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developing tumors (178, 179), and older patients with more accumulated mutations prior to cell expansion will also be at increased risk.

Expansion of T cells *ex vivo* presents additional risks. As most of the mechanisms that eliminate mutant cells *in vivo*, particularly those that are immune mediated, do not apply to cells in culture, there is an increased likelihood of retaining cells with new mutations. In addition, mutations that occur in any of the many genes that regulate DNA repair will compromise the repair processes and enhance the mutation rate. Furthermore, expanding T cells *ex vivo* entails removing them from their natural environment with percent oxygen levels between 5% and 10% to culture conditions where ambient oxygen is about 20%. As culture of cells at ambient oxygen versus culture at 3% oxygen significantly increases mutation rates (180. 181), *ex vivo* expansion of T cells under standard culture conditions for autologous transplant should be considered an additional risk factor. Clearly, *in vivo* and *ex vivo* T-cell expansion confers some risk for secondary T-cell malignancies. The risk is likely quite low but will vary according to individual genetic makeup (e.g., DNA repair capacity), patient age, magnitude and nature of genetic modifications, nature of vectors used (e.g., self-inactivating lentivirus versus conventional, retrovirus vectors), as well as the scale and methods employed for the *in vitro* culture and expansion of the transduced cells.

Adverse effects of immunotherapies and their management

Most, but not all, immunotherapy-related adverse effects are of the short-term type such as cytokine storm. However, there are concerns of longer-term effects such as the potential for increased risk of autoimmune disease following treatment with PD-1 or CTLA-4 inhibitors (23, 182). In addition, there are possible risks for acquiring somatic mutations during *ex vivo* and induced *in vivo* T-cell expansion that is necessary for autologous or allogeneic adoptive T-cell strategies. Possible mutational risks associated with large-scale expansion of universal CAR T cells for "off-the-shelf" CAR T-cell therapy must also be considered. For autologous CAR T-cell therapy, a patient's T cells are first recovered from peripheral blood, transduced with a retrovirus or lentivirus harboring a CAR against a tumor-specific antigenic determinant, expanded several fold, and infused into the patient. The concern arises when one considers the rate of mutation in somatic cells. Determining the mutation rate in mammalian cells *in vivo* is also relevant when considering the extent of T-cell expansion after activation.

CAR T cells and therapies

The intricacies of the immune signaling systems and their multiple regulatory feedback circuits are critical for maintaining a homeostatic balance in immune-mediated rejection and tolerance, which when perturbed can have serious consequences. The PD-1/PD-L1 inhibitory interaction, for example, serves not only the unwanted function of protecting the tumor, but is part of the mechanism that allows the body to discriminate between "self" as "non-self". As PD-1 is expressed in many tissues and cell types, alleviation of the PD-1/PD-L1 inhibitory mechanism runs the risk of inducing systemic adverse effects of varying degrees including autoimmune disease or "immune-related adverse events" (183). Similar concerns exist for suppressing immune inhibition mediated by CTLA-4 (184, 185).

Like overcoming the immune checkpoint for therapeutic purposes, the use of CAR T cells is not without its challenges and risks. Probably the most common adverse effect after CAR T-cell infusion is immune-mediated cytokine release syndrome (CRS) or "cytokine storm" which manifests as high fever, myalgia, anorexia, tachycardia, hypotension among other symptoms, and can sometimes be fatal (186–188). Although concerning, CRS can be fully reversed by corticosteroids, such as prednisone (95), which, however, runs the risk of compromising the therapeutic effect of the CAR T cells. An alternative approach based on the remarkable elevation of IL6 that has had considerable clinical success for managing and alleviating CRS symptoms is the administration of tocilizumab, a mAb directed to the IL6 receptor (189). Another challenge with CAR T-cell strategies is on-target (correct antigen target) off-target/off tumor (incorrect cell type target) concerns. Renal cell carcinomas (RCC), for example, express high levels of carboxy-anhydrase-IX (CAIX) at their cell surface (190). When patients with metastatic RCC were treated with CAIX CAR T cells, several developed liver toxicity due to CAR T-cell interaction with bile duct epithelial cells expressing CAIX (191). Similarly, a patient with a recurrent and metastatic ERBB2-expressing tumor, when treated with ERBB2 CAR T cells, displayed pulmonary toxicity due to ERBB2 expression in the lungs (192). Likewise, when patients with melanoma or myeloma expressing the MAGE-A3 antigen were infused with MAGE-A3 CAR T cells, they developed severe cardiotoxicity due to cross reactivity to a titin determinant that was expressed on cardiomyocytes (193).

