EDITORIAL

The challenges of solid tumors for designer CAR-T therapies: a 25-year perspective

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Designer chimeric antigen receptor (CAR) T cells (dTc) have come to be the latest designer ‘drug’ for treatment of cancer. This technology has generated multiple apparent cures in the treatment of B-cell malignancies, but has had much less impact in solid tumors. This perspective article considers the prior experience and the future possibilities for extending this technology’s potential to the much more numerous solid tumors for a broader clinical utility.

‘IMMUNE SURVEILLANCE’

T cells evolved to fight infection, specifically, to kill our own cells that harbor viruses or other pathogens.¹ This is clear. What is less clear is the role of T cells in the so-called ‘immune surveillance’ of cancer. If a cancer arises and is eliminated before it is clinically manifest, then how can we count the cures? I have been an immune surveillance nihilist, that if there was an autologous reaction against cancer, it was an incidental component. When looking at patients with primary or acquired T-cell deficiency as in HIV/AIDS, they have increased non-Hodgkin lymphomas, head and neck cancer and cervical cancer, but those which are largely virally mediated.^{2,3} So of course, these cancers, like the viruses that cause them, would be controlled with a competent T immune system, and they emerge disproportionately in immune deficiency. These are the cancers that immune surveillance prevents. As far as the garden variety cancers, lung, colorectal, breast and so on are concerned, there was little to suggest that these cancers are ‘seen’ by the immune system in any *effective* way. T cells may be present, and may even be cognate to antigens in the tumor, but they are maintained in an anergic or exhausted state.

One notable exception has been melanoma, where spontaneous complete remissions have been observed with primary cancers, and also, exceedingly rarely, in metastatic cases.⁴ Among these metastatic melanoma regressions, there is frequently a systemic immune stimulation from an associated infection, commonly *Streptococcus pyogenes*, but also other pathogens that became the foundation for Coley’s toxins,⁵ foreshadowing all immunotherapies of cancer.⁶ Another minor portion of these melanomas undergo remission when treated with interleukin-2 (IL-2), a T-cell growth factor that augments the activity of T cells in the tumor, supplying cytokine that exhausted T cells have lost.^{7,8} Similarly, complete regressions may be induced by *ex vivo* expansion of tumor-infiltrating lymphocytes (TILs) that have been extracted from patient tumors.⁹ These all speak to an antigen recognition that is subclinical in terms of not inducing tumor shrinkage, but which pre-exists and can be augmented by these manipulations to clinically important outcomes.

The class I antigens in melanoma have been characterized in some cases as being unmutated normal proteins.¹⁰ Some of these are melanocyte differentiation antigens (gp100, tyrosinase), and their targeting can induce tumor response but occasionally also vitiligo and uveitis, eliminating normal melanocytes in skin and retina.¹¹ Others are the so-called cancer testis antigens (melanoma-associated antigen (MAGE), G antigen (GAGE), B melanoma antigen (BAGE)) that are widely expressed in other tumors but

restricted in normal tissues to germ cells.¹⁰ The reason that there is a florid reaction in melanoma, relatively speaking, versus other tumors with the same antigens may have to do with the higher mutational load of melanomas, as discussed below.

Similarly, non-melanoma tumors may have T-cell infiltrates to a lesser degree that may correlate with improved prognosis that is also restrained when high levels of suppressor regulatory T cells (Treg) are present.^{12,13} Where prognosis is improved, survival could be related to some low-level T-cell activity that restrains the tumor growth without eliminating that growth, or the infiltrates may only be a marker of tumors whose characteristics were to be more indolent/less aggressive that elicits T-cell infiltration for other reasons.

Such T cells with ‘low-energy’ responses at baseline are presumed to be the mediators through which antibodies against immunoinhibitory receptors act, for example, cytotoxic T-lymphocyte-associated antigen 4 (CTLA4) and checkpoint receptors PD1, Tim3 and Lag3.¹⁴ These antibodies do not directly act on the cancer, but they act on the T cells to relieve blocks on the T-cell immune system. These agents have shown tumor responses in a significant minority of patients with select cancers. The idea that there is a subset of tumors with infiltration of T cells, presumably specific, but anergic, exhausted or in other ways immune hindered, is tantamount to acknowledging that the body recognizes cancer, even if the cancer may not be killed efficiently. With these interventions, solid tumors that were progressing under the patient’s own immune system are now able to be killed. Patients survive the treatment with relatively few side effects or they may experience autoimmune responses, depending on the particular agent, tumor and patient. These interventions yield significant numbers of solid tumor durable remissions, the challenge that is before us with designer chimeric antigen receptor T cells (CAR-T, dTc) in this essay. To understand this better, it bears recalling basic elements of T-cell ontogeny.

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T-cell precursors originate in the bone marrow and migrate to the thymus where they become CD4 CD8-double-negative (DN) pro-T cells. Next, as pre-T cells, they undergo T-cell receptor (TCR) rearrangement and become CD4 CD8-double-positive (DP) thymocytes. DP cells undergo positive and negative selection before maturation. Positive selection occurs for DP cells with TCRs that recognize self-major histocompatibility complex (MHC) plus self-peptide with low affinity. DP cells that do not recognize self-MHC are eliminated. With this, surviving DP T cells are now self-MHC restricted. Negative selection occurs against DP T cells with TCRs that recognize self-MHC plus self-peptide with high affinity, eliminating high-affinity self-TCRs. Following this, surviving DP T cells mature into single-positive CD4 or CD8 T cells. Only 2–3% of double-negative T cells entering the thymus survive this process, with the remaining 97–98% undergoing apoptosis and elimination by thymus macrophages.¹⁵

This process efficiently deletes T cells with high affinity for self-antigens and protects most of us from autoimmunity during our lifetimes. Low-affinity self-TCRs are passed through to allow a broader range of TCRs to confront pathogens with high-affinity anti-pathogen TCRs, whereas other mechanisms evolved to control these less vigorous effectors from self-reaction, for example, Tregs, myeloid-derived suppressor cells, anergy and

exhaustion.¹⁴ Yet, the activation of some of these low-affinity self-reactive T cells by pathogens may have a bystander effect in some autoimmune disorders in reacting to infections, as suggested in juvenile onset diabetes¹⁶ and as postulated in certain antitumor responses after bacterial exposures.¹⁷

On the other side of this equation, a correlation has been defined between response to checkpoint blockade and tumor mutational burden.^{18,19} Tumors such as melanoma, non-small-cell lung cancer and renal cell cancer have high levels of mutations that are thought to yield new antigens ('neoantigens') that are preferentially recognized versus the native proteins. One could speculate that low-affinity self-TCR may have a slightly higher affinity for altered peptide-MHC than was apparent with the native protein peptide. This could lead to modest degrees of T-cell expansion that presents as T-cell infiltrates but where the response is curtailed by T cell checkpoint receptors that have engaged ligand on tumor that checkpoint blockade unleashes. If there were no TCR affinity change, then the checkpoint blockade is unlikely to unleash full antitumor activity.

This prompts an interesting speculation. CD8 T cells ('cytotoxic' T cells, CTL) recognize peptide in the context of class I MHC, which is present on all nucleated cells. CD4 T cells ('helper' T cells, Th) recognize a structurally distinct peptide in the context of class II MHC, which is restricted to antigen-presenting cells, such as dendritic cells, monocytes and B cells. Neither a CD8 T cell nor a CD4 T cell can mount an effective immune response alone; their mutual collaboration is required. Because the structural aspects of the peptide for class I and class II MHC presentation are so divergent,²⁰ it is essentially impossible for a single mutational event to stimulate both types of T cells. Therefore, there must be a minimum two events for two newly altered peptide-MHC in the tumor cell to cross the threshold to be 'higher' affinity for antigen recognition on class I and class II MHC—now qualifying thereby as 'neoantigen', likely originating from different proteins. This could be considered a two-hit hypothesis for immunogenesis analogous to the two-hit hypothesis for tumorigenesis.²¹ This would mean that a high incidence of mutations increased the chance to achieve two mutations that would engender a heightened, functional immune recognition.

On the other hand, it has been demonstrated that CTL responses in melanoma against MAGE, GAGE and so on are against *unmutated* class I peptide.¹⁰ Yet, there still remains the information that the high mutation status of melanoma correlates with it being the most responsive cancer to immunotherapy.¹⁸ In the cases of a normal class I peptide, one may speculate that mutations in the tumor lead to selection of 'higher affinity' peptide for class II presentation that provides the CD4 help, albeit the combination is still insufficient in most instances to bring about tumor response except when alleviating inhibitory influences on the TILs.¹⁸ (Others have argued that the MAGE, GAGE and so on antigens are not important to clinical antitumor response, but that clinical response occurs solely with mutated genes that are not defined,²² which would bring us back to two mutations being required for melanoma as well.)

Yet, these *combinations* of normal and mutated self-peptides, or mutated and mutated, are truly selective for tumor, hence 'tumor-specific.' However, the incidence of autoimmunity²³ with tumor responses is still sufficient to invoke crossreaction, potentially with normal antigen in class I-directing CTL responses, but under the newly engaged class II reaction to promote CD4 helper cells.

The foregoing is all crucial background for conceptualizing the challenges of CAR-T in solid tumor therapies from the point of antigen definition.

CAR-T ANTIGENS

Every 'tumor antigen' in serum that oncologists use to follow the growth of cancer is a normal unmutated protein: CEA (carcinoembryonic antigen), PSA (prostate-specific antigen), CA19.9 (cancer

antigen 19-9), CA15.3 and so on. As CAR-T cells do not distinguish normal from tumor, only antigen-positive or antigen-negative, there is not an inherent basis for success against tumor and safety of the patient when killing all cells that express the antigen. On the other hand, there may be quantitative differences between tumor and normal tissues that may be exploited in some scenarios. For example, CEA is said to be 35-fold higher on colorectal tumors than on normal colonic epithelium, with similarly disparate expression of prostate-specific membrane antigen (PSMA) and mesothelin in tumor versus normal tissues.^{24–26}

Most importantly, with the exception of the EGFRvIII mutant protein in gliomas (below), we do not have any real, accessible neoantigens to broadly target. The strength of CAR-T as a therapeutic agent is also its handicap—CAR-T have been advertised as an off-the-shelf reagent for all patients with a diagnosis, not something that is personalized at the level of the tumor antigen that differs from one patient to another. Furthermore, the normal mode of CAR-T generation has been to create antigen-binding domains of uniformly high affinity, and in every case much higher than native TCR affinities for peptide-MHC. This may hinder the expectation of discriminating between high- and low-expressing cells in the body when a CTL can be activated with just a few TCRs being engaged.^{27,28}

There have not been tumor-specific antigens defined in a way that allows for broad targeting of patients with specific cancer types. To approximate this, genes with relatively restricted normal expression have attracted attention. These include the so-called cancer testis antigens, as mentioned under melanoma (above) and widely present in tumors—MAGE, GAGE, NY-ESO-1 and so on, whose normal expression is largely confined to gonadal tissue. Because these antigens are not surface expressed, they have been targeted with simple TCR transfers with encouraging responses,²⁹ but which omit the benefits of CAR technology to engage signals. Even in these instances, however, crossreactions with normal tissues are possible. A clinical trial using TCR to a MAGE peptide crossreacted with other proteins in the central nervous system that share peptide homology that yielded important clinical responses but also resulted in deaths and study termination.³⁰ Another approximation of a broad tumor antigen for CAR targeting has been to rely on alterations in differentiated functions of the normal tissue, e.g., as in where glycosylation differences were defined in specific proteins. This has been exploited in preclinical models to date,³¹ with utility in human testing still to be determined.

