

## Review

# The cancer-immunity cycle as rational design for synthetic cancer drugs: Novel DC vaccines and CAR T-cells



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## ABSTRACT

Cell therapy is an advanced form of cancer immunotherapy that has had remarkable clinical progress in the past decade in the search for cure of cancer. Most success has been achieved for chimeric antigen receptor (CAR) T-cells where CAR T-cells targeting CD19 show very high complete response rates for patients with refractory acute B-cell acute lymphoblastic leukemia (ALL) and are close to approval for this indication. CD19 CAR T-cells are also effective against B-cell chronic lymphoblastic leukemia (CLL) and B-cell lymphomas. Although encouraging, CAR T-cells have not yet proven clinically effective for solid tumors. This is mainly due to the lack of specific and homogeneously expressed targets to direct the T-cells against and a hostile immunosuppressive tumor microenvironment in solid tumors. Cancer vaccines based on dendritic cells (DC) are also making progress although clinical efficacy is still lacking. The likelihood of success is however increasing now when individual tumors can be sequenced and patient-specific neoepitopes identified. Neoepitopes and/or neoantigens can then be included in patient-based DC vaccines. This review discusses recent advancements of DC vaccines and CAR T-cells with emphasis on the cancer-immunity cycle, and current efforts to design novel cell therapies.

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## 1. Introduction

Cancer immunotherapy has taken huge strides forward in the last decade moving from preclinical evaluations to successful clinical trials and approval. The most successful drugs are checkpoint-inhibiting antibodies against CTLA-4 and PD-1, which function by releasing the breaks for T-cell activity against tumors.

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The success of checkpoint inhibitors relies on the presence of pre-existing T-cell response against the tumor or the ability to mount effective tumor targeting T-cell response [1]. Tumors can be broadly classified based on their immune cell infiltration status to immunologically ‘hot’ or ‘cold’. Hot tumors typically have high amount of effector immune cell infiltration, which is the case in for *e.g.* melanoma, lung cancer, head and neck cancer [2]. For instance high CD8<sup>+</sup> T-cell infiltration in the tumor and invasive margin of the tumor highly correlates with response towards PD-1 inhibition [3]. Cold tumors have poor immune cell infiltration or infiltration of suppressor immune cell, indicating lack of immune response against the tumors [2]. However, for both tumor types initiation of anti-tumor T-cell response, migration of T-cells to the tumor and subsequent inhibition of immune suppression in the tumor microenvironment is the key to successful immunotherapy.

The series of steps involved in mounting anti-tumoral T-cell responses is referred to as the cancer-immunity cycle (Fig. 1). The cycle is initiated by the capture of tumor-associated antigens (TAAs) from dying tumor cells by antigen-presenting cells like immature dendritic cells (DCs). Under optimal circumstances the DCs get stimulated to obtain maturation characteristics and (i) migrate to draining lymph nodes, which is characterized by expression of CCR7, (ii) present the antigens they have captured to naïve CD8<sup>+</sup> and CD4<sup>+</sup> T-cells via MHC-I and MHC-II (Signal 1), (iii) express T-cell co-stimulatory molecules *e.g.* CD40, CD80, and CD86 (Signal 2) and (iv) secrete critical cytokines that determine the nature of T-cell response (Signal 3). Naïve CD8<sup>+</sup> T-cells are primed and activated to become cytolytic T-cells, leave the lymphoid organs into the blood circulation, extravasate into the tissue and infiltrate the tumor bed. In the final steps the T-cell receptor (TCR) of cytolytic T-cells recognize antigens presented on MHC-I by tumor cells and kill the tumor cells by releasing granzymes and perforins. This leads to release of more TAA and renewed launch of the immunity cycle [4].

Genetically engineered cell-based therapies are being developed to initiate a successful anti-tumor T-cells response. This review focuses on recent advances in (i) Engineering DC-based vaccines to initiate a tumor-specific T-cell response. DCs are being modified either to express neoantigens (identified on a patient-specific basis) or common TAAs, and/or immune-potentiating molecules, and/or to silence negative modulators of DC function, with the goal of strengthening their ability to prime both CD4<sup>+</sup> and CD8<sup>+</sup> T-cell activation. (ii) Engineering patient-derived T-cells with TAA-specific receptors, either a TCR recognizing an antigenic epitope presented by MHC-I or a chimeric antigen receptor (CAR). (iii) Improving the T-cell trafficking and infiltration into the tumor bed.

## 2. Genetically modified dendritic cell cancer vaccines

### 2.1. Engineering DCs with TAAs or neoantigens

For a DC to initiate a TAA-specific T-cell response, the MHCs on mature DC should present the TAA-specific peptide (Signal 1). TAAs can be either proteins overexpressed in the tumor cells, or re-expressed embryonal antigens due to global loss of DNA methylation in tumor cells, viral proteins in virus-associated tumors or neoantigens arising from somatic mutations in tumor cells [5]. Several approaches have been explored to introduce these antigens into the DCs and the classical methods were to load *ex vivo*-cultured DCs with tumor cell lysates, total tumor RNA or synthesized antigenic peptides [6]. However, genetic manipulations of DCs offer means of more robust introduction of antigens into DCs, because antigens encoded from a vector or synthetic mRNA can be translated and presented by the endogenous antigen-processing machinery of DCs (Fig. 2a). This can provide a renewable source

of antigens, and allows for efficient antigen processing and presentation on MHC-I. Material from dying DCs can then be taken up by endogenous DC for presentation on MHC-II [7–9]. While CD8<sup>+</sup> cytolytic T-cells are the main killers of cancer cells, induction of CD4<sup>+</sup> helper T-cells is important, as it is known to be necessary to induce a long-lasting and sustaining antitumor CD8<sup>+</sup> T-cell response [10]. Clinical trials utilizing DCs genetically engineered with common TAA have shown mixed clinical results (Table 1), suggesting the need for larger clinical trials or improved methods to potentiate T-cell stimulatory capacity.

With advances in next generation RNA sequencing technologies, somatic mutations leading to expression of neo-antigens (binding to the patient’s MHC and recognized by autologous T-cells) can be identified within tumors. Neo-antigenic targets increase the possibility to initiate specific adaptive immune response and improve the safety profile because high-affinity CD8<sup>+</sup> T-cells recognizing these antigens have escaped the central tolerance and are specific to the tumor cells. This allows screening for patient-specific antigens for personalized vaccine development [11,12]. DCs engineered with dominant neo-antigen-pulsed peptides or mRNA is efficient in priming neo-antigen-specific T-cells in patients [13–15]. Post-vaccination TCR-β repertoire against both the dominant and subdominant neo-epitopes increased significantly, indicating that such vaccinations can also promote epitope spreading by revealing subdominant neo-antigens [13]. Such personalized DC vaccines have been shown to be feasible in the clinic however; neo-antigens are highly tumor and patient-specific and these vaccines are laborious to produce [16].

