Title: CAR-T cell therapy for triple-negative breast cancer: preclinical and clinical progress

Abstract (**187**/200 words)

Introduction. Triple-negative breast cancer (TNBC) accounts for most breast cancer-related deaths due to its aggressiveness and lack of effective therapies. Chimeric antigen receptor (CAR)-T cells emerged as a promising immunotherapeutic strategy both in TNBC preclinical models and early-phase clinical trials. These drugs combine the antigen specificity of an antibody with the effector function of T cells.

Areas covered. Here, we present the challenges that hamper the safety and efficacy of CAR-T cells in solid tumors, along with the most studied targets in TNBC.

Expert Opinion. A relevant challenge in the development of CAR-T cells for TNBC is the selection of the optimal target to minimize on-target/off-tumor toxicity, as well as to reduce tumor escape via antigen loss and intrinsic heterogeneity. To date, TROP2, GD2, ROR1, MUC1 and EpCAM represent promising targets. Persistence and trafficking to tumor cells may be enhanced by the implementation of CARs with a chemokine receptor and/or constitutively activated interleukin receptors. Fourth-generation CARs (TRUCKs) may redirect T-cells for universal cytokine-mediated killing. Finally, combinatorial approaches and the application of CARs to other immune cells might revert the suppressive immune environment that characterizes solid neoplasms.

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Highlights

1. Despite leading to relevant changes in the treatment of refractory hematologic malignancies, many challenges still hamper progress on the application of chimeric antigen receptor (CAR)-T cells to solid tumors.
2. Approaches to increase trafficking to the tumor and persistence of CAR-T cells are currently under investigation in triple-negative breast cancer (TNBC).
3. Numerous antigens have been identified as potential targets for CAR-T cell therapy in TNBC, both in preclinical models and early-phase clinical trials.
4. Strategies to improve CAR-T cell specificity for breast cancer cells are under evaluation to reduce off-tumor toxicity, thus increasing safety.
5. A better understanding of the immune environment of TNBC, as well as technological breakthroughs in CAR-T cell manufacturing, will be key to further clinical development.

Review

1. Introduction: current treatment landscape of TNBC

Triple-negative breast cancer (TNBC) is an aggressive type of breast cancer (BC), defined by lack of expression of estrogen receptor (ER), progesterone receptor (PgR), and human epidermal growth factor receptor 2 (HER2) [1]. Unlike other BC subtypes, for which endocrine and targeted treatments are available in different settings [2,3], systemic therapy for TNBC has historically been limited to chemotherapy [4,5]. Consistently, although TNBC accounts for only ~11% of new BC diagnoses, it is responsible for most BC-related deaths [6].

In recent years, improvements in survival outcomes have been observed for patients with TNBC thanks to advances in the diagnosis and management of these tumors [7,8]. For example, post-neoadjuvant capecitabine was demonstrated to improve both progression-free survival (PFS) and overall survival (OS) in patients with pathologic residual cancer burden after neoadjuvant chemotherapy [9]. However, more than ~35% of TNBC patients still relapse and survive less than 2 years in case of systemic metastases [10]. Multiple agents have been investigated to improve prognosis at early stages. Among these, the poly ADP ribose polymerase inhibitor (PARPi) olaparib has recently been approved as an adjuvant treatment for patients with high-risk TNBC harboring germline breast cancer type 1-2 susceptibility genes (BRCA1 and BRCA2) pathogenic variants [11]. The advanced setting, historically characterized by subsequent lines of palliative mono-chemotherapy, has witnessed the introduction of two PARPi and novel antibody-drug conjugates (ADCs) [12-14]. In this context, a recent trial showed that sacituzumab govitecan led to a consistent benefit over chemotherapy across all prespecified subgroups (i.e. patients 65 years of age or older, those with more than three previous therapies, those with previous use of programmed cell death protein 1 (PD-1) or programmed cell death ligand 1 (PD-L1) inhibitors, TNBC at initial diagnosis and other subtypes of BC at initial diagnosis, and those with liver metastases) [15]. Despite these achievements, the most significant innovation for the treatment of TNBC is represented by

immunotherapy with immune-checkpoint inhibitors (ICIs) [16,17]. This immuno-oncology (IO) approach is bringing the management of TNBC towards a biomarker-based level, both in the curative and the palliative settings [5].

The PD-1/PD-L1 axis can attenuate the host anti-cancer immune response to tumor cells (immune surveillance) [4]. Vice versa, by blocking either PD-L1 or PD-1, the co-inhibitory signal is interrupted, thus restoring an anti-cancer immune response. Therefore, the addition of ICIs, either pembrolizumab (anti-PD-1) or atezolizumab (anti-PD-L1), to first-line standard chemotherapy, now represents a standard of care in many countries for PD-L1-positive metastatic TNBC [18,19]. Noteworthy, these tumors account for 30%-40% of all TNBC [20]. In the curative setting, the addition of pembrolizumab to neoadjuvant chemotherapy produced a significant event-free survival benefit (hazard ratio (HR), 0.63; confidence interval (CI), 0.48-0.82; $p < 0.001$), compared with chemotherapy alone, at the median follow-up of 39 months in the phase III KEYNOTE-522 clinical trial [21]. As a result, in July 2021 the Food and Drug Administration (FDA) approved pembrolizumab in combination with chemotherapy for high-risk, early-stage TNBC as neoadjuvant treatment, and then continued as a single agent as adjuvant treatment after surgery. For an optimal identification of responders, biomarkers are of great importance. In this regard, in the KENOTE-522, the PD-L1 expression (clone 22C3) did not appear to differentiate responders. Thus, additional immune-related biomarkers are needed, including different PD-L1 thresholds and assays and other immune-related biomarkers, including tumor-infiltrating lymphocytes (TILs), and tumor mutational burden (TMB) [22]. Despite the previously mentioned significant results, some trials also reported negative finidngs [5]. The GeparNuevo trial (NCT02685059), which investigated the efficacy of durvalumab in addition to an anthracycline taxane-based neoadjuvant therapy in early TNBC, did not demonstrate an increased pathologic complete response (pCR) rate in the overall population [23]. Similarly, the addition of atezolizumab to neoadjuvant carboplatin/nab-paclitaxel followed by surgery and then adjuvant anthracycline/cyclophosphamide was evaluated in the NeoTRIPaPDL1 trial (NCT02620280), which failed to demonstrate differences in pCR rates among patients who did

