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Perspective

The Emerging Landscape of Immune Cell Therapies

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Cell therapies present an entirely new paradigm in drug development. Within this class, immune cell therapies are among the most advanced, having already demonstrated definitive evidence of clinical benefits in cancer and infectious disease. Numerous features distinguish these "living therapies" from traditional medicines, including their ability to expand and contract in proportion to need and to mediate therapeutic benefits for months or years following a single application. Continued advances in fundamental immunology, genetic engineering, gene editing, and synthetic biology exponentially expand opportunities to enhance the sophistication of immune cell therapies, increasing potency and safety and broadening their potential for treatment of disease. This perspective will summarize the current status of immune cell therapies for cancer, infectious disease, and autoimmunity, and discuss advances in cellular engineering to overcome barriers to progress.

The transformative potential of cells as therapeutic agents was first realized in the mid-20th century, when widespread availability of red blood cell transfusions dramatically improved outcomes following trauma, surgery, and some medical conditions. Subsequently, platelet transfusion and bone marrow transplantation enhanced the survival of patients with hematologic diseases. In the modern era, fundamental advances in immunology, molecular biology, and virology alongside technological advances in cell manufacturing and genetic engineering have led to exciting progress in the development of immune cell therapies, with T cell therapies emerging as the most advanced within this therapeutic class. Adoptive transfer of tumor-infiltrating T cells and T cells engineered to express recombinant T cell receptors recognizing tumor antigens mediate impressive response rates in some solid cancers, chimeric antigen receptor-modified T cells demonstrate impressive responses in B cell malignancies resistant to all standard agents, and virus-specific cytotoxic T lymphocytes (CTLs) potently control some viral infections in immunocompromised hosts (Figure 1). This success has catapulted immune cell therapies from treatments studied on a small scale at research institutions to a global commercial enterprise. The convergence of continuing progress in immunobiology and synthetic biology, rapid advances in technologies for genetic engineering and gene editing at clinical scale, and private sector investment has positioned immune cell therapies for a substantially greater effect on human health in the decades to come. In this perspective, we summarize the current status and future prospects for immune cell therapies for cancer, infectious disease, autoimmunity, and other conditions.

T Cell Therapies for Cancer

Adoptive Transfer of Tumor-Infiltrating Lymphocytes

Studies conducted in the late 1980s and early 1990s demonstrated that 25%–50% of patients with metastatic malignant melanoma treated with infusions of *ex-vivo*-expanded autologous tumor-infiltrating T cells (tumor-infiltrating lymphocytes [TILs]) plus recombinant human interleukin-2 (rhIL-2) experienced long-lasting complete remission (Rosenberg et al., 1988, 1994). These impressive results were seminal because they provided irrefutable evidence of tumor-specific T cell-mediated immunity in humans and led to the molecular definition of self- and neo-antigens that serve as the basis for T cell recognition of cancer (Brichard et al., 1993; Coulie et al., 1994; Kawakami et al., 1994a, 1994b, 1994c). Additional studies demonstrating a critical role of lymphopenia-induced elevations in homeostatic cytokines in supporting expansion of adoptively transferred T cells (Fry et al., 2001; Fry and Mackall, 2001; Gattinoni et al., 2005) led to the demonstration that lymphodepleting therapies are critical components of effective adoptive T cell regimens for cancer (Dudley et al., 2008; Kochenderfer et al., 2017; Turtle et al., 2016a, 2016b). Although the overall clinical effect of TIL therapy in oncology has been limited in part because adequate numbers of bioactive TILs have not been reliably generated from patients with non-melanoma cancers, promising results have also been observed in individual patients with metastatic colorectal or breast cancer following infusion of TILs enriched for patient-specific neoantigens (Tran et al., 2015; Zacharakis et al., 2018).

T Cells Expressing Engineered TCRs

Advances in genetic engineering enabled investigators to clone tumor-reactive T cell receptors (TCRs) from TILs in responding patients and express the TCR in T cells expanded from peripheral blood of other patients with cancer, providing a potentially limitless quantity of cells for therapeutic application (Figure 2). Although generating T cells expressing such "engineered TCRs" was technologically feasible, early experience in translating this approach into the clinic faced several challenges. One problem was the transgenic, tumor-specific TCR α and

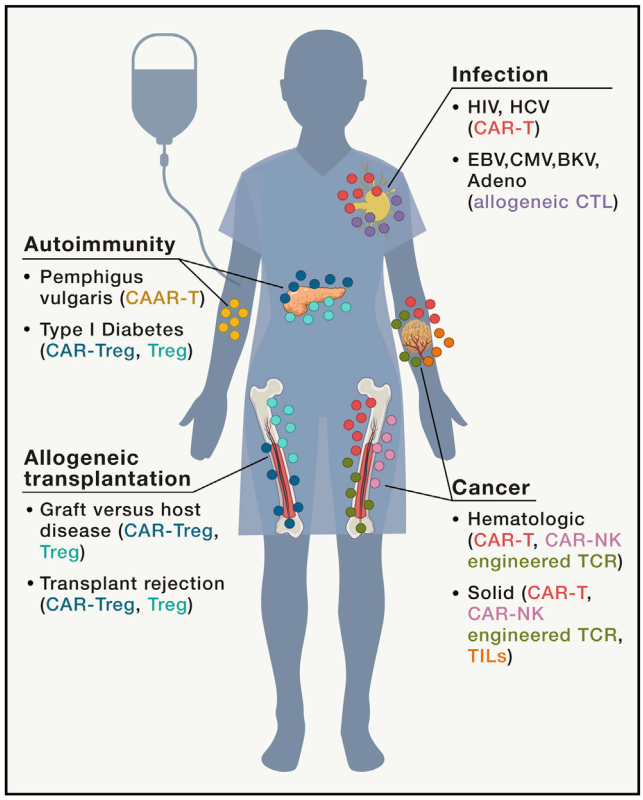

Figure 1. Immune Cell Therapies for the Treatment of Human Disease

Recent advances in synthetic biology and bioengineering have broadened the applicability of immune cell therapies to include cancer, infection, allogeneic transplantation, and autoimmunity. CAR-T or NK cells, engineered TCRs, and TIL therapy have been and continue to be tested in hematologic and solid cancers. Tregs, CAR-Tregs, and CAAR-T cells are being developed to treat various autoimmune diseases and prevent rejection of transplanted tissues.

β chains paired with endogenous receptors, which led to low levels of transgenic TCR expression and risks of off-target toxicity (Sarukhan et al., 1998). This challenge has been largely overcome by optimizing vector design (Jones et al., 2009) and incorporating cysteines (Cohen et al., 2007; Kuball et al., 2007) and/or mouse elements (Cohen et al., 2006; Hart et al., 2008; Voss et al., 2006) into the transgenic α and β chains to induce preferential pairing of the transgenic proteins.

