Review

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Synthetic biology in cell-based cancer immunotherapy

Deboki Chakravarti and Wilson W. Wong

Department of Biomedical Engineering, Boston University, Boston, MA 02215, USA

The adoptive transfer of genetically engineered T cells with cancer-targeting receptors has shown tremendous promise for eradicating tumors in clinical trials. This form of cellular immunotherapy presents a unique opportunity to incorporate advanced systems and synthetic biology approaches to create cancer therapeutics with novel functions. We first review the development of synthetic receptors, switches, and circuits to control the location, duration, and strength of T cell activity against tumors. In addition, we discuss the cellular engineering and genome editing of host cells (or the chassis) to improve the efficacy of cell-based cancer therapeutics, and to reduce the time and cost of manufacturing.

Emergence of cellular immunotherapy

The intricate relationship between tumors and the immune system has been the subject of intense research, providing both insight into cancer progression [1,2] and an arena for therapeutic intervention [3]. The immune system can directly attack tumors, and harnessing this power to eradicate tumors is a major goal in immunotherapy. The involvement of the immune cells in combating tumors was demonstrated when a lower rate of relapse was observed in cancer patients who underwent a hematopoietic stem cell transplant (HSCT) (see Glossary) to replace their bone marrow after chemotherapy [4–6]. This effect has been attributed to fresh T cells from the transplant engaging and killing the tumor in a graft-versus-tumor (GVT) response. However, this response is also correlated to graft-versus-host disease (GVHD), wherein the donor T cells begin to attack the tissue of the host. This potential autoimmune response has limited the use of stem cell transplants for cancer treatment as a universal solution. Instead, aiding the immune system of the patient to fight cancer may provide more viable and widespread therapies. However, cancer cells have also evolved strategies to oppose immune action [2,3,7]. As such, a major goal of cancer immunotherapy is to overcome these immunosuppressive mechanisms, including the use of cytokines to promote T cell proliferation [8–10] or antibody checkpoint blockers to prevent the signaling of inhibitory or apoptotic pathways of a T cell [11–13]. Cytokines and checkpoint blockers have shown great promise in therapy [14,15], and several high-profile drugs have been approved in recent years. These forms of immunotherapy aid the response of the body against cancer, but immune cells can also be directly used as therapeutic agents.

Cells are inherently capable of carrying out complex computations and responses, and the immune system in particular is composed of cells designed to perform cytotoxic tasks through careful assessment of targets. Adoptive T cell therapy, the use and engineering of a patient's T cells as therapeutic agents, has emerged as a promising branch of immunotherapy (Figure 1A). Much of the current success in adoptive T cell therapy is derived from the genetic engineering of tumor-targeting receptors. However, synthetic sensors, switches, and circuits are also being explored to improve efficacy and safety by providing greater control over the location, duration, and magnitude of T cell activity (Figure 1C). Synthetic biology, an emerging discipline aimed at reprogramming living organisms through the combined use of genetics, engineering principles, and systems and computational analysis [16–18], is primed to deliver the genetic tools necessary to enhance the control of these living therapies and explore T cell behavior [19].

Glossary

Chimeric antigen receptor (CAR): engineered receptor that fuses an extracellular single-chain variable fragment (scFv) of an antibody to intracellular T cell signaling domains.
Cytotoxic T lymphocyte-associated protein 4 (CTLA-4): an inhibitory receptor that downregulates T cell response.
Epitope: fragments of proteins expressed in a cell that are presented on the surface by the major histocompatibility complex (MHC) for detection by T cells. Epitopes that represent pathogenic organisms to the T cell trigger T cell activation upon binding.
Fatty acid oxidation: cascade of β-oxidation reactions that converts fatty acids in the mitochondria to produce acetyl-CoA.
Hematopoietic stem cell transplant (HSCT): transplant of blood cells from the bone marrow that give rise to all other blood cells. HSCT is usually performed in patients with blood or bone-marrow cancers.
Immunogenicity: the potential for a molecule to elicit an immune response. Proteins expressed in a cell can be processed into smaller fragments, termed epitopes, to be presented at the surface of the cell as potential antigens by the MHC. T cells assess these MHC-peptide complexes through their T cell receptor (TCR), which are selected to distinguish epitopes derived from self-proteins and those derived from foreign organisms. If the TCR recognizes an epitope as a foreign antigen, it will activate the T cell and drive the death of the antigen-presenting cell. In T cell therapy, engineered T cells can be targeted by other immune cells owing to the expression of foreign proteins as part of the receptors or circuit components of non-human origin.
Programmed cell death 1 (PD-1): a cell surface receptor that negatively modulates the T cell response by promoting apoptosis.
Ribozyme: RNA molecules that can act as catalytic agents.
T cell receptor (TCR): receptors expressed on the surface of T cells to drive recognition of pathogenic organisms through epitope–MHC binding.
Tumor-infiltrating lymphocytes (TILs): T cells that have been able to penetrate the tumor.

Corresponding author: Wong, W.W. (wilwong@bu.edu).
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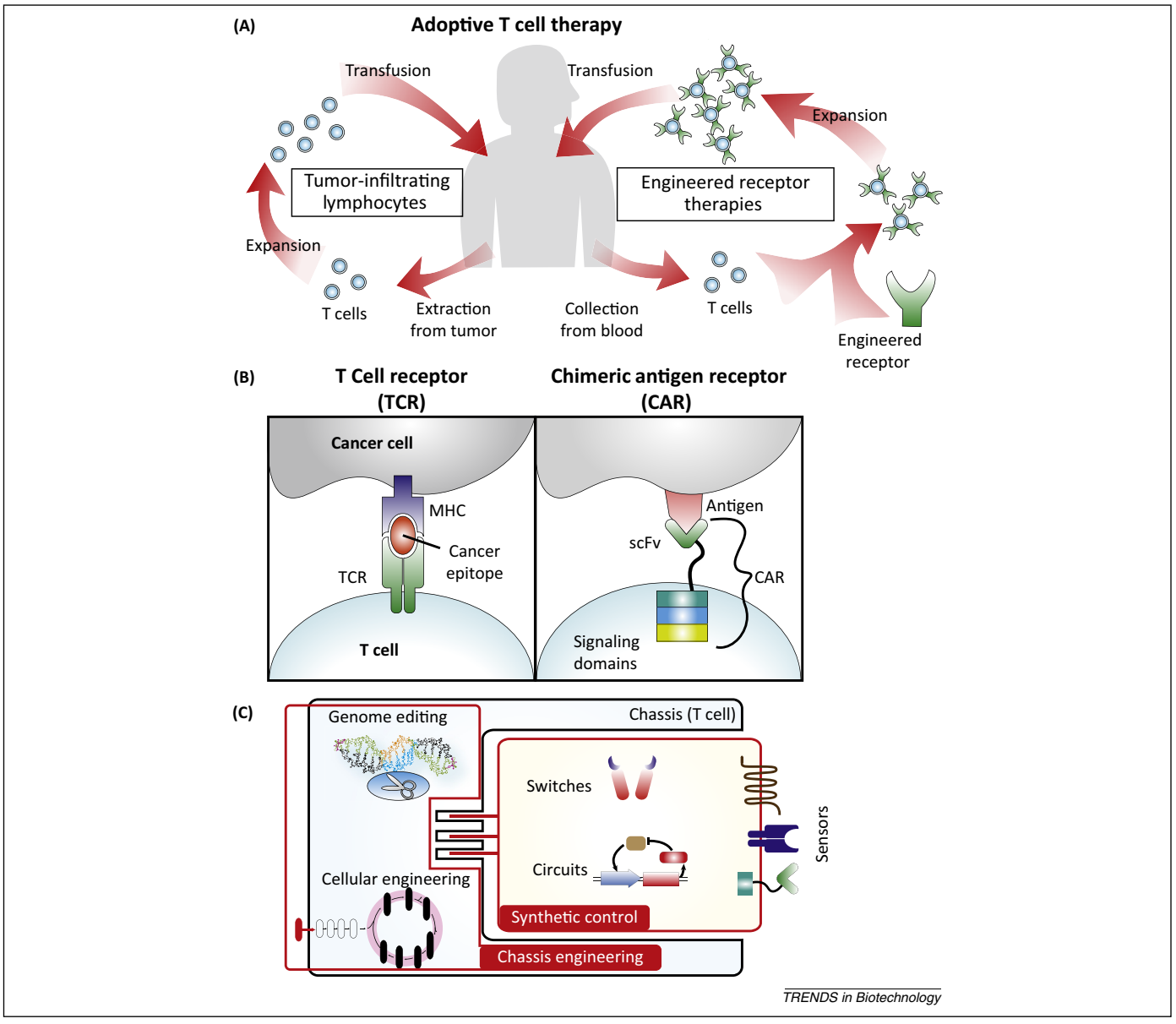