Most attention in clinical CAR T-cell experience has been with B-cell malignancies where CD 19 has been the redirected T-cell target. The approach has been relatively successful in eradicating the malignancy with the caveat that the strategy also eliminates most of the normal cells of B-cell lineage as they also express the CD19 marker (94). The loss of normal B cells and consequent off-tumor target toxicity, however, can be managed by immunoglobulin transfer to compensate for lost B cells (94). A modification of the single chimeric antigen receptor that enhances specificity is the development of a dual CAR T-cell strategy. This modification, which involves inclusion of a second chimeric antigen receptor targeting CD123 (IL3 receptor α chain), has been instrumental in overcoming evasion of CD19 CAR T-cell cytotoxicity or relapse by eliminating B-ALL cells that are CD19 negative (194). Evasion of CD19 CAR T cells by B-cell malignancies can arise due to a subset of cells that express an alternatively spliced CD19 variant that is not recognized by the CAR in the armed T cell. Inclusion of the CD123 chimeric antigen receptor eliminates the residual CD19-negative malignant B cells that also express the CD123 marker as well as those cells that have lost the CD 19 marker by acquired mutation and that contribute to CD19 CAR T-cell–resistant relapse (195). The CD123 marker is expressed on cells of the myeloid lineage and is a target for CD123 CAR T-cell therapy (196). One caveat with targeting CD123 for AML, for example, is that this marker is found on most myeloid cells and also on hematopoietic stem cells (197). Clinically, however, this may not be a serious problem (194), although this contention remains under debate (198, 199). In addition, in hematologic malignancies, where hematologic stem cell transplants (HSCT) are routinely employed to consolidate the often transitory remission following chemotherapy, CAR T-cell therapy could provide a so-called "bridge-to-transplant" allowing hematopoietic reconstitution by subsequent chemotherapy-mediated elimination of the CAR T cells and HSC transplantation.

The use of CAR T-cell approaches for solid tumors have been less successful than those for B-cell malignancies. This is in part

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because useful surface markers that are unique to the tumor are not common. To overcome the issue of specificity, CAR T cells with dual specificity have been designed with the idea that requiring both antigens to be engaged would increase specificity of the CAR T cells to the intended target (199–201). In a mouse glioblastoma xenograft model, optimal epitopes for HER2 and IL13 receptor α2 were designed by *in silico* modeling, and CAR T cells with chimeric antigen receptors targeting both epitopes were generated. The dual specificity CAR T cells appeared to have greater antitumor efficacy than CAR T cells expressing either CAR alone, and the animals survived for a longer time (202). While this dual specificity CAR T cell was efficacious in a murine xenograft model, its utility in an immunocompetent environment is unclear.

