Therapeutic Potential of T Cell Chimeric Antigen Receptors (CARs) in Cancer Treatment: Counteracting Off-Tumor Toxicities for Safe CAR T Cell Therapy

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Abstract

A chimeric antigen receptor (CAR) is a recombinant fusion protein combining an antibody-derived targeting fragment with signaling domains capable of activating T cells. Recent early-phase clinical trials have demonstrated the remarkable ability of CAR-modified T cells to eliminate B cell malignancies. This review describes the choice of target antigens and CAR manipulations to maximize antitumor specificity. Benefits and current limitations of CAR-modified T cells are discussed, with a special focus on the distribution of tumor antigens on normal tissues and the risk of on-target, off-tumor toxicities in the clinical setting. We present current methodologies for pre-evaluating these risks and review the strategies for counteracting potential off-tumor effects. Successful implementation of these approaches will improve the safety and efficacy of CAR T cell therapy and extend the range of cancer patients who may be treated.

INTRODUCTION: CHIMERIC ANTIGEN RECEPTORS

CAR: chimeric antigen receptor

MHC: major histocompatibility complex

TCR: T cell receptor

scFv: single-chain antibody variable fragment

HLA: human leukocyte antigen

NHL: non-Hodgkin's lymphoma

CLL: chronic lymphocytic leukemia

ALL: acute lymphoblastic leukemia

CR: complete response

ORR: overall response rate

Results from a growing number of clinical studies assessing the treatment of B cell malignancies with anti-CD19 chimeric antigen receptor (CAR) T cells show a remarkable rate of objective response and durable complete remission, creating great excitement in the field of cancer immunotherapy (1). When we pioneered the first CAR designs in the late 1980s and early 1990s (2–4), it was widely known that T cells are very powerful effectors in the fight against cancer, but the application of these cells to cancer patients suffered from two major limitations. First, T cell recognition depends on the expression of major histocompatibility complex (MHC) molecules and antigen processing machinery, and many tumors silence these pathways as part of their escape from immune recognition (5, 6). Second, many tumors that do not express costimulatory molecules required for triggering the full potency of T cells often render tumor-specific T cells nonfunctional (7). Researchers have designed CARs to offer an alternative to conventional T cell receptors (TCRs) and circumvent these hurdles.

The current antibody-based, single-chain CARs are based on the early, double-chain prototype (8). They are produced synthetically from chimeric genes encoding an extracellular single-chain antibody variable fragment (scFv) fused through a flexible hinge and transmembrane canonic motif to signaling components comprising immunoreceptor tyrosine-based activation motifs of CD3-ζ or FcRγ chains capable of T cell activation (3, 9–12). These CARs are modular by design, enabling the incorporation of the scFv of choice with different combinations of signaling and costimulatory components. Owing to this versatility, CARs can be used to redirect T cells to recognize practically any desired target antigen in an MHC-independent manner. Thus, a given antitumor CAR, unlike MHC-restricted TCRs, can be used universally regardless of the patient's human leukocyte antigen (HL) makeup and is not affected by tumor escape through downregulation or loss of HLA expression.

The modular design of the single-chain CARs allowed for sophisticated engineering of various signaling domains. Initially, our first-generation single-chain CARs included signaling motifs derived from the TCR or FcγRI complexes (2, 3) (**Figure 1**). These CARs were capable of activating T cells in the absence of costimulation by endogenous costimulatory receptors or their ligands on target cells. Subsequent generations of CAR designs incorporated cytoplasmic costimulatory domains from CD28, CD137 (4-1BB), and CD134 (OX40) (second generation) or their combinations, in addition to the ζ/γ chains (third generation). T cells expressing these CARs displayed superior functionality compared to first-generation constructs (13).

Indeed, recent Phase I clinical trials using second-generation anti-CD19 CARs yielded excellent outcomes, including high rates of long-term complete remission in patients with non-Hodgkin's lymphoma (NHL) (14–17), chronic lymphocytic leukemia (CLL) (15, 16, 18, 19), and acute lymphoblastic leukemia (ALL) (20–24). Researchers presented updates of these trials at the 56th annual meeting of the American Society of Hematology held in December 2014 (25). The combined reports showed complete responses (CRs) in all ALL patients who responded to treatment [55/65 (85%) pediatric patients and 33/38 (87%) adults]; a 10/16 (63%) overall response rate (ORR) in CLL patients with 3 CRs (19%); and a 38/53 (72%) ORR in NHL, 19 of which (36%) achieved CR.

As of the end of 2014, we were aware of 91 clinical trials registered at **http://ClinicalTrials.gov** employing scFv-CAR T cells for cancer treatment. In 58 trials, CARs target antigens in hematologic malignancies, and they target a wide range of antigens present on solid tumors in 33 trials. For detailed lists of these antigens and others in advanced preclinical examination, see **Table 1**. **Supplemental Table 1** lists all trials at **http://ClinicalTrials.gov** evaluating CAR T cells in cancer therapy (follow the **Supplemental Materials link** from the Annual Reviews home page at **http://www.annualreviews.org**).

Supplemental Material

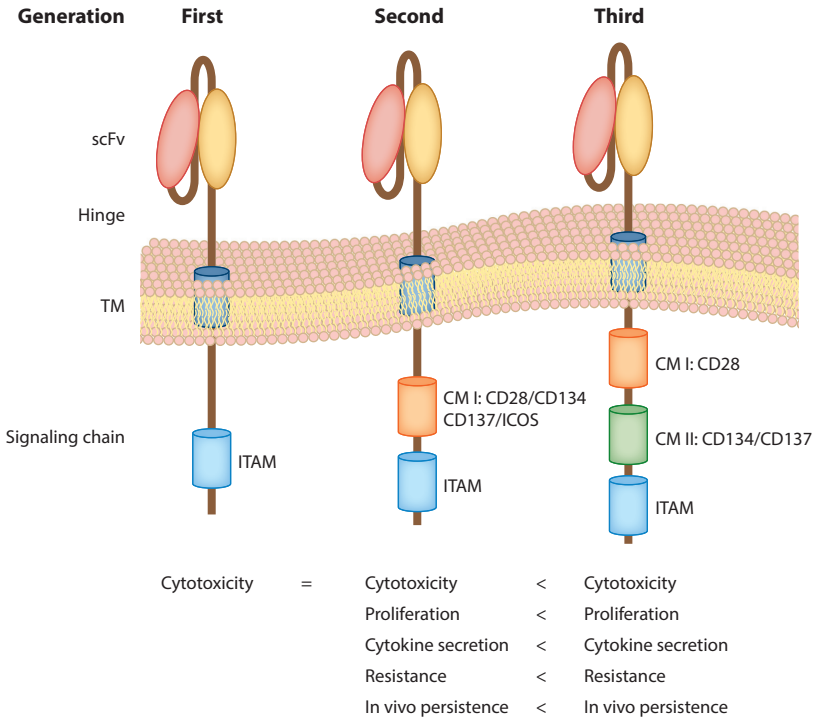

Figure 1

Generations of scFv-based CARs. Schematic illustration of the three generations of CARs and their relative activation features. Abbreviations: CAR, chimeric antigen receptor; CM, costimulatory motif; ITAM, immunoreceptor tyrosine-based activation motif; scFv, single-chain antibody variable fragment; TM, transmembrane. Modified from Reference 152.