or did not receive atezolizumab [24]. The KEYNOTE-119 demonstrated that pretreated metastatic TNBC did not present any improvement in PFS or OS with single-agent pembrolizumab versus single-agent chemotherapy [25].

Still, there is a relative paucity of therapeutic targets for TNBC that reached the clinical practice. In addition, the somehow unsatisfactory response rates obtained with ICIs plus chemotherapy are pushing researchers to focus on identifying novel immunotherapeutic approaches to further unleash the anti-cancer immune response. Such strategies include targeting immune-related targets via monoclonal antibodies, cytokines, oncolytic viruses, cancer vaccines, and adoptive cell therapy, such as chimeric antigen receptor (CAR)-T cells [10,26].

In this review, we describe the opportunities provided by CAR-T cell therapies in solid tumors, with a focus on emergent targets, ongoing clinical trials, and prospective clinical implications in TNBC. Special attention will be put on the major challenges related to the use of these new drugs with possible strategies to overcome these obstacles.

2. Current immune landscape in TNBC

Breast neoplasms are overall immunologically "colder" than other tumor types, mainly because of their lower TMB and their immune-suppressive tumor microenvironment (TME) [10]. However, BC is extremely heterogeneous. Hence, the HER2-positive and TNBC groups show higher TMB and TILs compared to hormone receptor (HR)-positive BC [27]. TILs, mononuclear immune cells discovered within tumor tissue in most types of solid tumors, consist of cytotoxic CD8+ T cells, helper CD4+ T cells, and Natural Killer (NK) cells [28]. The anti-tumor immune response of TILs can be activated by the release of cancer-related neoantigens in the microenvironment [29]. The presence of TILs in the tumor bed of tumor has been found to have a strong prognostic role in TNBC [30]. TNBC with high (>10%) TILs show a 15-25% decrease in risk of relapse and death [31,32]. Specifically, excellent survival rates have been observed in early TNBC with high TIL infiltration

particularly in the node-negative subpopulation compared to those with low TILs [33] . It has been suggested that TILs infiltration is less present in the advanced stages of BC compared to the early stage. Also, the metastatic TME appears to be colder compared to the early setting. It has been proposed that metastatic BC may evade immune surveillance by shifting the TME towards an inactive phenotype with depleted immune functions related to the downregulation of immune-activating molecules and the upregulation of immunosuppressive properties [33]. TNBCtype, a landmark classification at the gene expression level, identified six molecular TNBC subtypes [34]. Among these, immunomodulatory and mesenchymal stem-like types are enriched for TILs and stromal cells, respectively [35]. In line with these observations, a large cohort study demonstrated that each TNBCtype-4 category is associated with a specific TME profile [36]. Of note, the immune-rich TME is associated with a lower degree of clonal heterogeneity, fewer somatic copy number alterations, and a lower somatic mutation and neoantigen burden [37]. Individual genomic alterations can also affect the immune landscape. For example, p53 loss, the most frequent alteration in TNBC, can mediate Wingless-related integration site (WNT)-dependent inflammatory cascades, possibly favoring metastases and further influencing the interactions between immune cells and TNBC [38]. Another relevant alteration is DNA damage response (DDR) deficiency [20]. In this regard, germline mutations in BRCA1/2 can modulate the immune TME, with increased macrophage-predominant tumor infiltrates [39,40]. However, although current evidence suggests that the immune landscape of TNBC may affect both prognosis and cancer treatment outcomes, only a few patients with TNBC derive a long-term clinical benefit [10,41,42].

3. Manufacturing, structure, and function of CAR-T cells

CAR-T cell therapies encompass several classes of drugs characterized by engineered T cells targeting cancer-specific proteins. In the recent past, these drugs led to relevant changes in the clinical management of refractory hematologic malignancies [43]. To combine the T cell effector function

