Development of CAR T cells designed to improve antitumor efficacy and safety

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Abstract

Chimeric antigen receptor (CAR) T cell therapy has shown promising efficacy against hematologic malignancies. Antitumor activity of CAR T cells, however, needs to be improved to increase therapeutic efficacy in both hematologic and solid cancers. Limitations to overcome are 'on-target, off-tumor' toxicity, antigen escape, short CAR T cell persistence, little expansion, trafficking to the tumor and inhibition of T cell activity by an inhibitory tumor microenvironment. Here we will discuss how optimizing the design of CAR T cells through genetic engineering addresses these limitations and improves the antitumor efficacy of CAR T cell therapy in pre-clinical models.

Keywords

Chimeric antigen receptor; Armored CAR T cells; Genetic engineering; Cytokines; Costimulation; Tumor microenvironment

1. Introduction

Chimeric antigen receptor (CAR) T cell therapies show great promise for haematologic malignancies, but less so for solid cancers. CARs consist of an extracellular antigen recognition domain, often the single-chain Fragment variant (scFv) derived from an antibody, a transmembrane domain and the intracellular T cell activation domain of CD3ζ. Second generation CARs add to this a co-stimulatory domain, commonly CD28 or 4-1BB. The CARs are designed to target a tumor specific protein and engineered into autologous T cells, which are subsequently given back to the patient. The most well-studied and successful CARs thus far are targeted against CD19, which is present on normal B cells and B-cell malignancies. Second generation CD19-targeting CARs with either a CD28 (CD28/ CD3ζ, or 28z) or 4-1BB (4-1BB/CD3ζ, or BBz) costimulatory domain have shown

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antitumor activity in several clinical trials, most profoundly against B-cell acute lymphoblastic leukemia (B-ALL) (Brentjens et al., 2011, 2013; Kalos et al., 2011; Kochenderfer et al., 2012).

However, in spite of this success in B-ALL patients, a subset of patients that achieve complete remission relapses with CD19-negative leukemia (Grupp et al., 2013; Lee et al., 2015; Maude et al., 2014). Investigation of underlying causes for CD19 loss showed the presence of a *CD19* isoform that lacked exon 2 (ex2), caused by either genetic alterations or low expression of splicing factor SRSF3 (Sotillo et al., 2015). This CD19- ex2 was more stable than full-length CD19 and retains protein function, but cannot be detected by their flow cytometry antibody and fails to trigger a CD19-targeted CAR T cell response. Recently, two B-ALL patients with rearrangements in the mixed lineage leukemia (*MLL*) gene have been reported of which the relapsed tumor after CD19 CAR T cell therapy demonstrated lineage switching to a CD19-negative myeloid phenotype (Gardner et al., 2016). Jacoby et al. (2016) showed in syngeneic murine pre-B-ALL that CD19 CAR T cell therapy could drive lineage switching and as a result loss of CD19 expression.

In addition to antigen loss in relapsed tumors post CAR T cell therapy, it is expected that tumors are heterogeneous for expression of the targeted antigen. To achieve complete tumor eradication, epitope spreading (Beatty et al., 2014) and a more broad immune response need to be triggered by the CAR T cells, which would result in elimination of antigen-negative tumor cells as well. Heterogeneity might especially play a role in solid cancers. Several targets have been identified for CAR T cell therapy in a variety of solid malignancies (Jackson, Rafiq, & Brentjens, 2016). Thus far, however, clinical trials with CAR T cells in solid cancers have reported only limited antitumor efficacy. Adoptive cell therapy against solid cancers have, in addition to target antigen selection, T cell proliferation and persistence, also specific hurdles to overcome, such as trafficking of the T cells into the tumor and the ability to overcome an immune suppressive microenvironment (Beatty & O'Hara, 2016).

In this review we discuss how further genetic engineering of the CAR T cells can be used to improve their functionality, safety and antitumor efficacy, in both hematologic and solid malignancies (Fig. 1).

2. Specificity and safety of CAR T cell therapy

2.1. Targeting two tumor-associated antigens to reduce antigen escape

Expression of multiple tumor-specific targets reduces the chance of antigen escape by mutating or reducing expression of the target antigen, as both targets would have to be lost simultaneously. Two combinations have shown to prevent CD19-negative tumor escape: CD19/ C123 and CD19/CD20-dual targeting CAR T cells. CD123 is present on B-ALL blasts and CD19-negative relapsed tumors (Ruella et al., 2016). Simultaneous expression of CD123 and CD19-targeting CARs on T cells was more potent than treatment with pooled CAR T cells expressing either CAR. Zah, Lin, Silva-Benedict, Jensen, and Chen (2016) generated a bispecific CAR that gets activated upon binding of either CD19 or CD20 (so-called OR-gate CAR). This CAR showed enhanced antitumor efficacy in a pre-clinical B-

cell lymphoma model, as the outgrowth of CD19-negative tumor cells was abrogated. Co-expressing CD19 CARs with other CARs targeting B-cell malignancies, such as against CD22 (Haso et al., 2013), ROR1 (Hudecek et al., 2010) and immunoglobulin kappa chain (Igκ) (Vera et al., 2006), might have the potential to prevent CD19 antigen escape as well. CAR recognition of two TAA's potentially increases the risk of 'on-target, off-tumor' side effects. This extra toxicity will be minimal when both targets are restricted to the same cell type, such as CD19 and CD20. In addition, a lower affinity of the CAR to each target might lead to a strong CAR T cell activation when both targets are present and only weak against single-target expressing non-tumor cells. Duong, Westwood, Berry, Darcy, and Kershaw (2011) showed in *in vitro* models that T cells expressing two different CARs targeting folate-binding protein and HER2, were more responsive against tumor cells expressing both targets than against normal cells expressing only either one of the targets. This phenomenon was also demonstrated in glioblastoma xenograft tumors with HER2 and IL13Rα2-targeting CARs (Hegde et al., 2013). T cells expressing both CARs improved the antitumor response compared to pooled T cells expressing HER2 CARs or IL13Rα2 CARs. The same group generated a tandem CAR recognizing HER2 and IL13Rα2 simultaneously by inducing heterodimerization of the two targets (Hegde et al., 2016). This tandem CAR (tanCAR) further reduced antigen escape and had increased antitumor efficacy in pre-clinical models.

