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REVIEW

Immune cell engagers in solid tumors: promises and challenges of the next generation immunotherapy

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In the landscape of cancer immunotherapy, immune cell engagers (ICEs) are rapidly emerging as a feasible and easy-to-deliver alternative to adoptive cell therapy for the antitumor redirection of immune effector cells. Even if in hematological malignancies this class of new therapeutics already hit the clinic, the development of ICEs in solid tumors still represents a challenge. Considering that ICEs are a rapidly expanding biotechnology in cancer therapy, we designed this review as a primer for clinicians, focusing on the major obstacles for the clinical implementation and the most translatable approaches proposed to overcome the limitations in solid tumors.

Key words: immunotherapy, BiTEs, immune cell engagers, solid tumors, T cell redirection

INTRODUCTION

Immunotherapy emerged as a revolutionary weapon in the treatment of hematological and solid malignancies in the last decade.[1] Emblematic is the case of immune checkpoint inhibitors (ICIs), which have shown impressive clinical responses in certain histologies.[2,3] Notably, the antitumor immune response unleashed by ICIs largely relies on tumor immunogenicity, whereas not all the tumors may present appropriately immunogenic characteristics able to trigger a cell-mediated antitumor response.[4,5] Additionally, certain tumor types employ different strategies to overcome the immune response, as in the case of the loss/reduction in expression of class I major histocompatibility complex (MHC) molecules or alterations in the antigen processing machinery that prevent the appropriate antigen presentation to antitumor T cells.[6] Immune cell engagers (ICEs) have been specifically developed with the aim of redirecting immune cells against surface tumor-associated antigens (TAAs) for MHC-independent cancer cell elimination and generation of immune responses against poorly immunogenic tumors. Beyond adoptive cell therapy that requires an expensive and complex process of cell manufacturing,[7] ICEs could be seen as a feasible promise for the next generation immunotherapy. In this review we provide a concise outlook on ICEs with a focus on solid tumors, illustrate the available clinical data and discuss the challenges still open and the emerging advances.

DESIGN AND MECHANISM OF ACTION OF ICES

ICEs are molecules able to redirect immune effector cells (regardless of their antigen specificity) against cancer cells with the aim of triggering an efficient tumor cell killing.[8] Most ICEs are trans-binding bispecific antibodies (bsAbs) usually consisting of two linked single-chain fragment variables (scFvs) that originate from different monoclonal antibodies: one scFv recognizes a surface TAA, whereas the other is specific for a certain membrane molecule expressed on effector immune cells.[9] The scFv with specificity for the effector immune cell must be also able to trigger an appropriate signal transduction cascade to activate the killing machinery. The compact structure resulting from the link of these two different scFvs allows the formation of the immune synapsis between tumor and immune cells and eventually leads to tumor cell elimination. In order to avoid the risk of an uncontrolled triggering and subsequent toxicity, the activation of effector cells takes place only when both bsAbs 'arms' are engaged with their respective target antigens.[10]

The so-called 'bystander killing' effect (i.e. the killing of nearby target-negative cells via the release of molecules that diffuse locally upon ICEs-mediated effector cell activation in the presence of target-positive cells) also contributes to the antitumor activity of ICEs. Recent insights suggest that the diffusion of released cytokines leads to the

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upregulation of cell surface molecules (e.g. ICAM-1 and FAS) on bystander cells. The expression of these molecules makes bystander cells susceptible to effector cell-mediated killing even in the absence of a regular cytolytic synapse.[11] Notably, this phenomenon is of particular interest in solid tumors that are characterized by a marked heterogeneity of TAA expression and by an immune suppressive tumor microenvironment (TME): a bystander killing effect can indeed mitigate antigen escape and contribute to eliminate the pro-tumoral cellular compartment of TME.

Based on the type of immune effector engaged, ICEs developed up-to-now can be divided into three main categories: T cell, natural killer (NK) cell and cytotoxic/phagocytic cell engagers (Figure 1).