153 with antibody specificity, T cells are collected from the patient and activation through the introduction
154 of CARs either by viral vectors (i.e., lentivirus, retrovirus, or adenovirus) or non-viral vectors (i.e.,
155 synthetic DNA, mRNA transposons, CRISPR-Cas9, or plasmids) [44]. Then, the CAR-modified
156 patient T cells are expanded *in vitro* and finally reinfused into the patient after lymphodepleting
157 chemotherapy [44]. Most of the clinical studies take advantage of the viral transfer method, consisting
158 of the CAR-encoding gene transferring by the virus into the T cell, and subsequently integrated into
159 the genomic DNA. The CAR gene will be carried by the offspring of these transduced cells,
160 expressing the receptor on their surface [26,45]. For the CAR introduction phase, virus-specific T-
161 cell populations are employed, such as those specific for varicella-zoster virus, Epstein-Barr virus,
162 adenovirus, cytomegalovirus, or multivirus-specific T cells. Thus, these T cells can proliferate and
163 therefore increase their persistence and number through their endogenous virus-specific TCR [45].
164 Other strategies to enhance T-cell proliferation could be considered as virus vaccination (e.g.
165 varicella-zoster virus vaccination, or oncolytic adenovirus injected intratumorally). Memory T cells
166 could be alternatively used for increasing the persistence of the CAR-T cells [26,45].
167 Different generations of CAR-T cells have been developed over the years (**Figure 1**) [26]. Among
168 which, the second generation has been used more frequently in registered trials [45]. A prototypical
169 CAR consists of an extracellular tumor antigen-specific antibody-derived recognition motif, such as
170 single-chain antibody fragments (scFv), containing the variable region of the light chain (VL) and the
171 heavy chain (VH). Then, a flexible spacer bridges the extracellular segment with a transmembrane
172 domain, that is in turn linked to an intracellular CD3ζ chain of the T-cell receptor (TCR), which serves
173 as an activation domain (first generation). Second-generation CARs contain an additional co-
174 stimulatory domain (e.g., CD28) while the third generation contains two co-stimulatory domains
175 (e.g., CD28, 4-1BB, OX40, ICOS, DAP10, and CD27). More recently, CAR-T cells were engineered
176 to release a transgenic cytokine in the targeted tumor tissue to induce a proinflammatory milieu. Such
177 "T cells redirected for antigen-unrestricted cytokine-initiated killing" (TRUCKs) are also referred to
178 as 4th generation CAR-T cells and can provide a multifunctional treatment to the targeted tissue

which was so far not achieved by conventional CAR-T cells [46]. The upcoming 5th generation CARs rely on the exploitation of gene-editing to modify the expression of surface proteins like the TCR [47].

4. Challenges in the targeting of solid tumors with CAR-T cells

Limited intratumoral trafficking and multiple immunosuppressive signals within the TME of solid tumors limit the CAR-T cell efficacy [44]. Indeed, the neoplastic cells of certain hematologic malignancies can be identified through specific cell surface molecules, such as the clusters of differentiation (e.g., CD19). Similar ultra-specific tumor target antigens need to be identified in solid neoplasms in order not to have serious off-target toxic effects [48]. This observation is not trivial, as the optimal target should be highly immunogenic, highly expressed, and stable on tumor cells but absent on normal tissues [49]. Some interesting target candidates involve the TME, such as tumor vasculature or cancer-associated fibroblasts (CAF) [44]. Other challenges are related to insufficient immune trafficking, infiltration, CAR-T cell persistence in the tumor, and the suppressive TME [20]. Hence, tumor-associated macrophages (TAM), CAFs, myeloid-derived suppressor cells (MDSC) and regulatory T cells (Treg) are often recruited in solid cancers. As well, the production of immunosuppressive cytokines and soluble factors are highlighted [50]. The expression of immune checkpoint molecules on T lymphocytes further contributes to a suppressive TME [50]. All these factors can be used as biomarkers for predicting cytokine-release syndrome (CRS), and immune effector cell-associated neurotoxicity syndrome (ICANS) which are among the most common side effects in CAR-T cell therapy [26,51]. CRS, characterized by systemic inflammation, is triggered by inflammatory cytokines, (i.e., IL-6, IL-10, and IFN-γ) which are released by the activated T-cells, promoting tissue damage and multiorgan dysfunction. In addition, vascular endothelial activation is a risk factor associated with severe CRS. Moreover, biochemical parameters, such as C-reactive protein (CRP), ferritin, aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen

(BUN), and creatinine, are elevated in patients with CRS and ICANS. The levels of these proteins are useful means of predicting severe toxicity [52]. Still, an unsolved issue of CAR-T cell therapy in solid cancer is represented by clonal heterogeneity, which is perhaps the foremost mechanism of tumor escape from systemic therapies [20]. Due to the sub-clonal evolution of cancer cells, both tumor-associated antigens (TAA) and tumor-specific antigens (TSA) may show a wide spectrum of expression, potentially limiting CAR-T cell effectiveness and safety [49]. Finally, the identification of biomarkers for predicting the efficacy of CAR-T cell therapy still remains a major challenge. To date, there are several lines of evidence suggesting that higher CAR-T cell levels in the blood are associated with response [53,54]. It has also been reported that the pre-infusion of polyfunctional CAR-T cells can be significantly associated with clinical response to CAR-T cell therapy underscoring the potential of using biomarkers predicting response prior to infusion [55]. In terms of lymphodepletion, a recent study demonstrated that the administration of higher doses of lymphodepletion agents was associated with higher monocyte chemoattractant protein (MCP)-1 and IL-7 concentrations after T cell infusion being associated with a good prognosis [56].

5. CAR-T cell therapy in TNBC

Despite the difficulties for the implementation of CAR-T cell therapy in solid tumors, at least 12 early-phase clinical trials are currently assessing the efficacy and safety of this approach in TNBC, as summarized in **Table 1**.