Figure 1. Adoptive T cell therapy for cancer treatment. **(A)** Several approaches for the adoptive transfer of a patient's own T cells for cancer therapy. Tumor-infiltrating lymphocytes (TILs) involve extraction of T cells directly from the tumor, *ex vivo* expansion, and then transfusion back into the patient. For engineered receptor therapies, T cells are collected from the blood, genetically modified to express a cancer-targeting receptor, expanded, and then transfused back into the patient. **(B)** Receptors engineered to target cancer cells. T cell receptors (TCRs) naturally recognize protein epitopes presented by the major histocompatibility complex (MHC) of a target cell. Engineering a TCR to detect cancer epitopes 'teaches' the T cell to detect cancer cells. Chimeric antigen receptors (CARs) are composed of a single-chain variable fragment (scFv) from an antibody fused to intracellular T cell signaling domains that trigger activation and proliferation of the T cell. CARs recognize markers expressed at the surface of a cell, and, by choosing a cancer-specific scFv, can be made to trigger killing of the cancer cell upon binding to the target antigen. **(C)** Engineering T cells for improvement of adoptive T cell therapy. Generating novel receptors and circuits can enable increased control over cell-based therapies, and techniques to engineer the chassis, such as genome editing and cellular engineering, can drive the development of more powerful treatments.

In addition to the introduction of exogenous sensors and circuits, the endogenous machinery of the host cell (chassis) presents numerous opportunities for tinkering and optimization (Figure 1C). Therefore, cellular engineering and genome editing of T cells are also under active investigation [20,21]. Much akin to the role synthetic chemistry plays in transforming the development of small-molecule drugs, synthetic-biology approaches are becoming a major engine in driving the progress of adoptive T cell therapy.

Genetic engineering and cellular immunotherapy: a potent combination against tumors

One of the most promising and earliest forms of adoptive T cell therapy involves the use of a patient's tumor-infiltrating lymphocytes (TILs), which are T cells extracted from the tumor. These isolated TILs were expanded *ex vivo*, and then transfused back into the patient to treat cancer [22]. Owing to their inherent ability to locate and traffic to the tumor site, TILs have had some success against melanoma in clinical trials [23,24]. However, the identification and isolation of TILs in sufficient quantity from a patient is challenging, limiting their potential [25]. The shortcomings of TILs have accelerated efforts to redirect the specificity of T cells towards cancer rather than relying on the isolation of T cells with inherent tumor-targeting capability. T cells from a patient can be modified with genes that encode tumor-targeting receptors that will 'teach' the T cell to

bind to and kill cancer cells [25]. In this process, T cells (typically CD8 T cells) are collected from the patient, genetically modified *ex vivo* to express the receptor, and then transfused back into the patient. Two different types of receptors have been used for this purpose. One is a T cell receptor (TCR) that is engineered to detect cancer epitopes [26,27]. The other is a chimeric antigen receptor (CAR) that is composed of a cancer antigen-specific single-chain variable fragment (scFv) fused to T cell signaling domains that trigger activation and proliferation [28,29] (Figure 1B). The design of CARs has undergone some engineering through the choice and addition of different T cell signaling domains that can drive activation or proliferation, resulting in therapeutic variations between these different designs. TCRs and CARs are distinguished from one another by the type of cancer antigen they recognize. TCRs on CD8 T cells recognize protein epitopes derived from proteins expressed in the cell and presented on the surface by the major histocompatibility complex-1 (MHC-1). CARs bind to markers expressed at the surface of the cell.

Both TCR- and CAR-based therapies have been tested in clinical trials, with promising results. In one clinical trial treating 20 patients with melanoma using TCRs targeted towards MART-1 (melanoma antigen recognized by T cells 1), 33% of the patients demonstrated objective responses (Clinical Trials: NCT00509288, NCT00509496) [27]. Treatment of lymphoid leukemia with CD19-specific CARs has shown up to 90% complete response rates (NCT01044069, NCT01626495, NCT01029366, NCT01593696) [30–33], although similar clinical success in CAR-based targeting of myeloid leukemia has not been achieved yet (NCT01864902, CTX 08-0002) [34,35].

Although extremely encouraging, and commonly considered to be breakthroughs in the fight against cancer [36], toxicities have been observed in clinical trials associated with both forms of engineered T cell therapy [31,37]. The selectivity between tumors and vital organs is an especially significant safety issue that has emerged with both TCRs and CARs [37]. The identification of target epitopes and antigens for these therapies is limited by the potential for expression of these targets on non-cancerous cells, and this could lead to autoimmune responses against healthy tissue. MART-1 has demonstrated this 'on-target, off-tumor' autoimmune toxicity in TCR therapy [27]. Furthermore, in one trial using an ERBB2 (human epidermal growth factor receptor 2)-specific CAR to treat a patient with colon cancer, the patient died after the CAR-bearing T cells responded to low levels of ERBB2 in the vital organs (NCT00924287) [38].

Another major safety concern is the potential for an excessively strong, life-threatening T cell response. In clinical trials using CARs to treat leukemia, the release of large amounts of cytokines [30], or cytokine release syndrome (CRS), has led to severe symptoms including high fever, hypotension, and hypoxia [30]. CRS has been treated with immunosuppressive steroids and antibodies to temper the response of the immune system [33]. A recent clinical trial was also conducted to determine the maximum load of CAR-bearing T cells that can be given to a patient while minimizing the severity of CRS [33].

