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Manipulating the tumor microenvironment by adoptive cell transfer of CAR T-cells

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Abstract
T-cells expressing synthetic chimeric antigen receptors (CARs) have revolutionized immuno-oncology and highlighted the use of adoptive cell transfer, for the treatment of cancer. The phenomenal clinical success obtained in the treatment of hematological malignancies with CAR T-cells has not been reproduced in the treatment of solid tumors, mainly due to the suppressive and hostile tumor microenvironment (TME). This review will address the immunosuppressive features of the TME, which include the stroma, cytokine and chemokine milieu, suppressive regulatory cells and hypoxic conditions, which can all pose formidable barriers for the effective anti-tumor function of CAR T-cells. Some of the novel next generation CARs that have been developed and tested against the TME, will be discussed, to highlight the status of current research in CAR T-cell therapy for solid tumors.

Genetically modified T-cells expressing synthetic chimeric antigen receptors (CARs) have the potential to revolutionize the therapy of cancer (Kalos et al. 2011; Porter et al. 2011). Dramatic, complete, and durable remissions in patients with refractory acute lymphoblastic leukemia (ALL) and lymphoma have been reported, with CAR T-cells targeting CD19 (CAR19 T-cells) (Grupp et al. 2013; Maude et al. 2018; Park et al. 2018; Neelapu et al. 2017; Schuster et al. 2017). Complete remissions have also been reported in patients with multiple myeloma using CAR T-cells targeting the B-cell maturation antigen (BCMA), and in leukemia patients with CAR T-cells targeting CD22 (Ali et al. 2016, Fry et al. 2018, Frank (Xiaohu) et al. 2017, Berdeja et al. 2017). The FDA approval of tisagenlecleucel (Kymriah) and axicabtagene ciloleucel (Yescarta), for the treatment of precursor B-ALL and relapsed or refractory diffuse large B-cell lymphoma (DLBCL) (both CAR19 T-cell products), has brought the world of adoptive cell transfer into the limelight, and heralded in the era of CARs in cancer immunotherapy.

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In contrast to the dramatic responses seen for hematological malignancies, so far, the efficacy of CAR T-cell therapy for solid tumors has been limited, largely in part due to the immunosuppressive tumor microenvironment (TME). This review will highlight some of the key features of the TME and some of the CAR T-cells that are being developed to overcome TME barriers.

Adoptive T-cell therapy

Adoptive cell transfer or therapy (ACT) for treatment of malignancy involves isolation of immune cells, (for e.g., T-cells) from a healthy donor or a patient with malignancy, manipulating those immune cells ex vivo to generate anti-tumor function, and then infusing the modified cells into the patient (Fig. 1). The rationale behind ACT is that the infused T-cells will recognize and bind to tumor-associated antigens presented on the surface of tumor cells and destroy them. As a concept, it was first proposed in the mid-1950s by Mitchinson, but until the mid-1970s it was difficult to culture and expand T-cells in vitro, limiting its potential. Several ground breaking studies in 1988, by Rosenberg et al. where a 50% response rate was demonstrated in patients with metastatic melanoma after the administration of anti-tumor immune cells, rekindled the field of adoptive cell therapy for cancer (Rosenberg et al. 1988) (Dudley and Rosenberg 2003, 2007).

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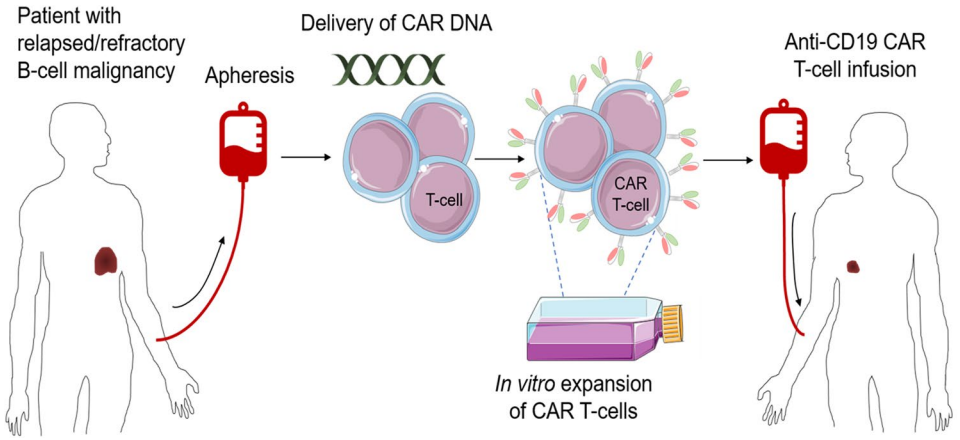

Fig. 1 Adoptive cell therapy involves collecting immune cells (e.g., T-cells) from a patient by apheresis, introducing the CAR DNA either by electroporation or by viral transduction, stimulating and expanding the T-cells in vitro, and infusing the genetically engineered cells back into the patient

ACT can be broadly classified into (a) Tumor infiltrating lymphocyte (TIL) therapy—TILs are anti-tumor T-lymphocytes found within tumors that can target unique epitopes—abnormally expressed antigens, non-mutated differentiation antigens, or mutated antigens (neoantigens) and are implicated in the elimination of tumor cells. TILs can be taken from metastatic lesions, cultured and expanded in vitro. While effective tumor-specific activity was exhibited by TILs that were obtained and cultured ex vivo, from melanoma tumors (Dudley et al. 2003), TIL therapy for other tumors like renal cell carcinoma, has been less effective (Andersen et al. 2018). Recently, successful application of TILs targeting mutant proteins along with administration of IL-2, and checkpoint blockade, in breast cancer patients has resulted in remarkable responses (Zacharakis et al. 2018).

(b) Transgenic T-cell receptor (TCR) therapy—A T-cell receptor recognizes processed peptides presented on the surface of a target cell, in the context of major histocompatibility complex (MHC). Engagement of the TCR with the peptide-MHC complex leads to activation of the T-cell and elimination of the cell expressing that protein. TILs and peripheral blood T-cells expressing TCRs specific for tumor antigens can be isolated and clonally expanded in vitro. TCRs from these tumor reactive clones can then be sequenced, cloned into a vector and used to transfer anti-tumor specificity to regular T-cells from the peripheral blood of healthy donors or cancer patients. ACT with engineered TCR against the NY-ESO antigen achieved significant responses in patients with melanoma and synovial cell sarcoma (Robbins et al. 2011, 2015) and those against the Wilms tumor antigen 1 (WT-1) and MART-1, MAGE-1 are under various stages of testing (Najima et al. 2016; Chodon et al. 2014). It is important to note that transgenic TCRs retain the need for interaction with antigen in the context of MHC. This means that the efficacy of transgenic TCRs is restricted to individuals with the same human leukocyte antigen (HLA) tissue type, limiting their application.

(c) Chimeric antigen receptor (CAR) therapy—Chimeric antigen receptors are engineered proteins that are created by bringing together the unique specificity of an antibody (to target a surface expressed tumor antigen) and the effector mechanism of lymphocytes (signaling and activation domains) (Maude et al. 2014; Kochenderfer et al. 2015). CAR expressing T-cells thus recognize antigens in a non-HLA restricted fashion, like an immunoglobulin, and carry out killing of the target like an effector T-cell. This broadens their application to individuals of any tissue type.