The discussion to this point has focused on the nature of antigens and how the immune system discriminates them from self and then targets them. To be an off-the-shelf reagent for many patients, however, antigen selection for CAR-T cells is of necessity restricted to shared normal cell constituents that will have its consequences, as we discuss below.

The other side of the CAR from the antigen selection and recognition is the machinery that drives the T-cell response. The majority of CAR research has been in this area, with the role of antigen specificity relegated to allowing a demonstration of targeting. The engineering of CARs and their evolution is captured in the following history that has focused on increasing the potency of the engineered T cells.

CAR-T HISTORY

I conceive that there have been five phases of designer CAR-T cell research.

Phase 1: The beginnings

Now 30 years ago, the first report of a chimeric antigen receptor was by Kuwana *et al.*³² in 1987 using an Fv-TCR $\alpha\beta$ chimera with antibody against a bacterial antigen (phosphoryl choline). Transduced T cells were activated in the presence of phosphoryl

choline-positive but not phosphoryl choline-negative bacteria, providing the first proof of principle that antibody-type antigen specificity could be engineered into T-cell signaling. This was followed by similar studies from a number of labs confirming grafting of VH+VL antibody domains onto TCR chains with hapten targets.^{33–36}

The first demonstration of a single molecule to engage recognition and signaling was by Romeo and Seed³⁷ in 1991 with an anti-HIV chimera that successfully directed transduced T cells to target and kill gp120-expressing cells. This involved linking antigen recognition via a CD4 extracellular domain to cytoplasmic domains of signaling molecules, CD3 ζ -chain of the TCR complex (e.g., CD4- ζ) and γ -chain of the Fc receptor γ -III molecule (e.g., CD4- γ) that is prominent on natural killer cells and mediates antibody-dependent cell-mediated cytotoxicity. These were the first true CARs in the modern sense of joining recognition and signaling in a single molecule.

The first single-chain antibody CARs against hapten and then tumor antigens emerged from the Eshhar group in 1993 and in many other labs shortly thereafter^{38–43} (Figure 1). These and ensuing studies helped to define the range of utility of 'T-body' or 'universal receptor' targeting, with a concentration on tumor targets. The focus of CAR research was on designing functions (hence 'designer T cells') that would rescue the range of reactivity and potency displayed by T cells. In an anti-Her2/neu (erbB2) xenograft model of human breast carcinoma in immunodeficient mice supplemented with IgTCR-modified T cells, tumor was delayed or cured.⁴¹ In a Mov18 human ovarian xenograft model,⁴² T cells with chimeric signaling molecules were similarly shown to delay or cure tumors. No toxicities were experienced by the animals.

While the first CAR, as noted above, actually used ligand (CD4) rather than antibody for target recognition (HIV gp120-expressing cells),³⁷ the first use of ligand for tumor targeting was the so-called IL-13 zetakine created by Jensen and co-workers⁴⁴ to engage IL-13Ra2 on gliomas. TCR-CARs with TCRab chains covalently linked to signaling chains were created for MAGE/A2 peptide-MHC for

melanoma therapy⁴⁵ and later by ourselves for peptide-MHC of JC virus that causes progressive multifocal leukoencephalopathy.⁴⁶

This period (1987–2004), which we can call Phase 1, was dominated by preclinical *in vitro* investigations and *in vivo* testing in animal systems, improving our understanding of the basic elements of the technology. Studies addressed the best chains for signaling (TCR CD3- ζ versus FcR γ III γ -chain), the use of Fab versus sFv antibody domains, the presence or absence of hinge spacers for optimal antigen engagement, the use of ligand versus antibody versus TCRab for antigen recognition, promoters for CAR expression, viral versus non-viral vectors, methods for vector production and T-cell transduction and finally methods for expansion and harvesting of the designer CAR-T cells for human use. See Ma *et al.*⁴⁷ for review of these early efforts.

Phase 2: Clinical trials, round 1

The next period, which we can call Phase 2, was marked by the beginnings of patient experience, all with what we now call first-generation agents. This spans the years 1994–2010. The lag from preclinical to clinical work was often 5 years or more. The first designer CAR-T in humans was not for oncology but for an HIV application with CD4- ζ CAR, which was tolerated but without persuasive benefit.^{48–50} The first oncology studies were by Hwu and colleagues,⁵¹ in ovarian carcinoma with an anti-MOV18 chimera. This was applied in 18 doses to a maximum of 5×10^{10} cells +IL-2 in eight patients without toxicity or clinical response.⁵² An anti-Tag72 IgTCR was applied in colorectal cancer, with doses to 10^{10} cells in 16 patients, and was well tolerated but without clinical responses.⁵³ (The initial report indicated potential responses by decline of serum Tag72 after treatment in several patients. However, this was later shown to be due to an anti-V region antibody response in patient sera that interfered with an ELISA (enzyme-linked immunosorbent assay) assay to give a false impression of decreased TAG72.) Our own Phase 1 tests applied 24 doses of up to 10^{11} anti-CEA designer T cells in an inpatient

Figure 1. Structure of an antibody-based CAR. The T-cell receptor/CD3 complex (TCR) is comprised of six proteins: α , β , γ , δ , ϵ and ζ . The structure of the $\alpha\beta$ receptor chains is analogous to the Fab portion of an antibody with variable (V) and constant (C) domains. γ , δ and ϵ also have Ig-like extracellular domains. Boxes in the cytoplasmic domains represent signaling motifs in all chains except α and β . Antibody V regions are engineered to create an sFv and then linked to the signaling portion of ζ , in this IgTCR CAR representation, via an intervening spacer derived from the hinge portion of CD8 α .

dose escalation in seven colorectal and breast cancer patients (five $-IL-2$ and two $+IL-2$ continuous infusion), with adequate tolerance and minor responses in two patients (one on each arm).⁵⁴ One of these patients, with liver metastases and retroperitoneal adenopathy, experienced a 50% CEA reduction and cancer pain remission, but also experienced fevers to 103.8 °F and relative hypotension reminiscent of later described cytokine release syndromes (CRS) (below). In this context, we speculated at that time: 'We believe it is likely that an effective immune response will correlate with flu-like symptoms, with ample cytokine production, inasmuch as flu symptoms are not caused by the virus during an infection, but rather by the immune system response to virus'.

Two Phase 1 studies at the City of Hope were initiated with designer T cells specific for CD20 in non-Hodgkin's lymphoma postautologous transplant⁵⁵ and for L1-CAM in neuroblastoma.⁵⁶ Doses to $1-2 \times 10^{10}$ cells were administered in seven patients in the first study and to 2×10^9 in six patients in the second, with good tolerance. Patients had T-cell persistence of 1–3 weeks in the CD20 study that was extended to 5–9 weeks in patients who also received low-dose subcutaneous IL-2. A partial clinical response was observed in 1/7 patients on the CD20 study, but the L1-CAM study was without objective responses. A later study was performed in prostate cancer with PSMA targeting using a first-generation CAR with single doses of 10^9 or 10^{10} T cells in conjunction with lymphodepletion (LD) (below) and continuous infusion IL-2. This showed CAR-T engraftment fractions of 5–52% and PR in 2/5 subjects with PSA declines of 50 and 70% and PSA delays of 78 and 150 days.⁵⁷ Depletion of IL-2 by high engrafted activated T-cell fractions was speculated to have limited the responses.

Building on a literature for regional therapy with cytokines and/or LAK/TIL (lymphokine activated killer/TIL) cytototherapy,^{58,59} this period also saw the first application of regional CAR-T infusions with the hope to overcome the apparent weakness of CAR-T to enter solid tumors. It had long been observed in TIL therapies that T cells administered intravenously are $>90\%$ retained initially in the lungs, and secondarily in the spleen and liver of the patients,^{60,61} and similarly with the designer T cells in one study.⁵⁰ To improve tumor targeting, strategies were devised to bypass the lung and secondary organs by directed site targeting (e.g., hepatic artery for liver metastases). (It is noteworthy that even for lung metastases, the tumor blood supply derives from the systemic circulation and not from the pulmonary vasculature. Hence, even in the setting of lung tumors, there is no advantage to the initial lung sequestration that follows intravenous administration.) Hege and co-workers⁵³ applied hepatic artery infusion with the Tag72-specific designer T cells in six patients with colorectal cancer liver metastases, with doses to 10^{10} cells, and 10 patients received the same doses peripherally. Treatments were tolerated, but clinical benefit was not apparent by either approach. Without responses in either of these intravenous or local infusion studies, it was not possible to make comparative judgments of the value of this strategy. Nevertheless, there is a viable rationale for intra-arterial administration that merits additional testing as newer designer T-cell agents are introduced into the clinic (below).

In a further regional intervention, IL-13 CAR directed against IL-13Ra2 was administered intracranially into glioma resection cavities in three patients with up to 12 doses of 10^8 CAR-T cells.⁶² Transient antiglioma responses were noted in two subjects, but without durable impact.

Finally, as a conceptual bridge to later engineering of costimulation into the CARs, an innovative approach was to piggyback the CAR specificity onto a competent immune response that engages a native costimulation. This was initially proposed to engineer an antitumor CAR onto alloreactive T cells in bone marrow transplantation settings.⁶³ The first clinical application of this concept took a first-generation, signal 1 GD2-specific CAR and expressed it in Epstein–Barr virus-specific CTLs and infused these back into patients

with neuroblastoma.⁶⁴ The CAR-T cells grafted onto Epstein–Barr virus T cells survived twice as long (>6 wks versus ~ 3 weeks) with an area under the curve 10 times greater for *in vivo* persistence compared with CAR-T cells created from non-immune OKT3-activated T cells. Tumor response was seen in 50% of subjects, a clear increase in benefit versus prior interventions.

Phase 3: The emergence of costimulation

The next period, we can call Phase 3, was marked by a return bedside-to-bench trajectory in the period 1996–2008. With the failure of first-generation products to induce major antitumor effects, evolving understandings of the normal role of costimulation in T-cell biology led to a belief that activation-induced cell death and inadequate local cytokine production was contributory to the inadequacy of these early products. These problems had the potential to be overcome by incorporating signals for costimulation.