A second challenge was identifying safe and effective TCRs contained within the TIL population to be used for genetic transfer. A significant fraction of TILs present in melanoma tumors can recognize self-antigens that are expressed at low levels on healthy tissues (Yee et al., 2000), underscoring the potential for engineered TCRs to elicit significant on-target, off-tumor toxicity (Table 1). Compared with TCRs recognizing foreign antigens, TCRs recognizing self-antigens generally manifest low potency, related in part to low affinity as a result of thymic selection (Bowerman et al., 2009; Chervin et al., 2009; Stone et al., 2009). To improve the potency of TCRs recognizing tumor-associated self-antigens, investigators enhance their affinity using yeast or bacteriophage display or by immunizing mice with human antigens (Robbins et al., 2008; Schmitt et al., 2013). Affinity-enhanced, tumor-reactive TCRs generally demonstrate increased potency but, in clinical trials, pose an increased risk of on-target, off-tumor toxicity because of recognition of low levels of antigen on normal tissues (Johnson et al., 2009; Yee et al., 2000). Affinity-enhanced TCRs are also prone to cross-reactivity (Zhao et al., 2007), as illustrated by two deaths occurring in one study where T cells engineered to express a high-affinity TCR targeting melanoma antigen gene (MAGE)-A3-derived peptide in the context of HLA-A*01 cross-reacted with titin expressed on cardiac tissue (Cameron et al., 2013; Linette et al., 2013). In a related instance, a high-affinity TCR designed to target MAGE-A3 induced lethal neurotoxicity, presumably because of cross-reactivity with a MAGE-12-derived peptide expressed in brain tissue (Morgan et al., 2013).

Approaches to predict off-target toxicity of engineered TCRs have now been developed, and some high-affinity TCRs demonstrate both safety and significant clinical activity. Most notable is a high-affinity TCR (c259) that recognizes a peptide derived from NY-ESO-1/LAGE-1 expressed on HLA-A2 (Zhao et al., 2007). Approximately 50% of patients treated with a lymphodepleting preparative regimen followed by 1–10 × 10^9 c259-expressing T cells with or without rhIL-2 experienced sustained antitumor effects (D'Angelo et al., 2018; Robbins et al., 2011, 2015). Despite the favorable response rate, complete tumor eradication was not observed in most patients, and work is underway to better understand the basis for resistance to this therapy. Favorable clinical outcomes were also observed in patients with NY-ESO-1/LAGE-expressing multiple myeloma treated with adoptive transfer of c259-expressing T cells following autologous hematopoietic stem cell transplantation (HSCT) (Rapoport et al., 2015). Similarly, T cells expressing an engineered TCR targeting the tumor-associated antigen WT-1 (Xue et al., 2005) demonstrate a favorable safety profile and have shown impressive results in preventing relapse of acute myelogenous leukemia when administered following HSCT (Chapuis et al., 2013, 2019).

To overcome the challenges associated with targeting tumor-associated self-antigens, several groups have sought to identify TCRs that mediate recognition of tumor-specific neoantigens (Cohen et al., 2015; Yossef et al., 2018; Table 1). Immune responses to neoantigens appear to play a major role in the antitumor response following treatment with immune checkpoint inhibition (Ribas and Wolchok, 2018), and TCRs recognizing neoantigens do not require affinity maturation and would be expected to demonstrate a safe profile. Autologous T cells expressing engineered patient-specific, neoantigen-targeting T cells have been generated on a small scale (Deniger et al., 2016), but the costs associated with such an individualized approach may be prohibitive for large-scale application. One strategy to overcome this challenge is to focus development on engineered TCR therapies that target shared neoantigens (Klebanoff and Wolchok, 2018; Table 1). Several recurring "hotspot" mutations have been identified in common oncogenes, such as phosphatidylinositol 3-kinase (PI3K), Ras, and p53 (Deniger et al., 2018; Lo et al., 2019), and some peptides derived from hotspot mutations are expressed on certain HLA alleles, as observed with the KRAS G12D mutation in colorectal cancer (Tran et al., 2016) and the H3K27M mutation in brain tumors (Chheda et al., 2018). Additionally, there is renewed interest in

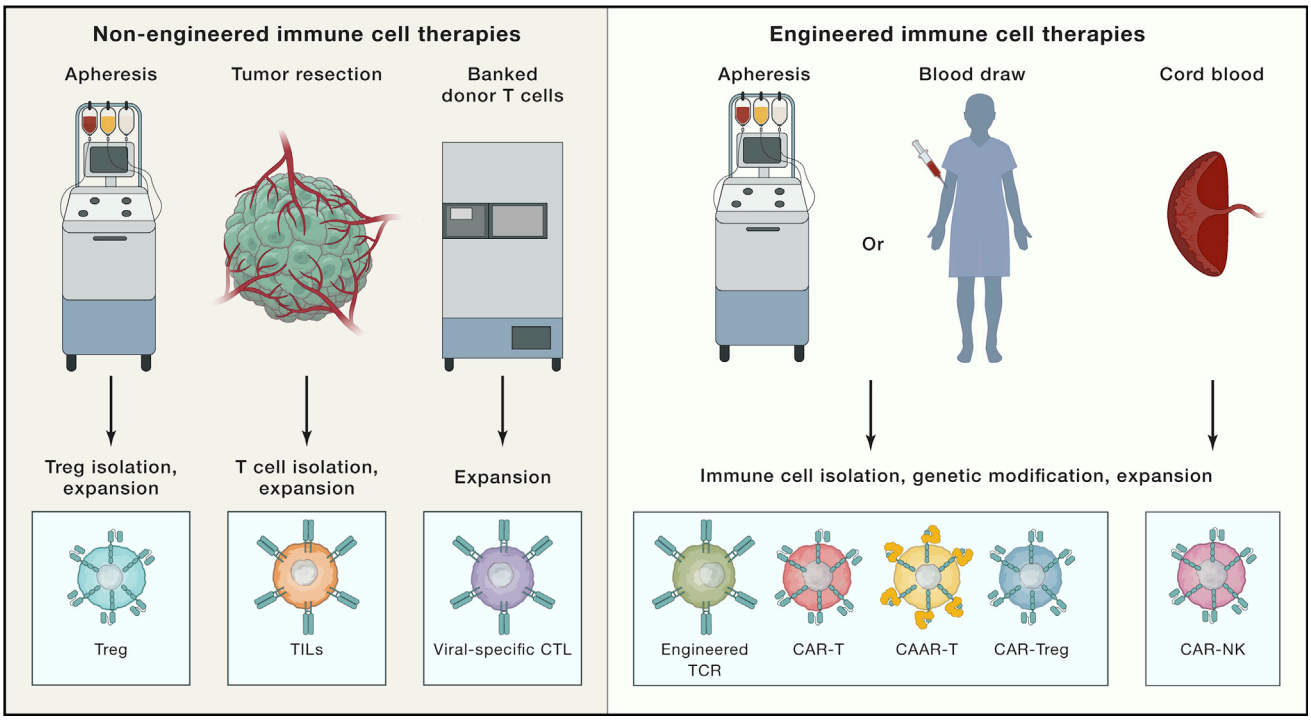

Figure 2. The Continuum of Immune Cell Therapies

Non-engineered immune cell therapies (left) have also exhibited clinical efficacy in various disease contexts, including peripheral Tregs isolated from patient apheresis that are expanded and reinfused into the patient to treat autoimmune disease, GVHD, or organ rejection. Tumor-infiltrating lymphocytes (TILs) are isolated from resected tumors (usually from melanoma patients), expanded *ex vivo*, and reinfused into the patient. Banked donor virus-specific CTLs are thawed, expanded, and reinfused into HLA-matched recipients for treatment of chronic infections. Engineered immune cell therapies (right) are generated by first apheresing or drawing blood from the patient, isolating T cells, and using viral or non-viral approaches to insert a transgene encoding a synthetic receptor. Examples of engineered T cells include (1) T cells expressing an engineered TCR consisting of TCR alpha and beta subunits; (2) CAR-expressing T cells (CAR-Ts) or NK cells (CAR-NKs), which consist of an extracellular antigen-binding domain fused to intracellular domains involved in TCR signaling; (3) CAAR T cells (CAAR-Ts), where the chimeric receptor is comprised of an antigen-binding domain that targets autoreactive B cells; and (4) CAR-Tregs, where Tregs are isolated from peripheral blood and engineered to express a CAR that redirects them to tissue affected by autoimmune disease. All engineered T cell types are further expanded *ex vivo* prior to re-infusion into the patient. Of note, T cells modified to express a CAR or TCR mainly comprise cytotoxic effectors since Tregs are not substantially enriched by current culture methods.