2.2. Targeting two tumor-associated antigens to increase specificity and safety

Targeting a specific combination of two antigens can also be exploited to increase specificity and reduce on-target, off-tumor side effects, even though the requirement of two antigens for CAR T cell activation increases the chance of antigen escape. CD19 CAR T cells target normal B-cells, leading to B-cell aplasia (Brentjens et al., 2011, 2013; Kalos et al., 2011; Porter, Levine, Kalos, Bagg, & June, 2011), which can be treated with monthly infusions of immunoglobulin. But a case report about a patient treated with HER2-targeting CAR T cells who experienced severe toxicity and died because of low levels of HER2 on lung epithelium (Morgan et al., 2010), demonstrates the need for careful design of CAR constructs, as targets that are completely tumor-specific are scarce.

Increased tumor specificity is achieved by separating the T cell activation signals over two antigen recognition molecules (Kloss, Condomines, Cartellieri, Bachmann, & Sadelain, 2013; Lanitis et al., 2013; Wilkie et al., 2012). The antigens do not need to be truly tumor specific, as long as the combination of the two garners tumor specificity. Kloss et al. (2013) describes CAR-mediated recognition of prostate stem cell antigen (PSCA) with an intracellular CD3ζ domain. Costimulation is provided by prostate specific membrane antigen (PSMA)-specific scFv coupled to CD28 and 4-1BB costimulatory domains (a chimeric costimulatory receptor). Lanitis et al. (2013) showed that transactivated CAR T cells (anti-mesothelin-CD3ζ plus anti-folate receptor-CD28) have similar antitumor efficacy against tumors expressing both antigens compared to cis-activated CAR T cells (anti-mesothelin-CD28-CD3ζ) and show less activity against single-positive tumors. This approach is expected to reach highest specificity when the CAR-mediated antigen recognition is relatively inefficient and T cells are only fully activated in presence of the antigen targeted by the chimeric costimulatory receptor.

Recently the lab of Wendell Lim has developed a different system in which two antigens are similarly needed for full CAR T cell activation: an AND-gate CAR, termed synNotch (Morsut et al., 2016; Roybal, Rupp, et al., 2016; Roybal, Williams, et al., 2016). This synthetic molecule consists of an engineered antigen-recognition domain, a Notch core and an artificial transcription factor, which gets cleaved off and activated upon antigen stimulation. This transcription factor specifically induces expression of the CAR, so the CAR and therefore the T cells only get activated when both antigens are present. This system works orthogonally and does not require an intermediate signaling molecule, creating a flexible tool to regulate specific signal-response cascades in a variety of applications. It remains to be investigated, however, whether the non-human transcription factors are immunogenic.

Another strategy to decrease on-target, off-tumor reactivity of CAR T cells is to co-express an inhibitory CAR (iCAR) that recognizes an antigen expressed on non-tumor tissues. The iCAR consists of an antigen-recognition domain coupled to the intracellular domain of T-cell checkpoint proteins programmed cell death protein 1 (PD-1) or cytotoxic T-lymphocyte-associated protein 4 (CTLA-4). Cells that express the iCAR target, even in the presence of the activating CAR antigen, do not activate T cells, thereby reducing damage to normal tissue (Fedorov, Themeli, & Sadelain, 2013). In this way, off-tumor toxicities are prevented rather than treated while the CAR T cells retain their antitumor activity. This is in contrast to the activation of suicide genes when toxicity is apparent (discussed below).

2.3. Suicide genes to control CAR T cell presence

CAR T cell therapy frequently results in severe side effects, such as the above-discussed on-target, off-tumor toxicity, neurotoxicity and cytokine release syndrome (CRS) with fevers and high levels of serum cytokines (Davila & Sadelain, 2016). CRS toxicity was found to correlate with tumor burden (Davila et al., 2014) and requires treatment with steroids and/or the anti-IL6 receptor antibody tocilizumab. CAR T cells may be selectively ablated by engineered expression of suicide genes, which can be activated in the case of acute toxicity or to reverse B-cell aplasia due to long-term CAR T cell survival as seen in the setting of CD19-targeted CAR T cells.

Transduction of allogeneic lymphocytes with herpes simplex virus thymidine kinase (HSV-tk) enables elimination of the lymphocytes by administration of ganciclovir when graft-versus-host disease (GVHD) developed in lymphoma patients (Bonini et al., 1997) and can be used for positron emission tomography (PET) imaging of the infused cells with a ^{18}F-radiolabeled ganciclovir analog (Keu et al., 2017). This virus-specific gene, however, might be immunogenic. Indeed, HSV-tk-specific CD8^{+} and CD4^{+} T cell responses developed in patients after hematopoietic cell transfer (Berger, Flowers, Warren, & Riddell, 2006). A similar immunogenic response can be anticipated against the new doxycycline-inducible Tet-On CAR expression systems (Sakemura et al., 2016). Another well-studied suicide gene for adoptive cell transfer is inducible caspase 9 (iCasp9), consisting of the intracellular domain of the pro-apoptotic human caspase 9 protein fused to a human FK506 binding protein (FKBP), allowing for dimerization induced by small molecules such as AP1903 (Straathof et al., 2005). Activation of iCasp9 in patients who developed GVHD after

genetically engineered T cell transplantation rapidly induced T cell apoptosis and reversed the GVHD (Di Stasi et al., 2011). Pre-clinical studies have observed efficient T cell elimination in the context of iCasp9-engineered CAR T cells (Budde et al., 2013; Hoyos et al., 2010; Minagawa et al., 2016) and the first clinical trials with iCasp9 in combination with anti-GD2 CAR T cell therapy are currently open to accrual (NCT02992210, NCT02107963).