CAR T-cells

Chimeric antigen receptors are fusion proteins, not occurring naturally, but synthetic molecules that are created by combining the single-chain variable fragment domain from an antibody (scFv), specific to a target antigen, with a signaling and activating domain of the zeta subunit of the CD3 complex, in a single multi-domain polypeptide. CAR expressing lymphocytes can thus acquire antibody-specific reactivity. The engineered lymphocytes can release cytokines in response to antigen bearing cells without the constraints of major histocompatibility recognition (MHC) and lyse the target cells in a highly specific manner. The development of such chimeric receptors originates from pivotal studies by Kuwana et al. and Eshhar et al. in the 1980s and early 90s, where fusion constructs made up of a MHC independent binding domain with T-cell activation moieties were described (Kuwana et al. 1987; Eshhar et al. 1993; Gross et al. 1989; Romeo and Seed 1991).

The antigen-specific (humanized or murine) single-chain variable fragment (variable F_{ab}–scFv) of an immunoglobulin (Ig) is followed by a hinge or spacer domain (frequently from the Ig constant heavy regions or from the CD8 molecule), a transmembrane domain to anchor the CAR, then, one or several co-stimulatory domains (from CD28 or 4-1BB or OX-40 proteins) which are fused finally to the signaling

or activation domain, usually the CD3ζ chain (Fig. 2). First generation CARs contained only the scFv and the CD3-zeta signaling domain. While these had anti-tumor activity in vitro, early clinical responses were disappointing, with poor expansion, persistence and anti-tumor activity. It was theorized that these first-generation CAR T-cells were not receiving necessary co-stimulatory signals to replicate physiological activation, partly because tumor cells downregulate co-stimulatory ligands to escape immune destruction. Subsequently, second and third generation CARs containing one or two co-stimulatory domains respectively were constructed with increased anti-tumor activity and persistence in vivo (Sadelain et al. 2013; Figueroa et al. 2015). All successful CAR19 T-cell trials to-date have used second or third generation CARs. This emphasizes the potential impact of proteins other than the target antigen on successful T-cell immunotherapy and highlights how engineering of CARs can overcome tumor immune evasion strategies. By extension, an understanding of the effects of the tumor microenvironment will enable us to develop CAR T-cells which can better penetrate the tumor and resist tumor immune suppression.

Multi-functional next generation CARs

Complex and multi-functional CARs need to be developed which can target the various challenges posed by solid tumors. Lessons learnt from the CAR19 paradigm have helped design novel structures (4th Generation or next generation CARs) (Fig. 3), some of which have been tested in the preclinical setting against the tumor microenvironment.

Incorporating molecular "suicide safety switches" to overcome toxicities such as cytokine release syndromes are important as the CAR T-cells can be turned "off" when required (Budde et al. 2013; Bonini et al. 1997). Some of these safety switches include the herpes simplex thymidine kinase gene (HSV-TK) (which can be induced with a treatment of ganciclovir), inducible Caspase9 to trigger apoptosis (induced by the addition of the inducer FK506) and truncated EGFR/CD20 ("marked" for elimination upon addition of Cetuximab/Rituximab). Alternatively, CARs can have a split structure and engineered to turn "on," in the presence of a titratable inducer molecule in addition to the antigen (Wu et al. 2015).

Targeting two antigens can increase specificity, reduce off-tumor toxicity (especially when target antigens are present at lower levels on non-tumor cells), and enable precision killing. The "AND-gate," CARs make use of the Synthetic Notch (SynNotch) system allowing for conditional expression of the CAR (Morsut et al. 2016; Roybal et al. 2016a, b). The intracellular domain of the synthetic Notch receptor is cleaved upon the binding of one antigen (1st signal), which can then translocate to the nucleus, serve as a transcription factor to activate the expression of the CAR that targets the 2nd antigen. Tandem CARs (TanCARs) contain two linked scFv with different specificities to be activated only when both the antigens are present (CD19 + HER2; HER2+ IL13Ra2) (Grada et al. 2013). Dual CARs have the primary signal and the co-stimulatory signals coming from different CARs—with one CAR against one antigen having only the CD3z signaling domain, and the second CAR against the second target providing the co-stimulatory function, providing increased specificity (Mesothelin + Fra; MUC1 + HER2) (Lanitis et al. 2013; Wilkie et al. 2012).

A switch receptor (PD-1 and IL-4/IL-7) CAR converts a negative signal to a positive one while Self-driving CARs co-expressing chemokine receptors (CCR2b, CCR4) can help in the homing of the T-cells to specific regions. Armored CARs or TRUCKS (T-cells redirected for universal killing) can deliver cytokines like IL-12, IL-18, either in a constitutive or induced manner (NFAT driven) to the tumor site to potentiate an effective anti-tumor response. In addition, self-decision-making CARs such as the HIF-CARs have domains that can sense the oxygen levels in the TME and function accordingly.

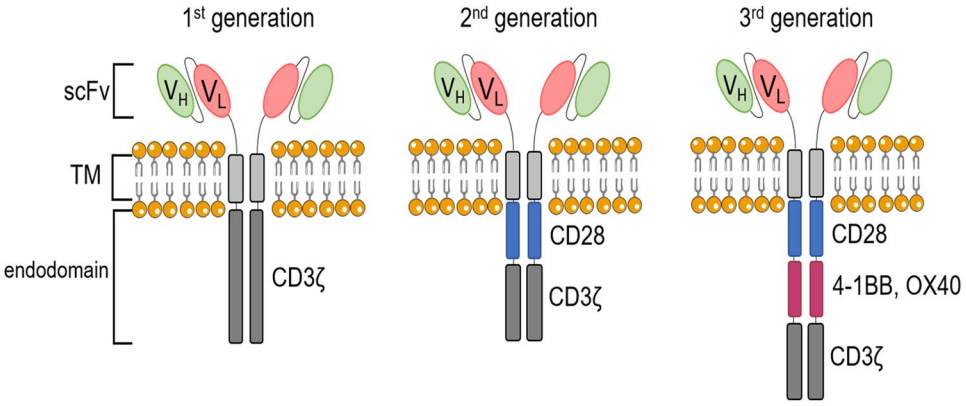

Fig. 2 Structure of CAR. The single-chain variable heavy chain is linked to the light making up the antigen binding moiety. This is fused to the transmembrane region of usually the CD28 protein via the spacer followed by the co-stimulation domain (CD28/4-1BB/OX40) and the cytoplasmic signaling domain from CD3ζ, while the 2nd generation included a single co-stimulatory domain. The 3rd generation CARs can include several co-stimulatory domains