T cell engagers

Bispecific T cell engagers (BiTes) are the most common class of ICEs and consist of a TAA-targeting scFv linked with an scFv usually activating a specific chain of the CD3 complex (mainly the CD3ε chain) that is associated with the T cell receptor (TCR) complex and participate in TCR-mediated signaling.[12] By this approach, T cells are physically redirected against tumor cells and at the same time activated. The formation of this 'artificial' immunological synapse is accompanied by the redistribution of signaling and secretory granule proteins in T cells, leading to the release of perforin and granzyme.[13] Such contact-dependent cytotoxicity is likely the main mechanism for BiTes-induced direct killing of tumor cells, as EDTA chelation of $Ca2^{+}$ (required for perforin multimerization and pore formation) leads to the complete inhibition of target cell apoptosis.[14] Activation of T cells also results in the secretion of cytokines and T-cell proliferation, which may be required to sustain a durable antitumor immune response.[15] Together with canonical cytotoxic T cells (CD8+ T cells), also CD4+ T cells, γδ T cells and NK T cells (NKT cells) can be activated by and contribute to the antitumor activity of BiTes specific for the CD3 complex.[16-18] A co-stimulation molecule (e.g. CD28 or 4-1BB) can also be exploited as a target to engage activated T cells, as showed using a trispecific antibody engaging CD3 and CD28 on T cells,[19] or 4-1BB engaging molecules.[20,21]

NK cell engagers

NK cells are cytotoxic innate lymphoid cells capable of recognizing viral infected or transformed cells by a set of germline-encoded receptors, and are characterized by the lack of TCR and CD3 molecules and by the expression of CD56 (also known as neural cell adhesion molecule) and CD16 (also known as FcγRIII).[22] NK cells activity is balanced by specific membrane receptors with activating (e.g. natural cytotoxicity receptors, like CD16) or inhibitory (e.g. inhibitory killer immunoglobulin-like receptors) functions.[23] CD16 is the most implemented NK cell target for the development of ICEs in the format of bsAbs. Data from preclinical studies showed that CD16-directed ICEs are able to activate NK cells and induce TAA-specific cytotoxicity with cytokine and chemokine production.[24] CD16-directed ICEs have shown antitumor activity in hematological malignancies and AFM13 (a CD30xCD16 bispecific compound) is currently in phase II clinical development for the treatment of Hodgkin's lymphoma.[25] In solid tumors, CD16-directed ICEs have preclinically been shown to induce effective responses in several solid tumor models.[26,27] Another approach to exploit NK cytotoxicity using ICEs involves the engagement of the activating NKG2D receptor. In preclinical models, NKG2D-directed bsAbs demonstrated activity both *in vitro* and *in vivo* against carcinoembryonic antigen (CEA)- and human epidermal growth factor receptor 2 (HER2)-positive tumors,[26] and a CD24xNKG2D bsAb demonstrated *in vivo* activity in a model of hepatocellular carcinoma in combination with sorafenib.[28]

Cytotoxic/phagocytic cell engagers

Cytotoxic/phagocytic immune cells (i.e. monocytes, macrophages, dendritic cells and cytokine-activated neutrophils) can be engaged via the non-ligand binding site of the high-affinity receptor for immunoglobulin G (FcγRI, also known as CD64) which is selectively expressed by these immune cells.[29] Chemically linked bispecific molecules engaging CD64 and targeting a TAA are able to trigger antibody-dependent cell-mediated cytotoxicity and cytotoxic lysis of tumor cells as shown in preclinical models of solid tumors targeting HER2 and epithelial cell adhesion molecule (EpCAM).[30,31]

CLINICAL EXPERIENCES WITH ICES IN SOLID TUMORS

ICEs demonstrated impressive clinical results in hematological malignancies, as demonstrated by the success of blinatumomab, a BiTE targeting CD19 and engaging CD3, which led to Food and Drug Administration and European Medicines Agency (EMA) approval for the treatment of adults and pediatric patients with certain relapsed or refractory acute lymphoblastic leukemia (ALL).[32-34] Unfortunately, translating these results in solid tumor patients is still challenging (Table 1).[29,35-61] Emblematic is the case of cytotoxic/phagocytic cell engagers. Despite the encouraging activity observed in tumor models, the clinical activity of HER2xCD64 and epidermal growth factor receptor (EGFR) xCD64 molecules in early phase clinical trials was scant, probably because of the high bsAb concentration and high effector-to-target cell ratio required for effective tumor cell elimination.[62] The first large-scale evidence about the clinical activity of a BiTE in solid tumors came with the approval of catumaxomab by the EMA in 2009 for the intraperitoneal treatment of malignant ascites in adult patients with EpCAM-positive carcinoma. EMA approval was based on the positive results of a large phase II/III trial in terms of time-to-next-paracentesis and signs and symptoms of ascites.[36,46] However, when attempts were made to shift from locoregional to systemic administrations, the results were not encouraging. A phase I study, which aimed to demonstrate the safety and tolerability of intravenous (i.v.) infusion of catumaxomab, revealed dose-dependent hepatotoxicity of different grades, with one patient experiencing fulminant fatal acute liver failure which led to the early termination of

