

# Combination of genetically engineered T cells and immune checkpoint blockade for the treatment of cancer

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## Abstract

Immune checkpoint (IC) blockade using monoclonal antibodies is currently one of the most successful immunotherapeutic interventions to treat cancer. By reinvigorating antitumor exhausted T cells, this approach can lead to durable clinical responses. However, the majority of patients either do not respond or present a short-lived response to IC blockade, in part due to a scarcity of tumor-specific T cells within the tumor microenvironment. Adoptive transfer of T cells genetically engineered to express chimeric antigen receptors (CARs) or engineered T-cell receptors (TCRs) provide the necessary tumor-specific immune cell population to target cancer cells. However, this therapy has been considerably ineffective against solid tumors in part due to IC-mediated immunosuppressive effects within the tumor microenvironment. These limitations could be overcome by associating adoptive cell transfer of genetically engineered T cells and IC blockade. In this comprehensive review, we highlight the strategies and outcomes of preclinical and clinical attempts to disrupt IC signaling in adoptive T-cell transfer against cancer. These strategies include combined administration of genetically engineered T cells and IC inhibitors, engineered T cells with intrinsic modifications to disrupt IC signaling, and the design of CARs against IC molecules. The current landscape indicates that the synergy of the fast-paced refinements of gene-editing technologies and synthetic biology and the increased comprehension of IC signaling will certainly translate into a novel and more effective immunotherapeutic approaches to treat patients with cancer.

**Keywords:** immune checkpoint inhibitors, chimeric antigen receptor, engineered T cells, cancer immunotherapy, gene editing

**Abbreviations:** A<sub>2A</sub>R: Adenosine A<sub>2A</sub> receptor; AML: Acute myeloid leukemia; APCs: Antigen-presenting cells; AT: Austria; BE: Belgium; CA: Canada; CAR(s): Chimeric antigen receptor(s); Cas9: CRISPR-associated 9; CD3ζ: T-cell surface glycoprotein CD3 zeta chain; CEACAM-1: Carcinoembryonic antigen cell adhesion molecule 1; CR: Complete response; CRISPR: *Clustered Regularly Interspaced Short Palindromic Repeats*; CTL019: Tisagenlecleucel; CTLA-4: Cytotoxic T-lymphocyte-associated protein 4; DCs: Dendritic cells; DE: Germany; DLBCL: Diffuse large B-cell lymphoma; DNR(s): Dominant negative receptor(s); ES: Spain; FDA: Food and Drug Administration; Flt-3L: FMS-like tyrosine kinase 3 ligand; Gal-9: Galectin-9; HLA: Human Leucocyte Antigen; HMGB1: High mobility group protein B1; IC(s): Immune/ immunological checkpoint(s); iCasp9: Inducible caspase 9 safety switch; IFNγ: Interferon gamma; IgSF: Immunoglobulin superfamily; IL: Interleukin; KTE-C19: Axicabtagene ciloleucel; LAG-3: Lymphocyte-activation gene 3; LSECtin: Liver and lymph node sinusoidal endothelial cell C-type lectin; mAb(s): Monoclonal antibodies(s); MHC: Major histocompatibility complex; N/A: Not available; NHL: Non-Hodgkin Lymphoma; NK: Natural killer; NL: Netherlands; NSCLC: Non-small cell lung cancer; ORR: Overall response rate; PD: Progressive disease; PD-1: Programmed cell death protein 1; PD-L1: *Programmed Death Ligand 1*; PD-L2: *Programmed Death Ligand 2*; PR: Partial response; PRAME: Preferentially expressed antigen in melanoma; PtdSer: Phosphatidylserine; r/r: relapsed or refractory; scFv(s): Single chain variable fragment(s); shRNA: Short hairpin RNA; siRNA: small-interfering RNA; Tc1: T cytotoxic 1; TCR(s): T cell receptor(s); Th1: T helper 1; Th17: T helper 17; TIGIT: T cell immunoglobulin and ITIM domain; TILs: Tumor infiltrating lymphocytes; TIM-3: T cell immunoglobulin and mucin 3; TNFα: Tumour Necrosis Factor alpha; T<sub>regs</sub>: Regulatory T cells; UK: United Kingdom; US: United States of America.

## Introduction

In the last few decades, immunotherapy has become one of the most promising cancer-treatment modalities. The extensions in survival rates, as well as the long-lasting responses achieved in various cancers, demonstrate the significant impact of immunotherapy versus standard treatments [1–3]. Distinct cancer immunotherapy strategies have emerged that include immunological checkpoint (IC) inhibitors, adoptive cellular immunotherapy, and cancer vaccines [4].

The American regulatory agency Food and Drug Administration (FDA) has approved several monoclonal antibodies which block ICs through PD-1, PD-L1, or CTLA-4 interactions in a variety of cancers [5]. However, even though IC inhibitors have revolutionized the landscape of therapy for

advanced disease in many solid tumors, not all respond in the same manner [6].

Adoptive transfer of genetically engineered immune cells such as chimeric antigen receptor (CAR)-T cells have shown excellent complete remission rates for some hematological malignancies. Nevertheless, obtaining the same results in solid neoplasms has been challenging. There are several factors that limit the performance of CAR-T cells in solid tumors, such as antigen heterogeneity, limited infiltration and trafficking, restricted recognition of cell surface antigens, and physical and metabolic barriers imposed by the tumor microenvironment [7]. Of special interest for this review, immunosuppressive mechanisms such as the upregulation of co-inhibitory ligands by malignant and non-malignant cells in the tumor

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microenvironment ultimately impair the antitumor immune response in favor of tumor survival. Despite the efforts to overcome these limitations, there is still no CAR-T-cell-based treatment approved for solid tumors so far [8, 9]. This review intends to summarize investigations of IC inhibition and adoptive transfer of genetically engineered T-cells, focusing on the effect of combining both strategies.

### Control of immune response: balance between co-stimulatory and co-inhibitory signals

The immune response is based on a balance between immunogenicity and tolerance, which are coordinated by multiple pathways and molecules that behave as co-stimulatory or co-inhibitory signals to control the immunity against foreign antigens and self-antigens. These molecules are classified into two groups: stimulatory, which are either constitutively expressed or inducible by T-cell activation, and inhibitory, usually expressed during or after cell activation.

The long-standing ‘two signal theory’ postulates that T-cell activation requires a first signal to initiate the immune response and this occurs through engagement between the T-cell receptor (TCR) on the T-cell surface and the major histocompatibility complex (MHC) in antigen-presenting cells (APCs). The second signal is provided by co-stimulatory molecules that interact with their respective ligands, resulting in cell proliferation and cytokine release thereby strengthening the immune response [10].

However, immune reactions should be fine-tuned to eliminate potentially harmful agents while preserving tissue integ-

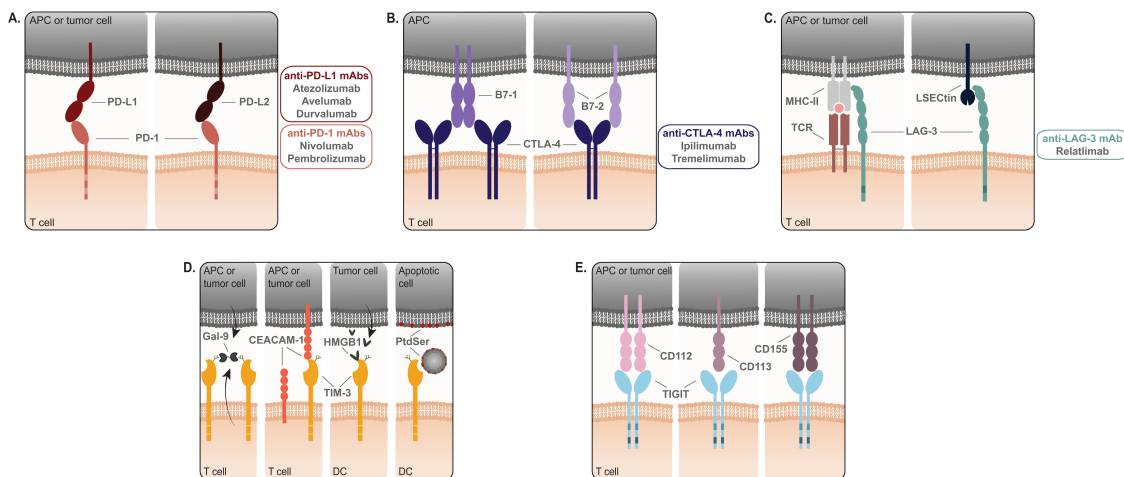
ity and maintaining self-tolerance. Thus, T cells evolved to be equipped with molecular circuitries responsible for providing repressive signals that restrain immune reactions. These signals are triggered by interactions between ligands and their corresponding receptors collectively known as immune checkpoint (IC) molecules. Engagement of these receptors on the surface of T cells modulates the spread and the length of T-cell response with the ultimate consequence of preventing an excess of inflammation, tissue damage and alloreactivity [11]. Among the IC receptors, PD-1, CTLA-4, LAG-3, TIM-3, and TIGIT are arguably the best characterized and many of their corresponding inhibitors are clinically available (Fig. 1). The key features of these molecules are described below:

#### 1. PD-1

The programmed cell death protein 1 (PD-1) is a type I transmembrane protein member of the immunoglobulin superfamily (IgSF), expressed on the surface of activated T cells, B-cells, and myeloid-cells after TCR stimulation or exposure to certain cytokines such as interleukin (IL)-2, IL-7, IL-15, and IL-21 [12–14]. Downregulation of immune response and induction of peripheral tolerance is triggered by binding of the programmed death ligand 1 (PD-L1) (expressed in APCs) and programmed death ligand 2 (PD-L2) (expressed in monocytes, dendritic cells, and activated endothelial cells) [15–17].