identifying TCRs that target breakpoint regions of oncogenic fusion proteins (Zamora et al., 2019). It may be possible to generate a bank of TCRs recognizing peptides expressed on a range of HLA alleles and use next-generation sequencing and major histocompatibility complex (MHC) typing to identify appropriate patients for such therapies. However, the laborious nature involved in identifying TCRs that exhibit potency and specificity as well as the relatively small fraction of patients who match both HLA type and tumor hotspot mutation limit the wide applicability of this strategy.

Avenues for Future Progress with Engineered TCR Therapeutics

Continued progress with T cell therapeutic agents incorporating engineered TCRs will require technologies to enhance potency, specificity, and safety. So far, the substantial effort required to develop potent and safe TCRs has limited the availability of such therapeutic agents to a small set of alleles, such as HLA-A2, resulting in significant barriers to access for patients with less common HLA alleles. Progress in this area is ongoing and includes a recent approach to modify the framework regions of the TCR variable domain, which increase expression levels and enhance potency while limiting the risk of cross-reactivity (Thomas et al., 2019), as well as new techniques to generate *de novo* TCRs (Sharma et al., 2019) or identify the antigenic targets of tumor-reactive T cells (Gee et al., 2018). Furthermore, a multitude of engineering improvements are under development to enhance the potency of adoptively transferred T cells for cancer (Figure 3), and integrating such improvements into cells expressing tumor-reactive TCRs could improve efficacy. Examples already in clinical trials include a trial testing NY-ESO-1/LAGE-1-specific TCR T cells in which PD-1, the inhibitory co-receptor, was eliminated using CRISPR/Cas9 (NCT03399448) (Stadtmauer et al., 2020) and trials administering engineered T cells with immune checkpoint inhibitors (NCT02775292 and NCT03709706).

Chimeric Antigen Receptor (CAR) T Cells for B Cell Malignancies

CARs combine an extracellular antigen-targeting domain that usually comprises a single-chain variable fragment (scFv), with intracellular signaling domains typically derived from the TCR. T cells expressing CARs are not restricted by MHC, enabling applicability across patients regardless of HLA type and also

Table 1. Challenges and Opportunities for Immune Cell Therapies for Cancer

Targeting Receptor	Opportunities	Challenges
TCR targeting overexpressed cancer-associated self-antigens	● Can target a wide range of intracellular or cell surface molecules overexpressed in cancer ● Antigens are prevalent in a wide range of cancer histologies ● Early evidence for a therapeutic window enabling safe targeting (e.g., NY-ESO-1/LAGE TCR, WT1 TCR)	● MHC restriction limits clinical applicability of any specific TCR ● Requirement for affinity maturation is labor intensive and increases the risk of off-target toxicity ● Risk of toxicity due to low-level antigen expression on normal tissues ● Most antigens in this class do not contribute to oncogenic fitness, increasing the risk of selection of antigen negative variants
TCR targeting individualized neoantigens	● TCRs do not require affinity maturation ● Likely to be safe because the antigen is not expressed in normal tissue	● Labor- and cost-intensive process to generate unique products for individual patients limits availability ● Most neoantigens are passenger mutations, increasing the risk of selection of antigen negative variants
TCR targeting public neoantigens in hotspot regions of oncogenes	● TCRs do not require affinity maturation ● Mutations likely contribute to oncogenic fitness, diminishing the risk of antigen negative variants ● Modern oncology sequencing platforms can identify subsets of patients with targetable mutations	● MHC restriction limits clinical applicability of any specific TCR ● Limited immunogenicity of most tumor-specific mutations renders it likely that TCR availability will be restricted to unique MHC alleles
CAR targeting overexpressed cell surface molecules	● Lack of MHC restriction enables applicability to all patients regardless of MHC allele expression ● MHC downregulation does not confer resistance ● Requirement for high antigen density for optimal CAR activation provides the potential for a therapeutic window even when targeting molecules expressed on normal tissues ● scFvs and other binders can be generated to specifically recognize a vast array of molecules, including post-translational modifications	● Risk of toxicity because of low-level expression on normal tissues ● Heterogeneous antigen expression increases the risk of antigen negative escape ● Requirement for high antigen density for optimal CAR activation increases the risk of antigen negative and antigen low escape

preventing resistance caused by MHC downregulation, which occurs commonly in cancer. Technologies are now readily available to generate scFvs and other binders to essentially any cell surface molecule (e.g., modified proteins, lipids, sugars, and MHC-restricted peptides) and to engineer binders with a broad range of biochemical properties.

Early studies utilized first-generation CARs, which incorporate the TCR zeta signaling domain without any co-stimulatory domain (Eshhar et al., 1993; Gross et al., 1989a, 1989b). Some first-generation CAR T cells show long-term persistence (Louis et al., 2011; Pule et al., 2008; Scholler et al., 2012), but they generally undergo limited expansion and fail to induce meaningful anti-tumor effects (Kershaw et al., 2006; Lamers et al., 2006; Park et al., 2007; Till et al., 2008). A major breakthrough ensued with the development of second-generation CARs, which integrate a co-stimulatory endodomain (usually CD28 or 4-1BB) upstream of CD3z (Brentjens et al., 2007; Imai et al., 2004). Beginning in 2010, second-generation CD19-CARs emerged as the most efficacious adoptive T cell therapy to date, mediating potent and long-lasting responses in high-grade B cell lymphoma in adults (Abramson et al., 2017; Kochenderfer et al., 2010, 2012; Neelapu et al., 2017; Schuster et al., 2017; Turtle et al., 2016b) and in B cell acute lymphoblastic leukemia (B-ALL) in both children and adults who are refractory to all standard therapies (Brentjens et al., 2011; Gardner et al., 2017; Grupp et al., 2013; Lee et al., 2015; Maude et al., 2015, 2018; Turtle et al., 2016a).

