (NCT03399448). However, although expression of inhibitory receptors is a hallmark of exhausted T cells, it is unclear whether signaling through these receptors directly causes T cell exhaustion. Genetic absence of PD-1 has been shown to promote accumulation of terminally exhausted CD8⁺ T cells, highlighting a novel role of PD-1 in preserving T cells from overstimulation, excessive proliferation, and terminal differentiation (139). A better understanding on how these inhibitory receptors affect exhaustion of engineered T cells, together with the identification of novel pathways implicated in T cell dysfunction, will be required to further enhance the effector functions and persistence of CAR-T cells in the tumor microenvironment. Moreover, whether the abrogation of negative immune regulators could lead to increased challenges of autoimmunity or transformation needs to be addressed in clinical trials. Relevant to the later possibility, a recent study has shown that PD-1 functions as a haploinsufficient tumor suppressor in mouse T cells (140). Whether this is true for human T cells remains unknown.

A distinct class of strategies to prevent or delay dysfunction in the context of CAR-T cells involves optimizing the design and regulatory expression of CAR constructs. The choice of the type and position of the costimulatory domain, the spacer length, and/or the promoter or vector used to express the CAR has been shown to influence the level of tonic signaling. Therefore, optimization of the CAR constructs is essential to avoid early T cell dysfunction. A newer approach to regulate CAR expression involves targeting the insertion of a CAR-coding sequence into the native TCR locus, placing it under the control of endogenous regulatory elements. Targeting the CAR to the TCR established effective internalization and reexpression of the CAR upon antigen recognition, reduced tonic signaling, delayed T cell differentiation and exhaustion, and enhanced potency of CAR-T cells (141).

Another way to curb environmental immunosuppression is to target non-cancer cell components of the tumor. Tumor stromal fibroblasts and tumor-associated macrophages can induce T cell dysfunction by secreting immunosuppressive proteins, expressing ligands for inhibitory receptors, and/or developing a fibrotic extracellular matrix that can impair T cell penetration and function. One approach currently undergoing clinical evaluation is to generate CAR-T cells targeting the fibroblast activation protein, which is overexpressed in nearly all solid tumors by a subset of immunosuppressive tumor fibroblasts (142–144). A more ideal, but also more challenging, strategy is to target an antigen that is overexpressed in both malignant and neighboring immunosuppressive cells. In this regard, CAR-T cells

targeting CD123 have been shown to overcome immunosuppression in preclinical models of Hodgkin lymphoma by simultaneous targeting of malignant cells and tumor-associated macrophages (145). A different approach to enhance the activity of CAR-T cells in patients with stroma-rich solid tumors is to further modify CAR-T cells to overexpress enzymes able to degrade the extracellular matrix (146).

Overcoming Loss and/or Heterogeneity of Antigen Expression

In both B-ALL (30) and glioblastoma (59) patients treated with CAR-T cells, antigen loss has been observed as a frequent escape mechanism. In B-ALL the majority of relapses after CD19-CAR-T therapy are characterized by the loss of CD19. While several mechanisms are being described (80, 87), targeting more than one antigen in leukemic cells might reduce antigen-negative escape. Possible targets expressed in both normal B cells and B-ALL blasts are CD22, CD20, and CD123. Several preclinical studies (88, 147–149) have been published using bi- or trispecific CAR-T cells, all showing that multiple-targeting is highly effective in preventing escape. Pools of CAR-T cells, i.e., mixtures of CAR-T cells with different specificities; or dual CAR-T cells where a single cell can engage several antigens with distinct CAR constructs; or a single CAR with two binding-domains in frame can serve this purpose (Figure 4). Ruella et al. (150) demonstrated in a clinically relevant preclinical model of CD19-negative leukemia escape that cotargeting CD19 and CD123 can treat and also prevent antigen loss, and dual CAR-T cells are more potent than pooled CAR-T cells. Other important targets in addition to CD123 that are being evaluated for cotargeting to prevent antigen escape are CD22 (10, 151) and CD20 (152). More recently, switchable CARs, such as biotin-binding immune receptors and UniCARs, have been developed by creating an array of soluble, modular, tagged scFv domains, equally fitting a constant CAR stalk and supplied as required, thus allowing extra flexibility and multispecificity in a timely controlled manner. A main drawback of these approaches is that selecting safe solid tumor targets can be challenging due to on-target, off-tumor side effects.