Trial ID	Ph	Estimated enrollment	Target	CAR design	Vectors	Route of administration	Primary endpoint(s)	Listed country
NCT02915445	I	30	EpCAM	3 gen	LV	Intra-venous	AE, DLT	China
NCT04348643	I/II	40	CEA	NA	NA	Intra-venous	AE	China
NCT04025216	I	112	TnMUC1	3 gen	LV	Intra-venous	DLT, ORR	USA
NCT02706392	I	60	ROR1	3 gen	LV	Intra-venous	AE	USA
NCT03635632	I	94	GD2	3 gen	RV	Intra-venous	MTD	USA
NCT04427449	I/II	100	CD44v6	4 gen	LV	Intra-venous	AE, ORR	China
NCT01355965	I	18	MSLN	4-1BB/ CD3ζ	mRNA	Intra-tumoral	AE	USA
NCT02414269	I/II	113	MSLN	iCasp9 CD28/	RV	Intra-tumoral	AE, clinical benefit	USA

				CD3ζ				
NCT02792114	I	186	MSLN	iCasp9 CD28/ CD3ζ	RV	Intra-venous	MTD	USA
NCT01837602	I	6	c-MET	4-1BB/ CD3ζ	mRNA	Intra-tumoral	SAE	USA
NCT02541370	I/II	20	CD133	4-1BB/ CD3ζ	LV	Intra-venous	AE	China

Table 1. Ongoing clinical trials investigating CAR-T cell therapy in TNBC. All of the studies have been assessing CAR-T cells in the metastatic setting; as yet, first and second-generation CAR-T cells have failed to enter the clinical practice. Abbreviations: ID, identifier; Ph, phase; EpCAM, epithelial cell adhesion molecule; CEA, carcinoembryonic antigen; TnMUC1, truncated Mucin 1; ROR1, tyrosine kinase-like orphan receptor 1; GD2, ganglioside G2; CD44v6, CD44 variant exon 6 isoform; MSLN, mesothelin; c-MET, tyrosine-protein kinase Met; LV, lentivirus; RV, retrovirus; NA, not available; AE, adverse events; DLT, dose-limiting toxicity; ORR, objective response rate; MTD, maximum tolerated dose; SAE, severe adverse event. Source: Clinicaltrials.gov; accessed on November 15, 2021.

5.1 Targets in early-phase clinical development

5.1.1 Disialoganglioside GD2

GD2 is a glycosphingolipid, typically upregulated in cancerous tissue [57]. This tumor-restricted target expression led to the development of antibody-based therapeutics, as exemplified by the FDA approval of dinutuximab beta and naxitamab for the treatment of neuroblastoma [58]. Consistently, GD2-CARs-T cells have been investigated for the treatment of neuroblastoma, with promising results (e.g., NCT03721068, NCT03635632) [59].

GD2 has been found highly expressed also in stem-like $CD44^{high}$ $CD24^{low}$ human BC cells [50]. Thus, third-generation CAR-T cells have been engineered with an scFv derived from dinutuximab to target TNBC cells, showing anti-cancer activity and increased persistence [50]. Besides, an effective antitumor immune response was also seen in a xenograft mouse model of TNBC [58]. Altogether, these preclinical data provided a rationale for investigating GD2 also at a clinical level.

5.1.2 Receptor tyrosine kinase-like orphan receptor 1

Receptor tyrosine kinase-like orphan receptor 1 (ROR1) is implicated in the neuronal growth that takes place in the central nervous system (CNS). Although ROR1 is limitedly expressed in healthy adult tissues, it appears to be highly and uniformly expressed in both hematologic malignancies and

242 solid tumors, including TNBC (~22%) [50,60,61]. Modification of the CAR spacer design and
243 increase of the affinity of ROR1-CARs have displayed ability in enhancing T-cell effector functions
244 [62]. More recently, in a three-dimensional *in vitro* model of TNBC, 4-1BB co-stimulated ROR1-
245 CAR-T cells were shown to infiltrate and migrate through TNBC cultures and cause significant
246 antitumor responses [60]. In this sense, ROR1-CAR-T cells have entered the clinic through a phase I
247 study (NCT02706392) (**Table 1**). In the early-phase assessment, from the 4 TNBC patients treated
248 with ROR1-CAR-T cells, 2 individuals showed stable disease and one participant had a partial
249 response after the second infusion, persisting for 14 weeks [63]. No safety signals were observed. A
250 strategy to avoid possible off-tumor toxicity has been implemented relying on the engineering of
251 ROR1-CAR-T cells with synthetic Notch receptors that are specific for EpCAM or B7-H3, which are
252 expressed on ROR1+ tumor cells but not on ROR1+ stromal cells. Synthetic Notch receptors can
253 induce ROR1 expression selectively within the tumor, thus sparing normal tissues [63].
254
255 **5.1.3 MUC1**
256 MUC1 is a glycoprotein that is expressed in healthy tissues on the luminal surface of epithelial cells,
257 and it is part of the mucosal barrier [64]. Serine and threonine residues present in the variable number
258 tandem repeats region of the MUC1 extracellular domain serve as attachment sites for O-glycans.
259 Consequently, post-translational modifications can be observed in the MUC protein [65]. TNBC
260 expresses a form of MUC1, namely tMUC1, with aberrant glycosylation in more than 95% of cases.
261 *In vitro* investigation of second-generation tMUC-CAR-T cells demonstrated anti-tumor activity and
262 significant cytokine production. Similar results were seen in a TNBC xenograft mouse model while
263 sparing normal breast epithelial cells [66]. The expression of a glycosylated biosynthetic isoform of
264 MUC1, namely TnMUC1, can be forced on TNBC cells [50]. In this regard, a CAR engineered with
265 a mouse anti-human scFv derived from the monoclonal antibody 5E5 recognizes the epitopes
266 TnMUC1, a CD8a transmembrane region and dual CD2 and CD3ζ intracellular signaling domain. In

the dose-escalation phase, no evidence of safety concerns or on-target/off-tumor toxicity was observed (NCT04025216) [67,68].