The safety of CAR T cell therapy is determined by its ability to discriminate between the tumor and healthy tissue. A major risk and the direct cause of adverse effects reported in clinical and preclinical trials is off-tumor, on-target toxicity due to expression of the target antigen on nontumor tissues.

Here we discuss safety issues associated with tumor antigens that are targeted by CAR T cells, with particular emphasis on tissue distribution and potential autoreactivity. We then review the strategies explored in the clinical and preclinical settings in an attempt to counteract off-tumor toxicity that can result from antigen recognition in healthy tissues.

TARGET ANTIGENS AND POTENTIAL TOXICITY IN CAR T CELL THERAPY

Candidate antigens for CAR therapy can be categorized according to their tissue distribution and the confirmed or predicted consequences of cross-reactivity with normal cells. True tumor-specific antigens that are expressed by a large proportion of patients with a particular cancer are rare. Shared antigens that are either coexpressed only in nonessential tissues or sequestered topologically from CAR T cells are more common, and targeting them does not pose life-threatening risks. In contrast, many tumor antigens are also expressed, at varying levels, on normal cells in essential tissues

Table 1 Target antigens of CAR T cells in clinical and preclinical studies

CAR target antigens evaluated in trials registered at **http://ClinicalTrials.gov**					
Type of malignancy	**Antigen**	**Key structural and functional features**	**Malignancy**	**Potential off-tumor targets**	**References**
Hematologic malignancies	CD19	Pan-B cell marker involved in signal transduction by the BCR	ALL, CLL, NHL, HL, PLL	Normal B cells	27, 110–112
	CD20	Tetra-transmembrane; regulation of Ca transport and B cell activation	CLL, NHL	Normal B cells	28, 29
	CD22	B lineage–specific adhesion receptor; sialic acid–binding Ig-type lectin family	ALL, NHL	Normal B cells	30, 31
	Igκ	Ig light-chain isotype expressed by approximately 65% of normal human B cells	CLL, NHL, MM	Normal B cells	33
	ROR1	Type I orphan-receptor tyrosine-kinase-like; survival-signaling receptor in tumors	CLL, NHL	Pancreas, adipose cells	113, 114
	CD30	TNFR member; pleiotropic effects on cell growth and survival involving NF-κB	NHL, TCL, HL	Resting CD8 T cells, activated B and Th2 cells	115–118
	$Lewis^Y$	Also called CD174; a membrane oligosaccharide harboring two fucose groups	AML, MM	Early myeloid progenitor cells	49
	CD33	Sialic acid–binding Ig-type lectin serving as adhesion molecule of the myelomonocytic lineage	AML	Hematopoietic progenitors, myelomonocytic precursors, monocytes	48, 68, 119
	CD123	The α chain of the IL-3 receptor	AML	BM myeloid progenitors, DCs, B cells, mast cells, monocytes, macrophages, megakaryocytes, endothelial cells	67–69
	NKG2D-L	Ligands for the NK and T cell activating receptor NKG2D; bear similarity to MHC class I molecules; upregulated during inflammation	AML, MM	Gastrointestinal epithelium, endothelial cells, fibroblasts	120–122
	CD138	Syndecan-1; cell surface heparan sulfate proteoglycan; ECM receptor	MM	Precursor and plasma B cells, epithelia	123
	BCMA	TNFR member; binds BAFF and APRIL; involved in proliferation signaling	MM	B cells	32

(*Continued*)

Table 1 (*Continued*)

CAR target antigens evaluated in trials registered at http://ClinicalTrials.gov					
Type of malignancy	**Antigen**	**Key structural and functional features**	**Malignancy**	**Potential off-tumor targets**	**References**
Solid tumors	GD2	Disialoganglioside	NB, sarcomas, solid tumors	Skin, neurons	59, 60
	FR-α	GPI-linked folate receptor; functions in the uptake of reduced folate cofactors	Ovarian cancer	Apical surface in kidney, lung, thyroid, kidney, and breast epithelia	38
	L1-CAM	CD171; neuronal cell adhesion molecule of the Ig superfamily	NB	CNS, sympathetic ganglia, adrenal medulla	61, 124
	ErbB2	HER2; member of the EGFR family of receptor tyrosine-protein kinases	Brain, CNS, gliomas, GBM, H&N, solid tumors	Gastrointestinal, respiratory, reproductive, and urinary tract epithelia; skin, breast, and placenta; hematopoietic cells	53–56, 58
	EGFRvIII	Splice variant; in-frame deletion in the amplified EGFR gene encoding a truncated extracellular domain that constantly delivers prosurvival signals	Brain, CNS, gliomas, GBM	None	98, 125–127
	VEGFR-2	Type III transmembrane kinase receptor of the Ig superfamily; regulates vascular endothelial function	Solid tumors	Vascular and lymphatic endothelia	94, 128, 129
	IL-13Rα2	The α chain of one of the two IL-13 receptors	Brain, CNS, gliomas, GBM	Astrocytes, brain, H&N tissue	90, 91
	FAP	Cell surface serine protease	Mesothelioma	Fibroblasts in chronic inflammation, wound healing, and tissue remodeling	72–74
	Mesothelin	40-kDa cell surface glycoprotein with unknown function	Mesothelioma; pancreatic, and ovarian cancers	Peritoneal, pleural, and pericardial mesothelial surfaces	93, 100
	c-MET	Hepatocyte growth factor receptor; disulfide-linked α-β heterodimeric receptor tyrosine kinase	TNBC	Liver, gastrointestinal tract, thyroid, kidney, brain	130
	PSMA	Type II membrane glycoprotein possessing N-acetylated alpha-linked acidic dipeptidase and folate hydrolase activity	Prostate cancer	Apical surface of normal prostate and intestinal epithelium and renal proximal tubular cells	131–135

(*Continued*)

Table 1 (*Continued*)