#### 2. CTLA-4

The cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), also known as CD152, a member of the IgSF, is upregulated



**Figure 1.** Immune checkpoint receptors and their ligands. (A) Programmed cell death protein 1 (PD-1) is expressed on activated T cells and, upon binding to one of its ligands (PD-1 ligand 1 (PD-L1) or PD-L2) on APCs or tumor cells, induces a state of exhaustion or anergy. Herein, the PD-1/PD-L1 inhibitors discussed are atezolizumab, avelumab, durvalumab, nivolumab and pembrolizumab. (B) CTLA-4 competes with CD28 costimulatory receptor on the T-cell surface for engaging with B7-1 (CD80) or B7-2 (CD86) ligands expressed by APCs, resulting in suppression of T-cell activation. The CTLA-4-blocking antibodies mentioned in this review are ipilimumab and tremelimumab. (C) LAG-3 protein on activated T cells interacts with several ligands, such as the major histocompatibility complex class II (MHC-II) expressed by APCs and tumor cells, and LSECtin expressed by tumor cells, leading to T-cell dysfunction. Relatlimab, which was cited in this review, is a LAG-3 inhibitor that interferes in the LAG-3/MHC-II interaction. (D) T-cell immunoglobulin- and mucin-domain-containing molecule 3 (TIM-3) is expressed on highly dysfunctional T cells and have multiple ligands. One of them is galectin-9 (Gal-9), expressed and secreted by many hematopoietic cells and some tumor cells. Another ligand is the adhesion protein CEACAM-1 expressed on tumor cells (for inhibitory function) and T cells itself (*cis* interaction for both TIM-3 stability and inhibitory function). Also, TIM-3 present in DCs engages with the alarmin HMGB1 released in the tumor microenvironment, mediating its sequestration from nucleic acid binding and, therefore, impairing innate immune responses. Phosphatidylserine (PtdSer) released from apoptotic cells (represented as apoptotic bodies) also interacts with TIM-3, a postulated important mechanism for antigen cross-presentation by TIM3<sup>+</sup> DCs. (E) T-cell immunoglobulin and ITIM domain (TIGIT) binds to three nectin or nectin-like proteins: CD112, CD113, and CD155. TIGIT is upregulated upon T-cell activation and interacts with increasing affinity with CD112, CD113, and CD155, respectively, expressed on DC and tumor cells.

after the early stages of T-cell activation through TCR signaling [18]. CTLA-4 downregulates T-cell responses upon the binding of B7-1 (CD80) and B7-2 (CD86). These ligands can also bind to the co-stimulatory molecule CD28 to consolidate T-cell activation. However, this process also leads to upregulation of CTLA-4, which will gradually outcompete CD28 for binding to B7-1 and B7-2. The consequent activation of CTLA-4 reduces IL-2 secretion and induces cell cycle arrest in T-cells [18–20].

### 3. LAG-3

The lymphocyte-activation gene 3 (LAG-3 or CD223) is expressed in activated T CD4, CD8, and natural killer (NK) cells, and encodes a type I transmembrane protein. It is another member of the IgSF with four extracellular immunoglobulin domains, structurally similar to the CD4 co-receptor [21]. Its major ligand is class II MHC expressed in APCs, to which LAG-3 binds with higher affinity than CD4 itself [22, 23]. It has been also postulated that LAG-3 binds to the C-type lectin receptor LSECtin expressed in the liver and some tumor cells, inhibiting interferon gamma (IFN $\gamma$ ) secretion in effector T cells [24]. LAG-3 signaling represses T-cell proliferation, pro-inflammatory cytokine release, and promotes exhaustion of effector T cells [22, 25].

### 4. TIM-3

The T-cell immunoglobulin and mucin 3 (TIM-3) was first identified on the surface of IFN $\gamma$ -producing CD4<sup>+</sup> T helper 1 (Th1) and CD8<sup>+</sup> T cytotoxic 1 (Tc1) T cells, followed by its detection on regulatory T cells (T<sub>regs</sub>) and on some innate immune cells, such as monocytes, NK cells, macrophages and dendritic cells (DCs) [26]. Until now, four TIM-3 ligands have been identified: galectin-9, high mobility group protein B1 (HMGB1), carcinoembryonic antigen cell adhesion molecule 1 (CEACAM-1), and phosphatidylserine (PtdSer). The engagement between TIM-3 and its ligands correlates with the suppression of Th1 and T helper 17 (Th17) responses [27], T cell exhaustion in chronic infection [28, 29] and in inducing peripheral tolerance [30].

### 5. TIGIT

The T-cell immunoglobulin and ITIM domain (TIGIT), is expressed predominately in T-cells and NK-cells. This receptor is a member of the IgSF and binds to CD155, CD112, and CD113 to exert a potent inhibitory effect on T cells [31] and to inhibit the cytotoxicity of NK-cells [32]. Also, the expression of TIGIT by T<sub>regs</sub> is associated with increased immunosuppressive capacity [33]. This inhibitory receptor competes with the co-stimulatory receptor CD226 (DNAM-1) for binding to the ligands CD155 and CD112 [34].

## Highjacking of immunological checkpoint signaling in cancer

Avoiding immune destruction is one of the hallmarks of cancer. The long-standing theory of immune surveillance postulates that immune cells are constantly monitoring the tissues and are able to identify and eliminate the majority of incipient cancer cells. This implies that established tumors have somehow managed to avoid this detection system, and

usurping the immune checkpoint signaling is one of the mechanisms by which some tumors succeed in avoiding immune cell-mediated killing.

Modulation of IC signaling in cancer is supported by a massive amount of data showing increased expression of IC receptors and ligands by non-malignant cells and neoplastic cells in the tumor microenvironment, respectively.

In melanoma patients, PD-L1 and PD-L2 are detected in 49% and 25% of the patients, respectively [35], while in non-small cell lung cancer (NSCLC) PD-L1 expression was reported in at least 68% of patients, with PD-L1 high expression in 28% of patients [36]. Notably, PD-L1 expression is associated with poor prognosis in different tumor types [37–39]. Similarly, the expression of CTLA-4 in the tumor microenvironment has also been related to poor prognosis and lower overall survival in several cancer types [40, 41].

As opposed to healthy individuals, advanced melanoma patients harbors TIM-3<sup>+</sup> NY-ESO-1-specific CD8<sup>+</sup> T cells that are less functional than the TIM-3<sup>-</sup> counterparts. Upon TIM-3 blockade, production of IFN $\gamma$ , tumor necrosis factor alpha (TNF $\alpha$ ), and IL-2 are enhanced upon stimulation with cognate peptide *ex vivo* [42]. A study on lung cancer reported that 30% of CD4<sup>+</sup> and CD8<sup>+</sup> tumour-infiltrating lymphocytes (TILs) expressed TIM-3. The frequency of TIM-3<sup>+</sup> CD4 T cells correlated with poor clinical parameters such as nodal metastasis and advanced cancer stage [43].

In head and neck carcinoma, LAG-3 mRNA is highly expressed in tumor cells when compared with the healthy oral mucosa, and its expression correlates with poor overall survival [44]. For NSCLC, 25.9% of patients display LAG-3<sup>+</sup> TILs. The recurrence-free survival for patients with LAG-3<sup>-</sup> TILs is 1.91 years versus 0.87 years for patients with LAG-3<sup>+</sup> TILs [45]. Also, TIGIT high expression has been observed in several tumors [46], such as melanoma [47], for example, and TIGIT upregulation in tumors is commonly associated with PD-1 overexpression.

The differential expression of IC molecules in cancer can be a result of a myriad of mechanisms implemented by neoplastic cells. For example, acute myeloid leukemia (AML) cells can upregulate the expression of CD86 and ICOS-LG, two potent co-stimulatory molecules. When co-cultured with T cells, continuous stimulation provided by AML cells induces the expression of CTLA-4, PD-1, TIM-3, and LAG-3 on the surface of CD4<sup>+</sup> helper T cells, leading to reduced proliferation and impaired cytokine production upon restimulation. These effects are not observed when T cells are co-cultured with healthy monocytes from peripheral blood [48].

These illustrative examples highlight the importance of IC signaling in enabling the immunological escape of cancer cells. These findings led to the development of IC inhibitors for the treatment of cancer and rendered a Nobel Prize in Physiology or Medicine for James Allison and Tasuko Honjo in 2018.

## Immunological checkpoint inhibitors: potential and limitations in cancer treatment

The recognition of IC molecules as negative regulators of T-cell activity led to the idea that blocking these signals could unleash an immune response against cancer. James Allison and colleagues were the first to demonstrate that CTLA-4 neutralizing monoclonal antibodies (mAbs) enhanced the antitumor immune response in mouse models of fibrosarcoma and colon

carcinoma [49]. Subsequent clinical trials of melanoma demonstrated the efficacy of targeting CTLA-4 and, in 2011, the FDA approved the CTLA-4 neutralizing mAb ipilimumab for non-resectable stages III/IV melanoma.

Hodi and colleagues demonstrated that ipilimumab improved the survival in patients with metastatic melanoma to 10.1 months, while the median overall survival with gp100 peptide vaccine was 6.4 months [3]. In 2015, Schadendorf and colleagues did a pooled meta-analysis evaluating 1861 patients and observed that 22% of patients with advanced melanoma experienced an extension of 3 years or more of life upon treatment with ipilimumab [50].