Approaches for antibody-mediated T cell ablation, through antibody-mediated cellular cytotoxicity (ADCC) and complement-mediated toxicity, include T cell transduction of CD20, targeted by rituximab (Griffioen et al., 2009; Vogler et al., 2010) and a truncated form of the epithelial growth factor receptor (EGFR), targeted by cetuximab (Koneru, O'Cearbhaill, Pendharkar, Spriggs, & Brentjens, 2015; Koneru, Purdon, Spriggs, Koneru, & Brentjens, 2015; Paszkiewicz et al., 2016; Wang et al., 2011). EGFRt-mediated CAR T cell elimination is incorporated in several CAR T cell clinical trials targeting CD171 (NCT02311621), CD19 (NCT02028455, NCT01865617, NCT02146924, NCT02051257), CD123 (NCT02159495) and MUC16^{ecto} (NCT02498912).

3. Armored CARs to improve T cell function

The antitumor activity of CAR T cell therapy is not only determined by antigen specificity; also persistence and expansion of the CAR T cells and an immune-suppressive microenvironment play a role. Persistence of second generation CAR T cells is dependent on many factors, including the costimulatory domain(s) (Zhao et al., 2015), tumor burden (Brentjens et al., 2011), lymphodepletion prior to CAR T cell therapy (Turtle et al., 2016) and stimulation of activation-induced T-cell inhibitory proteins, such as PD-1 (Cherkassky et al., 2016). Interestingly, ibrutinib, a Bruton tyrosine kinase (BTK) inhibitor used for treatment of CLL, was shown to reduce PD-1 expression on CAR T cells and to increase persistence and antitumor activity (Fraietta et al., 2016). The importance of CAR T cell persistence is demonstrated by its correlation with survival in both hematologic (Porter et al., 2015) and solid cancers (Louis et al., 2011).

To further improve their function, CAR T cells can be engineered to co-express other molecules, such as cytokines and costimulatory molecules. This not only stimulates these 'armored' CAR T cells in an autocrine manner, but may also have a pro-inflammatory effect on other immune cells within the tumor microenvironment, such as tumor-associated macrophages, dendritic cells (DCs), natural killer (NK) cells and tumor-infiltrating lymphocytes (TILs). Below we discuss various approaches that have been studied in the pre-clinical setting.

3.1. Armored CAR T cells secreting cytokines

Several cytokines have been investigated to enhance adoptive transfer efficacy, both for TILs and CAR T cells, mainly to increase the *ex vivo* expansion of the infusion product. Systemic administration of recombinant cytokine(s) to stimulate T cell activity after adoptive cell transfer is often associated with severe toxicities. To avoid systemic side effects, we and others have investigated expression of cytokines by the T cells themselves to locally

stimulate persistence and expansion in an autocrine manner. Here we will discuss expression of interleukin 12 (IL-12), IL-2 and IL-15.

IL-12 is most studied for its potent T cell activating effect. IL-12 is a pro-inflammatory cytokine that is produced by DCs, macrophages and neutrophils. It enhances cytotoxic activity of CD8^{+} T cells and NK cells and stimulates a Th1 helper T cell response. Because of its potent effect, cancer patients have been treated with systemic recombinant IL-12 (rIL-12). This led, however, to severe toxicity (Lacy et al., 2009) and treatment-related mortality (Leonard et al., 1997). To avoid these systemic side effects, several studies have engineered tumor-specific T cells to express IL-12, resulting in tumor-localized IL-12 secretion. In a B16 murine melanoma model, IL-12-secreting tumor-reactive pmel-1 CD8^{+} T cells still caused weight loss and decreased survival when more than 5×10^5 cells were administered (Kerkar et al., 2010). Lower cell numbers without side effects showed increased accumulation and antitumor activity in B16 melanomas. The pmel-1-reactive T cells, however, did not proliferate and, in fact, constitutive expression of IL-12 induced apoptosis. To circumvent this, the investigators generated inducible IL-12 under the promoter of nuclear factor of activated T cells (NFAT) (Zhang et al., 2011). These NFAT-IL-12 pmel-1 T cells retained antitumor activity without inducing toxicity. In the same pmel-1 model, local secretion of IL-12 was shown to increase cross-presentation of natural tumor antigens and reprogram myeloid-derived cells, including dendritic cells, macrophages and myeloid-derived suppressor cells (MDSCs), stimulating the CD8^{+} T cells (Kerkar et al., 2011). Fas expression on the tumor-resident myeloid-derived cells was shown to mediate the enhanced antitumor activity of IL-12-pmel-1 T cells (Kerkar et al., 2013). Patients with metastatic melanoma who received TILs transduced with NFAT-IL-12, however, experienced grade 3 and 4 toxicities (Zhang et al., 2015), similar to what has been observed after intratumor injection of rIL-12 (van Herpen et al., 2004). In the case of CAR T cells, inducible NFAT-IL-12 production also demonstrated improved antitumor activity in mice, at least in part mediated by macrophages, without apparent toxicities (Chinnasamy et al., 2012; Chmielewski, Kopecky, Hombach, & Abken, 2011).

