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FOCUSSED RESEARCH REVIEW

Targeting tumor vasculature: expanding the potential of DNA cancer vaccines

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Abstract Targeting the tumor vasculature with anti-angiogenesis modalities is a *bona fide* validated approach that has complemented cancer treatment paradigms. Tumor vasculature antigens (TVA) can be immunologically targeted and offers multiple theoretical advantages that may enhance existing strategies against cancer. We focused on tumor endothelial marker 1 (TEM1/CD248) as a model TVA since it is broadly expressed on many different cancers. Our DNA-based vaccine approach demonstrated that CD248 can be effectively targeted immunologically; anti-tumor responses were generated in several mouse models; and $CD8^+$/$CD4^+$ T cell responses were elicited against peptides derived from CD248 protein. Our work supports our contention that CD248 is a novel immunotherapeutic target for cancer treatment and highlights the efficient, safe and translatable use of DNA-based immunotherapy. We next briefly highlight ongoing investigations targeting CD248 with antibodies as a diagnostic imaging agent and as a therapeutic antibody in an early clinical trial. The optimal approach for generating effective DNA-based cancer vaccines for several tumor types may be a combinatorial approach that enhances immunogenicity such as combination with chemotherapy. Additional combination approaches are discussed and include those that alleviate the immunosuppressive tumor microenvironment induced by myeloid-derived suppressor cells and T regulatory cells. Targeting the tumor vasculature by CD248-based immunological modalities expands the armamentarium against cancer.

Keywords Tumor vasculature · Tumor endothelial marker (TEM) · DNA cancer vaccine · PIVAC-2014

Abbreviations

ACT	Adoptive cell therapy
ALL	Acute lymphocytic leukemia
APC	Antigen-presenting cells
bFGF	Basic fibroblast growth factor
CAIX	Carbonic anhydrase IX
CAR	Chimeric antigen receptor
CLL	Chronic lymphocytic leukemia
CTL	Cytotoxic T lymphocytes
CXCL	Chemokine (C-X-C motif) ligand
DC	Dendritic cells
E2	17β-estradiol
EC	Endothelial cells
EPC	Endothelial progenitor cells
G-CSF	Granulocyte colony-stimulating factor
HGF	Hepatocyte growth factor
IFN	Interferon
IL	Interleukin
IMG	Intussusceptive microvascular growth

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MDSC	Myeloid-derived suppressor cells
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinases
PDGF	Platelet-derived growth factor
scFv	Single-chain antibody
TAA	Tumor-associated antigen
TCR	T cell receptor
TEM	Tumor endothelial marker
TGF	Transforming growth factor
TNF	Tumor necrosis factor
Treg	T regulatory lymphocytes
TT	Tetanus toxoid
TVA	Tumor vasculature antigen
VDA	Vascular-disrupting agents
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

Introduction

Vasculature formation depends on the process of angiogenesis, which is responsible for the remodeling and expansion of the existing vasculature network and vasculogenesis, a process whereby endothelial precursor cells (EPC) migrate and differentiate in response to local signals (such as growth factors from stroma and extracellular matrix) to form new blood vessels. Vasculogenesis is a fundamental process during embryogenesis and in adults takes place during tumor growth, following trauma and in endometriosis [1]. In normal tissues, angiogenesis is a tightly regulated physiological process that comprises two different mechanisms: endothelial cells (EC) sprouting and intussusceptive microvascular growth (IMG), which are regulated by several endogenous activator (pro-angiogenic factors) and inactivator (anti-angiogenic factors) molecules. Pro-angiogenic factors include vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF), matrix metalloproteinases (MMP), transforming growth factor (TGF)-β and angiopoietins among others. Anti-angiogenic factors include interferon (IFN)-γ, CXCL9, CXCL10, thrombospondin 1, endostatin and angiostatin [2].

For cancer progression, angiogenesis is a necessary step in the uncontrolled proliferation, invasion, and metastatic dissemination of tumor cells, involving the formation and remodeling of new vessels to "feed" the tumor and promote its progression. Cancer angiogenesis begins when tumor nodules reach dimensions over a few millimeters, and nutrient/oxygen diffusion is no longer sufficient to sustain both the physiological cell cycle and basic cell metabolism. In response to these unfavorable conditions, cancer cells produce pro-angiogenic signals that are able to activate resting EC and stromal cells. The activated EC acquire the ability to proliferate, migrate, differentiate and remodel adjacent extracellular matrix in association with pericytes to stabilize the new blood vessels [3].