CAR target antigens evaluated in trials registered at http://ClinicalTrials.gov					
Type of malignancy	**Antigen**	**Key structural and functional features**	**Malignancy**	**Potential off-tumor targets**	**References**
	CEA	Surface glycoprotein; member of the Ig superfamily and the CEA-related family of cell adhesion molecules	Colorectal and breast cancers; solid tumors	Apical epithelial surface of colon, stomach, esophagus, and tongue	36, 78, 136
	EGFR	ErbB1; HER1; receptor tyrosine kinases signaling cell differentiation and proliferation upon ligand binding	Solid tumors	Tissues of epithelial, mesenchymal, and neuronal origin	137
Other CAR target antigens					
Type of malignancy	**Antigen**	**Key structural and functional features**	**Malignancy**	**Potential off-tumor targets**	**References**
Hematologic malignancies	CD38	A surface cyclic ADP ribose hydrolase involved in transmembrane signaling and cell adhesion	CLL, NHL, MM	PBMCs, BM, brain, eye, prostate, gut, pancreas, muscle, bone, kidney	138–140
	CS1	Cell surface signaling lymphocytic activation molecule	MM	PC, NK, NK-like T cells, $CD8^+$ T cells, activated monocytes, DCs	141
Solid tumors	PSCA	GPI-anchored membrane glycoprotein of the Thy-1/Ly-6 family	Prostate, bladder, and pancreatic cancers	Normal prostate	88, 142, 143
	CD44v6	Alternatively spliced variant 6 of the hyaluronate receptor CD44	H&N, liver, pancreatic, gastric, breast, and colon cancers; AML, NHL, MM	Skin keratinocytes, monocytes, activated T cells	144
	CD44v7/8	Alternatively spliced variant 7/8 of the hyaluronate receptor CD44	Breast and cervical cancers	Normal epithelia	145
	MUC1	Densely glycosylated member of the mucin family of glycoproteins	Colon, lung, pancreatic, breast, ovarian, prostate, kidney, stomach, and H&N cancers	Apical surface of most glandular epithelia	37
	IL-11Rα	The α subunit of the IL-11 receptor	Colon, gastric, breast, and prostate cancers; osteosarcoma	Stromal tissues in the gastrointestinal tract, endothelial cells, surface and gland epithelia, liver	146

(*Continued*)

Table 1 (*Continued*)

CAR target antigens evaluated in trials registered at http://ClinicalTrials.gov					
Type of malignancy	**Antigen**	**Key structural and functional features**	**Malignancy**	**Potential off-tumor targets**	**References**
	EphA2	EphA2 receptor; a member of the Eph family of receptor tyrosine kinases	Glioma; breast, colon, ovarian, prostate, and pancreatic cancers	Endothelia	147
	CAIX	Transmembrane zinc metalloenzyme	RCC, tumors under hypoxia	Pancreatobiliary epithelium, gastric mucosa, and small intestine crypt base	50–52
	CSPG4	High-molecular-weight melanoma-associated antigen; cell surface proteoglycan	Melanoma, TNBC, GBM, mesothelioma, H&N cancer, osteosarcoma	Epidermis basal cells, endothelial cells, activated pericytes	65, 148, 149

Abbreviations: ADP, adenosine diphosphate; ALL, acute lymphoblastic leukemia; AML, acute myelogenous leukemia; APRIL, a proliferation-inducing ligand; BAFF, B cell activation factor of the TNF family; BCMA, B cell maturation antigen; BCR, B cell receptor; BM, bone marrow; CAIX, carbonic anhydrase IX; CAR, chimeric antigen receptor; CEA, carcinoembryonic antigen; CLL, chronic lymphocytic leukemia; CNS, central nervous system; CSPG4, chondroitin sulfate proteoglycan 4; DC, dendritic cell; ECM, extracellular matrix; EGFR, epidermal growth factor receptor; EGFRvIII, variant III of the EGFR; EphA2, erythropoietin-producing hepatocellular carcinoma A2; FAP, fibroblast activation protein; FR-α, folate receptor-alpha; GBM, glioblastoma multiforme; GPI, glycophosphatidylinositol; H&N, head and neck; HL, Hodgkin's lymphoma; Ig, immunoglobulin; L1-CAM, L1 cell adhesion molecule; MM, multiple myeloma; NB, neuroblastoma; NF-κB, nuclear factor-κB; NHL, non-Hodgkin's lymphoma; NK, natural killer; NKG2D-L, NKG2D ligand; PBMC, peripheral blood mononuclear cell; PC, plasma cell; PLL, prolymphocytic leukemia; PSCA, prostate stem cell antigen; PSMA, prostate-specific membrane antigen; RCC, renal cell carcinomas; ROR1, receptor tyrosine kinase-like orphan receptor 1; TCL, T cell leukemia/lymphoma; Th2, T helper 2; TNBC, triple-negative breast cancer; TNFR, tumor necrosis factor receptor; VEGFR-2, vascular endothelial growth factor-2.

and require specific measures to counteract potential toxicities resulting from their off-tumor recognition. In this article, we review target antigens in trials listed in **http://clinicaltrials.gov** and other selected candidates for CAR therapy, referring to their distribution in healthy tissue and specific features in their expression patterns related to potential toxicity.

Low-Risk Antigens

CAR T cell recognition of antigens included in the different subgroups of this category is expected to cause practically no, or completely manageable, off-tumor on-target effects. Altogether, 60 clinical trials employ T cells redirected at such antigens, and advanced preclinical evaluation of additional important candidates is under way.

EGFR: epidermal growth factor receptor

GBM: glioblastoma multiforme

NSCLC: non-small-cell lung cancer

H&N: head and neck

Shared tumor-specific antigens. Cell surface antigens shared by many tumors that are truly tumor-specific and therefore do not raise particular safety concerns are scarce. One such antigen is variant III of the epidermal growth factor receptor (EGFRvIII). This splice variant arises from in-frame deletion in the amplified EGFR gene, resulting in a truncated extracellular domain of the receptor, which constitutively delivers tumorigenic prosurvival signals. The expression of EGFRvIII is strictly confined to tumor cells (26), and it is found in glioblastoma multiforme (GBM); non-small-cell lung cancer (NSCLC); and prostate, breast, head and neck (H&N), and ovarian cancers.

Researchers are already examining CARs targeting EGFRvIII clinically in central nervous system (CNS) tumors (**http://clinicaltrials.gov** identifier NCT01454596, NCT02209376).

CNS: central nervous system

mAb: monoclonal antibody

PSMA: prostate-specific membrane antigen

CEA: carcinoembryonic antigen

Antigens coexpressed by nonvital healthy tissue. Tumors affecting nonvital tissues such as B cells, prostate, and testes display some surface antigens that are shared only by their normal cell counterparts and can thus be targeted by CAR T cells with no life-threatening outcome. CD19 is a pan-B cell marker acquired very early in B cell differentiation and involved in signal transduction by the B cell receptor. CD19 is widely expressed in NHL, CLL, and ALL (1, 27) and is currently the target antigen in 43 clinical trials. Prolonged persistence of anti-CD19 CAR T cells preserves chronic B cell lymphopenia and hypogammaglobulinemia, which are manageable by the repeated administration of intravenous immunoglobulin (Ig). CD20 is expressed during most stages of B cell maturation, excluding early pro-B cells and plasma cells. It is present in a high percentage of NHLs, CLLs, ALLs, and hairy cell leukemias and is the target of clinically approved monoclonal antibodies (mAbs). Phase I evaluation of anti-CD20 CAR T cells in patients with NHL (28, 29) (NCT00621452) revealed minimal toxicities. CD22, a B lineage–specific marker, is expressed from the pre-B cell to mature B cell stage but not by plasma cells and is universally expressed in CLL, ALL, and NHL. An anti-CD22 CAR has recently entered clinical evaluation in ALL and NHL (30, 31) (NCT02315612). A potentially useful target is the B cell maturation antigen (BCMA) that is expressed by terminally differentiated B cells, including plasma cells, and by practically all multiple myelomas (MMs). An anti-BCMA CAR is now being tested clinically (32) (NCT02215967).