We generated constitutive IL-12 production by engineering a bicistronic vector encoding for a CD19-targeting CAR and a murine IL-12 α and β subunit fusion. In an immune-competent EL4 thymoma mouse model expressing human CD19, IL-12 production by hCD19-targeting CAR T cells increased the cytotoxicity and cytokine production of the CAR T cells and led to tumor eradication *in vivo* (Pegram et al., 2012). This required autocrine IL-12 signaling and subsequent IFNγ production. Importantly, hCD19/IL-12 CAR T cells were able to overcome inhibition of CAR T cell activity by regulatory T cells (Tregs), eliminating the need for prior conditioning chemotherapy to remove endogenous T and B cells (Pegram et al., 2012). Also a CAR targeting the MUC16[ecto] domain coupled to IRES-IL-12 expression demonstrated improved cytokine secretion, expansion and *in vivo* antitumor efficacy (Koneru, Purdon, et al., 2015). In both models no toxicities were observed. The MUC16[ecto] CAR cells secreting IL-12 are currently being tested in a phase I clinical trial (NCT02498912). To increase safety a low starting dose, 3×10^5 CAR T cells/kg, is used for dose escalation. In addition, expression of the suicide gene EGFRt has been included to alleviate potential on-target, off-tumor or other toxicity (Koneru, O'Cearbhaill, et al., 2015).

Genetic engineered secretion of IL-2 has been studied in the context of TIL therapy. T cells normally produce IL-2 upon activation and IL-2 is required for a good T cell response. IL-2 has been well studied to enhance the efficacy of adoptive transfer. Systemic high-dose rIL-2 treatment of cancer patients, however, demonstrated a wide range of toxicities and treatment-related deaths (Rosenberg et al., 1989). To achieve local IL-2 production by adoptively transferred T cells for autocrine IL-2 signaling, melanoma-derived TILs were transduced to constitutively express IL-2. This resulted in prolonged T cell proliferation after antigen removal by tumor cell death and in absence of exogenous IL-2, while retaining tumor specificity (Liu & Rosenberg, 2003). In metastatic melanoma patients, however, IL-2 producing TILs caused toxicity and an increased clinical response was not observed compared to untransduced TILs (Heemskerk et al., 2008).

Similar to IL-2, IL-15 stimulates activity and expansion of CD8^{+} cytotoxic T cells and NK cells and is produced by monocytes, macrophages and DCs (Waldmann, Dubois, & Tagaya, 2001). Autocrine IL-15 signaling largely increased the proliferative capacity of IL-15-transduced lymphocytes after external cytokine withdrawal, while retaining antigen recognition (Hsu et al., 2005). *Ex vivo* IL-15 stimulation of CAR T cells also increased proliferation and effector function and systemic treatment with rIL-15 after CAR T cell treatment in the SKOV3 ovarian cancer model slightly decreased tumor growth (Xu et al., 2016), potentially *via* reducing CD8^{+} T cell senescence (Weng et al., 2016). Further studies by Hsu et al. (2007), however, showed that IL-15-transduced T cells from one donor (out of 23) proliferated exponentially *ex vivo* for more than a year, indicating that expression of an IL-15 transgene could be leukemogenic. ShRNA-mediated knockdown of the IL-15 receptor showed a decrease in proliferation, demonstrating that the effect was indeed mediated by IL-15 signaling. To avoid the leukemogenic effect of IL-15 transduction, systemic treatment could be considered for this cytokine. Recombinant IL-15 could be safely administered to metastatic cancer patients (Conlon et al., 2015), although it is not clear whether intratumor IL-15 concentrations will be high enough to stimulate adoptively transferred TILs or CAR T cells. Co-expression of a suicide gene makes it possible to retain the local T cell-specific stimulation by IL-15 transduction, while preventing uncontrolled T cell proliferation (Hoyos et al., 2010; Quintarelli et al., 2007). Hoyos et al. (2010) generated a tricistronic vector expressing a CD19-targeting CAR construct combined with the IL-15 transgene and iCasp9 suicide gene. 19CAR/IL-15/ iCasp9 T cells produced IL-15 in response to antigen stimulation and demonstrated increased persistence, expansion and antitumor activity in the Raji (Burkit lymphoma) and Daudi (B cell lymphoma) xenograft models. Treatment with AP20187, the substrate for iCasp9, eliminated the CAR T cells in mice, indicating a safe approach to abolish uncontrolled T cell proliferation or other adverse effects.

3.2. Armored CAR T cells expressing (chimeric) cytokine receptors

Cytokines are powerful mediators to drive a pro or anti-inflammatory immune response. Responsiveness of CAR T cells to cytokines can be influenced by engineered expression of cytokine receptors. Taking this a step further, one can transform binding of an anti-inflammatory Th2 cytokine into a pro-inflammatory Th1 signal by fusing the ectodomain and endodomain of two different cytokine receptors. Combining the extracellular part of the Th2 cytokine Il-4 receptor α (IL-4Rα) with the intracellular β-subunit of the IL-2 and IL-15

receptors, creating the chimeric cytokine receptor 4αβ, has shown to largely increase T cell expansion *in vitro* in the presence of IL-4, more than with standard IL-2 treatment (Wilkie et al., 2010). 4αβ-T1E28z CAR T cells (called T4 immunotherapy), targeting ErbB dimers, also demonstrated *in vivo* antitumor activity (van der Stegen et al., 2013), although it is unclear how this compares to T1E28z CAR T cells. Interestingly, the toxicity profile of T4 immunotherapy depends on the route of CAR T cell administration; intraperitoneal administration induces CRS, whereas intratumoral and intravenous administration does not (van der Stegen et al., 2013). Intratumoral administration of T4 immunotherapy is currently being investigated in head and neck cancer patients (NCT01818323) (van Schalkwyk et al., 2013).