Despite these adverse side effects, the promising results of adoptive T cell therapy in clinical trials have generated enormous enthusiasm, which has led to numerous joint ventures, acquisitions, and collaborations within the pharmaceutical industry, as well as between industry and academia (Table 1). In particular, CARs have attracted the most attention because of their extraordinarily positive clinical trial results (Table 1). Both the success of these clinical trials and the significant financial investment from the industry heighten the urgency to engineer a cell-based therapy that is effective and safe, as well as to design practical strategies that will make manufacturing these therapies cheaper and faster.

Synthetic receptors and circuits for spatiotemporal control of T cell activity

Current T cell therapies, although promising, all share a similar design that triggers the same signaling pathways in response to a single target antigen. In this section we review the next wave of receptor designs that expand the signaling pathways triggered, enhance specificity, or provide inducible controls over the therapy. Furthermore, we also discuss the development of drug-inducible switches and circuits that will endow additional spatiotemporal control over the T cell response.

Receptors

Next-generation receptors. Understanding how receptors affect T cell response is particularly important to the implementation of CAR-based therapy given the novel nature of CARs. The design of CARs has undergone changes over time, and second-generation CARs contain an intracellular proliferative domain derived from either CD28 or 4-1BB (also known as TNFRSF9; tumor necrosis factor receptor superfamily member 9) (Figure 2A). The choice of proliferative domain has led to divergent therapeutic outcomes that have been further explored *in vitro* [30–33,39]. In clinical trials, the CD28 domain has been associated with faster short-term expansion, but also with shorter persistence compared to trials that use the 4-1BB domain. Developing CARs with different signaling domains could lead to the development of receptors with varied properties and, in turn, to treatments that are more complex and oriented around the specific needs of the patient. An inhibitory CAR (iCAR) has been developed using signaling domains from inhibitory pathways to suppress T cell activity upon binding to antigens from healthy cells [40], demonstrating the potential to reprogram the functionality of CARs using different signaling domains. A library approach has been used to test for different proliferative domains and their ability to drive antitumor activity [39,41]. Similar approaches to systematically map the effect of parameters that describe different signaling domains to therapeutic outcomes will provide valuable information for the optimal design of T cell therapy.

Combinatorial receptor system. A major concern in adoptive T cell therapy is the potential for severe side effects due to the inadvertent attack on healthy tissue by engineered T cells [37,38]. Improving the specificity of the T cell towards cancer represents a high priority in this field, and multiple strategies are being explored to identify tumor-specific antigens [42–47]. However, owing to the

Table 1. Advances in adoptive T cell therapy

Institutions	Date	Technology	Clinical trials	Details	Refs
University of Pennsylvania/ Novartis[a]	August 2012	CTL019	NCT02167360, NCT02228096, NCT02030834, NCT01626495, NCT01029366	CD19-specific CAR for B cell acute lymphoblastic leukemia (B-ALL) and non-Hodgkin lymphoma (NHL)	[32]
Juno Therapeutics[b,c]	December 2013	JCAR015	NCT01044069, NCT01840566	CD19-specific CAR for B-ALL and non-Hodgkin lymphoma (NHL)	[30,98–100]
		JCAR017	NCT02028455	CD19-specific CAR for leukemia	
		JCAR014	NCT01865617	CD19-specific CAR for chronic lymphocytic leukemia, NHL, ALL,CLL	
		JTCR016	NCT01640301, NCT00052520	WT1-specific TCR for leukemia, myelodysplastic syndrome	
		Exploration of other targets[d]	Preclinical	L1CAM: neuroblastoma, glioblastoma, lung cancer, pancreatic cancer, and ovarian cancer MUC-16: ovarian cancer ROR-1: lung cancer, triple negative breast cancer, pancreatic cancer, prostate cancer, ALL	
Juno/Opus Bio/National Cancer Institute (NCI)[e]	December 2014	CD22-CAR, CD123-CAR	NCT02315612, NCT02159495	Leukemia, lymphoma	
Celgene/bluebird bio/Baylor[f]	March 2013	Anti-BCMA CAR	Preclinical	Multiple Myeloma	
Kite Pharmaceuticals/National Cancer Institute[g,h]	October 2012	CD19-CAR	NCT00924326, NCT02348216	B cell leukemia, lymphoma (NHL, ALL, CLL)	[101,102]
		EGFRvIII-CAR	NCT01454596	Glioblastoma	[103,104]
		NY-ESO-1 TCR	NCT01967823	ESO-expressing tumors	
		HPV-16 E6 TCR	NCT0228081	Cervical/head and neck cancer	
		HPV-16 E7 TCR		Cervical/head and neck cancer	
		MAGE A3 TCR	NCT02111850	MAGE-A3-DP4-expressing tumors	
		SSX2 TCR	NCT02153905	MAGE-A3-expressing tumors, metastatic melanoma	
Kite Pharmaceuticals/Amgen[i]	January 2015	Exploration of other targets	Preclinical	Hematological cancers and solid tumors	
MD Anderson/Ziopharm Oncology/ Intrexon[j]	January 2015	RheoSwitch therapeutic system	Preclinical	Switches for control of dynamic range, spatial expression, and temporal expression	
		Non-viral integration		Sleeping Beauty transposon system for integration of CARs in T cells	
		Universal donor	Preclinical		
		CD19-specific CAR	NCT01497184, NCT01653717	Leukemia, lymphoma	

Table 1 (*Continued*)

Institutions	Date	Technology	Clinical trials	Details	Refs
Cellectis/Ohio State University[k]	January 2015	CS1-specific CAR	Preclinical	Multiple myeloma	
Pfizer/Cellectis[l]	June 2014	Working on several targets for CAR therapy			
GlaxoSmithKline/Adaptimmune[m]	June 2014	NY-ESO-1 TCR	NCT01350401, NCT01567891, NCT01892293	Multiple myeloma, melanoma, sarcoma, and ovarian cancer	
Advanced designs for adoptive T cell therapy					
Servier/Cellectis[n]	February 2014	UCART19	Filing for clinical trial authorization	Off-the-shelf T cell for leukemia	
Unum Therapeutics[o]	2014	ACTR + anti-CD20 antibody	NCT02315118	Leukemia, non-Hodgkin's lymphoma	[51]
Bellicum Pharmaceuticals	2011	GoCAR-T	Preclinical	Prostate stem cell antigen-expressing solid tumors	[50]
Bellicum Pharmaceuticals/Texas Children's Hospital/Baylor College of Medicine	2011	CaspaCIDe	NCT01494103	Graft-versus-host disease	[67]
bluebird bio (Acquisition of Pregenen)[p]	June 2014	Homing endonucleases, MegaTALEs			
Novartis/Intellia[q]	January 2015	CRISPR			

[a]http://www.novartis.com/newsroom/media-releases/en/2012/1631944.shtml.

[b]https://junotherapeutics.com/leading-cancer-research-centers-team-up-to-launch-biotech-startup-focused-on-cancer-immunotherapy/.

[c]https://junotherapeutics.com/pipeline/clinical/.