The first application of a CAR with costimulation is credited to Alvarez-Vallina and Hawkins in 1996.⁶⁵ They separately and then coordinately engaged the so-called signal 1 (TCR CD3- ζ) and signal 2 (CD28) with hapten-specific CARs for markedly improved cytokine production. This discovery prompted our own studies that showed engagement of CD28 costimulation in conjunction with designer CAR-T cell blocked activation-induced cell death while significantly enhancing CAR-T survival, expansion and net tumor cell killing.⁶⁶ Following or contemporaneous with these demonstrations, a redesign of agents ensued in what we now term a second-generation collinear format with signal 1 and signal 2 in a single chain, first shown by Finney *et al.* in 1998,⁶⁷ and subsequently by Maher *et al.*,⁶⁸ with antibody-hinge-CD28-CD3 ζ (Figure 2). A critical result was shown that has guided the design of all later constructs: any configuration that moved the CD28 endodomain distally from its native juxtamembrane position lost function, whereas the activity of ζ was indifferent to its position. Since this time, all CD28 costimulatory constructs have been placed juxtamembrane with CD3 ζ in the distal position. Although CD28 was most widely used for costimulation, other groups used 4-1BB alone or in combination with CD28.^{69,70}

Phase 4: Clinical trials, round 2

The clinical testing of second-generation, two-signal designer CAR-T cells marked Phase 4, the period from 2008 to 2016. The first second-generation, two-signal designer T cells to enter the clinic were for CD19+ lymphomas (M Sadelain) and for CEA+ adenocarcinomas (R Junghans). Results were initially modest for CD19+ tumors with direct systemic infusion of CAR-T cells.^{71,72} These results

Figure 2. Structure of first- and second-generation CARs. The first-generation CAR is linked to the signaling domain of TCR ζ -chain, generating Signal 1 on antigen contact. The second-generation CAR incorporates the CD28 signaling domain together with TCR- ζ , generating Signal 1+2 on antigen contact.

seemed at odds with the promise from preclinical studies of the ability of added costimulation to mediate cures. One study with a mixture of first- and second-generation CAR showed superior persistence and activity of the second-generation CAR-T cells,⁷³ seemingly supporting some value to the second-generation modification, albeit not a home-run. No responses were observed in the CEA study, even with CAR-T doses to 10^{11} cells; when IL-2 was added in this study, major marker reductions were noted that were encouraging for response in a limited set of patients treated systemically (RPJ, unpublished results) or regionally.⁷⁴

Also arising in this period of CAR-T applications was the implementation of lymphodepletion (LD) conditioning. This innovation, unrelated to any CAR-T design, was originated for TIL therapies at the Surgery Branch/NCI. LD protocols were instituted that used non-myeloablative chemotherapies or myeloablative radiation exposures. These created a hematopoietic space with elevation of homeostatic cytokines IL-7 and IL-15 that could engender massive expansions and engraftment of infused activated T cells. Dudley *et al.*⁷⁵ showed that the more intensive the conditioning, the better the engraftment and the higher the response rates in melanoma, including many durable remissions.

With the application of LD before CAR-T cell infusion, there was similarly a marked improvement in response rates. Even first-generation products (e.g., PSMA CAR,⁵⁷ above) could generate meaningful responses with this maneuver. Nevertheless, the advantages to costimulation would be also advantageous (or dangerous, as discussed below) in the LD setting. This Phase 4 with institution of LD became the period of spectacular responses with second-generation CAR-T in the CD19 acute B-cell lymphoblastic leukemias (ALL) especially, with CR rates of up to 90% or higher, including many durable remissions (reviewed in Davila and Sadelain⁷⁶). Lesser but still impressive response rates have been noted in CLL and non-Hodgkin's lymphoma.⁷⁶ The results in solid tumors with second-generation CAR-T and engraftment protocols are discussed in the later sections.

With these CD19 CAR-T results, an interesting conversation has arisen around the selection of costimulatory molecules. Although the constructs with CD28 and 4-1BB were equally potent for ALL, the CD28 results were markedly less potent than 4-1BB with CLL and non-Hodgkin's lymphoma B-cell malignancies. ALL may be more sensitive due to the inherent fragility of blast cells that are readily destroyed by either CAR-T (see below). Features of 4-1BB in promoting memory have been touted as possible reasons for the difference in the harder-to-treat non-ALL B malignancies.^{77,78} At this point, it is not clear whether there will be a shift to using 4-1BB, or whether other accessory interventions may drive CD28 CARs to equivalent benefit (e.g., deeper LD conditioning).

The first application of conditioning with CAR-T in solid tumors was in 2008 with PSMA targeting,⁵⁷ as noted above. With LD, PSMA targeting generated clinical responses without evidence for normal tissue toxicity. This employed a first-generation CAR. A subsequent prostate protocol with a second-generation anti-PSMA CAR and a milder conditioning regimen had no toxicity but also no response that may have been limited by the observed lack of engraftment.⁷⁹

The first use of a second-generation CAR with a full LD protocol was with an anti-Her2 CAR-T treatment in a colon cancer patient.⁸⁰ This patient died in 5 days, with hemorrhagic enteritis, pulmonary edema and cardiac arrest. A dose of 10^{10} CAR-T cells had been infused and these cells massively expanded after infusion post LD. These CAR-T cells proved to be extremely potent, as with engrafted TILs, but now with targeting known sites of normal antigen expression (bowel, lung, heart) in what came to be known as on-target/off-tumor toxicity. This event generated much discussion regarding safety to normal tissues when targeting with CAR-T (below).

The LD protocol was identical for the PSMA study⁵⁷ and for the Her2 study. The higher hazard in the Her2 study may be because of the second- versus first-generation format of the PSMA study or

because of the inherent risk/safety of the tumor antigens being targeted. This is considered further in later sections of the essay.

Phase 5: Clinical trials, round 2, extended edition—solid tumors

The death of the Her2 CAR-T patient marked the beginning of Phase 5, which is where we are now. Where on-target/off-tumor toxicity in CD19 targeting leads to normal B-cell aplasia, this can be offset by administration of immunoglobulin preparations. In solid tumors, loss of tissues expressing antigen can lead to fatal consequences, as illustrated. This phase marks the arrival of CAR-T potency, whether by CAR design or by how the cells are administered or both, with consequences good and bad. It marks that point at which a balance had to be struck between tumor killing and tissue toxicity. This phase may be considered to have originated with the use of second-generation agents, contemporaneously with the first B-cell lymphoma treatments, but with a more protracted exploration necessitated by hazard to normal tissues with solid tumor antigens.

A prevailing belief has been that LD in conjunction with second-generation CAR-T will be required to induce cancer remissions, whether tumor is liquid or solid.⁸¹ A re-examination of this premise is necessitated, however, by the worries of safety against self-antigens in normal tissues. This can be a concern even without LD, but especially with LD where it is difficult to control exposures (which can vary 10-fold from the same administered dose⁵⁷) and it is difficult to reverse toxicity once those exposures are in place. These considerations will occupy the remainder of this essay with speculations about how we can get to a point of comparable benefit with solid tumors—with the potential for helping many thousands more patients than we can with CD19 targeting.

RESPONSES WITH CAR-T IN SOLID TUMORS

With the CEA clinical trials, one with first-generation CAR, one with CEA-specific TCR and three with second-generation CAR^{54,74,82–84} (RPJ, unpublished data) all showed significant marker reductions but without corresponding tumor shrinkage on imaging studies 1 month after treatment. The TCR transfer was not a CAR, that is, there was no direct conjugation with signaling chains but only through the normal TCR association with CD3 chains to generate signal 1. This would be analogous to a first-generation CAR. The TCR transfer was with LD and T cells engrafted, but lost activity over time. However, under the new immune response (iResponse) criteria,⁸⁵ which allow for the delayed tumor shrinkage with immune therapies, these studies could have missed responses with their early views.

PSMA targeting was performed in two trials with first- or second-generation CAR, showing in one study PR in 2/5 subjects in a dose escalation, with 50 and 70% PSA reductions and PSA delays of 78 and 150 days, and in the second study no responses (0/7).^{57,79} The PSMA study with partial responses used a first-generation CAR but with IL-2 supplementation (low dose) and a more intensive conditioning regimen with higher CAR-T engraftment. In the PSMA and CEA studies, IL-2 was suggested to be important to the responses that were obtained^{57,74} (RPJ, unpublished data). In a transient protocol in two subjects, mesothelin targeting with a second-generation 4-1BB CAR without LD similarly showed minor or mixed response in ascites and tissues.⁸⁷

In two glioma studies, systemically administered second-generation CAR-T at very low doses ($\leq 5 \times 10^8$ cells) against EGFRvIII⁸⁸ showed 0/9 objective responses, or against Her2⁸⁹ (piggybacked on CMV specificity analogous to Pule *et al.*⁶⁴) generated 1/16 PR. Both were well-tolerated. In a follow-up on the earlier first-generation IL13 zetamine trial,⁶² a second-generation CAR-T against the IL13Ra2 antigen showed limited activity in seven patients when administered to tumor resection cavities, but then complete tumor regressions in brain and spine in the one patient who subsequently

also received CAR-T cells intraventricularly.⁹⁰ This agent showed good safety despite wide expression of IL13Ra1 in the body with which this CAR-T cross-reacts, possibly protected by the regional treatment strategy. Although the patient ultimately relapsed, this result is highly encouraging for treating solid tumor, at least for this tumor that allows selective regional targeting.

Logically, the tumor selectivity of the mutant EGFRvIII, a true neoantigen,⁹¹ should permit much higher dosing (e.g., 10^{11} cells systemically) or LD engraftments to improve response rates with continued safety. In contrast, further systemic simple-infusion (SI) dose escalations will be required to judge safety versus efficacy for targeting the widely expressed normal Her2 antigen, especially given its known hazard.⁸⁰ To date, these tests have been performed cautiously and safely, without engraftment procedures.

The pattern over these early studies is that there is encouraging activity against solid tumors, but the depth and duration of responses have not approximated those seen in B-cell leukemia therapies. The only solid tumor studies with effective LD were with the first-generation PSMA CAR and CEA TCR transfer that would be analogous to the first generation as stated above, and in the Her2 trial with second-generation CAR-T. In the latter case, it was not possible to judge tumor response because the patient died quickly. It is likely that an increased benefit would be obtained by the combination of LD with second-generation CAR if toxicity could be avoided. However, it is possible, even in the case of avoiding toxicity, that equaling the results in liquid tumors will require additional measures.

Finally, there are features of the tumor microenvironment that may predominate more in solid than in liquid tumors. Such features include suppressor cell types (Treg, myeloid-derived suppressor cell and so on) that can affect CAR-T,⁹² issues of vascular access and tumor penetration as well as antigen overload that can lead to T-cell exhaustion or anergy.^{8,93} An excellent review of the potential for suppressive microenvironment issues recently appeared.⁹⁴

Solid versus liquid tumors

In the following, we contrast liquid tumors with these solid tumor results: understanding the relative ease of treating liquid tumors could be instructive to the challenges for solid tumor treatment. By liquid, we mean leukemias and lymphomas, more specifically, B-cell malignancies.⁹⁵ It is unclear at this point whether there will be a similar success with other liquid tumors: the myeloid cancers acute myelogenous leukemia, chronic myeloid leukemia and their variants, or with T-ALL.