Leen et al. (2014) generated another chimeric cytokine receptor with IL-4, using the intracellular signaling domain of the IL-7 receptor. IL-7 is important for T cell homeostasis and expansion, but IL-7R levels are low on stimulated cytotoxic T cells (Vera et al., 2009). Expression of this IL-4/7 receptor on Epstein-Barr Virus (EBV)-specific T cells promotes a Th1 response instead of an IL-4-induced Th2 response and increases antitumor activity in an IL-4 producing xenograft tumor model. Conversely, genetically engineered expression of the IL-7 receptor α (IL-7Rα) itself in T cells restores their responsiveness to IL-7 (Vera et al., 2009). Perna et al. (2014) generated a bicistronic vector encoding a GD2-targeting CAR and IL-7Rα. Expression of both molecules on EBV-specific T cells stimulates *in vitro* proliferation and *in vivo* antitumor activity in the presence of IL-7 and overcomes inhibition by Tregs, which do not respond to IL-7. Importantly, treatment with recombinant IL-7 is well tolerated in patients (Rosenberg et al., 2006; Sportès et al., 2008), in contrast to IL-12 and IL-2, making this a feasible strategy for the clinic.

3.3. CAR T cells armored with additional costimulation

T cell receptor (TCR)-mediated T cell activation is amplified *via* co-stimulatory signaling. This reduces T cell anergy (unresponsiveness) and exhaustion. Initially, CAR constructs contained an intracellular CD3ζ chain for T cell activation (first generation). Second generation CARs contain an additional costimulatory domain (van der Stegen, Hamieh, & Sadelain, 2015), mostly CD28 or 4-1BB, which results in CAR T cell expansion and cytokine secretion upon target antigen exposure (Imai et al., 2004; Maher, Brentjens, Gunset, Rivière, & Sadelain, 2002). Many costimulatory and coinhibitory molecules have been identified to date, which are tightly regulated in space and time to achieve different functional outcomes (Chen & Flies, 2013). In an immune suppressive microenvironment, however, T cells may not be exposed to sufficient costimulation. Tumor cells often do not express costimulatory ligands CD80 and CD86 and expression of costimulatory receptors on DCs is inhibited (Zou, 2005). Here we discuss two costimulatory molecules, CD40L and 4-1BBL, genetically engineered to be constitutively expressed on armored CAR T cells to provide additional costimulation independent of antigen-presenting cells (APCs).

CD40 ligand (CD40L, CD154) is a type II transmembrane protein and member of the tumor necrosis factor (TNF) receptor superfamily. CD40L was initially found to be expressed on activated T and B cells and later also identified on a variety of immune and non-immune cell types (Armitage et al., 1992; Schönbeck & Libby, 2001). CD40L binds to its receptor CD40,

which is also expressed on multiple cell types, including DCs, macrophages and lymphocytes (Schönbeck & Libby, 2001). CD40 activation on DCs by binding to CD40L upregulates the costimulatory molecules CD80 and CD86 and is essential for production of IL-12, which in turn stimulates a Th1 T cell response (Cella et al., 1996). CD40/CD40L interaction also stimulates the generation of CD8^{+} T cell memory (Bourgeois, Rocha, & Tanchot, 2002) and reverses CD8^{+} T cell exhaustion (Bhadra, Gigley, & Khan, 2011). Interestingly, CD40 is expressed on several hematological and solid tumors. CD40 agonists have a direct antitumor effect and activate APCs to generate an antitumor specific cytotoxic T cell response (Khong, Nelson, Nowak, Lake, & Robinson, 2012). CLL patients that were treated with CLL cells transduced with murine CD40L adenovirus demonstrated increased plasma levels of IL-12 and IFNγ, tumor-specific T cells and a reduced tumor burden (Wierda et al., 2000). Together, this indicates that the CD40/CD40L axis can be used to elicit a direct as well as an APC-mediated antitumor response. When T cells are transduced with CD40L and co-cultured with either CD40^{+} tumor cells or DCs, this increases the tumor immunogenicity, DC maturation and IL-12 secretion (Curran et al., 2015). Furthermore, a bicistronic vector was generated to constitutively express CD40L on CD19-targeting CAR T cells. CD40L expression increased the CAR T cell cytotoxicity *in vitro* and prolonged survival of DOHH2 lymphoma-bearing mice. CAR T cells armored with CD40L expression is a promising approach to increase the antitumor efficacy of CAR T cell therapy.

The costimulatory receptor 4-1BB is also a type II transmembrane protein and TNF receptor superfamily member (Schwarz, Tuckwell, & Lotz, 1993). It is expressed on activated T cells and binding of 4-1BB ligand (4-1BBL) increases T cell proliferation, cytokine secretion and cytolytic capacity (Shuford et al., 1997; Takahashi, Mittler, & Vella, 1999). 4-1BB plays a role in maintenance of CD8^{+} T cell memory (Sabbagh, Snell, & Watts, 2007). In addition to T cells, 4-1BB has been found on other lymphoid cells, monocytes, DCs and other non-hematological cells, but not on cancer cells (Drenkard et al., 2007; Melero, Johnston, Shufford, Mittler, & Chen, 1998; Schwarz, Valbracht, Tuckwell, von Kempis, & Lotz, 1995; von Kempis, Schwarz, & Lotz, 1997; Wilcox et al., 2002; Zhang et al., 2010). DCs are activated by 4-1BBL binding to 4-1BB and secrete pro-inflammatory cytokines, such as IL-12 and IL-6 (Futagawa et al., 2002). To provide 4-1BB costimulation to CAR T cells and the tumor microenvironment, two pre-clinical studies have investigated the effects of armored CAR T cells with constitutive 4-1BBL expression on CAR T cell activity and antitumor efficacy.