[d]https://junotherapeutics.com/pipeline/pre-clinical/.

[e]https://junotherapeutics.com/juno-therapeutics-executes-license-for-phase-i-car-t-product-candidate-targeting-cd22-for-hematological-malignancies/.

[f]http://investor.bluebirdbio.com/phoenix.zhtml?c=251820&p=irol-newsArticle&ID=1817134.

[g]http://genengnews.com/gen-news-highlights/kite-nci-partner-on-engineered-autologous-t-cell-therapies/81247488/?kwrd=Chimeric%20Antigen%20Receptors.

[h]http://www.kitepharma.com/c/pipeline/.

[i]http://www.amgen.com/media/media_pr_detail.jsp?releaseID=2002967.

[j]http://www.fiercebiotech.com/story/intrexon-and-ziopharm-buy-car-t-100m-deal/2015-01-14.

[k]http://www.cellectis.com/en/content/cellectis-and-ohio-state-university-through-ohio-state-innovation-foundation-enter-licensi-0.

[l]http://www.pfizer.com/news/press-release/press-release-detail/pfizer_and_cellectis_enter_into_global_strategic_cancer_immunotherapy_collaboration.

[m]http://www.adaptimmune.com/wp-content/uploads/2014/05/Adaptimmune_GSK_-release_2-June-2014.pdf.

[n]http://www.cellectis.com/en/content/cellectis-and-servier-announce-collaboration-allogeneic-cell-therapy-ucart19-treat-leukemi-0.

[o]http://www.unumrx.com/#!2014dec01-unum-starts-phase-1/cyn5.

[p]http://investor.bluebirdbio.com/phoenix.zhtml?c=251820&p=irol-newsArticle&ID=1943834.

[q]http://www.novartis.com/newsroom/media-releases/en/2015/1884844.shtml.

heterogeneous nature of tumors, a single antigen is unlikely to uniquely distinguish all tumors from healthy tissues. Requiring the T cell to recognize two targets for full activation and proliferation will increase specificity towards the intended cancer cells, and this has been achieved using a combinatorial activation system consisting of a low-activating CAR and a chimeric costimulatory receptor (CCR) [48,49] (Figure 2B). The CAR and CCR each recognize different antigens, and antigen recognition by both receptors is necessary to drive full activation and proliferation of the T cell. This combinatorial activation system has been used to target different combinations of targets [118,119], and it has demonstrated promising selectivity in mouse models [48,119].

Split-receptor systems. While specificity is crucial to the outcome of T cell therapy, the magnitude and duration of T cell response will also influence the severity of any side effects that arise. Novel split-receptor designs where full activation of the engineered T cell requires both the antigen target and an exogenous factor (such as a drug or antibody) are under investigation. These systems provide a method to titrate the response of the T cell through dosage of the secondary activating factors. For example, the GoCAR-T designed by Bellicum Pharmaceuticals contains a CAR that, similar to the combinatorial activation system, is split into the antigen-responsive activation domain and a co-stimulatory domain [50] (http://www.bellicum.com/technology/gocart/) (Figure 2B). The co-stimulatory domain is fused to a rimiducid-inducible homodimerizer domain. For full activation of the T cell, binding to both rimiducid and the antigen is required.

Unum Therapeutics is also developing an alternative 'universal CAR' design using their antibody-coupled T cell receptor (ACTR) system (http://www.forbes.com/sites/brucebooth/2014/10/21/cellular-immunotherapy-unum-therapeutics-out-of-many-one/). ACTR contains the same T cell signaling domains as the current CARs, but the scFv