CAR T cells incorporating a CD28 or 4-1BB endodomain demonstrate significant clinical activity; however, these products differ in the rate of expansion (CD28 expands more quickly), peak expansion level (CD28 expands to a higher degree), and the propensity for persistence (4-1BB demonstrates greater persistence) (Majzner and Mackall, 2019). Differences in expansion kinetics and persistence are associated with clinical outcomes, as CD19.28.z-CARs exhibit the highest response rates in lymphoma with higher rates of CAR-mediated toxicity, whereas post-CAR relapse appears to be lower in CD19.BB.z-CARs for B-ALL, likely because CAR persistence of at least 3–6 months is important for long-term control of this disease (Finney et al., 2019; Majzner and Mackall, 2019). These observations underscore the potential benefits of tailoring CAR T cell constructs based on the unique characteristics of the tumor being targeted. Although unproven, it is possible that more aggressive tumors may require CD28 co-stimulation because of rapid expansion kinetics, whereas those that progress more slowly could be controlled more safely with CARs T cells that integrate 4-1BB co-stimulation. Further, scFv affinity and/or CAR density could be optimized to target tumors

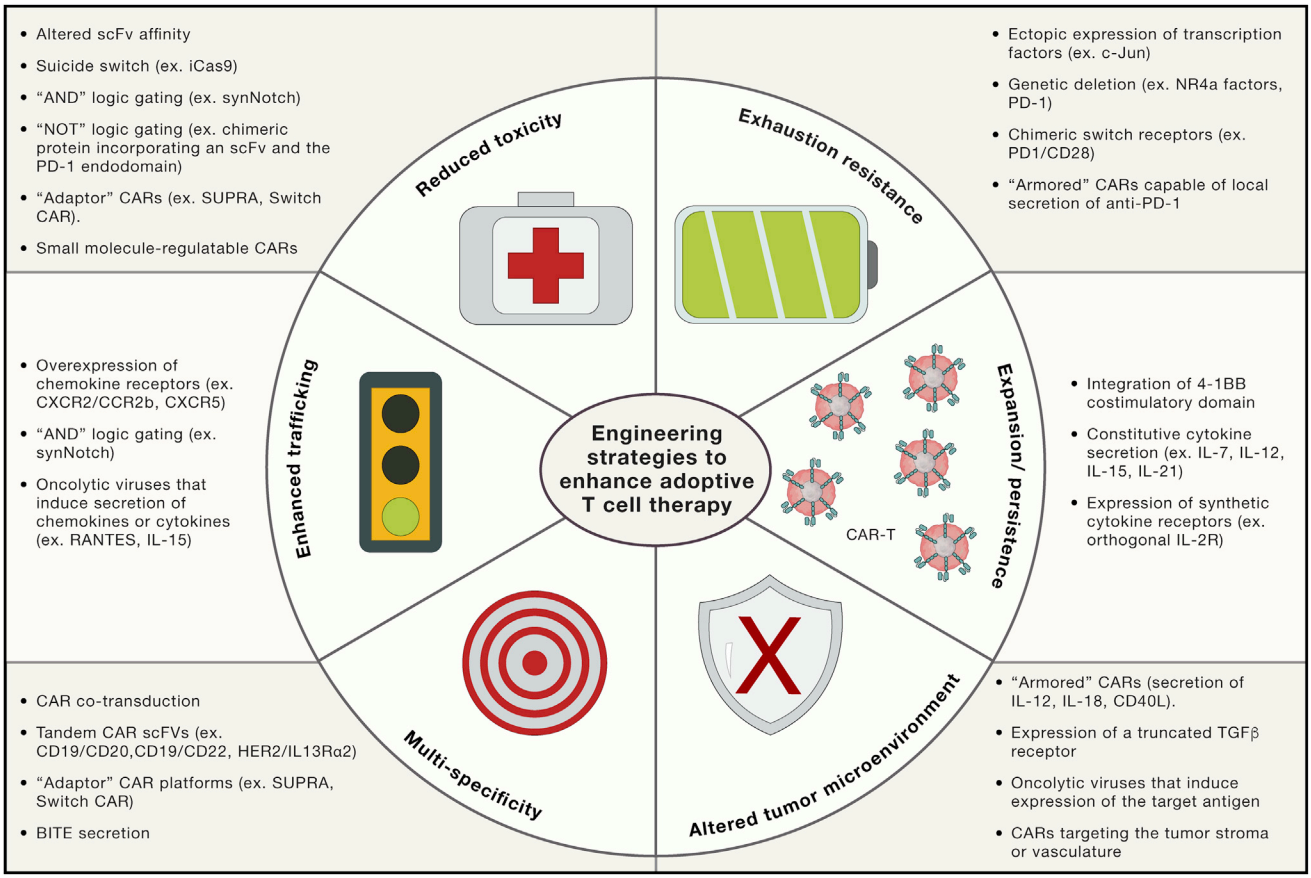

Figure 3. Engineering Strategies to Enhance Adoptive T Cell Therapy

Sophisticated bioengineering approaches are under development to enhance the potency, specificity, and safety of T cell therapies. Suicide switches, AND gating, and adaptor CAR platforms are being developed to mitigate CAR-mediated toxicity. Ectopic expression of c-Jun or genetic deletion of NR4a factors endows CAR T cells with exhaustion resistance, potentially improving efficacy in solid tumors and enhancing persistence. CAR T cells can also be engineered to secrete specific factors to augment expansion or persistence (e.g., IL-7, IL-12, IL-15, or IL-21), diminish the need for a lymphodepleting regimen, resist the suppressive tumor microenvironment (e.g., secretion of IL-18, expression of a truncated transforming growth factor β [TGF-β] receptor), or act as tumor-specific drug delivery vehicles (e.g., local secretion of anti-PD-1).

that express heterogeneous levels of antigen or to limit CAR-mediated toxicity (as discussed in more detail below).

Significant toxicities have been observed in patients treated with T cells expressing CD19 CARs, including cytokine release syndrome (CRS), whereby CAR T cell anti-tumor activity results in high levels of secreted IL-6, IL-1, and sepsis-like symptoms (Giavridis et al., 2018; Neelapu et al., 2017; Norelli et al., 2018), as well as immune effector cell-associated neurotoxicity syndrome (ICANS), which is associated with endothelial cell dysfunction at the blood-brain barrier induced by a hyperinflammatory milieu (Gust et al., 2017). Although these toxicities have resulted in serious or even fatal complications, lowering CAR T cell dose, and treatment with steroid therapy or antibodies blocking IL-6R (i.e., tocilizumab) has been highly effective. Notably, treatment-related mortality in large multicenter trials is currently less than 5% (Locke et al., 2019), which is not dissimilar from other standard treatment regimens for these refractory diseases. Additional pharmacologic strategies, like anti-IL1 receptor blockade (Giavridis et al., 2018) or the tyrosine kinase-inhibitory dasatinib, which potently and reversibly inhibits CAR T cell function (Mestermann et al., 2019; Weber et al., 2019), have been tested in preclinical models and may provide readily available, US Food and Drug Administration (FDA)-approved alternatives for patients who are refractory to standard CRS management therapies. Engineered safety switches, such as an inducible caspase 9 (Di Stasi et al., 2011; Diaconu et al., 2017), enable CAR T cell depletion in the case of severe toxicities but are irreversible by nature and have yet to be tested clinically. An alternative suicide switch that incorporates a thymidine kinase derived from herpes simplex virus initiated an immunogenic response leading to diminished survival of adoptively transferred T cells, illustrating the potential risk of enhanced immunogenicity following engineered expression of foreign proteins (Riddell et al., 1996).

The clinical success of CD19-targeting CARs has resulted in their approval by the FDA and the European Medicine Agency and has sparked widespread academic and private sector investment in CAR T cell research, including development of CAR T cells against other targets on B cell malignancies. CD22-targeting CARs also demonstrate remarkable success in

patients with ALL, with over 80% of patients treated at the highest-dose level achieving complete remission (Fry et al., 2018), and B cell maturation antigen (BCMA)-targeting CARs induce remissions in a high fraction of patients with multiple myeloma (Ali et al., 2016; Brudno et al., 2018; Cohen et al., 2019; Raje et al., 2019). The high response rates observed for CAR T cells targeting B cell malignancies are unprecedented, especially considering that most patients treated with these agents are refractory to all other therapies.