Cytokine Armed T Cells: Armored CARs and TRUCKs

One possible strategy to overcome some of the obstacles mentioned above is to further modify T cells to release cytokines, express costimulatory ligands, or secrete checkpoint-blocking scFvs. These novel engineered T cells are known as TRUCKs (T cells redirected for universal cytokine-mediated killing) or armored T cells. The selected transgene may be constitutively expressed or induced after T cell activation following recognition of the tumor-targeted antigen. Cytokine-secreting tumor-specific T cells would offer the opportunity to harness the adjuvant effect of recombinant cytokines as living pharmacies, with the advantages of dispensing the drug locally at the tumor site and obtaining maximal activity in situ with minimal systemic toxicity. Expression of IL-12 by TILs (153), virus-specific CTLs (154), and CAR-modified T cells (155, 156) has been proposed as a mechanism to avoid tumor escape. Because systemic administration of IL-12 has proven to induce severe side effects (157), it is important to limit its expression in the tumor microenvironment, ideally by using an inducible promoter. IL-12 expression in the tumor microenvironment has been shown to improve T cell cytolytic activity, mitigate regulatory T cell-mediated immunosuppression, and recruit and activate an innate immune cell response toward antigen-negative cancer cells that could otherwise escape T cell therapy. IL-12-

armored T cells are superior to their unarmored counterparts in a number of preclinical studies, thus providing the foundation for further clinical investigation (158). However, a first clinical trial infusing TILs modified to express IL-12 under the NFAT promoter reported lack of T cell persistence with severe dose-limiting toxicities (159). Given the potential IL-12 toxicity, expression of other cytokines, such as IL-15 (160) or IL-18 (161–163), has been proposed as a safer strategy to armor T cells. Both IL-15 and IL-18 have been shown to enhance the antitumor effects of infused T cells by prolonging T cell persistence and expansion.

Similarly, engineered T cells can be modified to constitutively express ligands for costimulatory molecules. Expression of CD40L in CAR-T cells has been proposed as a method to recruit an endogenous antitumor immune response (164). In a different strategy, Zhao et al. (165) tested different CAR designs and combinations of second-generation CARs with costimulatory ligands with the aim of clarifying the optimal costimulatory support to engineered CD19-CAR-T cells. Armoring a CD28-based CAR with 4-1BBL preserved the superior killing capacity of CD28-based CARs with the extended T cell persistence provided by the 4-1BB signaling.

Reducing Toxicity

The most obvious strategy to prevent toxicities is to target antigens that are expressed in tumor cells but not in normal tissue. In this regard, neoantigen-specific T cells have been shown to induce tumor regression in the absence of adverse effects (28). An elegant example of this was reported by Tran et al. (28), who showed that T cells modified to express a TCR against mutant KRAS G12D are able to kill such molecule-expressing tumor cells while ignoring HLA molecules that have wild-type KRAS peptides. Another promising strategy is to redirect CAR-T cells against cancer-specific neoepitopes formed by aberrant glycosylation, such as the Tn antigen on MUC-1 (166). However, the reality is that the majority of engineered T cells currently used, especially in the context of solid tumors, target overexpressed shared antigens. One alternative to target shared antigens is to generate affinity-optimized CAR variants that can discriminate between aberrant and healthy cells based on different levels of expression of the target antigen (167, 168). Specifically, low-affinity CARs have been proven to mediate strong cytotoxicity against transformed cells expressing high levels of the relevant antigen with minimal or no reactivity against antigen-low, normal cells (169, 170). Whether using low-affinity CARs will increase the chance of tumor escape should be further studied.

In case of T cell-mediated toxicities, the current clinical approach relies on the administration of tocilizumab and nonspecific pharmacological immunosuppression such as corticosteroid therapy. However, several approaches to actively eliminate toxic engineered T cells without killing beneficial immune cells are currently in clinical trials. Selective termination of CAR-T cell activity has been achieved by introducing suicide and/or elimination switches. Suicide genes include proteins like herpes simplex virus thymidine kinase or inducible caspase-9, which trigger apoptosis upon induction with ganciclovir or specific synthetic dimerizer drug, respectively (171, 172). Elimination switches are surface antigens, e.g., CD20 and EGFR-derived extracellular epitopes, that can mediate in vivo

depletion by clinical-grade monoclonal antibodies. Although powerful, the other side of *in vivo* CAR-T cell ablation would be the concomitant loss of immunosurveillance. Moreover, these strategies are reactionary, focused on treating rather than preventing toxicities. Prophylactic strategies to prevent severe toxicity due to inadequate T cell specificity are also being explored (Figure 4). The introduction of microcircuits and transcriptional loops to modulate CAR expression (173), the use of *trans* second-generation CARs with split costimulatory configuration or multi-CAR platforms, and combining conventional activating constructs with inhibitory CARs that carry CTLA-4- or PD-1-derived inhibitory domains could potentially add further precision and ensure that the effector cells reach full activation only against targets expressing a defined array of tumor-associated antigens (174, 175).