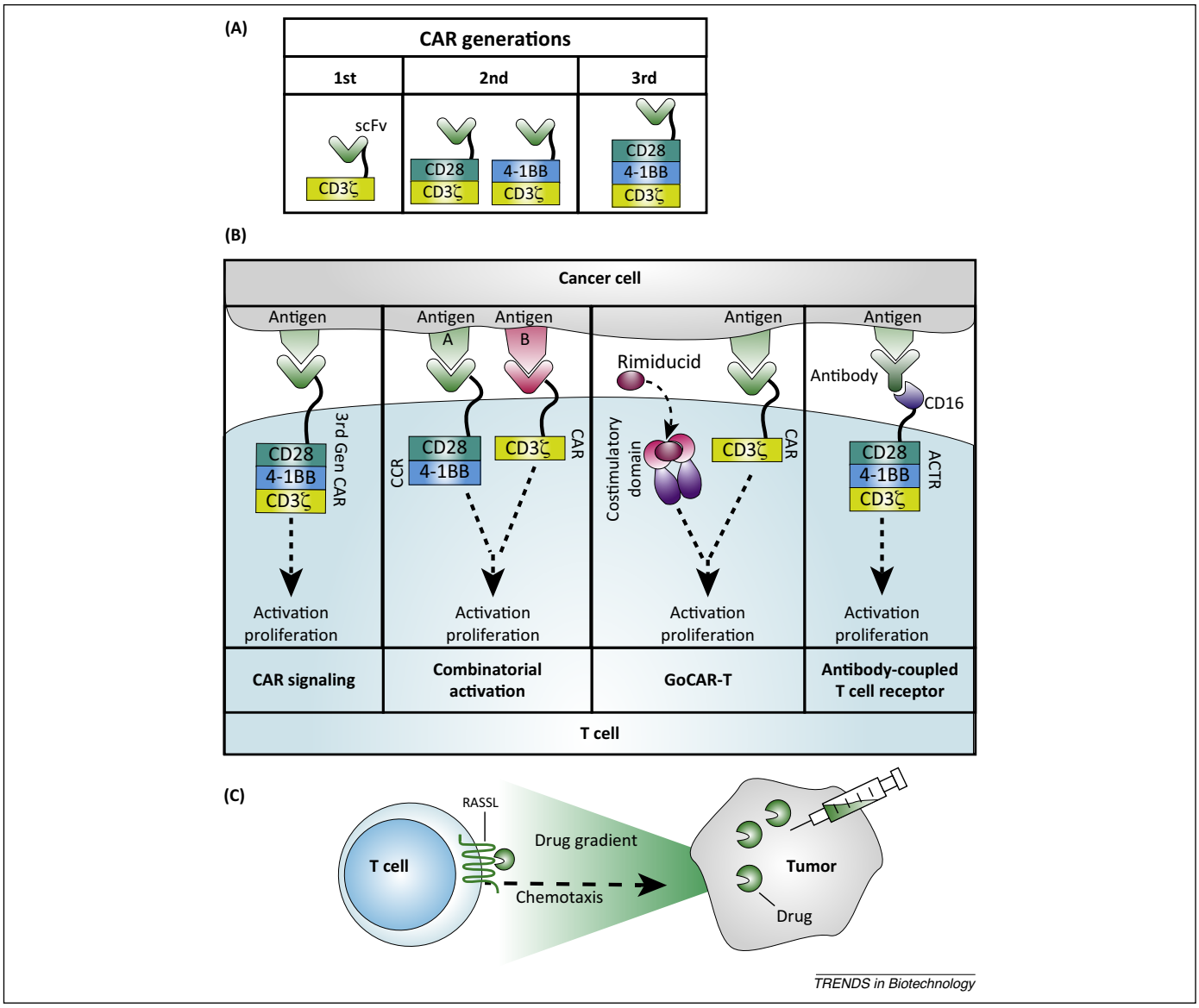

Figure 2. Receptor engineering for adoptive T cell therapy. **(A)** Development of chimeric antigen receptor (CARs) across three generations. The first-generation CAR consisted of a single-chain variable fragment (scFv) fused to the activating CD3ζ domain that drove activation upon antigen binding, but did not lead to sufficient persistence in clinical trials [105]. Second- and third-generation CARs have included the intracellular portions of proliferative signaling proteins, CD28 or 4-1BB. **(B)** Signaling of novel receptors for adoptive T cell therapy. (First panel) In second and third generation CARs, binding of the antigen to the scFv triggers proliferation and activation of the T cell. (Second panel) With combinatorial activation, binding to the CAR and chimeric costimulatory receptor (CCR) is required to drive both activation and proliferation of the T cell [48]. (Third panel) The GoCAR-T system requires binding to the target antigen for activation, but it also requires the addition of the drug rimiducid to dimerize the co-stimulatory domain for activation and proliferation [50] (http://www.bellicum.com/technology/gocart/). (Fourth panel) Antibody-coupled T cell receptors (ACTR) express a CD16 domain at the T cell surface instead of a scFv. CD16 binds to antibodies, and choosing antibodies that bind to the surface of cancer cells will drive T cell activity against the cancer cell [51]. **(C)** Engineered receptor for chemotaxis [59]. Engineered G-protein-coupled receptors activated solely by a synthetic ligand (RASSLs) are activated at the surface of the T cell by a drug, and drive chemotaxis of the cell along the drug gradient. Adding the drug to a tumor can direct T cells towards the tumor site.

is replaced with the extracellular portion of CD16, a receptor that binds to the constant fragment of antibodies [51] (Figure 2B). With ACTRs, any clinically relevant cancer-specific antibody can, in theory, be administered to the patients. The antibody binds to the T cell through CD16, which triggers T cell activation upon antigen binding. With the prevalence of commercially-available, cancer-specific antibodies, the ACTR system can rapidly expand the repertoire of potential targets for engineered T cells. These split systems illustrate the potential of separating target recognition from T cell activation, providing bedside 'on-demand' control of therapeutic strength and duration through the addition of a drug or antibody.

Chemokine receptors. A major barrier to the success of T cell-based therapy against solid tumors is the marked reduction of T cell trafficking to the tumor microenvironment [52]. The irregular blood flow and endothelium modification found in a tumor mass restricts T cell adhesion and infiltration [53]. Recent *in vivo* results have shown that localized delivery to the tumor can improve T cell activity against a solid tumor [54], and a biopolymer implant has been developed to provide localized delivery in conjunction with cytokines to improve T cell proliferation [55]. T cell localization can also be improved by directing their migration machinery toward signals derived from tumors. In T cells, the overexpression of CXCR2 (C-X-C motif chemokine receptor 2), a receptor that binds to the tumor-derived

chemokine growth-regulated oncogene-α (GRO1α, also known as CXCL1), drove preferential trafficking to the site of the tumor [56,57]. Another approach to direct T cell infiltration into tumors is to introduce small-molecule-inducible chemotaxis receptors into the T cell, which can be achieved using engineered G-protein-coupled receptors activated solely by a synthetic ligand (RASSLs) [58] (Figure 2C). The induction ligand can be added to the site of the tumor, directing T cell traffic towards the cancerous cells. Using this system, the migration of engineered T cells can be controlled in mice in response to localized delivery of the small molecule clozapine-*N*-oxide (CNO) [59].

Control switches and circuits

Kill switches. Designed to increase the safety of the therapy, inducible kill switches are simple circuits that provide a mechanism to terminate a T cell therapy if the patient exhibits severe side effects [60,61]. Several drug-inducible kill switches have been tested in clinical trials for this purpose. In patients who received allogeneic bone marrow transplants (allo-BMT), the transplanted T cells were modified to express herpes simplex virus thymidine kinase (HSV-TK), which drives apoptosis in the cell upon addition of ganciclovir [62]. Patients who developed GVHD were treated with ganciclovir, triggering apoptosis in the modified T cells. However, induced killing of T cells was not complete, and the viral origins of HSV-TK led to immunogenic responses [63]. CD20 and an inducible caspase 9 (iCasp9) (Figure 3A) are being explored as possible alternatives because of their human origins [63,64]. In particular, iCasp9 has been tested *in vivo* in conjunction with a CD20-specific CAR to demonstrate its potential to