### 2.2. Expression of positive regulators

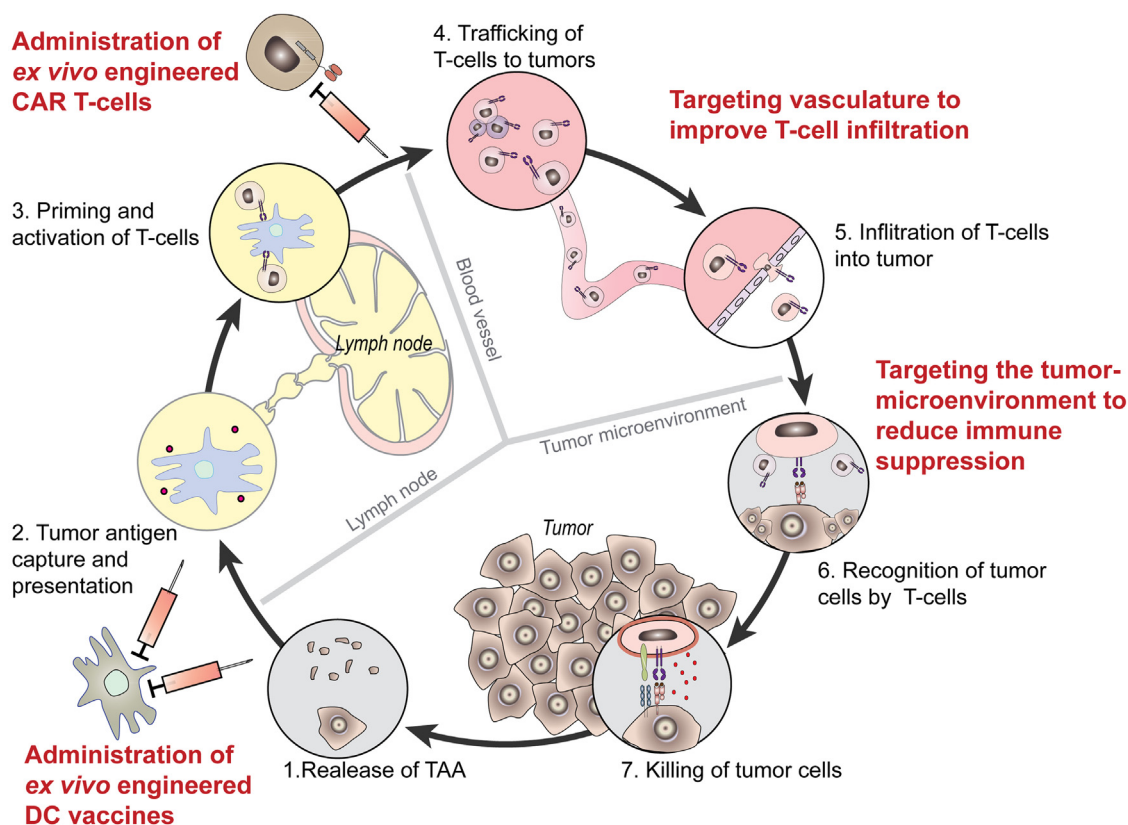
The ability of DCs to efficiently prime T-cells strictly depends on their ability to express surface-bound co-stimulatory molecules (Signal 2) and secrete appropriate cytokines (Signal 3) for T-cell activation and expansion. Viral vectors used to introduce TAA into DCs can by themselves serve as adjuvants providing maturation signal to DCs. For example, use of adenoviral vectors to introduce TAA also led to maturation of DCs with increased expression of costimulatory molecules (CD80, CD83, CD86 and HLA-DR), pro-inflammatory cytokines like interleukin (IL)-12, increased antigen-processing capacity and decreased expression of immune suppressive cytokines like IL-10 [17–19]. This positive maturation signal also correlates with the ability of DC vaccines to induce antigen-specific T-cells [17] (Fig. 2b).

DCs have been engineered to express CD40 ligand (CD40L), a potent activator of DCs, either by electroporation of mRNA [20] or transduction with adenoviral [21], lentiviral [22] or vaccinia viral [23] vectors. CD40L-engineered DCs mimic the licensing of CD40 ligation on DCs by activated CD4<sup>+</sup> T-cells. CD40L-engineered DCs have increased expression of co-stimulatory molecules (CD80 and CD86), are secreting IL-12p70 and promoting T-cell responses against poorly immunogenic tumor antigens both in wild type and CD4<sup>-/-</sup> mice [20,23,24]. Co-expression of CD40L along with active toll-like receptor (TLR)-4, CD70 and TAAs by a single step mRNA electroporation in DCs (TriMix DCs) further enhanced DCs ability to prime T-cells against MAGE-A3, MAGE-C2 and tyrosinase [25,26]. Besides CD40L, other co-stimulatory molecules such as OX40L [27], 4-1BBL [28], receptor-ligand pair RANK (receptor activator of NF-κB)/RANKL [29] have been used to engineer DCs in the preclinical setting to augment activation state of DCs.

In a similar fashion, DCs have also been engineered to constitutively express proinflammatory cytokines in order to improve DC function. IL-12 and granulocyte-macrophage colony-stimulating factor (GM-CSF) either alone or in combination are among the most commonly used cytokines to induce a Th-1 polarized milieu, which is ideal for priming of antitumor T-cell responses. IL-12-

**Table 1**  
Engineered DCs in the clinic.

Approach	Tumor type	TAA/Modification	Immune response to vaccination	Reference
RNA electroporated monocyte derived DCs	Neuroblastoma stage IV	Total RNA extracted from tumor cells	2/3 patients had a tumor-specific humoral immune response	[134]
	Recurrent brain tumors		2/7 patients had a tumor-specific immune response	[135]
	Uveal melanoma		Phase III, recruiting	NCT01983748
	Colorectal cancer	mRNA encoding CEA	3/24 patients had marginal increase in CEA-specific T-cells	[136]
	Melanoma Stage III/IV	Fusion of MAGE-A1, –A3, –C2, tyrosinase, MART-1, or gp100	4/10 patients had immune response against at least one TAA	[137]
	Recurrent stage III or stage IV melanoma	TriMix mRNA-CD40L, caTLR-4 and CD70 in combination with mRNA encoding tyrosinase, MAGE-A3, MAGE-C2, or gp100	1/4 gp100, 8/12 tyrosinase, 9/11 MAGE-C2, and 7/8 MAGE-A3- patients responding to vaccine had CD8 <sup>+</sup> T-cells	[138]
Neo-antigen peptide pulsed monocyte derived DCs	Recurrent Renal Cell Carcinoma	RNA-transfected CD40L	Recruiting	NCT02170389
	Melanoma	HLA-A*02:01 restricted neo-antigenic peptides	Neo-antigen specific T-cells were found in vaccinated patients	[13]
	Glioblastoma	Peptide containing EGFRvIII mutation	85% patients have vaccine-specific humoral response	[14]
DCs engineered with viral vectors	Lung adenocarcinoma	Peptide containing patients' individual RAS mutations	50% patients developed specific immune response mutant KRAS	[15]
	Metastatic melanoma	Adenovirus-encoded MART-1	6/11 patients developed CD8 <sup>+</sup> and 2/4 patients evaluated developed CD4 <sup>+</sup> T-cells to MART-1	[8]
	Epstein-Barr virus (EBV)-positive nasopharyngeal carcinoma	Adenovirus encoding a truncated LMP1 (Ad-ΔLMP1) and full-length LMP2 (Ad-ΔLMP1-LMP2)	No increase in frequency LMP1/2 specific T-cells detected after vaccination	[139]
DCs engineered with small interfering RNA	Ovarian cancer	siRNA IDO and mRNA encoding hTERT or survivin	Increased T-cell response to vaccine targets in all patients	[49]
	Metastatic melanoma	siRNAs targeting iP and mRNA encoding MART1, MAGE-3, gp100, and tyrosinase	Sustained antigen specific T-cells detected after 3–4 vaccinations	[52]
DCs engineered with cytokine-TAA fusion	Castration resistant prostate cancer	GM-CSF + PAP antigen fusion peptide (Sipuleucel-T)	46/63 patients developed T-cell response 100/151 patients developed antibody response	[57]