CAR T Cells for Non-hematologic Solid Tumors

In stark contrast to the success observed in B cell malignancies, CAR T cells have not demonstrated convincing evidence of activity in patients with solid tumors. Current concepts hold that this likely represents a convergence of several barriers. A major challenge is the dearth of identified cell membrane targets with high-level, homogeneous expression on solid tumors and limited expression on normal tissue. However, as investigators have focused more intently on cataloguing the surfaceome of cancer, molecules with significant differential expression have been identified, including the Tn glycoform of MUC1 on adenocarcinomas (Posey et al., 2016), GD2 ganglioside on diffuse intrinsic pontine glioma (Mount et al., 2018), GPC2 on neuroblastoma (Bosse et al., 2017), and PAPP-A on Ewing sarcoma (Heitzeneder et al., 2019). These results, combined with an emerging understanding that CARs require high levels of antigen density for optimal activation (Harris et al., 2018; Majzner and Mackall, 2018; Majzner et al., 2019; Walker et al., 2017) and that engineering approaches can tune the antigen density threshold (Liu et al., 2015; Majzner et al., 2020; Figure 3), provide optimism that a therapeutic window for CAR T cell targeting of cell surface molecules overexpressed on solid tumors can be identified.

A related issue is heterogeneity of antigen expression on cancer in general and on solid tumors in particular, which has fueled interest in developing multi-specific CARs, as discussed below (Figure 3). Effective treatment of solid tumors with cell therapies is also potentially hampered by limited trafficking. Recent work has demonstrated that regional delivery may mitigate this challenge in CNS tumors and other cancers, such as mesothelioma, which primarily demonstrate regional spread (Adusumilli et al., 2014; Brown et al., 2016; Priceman et al., 2018). Finally, solid cancers are well known to harbor a suppressive microenvironment, which inhibits CAR T cell functionality through multiple routes, including expression of checkpoint receptor ligands (e.g., PD-L1), hypoxia and nutrient depletion, and suppressive immune cells (e.g., regulatory T cells [Tregs], myeloid-derived suppressor cells [MDSCs]) (Chong et al., 2017; Juillerat et al., 2017; Long et al., 2016). As discussed below, numerous engineering approaches are in development to address these challenges and to enhance the potency of CAR T cells for solid tumors (Figure 3).

T Cell Therapies for Infectious Disease

Virus-Specific T Cell Therapy

Clinical investigators in the early 1990s observed that some patients with leukemia relapse following allogeneic HSCT could be rendered into remission following infusion of T cells from the donor (Bonini et al., 1997; Cullis et al., 1992; Drobyski et al., 1992; Kolb et al., 1990). These data ultimately provided convincing evidence for T cell-mediated antileukemic effects as well as the potential for such cells to treat uncontrolled viral infection, which occurred not uncommonly in this setting (Papadopoulos et al., 1994). In patients with severe immunosuppression, allogeneic virus-specific CTLs induce very high response rates against Epstein-Barr virus (EBV) infection and EBV-associated lymphomas, cytomegalovirus, adenovirus, BK virus, and human herpesvirus-6 infection, with limited evidence for graft versus host disease (GVHD) despite the use of minimally MHC-matched products (Heslop et al., 1996, 2010; McLaughlin et al., 2018; Melenhorst et al., 2010). Efficacy is dependent on limited rejection of the allogeneic cells because of recipient immunoincompetence, whereas the absence of GVHD is presumably due to the limited alloreactivity in CTL products that are devoid of naive T cells. ''Off-the-shelf'' banks of virus-specific CTLs are now available to treat multiple viruses covering the vast majority of HLA alleles such that an appropriate product is likely to be available for the majority of patients (O'Reilly et al., 2016; Tzannou et al., 2017; Figure 2), and private investment is seeking to commercialize this therapy. The success of these products has also raised the prospect of using virus-specific CTLs to target virus-associated cancers, including those driven by EBV (Bollard et al., 2014, 2018; McLaughlin et al., 2018), hepatitis B (Bertoletti et al., 2015), and others. However, because patients with virus-associated cancer retain sufficient immunity to reject allogeneic cell products, these efforts largely focus on adoptive transfer of autologous virus-specific CTLs or genetically engineered virus-reactive TCRs, and thus, are not dissimilar to the approaches discussed earlier that utilize non-virally directed T cells for cancer therapy.

Virus-Specific CAR T Cells for HIV Infection

Beginning in 1998, clinical trials of CAR T cells were launched to target HIV infection. T cells were engineered to express a first-generation CAR that utilized the CD4 exodomain recognizing the gp120 subunit of the HIV *Env* protein as its antigen-binding domain, enabling recognition of HIV-infected cells (Deeks et al., 2002; Scholler et al., 2012). These trials did not demonstrate efficacy in controlling HIV infection, but many patients exhibited long-term survival as a result of contemporaneous advances in antiretroviral therapeutic agents. Remarkably, 98% of samples tested more than 10 years following infusion demonstrated evidence of persistent CAR T cells, with no evidence of persistent clonal expansion or enrichment of cells in which the vector had integrated near oncogenes (Scholler et al., 2012), nor any evidence of the emergence of a replication-competent retrovirus/lentivirus (Marcucci et al., 2018). With more than 500 patient years of follow-up, this experience is the strongest evidence to date that adoptive transfer of retrovirally engineered T cells is safe and that such cells or their progeny are capable of persisting for more than a decade.

Numerous efforts are ongoing to improve the efficacy of HIV-specific CAR T cells (reviewed in Maldini et al., 2018), many of which overlap with efforts to enhance the functionality of CARs for cancer, including enhancing CAR T cell persistence and engineering multi-specificity to overcome viral heterogeneity (Hale et al., 2017). However, some challenges are uniquely relevant to CARs for HIV infection, including the need to engineer resistance to viral infection in the engineered T cells themselves,

which investigators are attempting via gene editing of CCR5 (Hale et al., 2017; Perez et al., 2008; Tebas et al., 2014) and overexpression of proteins that interfere with viral machinery (Maldini et al., 2018). Because the toxicity associated with lymphodepleting regimens is not likely to be acceptable in this clinical setting and because CAR bioactivity must be sustained for many years, engineering cells to ensure long-term persistence is a major focus. In one primate model, simian immunodeficiency virus (SIV)-reactive CAR T cells generated from HSCs compared with those generated from blood showed enhanced persistence and protection from rebound viremia upon cessation of antiretroviral therapy, raising the prospect that HSC-derived CAR-expressing T cells could be preferred in this setting (Zhen et al., 2017). Work is also underway to target the latent HIV reservoir by co-expressing CXCR5 (Ayala et al., 2017) to home HIV-specific CAR T cells to CD4+ T follicular helper (Tfh) cells in the B cell follicle of lymphoid tissues (Figure 3).

T Cell Therapies for Autoimmunity and Other Diseases

Despite the introduction of improved therapeutic agents for autoimmunity over the past several decades, more progress is needed. Small-molecule tyrosine kinase inhibitors and cytokine-targeting antibodies demonstrate remarkable clinical efficacy but are broadly immunosuppressive and not applicable to the full spectrum of autoimmune diseases. These challenges are driving efforts to develop more targeted approaches, such as adoptive transfer of Tregs (Figure 2), which play a major role in preserving self-tolerance, maintaining immune homeostasis, and preventing autoimmunity (Sakaguchi et al., 2008). Adoptive transfer of non-engineered Tregs mediates impressive results in a variety of murine models of autoimmunity and GVHD (Perdigoto et al., 2016), and adoptive transfer of human Tregs in clinical settings of GVHD, organ transplantation, and type I diabetes (T1D) have proven to be safe and feasible and have demonstrated persistence for up to 1 year post-infusion (Figure 1; Bluestone et al., 2015; Brunstein et al., 2011). However, the near- and long-term efficacy of these therapies remains unproven.