Anti-vascular strategies are mainly classified into two groups: anti-angiogenic and vascular-disrupting therapies. The VEGF–VEGF receptor (VEGFR) signaling axis has emerged as one of the most promising angiogenic targets because of its central role in tumor angiogenesis and growth. VEGFA (commonly referred to as "VEGF") has been identified as the predominant pro-angiogenic growth factor expressed by tumor cells and binds both VEGFR1 and VEGFR2 [4, 5]. Angiogenesis has become an accepted target for anticancer therapy since 2004 when bevacizumab, a VEGF-binding antibody, became the first angiogenesis inhibitor to be approved in the USA for the treatment of several metastatic cancer types (as a single or in combination with different cytotoxic chemotherapy) including colorectal cancer, non-squamous non-small cell lung cancer, renal cell carcinoma, cervical cancer, ovarian cancer and as a monotherapy for glioblastoma (Avastin Prescribing Information, 2015). Major side effects of bevacizumab result from the undesirable targeting of normal vasculature including gastrointestinal perforation, wound healing complications, hemorrhaging and effects on reproduction (Avastin Prescribing Information, 2015). The VEGFR signaling pathway as well as other angiogenic receptors (EGFR, PDGFR, FGFR) has also been targeted with the development of small molecule tyrosine kinase inhibitors such as vandetanib, cabozantinib, sorafenib, ponatinib and lenvatinib [6]. Vascular-disrupting agents (VDA), a relatively new class of drugs in clinical development, target and destroy preexisting tumor vessels, resulting in tumor cell death from ischemia and necrosis [7]. Preclinical data using VDA have confirmed the extraordinary potential of this approach to control tumor growth by inducing massive necrosis of cancer cells. VDA small molecules, such as tubulin-depolymerizing agents and flavonoids, have already produced promising preclinical results, although they lack robust specificity and induce neurological and cardiovascular side effects as observed in a phase I clinical trial [8]. The development of novel, effective and safe VDA for cancer therapy has been currently hindered by both the paucity of selective targets for different tumors and the lack of reagents with optimal affinity.

In the last several decades, anticancer vaccination strategies have been developed by different approaches preferentially based on targeting tumor cells expressing tumor-associated antigens (TAA) [9]; however, tumor cells are genetically unstable and when immune pressure is applied, can rapidly give rise to antigen escape variants, resulting in treatment failures. Development and delivery of immunotherapy directed against tumor vasculature antigens

(TVA) offer multiple theoretical advantages: (1) TVA are overexpressed on cancers of different histological types, whereas they are generally absent or poorly expressed on most normal tissue; (2) the tumor vasculature and stroma are genetically stable as compared to tumor cells, which can minimize escape variants [10, 11]; (3) TVA-targeting therapies can be broadly applicable to different cancers [12]; (4) TVA targeting is endowed with powerful effector lymphocytes that can persist in tumor-bearing hosts, leaving a long-lasting memory response that protects the host against tumor recurrence; (5) TVA-targeting efficacy can be enhanced in combination with different pharmacological/immunological strategies as we describe in the last section. In this review, we describe recent findings on the application of targeting the tumor vasculature with a CD248-specific DNA vaccine, highlighting its rationale, its therapeutic effectiveness and latest developments as well as potential future areas of clinical improvement.

CD248 background

As reported by Brad St. Croix et al. [13], colorectal tumor endothelium was found to up-regulate several genes compared to the normal intestinal endothelium; nine genes were overexpressed up to tenfold in tumor-derived versus normal-derived endothelium and were dubbed tumor endothelial marker (TEM). TEM overexpression in tumor vessels was confirmed by reverse transcriptase polymerase chain reaction and in situ hybridization [14]. These TEMs are found on the cell surface and although are not structurally related (TEM1, 3, 5, 7, 8), they are functionally conserved between mouse and human tumor endothelium. These two attributes make TEMs attractive candidates for immunotherapeutic interventions because these targets are directly accessible to the immune cells via the tumor vasculature. The most differentially expressed TEM, TEM1 (CD248) was found to be the same antigen, previously dubbed endosialin, a highly sialylated glycoprotein found on many different tumor types described first in the Rettig seminal paper [15]. Human CD248 mRNA and/or protein has been characterized in various human tumor settings including colorectal cancer, brain tumors and sarcomas [16–18]. CD248 has also been reported to be expressed by tumor vessel-associated pericytes [19] and stromal fibroblasts [20] on a broad variety of human tumors with different histology but not in normal vessels [21]. Moreover, CD248 has also been classified as a marker of tumor vessel-associated pericyte cells and tumor-associated myofibroblasts [22] as well as a selective EPC marker [23]. Pericytes provide structural support for EC and therefore stabilize the vasculature. CD248 plays a role in physiological processes such as cell adhesion, neo-angiogenesis, cell migration and tissue development [24] and has an essential role in promoting tumor growth; its overexpression negatively correlated with the clinical outcome of cancer patients [25]. Using a knockout mouse model for CD248 expression (*Tem*1$^{-/-}$ mice), researchers were able to identify the physiological and biological role of CD248 in promoting tumor progression. Tumor growth was inhibited in *Tem*1$^{-/-}$ mice, and metastatic dissemination was also reduced. Normal wound healing was observed in *Tem*1$^{-/-}$ mice compared to wild-type mice [24]. These data suggest that targeting CD248 may generate efficient therapeutic effects with minimal toxicity.

