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Approaches to improve chimeric antigen receptor T-cell therapy in solid tumors.

Focusing on current CAR models and the tumor microenvironment.

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Summary

Chimeric antigen receptor (CAR) T-cell therapy, which combines the recognition function of the single-chain variable Fragment (scFv) with the cytotoxic abilities of effector T-cells, has showed promising results and has been FDA approved for CARs targeting tumor-associated antigens (TAAs) of several hematologic malignancies, but similar positive results in solid tumors are lacking. Five generations with different intracellular domains have been conceived to improve effects of CAR T-cell therapy. These changes were aimed at improving CAR T-cell stimulation, increasing their persistence and/or activating intrinsic immune cells. In addition, advanced models of CARs have been created. These include universal CARs binding to targeting molecules that can target a wide range of TAAs, bi-specific or tandem CAR T-cells that combine the activation of two different CARs, inhibitory CAR T-cells that can be inactivated after recognition of a healthy tissue molecule and physiological CARs that lack the commonly used immunizing murine-scFv. CARs expressed in Natural Killer (NK) cells can enhance their inherently existing tumor-toxicity. Finally, suicide-genes can be used for quick elimination in the case of excessive toxicities.

The tumor microenvironment (TME) consists of the tumor cells, extracellular matrix (ECM) components, abnormal tumor vasculature, tumor-associated cells (TACs), cytokines, chemokines and is an acidic and hypoxic environment with specific metabolites present. All these factors are involved in the improvement of tumor proliferation and metastasis, while reducing the antitumor effectivity of the immune system and CAR T-cell therapy. Improved infiltration of the TME can be achieved by local injections near or in the tumor, with the additional proposed advantage of reduced systemic adverse effects. Several strategies targeting the altered tumor vasculature are options for improved trafficking of CAR T-cells towards tumor sites while also hindering the tumor-proliferative characteristics of the TME. The chemokine-network in the tumor can also be targeted to reduce tumor-proliferative properties, and increased tumor-infiltration of CAR T-cells can be achieved by expressing receptors in CAR T-cells for highly expressed TME chemokines. Improving ECM-degradation could also improve tumor infiltration. Targeting the TACs is another option to improve CAR T-cell response. Additionally, a strategy often applied is to target inhibitory mechanisms, using cell-extrinsic or -intrinsic strategies. Finally the metabolic environment can be targeted either altering the environment before CAR T-cell therapy or altering CAR T-cell metabolism itself.

In conclusion, although currently not very effective in solid tumors, many aspects of CAR T-cell therapies can be improved. These include choosing the right (next-) generation of CARs, maximizing infiltration and activity in the TME and need to be combined the optimal TAAs and concomitant (pre)treatment steps. This could potentially lead to CAR T-cell therapies becoming an addition to our current arsenal of treatments against solid tumors.

List of abbreviations:

CAR: chimeric antigen receptor; scFv: single-chain variable fragment; MHC: major histocompatibility complex; TAA: tumor-associated antigen; TME: tumor microenvironment; CRS: cytokine release syndrome; TLS: tumor lysis syndrome; NFAT: nuclear factor of activated T cells; TRUCKs: T cells redirected for universal cytokine-mediated killing; uniCAR: universal CARs; TM: targeting molecule; biCAR: bispecific CAR; tanCAR: tandem CAR; iCAR: inhibitory CAR; CAR-NK: CAR-natural killer cell; iCasp; inducible safety switch caspase 9; CID: chimeric inducer of dimerization; ECM: extracellular matrix; ET_BR: endothelin B receptor; VEGF: vascular endothelial growth factor; RGS5: G-protein signalling 5 ; GPCR: G-protein coupled receptor; TNF: tumor necrosis factor; HSPG: heparan sulphate proteoglycan; HPSE: Heparanase; MDSC: Myeoloid derived suppressor cell; Treg: regulatory T cell; ATRA: all-trans retinoic acid; PDE-5: Phosphodiesterase-5; NSCLC: non-small cell lung carcinoma; HNSCC: head and neck squamous cell carcinoma; ADCC: antibody dependent cellular cytotoxicity; PD-L: programmed death ligand; DNR: dominant negative receptor; KO: knock-out IDO: indoleamine 2,3 dioxygenase; PKA: protein kinase A; ROS: reactive oxygen species

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Introduction

The first ideas of engineered chimeric antigen receptor (CAR) T-cells came in the late '80s and early '90s (1,2). CARs consist of an extracellular single-chain variable fragment (scFv) region, corresponding to the antigen-recognizing domain of antibodies produced by B-cells, which is fused to an intracellular stimulatory domain and inserted in T-cells. The strategy behind CAR T-cells is to combine antigen-specificity of the antibodies that is independent of the major histocompatibility complex (MHC), with the cellular cytotoxic capabilities of T-cells (2). CAR T-cell based immunotherapies provided a form of adoptive T-cell therapy with potentially higher cytotoxic capabilities than tumor infiltrating lymphocytes (TILs) used in adoptive T-cell therapy, as they could be made to target (tumor) self-antigens with high affinity. While T-cells that harbor T-cell receptors (TCRs) with those properties are usually removed from circulation or suppressed in their function, adversely affecting their antitumor function (3). These CAR T-cells might have greater possibilities in cancer-therapy than or could be used in parallel to already well established antibody-based therapies aimed at, among others, tumor antigens or immune checkpoints (3).

The main positive clinical results of CAR T-cell therapy have come from hematological tumors. With the first 2 CAR T therapies targeting CD19 of B-cells in B-cell lymphomas approved in 2017, tisagenlecleucel (KYMRIAH™) and axicabtagen ciloleucel (YESCARTA™), with recently more CAR T therapies gaining approval for other hematological malignancies (4-9). Apart from an occasional positive result, CAR-T cell therapy in solid tumors has been rather disappointing (3)(10)(11). Various reasons exist. Firstly, dose-limiting toxicities do not allow for the concentrations of CAR T-cells needed for optimal antitumor results. In contrast, B-cell aplasia found after treatment of hematological malignancies is generally tolerable . Secondly, solid tumors are very heterogeneous, making it difficult to find optimal tumor-associated antigens (TAAs), while tumor antigen loss or mutation in antigens can lead to so-called antigen escape. Finally, it is very difficult to direct CAR T-cells to the tumor, to infiltrate the tumor microenvironment (TME) and to achieve desirable anti-tumor activities in the inhibiting TME (10)(11).

This report focuses on the different designs and adjustments that have been made to CARs and indicate their improvements in relation to their persistence, safety and improved activity. Furthermore, emphasis is put on strategies aimed at improving infiltration and antitumor effects of CAR T-cells in the TME. The selection of the right TAAs to optimize antitumor effectivity while minimizing on-target off-tumor is another big threshold in the way of solid tumor treatment, but it will not be treated in this report (Rev. in (12)).

CAR T-cell therapy

The general idea behind CAR T-cell therapy combines the inherent cytotoxic abilities of T-cells obtained from a cancer patient with the introduction of a CAR that targets specific TAAs (explained in "The chimeric antigen receptor", Fig. 1a). These CAR T-cells are subsequently expanded in vitro and infused back into the patient with the goal of eliminating the TAA-expressing tumor cells (Rev. in (3)). This therapy has yielded successful results and FDA-approval for multiple hematologic malignancies, but the results have been poor in solid tumors (3)(10)(11). Around 200 clinical trials using CAR T-cell therapy for solid tumors have been registered at *clinicaltrials.gov*, targeting many different organs, many different antigens and using many different strategies (Rev. up to 8/20/2020(13)). From these trials, only a few patients, 35 according to (13), have shown objective responses in solid tumor treatment (11). There are several reasons for limited success in solid tumors in contrast to hematological malignancies. Solid tumors are usually not in the same compartment as where the CAR T-cells are introduced -in the peripheral blood-. Tumor cells express antigens heterogeneously that are shared with normal tissues, and are surrounded by a difficult to infiltrate and immunosuppressive TME (10)(11).