CARs are being expressed in Tregs as a strategy to improve the potency and specificity of Treg therapies. In a landmark study, investigators engineered CAR Tregs specific for 2,4,6-trinitrobenzenesulfonic acid (TNBS) in a murine model of colitis (Elinav et al., 2009). CAR Tregs secreted suppressive factors, proliferated, and ameliorated disease symptoms in an antigen-specific manner. Similar results were observed in murine models of multiple sclerosis and transplant rejection (Boroughs et al., 2019; Fransson et al., 2012; MacDonald et al., 2016). CAR Tregs exhibited therapeutic effects at doses that were suboptimal for non-engineered Tregs, providing evidence that CAR expression improves the potency of Treg therapies in addition to enhancing specificity. Collectively, these studies provide a strong rationale for clinical testing of CAR Treg therapy.

A potential advantage of CAR Treg therapy over effector CAR T cell therapy is maintenance of the target population, which may result in sustained Treg expansion, persistence, and durable immunosuppression in the target tissue. Further, although off-target toxicity of effector CAR T cell therapies could result in substantial tissue damage, off-target effects of CAR Tregs are expected to be less severe and could include prolonged immunosuppression at non-diseased tissues, opportunistic infections, or suppression of local tumor immunity. Similar side effects could also manifest if Tregs were to express a CAR that exhibits antigen-independent tonic signaling (Long et al., 2015), resulting in constitutive and non-specific immunosuppression.

A distinct safety risk associated with CAR Tregs relates to the potential plasticity of the Treg lineage. In a murine model of T1D, Tregs exposed to inflammatory conditions *in situ* lost expression of FOXP3 and converted to an effector-like T cell (Zhou et al., 2009). Further, CAR Tregs incorporating a 4-1BB co-stimulatory domain converted to cytotoxic CAR T cells that lacked immunosuppressive capacity (Boroughs et al., 2019), raising the prospect that conversion of engineered Tregs into effector cells could potentiate autoimmunity rather than suppress it. A similar phenomenon could manifest in the event of effector CAR T cell contamination in the CAR Treg manufactured product. Given that effector T cells expand much more quickly and robustly upon activation compared with Tregs, a minor population of effector CAR T cells could quickly outcompete CAR Tregs and mediate devastating autoimmunity.

Cell engineering and synthetic biology provide potential opportunities to mitigate such toxicities (Figure 3). In addition to drug-inducible suicide switches like those used in effector CAR T cells (Diaconu et al., 2017), a cell-intrinsic suicide switch that activates autonomously upon loss of FOXP3 expression or in response to transcription of inflammatory cytokines could help protect against CAR Treg conversion (Maldini et al., 2018). Alternatively, ectopic expression of FOXP3 in CAR Tregs could mitigate conversion by maintaining FOXP3 expression and promoting suppressive function (Maldini et al., 2018). To prevent long-term immunosuppression of tumor immunity or opportunistic infections at target tissue sites, one could employ tunable platforms where CAR activity is dependent on a protein therapeutic agent infused into the patient (Cho et al., 2018; Leung et al., 2019; Pishali Bejestani et al., 2017; Rodgers et al., 2016; Wu et al., 2015). In autoimmune diseases that manifest periods of sudden and severe symptoms (i.e., flare-ups), like rheumatoid arthritis or relapsing remitting multiple sclerosis, such platforms could enable induction of CAR Treg activity only during these periods while keeping CAR Tregs dormant when disease symptoms are minimal.

Recently, investigators developed chimeric autoantibody receptors (CAARs) by using a creative strategy to exploit effector CAR T cells for treatment of autoimmune disease (Figure 1). CAARs are similar to typical CARs, except the extracellular antigen-binding domain targets the B cell receptor (BCR) of self-reactive B cells. Investigators utilized a murine model of pemphigus vulgaris, wherein self-reactive B cells that target desmogleins mediate skin damage. T cells expressing a CAAR with a desmoglein 3 extracellular domain specifically targeted pathogenic B cells, resulting in a cure (Ellebrecht et al., 2016). This study provides the important proof-of-concept that CAAR T cells could be utilized in B cell-mediated autoimmune diseases in which specific autoantigens are well-defined, such as rheumatoid arthritis and lupus erythematosus.

A provocative report recently utilized CAR T cells targeting fibroblast-activating protein (FAP) to prevent fibrosis in a murine model

of fibrosis-induced cardiomyopathy (Aghajanian et al., 2019). Fibrosis was induced over the span of 4 weeks, and CAR T cells were administered 1 week following the inducing stimulus. CAR T cells infiltrated the heart, induced killing of the reactive fibroblasts, and diminished fibrosis, which led to improved cardiac function. No apparent toxicity was observed over 12 weeks, presumably reflecting a therapeutic window between high levels of FAP expressed on fibroblasts within the inflamed cardiac tissue and low levels of FAP expressed on fibroblasts in normal tissues. Whether such time-dependent and antigen-dependent therapeutic windows can be identified in the context of human fibrotic diseases remains to be seen, but this study demonstrates the versatility of CAR T cells to treat a variety of human diseases and will surely be an area of future study.

Next-Generation Engineering to Address Major Barriers to Progress

Advances in genetic engineering, gene editing, cellular reprogramming, and synthetic biology provide an increasingly robust toolbox with which to engineer solutions to the problems of resistance and toxicity that currently limit the field. Many of these solutions are modular and can potentially be integrated in a multiplex fashion in individual cells and cell products, dramatically increasing the sophistication of immune cell therapeutic agents.

Antigen-Negative and Antigen-Low Escape following Treatment with Monospecific CAR T Cells

Similar to other targeted therapeutics in oncology or in infectious diseases like tuberculosis or HIV, selective pressure on any one target often leads to emergence of escape variants. Not surprisingly, antigen loss represents a major form of resistance to CAR T cell therapy (reviewed in Majzner et al., 2020; Majzner and Mackall, 2018; Maude et al., 2018; Sotillo et al., 2015). A related issue is the increasing recognition that, in contrast to TCR therapeutic agents which can recognize very low levels of antigen, high levels of antigen are required for optimal CAR T cell activation (Harris et al., 2018; Majzner et al., 2020; Majzner and Mackall, 2018, 2019; Walker et al., 2017). Although this property can provide a therapeutic window for targeting antigens with low expression in normal tissues, such as the GD2 ganglioside or mesothelin, resistance to CAR therapeutics can also occur by selection of variants with subthreshold levels of the targeted antigen, as observed with clinical trials of CD22-CAR (Fry et al., 2018). To address these issues, efforts are underway to engineer effective multi-specific CAR T cells (discussed below; Figure 3), modulate the antigen density threshold for CAR T cell activation by altering scFv affinity (Ahmed et al., 2015, 2017; Drent et al., 2016; Liu et al., 2015) or CAR architecture (Majzner et al., 2020), upregulate the antigen density on targeted cells (Pont et al., 2019; Ramakrishna et al., 2019), target tissue stromata to prevent escape of variant tumor cells (Spiotto et al., 2004), or engineer approaches to enhance induction of natural immunity and thereby broaden the CAR-induced immune response to include bystander, antigen loss variants (Beatty et al., 2014; Slaney et al., 2017).