Stephan et al. (2007) transduced PSMA-targeting first generation CAR T cells (PSMA/CD3ζ, Pz1) with CD80 and/or 4-1BBL. Constitutive coexpression of CD80 and 4-1BBL on Pz1 CAR T cells showed increased proliferation upon stimulation with PSMA^{+}CD80^{−}CD86^{−}4-1BBL^{−}LNCaP prostate cancer cells. This increase was more modest when CD80 and 4-1BBL were expressed on the cancer cells instead of on the CAR T cells. CAR T cells armored with both CD80 and 4-1BBL were able to eradicate PSMA[+] tumors *in vivo*, whereas CAR T cells expressing either CD80 or 4-1BBL showed no benefit compared to Pz1 CAR T cells alone. Furthermore, the investigators were able to show that coexpression of CD80 and 4-1BBL provides costimulation to the CAR T cell itself and, importantly, transcostimulation to other tumor-reactive T cells, not expressing CD80 or 4-1BBL. This transcostimulation may enhance the antitumor response of TILs that are

reactive to other TAAs, thereby broadening the antitumor immune response and reducing the chance of tumor antigen escape.

In order to elucidate optimal costimulatory signaling in CD19-targeting CAR T cells, Zhao et al. (2015) investigated various combinations of CD28 and 4-1BB receptors and their respective ligands CD80 and 4-1BBL. The initial comparison between second generation CAR T cells with either CD28 (1928z) or 4-1BB (19BBz) costimulatory domains showed different kinetics for CAR T cell proliferation and antitumor efficacy. Four different possibilities to combine CD28 and 4-1BB signaling in the CAR T cells were investigated, where the receptors are incorporated in the CAR molecule and the ligands coexpressed: 1928BBz, 1928z/4-1BBL, 19BBz/CD80 and 19z1/CD80/4-1BBL. Of these, 4-1BBL-armored 1928z CAR T cells showed the highest CD8/CD4 ratio and the best *in vivo* antitumor activity, which was in part mediated by IRF7, a transcription factor for type I interferons. The resulting increased IFNβ production could potentially increase antigen cross-presentation on intratumoral DCs (Yang et al., 2014), disrupt the tumor vasculature (Spaapen et al., 2014) and inhibit activation and proliferation of Tregs (Srivastava, Koch, Pepper, & Campbell, 2014). The authors hypothesize that together with transcostimulation in the tumor microenvironment (discussed above (Stephan et al., 2007)) this may be responsible for the increased antitumor activity of 1928z/4-1BBL armored CAR T cells. Together, these studies demonstrate how smart design of engineered costimulation has a big impact on behavior and antitumor efficacy of CAR T cells.

3.4. Armored CAR T cells to overcome an inhibitory tumor microenvironment

CAR T cell antitumor activity may be limited by an immune suppressive TME; not only through inhibitory cell types, such as MDSCs and Tregs (which may be targeted by IL-12, see 2.1), but also the expression and stimulation of inhibitory receptors. Normally, activated T cells express these checkpoint proteins, such as PD-1, CTLA-4, T-cell membrane protein 3 (TIM-3) and lymphocyte activating gene 3 (LAG-3), to control T cell activation and prevent autoimmunity, and are therefore often used as markers of T cell exhaustion. Cancer cells frequently express the PD-1 ligands PD-L1 and PD-L2, thereby preventing an effective anti-tumor immune response. PD-L1 can also bind to CD80, and thus prevent it from binding to CD28, adding another layer to T cell inhibition. Antibodies that block CTLA-4 (ipilimumab) or PD-1 (nivolumab and pembrolizumab) have been successful in patients with metastatic melanoma (Larkin et al., 2015; Wolchok et al., 2013) and non-small cell lung cancer (Borghaei et al., 2015; Brahmer et al., 2015; Garon et al., 2015) and are being tested in many other cancer types.

In syngeneic pre-clinical models, adoptively transferred T cells show upregulation of PD-1, TIM-3 and LAG-3 and combining cell therapy with PD-1 blockade increases antitumor efficacy of CAR T cells (John et al., 2013) and TILs (Moon et al., 2016). Important results are expected from the first clinical trial combining CAR T cells with CTLA-4 blockade (NCT00586391). A recent case study reported a patient with PD-L1[+] diffuse large B cell lymphoma who progressed after CAR T cell therapy and received pembrolizumab 26 days after CAR T cell infusion (Chong et al., 2017). PD-1 blockade led to an increase in circulating CAR T cells and reduced size of tumor lesions. Although it is unclear whether

this was mediated by CAR T cells or endogenous TILs or both, it is a promising response and a phase I/II clinical trial has been initiated for pembrolizumab treatment in CD19 CAR T-cell refractory or relapsing patients (NCT02650999).

Treatment with checkpoint inhibitors can cause severe immune-related adverse events, which can be treated with steroids or anti-TNFα antibodies (Michot et al., 2016). Even though treatment of the adverse events does not affect treatment efficacy or overall survival (Horvat et al., 2015), local checkpoint blockade might be able to prevent systemic side effects and further improve CAR T cell efficacy. Several approaches have been developed to prevent inhibitory signaling *via* the PD-1 receptor on CAR T cells. Secretion of anti-PD-L1 antibodies by armored CAR T cells against carbonic anhydrase IX (CAIX) increases the antitumor CAR T cell efficacy in an orthotopic xenograft model (Suarez et al., 2016). Cherkassky et al. (2016) used an anti-mesothelin CAR in an orthotopic pleural mesothelioma model and tested CAR T cells with either CD28 (M28z) or 4-1BB (MBBz) costimulation, of which the latter performed better than M28z at lower doses. M28z CAR T cells showed stronger PD-1 upregulation *in vivo* and they used three different approaches to disrupt PD-1-mediated CAR T cell inhibition. Both systemic administration of an αPD-1 antibody and especially coexpression of a dominant negative PD-1 receptor, which binds to PD-L1 but does not signal, improved tumor control by CAR T cells. Thirdly, coexpression of a PD-1-targeting short hairpin RNA improved CAR T cell function *in vitro*, but not *in vivo*, possibly due to incomplete knockdown of PD-1. In addition, disruption of PD-1 with the CRISPR/Cas9 system enhanced antitumor activity of PSCA CAR T cells and also in CD19 CAR T cells with additional disruption of TCR and beta-2-microglobulin (required for HLA class I expression) to allow for allogeneic use of the CAR T cells (Ren et al., 2016).