5.1.4 CD44v6

The adhesive receptor CD44 is widely expressed in hematologic and epithelial tumors, as it is thought to contribute to cancer stem/initiating phenotype [69]. Silencing of its variant exon 6 isoform (CD44v6) has demonstrated engraftment of human acute myeloid leukemia (AML) and multiple myeloma (MM) cells in immunocompromised mice [69]. Consistently, CD44v6-CAR-T cells showed significant anti-tumor activity against primary AML and MM while sparing normal hematopoietic stem cells and CD44v6-expressing keratinocytes [44]. The expression of CD44v6 has been investigated in several solid tumors, including squamous cell carcinomas and adenocarcinomas of differing origin, as well as in melanomas [70]. Such expression pattern has made CD44v6 an attractive target for the therapy of various types of CD44v6-positive cancers, including TNBC. In this regard, fourth-generation CAR-T cells are currently being investigated in solid tumors in a phase I/II clinical trial (NCT04427449).

5.1.5 EpCAM

Epithelial cell adhesion molecule (EpCAM) is a cell surface molecule involved in cell-to-cell adhesion and it is known to be highly expressed in colon and other epithelial carcinomas [71]. Recently, a real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed to quantify the level of EpCAM mRNA expression in normal breast tissue as well as primary and metastatic BCs. EpCAM resulted overexpressed 100- to 1000-fold in primary and metastatic BC. Moreover, silencing of EpCAM gene expression with short interfering RNA (siRNA) resulted in a 35-80% decrease in the rate of cell proliferation in four different BC cell lines [71]. EpCAM siRNA treatment was associated with decreased cell migration (~91.8%) and reduced cell invasion (~96.4%)

in BC MDA-MB-231 cell line [71]. Such results provide a rationale for exploiting EpCAM as a target for BC. In this regard, a phase I clinical trial is currently investigating third-generation EpCAM-CAR-T cells for the treatment of breast cancer (NCT02915445).

5.2 Targets in pre-clinical development

5.2.1 AXL

Receptor tyrosine kinase AXL contributes to signaling pathways involved in tumor progression and resistance to systemic therapies, such as phosphoinositide 3-kinase (PI3K), MAPK, and NF-kb [72]. In healthy tissues, AXL is expressed in capillary endothelium and vascular smooth muscle cells, with restored expression in cancer tissues with an associated poor prognosis. Several studies explored the role of AXL as a therapeutic target and predictive biomarker in TNBC [73,74]. Furthermore, AXL-CAR-T cell may be able to convert a 'cold' TME into a 'hot' one, by suppressing TAM-related production of cytokines and by causing myeloid-derived suppressor cells (MDSCs) depletion from the TME [75,76]. More recently, *in vitro* findings supported an antitumor activity and prolonged survival for IL-7-expressing AXL-CAR-T cells in a TNBC xenograft model [76]. These data provide a rationale for the investigation of AXL-CAR-T cells for the treatment of TNBC.

5.2.2 Folate receptor alpha

Folate receptor alpha (FRα) is a glycosylphosphatidylinositol (GPI)-linked membrane protein that binds to folic acids and mediates their intracellular transport [77]. This molecule is expressed in 70-86% of metastatic TNBC and it is related to a poor prognosis [10,44]. Conversely, a recent study highlighted a prevalence of FRα expression in ~71% of early TNBC samples being associated with improved disease-free survival (DFS). These findings led to the initiation of phase II clinical study investigating an FRα-directed peptide-based vaccine candidate in patients with high-risk, early TNBC

(NCT03012100) [78]. Coherently, FRα-CAR-T cells have shown significant anti-cancer activity in TNBC cell lines and in a xenograft mouse model, which correlates with FRα expression levels on tumor cells [79]. In this sense, a phase I clinical trial of FRα-CAR-T cells has been initiated for recurrent high-grade serous ovarian or primary peritoneal cancer (NCT03585764). Evidence from this trial is awaited, also given the potential translation of this result to TNBC. In addition, the co-targeting of FRα and mesothelin has been developed to help tackle the issue of potential on-tumor/off-target toxicity associated with FRα-CAR-T cell therapy [80]. Moreover, folate-fluorescein isothiocyanate (FITC) bispecific adaptor molecule has been shown to redirect universal anti-FITC-CAR-T cells to target tumor cells expressing the folate receptor [50,81].

5.2.3 Trophoblast cell-surface antigen 2

Trophoblast cell surface antigen 2 (TROP2) was first described as a surface marker of trophoblast cells. It subsequently showed to be increased in several tumors, including BC, resulting in poor prognosis [82]. This glycoprotein is overexpressed in ~90% of TNBC. Recently, the FDA approved sacituzumab govitecan, an ADC targeting TROP2, for the treatment of relapsed or refractory metastatic TNBC [15,48]. The targeting of TROP2 did not provide safety concerns, thus it has emerged as a good candidate for CAR-T cells as well. Dual targeting of TROP2 and PD-L1 using CAR-T cells showed *in vitro* and *in vivo* anti-tumor activity in gastric cancer [83]. TROP2-CAR-T cells have been engineered for use in TNBC as well [50]. Moreover, to address tumor heterogeneity, typical of solid tumors, exosomes from TROP2-expressing tumors were transferred to TROP2-negative tumor cells to increase the proportion of targetable cancer cells by TROP2-CAR-T cells [50].