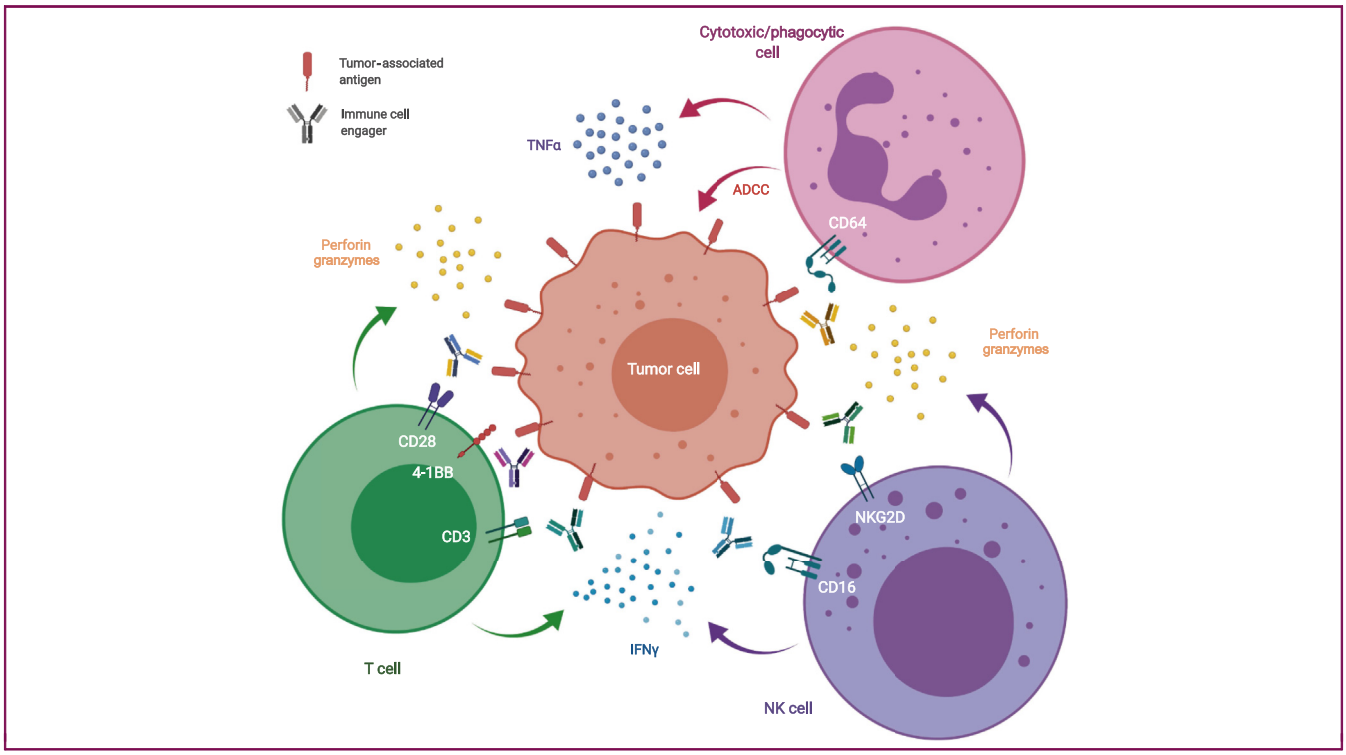

Figure 1. Mechanism of action of immune cells engagers.
Immune cell engagers are able to redirect immune effector cells and create an artificial immune synapse between tumor cells (targeting a tumor-associated antigen) and T cells (engaging CD3 or costimulatory molecules such as CD28 or 4-1BB), NK cells (engaging CD16 or NKG2D) and cytotoxic/phagocytic cells (engaging CD64).
IFN, interferon; NK, natural killer; TNF, tumor necrosis factor alpha.