After several promising preclinical data, a seminal clinical trial in 2010 demonstrated that blocking the PD-1 axis was also well-tolerated and promoted antitumor responses [51]. Four years later, the FDA approved the two first humanized anti-PD-1 mAbs (pembrolizumab and nivolumab) for refractory and unresectable melanoma. Since then, several new mAbs targeting the PD-1 axis were developed, such as atezolizumab (anti-PD-L1), avelumab (anti-PD-L1), and durvalumab (anti-PD-L1). Notably, combined neutralization of PD-1 and CTLA-4 with nivolumab and ipilimumab, respectively, extended the overall survival of advanced melanoma patients in comparison to ipilimumab alone [52].

Currently, the vast majority of available IC inhibitors focus on the PD1 and CTLA-4 axis. However, there are other promising targets such as LAG-3, whose blockade with relatlimab combined with nivolumab (NCT03470922) improved progression-free survival in melanoma patients compared with nivolumab alone [53]. In addition, several clinical trials are underway to assess both the safety and efficacy of TIM-3 inhibitors administered alone or combined with other mAbs (NCT03489343, NCT03099109, and NCT03652077) and TIGIT inhibitors alone or in association with different IC inhibitors (NCT02964013, NCT02794571, and NCT02913313).

Despite the important clinical responses in a considerable subset of patients, there are some limitations in the use of IC inhibitors to treat cancer. Firstly, several adverse events have been associated with use of IC inhibitors. Topalian and colleagues described grade 3 or 4 immune-related adverse events in 14% of cancer patients treated with anti-PD-1 antibody and reported three deaths from toxicity [54]. Also, in a clinical trial using ipilimumab previously cited here, 60% of patients treated with this IC inhibitor had immune-related adverse events and 10–15% had grade 3–4 immune-related adverse events, whereas in the gp100 treatment group these frequencies were much lower: 32% and 3%, respectively [3]. Secondly, the majority of patients are not responsive to IC inhibition [55] and the factors that predict clinical response are under thorough investigation. In the meantime, several investigators have been trying to potentiate clinical response by adopting a combinatorial approach between IC inhibitors and the adoptive transfer of genetically engineered T cells.

### Adoptive transfer of genetically engineered T cells

Adoptive transfer of anti-CD19 CAR-T cells has led to remarkable clinical results for B-cell malignancies [56]. The therapeutic potential of CAR-T cells relies on the redirection of the cytotoxic capacity of T cells towards defined molecular targets through the CAR. The CAR is a modular receptor

composed of an extracellular antigen-binding domain, which is commonly a single-chain variable fragment (scFv) from a monoclonal antibody, followed by a hinge, a transmembrane domain, and an intracellular signaling domain (Fig. 2A).

Over the years and with greater knowledge about CAR-T cells functionality, CAR structure has been gradually diversified, so that they are currently categorized into at least three generations. Briefly, first-generation CARs harbor only the CD3 $\zeta$  as the intracellular domain, while second-generation CARs also have a co-stimulatory domain (such as CD28 and 4-1BB), third-generation CARs harbor two co-stimulatory domains and fourth-generation CAR-T cells are engineered to simultaneously express at least a second-generation CAR and a cytokine that presumably boosts their immune activity.

Although CAR-T cells have been providing surprisingly positive results for the treatment of hematological neoplasms, the use of these engineered T cells in solid tumors remains challenging. In a meta-analysis published in 2020, for instance, authors could retrieve information from 42 clinical trials using CAR-T cells against solid tumors. Of the total patients of which the clinical outcome was disclosed (295 subjects), only 4.4% (13 subjects) had a complete response [57].

To respond to this challenge, a wide range of creative strategies to improve the therapeutic efficacy of CAR-T cells is under intense investigation [58]. One of the factors limiting the use of CAR-T cells to treat solid neoplasms is that conventional CARs are unable to bind intracellular antigens, which represent the majority of viable tumor-associated antigens in solid neoplasms. In addition, the microenvironment of solid tumors is complex and equipped with several immunosuppressive mechanisms that pose considerable challenges for T-cell performance.

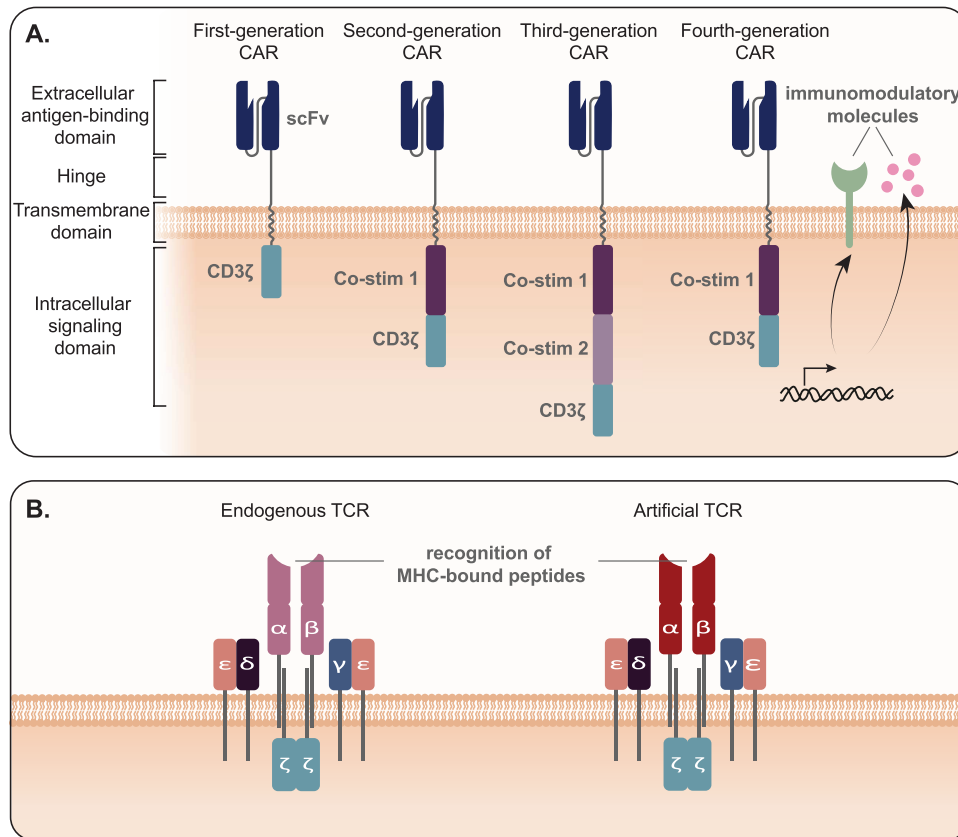
To allow the recognition of intracellular tumor antigens, T cells can be modified to express artificial TCRs instead of CARs (Fig. 2B). This class of engineered T cells, referred here as TCR-T cells, was developed virtually at the same time as CAR-T cells and is widely used in preclinical and clinical studies. Similar to CAR, the activity of T cells can be redirected to any MHC-displayed antigen (intracellular or not) through the ectopic TCR [59]. Adoptive transfer of TCR-T cells has also led to promising results in the clinical scenario [60, 61].

When comparing CAR versus TCR, one of the advantages of CAR-T cells is the antigen recognition in a human leucocyte antigen (HLA)-unrestricted way, while HLA-dependent antigen recognition by TCR-T cells may represent a limitation in the applicability of this therapy, as well as a mechanism for tumor escape mediated by the loss or downregulation of HLA. On the other hand, TCR-T cells are able to recognize intracellular proteins, unlike CAR-T cells, greatly increasing targeting options [62].

Despite the encouraging results observed with the adoptive transfer of both CAR and TCR-T cells, much of the therapeutic efficacy of these approaches are limited by immunosuppressive mechanisms in the tumor microenvironment such as induction of IC signaling. The exhaustion/energy resulting from IC signaling and observed in endogenous T cells [63] are also recapitulated in genetically engineered T cells.

Preclinical studies have reported the relevance of IC on CAR-T-cell functionality. For example, Falla-Mehrdardi and colleagues demonstrated that the expression of IC adenosine A<sub>2A</sub> receptor (A<sub>2A</sub>R) increased after stimulation of anti-CD19





**Figure 2.** Artificial receptors. (A) CAR encompass an extracellular antigen-binding domain, a hinge region, a transmembrane domain and an intracellular signaling domain. The extracellular binding domain is usually a scFv derived from a monoclonal antibody for targeting a specific antigen. The intracellular signaling domain typically involves the zeta chain of the CD3 complex (CD3 $\zeta$ ) in the first-generation CAR. Further modifications were made to enhance CAR-T-cell efficacy and proliferation by adding one or two co-stimulatory molecules (such as CD28 or 4-1BB) on second- and third-generation CARs, respectively. In addition, the fourth-generation CAR, named as TRUCK or armoured CAR, combines the receptor structure to the expression of a cell-surface or secreted immunomodulatory molecules that enhances T-cell function or helps to overcome the hostile tumour microenvironment. (B) T-cell receptor (TCR) is a heterodimer of two highly variable chains, being  $\alpha$  and  $\beta$  the most abundant in T cells. The TCR heterodimer forms a complex with CD3, which initiates a signaling cascade after TCR recognition of major histocompatibility complex (MHC)-bound peptides. CD3 comprises invariant dimers of CD3 $\epsilon$  and CD3 $\delta$  (CD3 $\epsilon\delta$ ), CD3 $\epsilon$  and CD3 $\gamma$  (CD3 $\epsilon\gamma$ ) and CD3 $\zeta$  homodimer (CD3 $\zeta\zeta$ ). On engineered T cells, artificial TCR comprises transduced  $\alpha/\beta$  heterodimer specific to a selected intracellular antigen. Co-stim, co-stimulatory molecule.