5.2.4 Tissue Factor

Tissue Factor (TF), also known as CD142 and coagulation factor III, is a membrane-bound cell surface receptor involved in the initiation of blood coagulation upon disruption of vessel wall integrity [84]. This surface target is expressed in 50-85% of TNBCs [84]. Recently, second-generation antibody-like immunoconjugate (L-ICON) targeting TF in TNBC has been developed in pre-clinical settings [84]. Consistently, drug development is moving towards the development of TF-targeting CAR-engineered Natural Killer (NK) cells, that co-express CD16 (FcγIII) to mediate antibody-dependent cellular cytotoxicity (ADCC) in TNBC as a single agent or combination with L-ICON. Preliminary results demonstrate that TF-CAR-NK cells alone can destroy TNBC cells, with enhanced efficacy by the addition of L-ICON *in vitro* [84,85]. Furthermore, TF-CAR-NK cells displayed anti-cancer activity also in cell lines and patient's tumor-derived xenograft mouse models [84]. Given the promising data that emerge from the use of this target, in-depth pre-clinical research is needed to assess the safety and feasibility of this strategy in TNBC patients.

5.2.5 Epidermal growth factor receptor

Epidermal growth factor receptor (EGFR, also called HER1), is a glycoprotein of 170 kDa, encoded by a gene located on chromosome 7p and a member of the HER family of tyrosine kinases. EGFR is widely expressed in several epithelial tumors and overexpressed in approximately 45-70% of TNBCs [50,86]. Despite EGFR antagonists (i.e., small-molecule kinase inhibitors such as gefitinib, erlotinib or monoclonal antibodies including cetuximab, panitumumab) being used in the clinical setting, the application of anti-EGFR as monotherapy has shown limited efficacy due to drug resistance or poor response [87]. On the other hand, it has been shown in both in vitro and in mouse models that EGFR-specific CAR-T cells may represent a promising therapeutic strategy against high-EGFR-expressing TNBC [88]. After being tested both in TNBC cell lines and in patient-derived xenograft mouse models, third-generation EGFR-targeting CAR-T cells (CD28/4-1BB) showed encouraging activity not only in terms of cytokine secretion but also of cytolytic activity [86]. A promising strategy for

future next generation CARs is represented by the engineering of T cells that incorporate dual or tandem CARs which are able to recognize multiple antigens. This can address the current limitation of tumor associated antigens, including EGFR, being expressed on normal tissue [50]. Interestingly, affinity-tuned scFvs in EGFR-CAR-T cells demonstrated better anti-tumor efficacy compared to high-affinity cells [88]. Of note, targets with a more-restricted expression, such as EGFR variant III, could reduce on-target/off-tumor toxicity [50]. Finally, novel CAR-T cells that integrate immune checkpoint blockade properties, such as anti-cytotoxic T lymphocyte-associated protein 4 (CTLA4), into EGFR-CAR-T cells are being explored, to restore a 'hot' TME [88].

5.2.6 FcγR-CAR-T cells in combination with therapeutic antibodies

Universal CAR-T cells that express a FcγReceptor(R)-CAR can be used to redirect the immune response to virtually any antigen employing specific antibodies against antigens expressed on tumor cells [89]. In this case, CAR is constituted of the extracellular domain of an FcγR (CD16a and CD32a, especially) that is linked with signaling and co-stimulatory domains. Then, FcγR-CAR-T cells bind the Fc portion of an antibody, resulting in the activation of CAR-T cells leading to ADCC and consequent target cell depletion [50,88]. In this regard, recent results of $CD16a^{158Phe}$ (low-affinity receptors) engineered CAR-T cells, in combination with the anti-EGFR cetuximab, showed relevant *in vitro* anti-cancer activity against EGFR-positive TNBC. Similarly, low-affinity $CD32a^{131Arg}$ engineered CAR-T cells administered in association with cetuximab or panitumumab determined the elimination of EGFR-positive MDA-MB-468 TNBC cells and related pro-inflammatory cytokines [90].

5.3 Other targets

Several other targets have been explored in both pre-clinical and early-phase clinical settings, such as Intercellular Adhesion Molecule 1 (ICAM-1), mesothelin, c-Met, Natural Killer Group 2D (NKG2D), Stage-specific embryonic antigen-4 (SSEA-4), Chondroitin Sulfate Proteoglycan 4