Light-chain isotype exclusion is maintained by most B cell malignancies. CAR-mediated targeting of Igκ chains on Igκ$^+$ B cell tumors can spare normal Igλ$^+$ B cells and prevent the loss of the entire B cell compartment resulting from targeting pan-B cell markers. A clinical trial evaluating an anti-Igκ CAR is under way (33) (NCT00881920). An update on this trial presented in 2013 (34) reported no major toxicities in 10 NHL or CLL treated patients.

Epithelial antigens exhibiting polarized membrane distribution. Malignant transformation of cells composing glandular or intestinal epithelia often abrogates the polarized distribution of surface proteins. As a result, apical surface antigens that would normally be sequestered from CAR (although not conventional) T cell recognition redistribute to the entire plasma membrane, where they become detectable by CAR T cells. This topological selectivity applies to numerous CAR antigens and diminishes the likelihood of adverse off-tumor effects. Normal expression of prostate-specific membrane antigen (PSMA) is restricted to the apical surfaces of prostate and intestinal epithelia and renal proximal tubular cells. PSMA is overexpressed in prostate cancer and in the neovasculature of many solid tumors, and its level is further elevated in metastatic lesions and hormone-refractory disease. Polarization of PSMA expression is abolished in prostate cancer (35), and this newly gained accessibility is exploited by anti-PSMA CARs (NCT00664196, NCT01140373, NCT01929239).

Carcinoembryonic antigen (CEA) is expressed in nearly all colorectal cancers; 70% of NSCLCs; approximately 50% of breast cancers; and ovarian, gastric, pancreatic, and endometrial cancers. In normal adult tissue, CEA is detected mostly at the apical surface of digestive tract epithelia. Although CEA expressed on normal epithelia is largely inaccessible to CAR T cells, this was not the case in a study with TCR-redirected CD8 T cells, which were capable of recognizing evenly distributed CEA peptide/HLA complexes in the normal gut epithelium and induced severe colitis in patients with colorectal cancer (36). Two clinical trials evaluating CEA-specific CARs against various solid tumors have been completed (NCT00004178 and NCT01373047), two are currently under way (NCT00673829 and NCT01723306), one was terminated for incorporation into another study (NCT00673322), and another (NCT01212887) was terminated owing to treatment-related adverse effects and lack of efficacy.

The mucin antigen MUC1 is normally expressed at relatively low levels on the apical surface of most glandular epithelia. In tumors, it is highly upregulated, is redistributed to the basal epithelium surface, and displays aberrant glycosylation resulting in new glycoforms. MUC1 is commonly found in many cancers, including colon, lung, pancreas, breast, ovarian, prostate, kidney, stomach, and H&N cancers. Anti-MUC1 CAR T cells selectively inhibited tumor growth in a xenograft mouse model without provoking any discernible off-tumor toxicities (37). Folate receptor-alpha (FR-α) is overexpressed on a variety of solid tumors, and its normal expression is restricted to the apical surfaces of a subset of polarized epithelia in the kidney, lung, thyroid, and breast. An early Phase I clinical trial assessing anti-FR-α CAR T cells in metastatic ovarian cancer did not reveal any adverse off-tumor effects, yet the CAR T cells failed to elicit any objective antitumor response (38) (NCT00019136).

Tumor antigens targeted by T cell receptor–like CARs. Unlike conventional CARs, TCR-modified T cells can also target intracellular tumor antigens, including the large family of cancer/testis antigens, which are normally expressed during fetal development and in the germline and are re-expressed in many epithelial tumors. Although most cancer/testis antigens are also expressed in various normal tissues, NY-ESO-1 and MAGE-A3 appear to be strictly confined to cancer cells and testis. One intriguing approach for extending the repertoire of tumor-specific CARs to these antigens is to employ scFvs prepared from a unique family of antibodies specific to selected peptide/HLA complexes. Such TCR-like antibodies, which can bind their antigens with high affinity and apparent selectivity, are typically generated by selection from recombinant antibody libraries (39). When their scFvs were engrafted onto different CAR scaffolds, the CAR T cells possessed high avidity and were redirected against selected tumor-associated peptides derived from MAGE-A1, NY-ESO-1, and gp100 in vitro (40–43). Although it falls into the broader definition of bona fide CARs, this unique design is in fact analogous to the use of genes encoding affinity-enhanced TCRs, which are HLA restricted, so that the benefit of MHC independence of CAR T cells is lost. Yet the accessibility to safe intracellular protein antigens, which are otherwise invisible to conventional CARs, comes along with two potential advantages over the use of TCRs: obviating the problem of mixed pairing with endogenous TCR chains and achieving a higher binding affinity for the target peptide/HLA complex. However, apart from off-tumor, on-target antigen recognition, cross-reactivity of such TCRs with nonidentical yet sequence-related HLA-I-binding peptides presented by vital cells can lead to fatal, off-target, off-tumor toxicity (44, 45). Binding of high-affinity TCR-like antibodies is likely to be even more promiscuous than that of mutated TCRs, which still possess a canonical Vα-Vβ structure. Moreover, researchers recently demonstrated affinity thresholds for antitumor activity of both TCR-modified T cells and TCR-like recognition, arguing that high-affinity TCRs do not necessarily improve efficacy but can enhance autoimmunity (46, 47). Preclinical safety assessment of TCR-like CARs will therefore require vigilant examination for potential cross-reactivity of such constructs with peptides presented by healthy tissue.

Antigens Expressed on Essential Cells: Evidence Concerning Off-Tumor Toxicity

Targeting CAR T cells against antigens in this category, which includes the vast majority of new candidates for future clinical evaluation, may lead to detrimental consequences. The advance of these CARs to the clinic therefore entails particularly meticulous preclinical assessment and painstaking design of clinical trials, regardless of the actual strategy employed for counteracting the predicted off-tumor effects.