CD248 targeting by DNA vaccine as a useful approach to strike tumor vasculature

To test the hypothesis that CD248 is a suitable candidate for cancer immunotherapy, we designed an active immunotherapeutic approach based on a DNA-based vaccine in conjunction with in vivo gene transfer [26]. To increase the immunogenicity of CD248 and break murine tolerance, we developed a DNA vaccine based on fusing mouse CD248 cDNA with the cDNA of the amino terminal domain of fragment C of *tetanus toxoid* (TT). The TT fragment DNA was introduced at the 3′ end of the CD248 coding sequence, generating the plasmid called pcDNA3.1/TEM1-TT. The TEM1-TT DNA sequence was codon-optimized to increase expression yields up to 100-fold as well as inclusion of CpG motifs which activate TLR9 to increase immunogenicity of the DNA vaccine. As an adjuvant, TT may enhance immunogenicity through several mechanisms; the whole C fragment of TT activates dendritic cells (DC) to secrete cytokines involved in CD4$^+$ T cell activation and Th_1 polarization, such as interleukin (IL)-12, IL-23 and tumor necrosis factor (TNF)-α [27]. In addition, fragment C contains universal T helper epitopes (p30, p2), which are effective across different major histocompatibility complex (MHC) class II haplotypes in mice and humans and elicits strong CD4$^+$ T responses [28]. Lastly, electrogene therapy induces local inflammation and promotes a favorable microenvironment that is able to stimulate T cell fitness and functionality [29].

We have reported that mice vaccinated with TEM1-TT plasmid vaccine were able to develop T lymphocytes with strong reactivity against a CD248-specific peptide; vaccinated C57BL/6 mice generated a specific CD8$^+$ T cell response toward the $TEM1_{696–710}$ peptide (GQSQRDDRWLLVALL). TEM1-TT-vaccinated BALB/c mice responded with elicitation of a CD8$^+$ and a CD4$^+$ T cell response against two CD248 peptides: $TEM1_{516–530}$ (ITSATHPARSPPYQP) and $TEM1_{511–525}$ (GHKPGITSATHPARS). Moreover, the identified CD248-derived