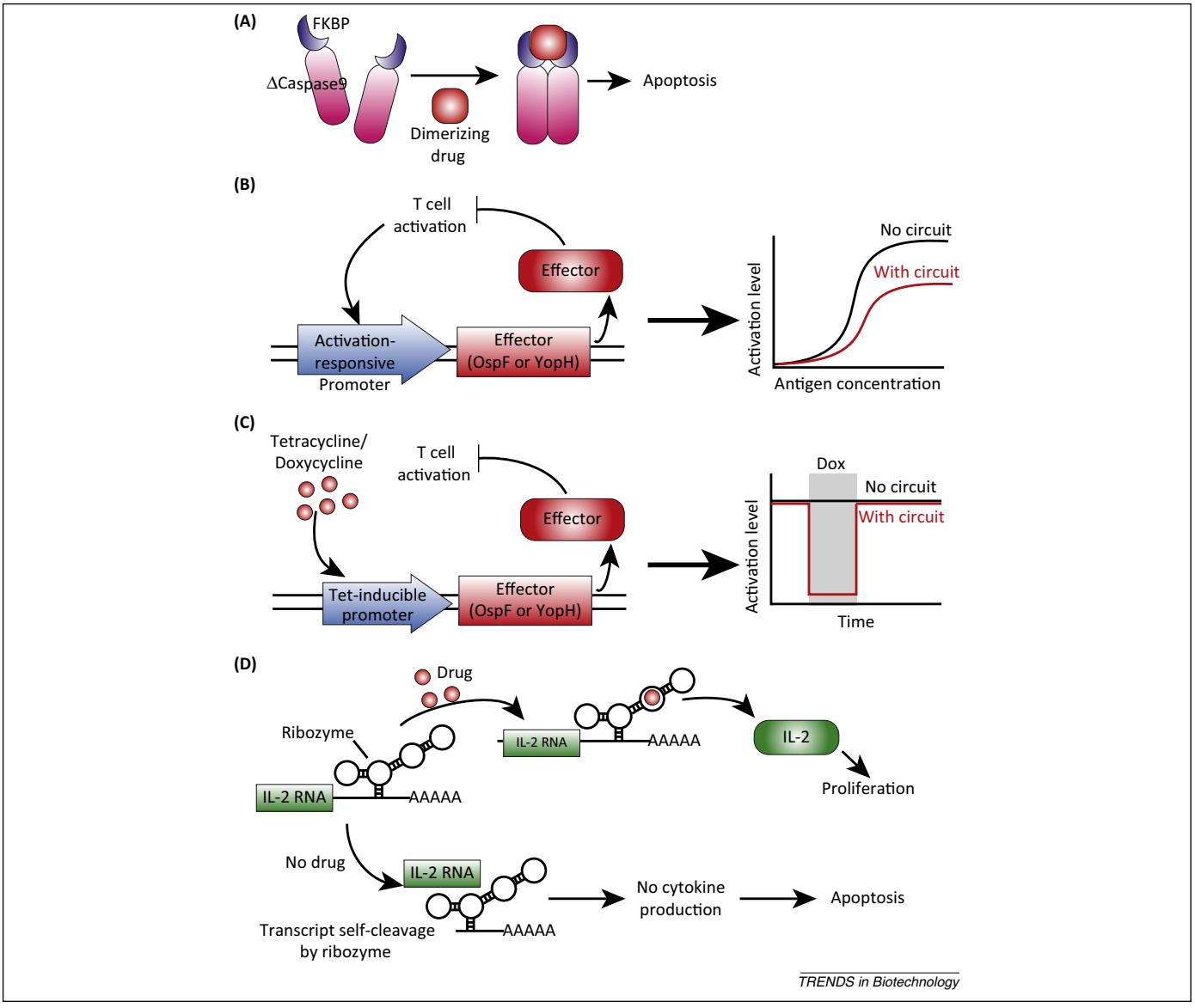

Figure 3. Synthetic genetic circuits to regulate T cell activity in patients. **(A)** Inducible suicide gene using iCasp9, a caspase 9 mutant (Δcaspase9) fused to FKBP dimerizing domains [63,66]. When the dimerizing drug AP1903 is added, Δcaspase9 dimerizes and drives apoptosis. **(B)** Amplitude limiter using the bacterial virulence proteins OspF and YopH as effectors [70]. These effectors reduce T cell activation, and expressing them from an activation-responsive promoter creates a negative feedback loop that reduces T cell activation. **(C)** Pause switches using OspF or YopH as effectors [70]. Using a tetracycline-inducible promoter to control effector expression, the addition of the drug will drive effector production, which will in turn shut off activation until the drug is removed. **(D)** A ribozyme switch to control T cell proliferation [71]. Cytokine RNA is expressed with the ribozyme switch, which will drive self-cleavage of the transcript and lead to no cytokine expression without the addition of the appropriate drug. When drug is added, the cytokine transcript is preserved, leading to cytokine production and proliferation. Abbreviations: Dox, doxycycline; FKBP, FK506 binding protein; IL-2, interleukin 2; OspF, *Shigella* outer surface protein F; YopH, *Yersinia* outer membrane protein.

eliminate CAR-bearing T cells [65]. In a clinical trial to treat leukemia patients receiving stem cell transplants, donor T cells were modified to express iCasp9. In patients who developed GVHD, induction of iCasp9 activity killed more than 90% of the transgenic T cells within 30 min of induction, successfully controlling GVHD with no observed immunogenicity [66]. This system is under commercial development by Bellicum Pharmaceuticals (Table 1) [67].

Pause switch and amplitude limiter. While inducible kill switches provide vital control over the safety of adoptive T cell therapy, they ultimately limit the benefit a patient might receive from the treatment. Modulating the T cell response through other circuits can enable physicians to fine tune the immune response before resorting to termination of the therapy.

The bacterial virulence proteins OspF (outer *Shigella* protein F) and YopH (*Yersinia* outer membrane protein) can modify the activity of a key kinase in TCR signaling pathways to reduce T cell activation [68,69]. With these proteins as effectors in genetic circuit design, the behavior of the T cell can be further controlled. For example, a library expressing OspF or YopH under a series of TCR-responsive promoters were designed as a negative feedback loop to reduce the amplitude of T cell activation [70] (Figure 3B). The amplitude could be furthered adjusted by tagging the OspF or YopH with a degradation tag. This circuit could be used to lower the activation of an engineered T cell, potentially reducing the severity of CRS. OspF and YopH were also used to design an inducible pause switch for T cells [70] (Figure 3C). By expressing the proteins under a doxycycline-inducible promoter, the T cell activity could be paused upon addition of doxycycline. This circuit presents an alternative to the inducible kill switch, allowing for the therapy to stop without completely destroying the cells involved such that they may be used again.

Growth switch. Increasing the growth of T cells will lead to greater persistence of the therapy, whereas decreasing the growth can potentially limit the severity of CRS. Therefore, a drug-inducible controller for the growth of engineered T cells can provide a powerful 'dial' to regulate the efficacy and safety of the therapy. A ribozyme switch to regulate the expression of the cytokines interleukin (IL) 2 or IL15 has been developed such that, without the addition of a drug, the cytokine ribozyme mRNA self-cleaves and no cytokine is produced [71] (Figure 3D). The addition of a drug prevents self-cleavage, allowing cytokine production that drives proliferation of the T cell. While T cell expansion can conceivably be controlled by adding cytokines directly to the patient or by expressing transgenes under inducible promoters, a large cytokine dosage can lead to systemic toxicity in the patient [72], and the packaging of large amounts of transgenes can be challenging [73]. An RNA-based system allows specific control that is easy to deliver and non-immunogenic.

Host cell (chassis) engineering and genome editing in adoptive T cell therapy

Another important design criterion of adoptive T cell therapy is deciding the best cell type for therapy because the type of T cell used has a direct role in the efficacy of the treatment. In particular, naïve and early effector T cells are more effective at treating tumors in mice than are differentiated effector T cells [74]. This effect was attributed to several characteristics, including the entry of differentiated effector T cells into a proapoptotic state and an inability to produce IL-2. Therefore, using T cells that are less differentiated could potentially increase their efficacy.

Cellular engineering of chassis

Engineering T cells to bias a population towards the naïve phenotype before transfusion back into the patient is an attractive possibility to boost the antitumor effect of the therapy [21]. Increasing evidence illustrates the importance of metabolism on T cell development and differentiation. In particular, differentiation into effector T cells is accompanied by a transition from oxidative phosphorylation to aerobic glycolysis [75]. Limiting cell dependence on glycolysis can prevent T cells from differentiating into effector cells [76]. Hence, a metabolic engineering strategy can be employed to limit glycolysis by lowering the expression of the glucose transporter Glut 1 or by reducing the activation of the protein kinase Akt (protein kinase B), a glycolysis enhancer [77] (Figure 4A).

In addition to using more naïve T cells, promoting the development of the memory T cell phenotype (particularly central or stem cell memory) may help to prevent relapse of the disease [78]. Limiting glycolysis can also help to promote memory T cell formation, as can the enhancement of fatty acid oxidation (FAO) [79,80]. For instance, the addition of metformin, an activator of AMP-activated protein kinase (AMPK) that promotes fatty acid oxidation, limits $CD8^+$ effector T cell differentiation while increasing $CD8^+$ memory T cell development (Figure 4A) [79]. Moreover, the overexpression of carnitine palmitoyl transferase 1a (CPT1A), a key enzyme in the transportation of fatty acids into the mitochondria, had been shown to increase FAO and promote memory T cell formation in mice [80].