Side-effects of CAR T-cell therapy

Various side-effects occur in patients treated with CAR T-cells, in both hematological and solid tumor cases (Rev. in (14)). Most common is cytokine release syndrome (CRS), caused by the massive

and immediate release of cytokines by activated CAR T-cells, resulting in various symptoms including fever, tachycardia, hypoxia and even death (14). Another serious side effect, especially apparent in hematological malignancies, is tumor lysis syndrome (TLS), which after release of intracellular substances from lysed tumor cells can lead to many metabolic disorders and can also be life-threatening (14). In addition, TAAs are almost always also (weakly) expressed on normal tissues, which leads to on-target off-tumor toxicities when CAR T-cells are activated by these antigens (14). In both hematological and solid tumors, TAAs are often (merely over-)expressed compared to normal tissues (15). In hematological tumor-patients this is generally tolerable, can be targeted using replacement immunoglobin therapy and B-cells are usually reproduced within 2 months to 2 years (16). While in solid tumors on-target adverse effects might be more debilitating, as indicated by a lethal consequence of a patient treated against metastatic colon cancer (Expl. In (13)). Neurotoxicity is also often observed after treatment with CAR T-cells, with symptoms such as confusion, delirium and seizures (14). Administration of CAR T-cells at smaller doses, or treatment at earlier stages are ways to control toxicity (15). Other methods involve different CAR designs and will be elaborated in "Advanced models of CARs"(14). Donor-derived CAR T-cells may induce graft-versus-host-disease, while the often murine-derived antibody used in the CAR (see "The chimeric antigen receptor") can cause immune responses against the CAR T-cells (14). Universal and physiological CARs are methods of improving protection against these adverse effects, respectively ("Advanced models of CARs")(14).

The chimeric antigen receptor

In principle, a CAR is made up of three parts (Fig. 1a)(e.g. (10), (15)). The first extracellular part is the TAA-recognizing extracellular unit, usually a scFv consisting of the variable heavy (V_H) and variable light (V_L) chain fused together with a peptide spacer. In general, it seems that the epitopes and tumor-density of and the binding-affinity to the TAAs can influence the activity of CAR T-cells, providing another way to adjust the CAR T-cell response (10). Second is the transmembrane hinge subunit, which connects the extracellular TAA-recognizing part to the intracellular T-cell activating subunit(s), the length of this hinge can influence optimal target-binding and T-cell activation (17). The last, intracellular, part consists in principle of subunits capable of activating the desired responses in T-cells, such as activating cytotoxic, proliferative or cytokine-releasing pathways.

Generations of CARs

Several adaptations have been made to the intracellular part of the CAR receptor to alter and optimize the desired effects of CAR T-cells after TAA-recognition, leading to the current 5 generations of CARs (Fig.1b)(Rev. in (18)).

The intracellular domain of the first generation of CAR T-cells, first explored in 1993, consists of the signal-transducing subunit of the T-cell receptor, namely CD3ζ (2). However, the proliferation and cytotoxicity of these cells was not adequate (18). Still, the CD3ζ remained central in following generations of CARs.

Second-generation CAR T-cells were employed, around the year 2000, with additional stimulatory subunits such as, but not limited to, CD28 or 4-1BB (19)(20). These so-called co-stimulatory molecules were added with the purpose of increasing persistence and cytotoxicity of CAR T-cells after introduction in the patient (18).

The idea behind the third generations of CAR T-cells, making their presence in the second half of the 2000s (21)(22), was to combine effects of co-stimulatory domains by fusion of multiple of these domains to the intracellular domain. Possibly obtaining enhanced proliferation, cytokine secretion, survival and cytolytic activities (18)(15).

The fourth generation added a nuclear factor of activated T cells (NFAT) domain to the second-generation domain, that induces cytokines such as IL-12 or IL-15 after CAR activation (23)(18). These were called TRUCKs, which stands for "T cells redirected for universal cytokine-mediated killing" and is also descriptive as these CAR T-cells "deliver transgenic payloads" to the tumor (18) (23). The cytokines released by these TRUCKs can stimulate the functioning of other immune cells, in fact targeting antigens that are not targeted by the CARs, thus inhibiting antigen escape, targeting

antigen heterogeneity and reducing the inhibitory aspect of the TME (18)(23). In addition, they can increase persistence of CAR T-cells themselves (18). A major point of attention is the possibility of additional toxicities (18)(23).

The most recent 5th generation was first designed and expressed in T-cells in 2018 (24) and adds a IL-2 Receptor-B (IL2RB) domain between the two domains of the second generation CAR (25). This domain activates the JAK-STAT pathway leading to promotion of proliferation and prevention of terminal differentiation of CAR T-cells (25).

From the portion of clinical trials in solid tumors that indicated usage of a specific generation of CAR, 10 use or have used the first, 58 the second, 6 the third and 5 the fourth generation of CAR T-cells (7 the fourth generation safety CAR, explained in “suicide genes”). No trials currently make use or have made use of the fifth generation (13).

Advanced models of CARs

In addition to the intracellular modifications that have been performed, alterations to the extracellular domain which bind the specific targets, have also been carried out (Fig. 2)(Rev. in (26)).

UniCAR T-cells (uniCAR) or switching CAR T-cells are an interesting model for off-the-shelf therapy (Fig. 2a)(Rev. in (25)). It makes use of an extracellular CAR-domain, for instance biotin or anti-FITC (fluorescein isothiocyanate), that can bind target molecules (TM), often TAA-recognizing antibodies, leading to activation of CAR T-cells (25). TMs have the advantage of being accurately dose-controllable, and can thus control T-cell activity, which could improve side effects. Moreover, they allow for easier switching between TAAs, increasing efficacy against antigen escaping and heterogeneous tumors. This ease of switching also indicates a possible use as general