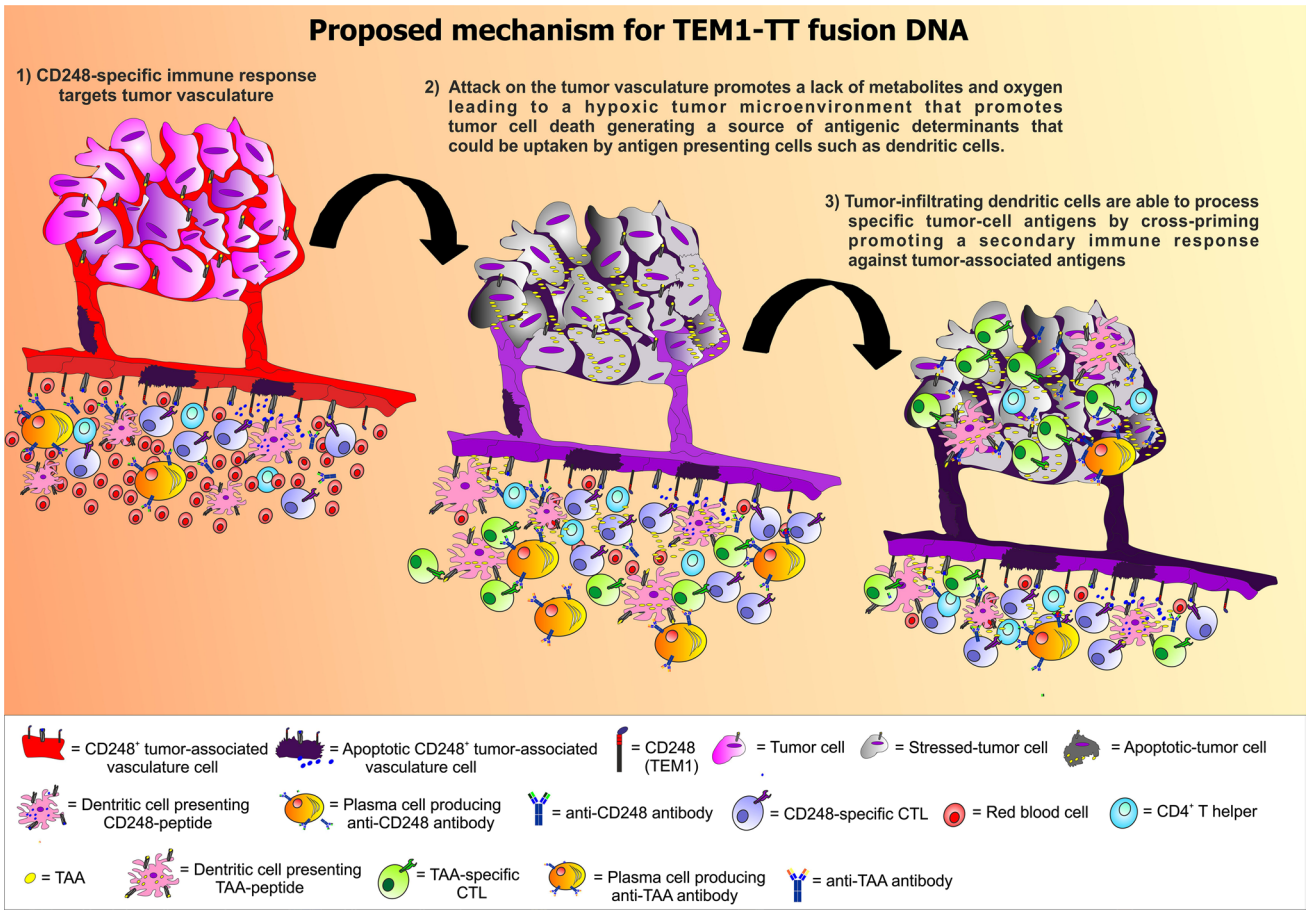

Fig. 1 Proposed mechanism of the therapeutic effectiveness of TEM1-TT vaccination. (*1*) TEM1-TT vaccine stimulates the elicitation and expansion of CD248-specific CTLs. Cytolysis of $CD248^+$ tumor-derived EC/pericytes results in blood vessel fenestration inducing vessel collapse as well as difficulty assembling new vasculature. (*2*) The lack of a constant supply of metabolites and oxygen promotes a hypoxic tumor microenvironment. This detrimental condition promotes both a tumor cell non-proliferative state and tumor cell apoptosis. Tumor apoptosis generates a source of TAA that can be engulfed by APC such as DC and macrophages recruited by the inflammation near the tumor mass. (*3*) These APC are able to process and present TAA peptide in association with MHC-I molecules. This secondary cross-priming event produces the expansion of both specific anti-TAA CTLs and plasma cell producing anti-TAA antibody. This second immune response assists the CD248-specific immune attack to control and limit tumor progression

peptides were able to expand CD248-specific cytotoxic T lymphocytes (CTL) in vitro that selectively recognized CD248-expressing endothelium immortalized cells such as H5V cells. Our data are the first demonstration of CD248 immunogenicity and establishes a foundation upon which to develop selective immunotherapy against this potential TVA. TEM1-TT vaccine was able to control in vivo tumor progression of different transplantable tumor models by both prophylactic and therapeutic immunization [26]. The therapeutic effectiveness of our vaccine can be summarized in three parts as schematically shown in Fig. 1. Firstly, TEM1-TT vaccine elicits anti-CD248 CTLs that specifically attack the tumor vasculature, resulting in significant inhibition and ablation of the tumor vasculature [26]. The TEM1-TT vaccine modified the tumor vessel architecture as demonstrated by reduction of $CD31^+$ EC and/or pericytes in the CT26 tumor model. By RNA FISH, CD248 mRNA was found to localize with, or to be in close juxtaposition to, CD31 mRNA, suggesting that EC and/or pericytes are expressing CD248 [26]. We also show that in TEM1-TT-vaccinated mice, CT26 tumor contained $CD31^+$ cells that were also TUNEL positive, suggesting that $CD31^+$ EC/pericytes were in an apoptotic state [26]. Pericyte coverage on EC provides the structural support for the vasculature. Since CD248 is expressed by tumor vessel-associated pericyte cells [22], TEM1-TT vaccine may also target pericytes directly, with subsequent destabilization and inhibition of the tumor endothelial cell network. This selective inhibition of tumor vascularity was also verified by in vivo ultrasound imaging which allowed us to define physical vascular parameters of the tumor mass such as the perfused tumor area and the red blood cell flux per unit area

of tissue. Collectively, our data demonstrate that TEM1-TT vaccine functionally destabilizes the tumor vasculature, induces vessel collapse, as well as possibly impedes the assembly of new vasculature [26].