Leukemia safety. CD19 targeting has been called the “low-hanging fruit” for CAR-T therapy, because we can do without the organ (‘B cells’) and its antigens are highly restricted. The same can be said of targeting CD20, CD22 and BCMA (CD269). To be clear, B cells are an essential organ. They are the progenitors to plasma cells that make antibody without which we cannot survive. All patients treated with anti-CD19 CAR-T with resulting B-cell aplasia would die if left without other intervention. Only modern medicine has provided the means to dispense with this organ by supplementing with clinical immunoglobulin prepared from donors who still have normal B cells. However, it is one of the few organs for which antigens are so selectively restricted that can also be so readily ‘dispensed’ with, i.e., substituted for. (Thyroid is a second example.) Other organs can also be ‘dispensed’ with, such as breast, prostate and reproductive organs, but antigens are rarely perfectly restricted to these organs.

Leukemia response. It is worth considering further differences and possible rationales for deeper responses in the CD19 studies versus solid tumors.

1. Liquid/lymphoid tumor versus solid:

a. Distribution: Liquid/lymphoid tumors are thought to be therapeutically more amenable to trafficking and killing than

solid tumor (e.g., prostate cancer), inasmuch as T cells will go where lymphocytes go and where B-CLL and ALL are found—blood, bone marrow, spleen and lymph nodes.

b. Sensitivity: There is also the matter of intrinsic sensitivity of lymphoid malignancies to therapeutic agents. For example, ‘single-agent’ prednisone can lyse 50% of CLL cells with no effect at all on solid tumors.^{96,97}

2. Three signals versus two signals: The CLL treatment setting is stated as using two signals (4-1BB or CD28 plus ζ engineered into CAR). Both the configurations worked well with B-ALL, with complete response rates of 90 and 87%,^{98,99} but the 4-1BB CAR was superior to CD28 for clearing B-CLL, with overall and complete response rates of 57% and 29% for 4-1BB versus 20% and 0% for CD28, respectively.^{71,100} The blasts of ALL are inherently more fragile and may be more susceptible to killing than small, more mature cells of CLL so that differences in the costimulatory molecule do not matter for lysing ALL but differences are important for killing CLL. However, the differences favoring 4-1BB in for CLL could derive from the 4-1BB CAR actually being three signals because of additional stimulation of CAR-T CD28 by B7 on CLL that is lacking on solid tumors—which we and others showed can be effective *in trans*.^{65,66} Nevertheless, significant differences exist in the activities of 4-1BB versus CD28 that may translate into superior resistance to exhaustion,⁷⁸ for example, and a favorable memory generation profile¹⁰² that could work in favor of this configuration even if just two signals. These are points that will be of interest to answer in solid tumor studies (in which B7 is normally absent). The CLL and ALL studies did not use IL-2, but effectiveness was correlated with CRS,¹⁰³ in which diverse T-cell cytokines were prominently detected.¹⁰⁴ Although two signals are superior to one signal for IL-2 secretion,¹⁰⁵ the third signal may be superior to two signals. Whether IL-2 would rescue the effectiveness of the two-signal CD19 CD28- ζ CAR (as for solid tumors¹⁰⁶) to equal that of the “three-signal” 4-1BB setting in lymphoma without IL-2 would need a further clinical trial.

3. Dispersed tumor sustaining bulk CAR-T activation: The dispersed nature of liquid tumors and the persisting normal B-cell emergence means that a large fraction of the CAR-T will undergo recurrent, continuing antigen stimulation, thereby maintaining the activation state of the bulk CAR-T, sustained by continued cytokine production, including IL-2, for T-cell support and expansion. In contrast, in the solid tumor patients who have small volume, focused sites of disease, most CAR-T in circulation will never see tumor, and bulk CAR-T will thus pass to resting state over time.

This dispersed or focused status could be important for recirculating CAR-T that may continually enter tumor sites. Recirculating CD19 CAR-T will still be activated to kill tumor on tumor re-entry, whereas the newly resting CAR-T that re-enter solid tumors will likely have lost their potency. This supposition accords with the long-ago observation that a prominent predictor of response was the rate of expansion of the TILs before dosing;¹⁰⁷ more activated and highly proliferative T cells had a longer duration of activity *in vivo* with greater clinical impact before passing to resting state. Validity of this scenario would add impetus to obtaining higher CAR-T exposures (engraftments) in that critical early time period of activation supported with adequate IL-2, providing a deeper suppression of the tumor.

SAFETY WITH CAR-T IN SOLID TUMORS

In general, there has been a significant concern for safety of CAR-T targeting, particularly in solid tumors.^{81,108} Because the antigens for CAR-T are inevitably shared by normal indispensable tissues, there is a potential for dose-limiting toxicity at ‘drug’ (CAR-T) exposures that will be insufficient for tumor eradication. Some examples are instructive.

When renal cell carcinoma was treated by a G250 CAR-T against CAIX (carbonic anhydrase 9), dose-limiting liver toxicity resulted from targeting the antigen on biliary epithelium,¹⁰⁹ ultimately leading to study suspension. It was not previously recognized that this antigen was expressed on bile ducts. CEA, the most widely expressed tumor antigen, with antigen-positive tumors accounting for ~150 000 deaths per year, was selected for targeting with CAR-T cells or with TCR transfer T cells (in which MHC-presented CEA peptide was recognized)^{54,74,83} (RPJ, unpublished data). In these studies, a prominent colitis developed. Two among the G250 and CEA studies were suspended,^{83,109} but no patient died as a result of these interventions; our own CEA studies are continuing to accrue patients. In the case of the patient treated with anti-erbB2/Her2 CAR, however, the patient experienced hemorrhagic enteritis, pulmonary edema and cardiac arrest with death in 5 days after treatment.⁸⁰ These toxicities correspond to known sites of Her2 expression—bowel, lung and heart. On the other hand, this death occurred in the setting of CAR-T engraftment after lymphodepleting chemotherapy and a high T-cell dose that led to massive CAR-T expansion.

With the CEA studies, as mentioned, patients showed marker responses and these correlated with colitis, on-target/off-tumor effects. In our studies, low-dose IL-2 was applied, and colitis was a feature of CAR-T toxicity only with IL-2, which also correlated with clinical response. (A PSMA protocol with the same IL-2 dosing did not have colitis,⁵⁷ thus implying that this was a CEA-specific CAR-T toxicity with CAR-T activity enhanced by IL-2.) For the colitis toxicities, we managed them with drugs used for inflammatory bowel disease (mesalamine, budesonide) and/or IL-2 dose reductions, with adequate control. However, a cure has not yet been achieved. Will colitis become more severe with a more effective intervention? Or have we seen the worst of it?—in which case cure with more potent T-cell deliveries could be achieved.

These are the types of questions that need to be addressed prospectively for each CAR-T agent. On the other hand, our PSMA targeting achieved clinical responses in prostate cancer without evidence of on-target/off-tumor adverse effects in central nervous system or kidney where the PSMA is also expressed. Is this because the loss of the subset of PSMA+ type II astrocytes is inconsequential, or were they shielded from attack from the blood–brain barrier? I do not generally believe that the so-called blood–brain barrier is any barrier to T cells, and it has not been a barrier to responses of melanoma brain metastases in TIL therapies.¹¹⁰ If brain had a barrier to T cells, then there would be no multiple sclerosis (MS) and blocking T cells from traversing blood to brain parenchyma with natalizumab¹¹¹ would not be effective in MS.

Other aspects make solid tumors different. We consider the origins and nature of the cytokine release syndrome. The very same aspect that makes liquid tumors susceptible to cytokine release syndrome also makes them an ideal environment for persistence and expansion of CAR-T. Although IL-2 seems to be an important adjunct to solid tumor CAR-T therapies^{57,74,106} (RPJ, unpublished results), it has not been as clearly needed in liquid tumors. In liquid tumors, virtually every CAR-T cell is exposed to tumor and undergoes stimulation with cytokine release. The summation systemically of all of these events can be sufficient to generate measurable levels of IL-2,¹⁰⁴ which is normally undetectable in human serum. By contrast, in solid tumors, with only a few CAR-T in contact with tumor at any given time, cytokine release syndrome is not generally observed in CAR-T therapies.

Safety in CAR design ('safer CARs')

Prompted by the severe adverse event in the Her2 trial⁸⁰ and an unrelated event in a lymphoma patient on a CD19 trial,¹¹² recommendations emerged to make the treatments safer. One such approach was to create 'safer CARs', with this section heading borrowed from an editorial on this topic by Dr Helsop,¹¹³

to reduce the impact of off-tumor toxicities. One approach is to include a suicide gene, such as the inducible caspase-9 that has been shown to rapidly abrogate graft-versus-host disease in transplant settings.¹¹⁴ Inducing this agent will effectively ablate the antihost response, and also any antitumor benefit. In an attempt to 'tune' or attenuate the risk to the patient, more advanced designs have been proposed. One such approach attempts to target tumor more selectively versus normal tissue via a combinatorial approach.¹¹⁵ Two methods are described: (1) requiring the presence of two antigens on the tumor cell to be targeted as a positive selection, or (2) negative selection where an antigen is expressed on a normal cell but not on a tumor. This depends, in the first instance, on the presence of two antigens together on a tumor that do not exist together on sensitive normal tissue, and in the second instance, the presence of a second protein on normal tissue that is not also present on the tumor. One or the other of these methods may be fruitful in select settings and awaits their first development for clinical use.

Safety in CAR-T dosing ('safer rules of the road')

As suggested by Dr Helsop¹¹³ and then by myself in a follow-up editorial from which this section title has been borrowed,¹¹⁶ controlling how the cells are administered may be as important as CAR design for safety in targeting. Because of the shared normal tissue antigens, I have advocated a cautious approach in solid tumors, so that a table of AEs can be created with a management plan for each while minimizing loss of patient lives in the meantime. Although the traditional 3+3 design for drug exposures in Phase 1 testing has been time-tested and durably reliable, this approach and the concept of 'managed risk' have been undermined with the advent of new preparative LD procedures. Based on the success of engraftment protocols with TILs,⁷⁵ pioneered in the Surgery Branch/NCI, there has been an impetus for applying the same with CAR-T under the perception that there will be less than maximum clinical impact without lymphodepletion/engraftment. However, the hazard of this approach was well demonstrated in the first use of LD with a second-generation Her2 CAR.⁸⁰ The patient underwent LD followed by a substantial CAR-T exposure (10^{10} cells), with an aggressive on-target/off-tumor toxicity that was impossible to reverse because of the high driver for CAR-T expansion from homeostatic cytokines post LD and the vibrancy of second-generation, two-signal CAR-T to resist immunosuppression.¹¹⁷ With this new means of generating 'drug exposures', new thinking was needed for managed risk to guide the patient exposures during safety testing.