the study. The severe adverse events were attributed to the off-target binding of a catumaxomab active Fc region to Fcγ receptors expressed by Kupffer cells in the liver, inducing local cytokine release and T cell-mediated hepatotoxicity.[63] Solitomab (MT110, AMG110), another EpCAMxCD3 BiTE, has been investigated in 65 patients with relapsed/refractory advanced-stage solid cancers in a phase I dose-escalation study, with administration by continuous i.v. infusion. The treatment was associated with dose-limiting toxicities in 15 patients, including elevation of serum liver enzymes in 8 patients and severe diarrhea in 6 patients (with one fatal outcome) which precluded dose escalation to potentially therapeutic levels.[49] Regardless of the Fc/Fcγ receptors interaction, the toxicity observed with the systemic administration of EpCAM-targeted BiTEs can be explained by EpCAM expression in both non-malignant and malignant epithelial cells,[64] thus lowering the specificity for cancer cells and the therapeutic window. More encouraging results emerged in a phase I clinical trial evaluating the safety of ertumaxomab, an HER2xCD3 BiTE, in patients with metastatic, HER2-positive breast cancer. Five out of 15 assessable patients showed a clinical benefit, including 1 complete response, 2 partial responses and 2 disease stabilizations.[51] Fourteen patients with HER2-positive advanced solid tumors were enrolled in another phase I trial with ertumaxomab. Clinical benefit was seen in 3 out of 11 assessable patients, including 1 partial response and 2 disease stabilizations.[50] Pasotuxizumab is a prostate-specific membrane antigen (PSMA)xCD3 BiTE investigated in a phase I trial. An interim analysis showed the ability of pasotuxizumab to reduce serum prostate-specific antigen levels and the numbers of circulating tumor cells.[61] At the higher dose levels investigated, one patient achieved a long-lasting near complete response as assessed by PSMA-position emission tomography.

GOING BACK FROM BED TO BENCHSIDE: IMPROVING ICES

In recent years, major efforts have been made to improve the toxicity profile and clinical activity of ICEs, and several ongoing clinical trials are actually recruiting patients (Table 2), portraying a promising future for ICE-based cancer immunotherapy.

Fine tuning of ICEs design

As mentioned before, the systemic administration of catumaxomab was severely limited by toxicity.[63] Of note, catumaxomab is a trifunctional ICE: beyond the binding with EpCAM on malignant cells and CD3 on T cells, this bsAb is able to recruit accessory cells via binding of its Fc region to Fcγ receptors, further promoting antibody-dependent cell-mediated cytotoxicity in target cells, but also causing hyperactivation of FcγR-expressing cells, as in the case of Kupffer cells in the liver. Since the high toxicity rate in case of systemic administration, catumaxomab has not been widely adopted and most of the subsequent BiTEs were designed with engineered Fc domains to reduce Fcγ receptor binding or as bsAbs fragments lacking the Fc region.[65] With few exceptions, nowadays most BiTEs incorporate monovalent CD3-binding scFvs (i.e. with affinity