**Fig. 1.** The cancer-immunity cycle as a rational for improved cell therapies: The sequential and iterative processes that occur naturally in our body to initiate an effective anti-tumor immune response is referred to as the cancer-immunity cycle. Cancers successfully interfere at all steps of the cycle. Therefore the cancer-immunity cycle should be taken into consideration for rational design of cell therapy-based immunotherapies. a) DC vaccines can be infused either into regional lymph nodes, (intranodally), intradermally subcutaneous or the tumor microenvironment. They should have a mature phenotype, and be able to induce T-cells with potent anti-tumor activity. b) CAR-engineered T-cells are infused into the blood stream. They should be equipped to efficiently extravasate into tissues and enter the tumor bed where they need to effectively kill tumor cells despite an immunosuppressive microenvironment. Image adapted from Chen and Mellman [4].

secreting DCs have increased activation state, antigen processing, antigen specific T-cell priming capacity and are subsequently inducing regression of poorly immunogenic tumors in mice [30,31]. Similarly, DCs constitutively expressing GM-CSF have increased resistance to apoptosis, by upregulating Bcl-xL, and expression of co-stimulatory molecules and decreased sensitivity to immune suppressive cytokines like TGF- $\beta$  [32,33]. When IL-12 and GM-CSF were used together with gp70 (TAA), higher frequencies of TAA-specific CD4<sup>+</sup> and CD8<sup>+</sup> T-cells were observed [34]. Apart from IL-12 and GM-CSF other cytokines, chemokines and chemokine receptors like TNF- $\alpha$  [35], IL-15 [36], CXCL10 [37], CCL21 [38], and CCR7 [39] have been used to improve maturation, migration and T-cell priming capacities of DCs.

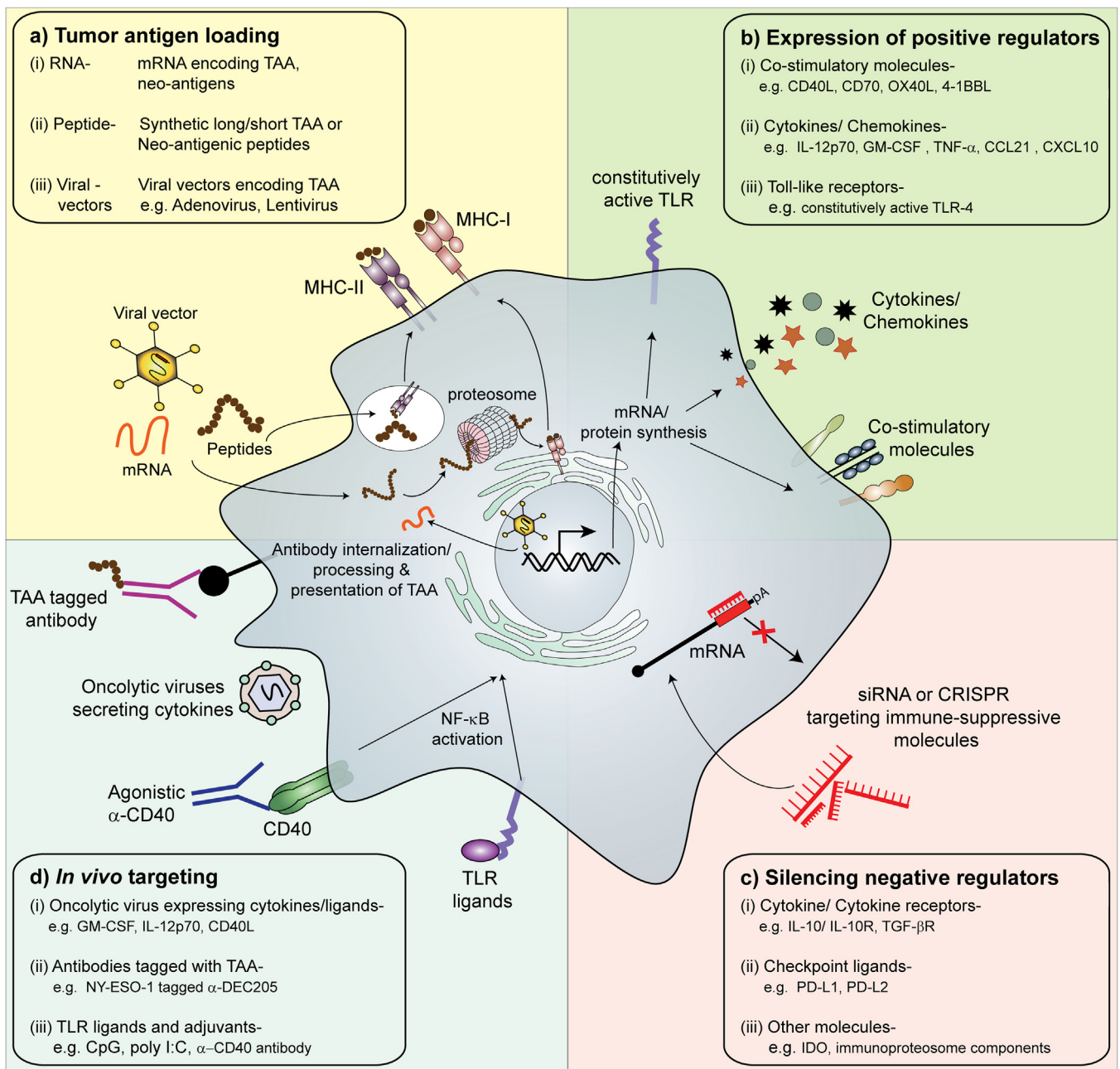
### 2.3. Silencing negative regulators

Tumors develop mechanisms to evade immune recognition by upregulating immune suppressive factors in the microenvironment like IL-10, TGF- $\beta$ , indoleamine 2,3-dioxygenase (IDO) and PD-L1/L2, which are ligands to the T-cell inhibitory receptor PD-1. DCs reprogrammed in the tumor microenvironment are often highly apoptotic or have a tolerogenic phenotype, which limits the therapeutic potential of vaccines [40,41]. Therefore, the focus has been made to silence inhibitory molecules to potentiate effective DCs function in the harsh immunosuppressive tumor microenvironment (Fig. 2c). Either IL-10-silenced or IL-10R-silenced DCs have increased expression of co-stimulatory molecules, IL-12 and MHC-II molecules in the presence of IL-10 [42,43]. These DCs also induced a stronger HPV E7-specific CD8<sup>+</sup> T-cell response and improved sur-