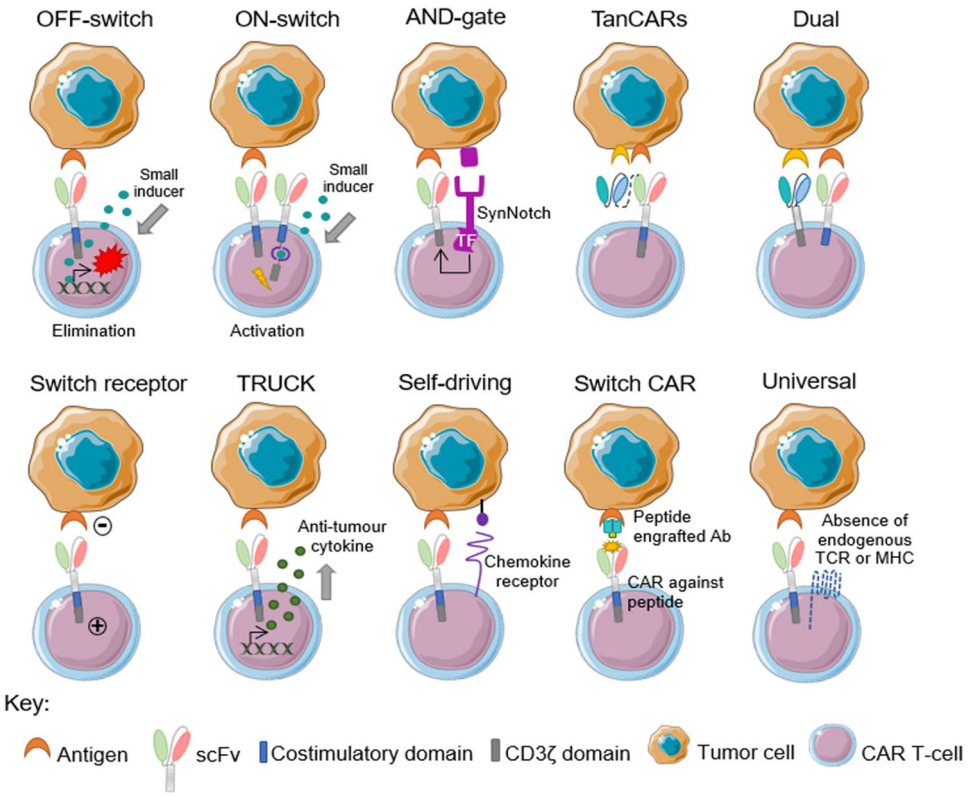

Fig. 3 Next generation CARs. OFF-switch CARs incorporate either an include caspase 9, (triggering apoptosis with the inducer FK506) herpes simplex thymide kinase gene (induced by ganciclovir) or truncated EGFR/CD20 (eliminated by Cetuximab/Rituximab) to stop CAR function, while ON-switch CARs have a split structure with the need for a small titratable molecule inducer, in addition to the antigen for effector function. Recognition of more than one antigen can increase specificity and enable precision killing. The "AND-gate" CARs make use of the synthetic Notch system, where binding of one antigen triggers cleavage of the intracellular domain of the Notch transcription factor, which can then activate expression of the CAR against the second antigen. Tandem CARs have linked scFV against two antigens (e.g., HER/CD19 or HER2/L13Ra2), while Dual CARs have the activating and stimulatory functions separated in two constructs, each targeting an independent antigen (e.g., HER2+FR+ or HER2+MUC1+). Switch receptors convert a negative signal to a positive one (PD1 switch), by substituting the negative intracellular signaling domain to an activating domain. TRUCKS (T-cell redirected for cytokine-mediated killing) are engineered to secrete cytokines like IL-12 or IL-18. Chemokine receptor expressing CARs (CCR4/CCR2b) are self-driven as they can traffick to the tumor in response to chemokine peptide, which can be linked to any antibody targeting any antigen. Universal CARs have their TCR and/or MHC disrupted for allogeneic applications

Currently, CARs with broad applications are being desired. An adapter/peptide specific Switch CAR can have multiple applications, as it is turned "on" upon application of a secondary antigen-specific antibody conjugated to the adapter (Rodgers et al. 2016). This single CAR can be "switched" to target various antigens and can be useful to overcome tumor escape variants. A single donor-derived T-cells can be used in allogeneic transfers, by the creation of Universal CARs with disruption in T-cell receptor (TCRα), and/or MHC loci to prevent graft vs. host disease or rejection (Eyquem et al. 2017; MacLeod et al. 2017).

With the development of genome engineering techniques like CRISPR/Cas9, it is possible to create a variety of CARs with an array of genes that can be incorporated in or knocked out. Transposon systems like the piggyBac and Sleeping Beauty are increasingly sought after, as they can overcome the limitations of viral vectors, in terms of cargo loads, that can accommodate several 100 kb, enabling the incorporation of large CAR DNA constructs, that allow targeting several factors simultaneously. Since multiple signals are needed to overcome the multiple suppressive factors in the TME, it is likely that standard viral vectors will not be as effective in delivering the complex next generation combination CARs.

Targeting solid tumors

Unlike B-cell malignancies where there are a few highly specific antigens to target, identifying safe target antigens with a restricted expression only on tumor cells and not on normal tissues is a tremendous challenge in the solid tumor field. In

addition, the level and pattern of antigen expression within solid tumors is often heterogenous, limiting the effectiveness of a single CAR targeting a single antigen. Immuno-editing with subsequent removal of immunogenic epitopes and antigen spreading leads to escape variants limiting the choice of available targetable antigens. Nevertheless, several antigens have been targeted for developing CAR T-cells against solid tumors and are discussed in Chen and Yang (2017) and Yong et al. (2017).

Clinical success of CAR19 T-cells in hematological malignancies

-ELIANA trial (Novartis) with tisagenlelecleucel (previously CTL019 developed by UPenn) in 75 patients (children and young adults) with long term median follow-up of 13.1 months, showed an overall remission rate if 81%, where 60% of patients achieved complete remission (Maude et al., 2018); JULIET trial with CTL019 reported a 64% response (18 out of 28) in adult patients with diffuse large B-cell (DLBCL) or follicular lymphoma (Schuster et al., 2017).

-ZUMA-1 phase2 trial (KTE-C19, KITE) with a median follow up of 15.4 months, where Axicabtagene ciloleucel was administered successfully to 101 patients with DLBCL, primary mediastinal B-cell lymphoma or follicular lymphoma, showed an objective response rate of 82% (Neelapu et al., 2017)

-JCAR014 (JUNO) early-phase trial enrolled 32 patients B-cell non-Hodgkin's lymphoma. 82% of the patients showed an overall response after lymphodepletion with cyclophosphamide and fludarabine (Turtle et al., 2016)

The ovarian cancer-associated alpha-folate receptor was among the first solid tumor antigens to be evaluated with a murine scFv against the antigen being linked to the Fc receptor γ chain. However, the lack of response was attributed in part due to the lack of persistence of the modified T-cells in patients beyond 3 weeks and the development of inhibitory factors in the serum (Kershaw et al. 2006). Some of the CAR T-cells being trialed in the clinic and in preclinical studies include those against the Carbonic anhydrase IX (renal cell carcinoma) (Lamers et al. 2006), carcinoembryonic antigen (CEA) (gastrointestinal tumors and colon cancer) (Beecham et al. 2000), HER2/neu (breast cancer) (Morgan et al. 2010; Zhao et al. 2009; Ahmed et al. 2015), Ganglioside GD2 (neuroblastoma and sarcoma) (Pule et al. 2008), mesothelin (mesothelioma) (Beatty et al. 2014), IL-13Rα2 (gliomas) (Brown et al. 2015; Zuccolotto et al. 2014), prostate-specific membrane antigen (PSMA) (prostate cancer) (Zuccolotto et al. 2014; Junghans et al. 2016), MUC-1 (Wilkie et al. 2008; You et al. 2016), MUC-16 (Ovarian cancer) (Chekmasova et al. 2010) EGFRvIII (glioblastoma) (Morgan et al. 2012), CD70 (renal cell carcinoma, thymic malignancies) (Shaffer et al. 2011; Wang et al. 2017), prostate stem cell antigen (PSCA) (Wei et al. 2017), CD171(Park et al. 2007), NKG2D (Lehner et al. 2012; Barber et al. 2007), Lewis Y (LeY+) Ag (Westwood et al. 2005), and IL13Rα2 (Pituch et al. 2018).