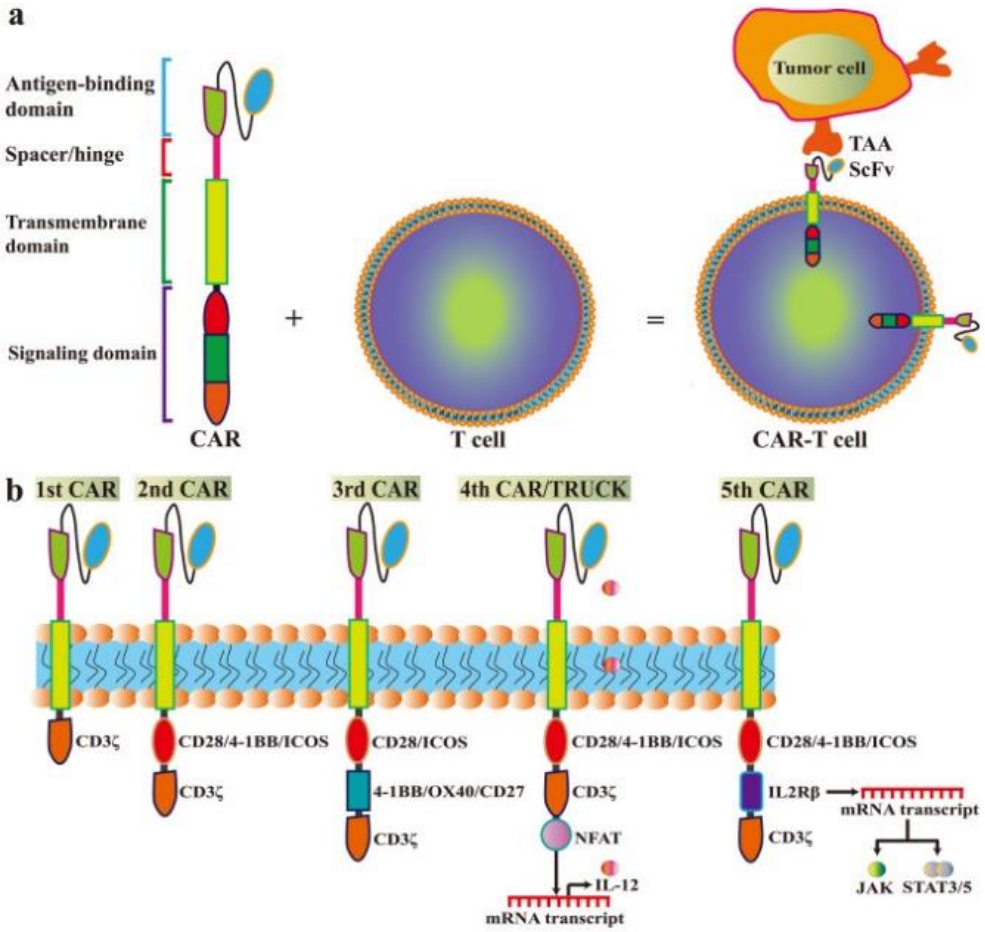

Figure 1. The principle CAR design and existing generations. a. The domains of a CAR and introduction into a T cell form a CAR T-cell. b. The five generations with different intracellular domains. CD3ζ=stimulatory domain, CD28/4-1BB/ICOS/OX40/CD27=co-stimulatory domain, NFAT=Nuclear factor of activated T-cells, IL2Rβ=IL-2 Receptor domain. (15)

off-the-shelf therapies, after the necessary steps have been taken to minimize GVHD (25). UniCARs have been used in different preclinical solid tumor models, using multiple different TMs, finding positive results (e.g. (27-30)). Several clinical trials are investigating uniCARs directed against hematologic Malignancies (Rev. in (31)), and a proof-of-concept has been achieved in Acute Myeloid Leukemia patients (32). A UniCAR T-cell therapy targeting PSMA in different solid tumors is in a phase 1 clinical trial (NCT04633148). The real potential of uniCARs in solid tumors is still unknown (25).

Bispecific CAR T-cells (biCAR) contain two antigen recognition domains, linked to two distinct intracellular domains (Fig. 2b). This can create both a synergistic effect when both CARs bind their TAA, which hinders antigen escape as CAR T-cells are activated when only one of the two CARs binds its TAA, or a protective effect, when CAR T-cells are only activated when both antigens are bound. This depends largely on the antigen affinity of the CARs and their target TAA abundance ((26)(14)). Preclinical tests of bispecific CAR T-cells have shown cytotoxic abilities (33)(34) and several clinical trials are currently performed against hematological malignancies, with some promising results (35). Bispecific CAR T-cells inhibited antigen escape and increased cytotoxicity against glioblastoma models (36). Currently no solid tumor clinical trial results are available (13).

Tandem CAR-T cells (tanCARs) also consist of two antigen recognition domains, however these are interlinked (in "tandem") and fused to the same intracellular domain (Fig.2c)(37). These were developed to target antigen loss. When both targets are bound, the stimulation of T cells is further enhanced, while TanCARs can also be activated when only one target is bound (37). TanCARs used against two antigens and even three antigens in preclinical models of glioblastoma have shown mitigation of antigen escape, improved cytotoxicity and more comparable interpatient cell-culture results, which because of the interpatient heterogeneity had previously complicated monovalent CAR design and efficacy (38)(39). Depending on the amount of the two (or three) antigens present on normal cells the on-target off-tumor toxicity can also be reduced, opening an avenue for improved safety (14).

Inhibitory CAR T-cells (iCARs) are designed to control on-target off-tumor responses using a CAR that inactivates T-cell responses after binding of an antigen expressed on normal cells (Fig.2d)(14). The intracellular domain is derived from immunoinhibitory receptors, such as PD-1 and CTLA-4, which after activation inhibits T-cell activity even if activating receptors are bound to their target as well (14). The effect of this inhibition can be temporary (40). One of the major requirements of inhibitory CARs are the identification of specific antigens that have relatively high and low expression in normal and tumor tissues respectively (14).

Physiological CAR T-cells were developed to prevent immune response and subsequent anaphylaxis to the CAR T-cells, which can happen when conventional CARs are used, as these contain scFv of murine origins (Fig.2e)(26). In this way physiological CAR T-cells, also known as

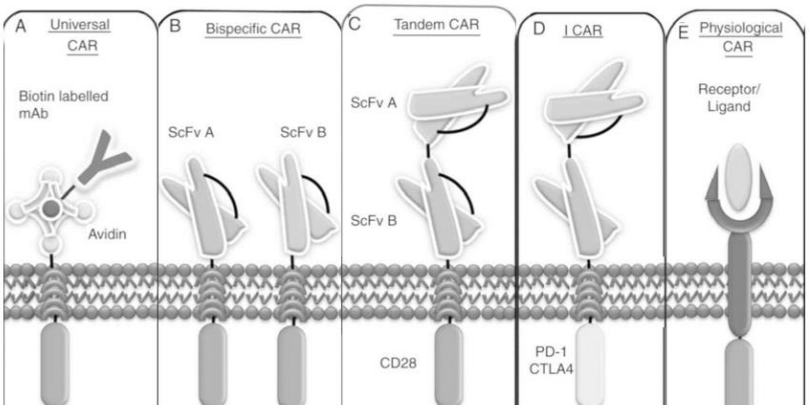

Figure 2. Advanced models of CARs. A. Universal CARs use a CAR that can bind a TAA-binding TM. B. Bispecific CARs uses two CARs fused to two distinct intracellular domains. C. Tandem CARs uses two CARs on fused to the same intracellular domain. D. Inhibitory CARs fuse a normal tissue-specific CAR to an inhibitory intracellular domain. E. Physiological CARs are not use murine derived and thus less immunogenic. (adapted from (24)).

receptor-ligand CAR T-cells, can improve persistence. Testing of physiological CAR T-cells in solid tumors is limited (26).

CAR Natural Killer-cells (CAR-NKs), not the main focus of this report, are another CAR-recipient cell-type of interest. NK-cells have been shown to be major safeguards against tumor growth and metastasis (Rev. in (41)). This inherent effector function of NK cells against metastases has led to several immunotherapeutic strategies aimed at improving NK-function, including CAR-NKs (Rev. in (42)). CAR-NKs have shown tumor-lytic abilities in models of both solid and hematological tumors (43)(44) (Rev. in(45)). Most clinical trials regarding NK cells focus on hematologic malignancies. Early results have shown good safety profiles, with no evidence of neurotoxicity, CRS or GvHD while some antitumor responses have been observed (46). Additional advantages of CAR NK- over CAR T-cells, aside from the safety profile, include higher antitumor toxicity, low expression of inhibitory receptors, better viability and lower costs (45). Several current trials focus on different solid tumors that have not shared results yet: (e.g. (NCT03692637)(NCT03692663)(NCT03941457)(NCT03415100)(NCT03940820)). Certainly, the development of CAR NK-cells is still in early stages, as compared to CAR T-cells, and many features need to be improved (46).