vival of mice bearing E7-expressing TC-1 tumor upon vaccination [43]. When the TGF- $\beta$ R was also silenced along with the IL-10R in a DC vaccine, even stronger CD8<sup>+</sup> T-cell responses and anti-tumor efficacy were observed in TC-1, which secretes high levels of TGF- $\beta$  and IL-10 [44]. PD-L1 and PD-L2-silenced DC vaccines have also shown promise in preclinical settings to boost CD8<sup>+</sup> effector and memory T-cell responses, especially to minor histocompatibility antigens [45,46]. IDO has recently gained interest as a factor produced by DCs inducing apoptosis in effector T-cell but not in immunosuppressive regulatory T-cells (T<sub>regs</sub>) [47]. IDO-silenced DC vaccines inhibited T<sub>reg</sub> responses and improved effector T-cell resistance to apoptosis, leading to inhibited tumor growth in a murine breast cancer model [48]. IDO-silenced DC vaccines were proven safe in the clinic, with indications of achievable disease control [49]. Other molecules like Bax and Bak apoptosis inducers [50], immunoproteasome (iP) [51,52], A20 [53], CCL22 and CCL17 [54] have all been silenced in DCs to improve survival, CD8<sup>+</sup> T-cell priming capacity and resist immune suppression (Fig. 2c).

### 2.4. Translating DC vaccines into the clinic

The preclinical success achieved by the use of *ex vivo* gene-modified DCs have unfortunately not translated into clinical efficacy. A brief summary of genetically modified DCs used in clinical trials is listed in Table 1. Most of the clinical trials are phase-I/II exploratory studies mainly looking for safety and immunological responses. The safety of DC vaccines is well documented and most common grade 1–2 adverse events reported are local pain and rash at the site of injection, systemic toxicities like pyrexia,



**Fig. 2.** Engineering strategies to improve efficacy of DC vaccination: Strategies used to improve function of *ex vivo*-engineered DCs are (a) Loading of tumor antigens in the form of mRNA or a viral vector encoding TAA/neo-antigens or in the form of synthetic TAA/neoantigenic peptides. (b) Expression of positive regulators of DC function such as T-cell co-stimulatory molecules (CD40L, CD70, OX40L, 4-1BBL), T-cell-stimulating cytokines/chemokines (IL-12p70, TNF- $\alpha$ , CCL21, CXCL10) and DC-activating receptors (TLR-4). (c) Silencing of negative regulators of DC function such as immune suppressive cytokines/cytokine receptors (IL-10/IL-10R, TGF- $\beta$ R), checkpoint inhibitor ligands (PD-L1, PD-L2) and other suppressive molecules such as IDO. (d) Alternatively, DCs can be targeted *in vivo* by administration of oncolytic viruses encoding DC/T-cell-stimulating cytokines and co-stimulatory molecules (IL-12p70, GM-CSF, CD40L), administration of DC-targeting antibodies tagged with TAA, administration of TLR-activating ligands (CpG, polyI:C) or administration of agonistic DC-activating antibodies ( $\alpha$ -CD40 antibody).

malaise, and other influenza-like symptoms. Grade 3–4 adverse events associated with DC therapies are uncommon [55]. Most DC therapy studies report immune responses to the vaccination (either a T-cell or antibody response), however it rarely correlates with clinical responses. The objective clinical responses are relatively low, rarely exceeding 15% [56]. To date only one DC vaccine (Sipuleucel-T) consisting of autologous DC pulsed with a fusion protein between prostatic acid phosphatase (PAP) and GM-CSF, has been approved for treatment of metastatic castration-resistant prostate cancer on the basis of a phase III study reporting 4.1-month extension of patient survival [57]. However, more phase III clinical

trials are currently ongoing with focus on efficiency and patient survival (NCT00045968/Glioma, NCT01983748/Uveal melanoma, NCT01875653/Melanoma).

An alternative to *ex vivo*-engineered DCs is to target DCs *in vivo* in the patient. This would overcome the drawbacks associated with the laborious and costly processes associated with producing patient specific vaccines. Also the poor outcome of *ex vivo*-cultured DCs could be attributed to their poor migratory capacity to the lymph node where they prime T-cells after re-administration [58]. Several approaches have been explored to target DCs *in vivo*, (Fig. 2d), e.g., TAA-bound antibodies targeting receptors on DCs

[59], oncolytic viruses expressing GM-CSF [60], CD40L [21] and other immune stimulatory molecules like bacterial proteins (HP-NAP) [61]. A phase-I trial with an antibody against a molecule expressed on DC (DEC205) tagged with NY-ESO-1, co-administered with TLR agonists as adjuvants was shown to be feasible and safe [59]. Another attractive strategy is to use oncolytic viruses secreting GM-CSF. The rationale for the strategy is that viral oncolysis will release neoantigens from the cancer cells that can be picked up by the tumor-residing DCs, which are then activated by GM-CSF secreted by the virus. An oncolytic Herpes Simplex virus secreting GM-CSF (T-Vec) was recently approved for the treatment of inoperable melanoma [62].

### 3. Genetically modified T-Cells for cancer therapy

Tumor-infiltrating lymphocytes (TILs) isolated from the tumor, expanded *ex vivo* and reinfused into the patient have shown efficacy for the treatment of melanoma in particular [63]. To overcome the difficulties associated with the isolation and access of tumor biopsies, T-cells isolated from the peripheral blood have been modified with new receptors. Adoptive transfer of T-cells genetically engineered with a receptor targeting a TAA essentially bypasses the steps involved in education of antigen-specific T-cells by DCs. T-cells are also modified with expression of stimulatory receptors and silencing of inhibitory molecules to improve their anti-tumor activity.

#### 3.1. Redirecting T-cells against TAA using artificial receptors

The two most commonly used receptor molecules to redirect T-cells towards TAAs are the natural T-cell receptor (TCR) and a chimeric antigen receptor (CAR).

TCR-engineered T-cells are generated by transferring the TCR  $\alpha$  and  $\beta$  chains from a TCR known to react with an epitope from a TAA that is presented by a specific MHC-I. TCRs used so far are often binding to HLA-A2-restricted epitopes from cancer-testis antigens. *In vitro* and *in silico* technologies have been used to enhance TCR avidity and affinity to increase anti-tumor activity against weakly immunogenic TAAs [64]. The approaches developed to isolate and enhance TCR function are reviewed elsewhere [65]. The first clinical study using TCR-engineered T-cells was in melanoma patients targeting an HLA-A2-restricted MART1 peptide. The TCR used here was isolated from TILs, had weak affinity to MART1 and this study had relatively few responders (2/17 with partial remissions) [66]. Other clinical trials using TCR-modified T-cells are listed in Table 2. To summarize, TCR-engineered T-cells have demonstrated responses in patients having melanoma, multiple myeloma, synovial cancer and colorectal carcinoma but also have off-target toxicity issues.