Comprehensive reviews of clinical trials using CAR T-cells are given in Holzinger et al. (2016), Fournier et al. (2017), and Hartmann et al. (2017), but suffice to say, the hunt for ideal solid tumor targets is still ongoing.

Overcoming the tumor microenvironment

The tumor microenvironment (TME) is made up an assortment of non-neoplastic cells recruited to nurture the neoplasm and the various matrix proteins, chemokines, cytokines, and growth factors they synthesize. The TME along with the stromal compartment helps in the establishment of the vascular network required to support the development of the tumor and can conversely suppress the effectiveness of CAR T-cell therapy. The challenges posed to CAR T-cells by the TME can be broadly divided into those posed by the suppressive cells and their secretory cytokines, the chemotactic environment preventing trafficking of the T-cells, physical barriers (stroma), and the functional inhibition due to physical contact between the tumor cells and the T-cells (Fig. 4). Furthermore, the hypoxic environment causes disorganization of vessels, leading to poor permeability and trafficking of T-cells. A shift in energy metabolism can further impair function and survival of the infused T-cells.

Suppressive cells and secretory factors

Immune suppression can be mediated by cells like T regulatory lymphocytes (Tregs), myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs), tumor-associated neutrophils and stromal fibroblasts, secreting suppressive factors and upregulating inhibitory ligands like PD-L1 (Pandiyan et al. 2007).

Tregs play important roles in immune responses. In addition to secreting TGFβ and IL10, activated Tregs can also contribute to the elimination or decrease of effector T-cells by rapidly taking up IL-2. MDSCs mediate T-cell suppression through a combination of various factors like inducible nitric oxide synthase (iNOS), arginase catalyze1, cyclooxygenase-2 (COX-2), prostaglandin E2, TGFβ, IL-10

indoleamine 2,3-dioxygenes (IDO), oxygen and nitrogen radicals. In addition, they support the growth of cancer cells (Khaled et al. 2013).

Tumor-associated macrophages (TAM) can express various chemokines and cytokines, including IL-10, matrix metalloproteinases (MMPs), urokinase-type plasminogen activator (uPA), fibroblast growth factor, vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), granulocyte-macrophage colony stimulating factor (GM-CSF), migration stimulating factor (MSF) and ornithine and polyamine due to the consumption of arginine, (all of which aid in the proliferation of tumor cells) (Solinas et al. 2010), in addition to depleting tryptophan. The MMP-2 and MMP-9 can specifically degrade the extracellular matrix, promoting migration of tumor cells (Condeelis and Pollard 2006).

MDSCs in the TME can express high levels of granulocytic markers (GR1). Depletion of GR1+cells was shown to augment the ability of CEA-CAR T-cells to control liver metastases in a murine model (Burga et al. 2015), suggesting a rationale to target MDSCs to increase the anti-tumor effect of CAR T-cells. Function of adoptively transferred CTLs was reduced by AML-associated Tregs in AML murine models. Even though depletion of Tregs alone did not have significant effects, a combination of Treg depletion and PD-L1 blockade showed improved anti-tumor responses (Zhou et al. 2010). Following the same approach, the efficacy of mesothelin CARs is currently being tested in combination with Treg depletion and PD1 block (Newick et al. 2016).

TGFβ is a common immunosuppressive factor present in the TME and can be overcome by the expression of T-cells with a dominant negative receptor. EBV-specific T-cells were modified to express a dominant negative (dn) TGFβ-receptor and Herceptin and were able to effectively lyse HER2 + tumor cells and overcome the inhibitory effects of the tumor-derived TGFβ both in vitro and in vivo (Foster et al. 2008; Bollard et al. 2002). In PSMA CAR T-cells

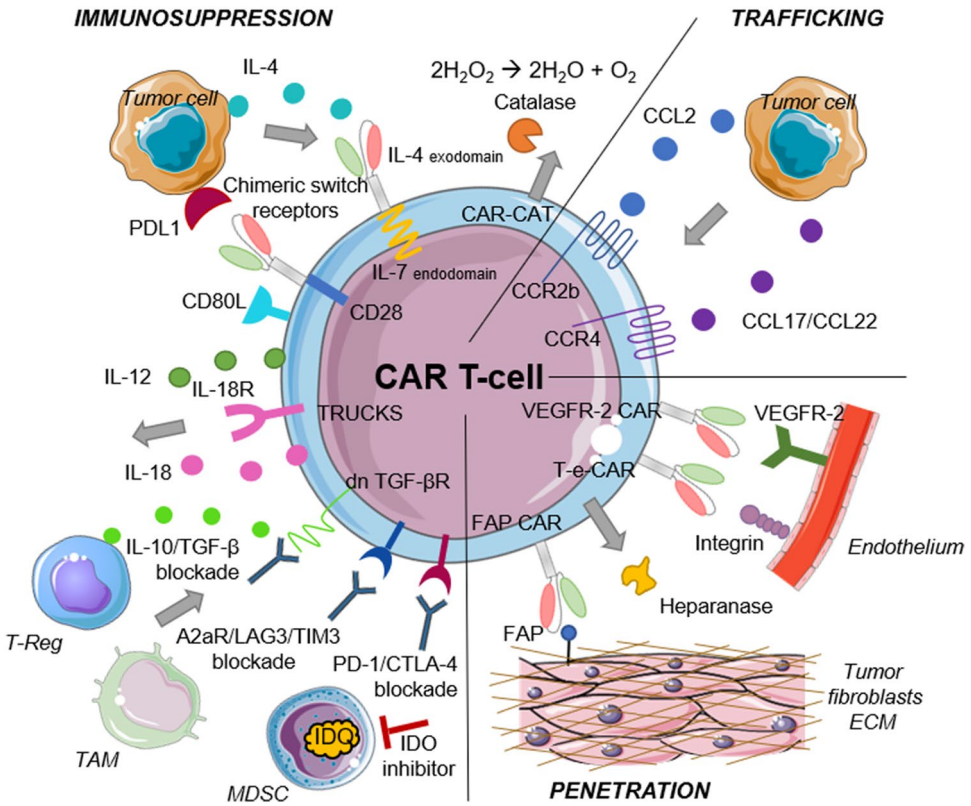

Fig. 4 The tumor microenvironment (TME). A diagrammatic representation of the immunosuppressive environment CAR T-cells need to overcome for an effective anti-tumor response. Trafficking to the tumor involves the expression of chemokine receptors like CCR2b, CCR4, corresponding to the chemokines secreted by the tumors and present in the TME. Penetration involves infiltrating the stroma and vasculature and overcoming the cytokine milieu. CAR T-cells expressing heparanase, or targeting FAP, VEGFR-2 and integrin are depicted. Suppressor cells [T-reg, Tumor-associated macrophages (TAM), Myeloid-derived suppressor cells (MDSC)] secreting adenosine, indoleamine 2,3 dioxygenase (IDO) are shown. CAR T-cells secreting cytokines (TRUCKS) like IL-12, IL-18, overcoming checkpoint inhibition, expressing anti-PD-1/anti-CTLA4/anti-PDL1 antibodies, a dominant negative signals to positive ones (IL-4 binding ectodomain fused to the IL-7 signaling endodomain, PD-1 extracellular with CD28 endodomain) and Catalase CARs (CAR-CAT) to breakdown hydrogen peroxide to overcome hypoxia are represented

expressing the dnTGFβ receptor II, effective block of signaling reduced the up-regulation of CD25 and CTLA4 and increased the CAR T-cell persistence, activity, and function in mice studies (Kloss 2016).