A very elegant CAR T-cell model that overcomes this reservation utilizes a synthetic modular Notch receptor (203) designed to recognize one ligand on a tumor cell and a third-generation CAR that recognizes a second antigen on the same tumor cell (204). The extracellular domain of the synthetic Notch receptor, whose normal ligand is Delta, was replaced with an extra cellular domain that recognizes a tumor antigen (antigen A). The intracellular Notch domain that is cleaved after ligand binding to the extracellular domain was replaced with a fragment that acts as a transcription factor that induces CAR expression to engage a second tumor antigen (antigen B) to initiate tumor cell cytolysis. To be killed selectively by the CAR T cell, therefore, the tumor must express both antigens. Any nontumor cell that expresses only one of the antigens will be spared. Of course, given tumor heterogeneity, it would not be surprising if some tumors contained a subpopulation of cells expressing only one or the other of the antigens, thereby escaping cytolysis by this approach and providing the seed for local tumor recurrence or metastasis. This concern has been addressed by the use of a bispecific CAR that enabled complete cytolysis of malignant B cells with no evidence of escape (205). These CAR T cells harbor reengineered single-chain bispecific antibodies that target both CD19 and CD20. Thus, malignant B cells that have lost CD 19, which normally would render them resistant to CAR T19 cell therapy, retain CD20 and are killed. It is also of course possible, though not yet directly demonstrated, that CAR-mediated lysis of a substantial population of tumor cells may in turn stimulate further immunologic responses against other tumor-associated antigens. This would produce an "antigen spread" and promote elimination of tumor cells that lack the CAR-targeted antigen(s). A related strategy involves direct administration of single molecule bispecific antibodies that target tumor cells (e.g., CD19) and T cells [e.g., a CD3 subunit TCR) to promote the engagement of the two cell types, activation of the coupled T cells and cytolysis of the target cells (206). This type of bispecific antibody, designated bispecific T-cell engager (BiTE), is currently under assessment by several clinical trials (206, 207).

Overcoming a hostile tumor environment

Overcoming a hostile immunosuppressive tumor microenvironment represents a major hurdle for CAR T-cell therapy. The tumor microenvironment is a highly complex network of tumor cells, stromal cells, activating and inhibitory cells of the immune system, vasculature, cytokines, and intercellular milieu that is generally immunosuppressive and an environment that favors tumor growth. As previously discussed, elevated local adenosine levels are inhibitory to CTLs and their ability to infiltrate the tumor. Reducing the local adenosine concentration by inhibiting ectonucleotidases (126–128) or suppressing adenosine uptake by blocking the adenosine receptor (122–124) represent alternative approaches to restoring $CD8^+$ T-cell, or CAR T-cell infiltration and activity. An additional approach for modifying the local tumor environment utilizes CAR T cells armed with the ability to secrete a cytokine (208). A major focus of this approach has centered on IL12 which exerts its antitumor activity by acting on NK cells and $CD8^+$ cells, and inducing the local production of IFNγ (209). CAR T cells that were modified to secrete IL12 have enhanced antitumor activity in a preclinical murine model (210, 211). However, a clinical trial in which melanoma patients were treated with TILs genetically modified to secrete IL12, encountered significant toxicities (212). Another recent report describes a phase I clinical trial in which MUC16 CAR T cells were further modified to secrete IL12 for treatment of patients with advanced, recurrent ovarian cancer (213), but no adverse effects have been reported to date. Tumor-associated stromal cells also contribute to the immunosuppressive microenvironment, mediated in part by elevated expression of fibroblast activation protein-α (FAP; ref. 214). When FAP expressed in stromal cells was targeted with CAR T cells in a preclinical cancer model, tumor cell growth was inhibited (215). Similarly, when stromal cells that express FAP in a murine model were eliminated, tumor-infiltrating $CD8^+$T cell activity and longer survival of mice harboring tumors were enhanced (216). Thus, adapting the tumor microenvironment to favor tumor eradication is fraught with obstacles but presents an encouraging approach.

Patient safety is paramount as evidenced by the recent temporary halting of a phase II clinical trial by the FDA. Patients with refractory ALL had received fludarabine plus cyclophosphamide prior to CD19 CAR T-cell infusion. Three patients less than 25 years of age developed cerebral edema and died while treated under this protocol and the FDA halted the trial. The fludarabine had been added to the preconditioning protocol and appeared to be the cause of the deaths. Shortly thereafter, the FDA allowed the trial to continue, but only after fludarabine was removed from the preconditioning protocol.