Table 1. Safety and activity data from published studies on ICEs in solid tumors

Drug name	ICE design	Phase	Condition	Number of patients treated	Administration	Toxicity	Objective responses	Reference
Catumaxomab	EpCAMxCD3	I	Solid tumors	16	i.v. (8 h)	Liver toxicity, fatal acute hepatic failure	Not reported	[35]
Catumaxomab	EpCAMxCD3	I	Lung cancer	21	i.v. (6 h)	Liver toxicity, lymphopenia	Not reported	[36]
Catumaxomab	EpCAMxCD3	I/II	Epithelial tumors	24	i.p. (3 h)	Liver toxicity, lymphopenia, anemia, pleural empyema	1 CR 4 PR	[37]
Catumaxomab	EpCAMxCD3	I/II	Colon, gastric, pancreatic cancer	24	i.p. (6 h)	SIRS, pulmonary edema, liver toxicity, lymphopenia	1 CR 3 PR	[38]
Catumaxomab	EpCAMxCD3	I/II	Ovarian cancer	23	i.p. (6 h)	Liver toxicity, lymphopenia, hypercalcemia	Not reported	[39]
Catumaxomab	EpCAMxCD3	II	Solid tumors	13	i.p. (6 h)	Transient increases in liver enzymes	Not reported	[40]
Catumaxomab	EpCAMxCD3	IIa	Ovarian cancer	45	i.p. (6 h)	Anemia, erythema induratum, liver toxicity	1 PR	[41]
Catumaxomab	EpCAMxCD3	II	Gastric cancer	54	Intraoperative i.p. bolus + i.p. (3 h)	Not applicable	Not reported	[42]
Catumaxomab	EpCAMxCD3	II	Gastric cancer	64	Intraoperative i.p. bolus + i.p. (3 h)	Hypotension, liver toxicity, SIRS, abdominal pain, hypertension	3 CR 13 PR	[43]
Catumaxomab	EpCAMxCD3	II	Ovarian cancer	32	i.p. (3 h)	Liver toxicity, dehydration, abdominal pain, chills, fatigue, nausea, vomiting	Not reported	[44]
Catumaxomab	EpCAMxCD3	II	Ovarian cancer	41	Intraoperative i.p. bolus + i.p. (3 h)	Liver toxicity, pleural effusion, pulmonary embolism	Not reported	[45]
Catumaxomab	EpCAMxCD3	II/III	Epithelial cancers	258	i.p. (6 h)	Liver toxicity, ileus, gastric hemorrhage, lymphopenia, anemia	Not reported	[46]
Catumaxomab	EpCAMxCD3	IIIb	Epithelial cancers	219	i.p. (3 h)	Liver toxicity, fatigue, nausea, pyrexia, abdominal pain	Not reported	[47]
Catumaxomab	EpCAMxCD3 (rechallenge)	I/II	Epithelial cancers	8	i.p. (3 h)	Liver toxicity, fatigue, nausea, pyrexia, abdominal pain	Not reported	[48]
Solitomab (MT110, AMG110)	EpCAMxCD3	I	Solid tumors	65	i.p. (3 h)	Liver toxicity, diarrhea, lipase increase, peripheral edema	1 PR	[49]
Ertumaxomab	HER2xCD3	I	Solid tumors	14	i.v. (3 h)	Infusion reaction, CRS, cephalgia, tumor pain, hypertension	1 PR	[50]
Ertumaxomab	HER2xCD3	I	Breast cancer	17	i.v. (6 h)	Hypotension and ARDS, SIRS, liver toxicity	1 CR 2 PR	[51]
MDX-H210	HER2xCD64	I	Solid tumors	13	i.v. (2 h)	Fever, chills, rigor	1 PR	[52]
MDX-H210	HER2xCD64	I	Solid tumors	24	i.v. (2 h)	Neutropenia, thrombocytopenia, monocytopenia	Not reported	[53]
MDX-H210	HER2xCD64	I	Prostate cancer	7	i.v. (2 h)	Flu-like symptoms, hematological, liver toxicity	Not reported	[29]
MDX-H210	HER2xCD64	I	Breast cancer	30	i.v. (2 h)	Tumor pain, hypotension, fever, chills, liver toxicity	Not reported	[54]
MDX-H210	HER2xCD64	I	Breast cancer	23	i.v. (8 h)	Diarrhea, infusion reaction	Not reported	[55]
MDX-H210	HER2xCD64	Ia/Ib	Breast and ovarian cancer	15	i.v. (2 h)	Hypotension, nausea	2 PR	[56]
MDX-H210	HER2xCD64	II	Prostate cancer	25	i.v.	Flu-like symptoms, infusion reaction	Not reported	[57]
2B1	HER2xCD16	I	Solid tumors	15	i.v. (1 h)	Hypotension, leucopenia, thrombocytopenia	1 PR	[58]
AMG211, MEDI-565	CEAxCD3	I	Gastrointestinal cancer	39	i.v. (3 h)	Hypoxia, diarrhea, CRS, liver toxicity	Not reported	[59]
RO6958688	CEAxCD3	Ia	Solid tumors	36	i.v.	Infusion reaction, pyrexia, diarrhea, hypoxia, colitis	2 PR	[60]
RO6958688	CEAxCD3	Ib	Solid tumors	10	i.v.	Infusion reaction, pyrexia, diarrhea, hypoxia, colitis	2 PR	[60]
Pasotuxizumab, BAY2010112	PSMAxCD3	I	Prostate cancer	16	i.v.	Lymphopenia, infections, CRS, fatigue	1 PR	[61]

ARDS, acute respiratory distress syndrome; CEA, carcinoembryonic antigen; CR, complete response; CRS, cytokine release syndrome; EpCAM, epithelial cell adhesion molecule; HER2, human epidermal growth factor receptor 2; ICE, immune cell engager; i.p., intraperitoneal; i.v., intravenous; PR, partial response; PSMA, prostate-specific membrane antigen; SIRS, systemic inflammatory response syndrome.