AML: acute myelogenous leukemia

CAIX: carbonic anhydrase IX

RCC: renal cell carcinoma

NB: neuroblastoma

MPM: malignant pleural mesothelioma

TNBC: triple-negative breast cancer

Antigens in hematologic malignancies. Unlike B cell–specific antigens, the cotargeting of antigens expressed by other hematopoietic cells may lead to adverse long-term consequences. CD33 is expressed by blasts and leukemic stem cells in a large majority of acute myelogenous leukemia (AML) patients and is normally expressed by early multilineage hematopoietic progenitors and myelomonocytic precursors and on blood monocytes. The anti-CD33 drug-linked mAb gemtuzumab ozogamicin was voluntarily withdrawn from the market in 2010 after 10 years of use owing to severe toxicity, including myelosuppression and the apparent targeting of $CD33^+$ Kupffer cells. Nevertheless, a report on one patient enrolled in NCT01864902 describes a transient and partial response and treatment-related effects but no adverse off-tumor, on-target toxicity (48). $Lewis^Y$ antigen (CD174) is expressed by approximately half of MM and AML tumors; on a subset of lung, colorectal, and gastric cancers; and on early myeloid progenitor cells. A clinical study with anti-$Lewis^Y$ CARs in AML (NCT01716364) did not reveal major toxicity (49).

Antigens in solid tumors. Transmembrane carbonic anhydrase IX (CAIX) is expressed highly in clear cell renal cell carcinomas (RCCs) and under hypoxia in several solid tumors and is also expressed by normal pancreatobiliary epithelium, gastric mucosa, and small intestine crypt base. A first clinical trial with anti-CAIX CAR T cells in RCC patients (DDHK 97-29/P00.0040C) resulted in severe disturbances in liver enzymes and autoimmune cholangitis caused by T cell infiltration around the bile ducts, where epithelial CAIX expression could be demonstrated (50–52).

One of the first tumor antigens we investigated was ErbB2 (HER2/neu) (53–57). The ErbB2 gene is amplified and overexpressed in a variety of solid tumors, and its overexpression has been associated with tumorigenesis; therefore, escape mutants are less likely to emerge. ErbB2 is normally found in epithelial cells in the gastrointestinal, respiratory, reproductive, and urinary tracts and in skin, breast, and placenta, as well as in normal hematopoietic cells. Five clinical trials are examining anti-ErbB2 scFv-CARs (NCT00889954, NCT00902044, NCT01109095, NCT01818323, NCT01935843) in brain tumors, H&N tumors, and other solid tumors. The death of a patient with metastatic colon cancer led to premature termination of NCT00924287, and investigators speculated it resulted from off-tumor, on-target reactivity against ErbB2 expressed at low levels in the cardiopulmonary epithelia (58).

The ganglioside GD2 is expressed on neuroectodermal tumors, including neuroblastomas (NBs) and a subset of melanomas, and in normal tissues, it is mainly restricted to the skin and neurons. Five clinical trials (NCT00085930, NCT01460901, NCT01822652, NCT01953900, NCT02107963) evaluate anti-GD2 CAR T cells against NBs, sarcomas, and different solid tumors. Clinical responses achieved in NCT00085930 were not associated with major off-tumor toxicities (59, 60).

L1 cell adhesion molecule (L1-CAM, CD171) is expressed in NBs, glioma, melanoma, and other solid cancers and in the healthy CNS, adrenal medulla, and sympathetic ganglia. In a first study with six NB patients (NCT00006480), no overt off-tumor toxicities were observed (61).

Mesothelin is overexpressed by the majority of malignant pleural mesotheliomas (MPMs) in pancreatic, ovarian, lung, and triple-negative breast cancers (TNBCs) and at low levels on normal peritoneal, pleural, and pericardial mesothelial surfaces. Interestingly, endogenous antimesothelin immune responses could be detected in patients with MPMs and ovarian and pancreatic cancers (62, 63), further supporting the safe targeting of this antigen. Two ongoing trials are testing antimesothelin CARs expressed from electroporated mRNA in the treatment of pancreatic cancer (NCT01897415) and MPM (NCT01355965); no evidence for adverse on-target, off-tumor toxicity could be detected in two patients, one in each trial (64).

STRATEGIES FOR PREVENTING OFF-TUMOR REACTIVITY IN CAR T CELL THERAPY

Preclinical Assessment of the Safety of CAR Treatment

IHC: immunohistochemistry

BM: bone marrow

Predicting whether a planned CAR treatment can result in autoreactivity against normal, vital cells expressing the target antigen is a safety issue of cardinal importance. Given that a false positive alert could exclude a valuable candidate antigen from the short list of clinically useful targets, the issue becomes increasingly critical. This preassessment can be performed at several levels.

Analyzing information on surface expression of the target antigen on vital cells. Protein-based prediction can be more clinically relevant than gene expression data sets, which may fall short of predicting actual surface expression and the degree of accessibility to CAR T cells. The Human Protein Atlas (**http://www.proteinatlas.org/**) contains protein expression profiles assembled on the basis of immunohistochemistry (IHC) staining from 44 normal and 20 cancer tissues, including subcellular localization of 83% of all human protein-coding genes. These data cannot possibly cover all the diverse cell types in human tissues and organs. Furthermore, conventional IHC analysis may fail to pinpoint the exact cell type stained in a given tissue sample or identify proteins expressed under abnormal conditions such as stress, hypoxia, or inflammation or in response to a multitude of other physiological stimuli. For example, the melanoma tumor marker chondroitin sulfate proteoglycan 4 is also overexpressed in TNBC, osteosarcoma, mesothelioma, H&N cancer, and GBM. Lack of any significant expression on a wide range of normal tissues, as judged by IHC staining (65), does not coincide with the IHC profile of this antigen in the Human Protein Atlas, showing high or medium expression levels in 59 of 83 normal tissues. Additionally, different antibodies used for IHC staining can lead to differing conclusions as to the actual tissue distribution of a given antigen (65).

In vivo performance of the source monoclonal antibody. The inherent lytic machinery of CAR T cells, their superior ability to penetrate solid tissues compared with soluble antibody, and their natural capacity to proliferate and persist for extended periods limit the relevance of safety conclusions drawn from mAb studies to CAR therapy. The death of the first patient treated with anti-ErbB2 CAR T cells owing to recognition of low levels of the antigen on lung epithelial cells in the NCT00924287 study led to an early termination of this trial. Yet noninfectious adverse pulmonary reactions associated with the administration of trastuzumab, the source mAb for this CAR, are rare (66).

Other CAR T cell studies targeting the same antigen. Each CAR possesses a characteristic profile reflecting epitope specificity, binding affinity, signaling elements, and distinct structural features. These, along with the influence of the particular vector employed for gene transfer and the number, differentiation status, and composition of the transferred T cells, determine the level of CAR expression and T cell functionality. As a result, targeting the same antigen with different CARs can lead to substantially different outcomes concerning both treatment efficacy and off-tumor reactivity. CD123 is widely expressed in AML and B cell ALL and on normal bone marrow (BM) myeloid progenitors and other lymphoid and myeloid cells. Several studies recently demonstrated potent in vivo eradication of human AML with anti-CD123 CAR T cells (67–69), and the first clinical trial is under way (NCT02159495). In addition to suppression of primary AML, Gill et al. (69) showed the ablation of normal human myelopoiesis, which was observed with neither the source mAb nor a different anti-CD123 CAR carrying the same scFv (67). As AML is thought to evolve from preleukemic hematopoietic stem cells, the authors proposed the

use of the anti-CD123 CAR as both a viable AML therapy and a novel conditioning regimen for myeloablation in hematopoietic cell transplantation. This is a unique example of harnessing an otherwise potentially adverse autoimmune effect to achieve a clinically favorable outcome.