CAR-T cells. Additionally, the authors co-cultured CAR-T cells with target cells in the presence of an adenosine analog, to recapitulate an immunosuppressive tumor microenvironment. In this setting, the use of an A2aR inhibitor improved cell proliferation and IFN $\gamma$  and IL-2 production by CAR-T cells [64].

Moreover, PD-1 or PD-L1 upregulation has been reported in CAR-T cells after co-culture with target cells [65] and post-infusion into patients [66]. Also, a comparison between CAR-T cell profile at infusion and at time of peak in peripheral blood revealed an increase in PD-1 expression [67]. Furthermore, increases in CTLA-4, TIM-3, LAG-3, and TIGIT expression in stimulated or infused CAR-T cells have also been described [68–72], and increased levels of PD-1, TIM-3, and LAG-3 have also been reported in TILs isolated from tumors after TCR-T-cell infusion [73]. In addition to the IC high expression, other exhaustion characteristics were described in CAR-T cells even before infusions, such as decreased cell proliferation, impaired cytokine production, effector-like phenotype, and epigenetic and transcriptional modifications consistent with functionally exhausted cells [74].

Based on these evidences, inhibiting IC signaling in engineered T cells to improve antitumor response and persistence

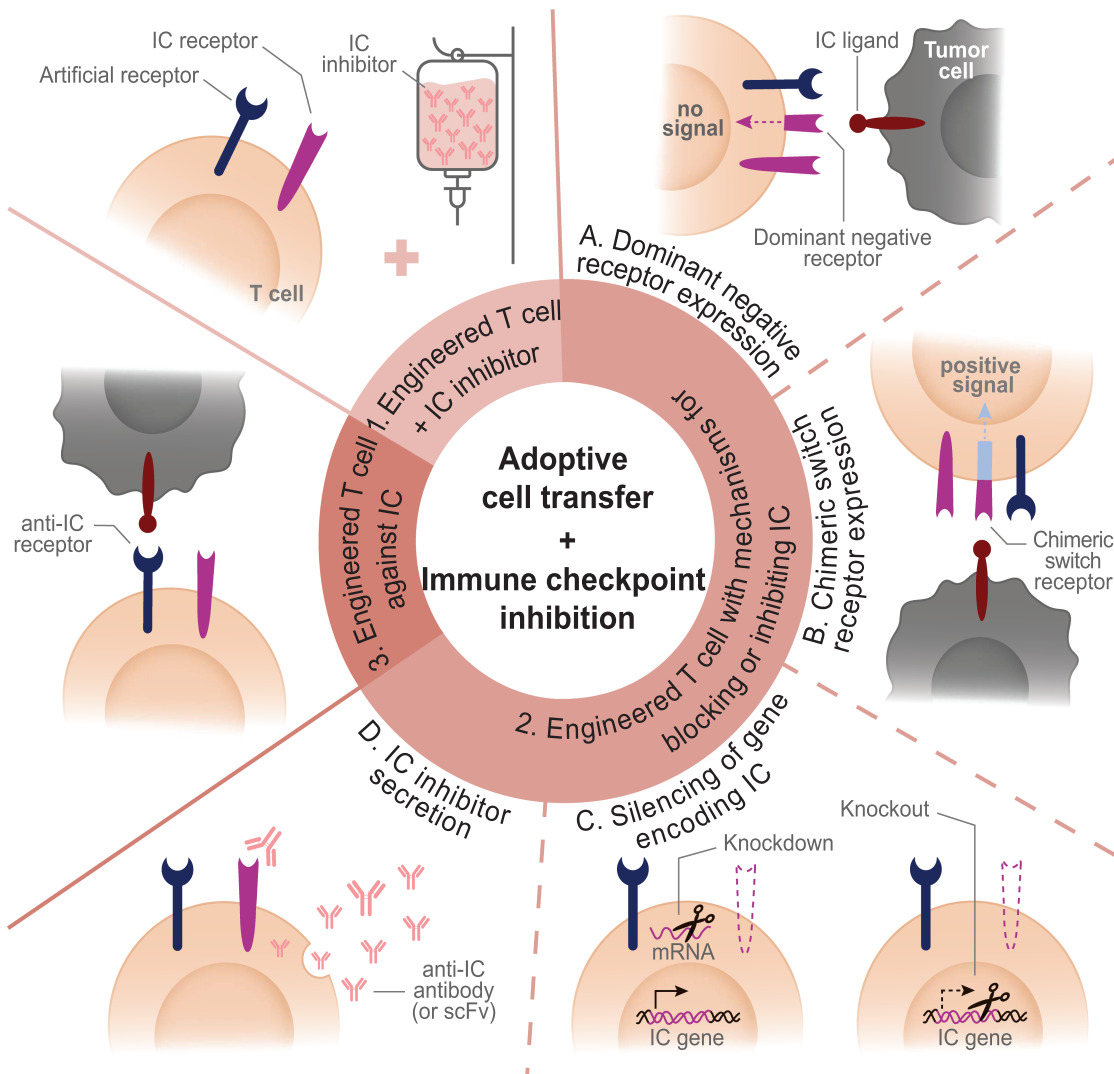
have been explored in different ways in several preclinical and clinical studies. The approaches of combining adoptive cell transfer with IC inhibition discussed in this review are summarized in Fig. 3. For a better presentation, we organized these strategies into the following sections: combined administration of genetically engineered T cells and IC inhibitors, engineered T cells with mechanisms for blocking or inhibiting IC and CAR-T cells against IC molecules.

## Combining adoptive T-cell transfer with immunological checkpoint inhibition

### Combined administration of genetically engineered T cells and IC inhibitors

Infusion of genetically engineered T cells followed by administration of IC inhibitors (Fig. 3.1) have shown promising results in preclinical studies and there are several clinical trials under way to evaluate the benefit of this combined strategy (Table 1).

PD-1 inhibition has been extensively explored in this combination therapy. John and colleagues provided clear evidence that PD-1 blockade in CAR-T cells, mediated by anti-PD-1 antibody addition in cell culture, was able to im-



**Figure 3.** Combination strategies of adoptive cell transfer and immune checkpoint inhibition. In this review, we focused on three main combination approaches. In the first one (1), engineered T cells are associated with the systemic infusion of an immune checkpoint (IC) inhibitor (monoclonal antibody). In the second one (2), engineered T cells are genetically boosted through different mechanisms. These mechanisms encompass: (A) the expression of a dominant negative receptor that does not transmit the negative signal upon binding to the IC ligand due to the lack of the intracellular signaling domain; (B) expression of a chimeric switch receptor, which reverses the IC inhibitory signal into a co-stimulatory (positive) one; (C) IC gene silencing through genome editing (for gene knockout) or RNA interference (siRNA or shRNA for gene knockdown); and (D) modification of engineered T cells to express an anti-IC antibody or scFv for *in situ* blocking of IC signaling. Finally, the third approach (3) discussed here relies on T cells engineered to express an artificial receptor against the IC ligand. Artificial receptor refers to the CAR or artificial TCR; IC; mAb; scFv.

prove proliferation, IFN $\gamma$ , and granzyme B expression and *in vivo* antitumor activity [65]. Furthermore, the addition of pembrolizumab in anti-GD2 CAR-T cells culture restored the levels of IFN $\gamma$  and TNF $\alpha$  cytokine secretion and cell viability after repeated stimulation [66]. The use of this inhibitor in combination with anti-NY-ESO-1 TCR-T cells in a murine model enhanced the efficacy of infused T cells [73].

In line with these promising preclinical data, some results observed in clinical trials are encouraging and suggest a synergy between adoptive cell transfer and IC inhibitors. In a case report, it was observed that pembrolizumab administration after 26 days of anti-CD19 CAR-T cells infusion in a patient with refractory diffuse large B-cell lymphoma (DLBCL) was followed by an increase in CAR-T cell number, high serum IL-6 levels, and a decrease in PD-1<sup>+</sup> CAR-T cells [80]. Other combination possibilities involving pembrolizumab-mediated PD-1 blockade are currently being explored along with

CD19/CD22 dual targeting CAR-T cells for treating patients with DLBCL (NCT03287817), and in combination with TCR-T cells targeting NY-ESO-1 for patients with NSCLC (NCT03709706).

Positive results were also observed in treatment with anti-CD19 CAR-T cells followed by nivolumab administration in a patient with DLBCL. Despite the progressive disease that led to the termination of the study, during the period after nivolumab infusion a partial response was observed, with a constant considerable reduction in tumor volume. Comparing these results with a previous clinical trial (NCT02348216), without nivolumab infusion, the authors suggested that PD-1 inhibitor administration significantly favors CAR-T-cell expansion [81]. Also, young patients with relapsed B-cell acute lymphoblastic leukemia or B lymphoblastic lymphoma received CAR-T cells targeting CD19 in combination with pembrolizumab or nivolumab and, despite the observed re-

**Table 1.** Registered clinical trials using combination strategy with engineered T cells and checkpoint inhibitors to treat cancer.