CARs are chimeras of domains derived from antibodies and T-cell receptors. A CAR molecule has 3 essential domains. (a) An extracellular antigen-binding domain that confers antigen-specificity to the engineered T-cell. A majority of the engineered CARs for cancer therapy have antibody-derived antigen-binding domains called single-chain variable fragment (scFv). CARs containing an scFv extracellular domain retain the specificity of an antibody and bypass the need for antigen presentation by MHC-I. (b) The spacer and transmembrane domain gives flexibility to allow proper dimerization of scFv and allows for stable CAR expression on the cell surface. (c) The cytoplasmic signaling domain(s) provides the necessary downstream signaling for T-cell effector function (Fig. 3a). CARs are classified into different generations based on the number of cytoplasmic co-stimulatory signaling domains it contains namely first, second and third generation. The most commonly used signaling domains are CD3 $\zeta$ , CD28, 4-1BB, OX40 and ICOS. The detailed design, construction and elements involved used

in CAR molecule are reviewed elsewhere [67]. In the clinic second generation CAR T-cells targeting CD19 have shown remarkable success for the treatment of pediatric acute lymphoblastic leukemia (ALL, 60–90% CR) [68–71], chronic lymphoblastic leukemia (CLL, 10 CR, 10 PR and 5 SD of the 40 patients treated in different centers) [72–75], to some extent for B-cell lymphomas [76,77] and multiple myeloma [78]. The observed complete remissions are in some cases sustained and long lasting, indicating significant clinical benefit. CD19 CAR T-cells are on the track to be approved as a drug for ALL. CARs targeting other antigens have also been evaluated in the clinic for other hematological and solid tumors, e.g., mesothelin for mesothelioma and pancreatic cancer [79], Lewis-Y antigen for acute myeloid leukemia [80], GD2 for neuroblastoma [81], HER2 for colorectal cancer and sarcoma [82], ERBB2 for metastatic colon cancer [83], and carbonic anhydrase IX (CAIX) for renal cell carcinoma [84]. CAR T-cells tested in the clinic are listed in Table 2. However, no other target has been as successful as CD19, which can be attributed lack of homogeneity for these tumors as compared to the uniform expression of CD19 on all B-cells (normal as well as malignant), but not in any other normal tissues. More about this topic is discussed in the section improving safety of engineered T-cells.

The development of CAR T-cells for solid tumors is still at a very early stage and very little success has been achieved in the clinic. This can be attributed to several characteristics of solid tumors (i) trafficking and infiltration of T-cells in to solid tumor mass, (ii) harsh tumor microenvironment characterized by oxidative stress, nutritional depletion, hypoxia, presence of suppressive soluble factors, cytokines and suppressive immune cells (T<sub>regs</sub>, MDSCs, TAMs and TANs), and (iii) negative regulators of T-cell function (e.g., upregulation of cytoplasmic and surface inhibitory receptors such as PD-1 and CTLA-4) [85]. With more understanding of tumor immunology and improvements in molecular biology for cell engineering, new strides are being taken to overcome these limitations.

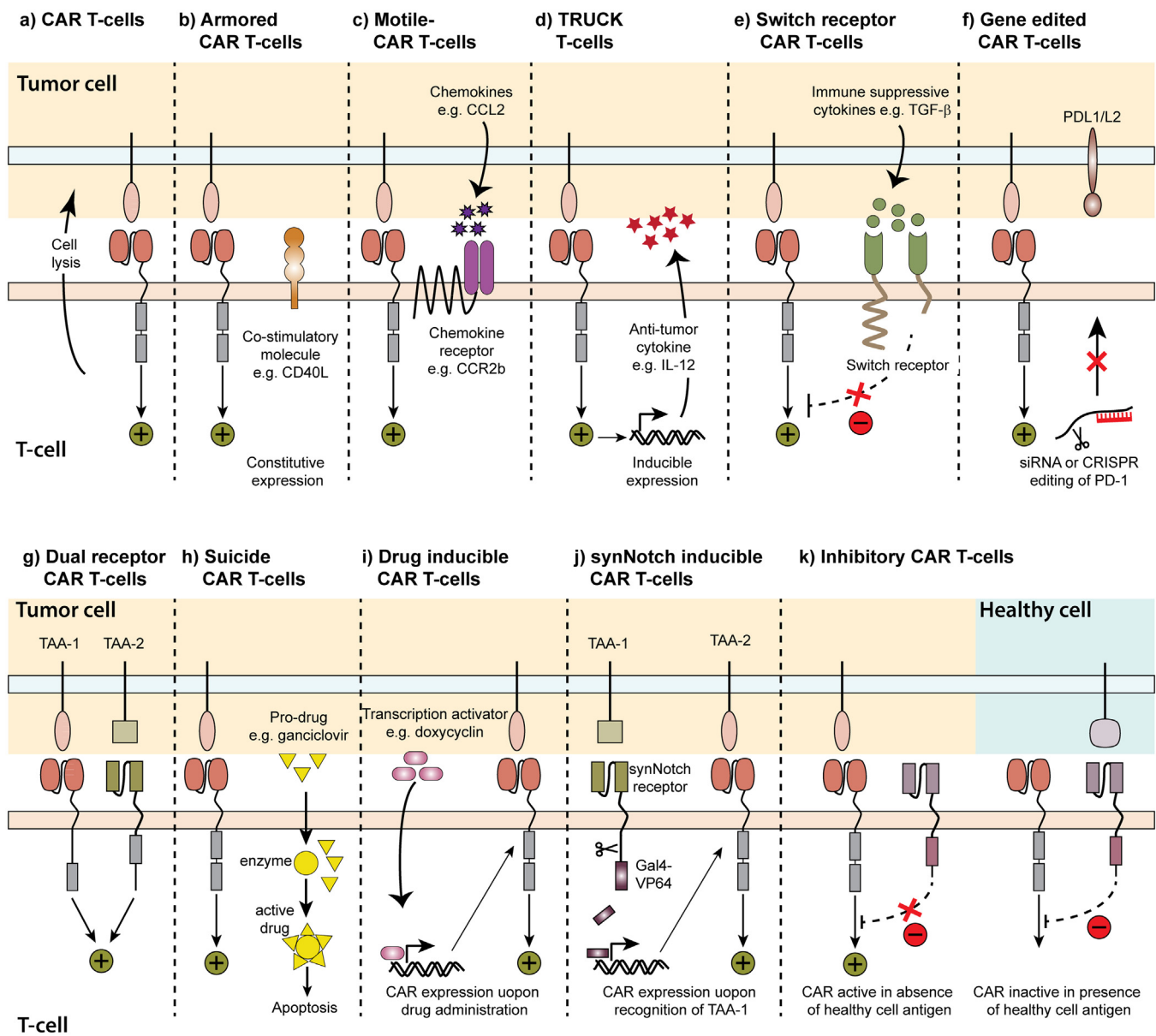
#### 3.2. Improving trafficking and infiltration of T-cells

After re-administration of *ex vivo*-engineered and expanded T-cells, they need to migrate through the blood circulation, extravasate to and infiltrate the tumor mass to initiate their effector functions. Extravasation of T-cells depends on interaction with specific adhesion molecules and chemokines expressed on the luminal side of blood vessels, mediating capture, rolling, firm adhesion and transendothelial migration of leukocytes [86]. Unfortunately, tumor endothelial cells are anergic due to tumor secretion of pro-angiogenic factors such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) and respond poorly to pro-inflammatory cytokines [87,88]. Mechanistically, VEGF-induced signaling inhibits NF- $\kappa$ B activation in endothelial cells leading to reduced expression of adhesion molecules and T-cell attracting chemokines such as CXCL10 and CXCL11 [89]. Endothelial anergy can be reversed by anti-angiogenic therapy, leading to increased expression of adhesion molecules and chemokines and enhanced T-cell infiltration into the tumor [89,90]. Consequently, neutralizing antibodies to VEGF or vascular endothelial growth factor receptor (VEGFR)-2 as well as small molecule kinase inhibitors targeting VEGFR-signaling have successfully been used to increase the efficacy of adoptive T-cell therapy and other T-cell based immunotherapies in animal models [91–93].