Suicide genes are used to be able to control CAR T-cell toxicity after introduction in the patient, as they allow rapid removal of CAR T-cells in the presence of an administered inducer (Rev. in (14)). Multiple suicide genes have been used and tested in (pre-) clinical trials, one example is inducible safety switch caspase 9 (iCasp9), which consists of a modified caspase 9 fused to a protein called FK506 binding protein. When a chemical inducer of dimerization (CID) is administered, iCasp9 is dimerized and activates a pathway resulting in apoptosis. In hematological preclinical models, administration of CID has shown to be a rapid and long-lasting elimination of the iCasp9-positive CAR T-cells (e.g. (47)(48)). Clinical trials for iCasp9 are ongoing for hematopoietic stem cell transplantations and this and other suicide genes could in the future be used in the CAR T-cell treatment of solid tumors to limit toxic side effects (14). 7 clinical trials against solid tumors make use of the iCasp9 in fourth generation CAR T-cells (13). Another suicide gene of interest is the herpes simplex virus-thymidine kinase with ganciclovir as inducing molecule (14).

The tumor microenvironment

The solid TME is a big threshold that needs to be overcome to achieve effective CAR-T cell therapy. The TME consists of the tumor cells, extracellular matrix (ECM) components, abnormal tumor vasculature, various non-malignant tumor-associated cells (TACs), such as effector immune cells, inhibitory immune cells and stromal cells like fibroblasts, various molecules, such as chemokines and cytokines, and has specific physiological and biochemical characteristics. (Fig.3)(Rev in (11)). CAR T-cells injected intravenously are not in the same compartment as the tumor cells, and the ECM together with the tumor vasculature form a physical barrier to CAR-T-cell entry (11). Some of the soluble molecules, such as vascular endothelial growth factor (VEGF) or andothelin-1, serve as regulation of tumor vessels, including promoting angiogenesis or downregulating the expression of adhesion molecules in the vessels, which further inhibits extravasation by effector (CAR) T-cells (11). The expression of chemokines is altered and can reduce or increase trafficking of effector (CAR) T cells and inhibitory immune cells (11). Tumor cells themselves often express ligands or receptors with inhibitory effects on T-cell activity, of which PD-L1/L2 is the most infamous example (11). This inhibitory activity is supported by inhibitory immune cells, like myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs) (11). The cytokines can have both effector functions, for instance activating effector immune cells, or inhibitory functions, such as the cytokines IL-4, IL-10 and TGF-β released by tumor cells and TACs (11). Finally the altered tumor metabolism results in the release of specific metabolites, acidification of the TME and low oxygen levels, which all negatively affect effector (CAR) T-cell functioning (11).

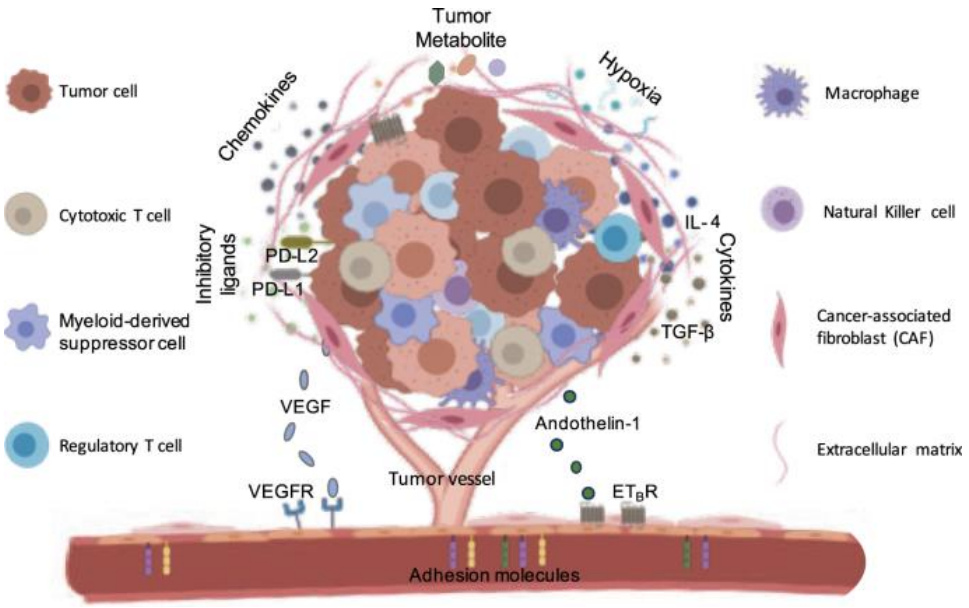

Figure 3. Schematic representation of the TME. All components of the TME contribute to tumor-proliferative conditions, while inhibiting the effector immune cells, such as CAR T-cells. PD-L= Programmed death ligand, VEGF=vascular endothelial growth factor, ET_BR=endothelin B receptor (11)

Infiltrating the TME

Many different strategies have been deployed targeting these different aspects have been devised that could improve CAR-T cell effectivity (Fig.4)(Rev. in (10)(11)(15)).

Local delivery of CAR-T cells

Local CAR T-cell injection makes sure that a high amount of CAR T-cells is present at the tumor, and it might also help in reducing the adverse side effects as it limits systemic distribution and thus reduces the antigen recognition of normal tissues by CAR T-cells (10). Examples of injection sites are intracranial for brain tumors or infusion of the hepatic artery for liver cancers. If the tumor itself can be reached relatively easily, then a direct intratumoral injection is another option, for example in breast or seminal vesicle cancer. Even though CAR-T cell accumulation in the systemic distribution is more limited, systemic circulation of CAR T-cells can still be detected. This is for instance shown in a phase 1 clinical trial by Zauderer et al, summarized by (49), which tested intrapleurally injected CAR T-cells against pleural mesothelioma and metastasic primaries to the pleura, and circulating CAR T-cells were still detectable months following the infusion. They propose that this could give effective results also for those metastatic tumors that are difficult to reach. Out of the CAR T clinical trials in solid tumors that indicate their injection sites, 9 are injected intraperitoneally, 19 in areas of the brain, 10 in the intrahepatric artery, 19 intratumorally, 3 transcatheter arterial infused, and 1 in both the pancreatic artery and vein (13).

Tumor vasculature

Normally, activated T cells express certain molecules, such as E- and P-selectin ligands that mediate T cells “rolling” on the endothelium of the blood vessels (15). Thereafter chemokine receptors can be activated by their ligand, of which CXCR3, CXCL9 and CXCL10 are important examples (15). This leads to the expression of the integrins, LFA-1 and VLA-4, which allow cell adhesion through ICAM-1 and V-CAM-1 respectively (15).

Tumor vasculature is different from the vasculature in normal tissues and is an important part of the low amount of T-cells precipitating in and near the solid tumor (11). There are three major differences in vasculature (11). Firstly, the absence or loose attachment of pericytes, a cell type of the vascular system, can cause leaky vessels and non-uniform bloodflow, reducing the movement of T-cells to the tumor. In addition, endothelial cells, another celltype of the vascular system, can obtain different morphologies. An example is upregulation of endothelin B receptor (ET_BR), that after interaction with tumor-secreted endothelin-1 inhibits expression of ICAM-1, and

as such reduces adhesion of effector T-cells. The final difference is called “endothelial cell anergy”, which describes the downregulation in endothelial cells of adhesion molecules, including ICAM- and V-CAM-1, due to overexpression of angiogenic factors like fibroblast growth factors (FGFs) or vascular endothelial cell growth factors (VEGFs), leading to reduced T-cell infiltration in tumors.

A variety of strategies targeting the tumor vasculature exist (Fig.4a)(11).