The second part of our proposed mechanism includes the consequences of tumor vessel destruction mediated by the TEM1-TT vaccine. We hypothesize that the lack of a constant stream of metabolites and oxygen leads to an increased hypoxic and inflammatory tumor microenvironment that promotes tumor cell death. Tumor cells from TEM1-TT-vaccinated mice showed an increase in tumor cell apoptosis. We verified that tumor masses isolated from TEM1-TT-vaccinated mice showed higher levels of carbonic anhydrase IX (CAIX), a cellular biomarker of hypoxia, which correlated with an elevated presence of apoptotic tumor areas. Furthermore, we evaluated tumor cells of TEM1-TT-vaccinated mice for Ki67 expression and found Ki67 down-regulation, indicating a less proliferative state compared to tumor cells from control-treated mice (Andrea Facciabene, unpublished results).

The ability of our vaccine to provide a secondary tumor-specific immune response represents the third and last step of the therapeutic effectiveness of TEM1-TT vaccine. All of these above events (hypoxia, tumor EC/pericyte apoptosis, tumor cells' proliferative status/apoptosis) can generate a source of antigenic determinants that can be engulfed by antigen-presenting cells (APC) such as DC and macrophages. These APC are able to process specific tumor cell antigens by cross-priming, promoting a secondary immune response [30] against the tumor-specific antigens. In our tumor models, CT26- and TC1-associated tumor apoptotic/necrotic bodies were processed by APC inducing a secondary immune response against specific TAA; the immunodominant antigen of CT26, $gp70_{423–431}$ (AH1 peptide) is derived from viral antigen Gp70 encoded by the genome of BALB/c mice [31]. Gp70 is poorly expressed in normal tissues [32]; AH1-specific T cells escape negative selection that can therefore be activated and expanded by inflammation associated during tumor progression [33]. In the TC-1 tumor model, a C57BL/6 epithelial lung tumor, tumorigenesis is maintained by the expression of the viral proteins E6 and E7 of HPV origin. E7 is recognized by the immune system and elicits a selective CTL response against the E7 immunodominant peptide $E7_{49–57}$ [34]. Our data clearly demonstrate that splenocytes from TEM1-TT-vaccinated mice contained $CD8^+$ T cell specific immune responses against CD248 immunodominant peptide (but not against the control CD248 peptide), as well as against the AH1 epitope in CT26-tumor-bearing mice or $E7_{49–57}$ epitope in TC1-tumor-bearing mice [26]. Moreover, the frequency of the specific TAA-related immune response correlated with the tumor volume, suggesting that the magnitude of the secondary immune response was important to control tumor progression. The induction of this secondary TAA-specific anti-tumor response amplifies the magnitude of the therapeutic effectiveness of the CD248-targeting strategy.

To define the immune system component (adaptive and/or humoral) that was responsible for the anti-tumor effects, we performed $CD3^+$ T cell adoptive transfer or adoptive serum transfer from TEM1-TT-immunized naïve donor mice into naïve mice that were irradiated and challenged with CT26 tumor and found that $CD3^+$ T cells exclusively mediated the anti-tumor effects [26]. To characterize the potency of the TEM1-TT vasculature-targeting component with the secondary TAA immune response component, we performed $CD3^+$ T cell adoptive transfer from either TEM1-TT-immunized mice (vaccinated only) donor mice or TEM1-TT-immunized tumor-bearing (vaccinated followed by tumor challenged) donor mice into recipient mice that were challenged with CT26 tumor. Both types of adoptively transferred donor cells significantly increased the survival of recipient mice once challenged with CT26. These data demonstrate the anti-CD248 immune response in the recipient mice adoptively transferred with $CD3^+$ T cells from vaccinated only donor mice (without concomitant cross-priming and epitope spreading) was sufficiently potent to control tumor growth [26]. This observation suggests that the therapeutic ability of TEM1-TT vaccine is not secondary to development of an anti-TAA response but is solely capable, on its own, to limit tumor progression. We hypothesize that the anti-CD248 cellular immune response is the initial triggering event that generates and sustains a secondary TAA cross-priming event, expanding TAA tumor-specific cytotoxic $CD8^+$ T cells that control tumor growth.

Since neo-angiogenesis is also a physiological process that occurs during wound healing and reproduction, we investigated whether these two physiological processes were negatively affected by the TEM1-TT vaccine. Our results demonstrate that CD248 targeting did not mediate normal vessel damage in the skin by inducing toxicity during wound healing. Moreover, we showed that TEM1-TT immunization did not alter the pregnancy success rate, the time to gestation, the total litter size and the pup weight at birth compared with control immunization; there were also no anatomical or histological abnormalities in embryos from TEM1-TT- or control-immunized groups during early or late gestation. Lastly, our vaccine did not affect normal luteogenesis; in fact, TEM1-TT-immunized mice presented the same serum levels of 17β-estradiol, progesterone, luteinizing hormone and follicle-stimulating hormone during their estrus cycle compared to control-vaccinated mice [26]. These results are critical to demonstrate that TEM1-TT vaccine is a safe immunization strategy because it does not induce an immune response against physiological neo-angiogenesis compared with other anti-vascular therapy

such as bevacizumab, which is associated with important safety concerns, including wound healing [35] and pregnancy [36]. Overall, our data demonstrate that targeting the vasculature with a TEM1-TT DNA-based vaccine is a safe immunotherapeutic approach and further investigations are warranted.