Finally, the assessment of the potential toxicity of novel CAR-T cells can be conducted in clinical trials by infusing T cells with transient expression of the CAR construct by means of RNA-electroporation (176). In the context of solid tumors, local delivery of genetically modified T cells could also mitigate toxicities while increasing efficacy (57, 177, 178).

Allogeneic Cell Infusions to Increase Access

An interesting alternative to current clinical approaches using autologous T cells is to develop universal CAR-T cells or TCR-engineered T cells that could be used “off the shelf” (179). An off-the-shelf product would allow one to start with T cells from healthy donors to create large quantities of universal tumor-specific T cells that could be used in any patient without the need for HLA matching. This strategy would reduce costs, speed drug administration, and make the T cell products accessible to lymphopenic and critically ill cancer patients that often do not have sufficient numbers of healthy T cells for treatment. To this end, strategies to avoid donor T cell rejection by the recipient immune system and to reduce the graft-versus-host disease (GVHD) caused by donor T cells are needed. Zinc-finger nucleases (180), TAL effector nuclease (TALEN) (181–183), or CRISPR/Cas9 (137, 138, 184) gene-editing techniques have been used to disrupt the cell surface expression of TCRs (to prevent GVHD) and β_2m or HLA-I molecules (to prevent rejection). An initial clinical trial testing universal CAR-T cells targeting CD19 showed effective separation of graft-versus-leukemia and GVHD effects in B-ALL pediatric patients (182), and other clinical trials to test universal CAR-T and TCR-T cells are underway. A limitation of this approach is that the absence of HLA molecules may trigger a natural killer cell response against universal T cells, and therefore strategies to avoid this eventual rejection are being explored. Finally, preclinical studies suggest that CAR-TCR interactions are a prerequisite for optimal CAR-driven T cell activation (185, 186). A better knowledge of the interaction between the CAR and the endogenous TCR is required to understand whether absence of TCRs might impair the function of universal CAR-T cells.

Lastly, another strategy that is currently being explored is the use of induced pluripotent stem cells to generate universal CAR-T cells. By inserting the CAR in the TCR locus of a single pluripotent cell, one can obtain and bank a TCR-deficient CAR-T cell product with unlimited capacity to self-renew. While this promising strategy is still in its infancy, it could offer several advantages over current strategies and warrants further evaluation.

Combination Therapies

Despite the promising results of ACT, a single optimized T cell therapy may not be sufficient to induce sustained complete responses in the majority of cancers, and therefore combination therapies may be needed to treat cancer. Combining treatments that have different mechanisms of action has the potential to increase antitumor effects and reduce the chance of resistance to ACT. However, while new therapies emerge, the options to combine therapies are many. To ensure the best possible responses, rational combinations will need to be designed upon an understanding of the mechanisms underlying tumor resistance to ACT. Here, we review three emerging therapies with special potential for synergy with ACTs.

A first logical strategy to increase the potency of T cell therapies is to take advantage of checkpoint inhibitors. Adoptively infused tumor-specific T cells are susceptible to immune-checkpoint inhibitory signals and combinatorial therapy with anti-PD1 monoclonal antibodies has shown the ability to relieve immunosuppression and restore T cell function in preclinical studies (136, 187, 188). A phase 1/2 clinical trial evaluating the effect of a PD-1-blocking antibody in patients with B cell lymphoma who failed treatment with CD19-CAR-T cells showed clinical efficacy in a subset of patients, with reexpansion of the CD19-CAR-T cells ([NCT02650999](#)) (189). Multiple clinical trials investigating the combination of ACT and checkpoint blockade in both hematologic malignancies and solid tumors are ongoing.