Table 2. Ongoing clinical studies with ICEs in solid tumors registered on ClinicalTrials.gov

Identifier	Drug name	ICE design	Condition	Phase	Note	Status
NCT03927573	GEM3PSCA	PSMAxCD3	Solid tumors	I		Recruiting
NCT04104607	CC-1	PSMAxCD3	Prostate cancer	I		Recruiting
NCT04496674	CC-1	PSMAxCD3	Squamous NSCLC	I/II		Recruiting
NCT03792841	AMG160	PSMAxCD3	Prostate cancer	I	± Pembrolizumab	Recruiting
NCT03983395	ISB1302	HER2xCD3	Breast cancer	I/II		Recruiting
NCT03406858	-	HER2xCD3	Prostate cancer	II	+ Pembrolizumab	Recruiting
NCT03661424	-	HER2xCD3	Breast cancer	I		Recruiting
NCT03272334	-	HER2xCD3	Breast cancer	I/II	+ Pembrolizumab	Recruiting
NCT03330561	PRS-343	HER2x4-1BB	Solid tumors	I		Recruiting
NCT03269526	-	EGFRxCD3	Pancreatic cancer	I/II		Recruiting
NCT03344250	-	EGFRxCD3	Glioblastoma	I	+ Temozolomide	Recruiting
NCT03296696	AMG596	EGFRvIIIxCD3	Glioblastoma	I		Active, non-recruiting
NCT04009460	ES101	PD-L1x4-1BB	Solid tumors	I		Recruiting
NCT03922204	MCLA-145	PD-L1x4-1BB	Solid tumors	I		Recruiting
NCT03809624	INBRX-105	PD-L1x4-1BB	Solid tumors	I		Recruiting
NCT03484962	-	MUC1xCD3	Hepatocellular carcinoma	II	+ Activated cytokines-induced killer cells	Recruiting
NCT03564340	REGN4018	MUC16xCD3	Ovarian cancer	I/II	± Cemiplimab	Recruiting
NCT04117958	AMG199	MUC17xCD3	Gastric cancer	I		Recruiting
NCT04260191	AMG910	CLDN18.2xCD3	Gastric cancer	I		Recruiting
NCT03319940	AMG757	DLL1xCD3	SCLC	I	± Pembrolizumab	Recruiting
NCT04221542	AMG 509	STEAP1xCD3	Prostate cancer	I		Recruiting
NCT03531632	MGD007	gpA33xCD3	Colorectal cancer	I/II	+ MGA012	Active non recruiting
NCT03860207	Hu3F8-BsAb	GD2xCD3	Solid tumors	I/II		Recruiting
NCT04424641	GEN1044	5T4xCD3	Solid tumors	I/II		Not yet recruiting
NCT03411915	XmAb18087	SSTR2xCD3	NET and GIST	I		Recruiting
NCT04128423	AMV564	CD33xCD3	Solid tumors	I		Recruiting

EGFR, epidermal growth factor receptor; GIST, gastrointestinal stromal tumor; HER2, human epidermal growth factor receptor 2; ICE, immune cell engager; NET, neuroendocrine tumor; PD-L1, programmed death-ligand 1; PSMA, prostate-specific membrane antigen; SCLC, small cell lung cancer.

for one epitope of CD3), since bivalent/multivalent anti-CD3 scFvs (i.e. with affinity for more than one epitope of CD3) were linked to an excess of activation-induced cell death in effector cells (limiting the efficacy) and TAA-independent immune effectors activation (increasing the toxicity).[66] For example, side-effects in cynomolgus monkeys were dependent on the affinity of the anti-CD3 scFv part of a full-length CLL1xCD3 bsAb, with the high-affinity variant being poorly tolerated because of extensive cytokine release.[67] Moreover, high affinity for CD3 was also linked to a reduced systemic exposure and shifted biodistribution from tumors to CD3-rich tissues (e.g. spleen and lymph nodes), as demonstrated *in vivo* in different tumor models.[68,69]