Clinical trial ID1	Phase	Enrolled participants2	Status3	Start year; Location(s)	Combination strategy		Tumour type	Published results	Comment
					Checkpoint inhibitor4	Engineered T cell target5			
<b>CAR T-cell</b>									
NCT01822652	I	11	Ongoing, not recruiting	2013 US	Pembrolizumab	GD2 (anti-GD2.CD28.OX40.zeta + iCasp9)	Neuroblastoma	[8]	6 PD, 2 CR in the combination group after salvage therapy. All treatment regimens were well tolerated.
NCT02414269	I/II	179	Recruiting	2015 US	Pembrolizumab	Mesothelin (anti-mesothelin.CD28.zeta + iCasp9)	Malignant Pleural Disease from mesothelioma, lung cancer, or breast cancer	[75]*	2 CR, 5 PR (N = 14). CAR-T cell therapy proved to be safe.
NCT03287817	I/II	171	Recruiting	2017 US, UK	Pembrolizumab	CD19/CD22 dual targeting CAR	Diffuse large B-cell lymphoma	[76]*	ORR 64%, CR 55% (N = 11). No severe toxicities observed.
NCT03630159	Ib	12	Ongoing, not recruiting	2018 US, AT, CA	Pembrolizumab	CD19 (anti-CD19.4-1BB.zeta,CTL019)	Diffuse large B-cell lymphoma	[77]*	1 PR, 2 PD (N = 4). Combination had a safety profile.
NCT03726515	I	7	Completed	2019 US	Pembrolizumab	EGFRvIII (anti-EGFRvIII.4-1BB.zeta)	Glioblastoma	N/A	
NCT04991948	Ib	34	Not yet recruiting	2021 US, BE	Pembrolizumab	NKG2DL (NKGD2.zeta + TIM, CYAD-101)	Colorectal Cancer	N/A	TIM (TCR inhibitory molecule) is a truncated form of CD3 $\zeta$ that was generated to interfere in the endogenous TCR signaling. Thus, NKGD2-CART and TIM co-expression enables the development of an allogeneic CAR-T cell.
NCT04995003	I	25	Not yet recruiting	2021 US	Pembrolizumab or Nivolumab	HER2 (anti-HER2.CD28.zeta)	Sarcoma	N/A	-
NCT04003649	I	60	Recruiting	2019 US	Nivolumab and Ipilimumab	IL13Ra2 (anti-IL13Ra2.4-1BB.zeta)	Glioblastoma	N/A	-
NCT04539444	II	10	Recruiting	2020 CN	Tislelizumab	CD19/CD22 CAR	B-cell non-Hodgkin lymphoma	N/A	-
NCT04381741	I	24	Recruiting	2020 CN	Tislelizumab	CD19 (anti-CD19-CAR +IL-7 andCCL19 expression)	Diffuse large B-cell lymphoma	N/A	-

Table 1. Continued

Clinical trial ID1	Phase	Enrolled participants2	Status3	Start year; Location(s)	Combination strategy		Tumour type	Published results	Comment
					Checkpoint inhibitor4	Engineered T cell target5			
NCT02926833	I/II	37	Ongoing, not recruiting	2016 US	Atezolizumab	CD19 (anti-CD19, CD28, zeta, KTE-C19)	Diffuse large B-cell lymphoma	[78]*	ORR: 75%, CR: 46% (N = 28). Combination strategy had a controllable safety profile. CAR-T cell levels and efficacy results were similar to the CAR-T cell monotherapy (ZUMA-1 trial).
NCT02706405	Ib	30	Ongoing, not recruiting	2016 US	Durvalumab	CD19 (anti-CD19.4-1BB, zeta + EGFRt expression, JCAR014)	B-cell non-Hodgkin lymphoma	[79]*	ORR: 50%, CR: 42%. For a preliminary analysis, the combination appeared well tolerated.
NCT03310619	I/II	77	Recruiting	2017 US	Durvalumab, Nivolumab, Relatlimab	CD19 (anti-CD19.4-1BB, zeta, JCAR017)	B-cell non-Hodgkin lymphoma	N/A	The study has different arms to test JCAR017 in combination with several agents, among them are the checkpoint inhibitors mentioned. These combinations are being evaluated separately.
<b>TCR-T cell</b>									
NCT03709706	Ib/IIa	54	Recruiting	2018 US, CA, NL, ES, UK	Pembrolizumab	NY-ESO-1/LAGE-1a (HLA-A*02-restricted)	Non-small cell lung cancer	N/A	
NCT03747484	I/II	16	Recruiting	2019 US	Pembrolizumab or Avelumab	Merkel cell polyomavirus oncoprotein (HLA-A*0201-restricted)	Merkel cell carcinoma	N/A	
NCT04520711	I/Ib	24	Not yet recruiting	2020 US	Pembrolizumab	Autologous tumour-specific antigens	Epithelial neoplasms	N/A	The therapeutic strategy will also include a CD40 agonist antibody.
NCT04408898	II	10	Recruiting	2020 US	Pembrolizumab	MAGE-A4 (HLA-A*02-restricted)	Head and neck cancer	N/A	
NCT02775292	I	1	Completed	2017 US	Nivolumab	NY-ESO-1 (HLA-A*0201-restricted)	Advanced solid tumours	N/A	Additionally combined with a NY-ESO-1 peptide-pulsed autologous dendritic cell vaccine.
NCT03970382	Ia/Ib	148	Recruiting	2019 US	Nivolumab	Autologous neo-epitopes	Advanced/metastatic solid tumours	N/A	
NCT03686124	I	42	Recruiting	2019 US, DE	Atezolizumab	PRAME	Advanced/metastatic solid tumours	N/A	



Table 1. Continued

Clinical trial ID1	Phase	Enrolled participants2	Status3	Start year; Location(s)	Combination strategy		Tumour type	Published results	Comment
					Checkpoint inhibitor4	Engineered T cell target5			
NCT04639245	I/II	18	Recruiting	2021 US	Atezolizumab	MAGE-A1 (HLA-A*0201 restricted)	Triple-negative breast cancer, urothelial cancer, non-small cell lung cancer	N/A	

<sup>1</sup>The listed trials in the table were from ClinicalTrials.gov, based on the following research terms: CAR; TCR; T cell; checkpoint inhibitor; pembrolizumab; nivolumab; PD-1 inhibitor; atezolizumab; durvalumab; PD-L1 inhibitor; ipilimumab; tremelimumab; CTLA-4 inhibitor.  
<sup>2</sup>The number of enrollments (actual or estimated) was based on data available in August/2021.  
<sup>3</sup>The trials with 'terminated', 'suspended' or 'withdrawn' status were not included in this list.  
<sup>4</sup>Pembrolizumab/Nivolumab/tislelizumab; anti-PD-1. Atezolizumab/durvalumab/avelumab; anti-PD-L1. Ipilimumab; anti-CTLA-4. Relatlimab; anti-LAG-3.  
<sup>5</sup>For CAR-T cell, it was also provided the CAR design and the product name (if applicable).  
 \*Preliminary results available.

sponses to treatment, adverse effects possibly related to prolonged use of IC inhibitors were reported [82].

The combination of anti-CD19 CAR-T cells with anti-PD-L1 monoclonal antibody durvalumab has also been investigated. The safety and feasibility of this approach were evaluated with the administration of durvalumab at different doses 1 day before or 21–28 days after CAR-T-cell infusion in patients with relapsed or refractory (r/r) aggressive B-cell Non-Hodgkin Lymphoma (NHL). In general, complete and partial remission was observed in 42% and 8% of patients, respectively. Furthermore, despite some patients developing cytokine release syndrome and neurotoxicity, when adoptive cell transfer was followed by treatment with durvalumab the same did not happen [79]. Also, inhibition of PD-L1 during CAR-T cell treatment has been evaluated with the administration of atezolizumab in patients with r/r large B cell lymphoma. Initial results suggest that, despite the observed adverse effects, combination therapy appears to be safe and capable of improving CAR-T cell expansion [83].

CTLA-4 inhibition has also been assessed in engineered T cells. Nowicki and colleagues investigated the safety and antitumor capability of anti-NY-ESO-1 TCR-T cells associated with dendritic cell vaccination with or without ipilimumab in sarcoma and melanoma patients. Ipilimumab increased serum levels of FMS-like tyrosine kinase 3 ligand (Flt-3L), which plays a fundamental role in dendritic cell development and differentiation. However, this approach did not improve tumor eradication [84]. Thus, CTLA-4 blockade in edited T cells has been explored concurrently with other IC inhibitors, such as anti-PD-1 mAbs (NCT04003649). Also, an ongoing clinical trial is evaluating the safety and efficacy of using CAR-T cells in association with several IC inhibitors, including the LAG-3 inhibitor relatlimab for the treatment of B-cell malignancies (NCT03310619). Additionally, combined pharmacological blockade of PD-1 plus TIM-3 or PD-1 plus TIGIT enhanced the capacity of anti-NY-ESO-1 TCR-T cells to control tumor growth in mice when compared to the single PD-1 blockade [85].

In general, the PD-1/PD-L1 axis has been the main target in current clinical trials exploring the combined approach of CAR/TCR-T cells and IC inhibitors. The benefit of targeting multiple IC was highlighted by Padmanee Sharma and James Allison, in the sense that by targeting a single IC, other inhibitory signaling pathways are induced, as suggested for CTLA-4-blockage that could contribute to increased PD-L1 expression [86]. So, by targeting multiple ICs, resistance to a single IC inhibitor could be overcome, potentiating the combination therapy.