Induced expression of the regulator of G-protein signaling 5 (RGS5), due to hypoxic circumstances, has been shown to induce abberant maturation of pericytes and in turn abberant vasculature development (50). Whereas improvement of vasculature in tumors of RGS5-deficient mice showed enhanced immune effectivity, with significant increases of infiltration by adoptively transferred T-cells, and prolonged survival in mice (50). No RGS5 inhibitors have been approved for human use as of yet (51), but this RGS5 and other regulators of pericyte function could be interesting targets for improving CAR T-cell infiltration (11). G-protein coupled receptors, with often dysregulated expression or functioning in cancers, are important targets in their treatment (Rev in. (52)).

Upregulation of ET_BR, also from the family of GPCRs, in endothelial cells has also been targeted using an inhibitor called BQ-788 (53). This led to improved adhesion of T-cells in vitro and homing to tumors in mice models of ovarian and cervical cancer (53). Several drugs such as BQ788 mentioned before and the monoclonal antibodies Rendomab B1 and B4 can inhibit the ET_BR and have shown tumor growth-inhibition in some melanoma patients and cell lines respectively (54). These results suggest that targeting ET_BR could possibly help as a neo adjuvant therapy to immunotherapies like CAR T-cell therapies (53)(11).

The VEGF-activated pathway is another example of a possible target to improve CAR T-cell infiltration into the tumor (11). In mice and murine cancer models respectively, (55) and (56) showed that VEGF inhibition, by anti-VEGF antibodies, lead to increased tumor infiltration by the (adoptively transferred (56)) T-cells. It has also been demonstrated that anti-VEGFR-2 antibodies caused the tumor vasculature to normalize and enhanced the effect of vaccine therapy (57). Again in mouse models, some studies have shown antitumor and antiangiogenic effectivities of VEGFR-targeting CAR-T cells (e.g. (58)(59)). A clinical trial of VEGFR2-CAR T-cells only showed a partial response in 1 out of 24 patients treated (NCT01218867).

As with VEGFR, many other CARs have been designed against markers overexpressed in the blood vessels of many solid tumors, showing tumor inhibition in mouse tumor models (Rev. in (11)). However, in some cases toxicity has been reported (e.g. (59)(60)). So the recognition of VEGFR or other targets on normal vessels might be toxic and could prove to be an issue (11).

Targeting tumor blood vessels using a specific peptide fused to TNFα, called NGR-TNF, can potentially improve CAR T-cell infiltration into the TME. This fusion-product can target a particular peptide selectively expressed by the endothelial cells of angiogenic tumor vessels (61). Calcinotto et al. (2012) showed that low doses of this drug in prostate or melanoma tumor-bearing mice caused an upregulation of adhesion molecules in endothelial cells of the tumor vessels and an increase in local secretion of immunomodulating cytokines. These two effects reduced endothelial cell anergy and allowed an increased amount of immune cells to reach the tumor site (61). They also tested the efficacy of tumor vaccines or adoptive T-cell transfer and found significant increased survivaltime of the mice, even though complete regression was not found (61). A phase 2 study using NGR-TNF in combination with doxurubicin, found increased rates of progression-free survival and overall survival in relapsed ovarian cancer patients (62). These results suggest that NGR-TNF can aid the effectivity of immunotherapies (61), possibly also CAR-T cells. NGR can also be fused to other molecules with the possibility to alter the desired effects, with many options for tumor imaging as well (Rev. in (63)).

Chemokine-network in TME

The balance of chemokine networks is often altered in tumors, caused by tumor and associated cells. This induces recruitment of inhibitory immune cells and angiogenesis, prevents recruitment of effector immune cells

and induces a hypoxic environment. All contributing to tumor proliferation and metastasis (15)(11). As chemokine networks are used in many ways to increase tumor proliferation, they have been treated as major targets of experimental therapies (Rev. in (11)).

One example is the lack of secretion of chemokines CXCL9 and CXCL10, suppressing the attraction of effector T cells and NK cells to tumors (11). Using a murine model of ovarian cancer, it was shown that increasing the expression of these chemokines and other cytokines, such as IFNγ and TNFα, using a clinically approved DNA methyl transferase inhibitor, decitabine, lead to increased duration of survival of the mice. This was ascribed to increased homing of and differentiation into effector T-cells (64). Decitabine also increased the effectivity of checkpoint inhibitors CTLA4 (64) and PD-1 (65) (see "targeting checkpoint inhibitors") when used in combination in mice models of ovarian and colorectal cancer respectively. Decitabine treatment in-vitro of CAR T-cells inhibited T-cell exhaustion through inhibition of methylation of DNA, which promoted anti-tumor effectivity of CAR T-cells in in vitro and murine tumor models (66). Although not only centered around CXCL9 and CXL10 (65) (66), it does show the possibilities of using decitabine combined with (immuno)therapy.

CXCL12 enrichment in the TME leads to advantageous circumstances for tumor proliferation and metastasis (11). Through similar reasoning as with CXCL9/10 described earlier but in reverse, antagonists of the action of CXCL12 on its receptor CXCR4 have been tested and found to be effective in inhibiting tumor development in mouse models including breast cancer metastasis (67), using small interfering RNAs targeting CXCR4 mRNA. A CXCR4 antagonist in combination with a PD-1 inhibitor in advanced refractory solid tumors showed a satisfactory safety profile, with stable disease in 4 out of 9 patients (68). (CXCL12/CXCR4 axis Rev. in (69), targeting CXCL12-secreting fibroblasts treated in "tumor-associated cells").

Many other chemokine networks of the TME with various functions have been targeted (explored further in(11)). Some clinical trials have been conducted, for instance testing the safety of targeting CCR2 for pancreatic cancers (70). Testing of these chemokine-network altering drugs in combination with CAR T-cells is limited (11), but it could aid in CAR T-cell therapy, either indepently targeting different tumor-associated pathways, improving effector (CAR T) immune cell homing or both.

CAR T-cell chemokine receptors

There is a mismatch between chemokines secreted by the tumor and tumor-associated cells and the chemokine receptors expressed on CAR T-cells (11)(15). This inhibits "homing" of CAR T-cells to the tumor (11,15). Many strategies have been deployed targeting this inequilibrium, by engineering the expression of chemokine receptors in CAR T-cells (11)(Fig.4b).

On the basis of promising results with chemokine receptors engineered in CAR T cells for hematological malignancies (71), similar strategies have been developed for solid tumors (11). For example, CCR2b expression was engineered, using retroviral or lentiviral vectors respectively, in CAR T-cells targeting GD2 expressed by neuroblastoma cells (72) and mesothelin expressed by pleural mesothelioma (73) in a human xenograft mouse model. These receptors of CCL2 increased migration and antitumor activity of the CAR T-cells in both models. Expression of CXCR2 ligands, like the pro-tumor IL-8, is high in hepatocellular carcinoma cells (74) or can be induced by radiotherapy, which also induces CXCR1 ligand expression (75). CXCR2, or either CXCR2 or CXCR1 (75), was introduced in CAR T-cells, and results showed increased migration and antitumor effectivities in hepatocellular (74), and radiation-treated ovarian, glioblastoma and pancreatic tumors (75) in-vitro and in-vivo xenograft tumor models.

Instead of chemokine receptors, chemokine ligands IL-7 and CCL19 were expressed in CAR T-cells, in an attempt to mimic T-zone reticular cells, which maintain and form T-cell zones from which T-cells and dendritic cells can be recruited from the periphery. They found increased antitumor activity and survival of mice models of humanized mastocyma and lung carcinoma, and pancreatic adenocarcinoma (76). Interestingly, T-cells and dendritic cells had increased infiltrations into the tumor, as measured using immunohistochemistry staining on resected tumor tissues, and depletion of the normal mice T-cells decreased the antitumor activity, suggesting that these T-cells

are activated by the cytokine-producing CAR T-cells (76).