Another strategy to increase the potency of CAR-T therapy is combination with other novel therapies that have proved efficacious in lymphomas and leukemias, such as ibrutinib, an irreversible inhibitor of Bruton's tyrosine kinase, a key component of B cell signaling and growth. Preclinical data have shown that concurrent ibrutinib treatment enhances CAR-T cell antitumor activity and engraftment in xenograft models of leukemia and lymphoma (190, 191). A clinical trial evaluating this combination is currently recruiting patients at the University of Pennsylvania ([NCT02640209](#)).

Oncolytic viruses (OVs) have received considerable attention recently due to their potential to synergize with cancer immunotherapies. OVs can specifically kill cancer cells by viral lysis without causing damage to normal cells. Similar to engineered T cells, these therapeutic agents are considered living therapies, as OVs can replicate in cancer cells and release their progeny, which results in an exponential increase of the virus dose. Another interesting aspect of OVs is that they can be genetically modified to deliver external transgenes locally in the tumor microenvironment, combining direct tumor debulking with gene delivery. Also, viruses provide a danger signal with the potential to overcome tolerance and revert immunosuppression, which makes them an appealing partner for cancer immunotherapies. In different preclinical studies, administration of an armed OV enhanced the immune functions of CAR-T cells. OVs expressing cytokines have been shown to enhance the survival of tumor-bearing mice by increasing CAR-T cell accumulation in the tumor and modulating the tumor microenvironment (192, 193). In a different strategy, expression of a PD-L1-blocking minibody from an OV could block the PD-1:PD-L1 interaction between CAR-T cells and tumor cells, resulting in enhanced tumor control (194). Finally, release of a bispecific-T cell engager by an OV enhanced CAR-T cell homing and activation in the tumor, addressing tumor escape caused by loss of antigen or heterogeneity of antigen expression and enhancing survival (90, 195).

CONCLUSIONS

The field of ACT is exponentially growing, and new strategies and ideas are being developed at a rapid pace. The scientific community is intensively studying strategies to increase T cell efficacy, to optimize T cell production, to develop off-the-shelf T cells, to reduce clinical toxicity, to reduce T cell exhaustion, to avoid tumor-microenvironment immunosuppression, and finally to test commercialization approaches. With the FDA approval of tisagenlecleucel in 2017, the ACT field has capitalized on the multitude of lessons learned as a result of the commitment, insights, and innovations of a multitude of scientists combined with the profound bravery and generosity of patients. In the last few years, ACT has progressively demonstrated dramatic potency in clinical trials of TILs and redirected TCR-T cells and CAR-T cells, leading to complete and durable responses in patients with late-stage and treatment-refractory disease. However, subsets of patients are still not responding, are relapsing, or do not have an adequate ACT strategy for their disease. There is therefore much to be done to ensure that most cancer patients respond in the long term.

Immunotherapy is now a pillar of cancer treatment and will likely be the backbone of future treatment algorithms. ACT has led to excellent results in some cancers, and ongoing research efforts will hopefully broaden its application to most cancers. For cancer with high mutational burden, checkpoint inhibitors and TILs are viable options (196). When the mutational burden is low, redirected high-affinity TCR-T cells might be a strategy, together with CAR-T cells when an appropriate surface marker is available.

Looking at the broader history of cancer therapy, where it is rare for a single agent to achieve long-term remission—with the exception of tyrosine kinase inhibitors for a subset of patients with chronic myeloid leukemia—it is likely that CAR-T therapy and indeed ACT will ultimately be of the most clinical utility in combination with novel therapies. The combination of CAR-T therapy, in particular with immune-based therapies, is eagerly awaited.

Finally issues of scale-up, automation, commercialization, intellectual property (197), and regulatory hurdles unique to cell therapy (198) need to be addressed, as more ACT products are being approved for clinical use and patients treated at large scale.

DISCLOSURE STATEMENT

S.G., M.R., and C.H.J. are inventors on patents related to CAR-T cell therapy, filed by the University of Pennsylvania and licensed to Novartis. C.H.J. and M.R. report receiving commercial research grants from Tmunity. C.H.J. has ownership interest (including patents) in Tmunity, and is a consultant/advisory board member for Tmunity, Immune Design, Carisma, and Celldex Therapeutics. M.R. reports receiving commercial research grants from Tmunity. C.H.J. has sponsored research grants from Novartis.