### Engineered T cells with mechanisms for blocking or inhibiting immunological checkpoint signaling

Another approach to combine adoptive T-cell transfer with IC inhibition is to further genetically engineer CAR or TCR-T cells to intrinsically disrupt or downregulate IC signaling. In addition, to stably prevent IC signaling, this approach has the potential to bypass the adverse effects associated with prolonged use of pharmacological IC inhibitors.

After observing high PD-L1 expression in an orthotopic model of pleural mesothelioma, Cherkassky and colleagues compared three strategies to inhibit PD-1 signaling and increase anti-mesothelin CAR-T-cell performance: administration of a PD-1-inhibitor, PD-1 downregulation using short hairpin RNA (shRNA) and expression of a PD-1 dominant

negative receptor (DNR) in CAR-T cells. Despite the improved CAR-T-cell performance upon anti-PD-1 administration, the maintenance of this response required multiple applications of the mAb. On the other hand, the expression of the PD-1 DNR in CAR-T cells controlled tumor burden and improved the survival of mice upon a single administration [87].

Unlike the endogenous receptor, PD-1 DNR lacks the intracellular inhibitory signaling domain, so cellular inhibition is not triggered upon ligand binding (Fig. 3.2A). As both receptors compete for the same ligand, DNRs contribute to attenuate CAR-T-cell exhaustion. The use of CAR-T cells co-expressing the PD-1 DNR has been shown to be safe and effective in a clinical trial involving patients with r/r B cell lymphoma (ChiCTR1900021295) [88]. Also, clinical results suggest that this approach is efficient against both PD-L1 positive and negative tumors [89].

Another strategy used to inhibit PD-1 signaling by a designed receptor is the expression of a PD-1:CD28 chimeric receptor, also called chimeric switch receptor. This is composed of the fusion of a truncated extracellular domain of PD-1 with transmembrane and cytoplasmic portions of the CD28 co-stimulatory molecule (Fig. 3.2B). Thus, the binding of PD-L1 to the PD-1 domain of this receptor provides T cell co-stimulation instead of an inhibitory signal [90].

Preclinical studies of CAR and TCR-T cells demonstrated that expression of PD-1:CD28 chimeric receptors enhanced antitumor activity [91, 92]. Notably, PD-1:CD28 receptors indeed provided an additional co-stimulatory signal especially in the immunosuppressive tumor microenvironment, where such ligands are overexpressed [92]. Recently, the efficacy and safety of this strategy were confirmed in patients with PD-L1<sup>+</sup> DLBCL (NCT03258047). Consistent with preclinical tests, a clinical trial showed that after 3 months of anti-CD19 CAR/PD-1:CD28-T-cell infusion, 58.8% of patients had clinical responses, and complete remission was reported in 41.2% of cases. Additionally, no cytokine-release syndrome and severe neurological toxicity were reported [93].

The conversion of an inhibitory signal into a co-stimulatory one using a chimeric receptor was also applied to CTLA-4 in tumor-specific T cells. Overexpression of CTLA-4:CD28 chimeric receptor increased T-cell reactivity. This increase in response was observed mainly in CD4<sup>+</sup> T cells, boosting the antitumor effect of CD8<sup>+</sup> T cells [94]. Increased CAR and TCR-T-cell functions were also described using TIGIT:CD28 chimeric switch receptor. The co-expression of this receptor with CAR or TCR was able to mediate an increase in TNF $\alpha$ , IFN $\gamma$ , and IL-2 secretion. Furthermore, it was observed in a xenograft tumor model that TCR-T TIGIT:CD28 cells were able to delay tumor growth, as well as increase the animal survival compared to TCR-T cells [95].

Gene knockout approaches have also been explored to abrogate IC signaling in engineered T cells (Fig. 3.2C). For instance, upon shRNA-mediated A<sub>2A</sub>R downregulation, CAR-T cells presented a modest improvement in antitumor capacity *in vivo*. However, this modification also reduced CAR-T-cell persistence. This collateral effect was not observed upon A<sub>2A</sub>R knockout using clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated 9 (Cas9), which increased CAR-T-cell function without compromising persistence. This approach also led to a superior antitumor activity when compared to pharmacological blockade of A<sub>2A</sub>R [96]. Although interesting, the authors did not address the reason

for these differences. Even though the authors used four distinct shRNAs, the possibility of shRNA-mediated non-specific off-target effects affecting CAR-T cell persistence cannot be completely ruled out. However, it was already reported that some shRNAs might induce distinct gene signatures in comparison to CRISPR/Cas9 editing, even when both techniques have been demonstrated to be on-target [97]. Thus, future studies incorporating the profiling of persistence-related genes could shed light on these intriguing results.

Disrupting PD-1 expression has also been consistently associated with enhanced cytotoxic capacity in tumor-specific T cells [98]. Similarly, knocking out PD-1 using CRISPR/Cas9 in anti-Glypican-3 CAR-T cells increased cytotoxic activity *in vitro*, IL-2 and IFN $\gamma$  secretion, Akt activation, and Bcl-xL anti-apoptotic protein expression after co-cultivation with tumor cells. These effects were recapitulated in a mouse model, resulting in increased CAR-T cell infiltration, persistence, and antitumor activity [99]. Similar results were consistently observed in other studies using anti-mesothelin and anti-CD19 CAR-T cells [100, 101]. Furthermore, this approach is already being evaluated in a clinical trial (NCT03525782).

In contrast with the results of A<sub>2A</sub>R and PD-1 disruption, a pioneer study on CRISPR/Cas9-based LAG-3 knockout in human T cells reported no differences in anti-CD19 CAR-T-cell performance both *in vitro* and *in vivo* [102]. As discussed by the authors, these results are in apparent contradiction to other studies showing increased cytotoxicity upon LAG-3 blockade using mAbs. The reason for these discrepancies is attributed to the limited recapitulation of an immunosuppressive environment in the Raji xenograft model using immunodeficient mice. The authors also recall that LAG-3 acts synergistically with PD-1, thus disrupting only LAG-3 would not suffice to enhance CAR-T performance. These speculations have not yet been addressed and this is an area of active investigation.

Knockdown of IC molecules through small-interfering RNA (siRNA) was also evaluated in engineered T cells. Concomitant downregulation of PD-1 and CTLA-4 or of PD-1 alone resulted in enhanced CAR-T-cell cytotoxicity *in vitro* [70]. Furthermore, downregulation of PD-1 ligands in MAGE-A4-specific TCR-T cells enhanced the lytic capacity and IFN $\gamma$  production [103]. Interestingly, Condomines and colleagues demonstrated that CTLA-4 downregulation in CAR-T cells using shRNA enhanced antitumor activity of first-generation CAR-T cells, which are dependent of natural CD28/CD80 co-stimulation, whereas the same did not occur with the second-generation CAR-T cells [104]. Since CTLA-4 competes for CD28 ligands, its immunosuppressive effects are likely to be higher when activation of CAR-T cells depends on endogenous CD28 activation and are less prominent when the costimulatory signal is already integrated in the CAR (i.e. second-generation CARs). These findings demonstrate that the effect of CTLA-4 inhibition on edited T cells may depend on the expressed artificial receptor.

Additionally, it was recently demonstrated that PD-1 and TIGIT simultaneous downregulation in CAR-T cells using shRNA promoted a robust antitumor response and improved *in vivo* CAR-T-cell persistence [72]. Currently, there is a clinical trial underway to evaluate the use of PD-1/TIGIT-downregulated anti-CD19 CAR-T cells in patients with relapsed or refractory DLBCL (NCT04836507).

Still aiming at attenuating inhibitory effects on engineered T-cell function, these cells can be modified to secrete

IC-blocking antibodies (Fig. 3.2D). CAR-T cells engineered to secrete anti-PD-L1 antibodies downregulated by 50% the expression of LAG-3, TIM-3, and PD-1 in comparison with cells expressing CAR alone. Also, a considerable decrease in PD-L1 expression was observed in tumors when animals were treated with CAR-T cells secreting anti-PD-L1 antibodies, as well as a better reduction in tumor size [105].

Additionally, CAR-T cells secreting anti-PD-1 antibodies showed improved effector function [106]. Accordingly, T cells engineered to secrete PD-1-blocking scFvs displayed improved antitumor activity in PD-L1<sup>+</sup> murine tumor models, with results comparable to those obtained using IC inhibitors. Notably, the authors suggested that scFv-secreting CAR-T cells may have an advantage in relation to safety, as scFvs were mainly detected in the tumor microenvironment, thereby avoiding systemic toxicity [107]. Currently, CAR-T cells secreting scFvs against PD-1/CTLA-4/TIGIT have been evaluated in clinical trials (NCT03198546, NCT03198052).

### CAR-T cells against immunological checkpoint molecules

CAR-T cells can be directed against IC molecules in order to reduce the immunosuppressive effect of the tumor microenvironment and allow the antitumor activity of T cells (Fig. 3.3). An important point to consider in this approach is that the IC target can be expressed in CAR-T cells themselves, which could result in fratricide.

Qin and colleagues compared two anti-PD-1 CAR designs: the first composed of the extracellular and transmembrane domains of PD-1 and the intracellular domains of 41BB, TLR2, and CD3 $\zeta$ ; the second construct consisted of a CAR harboring a high-affinity anti-PD-L1 scFv with the same cytoplasmic domain as the first construct. CAR-T cells expressing both constructs specifically lysed PD-L1<sup>+</sup> cell lines *in vitro* and *in vivo*, with anti-PD-L1 CAR-T cells showing better performance. However, combined administration of anti-PD-1 CAR-T cells and anti-mesothelin CAR-T cells did not improve antitumor capacity. Apparently, anti-mesothelin CAR-T cells, which overexpressed PD-L1, became targets of anti-PD-L1 CAR-T cells, limiting the expected synergism between them [108]. Nevertheless, the use of CAR-T cells targeting PD-L1-positive malignant and non-malignant cells in the tumor microenvironment has shown promising results [109,110].