The concept of 'Strategy Escalation' was proposed to address this setting.¹¹⁸ In this, the CAR generations were contrasted: first-generation versus second-generation, and the administration methods were contrasted: simple infusion (SI) versus prior LD.

The rationale for these categories is as follows:

Infusion method: The difference is in (a) the 'drug' exposure, AUC, in which the T cells decline in a monotonic manner with SI versus a massive expansion after LD, (b) cells after SI disappear over 2–4 weeks, whereas engrafted CAR-T after LD persist indefinitely and (c) exposure levels after SI are predictable and declining, whereas the exposures after LD are not predictable, with 10-fold differences between individuals with the same doses of CAR-T cells.⁵⁷

CAR generation: The difference is in (a) the proliferative potential, which is greater for second-generation, potentially amplifying toxicities if present, and (b) the higher resistance of the second generation to therapeutic immune suppression to control toxicity.¹¹⁷

By this, first generation by SI is the least aggressive and the strategy of second generation with LD is most aggressive.

For the current predominance of second- or third-generation CAR-T with incorporated costimulation, it was recommended that SI with standard 3+3 dose escalation to high levels (e.g., 10^{11} cells) would allow a controlled safety test and also provide

Table 1. Improving CAR-T potency

CAR-T 'drug' exposure	Cytokine support	Resistance to suppression
Higher infused doses	IL-2 (<i>in vivo</i>)	Anticheckpoint Abs (PD1, PDL1, etc.)
LD with engraftment	IL-15 (<i>in vivo</i>)	Anti-CTLA4
Higher-intensity LD	IL-12 (<i>ex vivo</i>)	CAR with 4-1BB
	IL-12 (<i>in situ</i>)	Treg depletion/anti-TGF- β
More signals (costimulation)		MDSC blockade
Stimulatory ligands (antigen-Fc)		IDO inhibitors

Abbreviations: Abs, antibodies; CTLA4, cytotoxic T-lymphocyte-associated antigen 4; IDO, indoleamine-pyrrole 2,3-dioxygenase; LD, lymphodepletion; MDSC, myeloid-derived suppressor cell; PD1, programmed death 1; PDL1, programmed death-ligand 1; TGF, tumor growth factor; Treg, regulatory T cell

response data. This has the further advantage that intrapatient dose escalation may be applied as we did in two prior trials,^{54,74} which is not practical with LD. With intrapatient escalation, the side effects and range of safety can be determined quickly and with a few patients. Then, if adequate safety is shown and responses could be improved, the rationale to advance to LD is supported, potentially being offered to the same patients who participated in the SI dose escalation.

This has been our procedure with the second-generation CEA CAR-T, which has generated interesting response data as well as GI toxicities—but without CAR-T deaths. Yet, we are not ready to advance to LD and engraftment until we have more confidence that the toxicity will not be significantly worse than that in the present, where we consider it manageable.

On the other hand, there are other ways to increase the potency with the SI method, still in a controlled manner, with cytokines, dosing and antibodies to inhibitory receptors/ligands, as discussed below. It may be that the costly and sometimes hazardous approach of LD can be avoided with meaningful clinical responses at the same time as our understanding of the underlying immunology improves. These options are all revealed by this incremental strategy of CAR-T testing.

SUMMARY

Every chemotherapy agent approved in use in cancer patients has proven its utility against cancer, to varying degrees, but most have significant side effects that can be found in the package insert. Similar to these chemotherapy agents, each with a set of toxicities that accompany its success, we must chronicle the adverse impacts of each CAR-T therapy and balance it against the benefits. In every instance, this is learned with the dose escalations that have been traditionally mandated by Food and Drug Administration and agencies. During such escalations, events may arise that were not anticipated *a priori*. For example, when CAIX was targeted by a first-generation G250 CAR-T, there was liver toxicity that was not anticipated because the antigen was not previously recognized on biliary epithelium.¹⁰⁹ Similarly, when applying a MAGE-3 TCR, MAGE-3 peptide targeting was not anticipated to yield cross-reactive neurotoxic effects.³⁰

It has been said (by me) that any immunologist can treat a B-cell cancer with CAR-T, but it will take an oncologist to treat a solid tumor. That is not to say that there are not hazards to treating a B-cell malignancy. A B-cell cancer generates cytokine release syndrome, which can be treated with antibodies.¹⁰³ But then one can sit back and watch the tumor disappear. Even this toxicity is not from targeting the normal tissue; it is systemic toxicity from the T cells themselves, undergoing an exuberant stimulation and cytokine production.

In the case of solid tumors, however, vulnerable normal tissue may be targeted and must be closely monitored during therapy to be protected. This is what every oncology fellow is taught from his first day in training. Monitor white count, platelets, hand-foot syndrome, desquamation, nausea, gastritis, colitis, neuropathy, diarrhea, ecchymoses and so forth. In T-cell therapies, listing the effects of normal tissue targeting generates the table of on-target/off-tumor toxicities for the particular CAR.

Can CAR-T have an important impact in solid tumors? I believe the answer is yes. We must exhibit patience to develop the data on responses and on safety in parallel. The drawback of the death with the first Her2 application is not just the premature loss of life, but that it essentially blocked the development of a promising agent that might have been useable and response-generating if administered by SI at high doses, with the right combination of costimulatory signals, cytokines or other mediators. Indeed, this target was only recently readdressed, this time by SI infusion with very low CAR-T doses ($< 3 \times 10^8$ cells versus 10^{11} in studies with TILs⁹ and CEA CAR⁵⁴ [RPJ, unpublished]) that showed early indications of clinical activity and, as yet, no dose-limiting toxicities.¹¹⁹ Higher doses by SI, with or without adjunctive measures, may yet bring about the remissions we seek.

It is possible to improve CAR-T potency, by any of several interventions other than LD (Table 1). These need to be considered part of the 'drug' escalation and managed with patient safety in mind. These interventions can be for *higher CAR-T 'drug' exposure*: higher dose of T cells; the choice of LD with engraftment versus SI; higher-intensity LD for higher engraftment;⁷⁵ *cytokine support*: coadministration of systemic cytokines IL-2 (refs 9, 57, 74, 106) or IL-15;¹²⁰ treatment of dose *ex vivo* with IL-12 to shift the T cells to Th1 phenotype;¹²¹ or IL-12 or other cytokines generated locally *in situ* (TRUCKs, armored CARs);^{122,123} *confer resistance to immunosuppression*: anticheckpoint antibodies (PD1, programmed death-ligand 1 and others),^{14,124} anti-CTLA4,^{14,125} incorporate 4-1BB for costimulation to make T cells resistant to PD1 suppression;⁷⁸ Treg depletion/anti-TGF- β ;¹²⁶ myeloid-derived suppressor cell blockade;¹²⁷ IDO (indoleamine-pyrrole 2,3-dioxygenase) inhibitors;¹²⁸ incorporation of other costimulatory molecules that will better support T-cell activation and proliferation at a higher level and/or to resist apoptosis; application of stimulatory ligands for *in vivo* activation.¹²⁹

Apart from the cited hazards and costs of LD and engraftment, there is a disadvantage to science of going reflexively to LD without first performing graded SI exposures. The conditioning procedure obscures any chance to test the core driving hypotheses of current research, for example, that additional signals, as embodied in the advanced generation designer T cells or in complementary interventions (e.g., Table 1), can promote a fully competent T-cell response with *in situ* expansion until tumor elimination. *My personal bias is that LD should be viewed as an intervening measure to compensate for our still-imperfect T-cell engineering, applied only until we become better at immunology.* T-cells engaged by antigen-presenting cells can eliminate infections and with very few starting cognate effectors, and when we have successfully adopted the requisite features into our engineering, I believe that we will similarly be able to eliminate cancers as efficiently, without engraftment and its attendant hazards and costs.

Balancing safety against these more potent CAR-T cells, however created and applied, methods may also be considered to attenuate or manage side effects in a controlled manner (Table 2). These include choice of antigen to minimize risk to sensitive normal tissue, choice of vector structure with combinatorial CARs or CAR+CAR,¹¹⁴ with suicide gene as last resort (inducible caspase).¹¹⁵ Safety may also be enhanced by choice of treatment design with regional versus systemic infusion, and by choice of escalation method with SI and dose escalation versus LD and engraftment. Finally, safety ultimately requires attention to the specifics of the side effects (fever, diarrhea and so on) that may be ameliorated with established medical interventions, or by newly devised interventions in the

Table 2. Improving safety in solid tumor therapy

Choice of antigen
Restricted expression
Cancer glycosylation
Vector structure
Combinatorial CAR
Suicide gene (iCaspase9)
Treatment design
Regional versus systemic administration
Escalation method
Simple infusion versus lymphodepletion
Evaluate toxicities for management

Abbreviation: iCaspase, inducible caspase.

same way that MESNA was developed to abate bladder toxicity in high-dose cyclophosphamide therapy.¹³⁰ In the same way that we apply chemotherapy agents with a balance between tumor and tissue activity, we need to be prepared for the same balance with these agents; the concepts are the same.

Finally, whereas Phase 1 and 2 studies focus on patients with large volume disease who have failed standard therapies with a certainty of death ahead of them, these are not necessarily the patients who will have the most benefit. Nevertheless, initial treatment of Stage IV patients is the same path all chemotherapy and biologic agents have followed prior to approval. However, when applied in the adjuvant setting, with minimal residual disease, cures have been obtained with chemotherapies when no cures were ever achieved with the same agent in macroscopic metastatic disease. This may be an important opportunity even for those CAR-T agents that cannot be safely administered with LD (e.g., Her2) but only under controlled SI dosing—which will be revealed by this more patient approach.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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REFERENCES