Conversely, a high valency of the anti-TAA scFv is generally desirable, since it leads to increased binding avidity and antitumor activity, as shown in a functional comparison using anti-GD2 BiTEs.[70]

A limitation that emerged from early clinical trials with certain ICEs was the development of anti-ICE antibodies that seriously affected the clinical development of the drugs.[71] This problem became evident in a phase I study that tested MEDI-565, a CEAxCD3 BiTE.[59] The study was discontinued after the observation of anti-MEDI-565 antibodies production in all patients treated at high doses. Current strategies are focused on improving the design of ICEs to limit their immunogenicity, for example using humanized scFvs.[72]

The very small size of certain BiTEs (due to the lack of the Fc domain) is responsible for a short half-life and the requirement for continuous infusion.[12] Several strategies have been explored to overcome this issue, such as conjugation with an engineered IgG Fc domain, human serum albumin or polyethylene glycol.[73] Moreover, Leconet et al.[74] developed an injectable *in situ* biodegradable polymer-based protein delivery system that was successfully designed to prolong the *in vivo* elimination half-life and the antitumor activity of a BiTE targeting PSMA in prostate cancer.[74]

Choosing the right TAA

High tumor specificity is a fundamental prerequisite for a TAA to be an appropriate ICE target. The toxicity observed with the systemic administration of catumaxomab was, at least in part, driven by the expression of the targeted TAA (i.e. EpCAM) in normal tissues (e.g. colon, liver). In addition, the density, size and mobility of TAAs on the membrane of tumor cells are crucial determinants for the choice of a good TAA.[75-77] All these properties have been taken into consideration to explore 'highly-fit' TAAs as novel targets. For example, EGFRvIII is a mutated variant of EGFR (deletion of exons 2-7) expressed solely in malignant cells of glioblastoma. An EGFRvIIIxCD3 BiTE demonstrated a potent *in vivo* antitumor effect against glioblastoma models,[77] and a phase I clinical trial is being conducted in patients with EGFRvIII-positive glioblastoma (NCT03296696).[78] Glypican-3 (GPC3) is a heparan sulfate chain proteoglycan expressed in fetal tissues (e.g. liver, lung, kidney and placenta) but not detected in normal postnatal tissues due to methylation-induced epigenetic silencing.[79,80] GPC3 is expressed in a wide range of solid

tumors (including hepatocellular carcinoma, melanoma, ovarian clear cell carcinoma and lung squamous cell carcinoma).[80] Ishiguro et al.[81] reported that ERY974, a GPC3xCD3 BiTE, was highly effective *in vivo* in immunogenic and non-immunogenic tumor models expressing GPC3, and the results of a phase I clinical trial of ERY974 in solid tumors are eagerly awaited (NCT02748837).[82] The disialoganglioside GD2 is another interesting oncofetal surface antigen expressed by several solid tumors.[83] A preclinical study exploring hu3F8-BsAb (a GD2xCD3 BiTE in which the IgG backbone was aglycosylated to prevent Fcγ receptor-mediated toxicity) showed high tumor killing potency, absence of neurotoxicity and ability to drive circulating T cells into solid tumors.[84] These encouraging preclinical results warranted the initiation of a clinical trial in patients with relapsed/refractory neuroblastoma, osteosarcoma and other solid tumors (NCT03860207). A promising non-conventional TAA is TRAIL-R2 (also known as DR5), a member of the tumor necrosis factor α superfamily. Indeed, a TRAIL-R2xCD3 BiTE demonstrated preclinical antitumor activity both *in vitro* in melanoma and breast cancer and *ex vivo* in a model of ascitic fluid freshly isolated from ovarian cancer patients.[85,86] Actually, several other promising targets are being studied, including SSTR2 in gastrointestinal stromal tumors and neuroendocrine tumors (NCT03411915), MUC-1 in hepatocellular carcinoma (NCT03484962), MUC-17 in gastric cancer (NCT04117958) and STEAP-1 in prostate cancers (NCT04221542).