Studies of CAR T cells targeting human tumor xenografts in immunodeficient mice. This experimental approach for risk assessment can only be informative if the tested CAR recognizes the corresponding mouse antigen. Fibroblast activation protein (FAP) is a membrane serine protease almost invariably expressed by tumor-associated fibroblasts in different carcinomas and by fibroblasts in chronic inflammatory disorders associated with fibrosis, but only at low to undetectable levels in resting fibroblasts of normal adult tissues. Targeting FAP-expressing tumor fibroblasts can potentially suppress tumor growth by inhibiting stromagenesis (70, 71). The humanized anti-FAP mAb F19 (sibrotuzumab) entered a Phase II clinical trial against metastatic colorectal cancer but could not produce the minimal clinical response required for continuation. Nevertheless, researchers recently evaluated T cells redirected with an F19-based anti-FAP CAR ex vivo (72) and in vivo (73) in the context of MPM, and the first clinical trial is now recruiting patients (NCT01722149). In preparatory experiments, analysis of normal human tissues detected FAP in specific areas in the pancreas and placenta and very weakly in the cervix and uterus. Another preclinical study with a different CAR, cross-reactive with mouse FAP, demonstrated cachexia and lethal bone toxicities resulting from FAP-expressing multipotent BM stromal cells (74). Yet recent in vivo reports evaluating two additional anti-FAP CARs revealed only minimal (75) or no (76) off-tumor toxicity, providing an additional illustration of the functional variability between different CARs targeting the same antigen.

Transgenic mice expressing the human target antigen. To increase the relevance of safety assessment to the human setting, investigators generated lines of transgenic mice expressing selected human antigens orthotopically. One such line carries a cosmid clone containing the complete structural human CEA gene and flanking regulatory elements (77). These mice express human CEA highly in epithelial cells along the entire gastrointestinal tract and at low levels in the lung, testis, and uterus and have been used as a model for transplantable CEA^+ human pancreatic cancer. No evidence was seen for destruction of healthy human CEA^+ mouse tissue despite a robust antitumor activity of mouse CAR T cells specific for human CEA (78, 79). Researchers generated two other human CEA transgenic lines from a bacterial artificial chromosome spanning the entire human CEA gene and additional CEA family genes, one harboring two and the other ten transgene copies (80). These mice exhibit a spatiotemporal expression pattern of CEA that is remarkably similar to that seen in humans. Recently, we were able to show that antihuman CEA CARs could induce colitis in the ten-copy transgenic mice but not in two-copy ones (81), underscoring the level of target gene expression in healthy tissue as a critical factor governing off-tumor toxicity. A mouse model for human ErbB2 carries the full wild-type gene under the whey acidic promoter, which targets the expression of heterologous genes to the mammary gland and brain (82). Investigators used this model to demonstrate the safety of anti-ErbB2 CAR T cells for treating an $ErbB2^+$ tumor, ascribing the lack of adverse autoreactivity to the lower expression level in the normal tissues (83, 84). Transgenic mice of a second line overexpress human ErbB2 under the murine mammary tumor virus promoter and develop mammary tumors spontaneously (85). Recently, we demonstrated that repeated systemic administration of relatively low doses of anti-ErbB2 CAR T cells could effectively eradicate existing tumors and protect these mice from the appearance of new tumors with no toxicity in healthy tissues expressing the human transgene (57).

As neither of these preassessment levels, nor combinations thereof, can fully guarantee clinical safety, cautionary measures, such as preliminary evaluation of the effect of escalating CAR T cell

doses on small cohorts of patients, should be considered. Future clinical results will undoubtedly extend the list of at-risk organs and tissues in given CAR therapies. Scientists in the field of CAR immunotherapy are therefore directing great effort at devising clinically applicable strategies for minimizing off-tumor responses. These can be divided into strategies that aim to prevent potential damage in advance (proactive measures) and those devised to eliminate, or suppress, transferred T cells once related adverse effects are already evident (reactive measures) (**Table 2**).

Proactive Measures

Proactive measures comprise different approaches, all aimed at obviating, or minimizing the risk of adverse autoreactivity prior to or at the onset of, any clinical manifestation. The distinct antigens currently being targeted in clinical and preclinical studies reflect the great efforts to avoid off-tumor toxicity in the face of the apparent scarcity of genuine, shared, tumor-specific antigens.

Inhibitory CARs. Off-tumor reactivity occurs when the target antigen of CAR-redirected T cells is shared with normal tissue. If this normal tissue expresses another surface antigen not present on the tumor, then coexpressing in the CAR T cells additional CARs targeting this nonshared antigen, which harbors a T cell inhibitory signaling moiety, can prevent T cell activation by the normal tissue. Fedorov et al. (86) recently demonstrated the protective capacity of such inhibitory CARs (iCARs); they successfully employed the intracellular domains of the T cell inhibitory molecules programmed cell death protein 1 (PD-1) and cytotoxic T lymphocyte–associated protein 4 (CTLA-4). Importantly, the initial regulatory effect of iCARs was selective and temporary, allowing full T cell activation upon subsequent encounters with target cells exclusively expressing the antigen recognized by the activating CAR.

Combinatorial antigen recognition. Although true tumor-specific surface antigens are rare, combinations of two different antigens, not necessarily classified as tumor-associated antigens that are coexpressed by a given tumor, can define a new tumor-specific signature. Restricting the activity of CAR T cells to such antigen pairs provides a critical safety mechanism, consequently extends the spectrum of tumor-specific targets, and may be of substantial therapeutic value.

Second- and third-generation CARs (see **Figure 1**) have been designed to provide therapeutic T cells with activation (signal 1) and costimulation (signal 2) signals upon engaging a single antigen through the tethering of two or more signaling portions at the CAR endodomain. However, if activation and costimulation are split in the same T cell between two CARs, each specific for a different antigen, then a full-blown response would require the cooperation of the two complementary signals that could only be accomplished in the presence of the two antigens. Keeping the activation signal at bay to ensure dependence on costimulation is crucial for preventing off-tumor damage and is a highly demanding technical challenge. Wilkie et al. (87) reported an attempt to implement this principle by exploring ErbB2/CD3-ζ for activation and MUC1/CD28 for costimulation. These authors demonstrated functional coexpression of the two CARs and an in vitro response to the complementary cues in the presence of target cells expressing different combinations and densities of the two antigens. Yet their results also underlined the inherent difficulty in achieving proper control over the relative strengths of the two signals, which is necessary for preventing unintended T cell activation against single-positive cells.