(CSPG4) and Tumor endothelial marker 8 (TEM8), with different results in terms of safety, data maturity and significance [26]. Concerning c-MET-CAR-T cells, a recent study highlighted that intratumoral injections of such cells were well tolerated and able to provoke an inflammatory response within cancer cells [44]. Although the results of a preliminary phase I study confirmed safety for intravenous injection of c-MET-CAR-T cells in advanced TNBC, the clinical trial was closed due to a halt in funding (NCT03060356). On the other hand, CSPG4-CAR-T cells can target various molecules simultaneously, including TNBC cells, stromal cells, and blood vessels. Indeed, a primary safety issue involving CSPG4 is related to potential on-target, off-tumor toxicity, especially in the form of severe bleeding [44,91]. Moreover, antigen loss could be a potential challenge considering the high degree of genetic instability in TNBC [92]. Finally, CSPG4 expression is not uniform on TNBC cells [44,92]. In a clinical trial, an optimized strategy to produce mRNA-based CSPG4-specific CD28/CD3ζ-CAR-T cells led to a sufficient number of highly pure engineered cells [93]. As for the surface adhesion molecule ICAM-1, which is highly expressed on TNBC cells, affinity-variant CD28/4-1BB co-stimulated ICAM-1-CARs have recently demonstrated that lower affinity has superior anti-tumor efficacy, with acceptable safety, compared to their higher affinity counterpart [94,95]. Overall, ICAM-1-CAR-T cells showed significant cytotoxicity against TNBC cells, providing a rationale for early-phase clinical development. TEM8 is a cell surface protein that is preferentially expressed in areas of aberrant neoangiogenesis within tumors [96]. TEM8 overexpression has been linked to increased tumor growth as well as a higher risk of metastatic spread; conversely, TEM8 knock-out resulted in reduced tumor growth [97,98]. In BC, TEM8 is widely expressed on TNBC cells and revealed itself as a possible marker of stem-like cells [99-101]. Of note, TEM8 is also expressed on tumor-associated perivascular stromal cells within the TME [102]. Altogether, these findings provide a rationale for the implementation of TEM8-CAR-T cells to treat TNBC. In particular, second-generation (CD28/CD3ζ) and third-generation (CD28/4-1BB/CD3ζ) TEM8-CARs have been engineered using scFv of the TEM8-directed antibody L2. Both these CAR-T cell products can co-target TNBC cells expressing TEM8 as well as tumor-associated

417 vessels *in vitro* [103]. In addition, CD28/4-1BB co-stimulated TEM8-CAR-T cells demonstrated the
418 ability to induce TNBC cells regression, as well as to reduce tumor neoangiogenesis [104].
419 Importantly, similar CD28 co-stimulated L2-based TEM8-CAR-T cells demonstrated loss of CAR-
420 expressing cells in the circulation, lung inflammation and spleen phlogosis in murine models. Such
421 phenomena did not appear in TEM8-knockout mice (off-tumor, on-target toxicity) [104]. Toxicity
422 was also reported by using the L2 antibody in a treatment strategy based on a bispecific T-cell
423 engager. Therefore, TEM8 exploitation for targeted immune therapy needs caution when planning to
424 move into the early clinical setting [44]. NKG2D ligands are generally absent on cells from healthy
425 tissues; however, they are often induced when the cells undergo biological stress, such as tumoral
426 transformation, including BC [105]. To effectively design NKG2D-CAR-T cells, the full-length
427 NKG2D has been fused to the intracellular domain of CD3ζ in reverse orientation - NKG2D is a type
428 II protein -, possibly with the co-stimulatory signal provided by DAP10 [105]. Another CAR design
429 exploited the extracellular domain of NKG2D, fused to CD18, 4-1BB or CD27 signaling domain, to
430 retain the ligand-binding function [106]. In this regard, 4-1BB/CD27 co-stimulated NKG2D-CAR-T
431 cells have been implemented to target TNBCs [44]. Such NKG2D-CAR-T approaches demonstrated
432 significant anti-tumor activity in TNBC both *in vitro* and *in vivo*, thus representing a promising
433 immunotherapeutic approach. Consequently, NKG2D-CAR-T cells are currently being investigated
434 in early-phase clinical trials, beginning with hematologic malignancies (AML, MM) and metastatic
435 colorectal cancer [107]. Provided that no safety signals would arise from such early-phase
436 developmental stages, the next step would be the implementation of this strategy in other
437 malignancies, such as TNBC [44].
438
439 **6. Conclusion and future perspectives**
440 TNBC is a heterogeneous and complex disease, but it is also the most immunogenic BC subtype [20].
441 To overcome the various challenges that are limiting the efficacy of CAR-T cells in solid tumors,
442 different novel strategies have been developed (**Figure 2**). By far, the most relevant obstacle is

represented by the selection of the optimal target, to minimize on-target, off-tumor toxicities, as well as to reduce tumor escape via antigen loss and intrinsic heterogeneity [10]. Suboptimal efficacy in solid tumors could also be related to difficult migration and reduced persistence. In this regard, implementation of CAR-T cells with chemokine receptors (e.g., CCR-2 and CCR-4), which largely account for directing the migration of T cells, may improve CAR-T cell migration to the tumor site and homing potential [44,108,109]. In the future, additional approaches can be explored to reprogram the hostile TME in TNBC to a proinflammatory state by armoring CAR-T cells with dominant-negative TGF-β receptors or inverted cytokine receptors [110]. From the clinical perspective, given the promising findings from the combination of CAR-T cell therapy with immune checkpoint blockade (e.g., anti–PD-1) at the pre-clinical level [111], such combination strategies could represent future therapies in the context of TNBC. Moreover, a detailed understanding of the strategies to mitigate toxicities related with CAR-T cell therapy (i.e. the early recognition of CRS and neurotoxicity through predictive biomarkers, prevention of on-target/off tumor effect, diminishing CARs' activity in case of severe toxicity) are essential for the safe use [112]. In conclusion, even if CAR-T cell therapy is emerging as a strategy worth investigating in solid tumors, current evidence is still too scarce to predict possible future implementation in the therapeutic algorithm of TNBC.