IL-4 and IL-10 are other suppressive cytokines that are frequently present in the TME. Chimeric switch receptors that can convert a negative signal of IL-4 to a positive one of IL-7 have been used to design prostate stem cell antigen (PSCA)-CAR T-cells targeting pancreatic cancer (Mohammed et al. 2017). The IL-4 ectodomain was fused to the prototypic Th1 cytokine, IL-7 receptor's endodomain to result in 4/7 inverted CARs that can now signal in an IL-7 fashion. This led to T-cell persistence in the presence of an otherwise suppressive IL-4 environment. This switch receptor thus conferred the added benefit of depriving the tumor cells of a growth factor while simultaneously transforming an inhibitory signal to one that will promote T-cell function. Recently, IL-7 and CCL19 expressing CAR T-cells targeting the CD20 antigen also showed improved anti-tumor responses in murine studies (Adachi et al. 2018).

Introducing effector cytokines using Armored CARs or TRUCKS (T-cells redirected for universal cytokine killing) that secrete IL-12 can enhance anti-tumor responses by counteracting the suppressive effects of Tregs, MDSCs and TAM (Yeku et al. 2017) (Zhang et al. 2011). IL-12 is a potent anti-tumor cytokine which helps in Th1 polarization, attracting endogenous T-cells and innate immune cells, and helps clear other cancer cells that are CAR antigen negative. IL-12 secreting TRUCKS targeting antigens like MUC16 (Koneru et al. 2015a, b), CD19, VEGFR2 (Chinnasamy et al. 2012) with enhanced activity have been described. Recently, anti-CEA CARs secreting IL-18 demonstrated superior anti-tumor activity as compared to the corresponding CARs without secretory cytokines. The IL-18 expression in the CAR T-cell favored a $FoxO1^{low}$ and $T\text{-}bet^{high}$ transcription factor profile, which enabled the CD8+ cells to maintain an effector phenotype and served to decrease suppressive cells in the TME. Moreover, the cytokine was produced in an inducible, CAR T-cell-dependent manner as it was under the control of the CAR activation-inducible NFAT promoter. These kinds of next generation TRUCKs ensure that "on demand" cytokines are produced only at the tumor site and controlled by the CAR signaling (Chmielewski and Abken 2017). Co-stimulatory signals can enhance CAR T-cell survival, proliferation, persistence and function, and this has been demonstrated in PSMA-specific CAR T-cells which have been modified to constitutively express co-stimulatory ligands like CD80 and 4-1BBL (Stephan et al. 2007).

Adenosine when produced at immunosuppressive concentrations in the TME can suppress T-cell responses by interacting with the Adenosine receptors (A2aRs) on activated T-cells (Raskovalova et al. 2007). CD73 expressed by Tregs, MDSCs or on tumor cells enables the conversion of adenosine monophosphate (AMP) to adenosine (Allard et al. 2016; Fallarino et al. 2006). Expression of CD73 limited responses to checkpoint blockade, (anti-PD1) which could be overcome with adenosine receptor antagonists (Beavis et al. 2015). Recently, CD73 expression by tumor and host cells has also shown to suppress immune responses mediated by anti-ErbB2 antibody in breast cancer (Turcotte et al. 2017). CD73 is thus becoming an important protein to target, in overcoming adenosine-mediated immunosuppression. Beavis et al. demonstrated that CAR T-cells upregulate A2aRs upon antigen stimulation and A2aR-deficient HER-2 CAR T-cells exhibited better therapeutic efficacy and enhanced responses in murine models (Beavis et al. 2017).

Tumor cells and myeloid cells can express indoleamine 2,3 dioxygenase (IDO), the enzyme that catalyzes the conversion of tryptophan to kynurenine which can inhibit CAR T-cell function (Munn and Mellor 2007). Lack of nutrients, like the amino acid tryptophan activates a stress response that regulates T-cell activity. In lymphoma xenograft models that contained cells expressing IDO, CAR19 T-cells were not effective in clearing the tumor cells (Ninomiya et al. 2015). However, treatment with fludarabine and a cyclophosphamide derivative, mafosfamide altered the levels of kynurenine and tryptophan, suggesting that, preconditioning chemotherapy before CAR T-cell infusion can be beneficial in combatting the suppressive TME by reducing IDO levels in addition to reducing the levels of Tregs.

The stroma creates physical barriers

The tumor stroma consists of non-malignant fibroblasts and mesenchymal cells and can form dense compact structures. Along with blood and lymph vessels, immune cells, matrix and inflammatory mediators, the stroma can thus pose formidable physical barriers for the penetration of CAR T-cells into the solid tumor (Mueller and Fusenig 2004; Orimo and Weinberg 2007).

The extracellular matrix, which includes glycopeptidases and proteoglycans, plays a major role in the remodeling of the immune response. Heparan sulfate proteoglycans have been described to play essential roles in tumor cell proliferation and migration (Theocharis et al. 2016). Remodeling of the matrix is a continuous and dynamic process during tumor progression. Fibroblasts can differentiate into cancer-associated fibroblasts (CAF), expressing markers like fibroblast activating protein (FAP), alpha-smooth muscle actin (aSMA), and stromal cell derived factor1a (SDF1A) (Vignali and Kallikourdis 2017). The cancer-associated fibroblasts and FAP can recruit endothelial cells and pericytes, cause collagen crosslinking (by releasing enzymes like lysol oxidase) and matrix degradation [by the secretion of matrix metalloproteinases (MMPs)] which can favor cancer progression. The secretion of CXCL12 by

the fibroblasts can also play a role in their differentiation (Kojima et al. 2010). Complex collagen structures resulting in dense matrix creating physical barriers for T-cell homing have been demonstrated in lung cancer models (Salmon et al. 2012).

The fibroblast activating protein (FAP) which is expressed on stromal cells in over 90% of epithelial cancers is an attractive target to overcome stromal barriers. Although there is some conflicting data with the anti-FAP CAR T-cells, with respect to efficient degranulation, production of effector cytokines and bone toxicities in mice, an intra-peritoneal model of FAP-CAR T-cells transfer in a mesothelioma xenograft had encouraging anti-tumor responses (Schuberth et al. 2013). Lo et al. have been able to demonstrate that FAP CAR T-cells can inhibit stromagenesis, reduce vascular density, and disrupt the spatial orientation of tumor cells in a pancreatic ductal adenocarcinoma tumor model (Lo et al. 2015). Some CAR T-cells can lose the expression of the Heparanase enzyme during the manufacture process, leaving them with an impaired ability to degrade heparin sulfate proteoglycans, which is a key component of the extracellular matrix, especially in stroma-rich tumors. A GD2-Heparanase CAR showed improved tumor infiltration, survival, and activity in vivo in neuroblastoma xenograft models (Caruana et al. 2015). Integrin αvβ3 is another protein that is highly expressed on endothelial cells of the tumor vasculature. Fu and colleagues described an echistatin-containing CAR (T-e-CAR) specific for αvβ3 integrin. Injection of these CAR cells led to significant tumor shrinkage in mice (Fu et al. 2013). Other groups have also trialed αvβ3-targeted CARs in ovarian, breast, and pancreatic tumor models (Whilding et al. 2017). Aberrant overexpression of VEGFR-2 in the tumor vasculature is associated with tumor angiogenesis (Ferrara and Alitalo 1999). Normalization of the vasculature by specific anti-VEGF antibodies led to increase in tumor-specific T-cell recruitment, indirect attenuation of FasL, improved efficacy, and clinical responses as seen in metastatic renal cell carcinoma (Shrimali et al. 2010). VEGFR-2-specific CAR T-cells are showing promising effects in several cancers and in combination with IL-12 secretion, altered the number and effect of MDSCs in the TME, and increased anti-tumor effects in murine models (Chinnasamy et al. 2012). The focal adhesion kinase (FAK) has shown to play an important role in tumor fibrosis formation resulting in an immunosuppressive TME in pancreatic ductal adenocarcinoma. FAK inhibition is being trialed clinically as a single agent and in combination with checkpoint inhibitors and chemotherapy (Jiang et al. 2016).