Extracellular Matrix

(CAR) T-cells need to actively degrade the ECM before being able to reach the tumor site (15). One important component of the ECM surrounding the tumor are the heparan sulphate proteoglycans (HSPGs) (15). To degrade these HSPGs the enzyme heparanase (HPSE) is needed, which itself has a dual tumor-promoting and -inhibiting function, with the latter being of interest in this case (Rev. in (77)). Caruana et al. (2015) found HPSEs to be underexpressed in in-vitro cultured T-lymphocytes, in contrast with "fresh" T-cells (78). They proceeded to engineer HPSE expression in CAR T-cells and found improved abilities of the CAR-T cells to degrade the ECM, infilitrate the tumor and antitumor activity in vitro and in vivo mice models, suggesting that engineering HPSE expression might improve the CAR T-cell activity, especially in tumors with stroma-rich ECMs (Fig.3c). The ECM is very complex, thus other strategies targeting other components of the ECM could also be interesting options, allowing better infiltration of CAR T-cells to the tumor sites, but also destabilising the tumor growth-enabling environment that is created by the interaction of the ECM with other components in the TME (10). CAR T-cells that target both the ECM and tumor antigens may eventually lead to improved results, however additional toxicity on ECM of healthy tissues needs to be closely monitored, and results from clinical trails are still lacking (11).

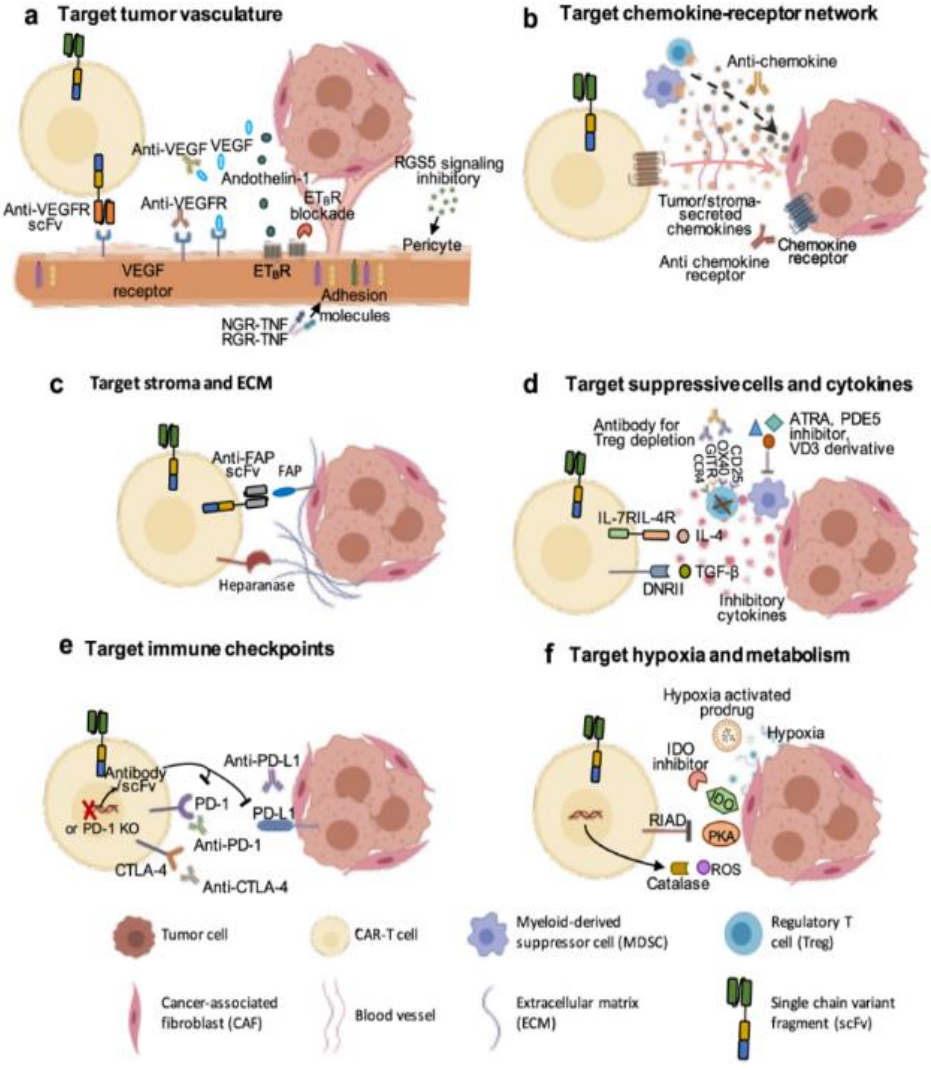

Figure 4. Strategies targeting the TME for improving CAR T-cell effectivities. a. Targeting the tumor vasculature can improve CAR T-cell trafficking, while reducing the tumor-beneficial TME. VEGF=Vascular endothelial growth factor, ET_BR=endothelin B receptor, RGS5= G-protein signalling 5. b. Targeting the chemokine network or expressing receptors can increase trafficking of CAR T-cells towards the tumor, while reducing inhibiting cells. c. Targeting of the ECM and of the stroma can increase CAR T-cell infiltration and reduce the amount of inhibiting cells. d. Targeting inhibitory immune cells and cytokines can enhance effector T-cell activities. ATRA= all-trans retinoic acid, PDE5= Phosphodiesterase 5, DNR= dominant negative receptor. e. Intrinsic and extrinsic strategies exist targeting checkpoint inhibition. f. Altering of the metabolic TME or altering metabolism of CAR T-cells can improve activities. IDO= indoleamine 2,3 dioxygenase, PKA= protein kinase A (inhibited by RIAD), ROS=Reactive oxygen species. Adapted from (11).