Another approach to not only abolish PD-1-mediated inhibition, but even turn inhibitory PD-1 signaling into a pro-inflammatory signal, Liu et al. (2016) coexpressed a "switch receptor" with the PD-1 extracellular domain and CD28 intracellular domain (Prosser, Brown, Shami, Forman, & Jensen, 2012) in CAR T cells. In two different solid tumor models PD-1/CD28-armored CAR T cells with 4-1BB costimulation showed increased antitumor activity and less exhaustion, compared to mice that received CAR T cells plus the PD-1 inhibitor pembrolizumab, or armored CAR T cells with a truncated non-functional PD-1.

Together, these studies demonstrate an increased CAR T cell antitumor activity when the PD-1/PD-L1 inhibitory axis is blocked or converted into a pro-inflammatory T cell signal. Most approaches, except the secretion of an anti-PD-L1 antibody (Suarez et al., 2016), do not affect inhibitory checkpoint signaling on endogenous TILs. Secreting a checkpoint inhibitor in the local tumor microenvironment might trigger a broader antitumor T cell response and increase efficacy of the CAR T cell therapy. Thus far, targeting other checkpoint proteins, such as CTLA-4, LAG-3 or TIM-3, have not been studied in the context of CAR T cell therapy. Dual blockade of PD-1 and a second checkpoint protein, however, might further optimize therapy as upregulation of TIM-3 has been demonstrated on PD-1-blocked T cells in murine and human lung tumors that acquired resistance to anti-PD-1 therapy (Koyama et al., 2016).

4. Other applications of armored CAR T cells

4.1. Armored CAR T cells to improve trafficking to tumor

In order to reach their full potential of killing tumor cells, CAR T cells have to migrate to and infiltrate into the tumor tissue. Especially for solid tumors, CAR T cell infiltration can present a major obstacle, as endogenous T cells are often excluded from the tumor microenvironment as well (Joyce & Fearon, 2015). Local delivery of CAR T cell can greatly enhance their therapeutic efficacy (Adusumilli et al., 2014). This is, however, not feasible for all tumor sites and metastatic lesions. To enhance CAR T cell migration to the tumor several studies have expressed receptors of tumor-secreted chemokines on CAR T cells to enhance migration to the tumor and efficacy of CAR T cell therapy.

Di Stasi et al. (2009) showed that Reed-Stemberg cells of Hodgkin lymphoma produce CCL17 and CCL22. These bind to CCR4, which is expressed by Tregs and Th2 cells, but not by activated cytotoxic T cells. Co-expression of CCR4 on CD30-targeting CAR T cells improved CAR T cell migration to the tumor site and antitumor efficacy. A similar increase in CAR T cell infiltration and antitumor activity has been demonstrated for CCR2b expression in anti-GD2 (Craddock et al., 2010) and anti-mesothelin (Moon et al., 2011) CAR T cells and for CXCR2 expression in pmel-1 T cells (Peng et al., 2010). Collectively, these pre-clinical findings indicate that CAR T cell trafficking to the tumor can be enhanced through engineered expression of tumor-associated chemokine receptors.

4.2. CAR T cells as micropharmacies

As discussed above, CAR T cells can be engineered to express anything to stimulate an antitumor T cell function or manipulate the tumor microenvironment. In addition, the tumor-specific nature of CAR T cell therapy can be used to deliver molecules that target the tumor cells directly. Boice et al. (2016) found that in follicular lymphomas (FL) the interaction between the tumor suppressor HVEM and BTLA is frequently lost due to loss of one of the two molecules, leading to B cell proliferation, accompanied by an increase in BTLA-expressing follicular helper T cells. Treatment with soluble HVEM ectodomain protein (solHVEM) in HVEM-deficient FL activated the inhibitory BTLA receptor and reduced proliferation of lymphoma cells *in vitro*. Intratumoral injections of solHVEM in subcutaneous lymphomas inhibited tumor growth *in vivo*, but less so with 10-fold more systemic solHVEM administration. To address this limitation, the investigators generated CD19 CAR T cells that secrete solHVEM and showed reduced tumor growth compared to non-solHVEM-producing CAR T cells. This indicates that CAR T cells can be used for local delivery of tumor-targeting factors to increase the antitumor efficacy of both CAR T cell therapy and targeted cancer therapy.

5. Concluding remarks

Clinical studies have demonstrated great potential of CAR T cell therapy, especially in B-ALL. Further improving the antitumor activity of CAR T cells, however, will be important to be successful in other hematologic and solid malignancies. The generation of armored CAR T cells is a powerful strategy to achieve local expression and secretion of a variety of

molecules to stimulate CAR T cell function, manipulate tumor-infiltrating immune cells and target the tumor cells directly. Local production of cytokines and checkpoint inhibitors is expected to reduce side effects that are observed with systemic administration. Synthetic molecules such as the CAR itself, chimeric costimulatory receptors and synNotch increase tumor specificity and reduce antigen escape. Furthermore, genetic engineering of chimeric receptors allows for converting inhibitory signals into a stimulating response and *vice versa*, as discussed for the iCAR, switch receptor and chimeric cytokine receptors, thereby increasing tumor specificity, safety and reducing immune suppressive signaling. Pre-clinical studies in syngeneic tumor models will help to elucidate which of the discussed strategies will be optimal, which likely differs per target antigen and tumor (sub)type. Ultimately, clinical trials with armored CAR T cells will provide crucial information to make CAR T cell therapy more safe and effective in a broad range of malignancies.