- Paul WE. The immune system. Chapter 1. In: Paul WE (ed.). *Fundamental Immunology*, 7th edn. Lippincott: Philadelphia, PA, USA, 2013, pp 1–21.
- Fischer A. Recent advances in understanding the pathophysiology of primary T cell immunodeficiencies. *Trends Mol Med* 2015; **21**: 408–416.
- Levine AM. AIDS-related malignancies: the emerging epidemic. *J Natl Cancer Inst* 1993; **85**: 1382–1397.
- Kalialis LV, Drzewiecki KT, Klyver H. Spontaneous regression of metastases from melanoma: review of the literature. *Melanoma Res* 2009; **19**: 275–282.
- McCarthy EF. The toxins of William B. Coley and the treatment of bone and soft-tissue sarcomas. *Iowa Orthop J* 2006; **26**: 154–158.
- Chang AE, Shu S. Current status of adoptive immunotherapy of cancer. *Crit Rev Oncol Hematol* 1996; **22**: 213–228.
- Rosenberg SA, Lotze MT, Yang JC, Aebersold PM, Linehan WM, Seipp CA *et al.* Experience with the use of high-dose interleukin-2 in the treatment of 652 cancer patients. *Ann Surg* 1989; **210**: 474–484; discussion 484–485.
- Balkhi MY, Ma Q, Ahmad S, Junghans RP. T cell exhaustion and interleukin 2 downregulation. *Cytokine* 2015; **71**: 339–347.
- Rosenberg SA, Yannelli JR, Yang JC, Topalian SL, Swartzentruber DJ, Weber JS *et al.* Treatment of patients with metastatic melanoma with autologous tumor-infiltrating lymphocytes and interleukin 2. *J Natl Cancer Inst* 1994; **86**: 1159–1166.
- De Smet C, Lurquin C, De Plaen E, Brasseur F, Zarour H, De Backer O *et al.* Genes coding for melanoma antigens recognised by cytolytic T lymphocytes. *Eye (Lond)* 1997; **11**(Part 2): 243–248.
- Yeh S, Karne NK, Kerkar SP, Heller CK, Palmer DC, Johnson LA *et al.* Ocular and systemic autoimmunity after successful tumor-infiltrating lymphocyte immunotherapy for recurrent, metastatic melanoma. *Ophthalmology* 2009; **116**: 981–989.e1.
- Pagès F, Berger A, Camus M, Sanchez-Cabo F, Costes A, Molitor R *et al.* Effector memory T cells, early metastasis, and survival in colorectal cancer. *N Engl J Med* 2005; **353**: 2654–2666.
- Katz SC, Bamboat ZM, Maker AV, Shia J, Pillarisetty VG, Yopp AC *et al.* Regulatory T cell infiltration predicts outcome following resection of colorectal cancer liver metastases. *Ann Surg Oncol* 2013; **20**: 946–955.
- Suzuki S, Ishida T, Yoshikawa K, Ueda R. Current status of immunotherapy. *Jpn J Clin Oncol* 2016; **46**: 191–203.
- Rothenberg EV, Champhakar A. T cell developmental biology. In: Paul WE (ed.), *Fundamental Immunology*. Lippincott: Philadelphia, PA, USA, 2013, pp 25–54.
- Afonso G, Mallone R. Infectious triggers in type 1 diabetes: is there a case for epitope mimicry? *Diabetes Obes Metab* 2013; **15**(Suppl 3): 82–88.
- Krone B, Kölmel KF, Henz BM, Grange JM. Protection against melanoma by vaccination with Bacille Calmette–Guerin (BCG) and/or vaccinia: an epidemiology-based hypothesis on the nature of a melanoma risk factor and its immunological control. *Eur J Cancer* 2005; **41**: 104–117.
- Champiat S, Ferte C, Lebel-Binay S, Eggermont A, Soria JC. Exomics and immunogenics: bridging mutational load and immune checkpoints efficacy. *Oncoimmunology* 2014; **3**: e27817.
- Rizvi NA, Hellmann MD, Snyder A, Kvistborg P, Makarov V, Havel JJ *et al.* Cancer immunology: mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. *Science* 2015; **348**: 124–128.
- Davis MM, Chien YH. T cell antigen receptors. In: Paul WE (ed.), *Fundamental Immunology*. Lippincott: Philadelphia, PA, USA, 2013, pp 79–95.
- Knudson AG Jr. Prince Takamatsu memorial lecture. Rare cancers: clues to genetic mechanisms. *Princess Takamatsu Symp* 1987; **18**: 221–231.
- Hinrichs CS, Rosenberg SA. Exploiting the curative potential of adoptive T-cell therapy for cancer. *Immunol Rev* 2014; **257**: 56–71.
- Kähler KC, Hassel JC, Heinzerling L, Loquai C, Mössner R, Ugurel S *et al.* 'Cutaneous Side Effects' Committee of the Work Group Dermatological Oncology (ADO). Management of side effects of immune checkpoint blockade by anti-CTLA-4 and anti-PD-1 antibodies in metastatic melanoma. *J Dtsch Dermatol Ges* 2016; **14**: 662–681.
- Boucher D, Cournoyer D, Stanners CP, Fuks A. Studies on the control of gene expression of the carcinoembryonic antigen family in human tissue. *Cancer Res* 1989; **49**: 847–852.
- O'Keefe DS, Bacich DJ, Heston WD. Comparative analysis of prostate-specific membrane antigen (PSMA) versus a prostate-specific membrane antigen-like gene. *Prostate* 2004; **58**: 200–210.
- Hassan R, Ho M. Mesothelin targeted cancer immunotherapy. *Eur J Cancer* 2008; **44**: 46–53.
- Sykulev Y, Cohen RJ, Eisen HN. The law of mass action governs antigen-stimulated cytolytic activity of CD8⁺ cytotoxic T lymphocytes. *Proc Natl Acad Sci USA* 1995; **92**: 11990–11992.
- Manz BN, Jackson BL, Petit RS, Dustin ML, Groves J. T-cell triggering thresholds are modulated by the number of antigen within individual T-cell receptor clusters. *Proc Nat Acad Sci USA* 2011; **108**: 9089–9094.
- Rapoport AP, Stadtmauer EA, Binder-Scholl GK, Goloubeva O, Vogl DT, Lacey SF, *et al.* NY-ESO-1-specific TCR-engineered T cells mediate sustained antigen-specific antitumor effects in myeloma. *Nat Med* 2015; **21**: 914–921.
- Morgan RA, Chinnsamy N, Abate-Daga D, Gros A, Robbins PF, Zheng Z *et al.* Cancer regression and neurological toxicity following anti-MAGE-A3 TCR gene therapy. *J Immunother* 2013; **36**: 133–151.
- Posey AD Jr, Schwab RD, Boesteanu AC, Steentoft C, Mandel U, Engels B *et al.* Engineered CAR T cells targeting the cancer-associated Tn-glycoform of the membrane mucin MUC1 control adenocarcinoma. *Immunity* 2016; **44**: 1444–1454.
- Kuwana Y, Asakura Y, Utsunomiya N, Nakanishi M, Arata Y, Itaoh S *et al.* Expression of chimeric receptor composed of immunoglobulin-derived V regions and T-cell receptor-derived C regions. *BBRC* 1987; **149**: 960–968.
- Becker ML, Near R, Mudgett-Hunter M, Margolies MN, Hedrick SM. Expression of a hybrid immunoglobulin-T cell receptor protein in transgenic mice. *Cell* 1989; **58**: 911–921.
- Gross G, Waks T, Eshhar Z. Expression of immunoglobulin-T cell receptor chimeric molecules as functional receptors with antibody-type specificity. *Proc Natl Acad Sci USA* 1989; **86**: 10024–10028.
- Governan J, Gomez SM, Segesman KD, Hunkapiller T, Laug WE, Hood L. Chimeric immunoglobulin-T cell receptor proteins form functional receptors: implications for T cell receptor complex formation and activation. *Cell* 1990; **60**: 929–939.