Expert Opinion (**942**/1000 words)

TNBC is a biologically and clinically heterogeneous disease characterized by higher immunogenicity compared to the other BC subtypes [20]. To date, many barriers are preventing CAR-T cells from entering the clinic for solid tumors, including TNBC. The most relevant challenge is represented by the selection of the optimal target to minimize on-target, off-tumor toxicities, as well as to reduce tumor escape via antigen loss and intrinsic heterogeneity [10,113]. To date, TROP2, GD2, ROR1, MUC1, CD44v6 and EpCAM are among the most promising targets. Indeed, the developed CARs against these targets have infiltrated and migrated through TNBC cultures and caused significant antitumor responses. In addition, limited safety concerns or on-target/off-tumor toxicities have

emerged. Identification of new biomarkers could help in improving the quality of CAR-T cell products and establish a thorough understanding of the mechanisms associated with cytotoxicity and treatment response. In the next decade, the design of multi-specific CAR-T cell therapeutics able to simultaneously target multiple antigens may be a possible solution. Moreover, the optimal target should also be a tumor-specific antigen, rather than a tumor-associated antigen, even if the typical off-the-shelf nature of the CAR-T cell products represents another obstacle in tailoring this adoptive cell therapy approach on the patients' neo-antigens [78,114]. Suboptimal efficacy in solid tumors could also be related to difficult migration and reduced persistence. In this regard, implementation of CAR-T cells with a chemokine receptor (e.g., CCR-2 and CCR-4), which largely accounts for directing the migration of T cells, may improve CAR-T cell migration to the tumor site and homing potential [44,108,109]. Further efforts to prolong the persistence of CAR-T cells in solid tumors are ongoing and include the implementation of constitutively activated interleukin (IL)-15 and IL-7 receptors into CAR-T cells [115,116]. Additionally, novel TRUCKs, designed to redirect T-cells for universal cytokine-mediated killing, can release proinflammatory cytokines upon CAR engagement (**Figure 2**) [50]. Finally, efficacy may be impaired also by the immune suppressive TME that often characterizes solid neoplasms. A 'cold' TME can counterstain the activity of CAR-T cell-based products. Therefore, combination treatments with immune checkpoint blockade or cancer vaccines may further unleash the anti-cancer immune response [10]. To help reprogram the TME into a 'hot' counterpart, macrophages, NK cells, induced pluripotent stem cells (iPSC)-derived T/NK cells implemented with CARs are currently being explored in both preclinical and early-phase clinical stages [117]. Possibly, other combination strategies, like chemotherapy, radiotherapy and genetic engineering may help manipulate T-cell trafficking towards cancer cells, to increase the efficacy and applicability of CAR-T cell technology [44]. To improve the safety profile, different suicide genes have been engineered into preclinical models investigating CAR-T cell constructs [118]. Also, dual or synthetic notch CARs that utilize AND-gating logic or inhibitory CARs that utilize NOT-gating logic have shown success in reducing on-target/off-tumor toxicity in other solid tumors (**Figure 2**)

[50]. In conclusion, although CAR-T cell therapy is unlikely to replace chemotherapy in TNBC in the next future, it may be useful as part of combination strategies. Interestingly, the PARPi olaparib has recently been demonstrated to induce CD8+ T-cell infiltration via stimulation of interferon genes (STING) signaling in BRCA-deficient TNBC *in vivo*, thus providing the rationale for a possible combination of PARPi and CAR-T cell therapy for the treatment of TNBC [119]. Interestingly, PI3K inhibition during *ex-vivo* CAR-T cell expansion induced a memory phenotype, improving *in vivo* persistence and antitumor activity in leukemia [120]. However, the potential added benefit of a combinatorial strategy involving alpelisib and CAR-T cells is unknown. Finally, emerging platforms involving γδCAR-T cells and CAR-NK-cells therapies may be promising approaches that should be investigated for the treatment of solid tumors, including TNBC [50,84]. From a manufacturing standpoint, cell-based therapies now comprise a large proportion of the anticancer pipeline, namely activated autologous antigen-presenting cells, autologous expanded T cells, TILs, CAR-T cells, CAR-NK cells, engineered TCR, allogeneic genetically edited cells, using techniques like transcription activator-like effector nucleases (TALEN), CRISPR/Cas9, or zinc finger nucleases [121]. The production of patient-derived therapeutics is usually centralized to ensure standardization, optimization, automation, and scale-out [122]. To diminish some of the obstacles related to centralized manufacture, an alternate approach is to produce these cellular therapies at the academic centers where patients are treated [122]. In this sense, cryopreservation may be avoided as well as much of the logistics-related costs [123,124]. Other relevant advantages may be the reduction of transportation-related bottlenecks, a shorter time-to-final product, reduced environmental impact, and much greater flexibility in the designing of personalized therapies, based on the molecular characteristics of individual patients [122]. Of note, quality control and quality assurance would require the setting up of robust internal procedures with clearly defined legal responsibility, good manufacturing practices (GMP) standards of manufacture, testing and an audit trail [122]. To date, in addition to the fact that current evidence is still too scarce to predict possible future implementation in the therapeutic algorithm of TNBC, the most worrying ethical challenge in the field of adoptive

cell therapy remains inequity of access. Manufacturing these products involves very steep financial, knowledge, and logistic barriers, which are restricted to only a few countries or even regions within countries. Efforts should be made to expand access by knowledge-sharing, technology transfer, and funding assistance [122]. In conclusion, even if CAR-T cell therapy is emerging as a strategy worth investigating in solid tumors, however, there is still much evidence to be obtained from preclinical research and ongoing clinical trials investigating this approach in TNBC. In this regard, a collaborative path, where industry and academia work in partnership to experiment and manufacture licensed cellular therapies, would be a potential way to boost this emerging and promising field.

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