CAR-T cells targeting protein HLA-G were recently evaluated for their antitumor potential. This protein is not only an IC but also a tumor-associated antigen found in a few healthy tissues, making it an interesting target for CAR-T cells. Anti-HLA-G CAR-T cells showed specific cytotoxic capacity against HLA-G<sup>+</sup>-targeted cells and contributed to tumor regression *in vivo* [111].

Anti-TIM-3 CAR-T cells also demonstrated a potent anti-leukemic activity against AML cell lines and patient-derived blasts in a preclinical study. Noteworthy, TIM-3 is expressed in AML blasts and leukemic stem cells and is not present in normal hematopoietic stem cells, naïve lymphocytes, granulocytes, and most nonhematopoietic tissues [112]. Overall, these examples indicate that the expression of IC molecules by cancer cells as a mechanism to escape immunosurveillance can be leveraged for the development of tumor-specific CAR-T cells. In addition, these IC-directed CAR-T cells might provide additional anti-tumor benefits by eliminating non-malignant immunosuppressive cells in the tumor microenvironment.

## Concluding remarks

After many years of skepticism that immune cells could be harnessed to eliminate tumors, cancer immunotherapy is currently one of the most effective approaches for treating patients with cancer. The two most prominent approaches of the immunotherapy toolbox are inhibitors of IC molecules and the adoptive transfer of genetically engineered T cells. Despite the clinical success, not all patients respond to these therapeutic strategies or some of them only experience short-term responses or unacceptable toxic collateral effects. In this review, we highlight the efforts of combining both the adoptive transfer of genetically engineered T cells and the disruption of IC signaling through genetic and pharmacological approaches. Overall, these interventions have been resulting in promising therapeutic responses and at the same time revealing how and to what extent different IC molecules in different tumor types mediate the escape from the immune system. As a future perspective, lessons from these trials are advancing the field toward a complex and sophisticated genetic engineering of immune cells to make them resistant to tumor-associated immunosuppressive mechanisms while maintaining high specificity and cytotoxicity against cancer cells.

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## Author contributions

R.R. Writing – original draft, writing – review and editing. H.B. Writing – original draft. S.C.G.d.L. Writing – original draft, writing – review and editing. I.P.F. Writing – original draft. R.M.S. Writing – original draft. D.M.C.F. Writing – original draft. D.T.C. Funding acquisition, resources. L.E.B.d.S. Conceptualization, funding acquisition, resources, supervision, writing – original draft, writing – review and editing.

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## Competing interests

The authors have no competing interests to declare.

## Data Availability Statement

Original data related to this work is available upon request.

## References

1. Garon EB, Hellmann MD, Rizvi NA. et al. Five-year overall survival for patients with advanced non-small-cell lung cancer treated



- with pembrolizumab: results from the phase I KEYNOTE-001 study. *J Clin Oncol* 2019;37:2518–27.
2. Chong EA, Ruella M, Schuster SJ; Lymphoma Program Investigators at the University of Pennsylvania. Five-year outcomes for refractory B-Cell lymphomas with CAR T-Cell therapy. *N Engl J Med* 2021;384:673–4.
  3. Hodi FS, O'Day SJ, McDermott DF. et al. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med* 2010;363:711–23.
  4. Waldman AD, Fritz JM, Lenardo MJ. A guide to cancer immunotherapy: from T cell basic science to clinical practice. *Nat Rev Immunol* 2020;20:651–68.
  5. Murciano-Goroff YR, Warner AB, Wolchok JD. The future of cancer immunotherapy: microenvironment-targeting combinations. *Cell Res* 2020;30:507–19.
  6. Syn NL, Teng MWL, Mok TSK. et al. De-novo and acquired resistance to immune checkpoint targeting. *Lancet Oncol* 2017;18:e731–41.
  7. Newick K, O'Brien S, Moon E. et al. CAR T cell therapy for solid tumors. *Annu Rev Med* 2017;68:139–52.
  8. Heczey A, Louis CU, Savoldo B. et al. CAR T cells administered in combination with lymphodepletion and PD-1 inhibition to patients with neuroblastoma. *Mol Ther* 2017;25:2214–24.
  9. Yeku OO, Purdon TJ, Koneru M. et al. Armored CAR T cells enhance antitumor efficacy and overcome the tumor microenvironment. *Sci Rep* 2017;7:10541.
  10. Baxter AG, Hodgkin PD. Activation rules: the two-signal theories of immune activation. *Nat Rev Immunol* 2002;2:439–46.
  11. Long EO. Regulation of immune responses through inhibitory receptors. *Annu Rev Immunol* 1999;17:875–904.
  12. Ishida Y, Agata Y, Shibahara K. et al. Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. *Embo J* 1992;11:3887–95.
  13. Agata Y, Kawasaki A, Nishimura H. et al. Expression of the PD-1 antigen on the surface of stimulated mouse T and B lymphocytes. *Int Immunol* 1996;8:765–72.
  14. Kinter AL, Godbout EJ, McNally JP. et al. The common gamma-chain cytokines IL-2, IL-7, IL-15, and IL-21 induce the expression of programmed death-1 and its ligands. *J Immunol* 2008;181:6738–46.
  15. Freeman GJ, Long AJ, Iwai Y. et al. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J Exp Med* 2000;192:1027–34.
  16. Nishimura H, Nose M, Hiai H. et al. Development of lupus-like autoimmune diseases by disruption of the PD-1 gene encoding an ITIM motif-carrying immunoreceptor. *Immunity* 1999;11:141–51.
  17. Latchman Y, Wood CR, Chernova T. et al. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. *Nat Immunol* 2001;2:261–8.
  18. Brunner MC, Chambers CA, Chan FK. et al. CTLA-4-mediated inhibition of early events of T cell proliferation. *J Immunol* 1999;162:5813–20.
  19. Linsley PS, Greene JL, Brady W. et al. Human B7-1 (CD80) and B7-2 (CD86) bind with similar avidities but distinct kinetics to CD28 and CTLA-4 receptors. *Immunity* 1994;1:793–801.
  20. van der Merwe PA, Bodián DL, Daenke S. et al. CD80 (B7-1) binds both CD28 and CTLA-4 with a low affinity and very fast kinetics. *J Exp Med* 1997;185:393–403.
  21. Triebel F, Jitsukawa S, Baixeras E. et al. LAG-3, a novel lymphocyte activation gene closely related to CD4. *J Exp Med* 1990;171:1393–405.
  22. Huard B, Prigent P, Tournier M. et al. CD4/major histocompatibility complex class II interaction analyzed with CD4- and lymphocyte activation gene-3 (LAG-3)-Ig fusion proteins. *Eur J Immunol* 1995;25:2718–21.
  23. Huard B, Mastrangeli R, Prigent P. et al. Characterization of the major histocompatibility complex class II binding site on LAG-3 protein. *Proc Natl Acad Sci U S A* 1997;94:5744–9.
  24. Xu F, Liu J, Liu D. et al. LSECtin expressed on melanoma cells promotes tumor progression by inhibiting antitumor T-cell responses. *Cancer Res* 2014;74:3418–28.
  25. Blackburn SD, Shin H, Haining WN. et al. Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. *Nat Immunol* 2009;10:29–37.
  26. Monney L, Sabatos CA, Gaglia JL. et al. Th1-specific cell surface protein Tim-3 regulates macrophage activation and severity of an autoimmune disease. *Nature* 2002;415:536–41.
  27. Zhu C, Anderson AC, Schubart A. et al. The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. *Nat Immunol* 2005;6:1245–52.
  28. Jones RB, Ndhlovu LC, Barbour JD. et al. Tim-3 expression defines a novel population of dysfunctional T cells with highly elevated frequencies in progressive HIV-1 infection. *J Exp Med* 2008;205:2763–79.
  29. Takamura S, Tsuji-Kawahara S, Yagita H. et al. Premature terminal exhaustion of Friend virus-specific effector CD8+ T cells by rapid induction of multiple inhibitory receptors. *J Immunol* 2010;184:4696–707.
  30. Sabatos CA, Chakravarti S, Cha E. et al. Interaction of Tim-3 and Tim-3 ligand regulates T helper type 1 responses and induction of peripheral tolerance. *Nat Immunol* 2003;4:1102–10.
  31. Joller N, Hafler JP, Brynedal B. et al. Cutting edge: TIGIT has T cell-intrinsic inhibitory functions. *J Immunol* 2011;186:1338–42.
  32. Stanitsky N, Simic H, Arapovic J. et al. The interaction of TIGIT with PVR and PVRL2 inhibits human NK cell cytotoxicity. *Proc Natl Acad Sci U S A* 2009;106:17858–63.
  33. Joller N, Lozano E, Burkett PR. et al. Treg cells expressing the coinhibitory molecule TIGIT selectively inhibit proinflammatory Th1 and Th17 cell responses. *Immunity* 2014;40:569–81.
  34. Lozano E, Dominguez-Villar M, Kuchroo V. et al. The TIGIT/CD226 axis regulates human T cell function. *J Immunol* 2012;188:3869–75.
  35. Obeid JM, Erdag G, Smolkin ME. et al. PD-L1, PD-L2 and PD-1 expression in metastatic melanoma: correlation with tumor-infiltrating immune cells and clinical outcome. *Oncoimmunology* 2016;5:e1235107.
  36. Aggarwal C, Abreu DR, Felipe E. et al. Prevalence of PD-L1 expression in patients with non-small cell lung cancer screened for enrollment in KEYNOTE-001, -010, and -024. *Ann Oncol*. 2016;27 (Suppl\_6):vi363.
  37. Muenst S, Schaerli AR, Gao F. et al. Expression of programmed death ligand 1 (PD-L1) is associated with poor prognosis in human breast cancer. *Breast Cancer Res Treat* 2014;146:15–24.
  38. Shi SJ, Wang LJ, Wang GD. et al. B7-H1 expression is associated with poor prognosis in colorectal carcinoma and regulates the proliferation and invasion of HCT116 colorectal cancer cells. *Plos One* 2013;8:e76012.
  39. Thompson RH, Kuntz SM, Leibovich BC. et al. Tumor B7-H1 is associated with poor prognosis in renal cell carcinoma patients with long-term follow-up. *Cancer Res* 2006;66:3381–5.
  40. Paulsen EE, Kilvaer TK, Rakaee M. et al. CTLA-4 expression in the non-small cell lung cancer patient tumor microenvironment: diverging prognostic impact in primary tumors and lymph node metastases. *Cancer Immunol Immunother* 2017;66:1449–61.
  41. Huang PY, Guo SS, Zhang Y. et al. Tumor CTLA-4 overexpression predicts poor survival in patients with nasopharyngeal carcinoma. *Oncotarget* 2016;7:13060–8.
  42. Fourcade J, Sun Z, Benallaoua M. et al. Upregulation of Tim-3 and PD-1 expression is associated with tumor antigen-specific CD8+ T cell dysfunction in melanoma patients. *J Exp Med* 2010;207:2175–86.
  43. Gao X, Liu D, Fan Y. et al. The two endocytic pathways mediated by the carbohydrate recognition domain and regulated by the collagen-like domain of galectin-3 in vascular endothelial cells. *Plos One* 2012;7:e52430.