Patient safety and alleviation of therapeutic cost

While patient safety remains a constant concern, probably the biggest obstacle for autologous CAR T-cell therapy is the cost, which has been estimated to be as high as $400,000 to $500,000. Even if this price is exaggerated, the high cost should not be surprising given the degree of personalized clinical care required for current autologous CAR T-cell therapy protocols. As a first step, patient-derived T cells are recovered, often by leukapheresis, and the recovered T cells are stimulated to proliferate to facilitate their genetic modification by infection with a γ-retrovirus or lentivirus encoding the CAR construct. The ex vivo–transduced T cells are then expanded about 10-fold at which point the cell preparation is ready for patient infusion. This multistep process must be repeated for each patient, accounting in part for the very high cost.

To alleviate the high price of individualized CAR T-cell therapy, "universal" engineered T cells have been generated. These have the advantage that they can be expanded into large batches and used as "off the shelf" therapeutic allogeneic CAR T cells with broad applicability (216). The TCR has been disrupted by TALEN-mediated site-specific mutagenesis to eliminate the risk of graft versus host disease. Similarly, CD52, which is broadly expressed on multiple hematologic cell lineages, is also eliminated, thus rendering these cells resistant to the anti-CD52 mAb

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alemtuzumab, which is frequently employed in the treatment of hematologic malignancies. Therefore, these cells can be used in the context of alemtuzumab conditioning of the patient, which reduces the leukemic burden while concomitantly conferring a selective advantage to the allogeneic CAR T cells, including the reduced risk of rejection of these HLA-mismatched cells by the host (217). In brief, donor T cells are collected by leukapheresis, activated in culture, TCR is inactivated by site-directed mutagenesis, and transduced with a lentivirus vector harboring the CAR of interest. The cells are expanded about 10 fold to yield about 10^{10} genetically engineered "universal" T cells. From each batch prepared, aliquots are frozen, and thawed for use as needed. Treatment of an 11-month girl with relapsed $CD19^{+}$ B-ALL provided an opportunity for clinically relevant proof of principle. The patient received a single dose of the "universal" CAR T cells, designated UCART19 cells, and has undergone complete molecular and clinical remission, within the limits of detection (218).

The advances in our understanding of the biology of how our bodies distinguish "self" from "non-self" during the last 60 years have been remarkable, and the degree to which this understanding is being applied to anticancer therapies is encouraging. As Helen Keller once remarked, "Optimism is the faith that leads to achievement. Nothing can be done without hope and confidence" (219). Given the many distant and recent accomplishments, optimism is clearly applicable to the future of cancer immunotherapy. As described above, the hurdles that remain for achieving a uniformly curative anticancer immunotherapy strategy are significant but it is likely that all can be overcome or circumvented. Particularly encouraging is the idea that immunotherapy approaches can be complemented by or combined with surgery or with other developing strategies. These include targeting of biochemical pathways that are key to tumor survival or manipulating the tumor microenvironment rendering it hostile to tumor viability. Decisions by oncologists regarding optimal treatment strategies in the future will require a personalized or "precision" approach taking into account variations in genomic, transcriptomic, proteomic, and metabolomic profiles for a given tumor and patient. While currently in its infancy, the promise of such a comprehensive approach is enormous, and the expectation that cancer will be managed as a chronic disease, if not commonly cured, is becoming reality.

Disclosure of Potential Conflicts of Interest

J. Maher is a chief scientific officer at Leucid Bio. F. Farzaneh reports receiving a commercial research grant from Autolu, Cellectis, speakers bureau honoraria from Autolus and Cellectis, has ownership interest (including patents) in Autolus, and is a consultant/advisory board member for Autolus. No potential conflicts of interest were disclosed by the other authors.

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The views expressed are those of the authors and not necessarily those of the NHS, the NIHR or the Department of Health.

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