Future perspective and potential applications

Identification and characterization of TVA such as CD248 and other TEM family members have created strong rationale and motivation to develop cancer immunotherapy against the tumor vasculature. The feasibility of this approach has been verified by experiments based on the use of *Tem*1$^{-/-}$ mice, where it was demonstrated that the genetic ablation of CD248 was not lethal and was associated with both control of tumor progression, local invasion and a reduction of metastases [24]. Our strategy to break CD248 self-tolerance using the sequence of modified TT adjuvant lays the foundation for developing other potential fusion protein constructs using cDNA of CD248 or other TEMs in association with the cDNA of either other microbial-derived proteins, such as the heat-labile enterotoxin B subunit (LTB) of enterotoxigenic *Escherichia coli* [37, 38], or other immunogenic molecules such as pathogen-associated molecular patterns (PAMP) [39]. The therapeutic effectiveness of electrogene transfer with the fusion vaccine encoding human CD248 (hCD248) cDNA will be tested in guiding clinical trials since the safety of DNA-based modalities has already been demonstrated [40]. Identification of the immunodominant peptide(s) of hCD248 should be a source of innovative clinical trials in which patients will be treated with new formulations of either emulsionated peptide immunization in which CD248 peptide will be injected with effective adjuvants such as montanide [41] or CD248 peptide-loaded DC vaccination protocols (Fig. 2). The most effective immunotherapy approach today is based on adoptive cell therapy (ACT) [42]. To test the potential of CD248-specific T cells, we adoptively transferred T cells from CD248-immunized mice into tumor-bearing mice and clearly demonstrate CD248-specific T cells are capable to reject established tumors. Engineered high-affinity anti-CD248 T cell ACT is a potential innovative approach in which the gene sequences of TCR α and β chains can be isolated and inserted into a lentiviral/retroviral vector carrying a bidirectional promoter capable of robust and coordinated expression of the two transgenes. Transduction of donor peripheral blood mononucleated cells (PBMC) with the lentiviral vector leads to generation of specific anti-hCD248-positive T cells. This strategy can overcome the obstacles of ACT in the clinic including technical factors limiting the availability of sufficient numbers of tumor-specific T cells as well as the availability of an antigen shared by tumors of different histologic patterns and conserved during tumor progression [43]. The potential of engineered human anti-CD248 T cells for adoptive transfer could be useful to treat a broad spectrum of histologically different cancers since many tumor types express CD248 to varying degrees.

In addition to targeting CD248 with T cell-based immunotherapy protocols, other investigators have been developing both diagnostic and therapeutic antibodies against CD248 antigen. Radiolabeled ^{125}I-Fc78 antibody was designed and based on a CD248-specific fully human single-chain antibody (scFv) 78, shown to bind with high affinity to both human and mouse CD248 [44, 45]. An ideal antibody-based therapy platform should have an antibody that can be used for diagnostic and therapeutic use (theranostic). The diagnostic antibody should be amenable to complexing to an imaging agent as well as complexing the antibody to a cytotoxic agent. The theranostic potential of MORAb-004 as a PET imaging tracer and naked antibody therapy for CD248^{+} tumor has recently been demonstrated [46]. (124)I-MORAb-004, a humanized monoclonal antibody targeting an extracellular epitope of hCD248, was evaluated for its ability to specifically and sensitively detect vascular cells expressing hCD248 in vivo [47]. Lastly, since CD248 is a cell surface marker, MORAb-004 antibody is currently undergoing phase I clinical trials for the treatment of patients with various cancers [15, 48]. With great enthusiasm, it is possible to use CD248-scFV to create chimeric antigen receptor (CAR)-bearing T cells to recognize hCD248 on endothelial targets in vivo. For example, CAR T cells directed against VEGFR have been shown to destroy the tumor vasculature and impair tumor growth [49]. Strikingly, up to 90 % of acute lymphocytic leukemia (ALL) patients and up to 50 % of chronic lymphocytic leukemia (CLL) patients are in durable remission with T cells engineered with CARs [50]. All of the aforementioned potential immunotherapeutic strategies based on CD248 targeting are summarized in Fig. 2.