- 36 Kawasaki H, Becker ML, Hedrick SM. Specificity for molecules of the major histocompatibility complex mediated by a hybrid immunoglobulin-T cell receptor. *New Biol* 1991; **3**: 487–497.
- 37 Romeo C, Seed B. Cellular immunity to HIV activated by CD4 fused to T cell or Fc receptor polypeptides. *Cell* 1991; **64**: 1037–1046.
- 38 Eshhar Z, Waks T, Gross G, Schindler DG. Specific activation and targeting of cytotoxic lymphocytes through chimeric single chains consisting of antibody binding domains and γ or ζ subunits of the immunoglobulin and T cell receptors. *Proc Natl Acad Sci USA* 1993; **90**: 720–724.
- 39 Hwu P, Shafer GE, Treisman J, Schindler DG, Gross G, Cowherd R *et al*. Lysis of ovarian cancer cells by human lymphocytes redirected with a chimeric gene composed of an antibody variable region and the Fc receptor γ chain. *J Exp Med* 1993; **178**: 361–366.
- 40 Stancovski I, Schindler DG, Waks T, Yarden Y, Sela M, Eshhar Z. Targeting of T lymphocytes to neu/Her2-expressing cells using chimeric single chain Fv receptors. *J Immunol* 1993; **151**: 6577–6582.
- 41 Moritz D, Wels W, Mattern J, Groner B. Cytotoxic T lymphocytes with a grafted recognition specificity for erbB-2 expressing tumor cells. *Proc Natl Acad Sci USA* 1994; **91**: 4318–4322.
- 42 Hwu P, Yang JC, Cowherd R, Treisman J, Shafer GE, Eshhar Z *et al*. *In vivo* antitumor activity of T cells redirected with chimeric antibody/T cell receptor genes. *Cancer Res* 1995; **55**: 3369–3373.
- 43 Weijtens MEM, Willemsen RA, Valerio D, Stam K, Bolhuis RLH. Single chain Ig/ γ gene-redIRECTED human T lymphocytes produce cytokines, specifically lyse tumor cells, and recycle lytic capacity. *J Immunol* 1996; **157**: 836–843.
- 44 Kahlon KS, Brown C, Cooper LJ, Raubitschek A, Forman SJ, Jensen MC. Specific recognition and killing of glioblastoma multiforme by interleukin 13-zetakine redirected cytolytic T cells. *Cancer Res* 2004; **64**: 9160–9166.
- 45 Willemsen RA, Weijtens ME, Ronteltap C, Eshhar Z, Gratama JW, Chames P *et al*. Grafting primary human T lymphocytes with cancer-specific chimeric single chain and two chain TCR. *Gene Therapy* 2000; **7**: 1369–1377.
- 46 Yang W, Beaudoin EL, Lu L, Du Pasquier RA, Kuroda MJ, Willemsen RA *et al*. Chimeric immune receptors (CIRs) specific to JC virus for immunotherapy in progressive multifocal leukoencephalopathy (PML). *Int Immunol* 2007; **19**: 1083–1093.
- 47 Ma QZ, Gonzalo-Daganzo R, Junghans RP. Genetically engineered T cells as adoptive immunotherapy of cancer. Chapter 15. In: Giaccone R, Schilsky R, Sondel P (eds.), *Cancer Chemotherapy & Biological Response Modifiers—Annual 20*. Elsevier Science: Oxford, UK, 2002, pp 19–345.
- 48 Walker RE, Bechtel CM, Natarajan V, Baseler M, Hege KM, Metcalf JA *et al*. Long-term *in vivo* survival of receptor-modified syngeneic T cells in patients with human immunodeficiency virus infection. *Blood* 2000; **96**: 467–474.
- 49 Deeks SG, Wagner B, Anton PA, Mitsuyasu TR, Scadden DT, Huang C *et al*. A phase II randomized study of HIV-specific T-cell gene therapy in subjects with undetectable plasma viremia on combination antiretroviral therapy. *Mol Ther* 2002; **5**: 788–797.
- 50 Mitsuyasu RT, Anton PA, Deeks SG, Scadden DT, Connick E, Downs MT. Prolonged survival and tissue trafficking following adoptive transfer of CD4zeta gene-modified autologous CD4(+) and CD8(+) T cells in human immunodeficiency virus-infected subjects. *Blood* 2000; **96**: 785–793.
- 51 Parker LL, Do MT, Westwood JA, Wunderlich JR, Dudley ME, Rosenberg SA *et al*. Expansion and characterization of T cells transduced with a chimeric receptor against ovarian cancer. *Hum Gene Ther* 2000; **11**: 2377–2387.
- 52 Kershaw MH, Westwood JA, Parker LL, Wang G, Eshhar Z, Mavroukakis SA *et al*. A phase I study on adoptive immunotherapy using gene-modified T cells for ovarian cancer. *Clin Cancer Res* 2006; **12**(Part 1): 6106–6115.
- 53 Warren RS, Fisher GA, Bergsland EK, Pennathur-Das R, Nemunaitis J, Venook AP *et al*. Clinical studies of regional and systemic gene therapy with autologous CC49-z modified T cells in colorectal cancer metastatic to the liver. *Cancer Gene Ther* 1998; **5**: S1–S2.
- 54 Junghans RP, Safar M, Huberman MS, Ma Q, Ripley R, Leung S *et al*. Preclinical and phase I data of anti-CEA designer T cell therapy for cancer: a new immunotherapeutic modality. *Proc Am Assoc Cancer Res* 2000; **41**: 543.
- 55 Till BG, Jensen MC, Wang J, Chen EY, Wood BL, Greisman HA *et al*. Adoptive immunotherapy for indolent non-Hodgkin lymphoma and mantle cell lymphoma using genetically modified autologous CD20-specific T cells. *Blood* 2008; **112**: 2261–2271.
- 56 Park JR, Digiusto DL, Slovak M, Wright C, Naranjo A, Wagner J *et al*. Adoptive transfer of chimeric antigen receptor re-directed cytolytic T lymphocyte clones in patients with neuroblastoma. *Mol Ther* 2007; **15**: 825–833.
- 57 Junghans RP, Ma QZ, Rathore R, Gomes EM, Bais AJ, Lo ASY *et al*. Phase I trial of anti-PSMA designer T cells in prostate cancer: possible critical role of interacting interleukin 2-T cell pharmacodynamics as a determinant of clinical response in engraftment settings with solid tumor. *Prostate* 2016; **76**: 1257–1270.
- 58 Okuno K, Ohnishi H, Nakajima I, Akabane Y, Kurooka K, Koh K *et al*. Complete remission of liver metastases from colorectal cancer by treatment with a hepatic artery infusion (HAI) of interleukin-2-based immunochemotherapy: reports of three cases. *Surg Today* 1994; **24**: 80–84.
- 59 Sato T. Locoregional immuno(bio)therapy for liver metastases. *Semin Oncol* 2002; **29**: 160–167.
- 60 Fisher B, Packard BS, Read EJ, Carrasquillo JA, Carter CS, Topalian SL *et al*. Tumor localization of adoptively transferred indium-111 labeled tumor infiltrating lymphocytes in patients with metastatic melanoma. *J Clin Oncol* 1989; **7**: 250–261.
- 61 Griffith KD, Read EJ, Carrasquillo JA, Carter CS, Yang JC, Fisher B *et al*. *In vivo* distribution of adoptively transferred indium-111-labeled tumor infiltrating lymphocytes and peripheral blood lymphocytes in patients with metastatic melanoma. *J Natl Cancer Inst* 1989; **81**: 1709–1717.
- 62 Brown CE, Badie B, Barish ME, Wang L, Ostberg JR, Chang WC *et al*. Bioactivity and safety of IL13Ra2-redIRECTED chimeric antigen receptor CD8+ T Cells in patients with recurrent glioblastoma. *Clin Cancer Res* 2015; **21**(18): 4062–4072.
- 63 Kershaw MH, Westwood JA, Hwu P. Dual-specific T cells combine proliferation and antitumor activity. *Nat Biotechnol* 2002; **20**: 1221–1227.
- 64 Pule MA, Savoldo B, Myers GD, Rossig C, Russell HV, Dotti G *et al*. Virus-specific T cells engineered to coexpress tumor-specific receptors: persistence and antitumor activity in individuals with neuroblastoma. *Nat Med* 2008; **14**: 1264–1270.
- 65 Alvarez-Vallina L, Hawkins RE. Antigen-specific targeting of CD28-mediated T cell co-stimulation using chimeric single-chain antibody variable fragment-CD28 receptors. *Eur J Immunol* 1996; **26**: 2304–2309.
- 66 Beecham EJ, Ma Q, Ripley R, Junghans RP. Coupling CD28 co-stimulation to immunoglobulin T-cell receptor molecules: the dynamics of T-cell proliferation and death. *J Immunother* 2000; **23**: 631–642.
- 67 Finney HM, Lawson AD, Bebbington CR, Weir AN. Chimeric receptors providing both primary and costimulatory signaling in T cells from a single gene product. *J Immunol* 1998; **161**: 2791–2797.
- 68 Maher J, Brentjens RJ, Gunset G, Riviere I, Sadelain M. Human T lymphocyte cytotoxicity and proliferation directed by a single chimeric TCR/CD28 receptor. *Nat Biotechnol* 2002; **20**: 70–75.
- 69 Milone MC, Fish JD, Carpenito C, Carroll RG, Binder GK, Teachey D *et al*. Chimeric receptors containing CD137 signal transduction domains mediate enhanced survival of T cells and increased antileukemic efficacy *in vivo* [published erratum appears in: *Mol Ther* 2015; **23**:1278]. *Mol Ther* 2009; **17**: 1453–1464.
- 70 Zhong XS, Matsushita M, Plotkin J, Riviere I, Sadelain M. Chimeric antigen receptors combining 4-1BB and CD28 signaling domains augment PI3kinase/AKT/Bcl-XL activation and CD8+ T cell-mediated tumor eradication. *Mol Ther* 2010; **18**: 413–420.
- 71 Brentjens RJ, Riviere I, Park JH, Davila ML, Wang X, Stefanski J *et al*. Safety and persistence of adoptively transferred autologous CD19-targeted T cells in patients with relapsed or chemotherapy refractory B-cell leukemias. *Blood* 2011; **118**: 4817–4828.
- 72 Davila ML, Brentjens R, Wang X, Riviere I, Sadelain M. How do CARs work? Early insights from recent clinical studies targeting CD19. *Oncoimmunology* 2012; **1**: 1577–1583.
- 73 Savoldo B, Ramos CA, Liu E, Mims MP, Keating MJ, Carrum G *et al*. CD28 costimulation improves expansion and persistence of chimeric antigen receptor-modified T cells in lymphoma patients. *J Clin Invest* 2011; **121**: 1822–1826.
- 74 Katz SC, Burga RA, McCormack E, Wang LJ, Mooring JW, Point G *et al*. Phase I hepatic immunotherapy for metastases (HITM) study of intra-arterial chimeric antigen receptor modified T cell therapy for CEA+ liver metastases. *Clin Cancer Res* 2015; **21**: 3149–3159.
- 75 Dudley ME, Yang JC, Sherry R, Hughes MS, Royal R, Kammula U *et al*. Adoptive cell therapy for patients with metastatic melanoma: evaluation of intensive myeloablative chemoradiation preparative regimens. *J Clin Oncol* 2008; **26**: 5233–5239.
- 76 Davila ML, Sadelain M. Biology and clinical application of CAR T cells for B cell malignancies. *Int J Hematol* 2016; **104**: 6–17.
- 77 Kawalekar OU, O'Connor RS, Fraietta JA, Guo L, McGettigan SE, Posey AD Jr *et al*. Distinct signaling of coreceptors regulates specific metabolism pathways and impacts memory development in CAR T cells. *Immunity* 2016; **44**: 380–390.
- 78 Cherkassky L, Morello A, Villena-Vargas J, Feng Y, Dimitrov DS, Jones DR *et al*. Human CAR T cells with cell-intrinsic PD-1 checkpoint blockade resist tumor-mediated inhibition. *J Clin Invest* 2016; **126**: 3130–3144.
- 79 Slovin SF, Wang X, Hullings M, Arauz G, Bartido S, Lewis JS *et al*. Chimeric antigen receptor (CAR⁺) modified T cells targeting prostate-specific membrane antigen (PSMA) in patients (pts) with castrate resistant metastatic prostate cancer (CMPC). *J Clin Oncol* 2013; **31**(Suppl 6): abstract 72.
- 80 Morgan RA, Yang JC, Kitano M, Dudley ME, Laurencot CM, Rosenberg SA. Case report of a serious adverse event following the administration of T cells