Endowing Multi-specificity to Enhance Efficacy (OR Gates)

Engineering combinatorial antigen recognition could enhance CAR T cell efficacy by overcoming antigen escape and/or by increasing the repertoire of targetable antigens (Figure 3). Administration of multiple CAR T cell products is one strategy of multi-antigen targeting. However, this method imposes significantly increased cost and labor. Further, in preclinical models, this approach was less effective than engineering multispecific recognition into a single cell (Fry et al., 2018; Hegde et al., 2016), and in the only reported clinical trial to date, response rates were similar to patients treated with a single CAR T cell product (Yan et al., 2019). Several approaches are under development to engineer individual cells capable of targeting two antigens, where binding of either antigen would trigger CAR T cell activation (an "OR" gate, in terms of Boolean logic). One approach is to co-transduce a single population of T cells with vectors encoding two CARs (Ruella et al., 2016), whereas a related approach incorporates a bicistronic vector to express two separate chimeric receptors on every cell (Majzner and Mackall, 2018). An alternative approach is to create a bivalent or "tandem" construct, where recognition of antigen by either one of two binding domains on the extracellular portion of the CAR can trigger effector function. Hegde et al., 2016 reported a HER2/IL13Rα2 tandem CAR for treatment of glioblastoma and observed protection against antigen escape as well as a synergistic effect on CAR T cell activation when both antigens were present. Numerous tandem CARs have been developed and studied in preclinical models (Ormhoj et al., 2019; Scarfò et al., 2018; Schmidts et al., 2019; Schneider et al., 2017; Zah et al., 2016), and clinical trials of CD19/20 and CD19/22 CARs for lymphoma (Shah et al., 2019) and CD19/22 CARs for leukemia (Schultz et al., 2018) are underway. Interestingly, all of the tandem CAR designs have required systematic testing of various configurations to determine the optimal design for each antigen. For example, the CD19/20 CARs required testing of different lengths of linkers between specificities, where only short linkers preserved function (Zah et al., 2016), and the optimal CD19/22 CAR required a looped structure whereby the two variable regions of the CD19 component were interspersed with the variable regions of the CD22 binder component (Qin et al., 2018).

Dual- or multi-targeted OR CARs can also be generated using so-called "adaptor" CARs, which have an extracellular domain that can bind a variety of binders of different antigen specificities (Cho et al., 2018; Kudo et al., 2014; Rodgers et al., 2016; Tamada et al., 2012; Urbanska et al., 2012; Figure 3). A soluble adaptor must be administered for the CAR T cell to be activated, and OR gating can be achieved when multiple binders are administered simultaneously. Such platforms theoretically provide a safety switch because CAR T cell function is ablated by clearance of the soluble adaptor. The ability to regulate the activity of the CAR is dependent not only on the kinetics of T cell expansion and persistence, but also on the half-life and stability of the adaptor protein that confers antigen specificity. An alternative approach involves engineering CAR T cells to secrete a bispecific antibody-like molecule that triggers T cell activation on one end and binds a second antigen on the tumor with the other end (Bonifant et al., 2016; Iwahori et al., 2015; Velasquez et al., 2016). This approach was recently pioneered in the setting of glioblastoma, where CAR T cells directed to the oncogenic tumor antigen EGFR variant III also secreted bispecific molecules targeting EGFR (Choi et al., 2019).

"AND" and "NOT" Gating Strategies to Enhance Safety

CAR T cells can also be engineered to activate only in response to target cells expressing two antigens concurrently, enabling discrimination between tumor cells expressing antigen pairs versus healthy tissue expressing only one of the targets (Figure 3). In one strategy, one receptor incorporates a CD3 zeta endodomain and a second incorporates a co-stimulatory domain. To prevent an OR gate, the CAR incorporating CD3 zeta must be engineered to have a very low affinity so that it induces only subpar activation upon antigen binding (Kloss et al., 2013). A different approach involves the use of synthetic Notch (synNotch) receptors (Roybal et al., 2016b), where sensing of antigen 1 by the synNotch receptor induces transcription of a CAR with specificity for antigen 2 (Roybal et al., 2016a). This strategy was efficacious in the context of a pre-clinical model of anatomically separated solid tumors, where one tumor expressing both antigens was engrafted on one flank, whereas a second (control) tumor expressing only one antigen was engrafted on the second flank. However, in a liquid tumor model, where normal stroma expressing antigen 1 (ROR1) was co-mingled anatomically with tumor cells expressing antigen 1 (ROR1) and antigen 2 (EpCAM or B7-H3), the synNotch logic gate failed to spare the antigen 1-only expressing healthy cells (Srivastava et al., 2019). Some investigators have also sought to develop NOT gates, where antigen 1 is targeted only in the absence of antigen 2, by incorporating the intracellular domain of either CTLA-4 or, more effectively, PD1 on the CAR targeting antigen 2 (Fedorov et al., 2013). This has been demonstrated to be efficacious in a pre-clinical model of allogeneic rejection of fibroblasts but has not yet entered clinical trials.

Targeting T Cell Exhaustion and the Tumor Microenvironment to Enhance Potency

Chronic antigen stimulation leads to a state of T cell exhaustion, characterized by functional impairment (Wherry et al., 2003); surface expression of multiple inhibitory receptors, including PD-1, TIM-3, and LAG-3 among others; and a distinct transcriptional and epigenetic profile (Bengsch et al., 2018; Blackburn et al., 2009; Blank et al., 2019; Pauken et al., 2016; Quigley et al., 2010; Sen et al., 2016). There is ample pre-clinical and clinical evidence that CAR T cells are predisposed to exhaustion and that this limits efficacy. Canonical exhaustion markers on tumor-infiltrating CD19 CAR T cells were higher in non-responders versus those who exhibited a complete response (CR) (Schuster et al., 2017), and high exhaustion marker expression on the CAR T manufactured product was found to be predictive of non-response (Finney et al., 2019). The phenomena of tonic signaling caused by antigen-independent aggregation of CAR receptors in the cell membrane or exposure to high tumor burdens can also lead to exhaustion (Frigault et al., 2015; Long et al., 2015). In cases where exhausted T cells are present prior to engineering, selection of T cell subsets with greater proliferative capacity prior to genetic manipulation could improve outcomes (Busch et al., 2016; Sabatino et al., 2016; Sommermeyer et al., 2016; Turtle et al., 2016a; Wang et al., 2016). Interestingly, pre-clinical and clinical studies have shown that small molecule drugs such as dasatinib or ibrutinib may prevent or reverse T cell exhaustion (Fraietta et al., 2016; Long et al., 2017; Sagiv-Barfi et al., 2015; Weber et al., 2020). Finally, transient disruption of tonic CAR signaling via regulation of CAR protein can epigenetically reprogram exhausted CAR T cells and augment efficacy in pre-clinical models (Weber et al., 2020).