Abbreviations

28z	CD28 + CD3ζ
4-1BBL	4-1BB ligand
B-ALL	B cell acute lymphatic leukemia
BBz	4-1BB + CD3ζ
CAR	chimeric antigen receptor
CD40L	CD40 ligand
CRS	cytokine release syndrome
CTLA-4	cytotoxic T-lymphocyte associated protein 4
DCs	dendritic cells
GVHD	graft-*versus*-host disease
iCAR	inhibitory chimeric antigen receptor
iCasp9	inducible caspase 9
IL	interleukin
NFAT	nuclear factor of activated T cells
NK cells	natural killer cells
PD-1	programmed cell death protein 1
PD-L1	programmed cell death 1 ligand 1
PSCA	prostate stem cell antigen
PSMA	prostate specific membrane antigen

TAA	tumor-associated antigen
TILs	tumor-infiltrating lymphocytes
Tregs	regulatory T cells

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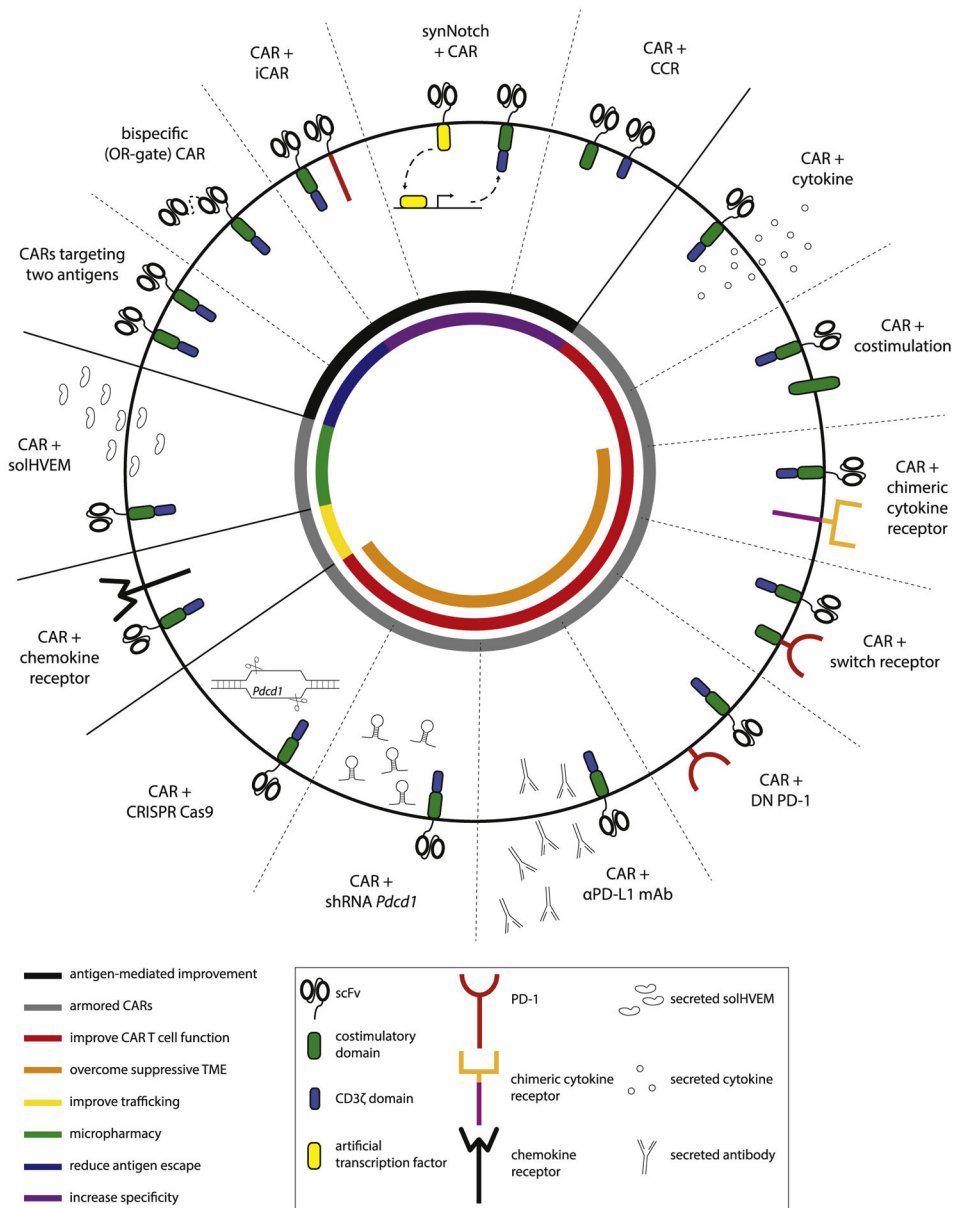
Fig. 1.
Summary of genetic strategies to improve CAR T cell function and antitumor activity. CAR: chimeric antigen receptor, iCAR: inhibitory CAR, CCR: chimeric costimulatory receptor, DN: dominant negative, PD-1: programmed cell death protein 1, αPD-L1 mAb: anti-PD-1 ligand 1 monoclonal antibody, shRNA: small hairpin RNA, solHVEM: soluble HVEM ectodomain protein, TME: tumor microenvironment.