Optimizing activity in the TME

Tumor associated cells

TACs include stromal cells, such as fibroblasts, and inhibitory immune cells, such as MDSCs and Tregs, that support the inhibition of effector immune function by tumor cells (11).
Tumor-associated fibroblasts secrete CXCL12, the effect of which, as described in "chemokine-network in TME", can be inhibitory for T-cell trafficking and stimulatory for tumor proliferation (11).
This pathway can be targeted, in addition to CXCL12 antagonists, by targeting the fibroblasts directly (79)(Fig.4c). Fibroblast activation protein (FAP) is upregulated in most tumor types with scarce expression in normal tissues, and its function is correlated with several hallmarks of cancers, thus forming a good target for fibroblast-directed treatment (79). Several approaches exist targeting FAP, such as low molecular weight inhibitors, FAP-activated prodrugs, FAP antibodies and FAP-targeted CAR T-cells (rev. in (79)). Anti-FAP antibodies have not shown impressive antitumor capabilities, but could be used to target FAP when conjugated with specific active drugs or molecules (79). FAP-CARs have shown cytotoxicity in in-vitro models and increased antitumor effects and improved survival in in-vivo models of lung cancer, especially when combined with cancer cell antigen specific CAR T-cells, and models of pleural mesothelioma (80,81). Another study found that this effect was shown to be largely due to activation of the intrinsic immune system, as no results were found for immune-deficient mice (82). In some models however, FAP-CAR treatment lead to high morbidity and mortality, where FAP-positive osteogenic cells and possibly other cells of other organs were targeted (83). A phase 1 clinical trial targeting FAP and a specific cancer-associated antigen "Nectin4" in the treatment of various solid tumors is currently underway (NCT03932565), while a phase 1 clinical trial of anti-FAP-CAR T-cells against malignant pleural mesothelioma showed no toxicities, increased persistence of introduced CAR T-cells and activity in vitro (NCT01722149)(84). In general, most studies indicate that FAP-targeted treatments are not seriously toxic, and that it might be useful in the treatment of solid tumors, especially in combination therapy, for example improving the effect of standard chemotherapeutics, as a permanent cure was usually not the result in preclinical models using FAP-CAR monotherapy (79).
Tumors utilize regulatory mechanisms to inhibit the immune system, with many immunosuppressive cells, such as MDSCs and Tregs, involved in the TME (11)(Fig.4c)
One strategy is administration of all-trans retinoic acid (ATRA), which has shown MDSC reduction and effector immune cells induction in patients with renal cell carcinoma (85). Phosphodiesterase-5 (PDE-5) inhibition, known for vaso-dilating drugs such as Viagra/Sildenafil, can also inhibit MDSC-immune suppression, increase tumor infiltration of effector immune cells and increase the immune response in patients with head and neck squamous cell carcinoma (HNSS) (86). Many pre-clinical and clinical trials have been performed, using ATRA or PDE-5 inhibition, for several different (models of) solid tumors (Rev. in (87)(88)), which mainly show MDSC inhibition and support for synergistic effects with other (chemotherapeutic) treatments. In general, MDSC-targeting strategies are expected to improve T cell trafficking and effectivity of adoptive immunotherapies (11).
Tregs have also been targeted in many different ways (11). Antibodies targeting expressed molecules specific for Tregs, have been shown to deplete Treg cells in an antibody dependent cellular cytotoxicity (ADCC)-manner (89). Depletion of Tregs, or inhibiting their recruitment, proliferation, or activity are popular targets for cancer therapies in clinical and pre-clinical trials (Rev. in (89)). This has shown enhancement of vaccine T-cell immunity in mice models, but has not yet been combined with CAR T-cells (11).

Inhibitory checkpoints and cytokines

Tumor cells together with the tumor associated cells express many inhibitory ligands, such as PD1- and CTLA-4-ligands, and many cytokines, such as TGF-β, IL-4 and IL-10, normally involved in regulation and balance of T cell response, that inhibit the antitumor-activity of CAR T-cells in the TME (11). Regardless of co-stimulation domain used in the CAR T-cells, the expression of PD1-

ligands PD-L1 and PD-L2 has been shown to inhibit CAR T-cell functioning (90).
Many strategies exist targeting these PD1-ligands, which can be divided in cell-extrinsic and -intrinsic strategies (Fig.4e)(Rev. in (49)). Extrinsic strategies use checkpoint blockades, with for instance antibodies against PD-1 or PD-L1, which are approved for the treatment of some cancers, such as NSCLC, HNSCC and melanoma, and apply these in combination with CAR T-cells to increase efficacy in preclinical models (49). PD-1 blockade enhanced CAR T-cells efficacy in vitro models of neurblastoma and melanoma (91) and in vitro and vivo models of sarcoma, breast cancer, pleural mesothelioma (92)(90). A clinical trial of a combination therapy of CAR T-cells and PD-1 inhibiting antibodies (Nivolumab, Pembrolizumab respectively) is underway for patiens with glioblastoma (NCT04003649) and metastatic pleural disease coming from mesothelioma, lung cancer or breast cancer (NCT02414269). Conclusions can not be drawn from completed clinical trials, as the groups of patients were too small, and further investigation is needed to assess safety and efficacy, with the short-life and variable TME infiltration of PD-1 antibodies and risk of systemic toxicity being possible limitations of this approach (49).
PD-L1 blockade on MDSCs using antibodies or adenovirus-expressing "mini-antibodies" has been effective in enhancing CAR T-activities in mice models of colorectal cancer with liver metastasis (93) and HER2-positive tumors (94).
One intrinsic strategy is the engineered expression of anti-PD-L or anti-PD-1 antibodies by CAR T-cells, with these CAR T-cell obtaining enhanced activities in a model of renal cell carcinoma (95) and PD-L1-positive models of solid tumors (96)(97) respectively.
Engineering a "dominant negative receptor" DNR of PD-1 that is missing the intracellular signalling domain in CAR T-cells, has shown enhanced antitumor activity in mesothelioma and lung cancer mouse models (90). DNR PD-1 receptors were similar in efficacy compared to anti-PD-1 antibodies (98). Combining the PD-1 extracellular domain with a CD28 intracellular signalling domain enhanced CAR T-cell activity against solid tumors in mice (99).
Another strategy used gene-editing to create "knock-out" (KO) PD-1 CAR T-cells, which improved cytotoxicity in vitro and in vivo (100)(101). However, this might create counterintuitively exhausted T cells and PD-1 editing might induce additional genotoxicity (49). PD-1 KO CAR T-cells are currently under clinical investigation in a variety of solid tumors (Rev. in (102)).
It is unclear whether CAR T-cell therapy in combination with checkpoint blockades can provide the necessary antitumor activity, while on-target off-tumor toxicities might become additional barriers (49).

To overcome the inhibition of the cytokine TGF-β, CAR T-cells can be made to express a DNR of TGF-β receptor. This practice has shown increased cytotoxic ability in vitro and prolonged survival in vivo in preclinical trials of several tumors, including renal cell carcinoma, melanoma (103) and prostate cancer (104). Based on the results of the preclinical trials for prostate cancer, a phase 1 clinical trial is underway using a DNR-TGFβ-receptor PSMA-targeting CAR T-cell therapy for relapsed and refractory metastatic prostate cancer (NCT03089203)(104). A risk of these types of adjustments is "lymphoproliferative syndrome", which is an antigen- and growth factor-independent massive expansion of CAR T cells (105)(Fig.4d).
Fusion of the extracellular IL-4 receptor domain to the intracellular signalling domain of IL-7, a so-called inverted cytokine receptor, has been applied in CAR T-cells targeting prostate stem cell antigen (106). In an IL-4-rich environment, mimicking the IL-4 rich TME of tumors such as pancreatic cancer, these CAR T-cells showed increased antitumor activity (106). A similar strategy has also been designed to convert TGFβ-inhibiting signals to IL-7-like activating signals in prostate cancer, showing improved effectiveness in vitro and vivo models (107).

Metabolic and hypoxic tumor environment

Inhibition of CAR T-cells is further achieved by the creation of an environment deprived of nutrients, with low oxygen levels, an acidic pH and many metabolites with immunosuppressive characteristics (11).
An essential nutrient for T-cell metabolism is tryptophan and upregulation of the tryptophan-degrading indoleamine 2,3 dioxygenase (IDO) by the tumor cells suppresses T-cells (11). IDO

inhibitors have been shown to activate intrinsic immune effector cells, suppress inhibotory immune cells and improve therapy with checkpoint blockades, inhibiting tumor growth in in vivo models of pancreatic ductal adenocarcinoma, melanoma and glioblastoma (108-110). In a lymphoma mouse model, it was shown that treatment with lymphodepleting drugs that are frequently administered before CAR T-cells, fludarabine and cyclophosphamide, decreased IDO expression and improved CAR T-cells efficacy (111) (Fig.4f).

The TME becomes hypoxic due to excessive oxygen consumption needed for the tumor proliferation and limited oxygen supplied due to impaired vasculature. This reduces CAR T-cell effectivity, mainly due to hypoxia-inducible factor (HIF) proteins that, among others, induce PD-L1 upregulation on tumor cells and MDSCs and the production of immunosuppressive metabolites (11). Hypoxia-activated prodrugs and other treatments that alter the amount of HIF present or the activities of HIF could be used in treatment of solid tumors (112). Targeting of these proteins has not yet been tested in combination with CAR T-cells in clinical trials (11) (Fig.4f).