CD248-based immunotherapy in combination with chemotherapy can be a suitable approach for translation into the clinic. The majority of chemotherapeutic compounds, when used at clinically useful doses, are endowed with intrinsic immunosuppressive properties owing to the fact that they preferentially kill rapidly proliferating cells. However, most of these drugs are able to stimulate the immune system against cancer by various different mechanisms: directly activating CD4^{+}, CD8^{+} or γδ T cells, promoting the production of IL-2, IFN-γ and IL-17, facilitating the maturation or activation of DC, inhibiting or depleting immunosuppressive cell subsets, triggering immunogenic cell death of tumor cells, up-regulating MHC-I molecules on cancer cells, increasing the permeability of tumor cells

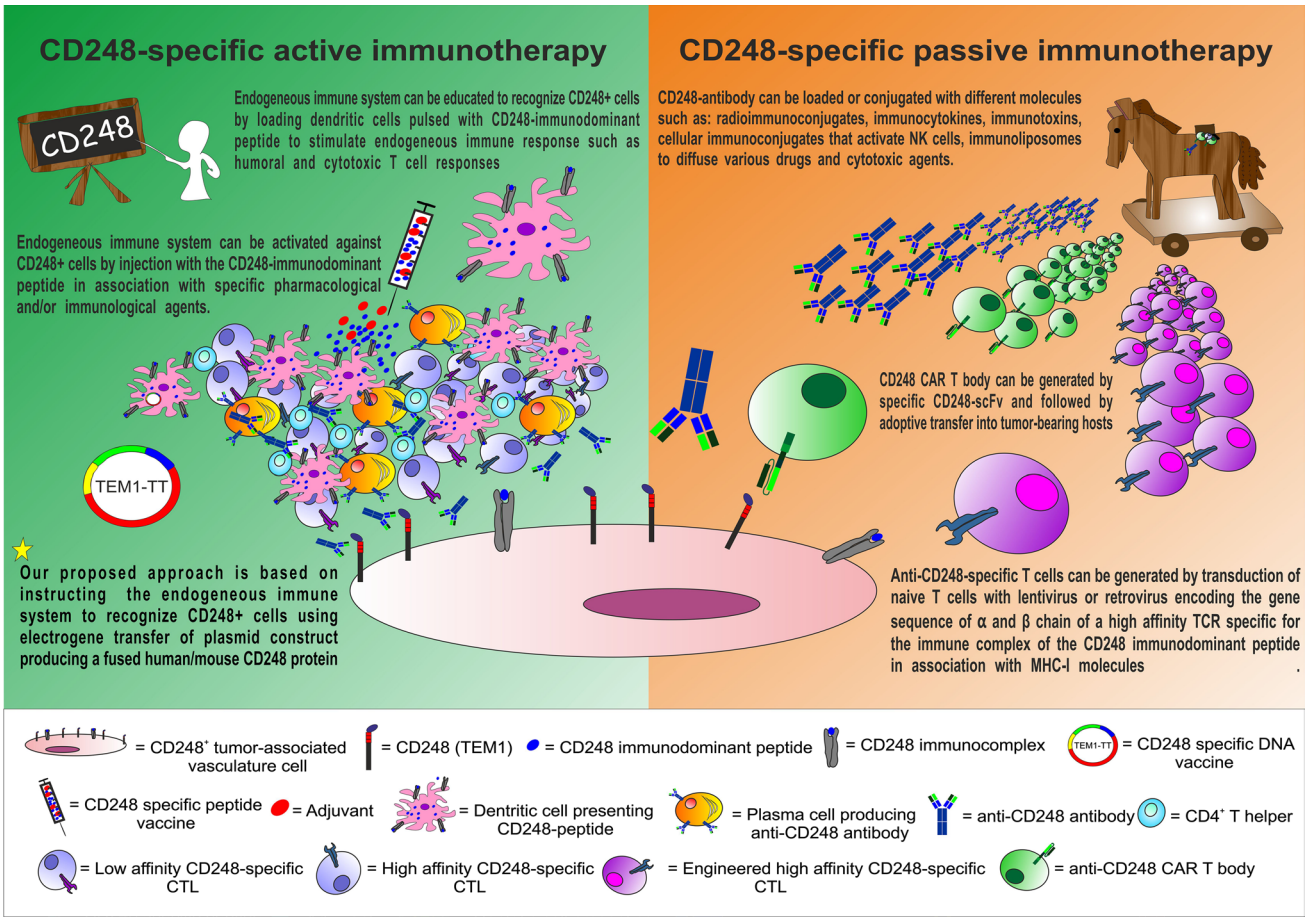

Fig. 2 Future development of CD248-targeting immunotherapy. Tumor-derived EPC/pericytes expressing CD248 antigen will be selectively eliminated by both active and passive immunotherapeutic approaches. The immune system of cancer patients can be educated to recognize the specific vascular antigen by several active immunotherapies such as DNA vaccines, based on fusing CD248 cDNA sequence with the cDNA of adjuvant molecules; peptide vaccines, in which the CD248 immunodominant peptide will be injected in association with specific adjuvants; and DC vaccines, in which syngeneic DC will be loaded with the immunodominant CD248 peptide and then injected into cancer patients. All or most of these strategies should be able to stimulate both an antibody- and cellular-mediated CD248-specific immune response. To overcome the immunosuppressive tumor microenvironment that limits the functionality of the immune response, resulting in protection of the transformed cells, the most successful clinical approach may likely be passive immunotherapy that, like a "Trojan horse," transfers a large group of highly potent effector immune agents into the tumor-bearing host. These Trojan horses can be in the form of anti-CD248 antibodies, anti-CD248 CAR T bodies or engineered high-affinity anti-CD248 T cells