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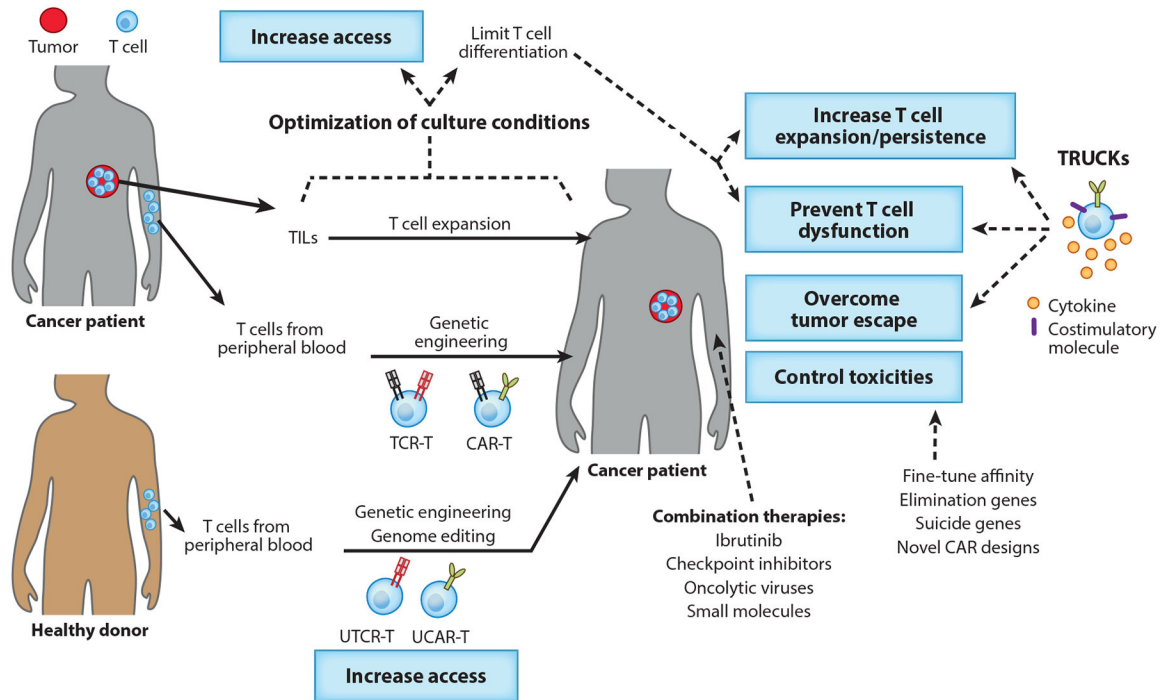


Figure 1.

Adoptive T cell transfer therapies. Tumor-specific T cells can be isolated from tumors or generated by genetic engineering of peripheral T cells. To eliminate the tumor, infused therapeutic T cells need to reach cancer cells, proliferate, and survive in a hostile tumor microenvironment. Research approaches to overcome these challenges while preventing toxicity and ensuring access to every patient are exemplified. Abbreviations: CAR, chimeric antigen receptor; TCR, T cell receptor; TIL, tumor-infiltrating lymphocyte; TRUCK, T cell redirected for universal cytokine-mediated killing; UCAR-T, universal CAR-T cell; UCR-T, universal TCR-T cell.

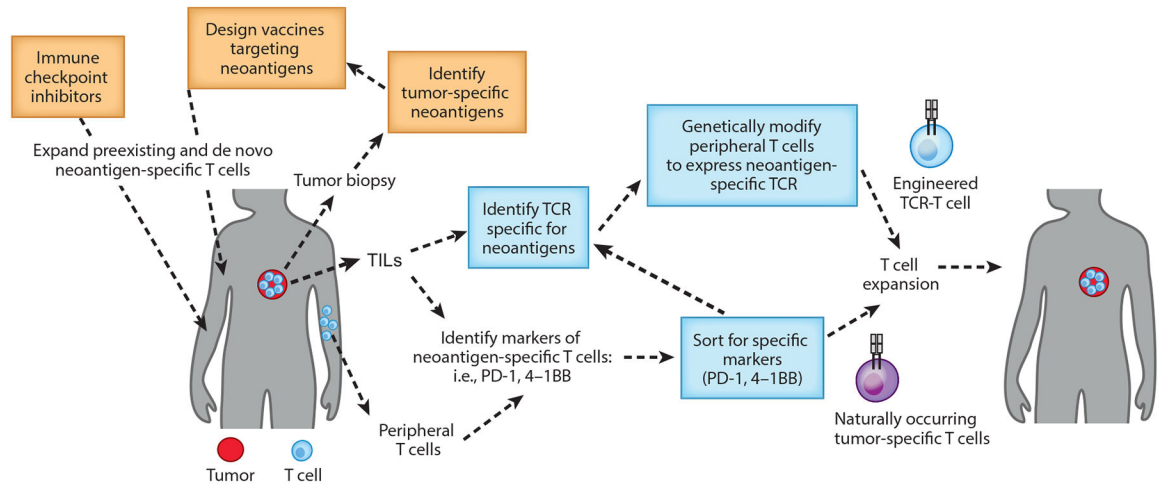


Figure 2.