Tumor endothelial cells can also act as a barrier to infiltrating effector immune cells by expressing molecules that directly promote tumor tolerance. For instance, Fas ligand is expressed specifically in tumor endothelial cells in solid tumors, but not in normal blood vessels, and mediates killing of effector CD8<sup>+</sup> T-cells, but not T<sub>regs</sub> [94]. Other molecules expressed by tumor endothelial cells that may significantly contribute to tumor immune suppress-

**Table 2**  
Engineered T-cells in the clinic.

Target antigen	Tumor type	Receptor construction	Clinical response	Comments	Reference
MART-1/HLA-A2	Melanoma	DMF4-TCR isolated from TIL	OR-2/17 (12%)	No adverse events reported	[66]
		DMF5-TCR isolated from TIL with high avidity	OR- 6/20 (30%)	9/36 patients experience toxicities. On-target toxicity on melanocytes- Skin rash, Uveitis and hearing impairment	[140] [141]
		1D3-TCR isolated from patient, codon optimized and murinized	SD- 7/13 at 3 months N/A	4/13 Skin rash and 2/14 respiratory impairment 1/1 lethal toxicity and death due to CRS	[142]
MAGE-A3/HLA-A2 MAGE-A3/HLA-A1	Melanoma, synovial cell sarcoma, esophageal cancer Melanoma, Myeloma	TCR isolated from immunized mice and affinity enhanced	OR- 5/9 (55%), CR-2/9 (22%)	2/9 Central nervous system toxicity and death, 1/9 Parkinson-like symptoms, 1/9 Aphasia. TCR also recognized epitopes in MAGE-A3/A9/A12. MAGE-A12 was expressed in brain	[120]
		a3a-TCR isolated from patient and affinity-enhanced	N/A	2/2 death. Cardiac toxicity due to recognition of unrelated epitope from Tintin	[143]
CEA/HLA-A2	Colorectal carcinoma	TCR isolated from immunized mice and affinity enhanced	OR- 1/3 (33%)	3/3 experienced inflammatory colitis. On-target toxicity on the colon	[144]
NY-ESO-1/HLA-A2	Melanoma, Myeloma	1G4-TCR isolated from patient and affinity-enhanced	OR- 11/20 (55%) OR- 16/20 (80%)	No toxicity, TCR recognizes only NY-ESO-1 positive tumor cells	[145] [146]
CD19	ALL	scFv-CD28-CD3 $\zeta$	CR- 91%	32 adults. Toxicity- B-cell aplasia, CRS and neurotoxicity	NCT01044069
		scFv-41BB-CD3 $\zeta$	CR- 70%	20 children and young adults. Toxicity- B-cell aplasia, CRS	NCT015936962
			CR- 90%	30 children and young adults. Toxicity- B-cell aplasia, CRS	NCT01626495, [68]
			CR- 83%	20 adults. Toxicity- CRS	NCT01865617
CLL		scFv-CD28-CD3 $\zeta$	CR-1, PR-2, SD-1	4 patients. Toxicity- reversible CRS	[127]
		scFv-4-1BB-CD3 $\zeta$	CR-4, PR-4	14 patients. Toxicity- CRS correlating with T-cell proliferation. CAR T-cells persisted beyond 4 years in 2 patients undergoing CR	[147]
Lymphoma (FL, DLBCL, NHL)		scFv-4-1BB-CD28-CD3 $\zeta$	CR- 50%	Cyclophosphamide, Fludarabine lymphodepletion	[77]
		scFv-CD28-CD3 $\zeta$	CR- 53%, PR-26%	CAR T-cells administered at a predefined ratio 1:1 CD4 <sup>+</sup> :CD8 <sup>+</sup> Acute toxicities including fever, hypotension, delirium, neurologic toxicities. One death due to unknown reasons	[76]
Multiple Myeloma		scFv-4-1BB-CD3 $\zeta$	CR-1, PR-4, SD-4	CR for 12 months following treatment was achieved despite the absence of CD19 expression	[78]
CAIX	Renal cell carcinoma	scFv-FcR $\gamma$	NR-12/12 (100%)	Grade 2–4 liver toxicities. Liver biopsies revealed CAIX expression on bile duct epithelium with CAR T-cells infiltration	[148]
GD2	Neuroblastoma	EBV specific T-cells transduced with scFV- CD3 $\zeta$ CAR construct	CR-3/11, PR-1/11, SD-1/11, PD-4/11, tumor necrosis-2/11	19 Patients. 8/19 has no disease at the time of infusion and 11/19 had active disease	[81]
	Neuroblastoma, Osteosarcoma, and Melanoma	scFv-OX40-CD28- CD3 $\zeta$ -iCasp9	Ongoing Phase I	3rd generation CAR with improved potency and safety (suicide gene inducible Caspase 9)	NCT02107963
HER2	Colorectal cancer Sarcoma	scFv-CD28-4-1BB-CD3 $\zeta$	N/A	1 patient died due to CRS and severe acute respiratory failure	[83]
		scFv-CD28-CD3 $\zeta$	SD- 4/17	2/2 evaluated patients had CAR T-cell infiltration in the tumor. Persistence 6-weeks and no toxicity reported	[82]
CD171	Neuroblastoma	scFv-CD3 $\zeta$	PR1/6, PD-5/6	Patients with bulky disease had short T-cell persistence (1–7d) than limited disease burden (42d)	[149]
CEA	Colorectal cancer	scFv-CD28- CD3 $\zeta$	OR-1/3	3/3 severe transient inflammatory colitis. CAR T-cells were infused i.v.	[144]
	Adenocarcinoma with liver metastases	scFv-CD28- CD3 $\zeta$	SD- 1/6, PD- 5/6	No toxicity. CAR T-cells administered via percutaneous hepatic artery infusion	[150]
Mesothelin	Mesothelioma	Murine scFv-4-1BB- CD3 $\zeta$	N/A	Transient mRNA CAR expression. 1 patient developed anaphylaxis and cardiac arrest due to immune response against murine scFv	[151]