Thus, the CARs targeting FAP, VEGFR-2, Integrin αvβ3, or expressing heparanase have shown tremendous promise in preclinical studies in effectively penetrating the stroma.

The chemokine/chemokine receptor axis affects T-cell trafficking

The vascular endothelium can be considered as a dynamic cellular organ that controls the passage of nutrients, maintains flow of blood, regulates the trafficking of leukocytes by controlling the chemokine and cytokine make-up of the TME and can act as a barrier preventing the entry of CAR T-cells (Kalluri and Zeisberg 2006). Chemokines are a family of small heparin-binding proteins with conserved tertiary structures which are overexpressed by cancer cells, immune cells and stromal cells, and play key roles in tumor growth and remodeling of the tumor microenvironment (Kitamura and Pollard 2015). They create chemotactic gradients and regulate the trafficking of cells expressing the corresponding receptors.

Tumor cells (e.g., melanoma) and stromal cells abundantly express and secrete chemokines like CXCL1, CXCL8, and CXCL12, which attract and activate endothelial cells, synergizing with pro-angiogenic molecules like VEGF-A (secreted by either tumor cells or the TAMs and MDSCs) (Martin et al. 2009). Macrophages can release CCL1, CCL2, CCL3, CCL5, CXCL1, CXCL10, CXCL12 and indirectly contribute to metastasis, while fibroblasts can release CXCL6, CXCL12, CXCL14, CCL2, CCL5, HGF, IL-6, IL-1b, IL-11 that support cancer cell growth, proliferation, and angiogenesis (Kitamura and Pollard 2015; Joyce and Fearon 2015). Cancer cells can also express receptors such as CCR1-4, CCR7, CCR9, CX3CR1, CXCR4 which help in their migration and metastasis to secondary sites. Signals from the collagen matrix and collagen fibers help the cancer cells access blood vessels aiding in their migration and evasion from effector T-cells (Provenzano et al. 2008).

Trafficking of T-cells into the tumor is governed by the expression of chemokine receptors corresponding to the chemokines present in the TME. Lymphocyte migration is a regulated process involving selectins, chemokine receptors, and integrins. Selectin-dependent T-cell contact with endothelial cells promotes lymphocyte tethering, and rolling on the endothelium, which triggers a cascade of molecular events culminating in trans-endothelial migration, (diapedesis) and results in the movement of the T-cell via the chemotactic gradients, towards the target tumor (Johnston and Butcher 2002). Extensive collagen deposits and abnormal vasculature can prevent T-cells from rolling on the endothelium and infiltrating the tumor. Thus, the chemokine environment needs to be targeted by expression of corresponding receptors along with tackling the other physical barriers.

While Th1 T-cells generally express CXCR3 and IL-17 and IL-4 producing cells express CCR6 and CCR4 receptors, it is unlikely that CAR T-cells will constitutively express the myriad of chemokine receptors required to traverse the TME. Forced expression of chemokine receptors is one way

to respond to the chemokine gradient and this was demonstrated in models of Hodgkin Lymphoma (HL), where the suppressive chemokines CCL17 and CCL22 were overcome by the co-expression of receptor CCR4 in CAR T-cells targeting the HL antigen CD30. This resulted in increased trafficking of effector cells towards the tumor and efficient tumor clearance in mice (Di Stasi et al. 2009). Similarly, neuroblastomas secrete high levels of CCL2. Co-expression of the corresponding receptor CCR2b in anti-GD2-CARs resulted in increased trafficking, infiltration and subsequent increased anti-tumor effects (Craddock et al. 2010). Epstein Barr virus (EBV)-specific T-cells modified with a CAR against GD2, and which co-expressed IL-7Rα also showed increased proliferation and resistance to Treg-mediated immune suppression, and restored responsiveness to IL-7 (Perna et al. 2014).

Checkpoint inhibition

In addition to the TME, tumors can directly abrogate the immune response by expressing negative ligands, such as programmed death ligand 1 (PD-L1) or carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), resulting in suppression of T-effector function. The inhibitory ligands interact with T-cell checkpoint receptors like PD-1, TIM3, LAG3, CD160, CTLA-4, VISTA, causing T-cell dysfunction (Zhang et al. 2018). CTLA-4 recruits phosphatases against the CD3 activation domain and abrogates T-cell function by dephosphorylation of the tyrosine-based activation motifs (ITAM). In addition, the inhibitory motif ITIM in the intracellular domain of CTLA-4 can also get phosphorylated, leading to increased expression of the protein on the cell surface. PD-1 (programmed death receptor-1) can bind to the ligands PD-L1 and PD-L2 and decrease effector function via the SHP-1 signaling pathway. Lymphocyte activation gene-3 (LAG3) can negatively regulate MHC class II molecule with a higher affinity than CD4, preventing anti-tumor responses, while the T-cell immunoglobulin mucin 3 (TIM3) receptor can bind to Galectin-9 and inhibit T-cell responses while promoting apoptosis. The V-domain Ig suppressor of T-cell activation (VISTA) can reduce the production of effector cytokines. Several approaches to combat checkpoint inhibition are underway (Yoon et al. 2018; Zhang et al. 2018).

Since checkpoint inhibition is one of the major barriers to the effector function of T-cells, several studies have focused on developing ways to overcome this inhibition. CAR T-cells, especially those specific for CD19, HER2, mesothelin, CAIX, GD2, and CEA, acquire a differentiated and exhausted phenotype associated with increased expression of the inhibitory receptors. CARs specific for HER2 with an additional co-expression of a PD-1 blocking antibody demonstrated increased activity against HER2+ tumors (John et al. 2013). Strikingly, a decrease in MDSCs in the TME of the mice that were treated was also observed. In other studies, CEA-CAR T-cells against lung cancer upregulated PD-1 and were suppressed in activity due to the engagement of PD-L1 from the tumor cells and MDSCs. It was shown that GM-CSF promoted the up-regulation of PD-L1 in a STAT3-dependent manner in the MDSCs. CAR T-cell efficacy was re-established when either PD-L1 was blocked, or when the MDSCs were depleted, or when GM-CSF was neutralized (Burga et al. 2015). A small phase I trial combining GD2-CAR and PD-1 blockade, however, did not exhibit any significant benefit. Clinical success of CAR19 T-cell treatment in combination with anti-PD1 pembrolizumab is limited, with only a few trials having been conducted, though several other trials are ongoing (Yoon et al. 2018).