RNA interference and genome editing

Clinical trials with CARs have demonstrated significant promise against hematological cancers such as leukemia, but solid tumors remain a major challenge owing to their immunosuppressive microenvironment [2]. For example, tumors can deplete the microenvironment of tryptophan [7] while also expressing factors to promote regulatory T cell development [81] and effector T cell death [82]. Antibody drugs such as ipilimumab (anti-CTLA-4) and pembrolizumab (anti-PD-1) attempt to address the challenges presented by the microenvironment by blocking inhibitory or apoptotic signals in T cells. However, given the complexity of the tumor microenvironment, a greater understanding of the T cell response to these challenges can help in the development of novel strategies to improve their performance in adoptive T cell therapy.

Analyzing how the disruption of specific genes within a T cell affects survival in a tumor can provide insight into the development of a stronger tumor-penetrating T cell. Given the large number of genes within the T cell, a library approach to gene disruption would be especially valuable for identifying novel factors at play in the T cell. One such

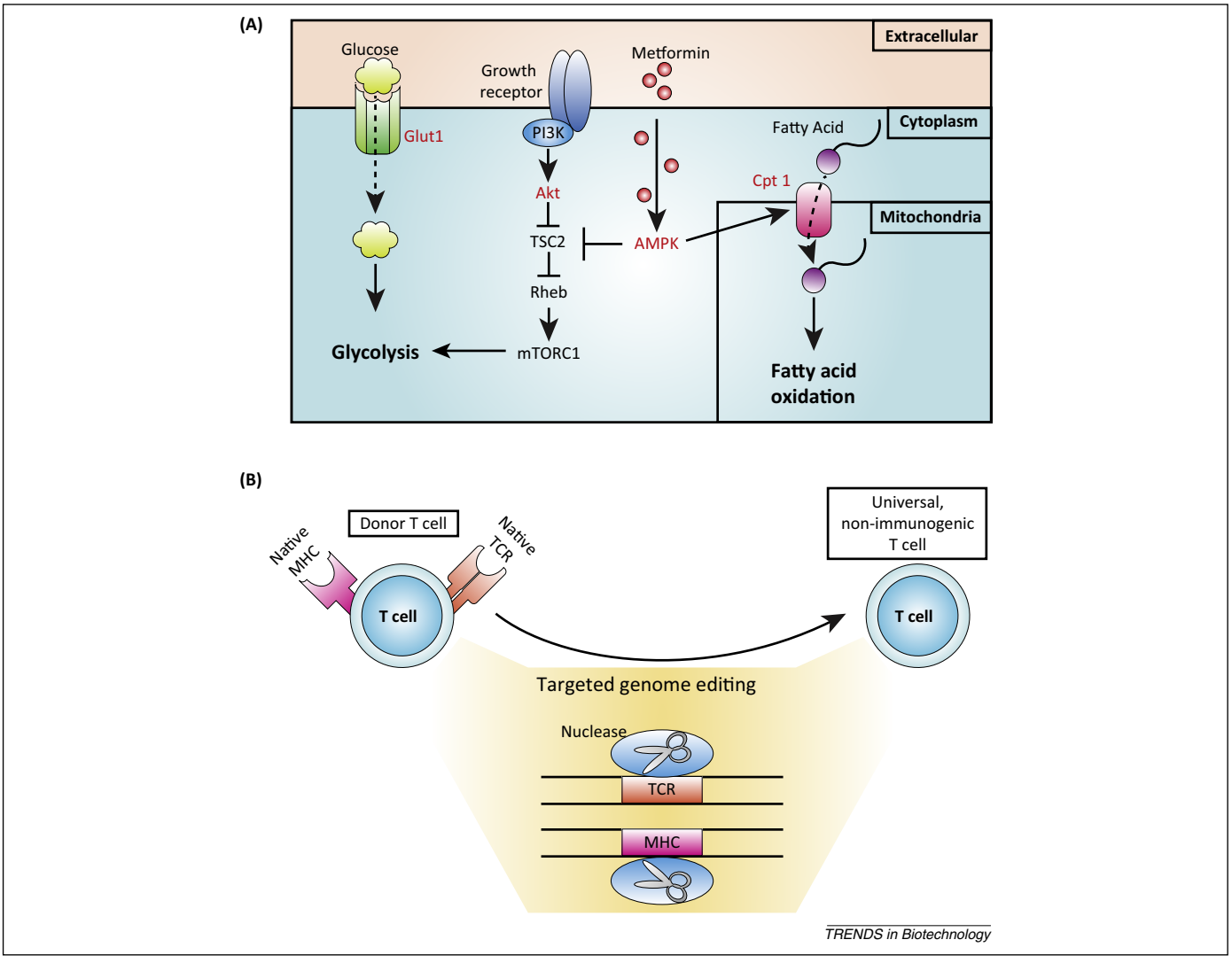

Figure 4. Cellular engineering and genome editing for chassis engineering. **(A)** Targets to bias T cells towards a naïve state or to the development of the memory T cell phenotype. Limiting glycolysis can prevent T cells from differentiating into effector cells and promote memory T cell development [75,79]. In addition, memory T cell development can be increased by promoting fatty acid oxidation. Different components of these metabolic pathways can be targeted for these aims (labeled in red). Reducing the expression of glucose transporter Glut1 limits the intake of glucose, while expressing an Akt inhibitor limits glycolysis as a result of Akt (protein kinase B) activation of mTORC1 (mechanistic target of rapamycin complex 1) through TSC2 (tuberous sclerosis 2) and Rheb (Ras homolog enriched in brain) signaling [76,77,106]. Fatty acid oxidation can be promoted by overexpressing the enzyme CPT1a (carnitine palmitoyl transferase 1a), which aids the transportation of fatty acids into the mitochondria [79]. Activating AMP-activated protein kinase (AMPK) with the drug metformin promotes fatty acid activation, both by repressing components involved in activating glycolysis and by indirectly overexpressing CPT1 [79]. **(B)** Genome editing for the production of an allogeneic, non-immunogenic T cell. Using targeted nucleases to disrupt the expression of the T cell receptor (TCR) renders the T cell unable to detect targets until the cell is further modified to express an engineered receptor [87]. This process could be used to produce universal T cells from healthy donors, that could be stored in a 'T cell bank' for use in patients, because the lack of endogenous TCR expression would prevent graft-versus-host disease (GVHD). The expression of chimeric antigen receptors (CARs) and genetic circuits can involve the expression of components that elicit an immunogenic response. Disrupting major histocompatibility complex (MHC) expression prevents the T cell from presenting epitopes from these components, and this would reduce the risk of an immunogenic response [88]. Abbreviation: PI3K, phosphatidylinositide 3-kinase.

small hairpin RNA (shRNA) knockdown library was used in an *in vivo* screen, revealing the potential of the *Ppp2r2d* (protein phosphatase 2, regulatory subunit Bδ) knockdown to promote antitumor activity [83]. Similar approaches using gene activation libraries could reveal important factors to promote T cell activity, and exploring tools for multiplexed activation and knockdown libraries could also illustrate the more complex responses in a T cell that would be beneficial to target in therapy [84–86].