Prostaglandin E2 and adenosine are two inhibitory metabolites that inhibit proliferation and activity of T-cells through protein kinase a (PKA). Disruption of PKA localization and thus of its functioning, using CAR T-cell coexpressing an anti-mesothelin CAR and a PKA-inhibiting peptide, resulted in increased antitumor activity in an in vivo model of mesothelioma (113)(Fig.4f).

Reactive oxygen species (ROS), such as H_2O_2, inhibit immune responses and are also possible targets of new strategies. Catalase-expression introduction into CAR T-cells reduced oxidative stress in the TME and enhanced in vitro antitumor effects of CAR T-cells while also improving protection and activation of intrinsic effector immune cells (11)(Fig.4f).

Overall, targeting of the metabolic and hypoxic TME could be an approach to improve therapeutic efficacy of CAR T-cell therapy. Although some preclinical trials show interesting results, currently clinical trial results are lacking (11).

Discussion

5 generations of CAR T-cells with different intracellular domains, leading to different effects, have been conceptualized. In current clinical trials, the second generation is most often used. In the case of new insights, the modular nature of the CAR will still allow for continuing refinement of CARs. Potentially allowing for the creation of specifically refined CARs for different tumor (sub)types or even patient-specific (3).

Universal CAR T-cells can be accurately dose-controlled and can quickly switch, using different doses and types of TMs, allowing for potential extra safety and increased efficacy against antigen heterogeneous tumors. The ability to quickly switch targets could also be used in general off-the-shelf CAR T-cell therapies. Preclinical trials against solid tumors showed positive results, while a proof-of-concept has been achieved in hematological tumor patients. Clinical trials investigating uniCARs against solid tumors are still limited, and the possible exciting use of universal allogeneic CAR T-cells is still uncertain.

Bispecific and tandem CAR T-cells show promise in either mitigating antigen escape or allowing a protective effect. Clinical trials against solid tumors still lack results, but solid tumor targeting preclinical trials and clinical trials investigating hematological malignancies have shown promising results.

Healthy tissue-specific inhibition of CAR T-cells could be achieved with inhibitory CARs, which would still allow for tumor-specific activation. Clinical trials using these CARs are still lacking and better knowledge of healthy tissue-specific markers is needed.

Physiological CARs could reduce host-immune responses, but clinical testing is still limited. In addition “humanized” scFv could also cause limited host-immune responses (14).

Early results of preclinical CAR-NK models have shown good safety profiles. CAR-NKs have many other possible advantages over CAR T-cells, such as higher cytotoxicity, lower inhibitory receptors expression and cheaper costs. Several clinical trials targeting solid tumors are underway. Compared to CAR T-cell therapy experience is more limited with CAR-NKs, with many features still in need of improvement.

Suicide genes, such as iCasp9, have shown reliable and quick inducer-dependent CAR T-cell degradation in pre-clinical trials. They are currently used in 7 clinical trials of solid tumor-targeting CAR T-cell therapies, indicating the confidence the scientific community has in these safety switches.

The tumor microenvironment is a demanding obstacle. Many different strategies have been designed to improve tumor infiltration and cytotoxicity of T-cells in the TME.

Local injection of CAR T-cells is already in use in clinical trials for a variety of solid tumors, indicating that this is a relatively easy and attainable method to possibly improve CAR T-cell homing in the TME of solid tumors. However not all solid tumors can be reached with a local injection.

Several drugs targeting the tumor vasculature have shown improved adhesion and infiltration of (adoptively transferred) immune cells in solid tumors. This indicates a possible use in combination with CAR T-cell therapy, although research combining these drugs with CAR T-cell therapy is still limited. A bit more advanced concerning the use of CAR T-cells; VEGFR- CAR T-cell therapy, and other tumor vasculature marker targeted CAR T-cells, have shown tumor growth inhibition in pre-clinical models, but also some toxic side-effects. In addition, a clinical trial only showed a partial response. Targeting tumor vasculature in combination with CAR T-cell therapy could potentially improve effectivity, but this potential is still not conclusively supported.

Agonists or antagonists of specific chemokines and their receptors, could be used with the purpose of inhibiting the tumor-proliferative TME. (pre-)Clinical results have shown some positive results in solid tumors, in combination with for instance checkpoint inhibitors. Research investigating their possible use in combination with CAR T-cells has not yet been performed.

Insertion of specific chemokine receptors that can bind ligands released by the tumor cells, or insertion of chemokine ligands themselves, in CAR T-cells has been tested and found promising results in pre-clinical models of several tumors. The identification of more potential chemokine receptors, and testing on a clinical scale is what is needed still to support this strategy.

Ensuring expression of necessary ECM-degrading enzymes, such as HPSE, is present in CAR T-cells could improve CAR T-cell therapy, but clinical trials are needed to confirm this potential. Targeting ECM components using a ECM-directed CAR is still under investigation in preclinical trials. AntiFAP-CAR T-cells targeting fibroblasts, could be used in the combination therapy with chemotherapeutics, as most clinical trials indicated an acceptable safety profile, but they have not shown curative results as monotherapy. Targeting MDSCs or Tregs has shown improved antitumor immune responses, inferring a possible combination therapy with CAR T-cell therapy, although this has not been investigated yet.

A lot of activity has been aimed at developing intrinsic or extrinsic strategies to reduce checkpoint inhibition and the action of inhibitory cytokines. The furthest developed, currently under investigations in clinical trials, for the treatment of solid tumors are combination CAR T-cell therapy with PD-1 inhibiting antibodies, PD-1 KO CAR T-cells and DNR-TGF-β receptors. Possible side effects need to be closely investigated, and results from clinical trials are still uncertain, but the use of checkpoint inhibitors in combination with CAR T-cell therapy holds a lot of promise.

Preclinical trials show promising results for the targeting of the specific molecular characteristics of the tumor environment in combination with CAR T-cell therapy. Clinical results are still lacking however, so whether using these strategies results in improved antitumor efficacies with tolerable safety profiles is still unknown.

To narrow the distance between the results obtained from preclinical and clinical trials, newer models are needed. As the often used cell-cultures and immunodeficient animal models do not replicate the complex TME. (11)

In addition, metastatic sites are different from the primary tumor sites, with respect to infiltration, sensitivity to therapy and immunosuppressive properties. Thus attention must also be paid to CAR T-cell efficacy in these metastatic sites as well (49).

A big challenge not examined in this report is the lack of specific tumor-associated antigens, more insight is needed into the specific antigens expressed by specific tumors, with no or very low expression in healthy tissues, for optimal

antitumor efficacy while minimizing toxicity to normal tissues (10). Using multiple target antigens, introducing healthy-tissue specific antigen inhibition or adjusting affinity are possible strategies to aid on-target off-tumor toxicity (10) (14).

Other issues not addressed in this report include the high costs of CAR T-cell treatment (14), which could at least partly be reduced if universal CAR T-cells can be adopted (25), and competition with other immunotherapies, such as CAR-NK, treated shortly, or T cell-engaging bispecific antibodies (BiTes)(Rev. in (114)).

In conclusion, although currently not very effective in solid tumors, many avenues of improving CAR T-cell therapies exist. Finetuning of all these different avenues, including choosing the right (next-) generation of CARs, maximizing infiltration and activity in the TME, in combination with finding the optimal TAAs and concomitant (pre)treatment steps, could potentially lead to CAR T-cell therapies becoming an addition to our current arsenal of treatments against solid tumors.

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