Overcoming resistance to ICEs

Studies into potential mechanisms of resistance to BiTes indicated that the reduction in expression of target antigens on cancer cells is a cause of tumor immune escape. CD19-negative relapses have been observed in ALL patients treated with blinatumomab, preventing further activity of the BiTE.[87] To obviate this hurdle, multispecific antibodies were developed to concurrently recognize multiple antigens on the surface of cancer cells, as in the case of the tetraspecific molecules FL518 and CRTB6 targeting EGFR, HER2, HER3 and vascular endothelial growth factor, that demonstrated the ability to avoid tumor antigen escape.[88]

The upregulation of inhibitory immune checkpoints by tumor and TME cells represents another mechanism of resistance to ICEs. Following BiTE-mediated effector activation and interferon-γ release, programmed cell death protein 1 (PD-1) and programmed death-ligand 1 (PD-L1) are upregulated in T cells and cancer/TME cells, respectively, whereas combining BiTE treatment with the inhibition of the PD-1/PD-L1 axis was shown to enhance the antitumor efficacy of BiTEs in preclinical models.[89,90] Consistently, preliminary clinical data showed an enhanced activity and a manageable safety profile for the CEAxCD3 BiTE cibisatamab when combined with the anti-PD-L1 antibody atezolizumab in patients with metastatic colorectal cancer,[60] similarly to what was observed in patients with ALL treated with blinatumomab in combination with the anti-PD-1 antibody nivolumab.[91] A different approach to deal with the overexpression of inhibitory immune checkpoints in the TME is the development of immune-checkpoint targeting BiTEs. The proof-of-concept for this type of T cell engager has been developed in a preclinical model of melanoma using a PD-L1xCD3 BiTE able to redirect T and NKT cells towards PD-L1-positive cells with a promising antitumor activity, especially when combined with the inhibition of other inhibitory immune checkpoints.[92] The direct attack of TME immunosuppressive cells, such as myeloid-derived suppressor cells, is under study using a CD33xCD3 BiTE (AMV564, NCT04128423). Inhibitory signals can be also converted into activating signals, for example using a simultaneous multiple interaction T cell engaging (SMITE) strategy. The SMITE approach consists of the combination of several BiTEs at a time, with each BiTe binding cancer cells and either CD3 or CD28 to provide T cell costimulation. A SMITE system encompassing a PD-L1xCD28 BiTE can convert inhibitory PD-L1 signals into positive costimulatory signals through engagement of CD28 on T cells that are activated via the second TAAxCD3 BiTE.[93]

FUTURE PERSPECTIVES AND CONCLUSIONS

Given the complex nature of solid tumors, exploiting the synergy between ICEs and other innovative therapeutics, such as oncolytic viruses (OVs) and chimeric antigen receptor (CAR) T cells, may be an ideal strategy to improve the efficacy of cancer immunotherapy. OVs are viruses able to selectively enter, replicate in and destroy cancer cells inducing systemic antitumor immune responses.[94] OVs can be genetically engineered to produce and deliver a functional BiTE, as in the case of ICOVIR-15K-BiTE, an oncolytic adenovirus engineered to express an EGFRxCD3 BiTE. Preclinical evidence showed that tumor-infiltrating T cells could be more effectively activated by ICOVIR-15K-BiTE compared with the OV ICOVIR-15K alone.[95] Other preclinical studies reported on the antitumor activity of the oncolytic group B adenovirus EnAdenotucirev engineered to express an EpCAMxCD3 BiTE,[96] and the ability of an oncolytic adenovirus engineered to express a FAPxCD3 BiTe to cause virolysis of cancer cells together with a depletion of the immunosuppressive cancer-associated fibroblasts.[97] With the aim of creating a synergizing system between ICEs and adoptive cell therapy, Choi et al.[98] designed a single-gene-modified T cell product for the treatment of glioblastoma, namely, CART.BiTE, consisting of an anti-EGFRvIII CAR T cell product modified to deliver an EGFRxCD3 BiTE. This novel design allowed dealing with heterogeneous EGFRvIII tumor expression resulting in promising *in vivo* antitumor activity and overcoming the drawbacks of their parental biotechnologies, such as the necessity of continuous injection for BiTEs or antigen escape for CAR T cells.

In conclusion, ICEs have the potential to enable a large-scale level strategy of immune effector cells redirection in patients with solid tumors, sparing the major infrastructural and pharmacoeconomic obstacles that characterize adoptive cell therapy. However, further preclinical and clinical research efforts are needed to obviate technical issues,

overcome resistance and eventually let ICEs hit the clinic in solid tumors.

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