Future strategies with adoptive transfer of neoantigen-reactive T cells. Tumor-reactive T cells are isolated from tumors or peripheral blood after sorting for specific markers ($PD-1^+$ and $4-1BB^+$ cells), expanded ex vivo, and reinfused into the tumors. Alternatively, neoantigen-specific TCRs can be identified and cloned in viral vectors and used to genetically modify peripheral T cells. These strategies can be combined with immunological-checkpoint inhibitors or neopeptide vaccination. Abbreviations: TCR, T cell receptor; TIL, tumor-infiltrating lymphocyte.

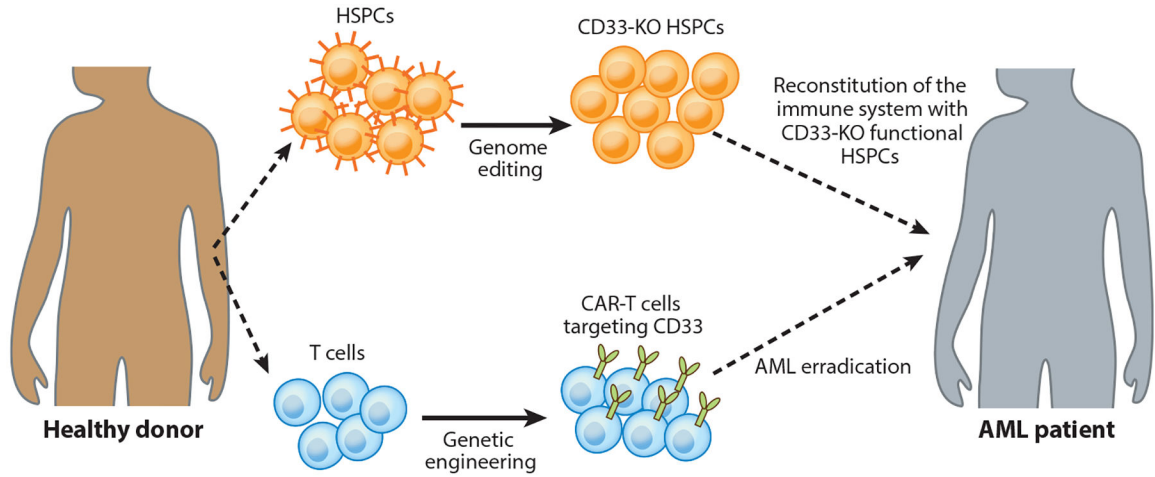
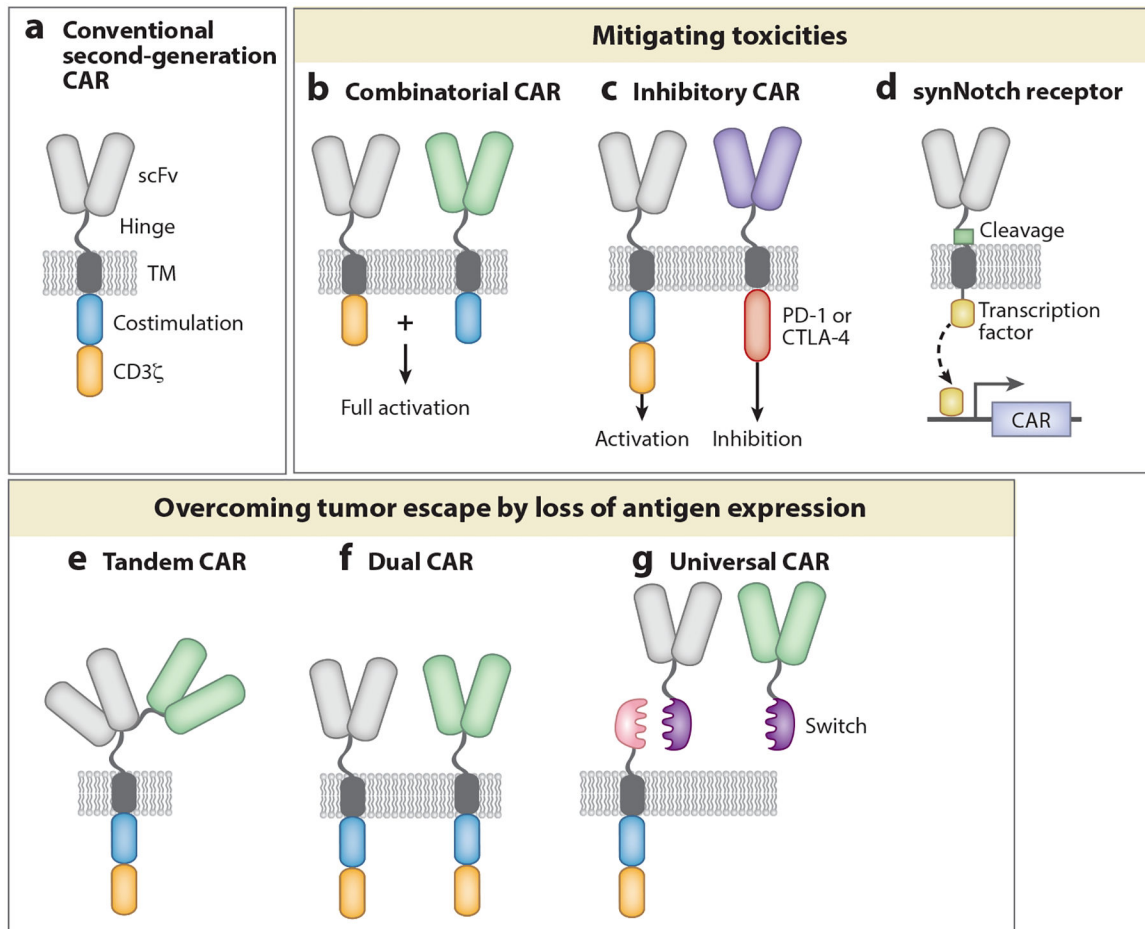