To overcome the PD-L1 activity in the TME, Suarez et al. designed a bi-cistronic CAR targeting carbonic anhydrase IX (CAIX) and secreting anti-PD-L1 antibody (Suarez et al. 2016). This resulted in a fivefold reduction of tumor growth compared to a CAIX CAR without PD-L1 Ab in a mouse model of clear cell and renal cell carcinoma. CAR19 T-cells incorporating anti-PD-1 Ab have also been described that showed a higher proliferation potential and decreased exhaustion phenotype as compared to the parental CAR (Li et al. 2017b). Mesothelin-CAR T-cells showed enhanced effector function when combined with PD1/PD-L1 blockade (using anti-PD1 Ab, or a dominant negative PD-1 receptor, or PD-1 shRNA) in murine models of mesothelioma. A PD-1 dominant negative receptor (DNR) contains the extracellular ligand binding domain of the receptor fused to a CD8 transmembrane domain to compete for PD-L1 binding. Using both these blocking strategies, the anti-tumor CAR function was improved (Cherkassky et al. 2016).

Chimeric switch receptors have been engineered by substituting the intracellular negative domain of PD-1 with the activating intracellular domain of CD28. The switch receptor containing CAR19 T-cells were able to overcome immunosuppressive effects (Liu et al. 2016) leading to increased anti-tumor activity. Using CRISPR/Cas9 technology, Rupp et al. generated PD-1-deficient CAR19 T-cells and showed enhanced clearance of PDL1+ tumor xenografts in vivo (Rupp et al. 2017). PD-1 knockdown using CRISPR/Cas9 was also demonstrated in prostate-specific cancer antigen (PSCA)-specific CAR T-cells with improved function (Ren et al. 2017).

Combining blocking of check point receptors and components of the TME have yielded promising results. Blocking PD-1 and IL-10 has been demonstrated to increase T-cell activity (Sun et al. 2015). Dual block of A2aR and PD1 has also shown to increase IFNγ and granzyme B release by CD8+ T-cells (Mittal et al. 2014). CD73 expression is a poor prognostic factor in head and neck squamous cell carcinoma

and is upregulated in the tumor infiltrating cells in this cancer type. Inhibition of CD73 was able to reverse exhausted phenotype via down regulation of PD-1 and CTLA4 (Deng et al. 2018). CD73 has been identified as a potential biomarker for response to anti-PD1 treatment in overcoming adenosine-mediated suppression and since the development of anti-CD73 antibodies have shown additive activity with anti-PD-1 antibodies (Beavis et al. 2015; Hay et al. 2016; Vijayan et al. 2017), it is likely that direct CAR constructs targeting CD73 when created will be efficacious. Similarly, dual IDO and PD1 block have been effective in patients and it is logical to expect the incorporation of these combinatorial blocks in CAR T-cells (Holmgaard et al. 2013; Spranger et al. 2014).

Combining TIM3 block with anti CEACAM1 antibodies have shown promising results in murine models of gliomas (Li et al. 2017a). Src homology 2 domain containing protein tyrosine phosphatase 1, (SHP-1) dephosphorylates kinases like Lck and ZAP70 and plays a crucial role in the downstream signaling from checkpoint receptors. Mesothelin-CARs with dominant negative SHP-1 showed a better response against PDL1+ tumors. The response was further augmented when combined with PD-1 blockade and is reviewed in (Yoon et al. 2018). Thus, SHP-1 blocking may present a novel way of preventing suppression mediated by the checkpoint receptors.

Hypoxia and nutrient starvation

Hypoxia and nutrient starvation are hallmarks of solid tumors. Hypoxia refers to the low oxygen concentration within tumors because of poor blood supply, aberrant vascularization and production of hydrogen peroxide. Hypoxic conditions can also activate unregulated angiogenic processes that favor abnormal distorted blood vessels (Ager et al. 2016), regulating abnormal T-cell trafficking and homing. Enhanced expression of FasL (inducing effector T-cell death), endothelin B receptor (that blocks the expression of ICAM-1, required for E-selectin based trafficking), CD276 (checkpoint molecule that can inhibit T-cell function), are detected in the vessels of triple negative breast cancer, glioblastoma, and pancreatic cancer [reviewed in (Vignali and Kallikourdis 2017)].

The distorted blood vessels result in poor permeability, leading to poor migration of the T-cells into the tumor (Ager et al. 2016). A high density of CD31+ CD134+ tumor vessels are generally associated with poor prognosis while the high endothelial vessels have better clinical outcomes (Dieu-Nosjean et al. 2016). Release of pro-inflammatory cytokines leads to endothelial venules (HEV) formation and maturation in murine models. HEVs in turn can lead to secretion of CCL21, the ligand for CCR7 and CXCR3 and can thus regulate the migration of naïve and memory T-cells (Peske et al. 2015). Targeting the hypoxic environment is a valid strategy to improve T-cell homing to tumors.

The hypoxia inducible factor (HIF) family of transcription factors are the best understood proteins involved in hypoxia-mediated gene regulation. The stabilization of the HIFs during hypoxic conditions results in the up-regulation of glycolytic genes like Glut1 and Glut3 and preferentially steers the cell down a glycolytic pathway, while blocking the oxidative phosphorylation pathway. Novel chimeric receptors based on the γ and β chains of the IgE receptor domains were fused to oxygen sensitive sub-domains of the hypoxia-inducible factor (HIF1α) and the scFv targeting CD19, to result in HIF-CARs whose function could be modulated by variations in the oxygen levels (Juillerat et al. 2017). This proof-of-principle in vitro study paves the way for designing oxygen sensitive, next generation "self-decision-making CAR" T-cells, that could be targeted to the hypoxic TME.

Reactive oxygen species (ROS) which can impair CAR T-cell function (Toyokuni et al. 1995) can be overcome by expressing the enzyme Catalase, which catalyzes the conversion of hydrogen peroxidase to water and oxygen. Catalase expressing CARs (CAR CATs) were able to overcome ROS and protected other infiltrating effector cells from the high hydrogen peroxidase levels (Ligtenberg et al. 2016).

Protein kinase A (PKA) is the downstream effector of the suppressor prostaglandin E2 and blocks TCR activation. Disruption of PKA anchorage to the lipid rafts by an inhibitory RIAD peptide (regulatory subunit I anchoring disruptor) hinders the interaction of PKA and ezrin, which is required for PKA tethering to adenylyl cyclase to restore TCR activation and T-cell function. Mesothelin-directed CAR T-cells expressing the RIAD peptide showed an increase in CXCR3 expression, and hence were able to display better adhesion and chemotaxis in a CXCL10 microenvironment. The CAR-RIAD cells displayed better persistence and function in vivo as compared to the Mesothelin CAR without the RIAD peptide (Newick et al. 2016).

Manipulating metabolism

Although manipulating cellular metabolism in the context of CAR T-cell therapy is still in its infancy, studies on the shifts of cellular energy status have gained prominence recently. Solid tumors consume large amounts of glucose and may create an anaerobic glucose-low environment that can cause metabolic barriers to effective T-cell function and activity (Vander Heiden et al. 2009). This raises the possibility that anti-tumor immunity can be improved through manipulation of tumor cell and CAR T-cell metabolism (Beezhold and Byersdorfer 2018).

Blocking PD-L1 can directly inhibit mTOR activity, decreasing glycolytic enzymes and dampening glycolysis. This can return glucose to the TME, resulting in increase of

T effector function. It would be rational to target the availability of glucose and check point blockade as a combination strategy. In addition to changing the metabolism within the tumor cells and the TME, it is possible to change the metabolism directly within the T-cell. Increased expression of phosphoenolpyruvate carboxykinase (PKC1) increases conversion of oxaloacetate to phosphoenolpyruvate (PEP), a glycolytic intermediate. PEP facilitates NFAT signaling and can increase T-effector function (Ho et al. 2015).