A recent case study reported enrichment of a single T cell clone in which the CAR transgene integrated into the TET2 locus, resulting in a loss-of-function mutation. This mutation endowed CAR T cells with increased potency, expansion, persistence, and a memory-like phenotype, ultimately leading to 5-year complete remission at the time of the report (Fraietta et al., 2018), raising the prospect that CAR T cells can be engineered to avoid or resist exhaustion (Figure 3). This has emerged as an active and promising area of research in the field. Insertion of the CAR transgene into the TRAC locus and endogenous control of CAR expression prevented exhaustion in pre-clinical leukemia models (Eyquem et al., 2017). Overexpression of the transcription factor c-Jun (Lynn et al., 2019) has been shown to protect T cells from exhaustion from even the most exhausting CAR designs. In murine models, expression of the nuclear receptor transcription factors NR4A1, NR4A2, and NR4A3 was also associated with CAR T cell exhaustion, but without available pharmacologic inhibitors of these, it is less clear how this strategy could be applied in human T cells, where 3 separate genes would require knockout (Chen et al., 2019).

Numerous approaches to modify the tumor microenvironment or confer resistance to it in CAR T cells are also under investigation. Some groups have focused on engineering blockade of the PD-1 axis by engineered secretion of anti-PD1 nanobodies (Rafiq et al., 2018), gene editing to delete the PD-1 protein altogether (Ren et al., 2017), or engineering a "switch" receptor composed of extracellular PD-1 fused to the intracellular domain of a costimulatory molecule like CD28, which converts suppressive signals induced by tumor PD-L1 into activation signals (Liu et al., 2016). Similarly, to overcome death signaling imposed by tumor overexpression of Fas ligand, investigators expressed a dominant-negative Fas receptor that conferred increased expansion and persistence in CD19-targeting CAR T cells (Yamamoto et al., 2019). Brentjens has a series of publications on "armored" CAR T cells, where a second transgene is meant to modify the tumor environment; this includes secretion of the inflammatory cytokines IL-12 (Koneru et al., 2015; Pegram et al., 2012) or IL-18 (Avanzi et al., 2018), or CD40L (Curran et al., 2015) to enhance antigen cross-presentation and promote epitope spreading. Other groups have also used a non-signaling form of the transforming growth factor β (TGF-β) receptor that outcompetes the endogenous receptor because of its constitutive and high expression. This transgene was first applied to human EBV-specific T cells (Foster et al., 2008) and then included as a second transgene in human CAR T cells targeting prostate cancer in preclinical models (Kloss et al., 2018) and in clinical trials (NCT03089203). Finally, preclinical models have indicated that targeting the tumor stroma and/or vasculature can enhance CAR-T efficacy and potentially limit escape of antigen-negative variants (Kakarla et al., 2013; Seaman et al., 2017; Spiotto et al., 2004; Wang et al., 2014).

Alternative Immune Cells (Natural Killer [NK] Cells, Gamma-Delta [γδ] Cells, NK T [NKT] Cells, and Induced Pluripotent Stem Cell [iPSC]-Derived Immune Effectors) and Allogeneic Immune Cell Therapies

Although this review has primarily focused on therapies that administer and engineer αβ T cells, there is an emerging body

of work demonstrating progress in using similar techniques to engineer other immune effector cells that could confer certain advantages. The principal advantage of NK, γδ T cells, and NKT cells is that they all possess cytotoxic capacity, but none express an endogenous TCR; therefore, they do not mediate GVHD when administered to MHC-mismatched hosts. However, adult peripheral blood NK cells are relatively resistant to retroviral and lentiviral transduction and exhibit poor persistence in the absence of high levels of IL-2 or IL-15. To circumvent this, Liu et al. (2018, 2020) pioneered an approach whereby NK cells contained in unrelated cord blood are transduced to express a CD19-targeting CAR and a transgene coding for IL-15, which, in a recent report, mediated CRs in 7 of 11 patients treated with this therapy. Such an approach could provide an off-the-shelf CAR-NK cell product, enabling unprecedented scaling of CAR-NK therapy. γδ T cells are rare populations in peripheral blood and require substantial enrichment and bisphosphonates during *ex vivo* culture (Xiao et al., 2018). Nonetheless, investigators have successfully transduced γδ T cells with CARs, which have demonstrated activity in pre-clinical models (Capsomidis et al., 2018; Harrer et al., 2017). Similarly, invariant NKT cells are very rare populations but demonstrate pre-clinical efficacy in solid tumors (Heczey et al., 2014) and show enhanced persistence when they are engineered to secrete IL-15 (Xu et al., 2019).

Improved approaches are emerging to generate immune effector cells from iPSCs (Li et al., 2018; Themeli et al., 2013), further opening the potential for scalability of engineered immune cell populations. It is not yet clear how the cytotoxicity and persistence exhibited by iPSC-derived cells compare with cell products engineered from mature αβ T cells and whether clinically relevant cell numbers can be produced using artificial cell culture systems; however, successful approaches to generate and deliver effective iPSC-derived engineered immune effector cells offer the tantalizing possibility of manufacturing hundreds of doses of therapeutic cells from an inexhaustible source.

A major barrier to the success of this approach is rejection of the allogeneic product; although, progress is being made in this area. Recent preliminary reports have demonstrated the feasibility and some clinical activity of allogeneic CAR T cells engineered to delete the TCR as well as CD52 (Qasim et al., 2017; Torikai et al., 2012), enabling selective depletion of lymphocytes in the host to prevent rejection using a CD52-directed mAb. Recent progress has also been reported in diminishing rejection by deletion of HLA class I and II combined with overexpression of CD47 (Deuse et al., 2019). If the dual challenges of GVHD and rejection can be overcome, the availability of banks of immune effector cells generated from healthy donors could transform the field of immune cell therapy by enabling more cost-effective therapies, reducing the time needed to provide such therapies to ill patients, providing a platform for more sophisticated multi-engineering, and enabling standardization of quality across products beyond what can be accomplished using the autologous platform.

Conclusions

Immune cell therapies are a rapidly emerging class of therapeutic agents that have already demonstrated a transformative effect in some B cell malignancies and are well positioned for increasing effects in cancer and beyond in the coming years. Seminal work conducted in the 1980s and 1990s established the foundational principles of immunotherapy and genetic engineering that are now being leveraged to engineer human immune cells into "living" drugs. Remarkable progress has culminated in FDA approval of CAR T cell therapy for treatment of B cell malignancies, but translation to solid tumors has proven to be immensely challenging, and these therapeutic agents have had a limited effect on other diseases. Sophisticated bioengineering approaches utilizing genetic deletion, ectopic overexpression of transcription factors, multi-specific binders, Boolean gating, and other synthetic systems will ultimately determine the extent to which next-generation immune cell therapies emerge as efficacious alternatives to traditional medicines.

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DECLARATION OF INTERESTS

C.L.M. is an inventor on numerous patent applications in the area of CAR T cell immunotherapy and has received royalties for the CD22-CAR from the NIH following licensure to Opus Bio and Juno Therapeutics. C.L.M. is a founder of, holds equity in, and receives consulting fees from Lyell Immunopharma, which develops cellular therapies for cancer. She is also a consultant for Nektar, Neoimmune Tech, and Apricity and holds equity in Apricity and Allogene. M.V.M. is an inventor on numerous patent applications in the area of CAR T cell immunotherapy and has received royalties. M.V.M. is a consultant or advisory board member for multiple companies developing cellular therapies and holds equity in TCR2 and Century therapeutics. E.W.W. is an inventor on numerous patent applications in the area of CAR T cell immunotherapy and holds equity in and receives consulting fees from Lyell Immunopharma.

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