Figure 3. Reconstitution of the patient’s immune system with genome-edited hematopoietic stem cells to prevent on-target, off-tumor toxicity after CAR-T cell treatment. CD33 is expressed in both AML cells and normal myeloid progenitors, and therefore CAR-T cells targeting CD33 could induce myelotoxicity. In this strategy, CD33 is genetically disrupted in hematopoietic stem and progenitor cells to regenerate an antigen-negative myeloid system that is resistant to CAR-T cell therapy. Anti-CD33 CAR-T cells can eradicate AML while sparing CD33-deficient hematopoietic stem and progenitor cells. Abbreviations: AML, acute myeloid leukemia; CAR, chimeric antigen receptor; HSPC, hematopoietic stem and progenitor cell; KO, knockout.

**Figure 4.**

Next-generation CAR-T cells. Novel CAR designs can mitigate toxicities and/or address tumor escape. (a) Second-generation CARs consist of an extracellular domain that recognizes the tumor antigen, a hinge region, a transmembrane domain, and two intracellular signaling domains that transmit the signal. Optimization of each module is required for optimal T cell function and persistence. (b) Combinatorial CARs consist of two separate receptors targeting two different tumor antigens. One receptor bears the CD3 ζ intracellular domain, and the other bears the costimulatory domain. Full activation is only achieved after recognition of both antigens. (c) Inhibitory CARs contain the intracellular domain from inhibitory molecules. Antigen recognition in normal cells by inhibitory CARs turns T cells off. (d) Synthetic Notch receptors involve a combination of two receptors. Activation of one receptor induces the expression of a second receptor (a CAR or TCR) that mediates cell killing. Efficient T cell activation occurs only when both antigens are recognized. (e) Tandem CARs contain two different scFvs in a single CAR construct. These receptors can be activated by two different cognate antigens. (f) Dual CARs consist of two separate second-generation CARs targeting two different tumor antigens. (g) Universal CAR constructs include T cells engineered to express the universal CAR, an antibody that will recognize the tumor antigen, and a switch that acts as a bridge between the target cell and the CAR, allowing targets to be switched without reengineering the T cells. Abbreviations:

CAR, chimeric antigen receptor; scFv, single-chain variable fragment; synNotch, synthetic Notch; TCR, T cell receptor; TM, transmembrane.

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