In B16 melanoma mouse models, it was shown that chronic AKT signaling in anti-tumor T-cells drives a loss of PGC1a expression, with a subsequent decrease in mitochondrial biogenesis and lowering of oxidative potential (Scharping et al. 2016). Overexpression of PGC1a increased oxygen consumption rates, increased effector function, and led to higher incidence of complete tumor regression. In a model of renal cell carcinoma, dysregulation of mitochondrial metabolism and impairment of glycolysis in CD8+ TILs showed decreased expression of activation markers (CD25, CD71) and effector function (Siska et al. 2017). Addition of pyruvate restored the activation suggesting that correction of metabolic impairments can improve tumor responses.

Constitutive expression of metabolic proteins is certainly feasible in CAR T-cells. Another approach would be to culture the CAR T-cells in conditions that favor oxidative metabolism, for example, in media supplemented with additional L-arginine and select for the most metabolically fit T-cells during the manufacture process (Sukumar et al. 2013). A recent study showed that selecting T-cells that had low mitochondrial membrane potential resulted in T-cells that had enhanced persistence and anti-tumor activity (Sukumar et al. 2016). Acute myeloid leukemia cells have low ROS levels, increased dependence on oxidative phosphorylation, and increased expression of Bcl-2. Bcl-2 has been shown to play a role in regulating oxidative and mitochondrial metabolism in tumor cells (Lagadinou et al. 2013; Gabriel et al. 2016). Inhibiting Bcl-2 with its inhibitor ABT-737 has also been shown to enhance in vitro killing of malignant B-cells by CAR T-cells (Karlsson et al. 2013), and hence, manipulating CAR T-cells to target Bcl-2 is a potential strategy.

From a CAR structure point of view, the 4-1BB domain containing CARs show higher rates of basal oxygen consumption and enhanced conversion of palmitate into acetyl CoA, which is a hallmark of beta-oxidation (Choi et al. 2017). The increased oxidative potential correlates with increase in mitochondrial biogenesis with increased fatty acid uptake. In contrast, CD28 domain containing CARs produce lactate, have increased expression of glycolytic genes, including glucose transporter 1 (Glut1) and pyruvate dehydrogenase kinase 1 (PDK1) (Kawalekar et al. 2016).

In conclusion, glycolysis favors early proliferation, but decreased long-term persistence (CD28 CARs), while oxidative phosphorylation correlates with increased persistence and memory formation. Balancing proliferation versus persistence is key to generating successful therapy. Limiting glucose concentrations, expanding CAR T-cells in the presence of glycolytic inhibitors, or silencing Glut1 in CAR T-cells by genome engineering could prevent the cells from being too dependent on glycolysis. Similarly, the use of glycogen synthase kinase 3-b inhibitors can facilitate an oxidative phenotype. It is possible to generate and selectively expand cells that have metabolically advantageous profiles.

Thus, conferring additional functionality to CAR T-cells can successfully overcome the multiple barriers posed by the hostile TME as indicated in the examples described in the preceding sections.

New delivery methods

Effective CAR therapy will depend on efficient delivery of the CAR T-cells to the tumor. Oncolytic viruses can selectively target the tumor cells, reduce vasculature, and inhibit tumor growth (Pikor et al. 2015). Combining CAR T-cells with oncolytic viruses is a promising strategy to tackle the TME. CAR T-cells combined with RANTES and IL-15 expressing oncolytic viruses have shown increased trafficking and a significant decrease in tumor growth (Nishio and Dotti 2015). Exosomes mediate cell–cell communication and can act as carriers of functional proteins secreted from cells into the extracellular milieu (Tang et al. 2015). CAR T-cell-derived exosomes have been used to target tumor cells and are effective in overcoming the effect of oncogenic KRAS in pancreatic cancer and increase overall survival (Kamerkar et al. 2017). Recently, nanomedicine-based immunotherapy for the elimination of tumor cells, with controlled and precise targeted delivery, has opened exciting possibilities. Research into biopolymers and nanoparticles that can be used to deliver CAR DNA along with immune adjuvants is gaining momentum (Smith et al. 2017a). T-cells took up nanoparticles carrying CAR19 DNA efficiently, expressed CAR effectively and provided long term remission (Smith et al. 2017b). CAR19 T-cells have been used as chaperones to deliver A2aR-specific antagonists to the tumor mass within the TME, by covalently attaching the liposomal vesicle nanoparticles carrying the antagonist. This resulted in reversing T-cell hypofunction and increase in anti-tumor responses in murine models (Siriwon et al. 2018). Nanotechnology offers novel, practical and simple methods to generate CAR T-cells and has tremendous potential for improving manufacturing and delivery options required for clinical applications.

At a glance

- Tumor Microenvironment is immunosuppressive, hypoxic and nutrient deprived – consists of
- Suppressor cells, chemokines, cytokines, stroma, vasculature
- CAR T-cells need to traffick to tumor, penetrate the stroma, and overcome immunosuppression
- Some next generation CARs can overcome TME barriers in preclinical settings

TME	CAR	Cancer model
Trafficking (Chemokine environment)	CCR4-CD30 CCR2b-GD2	Neuroblastoma, Hodgkin lymphoma
Penetration (Stroma)	FAP Heparanase-GD2 EGFR-2 Integrin	Ductal adenocarcinoma Neuroblastoma Renal cell carcinoma Pancreatic
Immunosuppression (Cytokines, Checkpoint inhibition, Hypoxia, Nutrient depravation)	dnTGFβR-HER2, PSMA IL4/7 switch-PSCA IL-12-CAR19, MUC16 IL-18-CEA A2aR-HER2 PD1/PDL1 block-CEA, CD19, CAIX, Mesothelin PD-1 switch-CAR19 Catalase-CAR-CAT-CEA HIF-CD19	Breast, Prostate Pancreatic Leukemia, Ovarian Colorectal Melanoma Leukemia, Lung, Renal cell carcinoma, Mesothelioma Leukemia Colorectal cancer Leukemia

Conclusion

CAR T-cells, the "game-changers," have revolutionized immuno-oncology and have heralded an entire novel era in cancer therapy. However, extending clinical success obtained in the treatment of hematological malignancies to solid tumors, has not been easy, due to several tumor-intrinsic mechanisms and the associated hostile tumor microenvironment. Poor trafficking of cells into the tumors, physical stromal barriers, suppression of CAR T-cells due to soluble inhibitory mediators, up-regulation of checkpoint receptors on the CARs, in response to the microenvironment and inhibitory ligands on the tumor, are some key factors that pose substantial challenges to an effective anti-tumor response. In addition, the heterogenous nature of antigen expression in solid tumors poses challenges in selecting suitable targets, and for several tumors, it is likely that more than one antigen will need to be targeted. Several suppressive factors will need to be targeted and overcome, simultaneously, for effective sustained CAR T-cell responses in solid tumors. This will no doubt increase the complexity of CAR designs. It is envisaged that advances in genome engineering, and vector designs, like the transposon systems (which enable large cargo loads, allowing for expression of multiple genes within the same CAR construct), will enable the successful development of smart, multi-functional CARs. The explosion of information and research findings in this area has already enabled the testing of potential combination therapies to overcome the effects of the TME. Several strategies have been promising in preclinical models and some of them are currently being tested in clinical trials. And lastly, with the development of nanomedicine/nanoparticles and biopolymers, exciting innovative and simple ways of manufacturing and delivering CAR T-cells to the tumors are likely to replace current protocols and enable them to be first-line treatment options for several cancers.

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