to granzyme B and inhibiting immunosuppressive cytokine release such as IL-10 or up-regulating the expression of type I IFN [51]. These chemotherapy immune-modulating properties can be used to improve the therapeutic efficacy of CD248-based immunization both in preclinical and in clinical settings as previously described for different tumor antigens [52]. CD248-based immunotherapy might be combined with other anti-vasculature agents such as small molecule inhibitors (sunitinib, axitinib, pazopanib, vandetanib, cabozantinib, sorafenib) that target VEGFR2 and other receptor tyrosine kinases [53].

Finally, there is emerging evidence that tumor resistance occurs in cancer patients treated with conventional anti-vascular therapy. Several hypotheses to explain this resistance have been already proposed, and they include an increased redundancy of other angiogenic factors that compensate for the specific pathway blockade [54], the presence of a hypoxic microenvironment that modifies signaling pathways in both tumor and vasculature cells [55] and epigenetic mechanisms [56]. Moreover, bone marrow myeloid cells recruited by tumor-derived factors (TDF) play an important role in promoting and sustaining tumor angiogenesis/vasculogenesis and include Tie2-expressing monocytes, neutrophils and myeloid-derived suppressor cells (MDSC), generally defined as $CD11b^{+}Gr\text{-}1^{+}$ cells [57]. A role for $CD11b^{+}Gr1^{+}$ cells in tumor resistance to anti-VEGF antibody treatment was recently proposed by Ferrara et al. [58]. This effect depends on the up-regulated

production and secretion of protein Bv8 (also known as prokineticin-2) by $CD11b^{+}Gr\text{-}1^{+}$ cells specifically induced by granulocyte colony-stimulating factor (G-CSF). Thus, G-CSF overproduction by tumors has a deleterious effect promoting vasculature alteration through a Bv8-dependent pathway that bypasses VEGF and renders tumors refractory to anti-VEGF therapy. Pharmacological/immunological MDSC inhibitors are able to either inhibit MDSC differentiation/maturation from precursors or inhibit MDSC accumulation in lymphoid organs [59] and can be used to synergize with CD248-targeting modalities. Moreover, as recently described in the literature, the accumulation of T regulatory lymphocytes (Treg) at tumor sites has been correlated with biomarkers of tumor-associated angiogenesis such as VEGF overexpression and increased microvessel density in endometrial [60] and breast cancers [61] providing clinical indication for an association between Treg and angiogenesis. Treg can contribute to tumor angiogenesis through both indirect and direct mechanisms. Treg sustain angiogenesis indirectly by eliminating effectors T lymphocytes that secrete angiostatic soluble molecules such as TNF-α, IFN-γ, CXCL9, CXCL10 and CXCL11. Moreover, Treg are able to directly promote angiogenesis in ovarian cancer by production of CCL28 that increases VEGF levels in the tumor microenvironment [62]. Depletion of Treg significantly down-regulates VEGF expression in the tumor, and subsequently angiogenesis within tumor sites, and therefore favors an anti-tumor immune response. Immunological/pharmacological anti-Treg treatments include cyclophosphamide, gemcitabine, mitoxantrone, fludarabine, thalidomide analogues, cyclooxygenase 2 inhibitors, as well as Denileukin diftitox (Ontak), a fusion protein of human IL-2 and diphtheria toxin, able to reduce the frequency of Treg in peripheral blood of chronic T cell lymphoma patients [63]. Combination treatment with any of the above anti-Treg treatments with a CD248-targeting strategy may form a complementary approach against the tumor vasculature network. Moreover, the CD248-targeting strategy can be used to treat non-cancer-related inflammatory diseases such as arthritis; in fact, it has recently been reported that CD248 genetic deletion using $Tem1^{-/-}$ mice promotes osteoblast-mediated bone formation and reduces local inflammation [64]. In conclusion, our DNA-based immunotherapy approach targeting the tumor vasculature with a novel TVA demonstrates that CD248 can be targeted immunologically and generates potent anti-CD248 T cell responses as well as anti-tumor responses. Alternate therapeutic approaches targeting CD248 and other modalities in combination with CD248 targeting are warranted.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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