- transduced with a chimeric antigen receptor recognizing ERBB2. *Mol Ther* 2010; **18**: 843–851.
- 81 Corrigan-Curay J, Kiem HP, Baltimore D, O'Reilly M, Brentjens RJ, Cooper L *et al*. T-cell immunotherapy: looking forward. *Mol Ther* 2014; **22**: 1564–1574.
 - 82 Junghans RP, Safar M, Huberman MS, Ma Q, Ripley R, Leung S *et al*. Preclinical and phase I data of anti-CEA 'designer T cell' therapy for cancer: a new immunotherapeutic modality. *Proc Am Assoc Can Res* 2001; **41**: 543.
 - 83 Parkhurst MR, Yang JC, Langan RC, Dudley ME, Nathan DA, Feldman SA *et al*. T cells targeting carcinoembryonic antigen can mediate regression of metastatic colorectal cancer but induce severe transient colitis. *Mol Ther*. 2011; **19**: 620–626.
 - 84 Guest RD, Kirillova N, Mowbray S, Gornall H, Rothwell DG, Cheadle EJ *et al*. Definition and application of good manufacturing process-compliant production of CEA-specific chimeric antigen receptor expressing T-cells for phase I/II clinical trial. *Cancer Immunol Immunother* 2014; **63**: 133–145.
 - 85 Wolchok JD, Hoos A, O'Day S, Weber JS, Hamid O, Lebbe C *et al*. Guidelines for the evaluation of immune therapy activity in solid tumors: immune-related response criteria. *Clin Cancer Res* 2009; **15**: 7412–7420.
 - 86 Slovin SF, Wang X, Hullings M, Arauz G, Bartido S, Lewis JS *et al*. Chimeric antigen receptor (CAR β) modified T cells targeting prostate-specific membrane antigen (PSMA) in patients (pts) with castrate resistant metastatic prostate cancer (CMPC). *J Clin Oncol* 2013; **31**(Suppl 6): abstract 72.
 - 87 Beatty GL, Haas AR, Maus MV, Torigan DA, Soulen MC, Plesa G *et al*. Mesothelin-specific chimeric antigen receptor mRNA-engineered T cells induce anti-tumor activity in solid malignancies. *Cancer Immunol Res* 2014; **2**: 112–120.
 - 88 O'Rourke DM, Nasrallah M, Morrisette JJ, Melenhorst JJ, Lacey SF, Mansfield K *et al*. Pilot study of T cells redirected to EGFRvIII with a chimeric antigen receptor in patients with EGFRvIII+ glioblastoma. *J Clin Oncol* 2015; **34**: 2067.
 - 89 Ahmed N, Brawley V, Hegde M, Bielamowicz K, Wakefield A, Ghazi A *et al*. Autologous HER2 CMV bispecific CAR T cells are safe and demonstrate clinical benefit for glioblastoma in a phase I trial. *J Immunother Cancer* 2015; **3**: 011.
 - 90 Brown CE, Alizadeh D, Starr R, Weng L, Wagner JR, Naranjo A *et al*. Regression of glioblastoma after chimeric antigen receptor T-cell therapy. *N Engl J Med* 2016; **375**: 2561–2569.
 - 91 Gan HK, Cvrljevic AN, Johns TG. The epidermal growth factor receptor variant III (EGFRvIII): where wild things are altered. *FEBS J* 2013; **280**: 5350–5370.
 - 92 Burga RA, Thorn M, Point GR, Guha P, Nguyen CT, Licata LA *et al*. Liver myeloid-derived suppressor cells expand in response to liver metastases in mice and inhibit the anti-tumor efficacy of anti-CEA CAR-T. *Cancer Immunol Immunother* 2015; **64**: 817–829.
 - 93 Baitsch L, Fuertes-Marraco SA, Legat A, Meyer C, Speiser DE. Exhaustion of tumor-specific CD8(+) T cells in metastases from melanoma patients. *J Clin Invest* 2011; **121**: 2350–2360.
 - 94 Newick K, Moon E, Albelda SM. Chimeric antigen receptor T-cell therapy for solid tumors. *Mol Ther Oncol* 2016; **3**: 16006.
 - 95 Park JH, Geyer MB, Brentjens RJ. CD19-targeted CAR T-cell therapeutics for hematologic malignancies: interpreting clinical outcomes to date. *Blood* 2016; **127**: 3312–3320.
 - 96 Levine EG, Peterson BA, Smith KA, Hurd DD, Bloomfield CD. Glucocorticoid receptors in chronic lymphocytic leukemia. *Leuk Res* 1985; **9**: 993–999.
 - 97 Sonpavde G, Pond GR, Templeton AJ, Kwon ED, De Bono JS. Impact of single-agent daily prednisone on outcomes in men with metastatic castration-resistant prostate cancer. *Prostate Cancer Prostatic Dis* 2016 (doi:10.1038/pcan.2016.44).
 - 98 Maude SL, Frey N, Shaw PA, Aplenc R, Barrett DM, Bunin NJ *et al*. Chimeric antigen receptor T cells for sustained remissions in leukemia. *N Engl J Med* 2014; **371**: 1507–1517.
 - 99 Davila ML, Riviere I, Wang X, Bartido S, Park J, Curran K *et al*. Efficacy and toxicity management of 19-28z CAR T cell therapy in B cell acute lymphoblastic leukemia. *Sci Transl Med* 2014; **6**: 224–225.
 - 100 Porter DL, Hwang WT, Frey NV, Lacey SF, Shaw PA, Loren AW *et al*. Chimeric antigen receptor T cells persist and induce sustained remissions in relapsed refractory chronic lymphocytic leukemia. *Sci Transl Med* 2015; **7**: 303ra139.
 - 101 Beecham EJ, Ma QZ, Ripley R, Junghans RP. Coupling of CD28 co-stimulation to IgTCR molecules: dynamics of T cell proliferation and death. *J Immunother* 2000; **23**: 631–642.
 - 102 Kawalekar OU, O'Connor RS, Fraietta JA, Guo L, McGettigan SE, Posey AD Jr *et al*. Distinct signaling of coreceptors regulates specific metabolism pathways and impacts memory development in CAR T cells. *Immunity* 2016; **44**: 380–390.
 - 103 Teachey DT, Lacey SF, Shaw PA, Melenhorst JJ, Maude SL, Frey N *et al*. Identification of predictive biomarkers for cytokine release syndrome after chimeric antigen receptor T-cell therapy for acute lymphoblastic leukemia. *Cancer Discov* 2016; **6**: 664–679.
 - 104 Kalos M, Levine BL, Porter DL, Katz S, Grupp SA, Bagg A *et al*. T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced leukemia. *Sci Transl Med* 2011; **3**: 95ra73.
 - 105 Emtage PCR, Lo ASY, Gomes EM, Liu DL, Gonzalo-Daganzo R, Junghans RP. 2nd generation anti-CEA designer T cells resist activation-induced cell death, proliferate on tumor contact, secrete cytokines and exhibit superior anti-tumor activity *in vivo*: a preclinical evaluation. *Clin Cancer Res* 2008; **14**: 8112–8122.
 - 106 Lo ASY, Ma Q, Liu DL, Junghans RP. Anti-GD3 chimeric sFv-CD28/T cell receptor zeta designer T cells for treatment of metastatic melanoma and other neuroectodermal tumors. *Clin Cancer Res* 2010; **16**: 2769–2780.
 - 107 Schwartzentruber DJ, Hom SS, Dadmarz R, White DE, Yannelli JR, Steinberg SM *et al*. *In vitro* predictors of therapeutic response in melanoma patients receiving tumor-infiltrating lymphocytes and interleukin-2. *J Clin Oncol* 1994; **12**: 1475–1483.
 - 108 Amos SM, Duong CP, Westwood JA, Ritchie DS, Junghans RP, Darcy PK *et al*. Autoimmunity associated with immunotherapy of cancer. *Blood* 2011; **118**: 499–509.
 - 109 Lamers CH, Sleijfer S, Vulto AG, Kruit WH, Kliffen M, Debets R *et al*. Treatment of metastatic renal cell carcinoma with autologous T-lymphocytes genetically retargeted against carbonic anhydrase IX: first clinical experience. *J Clin Oncol* 2006; **24**: e20–e22.
 - 110 Hong JJ, Rosenberg SA, Dudley ME, Yang JC, White DE, Butman JA *et al*. Successful treatment of melanoma brain metastases with adoptive cell therapy. *Clin Cancer Res* 2010; **16**: 4892–4898.
 - 111 Engelhardt B, Kappos L. Natalizumab: targeting alpha4-integrins in multiple sclerosis. *Neurodegener Dis* 2008; **5**: 16–22.
 - 112 Brentjens R, Yeh R, Bernal Y, Riviere I, Sadelain M. Treatment of chronic lymphocytic leukemia with genetically targeted autologous T cells: case report of an unforeseen adverse event in a phase I clinical trial. *Mol Ther* 2010; **18**: 666–668.
 - 113 Heslop HE. Safer CARs. *Mol Ther*. 2010; **18**: 661–662.
 - 114 Kloss CC, Condomines M, Cartellieri M, Bachmann M, Sadelain M. Combinatorial antigen recognition with balanced signaling promotes selective tumor eradication by engineered T cells. *Nat Biotechnol* 2013; **31**: 71–75.
 - 115 Di Stasi A, Tey SK, Dotti G, Fujita Y, Kennedy-Nasser A, Martinez C *et al*. Inducible apoptosis as a safety switch for adoptive cell therapy. *N Engl J Med* 2011; **365**: 1673–1683.
 - 116 Junghans RP. Is it safer CARs we need, or safer rules of the road? *Mol Ther* 2010; **18**: 1742–1743.
 - 117 Loskog A, Giandomenico V, Rossig C, Pule M, Dotti G, Brenner MK. Addition of the CD28 signaling domain to chimeric T-cell receptors enhances chimeric T-cell resistance to T regulatory cells. *Leukemia* 2006; **20**: 1819–1828.
 - 118 Junghans RP. Strategy escalation: an emerging paradigm for safe clinical development of T cell gene therapies. *J Transl Med* 2010; **8**: 55.
 - 119 Ahmed N, Brawley VS, Hegde M, Robertson C, Ghazi A, Gerken C *et al*. Human epidermal growth factor receptor 2 (HER2)-specific chimeric antigen receptor-modified T cells for the immunotherapy of HER2-positive sarcoma. *J Clin Oncol* 2015; **33**: 1688–1696.
 - 120 Conlon KC, Lugli E, Welles HC, Rosenberg SA, Fojo AT, Morris JC *et al*. Redistribution, hyperproliferation, activation of natural killer cells and CD8 T cells, and cytokine production during first-in-human clinical trial of recombinant human interleukin-15 in patients with cancer. *J Clin Oncol* 2015; **33**: 74–82.
 - 121 Emtage PCR, Clarke D, Gonzalo R, Junghans RP. Generating potent Th1/Tc1 T cell adoptive immunotherapy doses using human IL12: harnessing the immunomodulatory potential of IL12 without the *in vivo*-associated toxicity. *J Immunother* 2002; **26**: 97–106.
 - 122 Chmielewski M, Abken H. CAR T cells transform to trucks: chimeric antigen receptor-redirected T cells engineered to deliver inducible IL-12 modulate the tumour stroma to combat cancer. *Cancer Immunol Immunother* 2012; **61**: 1269–1277.
 - 123 Yeku OO, Brentjens RJ. Armored CAR T-cells: utilizing cytokines and pro-inflammatory ligands to enhance CAR T-cell anti-tumour efficacy. *Biochem Soc Trans* 2016; **44**: 412–418.
 - 124 Zarour HM. Reversing T-cell dysfunction and exhaustion in cancer. *Clin Cancer Res* 2016; **22**: 1856–1864.
 - 125 Yun S, Vincelette ND, Green MR, Wahner Hendrickson AE *et al*. Targeting immune checkpoints in unresectable metastatic cutaneous melanoma: a systematic review and meta-analysis of anti-CTLA-4 and anti-PD-1 agents trials. *Cancer Med* 2016; **5**: 1481–1491.
 - 126 Liu C, Workman CJ, Vignali DA. Targeting regulatory T cells in tumors. *FEBS J* 2016; **283**: 2731–2748.
 - 127 Holmgaard RB, Zamarin D, Lesokhin A, Merghoub T, Wolchok JD. Targeting myeloid-derived suppressor cells with colony stimulating factor-1 receptor blockade can reverse immune resistance to immunotherapy in indoleamine 2,3-dioxygenase-expressing tumors. *EBioMedicine* 2016; **6**: 50–58.
 - 128 Moon YW, Hajar J, Hwu P, Naing A. Targeting the indoleamine 2,3-dioxygenase pathway in cancer. *J Immunother Cancer* 2015; **3**: 51.
 - 129 Ma QZ, DeMarte L, Wang YW, Stanners CP, Junghans RP. Carcinoembryonic antigen-immunoglobulin Fc fusion protein (CEA-Fc) for identification and activation of anti-CEA chimeric immune receptor modified T cells: representative of a new class of Ig fusion proteins. *Cancer Gene Ther* 2004; **11**: 297–306.
 - 130 Shaw IC, Graham MI. Mesna—a short review. *Cancer Treat Rev* 1987; **14**: 67–86.