In addition to making T cells more adept at navigating the tumor microenvironment, T cells can be engineered to be both safer and easier to produce for therapy. Acquiring and modifying the T cells from a patient is a very involved process, and potential costs are estimated to be as high as US$ 500 000 (http://www.wsj.com/articles/new-costly-cancer-treatments-face-hurdles-getting-to-patients-1412627150). Currently, using a patient's own T cells is important to prevent GVHD. However, a cell-based therapy could mitigate some of the challenges of large-scale personalized therapy if, similar to a blood bank, healthy donors could provide T cells. This type of T cell bank would ideally provide 'off-the-shelf' cancer-killing cell products that can be manufactured at a large scale and implemented on demand.

T cells could be modified for this universal cell-based therapy by disrupting the endogenous TCR of the donor T cells, rendering them responsive only to targets programmed by the chosen cancer-targeting receptor

Box 1. Genome editing for adoptive immunotherapy

Genome editing for targeted disruption of genes, such as HLAs or inhibitory receptors [107,108], can render T cells safer and more powerful for adoptive immunotherapy. Several genome-editing systems are available that rely on the same underlying mechanism: a nuclease targets a sequence and creates a double-stranded break. The break is then repaired using either the error-prone non-homologous end-joining (NHEJ) or homology-directed repair (HDR), which disrupt expression of the gene [107]. To contend with potential of off-target cutting [109,110], tools have been developed to predict off-target cleavage for several of these systems [111,112].

Meganucleases: nucleases that belong to one of five families characterized by their sequence and structure motifs [113]. Owing to their large recognition sites, meganucleases are very specific. They can also be engineered for new targets by combining domains of other meganucleases or mutating residues [113], but these techniques and other approaches to expand the meganuclease repertoire require large screens to find the optimal meganuclease. To facilitate this process, computational tools are being developed to predict meganuclease design for new targets [114].

Transcription activator-like effector nucleases (TALENs): derived from the DNA-binding domain of TAL effectors, the base specificity of these bacterial proteins can be altered by changing two specific amino acids [107]. An array of these proteins can be generated to target a desired sequence: these are then fused to the catalytic domain of the *Fok*I nuclease to confer the ability to cut the target sequence [115].

Zinc-finger nucleases (ZFNs): composed of zinc-finger proteins that bind to DNA nucleotide triplets. By combining zinc fingers with known binding sequences, a larger desired sequence can be targeted. Similarly to TALENs, the zinc-finger protein is fused to the *Fok*I catalytic domain to enable targeted cutting [116].

Clustered regularly interspaced short palindromic repeats (CRISPR): an RNA-guided nuclease system derived from bacterial immune defenses. The nuclease Cas9 (CRISPR-associated 9) is targeted to a sequence through short complementary RNA sequences. The RNA guides Cas9 to the complementary target sequence, where it can then cut the DNA. RNA design with this system is very straightforward, making CRISPR a promising tool for genome editing [117].

(Figure 4B). A zinc-finger nuclease has been used to eliminate endogenous TCR chain expression in a T cell that also expressed a CD19-specific CAR [87] (Box 1). This potential for an 'off-the-shelf' therapy is valuable for pharmaceutical companies, and several companies with adoptive T cell portfolios are investing in companies with expertise in DNA nuclease technologies (Table 1).

A similar approach could be used to reduce the risk of immunogenicity of an engineered T cell by disrupting expression of the T cell MHC (or human leukocyte antigen, HLA), which is involved in presenting potential antigens on the surface of a cell for detection by other T cells [88] (Figure 4B). By removing HLA expression, a T cell would no longer be able to present potential epitopes at the surface, and this would prevent an immunogenic response to any of the components involved in CAR expression or other circuitry.

Concluding remarks and future perspectives

Living cells are increasingly viewed as an attractive platform for designing the ultimate smart therapeutics [19] in view of their extraordinarily sophisticated systems to sense and respond to challenges as well as their flexibility in accommodating genetic modification. Given that the genetic engineering strategies discussed in this review for improving adoptive immunotherapy are not mutually exclusive, an intriguing possibility is to combine several, or even all, of the technologies together to generate extremely sophisticated therapeutic agents for controlling when, where, how long, and how strong the therapeutic agents will engage tumors.

Designing and implementing genetic circuits in immunotherapy may provide powerful tools for control over the therapy. However, there are several challenges in designing genetic circuits for this purpose. One of the potential limitations is the need for methods that can efficiently integrate large amounts of DNA into T cells. Viral integration can become inefficient as the size of the insert increases [73], and circuits that require the expression of several components can become large, making efficient transduction of these genes very challenging. Transposon-based systems such as PiggyBac and Sleeping Beauty, which can integrate large sequences of DNA, are a potential alternative to viral transduction [89,90]. Sleeping Beauty has been used to integrate CARs into T cells in a clinical setting, making it a viable option for integrating larger circuits into cells for immunotherapy [91].

Many genetic circuits rely on proteins that are derived from other organisms. The expression of these foreign proteins in a cell has the potential to elicit an immune response against the engineered T cell, leading to the death of the cell and potentially reduced efficacy of the treatment. This immunogenic response was observed when HSV-TK was used as an inducible suicide gene in patients [63], and components in other circuits could also elicit this response. One option to avoid an immunogenic response is to disrupt the antigen-presentation process in the T cell such that it can no longer present the foreign protein as a potential threat to other immune cells. This disruption might be achievable through genome editing to knock out the expression of the MHC [88], that is used to present protein epitopes to other T cells. With MHC expression disrupted, the engineered T cell can effectively hide the presence of foreign proteins from other immune cells, preventing an immunogenic response.

Although T cells, which already possess powerful machinery to kill cancer cells or foreign invading organisms, have captured the most attention in cell-based cancer therapy, other cell types and organisms are also being explored as potential therapeutic agents. For instance, adoptive T cell therapy is not a viable option for patients with T cell deficiency. Induced pluripotent stem cells can potentially be used to derive cancer targeting T cells [92], and natural killer (NK) cells are a possible alternative to T cells. NK cells expressing a HER2 (human epidermal growth factor)-specific CAR are able to eliminate tumor cells *in vivo* [93,94]. These cells can also trigger cytotoxicity to tumor cells through multiple receptors, and their limited lifespan may make them less dangerous for patients. The availability of the NK-92 cell line from Conkwest provides a platform for an 'off-the-shelf' form of cell-based therapy that might provide further advantages to using NK cells for CAR-based therapy. In addition to NK cells, oncolytic viruses are showing promise in clinical trials against glioblastomas and multiple myeloma [95,96]. Their specificity toward tumors may also be improved with micro-RNA-based

classifiers [97]. Similarly to the engineering strategies outlined here to advance T cell therapy, these other cells and organisms can be further modified to improve their performance, and this parallel emergence of immunotherapy, cellular engineering, and synthetic biology is creating a unique interface to usher in a new era of cell-based cancer therapy.

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