**Fig. 3.** Engineering strategies to improve efficacy and safety of CAR T-cell therapy: (a-f) Strategies developed to increase the efficacy of CAR T-cell therapy. (a) CAR T-cells engineered with a CAR molecule that has scFv recognizing TAA (ecto-domain) and T-cell portion (endo-domain). The T-cell is activated upon TAA recognition by the CAR molecule and induces target cell lysis. (b) Armored CAR T-cells are engineered to constitutively express molecules that activate T-cells e.g. CD40L. (c) Motile CAR T-cells are engineered to co-express chemokine receptors that aid in T-cell trafficking to tumors expressing the corresponding chemokine e.g. C-C motif chemokine ligand 2 (CCL2). (d) TRUCK T-cells (T-cells redirected for universal cytokine killing), co-express an anti-tumor cytokine e.g. IL-12, which is induced upon T-cell activation. (e) Switch receptor CAR T-cells co-express dominant negative or switch receptors for immune suppressive cytokines/molecules to overcome tumor mediated immune suppression, e.g. TGF- $\beta$ R without signaling domain or PD1 with CD28 signaling domain. (f) Gene-edited CAR T-cells have T-cell intrinsic negative regulators silenced by using siRNA or edited with CRISPR e.g. PD-1 silencing. (g-k) Strategies developed to increase the safety of CAR T-cell therapy. (g) Dual receptor CAR T-cells expresses two separate CARs targeting two TAAs on a target cell; one CAR includes only the CD3 $\zeta$  domain and the other CAR includes only the co-stimulatory domain(s). Dual CAR T-cell activation requires co-expression of both targets on the tumor cell. (h) Suicide CAR T-cells express enzymes/molecules that activate a pro-drug to induce apoptosis of the CAR T-cell e.g. phosphorylation and activation of ganciclovir by thymidine kinase in gene-modified lymphocytes. (i) Drug inducible CAR T-cells have CAR expression controlled by a drug inducible promoter e.g. doxycyclin administration activates Tet-on promoter controlled CAR expression. (j) synNotch inducible CAR T-cells expresses active CAR molecule against TAA-2 only when a synNotch receptor binds TAA-1 on a target cell. (k) Inhibitory CAR (iCAR) T-cells co-expresses an iCAR (scFv fused to an intracellular inhibitory domain (for example, CTLA4 or PD1)). iCAR T-cells co-expressing a standard CAR is activated only when encountering target cells that possess the standard CAR TAA but lack the iCAR target (usually present on healthy cells).

sion include TNF-related apoptosis-inducing ligand (TRAIL), PD-L1, IL-10, TGF- $\beta$  and IDO [95]. The role of tumor endothelial cells in immune suppression is still under investigation, but it is clear that vascular targeting drugs that can specifically reverse endothelial energy and decrease endothelial expression of immunosuppressive molecules have the potential to improve future CAR T-cell therapies.

Engineering CAR T-cells to express specific chemokine receptors may enhance trafficking to the tumor. T-cell migration is strictly controlled by secretion of chemokines by the tumor cells and expression of the matching chemokine receptors by the T-cells (Fig. 3c). For example, CAR T-cells expressing CCR2b have improved migration to mesothelioma and neuroblastoma that naturally secrete large quantities of CCL2 [96,97]. Likewise, CD30-directed CART T-cells engineered with CCR4 had enhanced migratory

capacity in to Hodgkin's lymphoma in murine xenograft models [98]. However this approach is not a universal solution as chemokine profile can vary among tumors and the receptor pair has to be personalized.

### 3.3. Improving T-cell activation in the harsh tumor microenvironment

The actual number of malignant cells present in the tumor mass can be rather low, while the majority can be made up of stromal cells, fibroblasts and extracellular matrix (ECM). These cells provide support for tumor growth and act as physical barriers to immune cell infiltration. Fibroblast-activation protein (FAP)-directed CAR T-cells targeting FAP-positive fibroblasts [99] or CAR T-cells secreting heparanase, an enzyme degrading ECM [100] have been tested successfully in preclinical models to augment CAR T-cell activity. Constitutive expression of CD40L in CAR T-cells increased proliferative and cytokine secretion capacity of T-cells (Fig. 3b). They also altered the immunogenicity of CD40<sup>+</sup> tumor cells by inducing expression of CD80, CD86, MHC-I and Fas-death receptor [101]. Pro-inflammatory cytokine-secreting CAR T-cells have been engineered to improve the tumor microenvironment milieu and improve T-cell function. A classic example of this approach is CAR T-cells engineered to secrete T helper type-1 (Th1) polarizing cytokine IL-12 upon receptor engagement (Fig. 3d). IL-12 can augment the activation of both primary effectors of the adaptive and innate immune responses like DCs, NK cells and CD8<sup>+</sup> T-cells. IL-12-secreting T-cells have shown remarkable efficacy in eradicating tumor in animal models [102,103]. However, TILs engineered to secrete IL-12 upon TAA recognition tested in the clinic to treat metastatic melanoma patients resulted in severe IL-12-related toxicities [104]. So, engineering T-cells to overexpress cytokines must be carefully considered to reduce the risk of serious side effects.

Different combinations of co-stimulatory domains have been explored to get the optimal phenotype and activation status of CAR T-cells to achieve the best therapeutic agent. Efficacy of CAR T-cell correlates with *in vivo* persistence and differentiation state of T-cells (less differentiated memory T-cells survive longer) [105]. Mostly second generation CARs using CD3 $\zeta$  stimulatory and CD28 or 4-1BB co-stimulatory domains have been used in the clinic. CD19-CAR T-cells having a 4-1BB domain generally has shown better specific tumor cell lysis, *in vivo* persistence (up to 1 year after T-cell infusion), memory phenotype and lower exhaustion [105–108]. CAR T-cells have also been engineered with the ICOS co-stimulatory domain to promote T-cell persistence and these cells tend to survive longer than CD28 or 4-1BB co-stimulatory domain-containing CAR T-cells [109]. A recent study from Sadelain's group indicates that a third generation CAR splitting the two costimulatory domains performs better than a third generation CAR having both domains in tandem on the actual CAR [110].

### 3.4. Suppressing negative regulators of T-cell function

Recent focus has been to develop strategies to change the balance of an immunosuppressive microenvironment to a more favorable environment for T-cell function. A well-known characteristic of the tumor microenvironment is the presence of T-cell suppressive cytokines like IL-4, IL-10 and TGF- $\beta$ . CAR T-cells co-expressing dominant-negative receptors or switch receptors for these cytokines have been developed to overcome these suppressive factors. These receptors usually have an extracellular domain binding the cytokine and a non-functional intracellular domain or the intracellular domain replaced with a signaling domain to give positive signal to the T-cells (Fig. 3e). Examples of this approach are MUC1-CAR T-cells engineered with an IL-4 receptor with an IL-2 signaling domain [111] and expression of a TGF- $\beta$  receptor

without signaling domain [112] increased resistance of T-cells to these cytokines and improved anti-tumor efficacy.

To resist T-cell immune response tumors can express regulatory molecules like PD-L1 to suppress T-cell cytotoxicity. Checkpoint inhibiting antibodies ( $\alpha$ PD-1,  $\alpha$ CTLA-4,  $\alpha$ PD-L1) block the inhibitory signal to the T-cells and have been very successful in the clinic for the treatment of different tumor types [1]. However administration of such antibodies enhances T-cell function systemically and increases the chance of developing autoimmune reaction and potential side effects [113]. To restrict blocking of PD-1 to only tumor-reactive T-cells, siRNA or CRISPR-Cas9 mediated silencing approached has been adopted to selectively knockdown or knock-out PD-L1 on tumor cells [114] or PD-1 itself on the T-cells [115,116] (Fig. 3f). Both approaches have been very successful in preclinical models improving T-cell activity against the tumor and are also being tested in the clinic (NCT02793856, NCT02863913) [117]. T-cells expressing a PD-1 switch receptor with the negative signaling domain of PD-1 replaced with positive CD28 signaling can overcome PD-1 mediated inhibitory effects and demonstrate enhanced anti-tumor efficacy in preclinical models [118]. These approaches have the potential to improve the efficacy adoptive transfer of engineered T-cells.