Addressing this critical issue, Kloss et al. (88) chose to regulate the activating CAR through meticulous titration of its signaling capacity by testing a panel of scFvs possessing a wide range of binding affinities for the antigen. In this study, the authors first evaluated the CD19/CD3-ζ–PSMA/CD28/4-1BB pair and later replaced the prior with prostate stem cell antigen

Table 2 Strategies for counteracting on-target, off-tumor toxicities

Strategy			Details and antigens		http://ClinicalTrials.gov identifier and references
Proactive	Selecting safer antigens	Tumor-specific antigens	EGFRvIII		NCT01454596, NCT02209376
		Antigens expressed on nonessential tissue	B cells	CD19	43 clinical trials; see text and **Supplemental Table 1**
				CD20	NCT00012207, NCT00621452, NCT01735604
				CD22	30, 31
				Igκ	NCT00881920
				BCMA	NCT02215967
			Prostate	PSCA	88 (in vivo), 142, 143
				PSMA	NCT00664196, NCT01140373, NCT01929239
		Epithelial antigens normally confined to apical surfaces	PSMA		NCT00664196, NCT01140373, NCT01929239
			MUC1		37, 87
			CEA		NCT00004178, NCT00673322, NCT00673829, NCT01212887, NCT01373047, NCT01723306
			FR-α		NCT00019136
		CARs based on TCR-like Abs	MAGE-A1, NY-ESO-1, gp100		40–43
	Inhibitory CARs		CTLA-4 and PD-1 signaling domains		86
	Combinatorial antigen recognition		Dual recognition		150, 151
			Complementary signaling		87–89
	Mutated receptors		Mutant IL-13; limiting binding to IL-13Rα2(+) IL-13 receptors		NCT01082926, NCT02208362
	Limiting CAR expression		mRNA electroporation	Mesothelin	NCT01355965, NCT01897415
				CD19	NCT02277522
				c-MET	NCT01837602
			Transient DNA	VEGFR-1	94
	Limiting CAR function		No costimulation		94

(*Continued*)

Supplemental Material

Table 2 (*Continued*)

Strategy		Details and antigens		http://ClinicalTrials.gov identifier and references
	Avoiding systemic administration	Intratumoral administration	IL-13Rα2	NCT01082926, NCT02208362
			ErbB	NCT01818323
			CEA	78, 97
			Mesothelin	100
			EGFRvIII	98
			ErbB2	57
	Removing donor-derived T cells	Host-versus-graft	ErbB2	95
Reactive	Suicide genes	HSV-tk	IL-13Rα2	NCT00730613, NCT01082926
			PSMA	NCT01140373
			CD19	NCT00182650
		iC9	GD2	NCT01822652, NCT01953900, NCT02107963
			CD19	NCT02247609
	Ab-mediated depletion of CAR T cells	EGFRt + cetuximab	CD19	NCT01683279, NCT01815749, NCT02051257, NCT02146924
			L1-CAM	NCT02311621
		Truncated CD19	IL-13Rα2	NCT02208362
		CD20 + rituximab		105, 106
		Anti-CD19 anti-idiotypic mAb		108
	mAb blocking of target antigen	Anti-CAIX, G250 mAb		52

Abbreviations: Ab, antibody; BCMA, B cell maturation antigen; CAIX, carbonic anhydrase IX; CEA, carcinoembryonic antigen; EGFR, epidermal growth factor receptor; EGFRt, truncated EGFR; EGFRvIII, variant III of the EGFR; FR-α, folate receptor-alpha; HSV-tk, herpes simplex virus thymidine kinase; iC9, inducible caspase 9; Ig, immunoglobulin; L1-CAM, L1 cell adhesion molecule; mAb, monoclonal antibody; PSCA, prostate stem cell antigen; PSMA, prostate-specific membrane antigen; VEGFR-1, vascular endothelial growth factor-1.

(PSCA)/CD3-ζ as a more clinically relevant specificity. They eventually identified an scFv with sufficiently diminished affinity for PSCA that could transmit a suboptimal activation signal only in the absence of costimulation. Indeed, in a human xenograft model, T cells coexpressing this pair of activating and costimulatory CARs could discriminate between human tumor cells expressing the two antigens in *cis* and those expressing only one. Lanitis et al. (89) also described a similar design; they provided complementary CD3-ζ and CD28 signaling in *trans* via antimesothelin scFv-CD3-ζ and anti-FR-α scFv-CD28 CARs. Although it suggests an intriguing solution to combinatorial T cell activation, clinical implementation of this approach will likely require flexible adjustments to the varying levels of the antigens among patients, tumors, and healthy tissues and of the respective CARs in transduced T cells.

Mutating the CAR recognition domain. IL-13 binds both the IL-13Rα1 and IL-13Rα2 chains of IL-13 receptors. IL-13Rα1/IL-4Rα receptors bind IL-13 and IL-4 and are ubiquitously expressed in human tissues, whereas IL-13Rα2 binds only IL-13. IL-13Rα2 is overexpressed on a variety of solid tumors but is not detected at significant levels on most vital, nontumor tissues tested. Surface expression of IL-13Rα2 on normal pulmonary artery smooth muscle cells is

PSCA: prostate stem cell antigen

one exception, and binding of IL-13 to these cells is indeed associated with the pathogenesis of pulmonary arterial hypertension. For selective CAR targeting of IL-13Rα2-expressing tumors, researchers designed a mutated IL-13 to discriminate between IL-13Rα2 and IL-13Rα1 (90, 91). However, evidence for binding of mutant IL-13-based CARs to both IL-13Rα1 and IL-13Rα2 (91, 92) should be taken into account. Three clinical trials are examining such mutant IL-13 CARs in CNS tumors (NCT00730613, NCT01082926, NCT02208362).

GVHD: graft-versus-host disease

Limiting in vivo persistence and function of CAR T cells. Preventing prolonged CAR-mediated autoreactivity can be achieved by transient expression of the transgene, which is implemented primarily through the electroporation of in vitro–transcribed mRNA. This procedure is safe and exceptionally efficient, achieves high and uniform expression, and allows the transfer of predefined gene mixtures. Indeed, mRNA has been applied successfully in experimental adoptive T cell transfer and is currently being evaluated clinically with an antimesothelin CAR against MPM and pancreatic cancer (NCT01355965, NCT01897415) (93), an anti-cMET CAR in TNBC (NCT01837602), and an anti-CD19 CAR in HL (NCT02277522). Another effort in this direction harnessed the electroporation of plasmid DNA encoding an anti–vascular endothelial growth factor receptor-1 (VEGFR-1) CAR (94). A first-generation CAR (CD3-ζ only) lacking a costimulatory element was chosen to blunt the ensuing activity and further reduce the risk of damage to normal endothelia expressing low levels of the antigen.