### 3.5. Improving safety of genetically engineered T-cells

Heterogeneity in antigen-expression in tumors and lack of tumor-specific antigens has been a hindrance to the development of both TCR and CAR-engineered T-cells against solid tumors. It promotes escape of antigen negative clones, recognition of antigen expressed in healthy tissues (ON-target toxicity) and recognition of a highly similar antigen on healthy tissues (OFF-target toxicity) leading to severe side effects. Both ON-target and OFF-target toxicities have been reported in case of TCR T-cells, e.g., TCR T-cells targeting MART-1/HLA-A2 with severe inflammation of skin, eyes, and ear [119] and TCR T-cells targeting MAGE-A3, -A9/HLA-A2 with severe neurological toxicities [120]. In the case of CD19-directed CAR T-cells, B-cell aplasia is a well-known ON-target toxicity, however it can be managed by intravenous administration of gamma globulin. ERBB2-CAR T-cells have been reported to have toxicities in heart and pulmonary vasculature [83], CAIX-directed CAR T-cells have been reported to have severe toxicity and patients developed cholangitis and damage to the epithelial cells of the bile duct [84]. To overcome such antigen targeting issues, bispecific antigen receptors are being expressed in the same T-cell to target two TAAs (Fig. 3g). The rationale behind this approach is that T-cell signaling is active only when both receptors bind to their target antigens on the same tumor cell. Successful examples of this approach are CAR T-cells targeting HER2 and IL13R $\alpha$ 2 to treat experimental glioblastoma [121] and PSMA and PSCA to treat experimental prostate cancer [122]. Expression of two CAR molecules, one providing positive signal when it recognizes TAA and another inhibitory CAR (iCAR) providing negative signal when it recognizes an antigen expressed on a normal tissue, but not tumor would protect healthy cells from CAR T-cell attack (Fig. 3k). An example of this approach is co-expression of CD19-CAR carrying activating signaling (CD3 $\zeta$  and CD28) and PSMA-CAR carrying inhibitory signaling domains (from PD-1 or CTLA-4). iCAR T-cells were activated only when the target cells expressed CD19 and not when they expressed both CD19 and PSMA [123]. Another approach is to develop CARs against more common neoantigen-specific targets, as they are antigens arising due to somatic mutations in the tumors; their expression is confined to the tumor cells. Epidermal growth factor receptor variant 3 (EGFRvIII)-CAR T-cells is an example of this approach for treatment of glioblastoma [124]. Clinical testing of these CAR T-cells is underway (NCT02209376, NCT0145459).

Apart from ON and OFF-target toxicities other side effects of CAR T-cells (especially targeting CD19) are cytokine release syndrome (CRS) and neurotoxicity. CRS is associated with excessive release of pro-inflammatory cytokines like IL-6, IFN- $\gamma$  and TNF- $\alpha$  by activated CAR T-cells. CRS is controlled with anti-IL-6 receptor antibody (tocilizumab) or TNF- $\alpha$  antibody (infliximab) and in severe cases in combination with steroids. However, immunosuppressive interventions also affect CAR T-cell therapeutic efficacy [125–127]. Neurological toxicities have also been observed in ALL patients treated with CD19 CAR T-cells with symptoms like confusion, seizures, delirium aphasia [71]. The cause of neurotoxicity is still unknown but usually subsides without any intervention. To avoid such toxicities, safety features can be being built into CAR T-cell constructs, so that they can be selectively ablated. Suicide genes can be inserted into CAR T-cells to irreversibly eliminate them in case of a toxic event (Fig. 3h). Herpes simplex virus-thymidine kinase (HSV-TK) and inducible-caspase-9 (iCasp9) are the two currently validated suicide genes in the clinic [128,129]. Another approach is to induce CAR expression in T-cells only when needed, for example by using inducible promoter constructs (Fig. 3i). Tet-on inducible systems have been used to control CAR expression by administration of doxycyclin [130]. CAR-like synNotch receptors to control the expression of a CAR molecule targeting a TAA have been constructed to induce CAR expression based on combinatorial antigen recognition. The feasibility of this approach was demonstrated by using CAR-like synNotch receptor bearing Gal4-VP64 or TetR-VP64 domains against GFP. Gal4-VP64 and TetR-VP64 are transcription factors that activate transcription of a conventional CD19-CAR molecule. Essentially a functional CAR T-cell is generated only when both the synNotch ligand (GFP) and the CAR ligand (CD19) are both expressed on a target cell [131] (Fig. 3j).

#### 4. Conclusion and future prospects

Checkpoint blockade antibodies unleashing the cancer patient's own T-cells to attack and kill cancer cells are effective in cases when cytolytic T-cells directed against neo-epitopes or TAAs exist and are present in the tumor bed. Such tumors are classified as immunologically "hot". For immunologically "cold" tumors on the other hand, where the microenvironment is immunosuppressive, other interventions are needed as well. In those cases the cancer-immunity cycle can serve as a guide to improve therapies. Most importantly, emphasis on altering the immune and inflammatory landscape could make "cold" tumors become "hot" and accessible for checkpoint blockade antibody and/or cell therapies.

Therapeutic DC vaccination has the potential to become effective in all forms of cancer. DC vaccines with TAAs have in general failed to generate strong and long-lasting anti-tumor immune responses most likely because T-cells, especially high-affinity T-cells against TAAs are lacking. With the identification of patient-specific neo-epitopes the likelihood of success increases, as T-cells against the neo-epitopes should have escaped immune selection and be available for activation. It is also possible that patient-specific neo-epitopes identified by sequencing will turn out to be relatively common and therefore have a wider use. Genetic manipulation of DCs to reduce negative signals and augment positive signals may also increase their potency. Whether myeloid or plasmacytoid DC will be most in this context successful remains to be determined. It is more and more appreciated that endogenous DCs *in situ* are required for induction of potent T-cell responses, as bystander DCs have been found to be essential in priming specific CD8+ T-cell immunity after viral infections [132]. Furthermore, antigen transfer has been observed between injected and bystander host DCs and between different resident DC subsets [133]. It is therefore possible that allogeneic DCs could be used as adjuvant

relying on uptake and presentation by endogenous DCs as long as they provide neoantigens and a favorable cytokine profile. This would significantly improve the logistics for DC vaccinations.

In order for CAR T-cells to be effective against solid tumors smart design is needed to protect healthy tissues, as truly tumor-specific surface antigens for CARs are hard to identify. The CAR T-cells must also be able to induce bystander immunity, as antigen heterogeneity will always be an issue for solid tumors. Therefore, future CAR T-cells will likely be armed with inducible secretion of molecules that can alter immune suppression in the tumor microenvironment, change the immunological landscape within tumors and induce bystander immunity. Aspects not discussed in this review that are of great importance is selection of an appropriate T-cell phenotype and finding the appropriate ratio between CD8+ and CD4+ T-cells as starting material for CAR T-cell as well as optimization of the manufacturing methods to be able to deliver a viable and long-lasting CAR T-cell product to patients.

#### Conflict of interest

The authors declare no financial conflict of interest.

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