The use of off-the-shelf, ready-to-use, nonautologous T cells is a long-desired goal in the field of CAR T cell therapy. An interesting scenario is created upon the administration of HLA^+, TCR^+ allogeneic donor CAR T cells redirected against a tumor antigen expressed by the recipient. Using a mouse model, we recently showed that TCR-mediated graft-versus-host disease (GVHD) and potential off-tumor activity exhibited by the CAR T cells are terminated upon rejection of the transferred T cells by the recipient's immune system (95). To maximize the graft-versus-tumor effect and delay rejection, we used low-dose irradiation of recipients. Meanwhile, we achieved inhibition of both T cell rejection and GVHD but not the antitumor response by blocking the recipient lymphocyte egress from secondary lymphoid organs using the lymphocyte-sequestering agent FTY720. Rejection, albeit delayed, of donor CAR T cells thus also serves to control toxicity.

Alternative routes for T cell administration. Within a few hours of their intravenous administration—currently the standard clinical protocol in adoptive cell therapy—most T cells are cleared from the blood. They first redistribute to the lungs, where they can be detected as soon as 30 min post-transfer (49), and later to the liver and spleen, as seen in patients (38, 49) and in a mouse model using human T cells (96). This distribution pattern may explain the pulmonary and hepatic toxicities observed in the clinical trials employing CAR T cells directed at CAIX and ErbB2. To promote access to the target tissue and limit dissemination to off-tumor destinations, several laboratories have embarked on the intratumoral administration of CAR T cells, which has been shown to retain a large fraction of cells at the site of injection (96). Intrapancreatic injection of CEA-redirected T cells in an orthotopic xenograft model for pancreatic cancer resulted in T cell accumulation and persistence at the tumor site with no toxicity (78, 97). Researchers first performed intracranial injection of IL-13Rα2-specific T cells in a glioma xenograft model (91) and later employed it in two clinical trials (NCT01082926, NCT02208362) (92). Intracerebral injection of anti-EGFRvIII CAR T cells mediated safe therapeutic responses against EGFRvIII-expressing tumors in the brain of immunodeficient mice (98). Investigators have described intratumoral delivery of anti-ErbB CAR T cells in H&N cancer (99), and it is currently being evaluated clinically (NCT01818323). Intrapleural injection of antimesothelin CAR T cells in an orthotopic mouse model for MPM achieved enhanced localization and required 30-fold

fewer transferred cells to induce long-term complete remissions compared with intravenous injection (100). In our own study, we demonstrated the efficacy of intratumoral administration of anti-ErbB2 CAR T cells in the human ErbB2 mouse model for mammary tumors (57).

HSV-tk: herpes simplex virus thymidine kinase

iC9: inducible caspase 9

Reactive Measures

The different strategies for terminating deleterious off-tumor activity of CAR T cells are devised to achieve complete elimination of these cells once damage to healthy tissue necessitates such intervention. At present, 14 clinical trials have incorporated such tools for minimizing potential toxicities (**Table 2**).

Inducible suicide genes. Inclusion of suicide genes enables the controlled depletion of CAR-expressing cells. Researchers are currently testing two inducible suicide genes codelivered with CAR genes in different experimental settings. One encodes herpes simplex virus thymidine kinase (HSV-tk), which phosphorylates the guanosine analog ganciclovir, initiating a cascade of reactions that eventually leads to arrest of DNA synthesis and cell death. The ablation of HSV-tk-modified T cells was first demonstrated in the clinic in controlling GVHD that developed in patients after allogeneic BM transplantation (101) and is currently being tested clinically for safer targeting of IL-13Rα2 in CNS tumors (NCT00730613, NCT01082926), PSMA in prostate cancer (NCT01140373), L1-CAM in NB (NCT00006480), and CD19 in NHL (NCT00182650).

A second gene, inducible caspase 9 (iC9), encodes a fusion polypeptide comprising a truncated human caspase 9 and a mutated FK506-binding protein. The latter allows conditional dimerization via a small, nontoxic synthetic drug, initiating an apoptotic cascade that leads to cell death (102). The efficacy of the iC9 system was demonstrated first in the context of GVHD (103) and is currently being tested as a safety switch along with anti-GD2 CARs in NB (NCT01822652), sarcomas (NCT01953900), and solid tumors (NCT02107963), as well as an anti-CD19 CAR in NHL (NCT02247609). iC9 offers two important advantages over HSV-tk: Its activity does not depend on cell division, and it is less immunogenic, as its constituent proteins are of human origin.

Use of antibodies for selective CAR T cell depletion. Another approach for the selective elimination of the CAR-modified T cells exploits the coexpression on the T cell surface of an epitope recognized by clinically approved mAbs that can then be used for selective T cell depletion. Several such epitopes that investigators have tried include a truncated EGFR, recognized by the anti-EGFR mAb cetuximab, implemented along with CD19 CARs (104) (NCT01815749, NCT02051257, NCT02146924); CD20 and rituximab (105, 106); a CD19 derivative devoid of most of the cytoplasmic domain (so as to avoid potential signaling) to allow removal of T cells expressing anti-IL-13Rα2 CAR (NCT02208362); and a c-Myc tag examined in TCR-modified T cells (107). Another approach that should offer selectivity and does not require the expression of an additional antigenic epitope is the use of an scFv-specific anti-idiotypic mAb. A reported anti-idiotypic mAb to an anti-CD19 CAR can be used not only for T cell detection, as originally suggested (108), but also for their elimination.

CONCLUSIONS AND PERSPECTIVE

The CAR T cell approach targeting $CD19^+$ B cell malignancies has proved extremely efficient in the clinic, but treatment also eliminates normal B cells and causes B cell aplasia. In the long run, the B cell repertoire is regenerated in the treated patient from the hematopoietic stem cells. As emphasized in a recent editorial by Rosenberg (109) and illustrated throughout this review, the major

obstacle to using gene-modified T cells for the immunotherapy of additional cancers is the scarcity of antigens that can be targeted without injuring normal tissue. Despite the availability of advanced approaches for in vivo assessment of safety, their limitations should not be ignored and call for particularly careful design of clinical studies. Of the different approaches devised to minimize off-tumor toxicity, the use of iCARs and the exploitation of complementary signals for dual recognition are most suited for adding new antigens, or antigen combinations, to the list of safe CAR targets in the clinic. If these strategies are successfully employed, the discriminatory power of the resulting recognition machinery should allow the targeting of antigenic signatures of more tumors in a highly specific manner. Whether they utilize genes, antibodies, or adaptation of clinical procedures, all strategies presented in this review are designed to improve the prospects of preventing or curtailing adverse off-tumor reactivity of CAR T cells to allow safer treatment for many more cancer patients.

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