44. Deng WW, Mao L, Yu GT. et al. LAG-3 confers poor prognosis and its blockade reshapes antitumor response in head and neck squamous cell carcinoma. *Oncoimmunology* 2016;5:e1239005.
45. He Y, Yu H, Rozeboom L. et al. LAG-3 protein expression in non-small cell lung cancer and its relationship with PD-1/PD-L1 and tumor-infiltrating lymphocytes. *J Thorac Oncol* 2017;12:814–23.
46. Johnston RJ, Comps-Agrar L, Hackney J. et al. The immunoreceptor TIGIT regulates antitumor and antiviral CD8(+) T cell effector function. *Cancer Cell* 2014;26:923–37.
47. Chauvin JM, Pagliano O, Fourcade J. et al. TIGIT and PD-1 impair tumor antigen-specific CD8<sup>+</sup> T cells in melanoma patients. *J Clin Invest* 2015;125:2046–58.
48. Ozkazanc D, Yoyen-Ermis D, Tavukcuoglu E. et al. Functional exhaustion of CD4<sup>+</sup> T cells induced by co-stimulatory signals from myeloid leukaemia cells. *Immunology* 2016;149:460–71.
49. Leach DR, Krummel MF, Allison JP. Enhancement of antitumor immunity by CTLA-4 blockade. *Science* 1996;271:1734–6.
50. Schadendorf D, Hodi FS, Robert C. et al. Pooled analysis of long-term survival data from phase II and phase III trials of ipilimumab in unresectable or metastatic melanoma. *J Clin Oncol* 2015;33:1889–94.
51. Brahmer JR, Drake CG, Wollner I. et al. Phase I study of single-agent anti-programmed death-1 (MDX-1106) in refractory solid tumors: safety, clinical activity, pharmacodynamics, and immunologic correlates. *J Clin Oncol* 2010;28:3167–75.
52. Wolchok JD, Chiarion-Sileni V, Gonzalez R. et al. Overall survival with combined nivolumab and ipilimumab in advanced melanoma. *N Engl J Med* 2017;377:1345–56.
53. Lipson EJ, Tawbi HA-H, Schadendorf D. et al. Relatlimab (RELA) plus nivolumab (NIVO) versus NIVO in first-line advanced melanoma: primary phase III results from RELATIVITY-047 (CA224-047). *J Clin Oncol*. 2021;39(15\_suppl):9503.
54. Topalian SL, Hodi FS, Brahmer JR. et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med* 2012;366:2443–54.
55. Postow MA, Chesney J, Pavlick AC. et al. Nivolumab and ipilimumab versus ipilimumab in untreated melanoma. *N Engl J Med* 2015;372:2006–17.
56. Park JH, Rivière I, Gonen M. et al. Long-term follow-up of CD19 CAR therapy in acute lymphoblastic leukemia. *N Engl J Med* 2018;378:449–59.
57. Schaft N. The landscape of CAR-T cell clinical trials against solid tumors—a comprehensive overview. *Cancers* 2020;12:2567.
58. de Lima SCG, Fantacini DMC, Batista LC. et al. Strategies to enhance the therapeutic efficacy, applicability, and safety of genetically engineered immune cells. *Crit Rev Immunol* 2021;41:41–67.
59. Dembić Z, Haas W, Weiss S. et al. Transfer of specificity by murine alpha and beta T-cell receptor genes. *Nature* 1986;320:232–8.
60. Robbins PF, Morgan RA, Feldman SA. et al. Tumor regression in patients with metastatic synovial cell sarcoma and melanoma using genetically engineered lymphocytes reactive with NY-ESO-1. *J Clin Oncol* 2011;29:917–24.
61. Rapoport AP, Stadtmauer EA, Binder-scholl GK. et al. NY-ESO-1-specific TCR-engineered T cells mediate sustained antigen-specific antitumor effects in myeloma. *HHS Public Access*. 2016;21(8):914–21.
62. Getts D, Hofmeister R, Quintás-Cardama A. Synthetic T cell receptor-based lymphocytes for cancer therapy. *Adv Drug Deliv Rev* 2019;141:47–54.
63. Baitsch L, Baumgaertner P, Devèvre E. et al. Exhaustion of tumor-specific CD8<sup>+</sup> T cells in metastases from melanoma patients. 2011;121(6):23–5.
64. Fallah-Mehrjardi K, Mirzaei HR, Masoumi E. et al. Pharmacological targeting of immune checkpoint A2aR improves function of anti-CD19 CAR T cells in vitro. *Immunol Lett* 2020;223:44–52.
65. John LB, Devaud C, Duong CP. et al. Anti-PD-1 antibody therapy potently enhances the eradication of established tumors by gene-modified T cells. *Clin Cancer Res* 2013;19:5636–46.
66. Gargett T, Yu W, Dotti G. et al. GD2-specific CAR T cells undergo potent activation and deletion following antigen encounter but can be protected from activation-induced cell death by PD-1 blockade. *Mol Ther* 2016;24:1135–49.
67. Kochenderfer JN, Dudley ME, Carpenter RO. et al. Donor-derived CD19-targeted T cells cause regression of malignancy persisting after allogeneic hematopoietic stem cell transplantation. *Blood* 2013;122:4129–39.
68. Moon EK, Wang LC, Dolfi DV. et al. Multifactorial T-cell hypofunction that is reversible can limit the efficacy of chimeric antigen receptor-transduced human T cells in solid tumors. *Clin Cancer Res* 2014;20:4262–73.
69. Galon J, Rossi J, Turcan S. et al. Characterization of anti-CD19 chimeric antigen receptor (CAR) T cell-mediated tumor microenvironment immune gene profile in a multicenter trial (ZUMA-1) with axicabtagene ciloleucel (axi-cel, KTE-C19). *J Clin Oncol*. 2017;35(15\_suppl):3025.
70. Simon B, Harrer DC, Schuler-Thurner B. et al. The siRNA-mediated downregulation of PD-1 alone or simultaneously with CTLA-4 shows enhanced in vitro CAR-T-cell functionality for further clinical development towards the potential use in immunotherapy of melanoma. *Exp Dermatol* 2018;27:769–78.
71. Zolov SN, Rietberg SP, Bonifant CL. Programmed cell death protein 1 activation preferentially inhibits CD28.CAR-T cells. *Cytotherapy* 2018;20:1259–66.
72. Lee YO, Lee HJ, Kim HC. et al. PD-1 and TIGIT downregulation distinctly affect the effector and early memory phenotypes of CD19-targeting CAR T cells. *Mol Ther* 2022;30:579–92.
73. Moon EK, Ranganathan R, Eruslanov E. et al. Blockade of programmed death 1 augments the ability of human T cells engineered to target NY-ESO-1 to control tumor growth after adoptive transfer. *Clin Cancer Res* 2016;22:436–47.
74. Weber EW, Parker KR, Sotillo E. et al. Transient rest restores functionality in exhausted CAR-T cells through epigenetic remodeling. *Science (80-)* 2021;372(6537):eaba1786.
75. Adusumilli PS, Zauderer MG, Rusch VW. et al. Regional delivery of mesothelin-targeted CAR T cells for pleural cancers: safety and preliminary efficacy in combination with anti-PD-1 agent. *J Clin Oncol* 2019;37(15\_suppl):2511.
76. Osborne W, Marzolini M, Tholouli E. et al. Phase I Alexander study of AUTO3, the first CD19/22 dual targeting CAR T cell therapy, with pembrolizumab in patients with relapsed/refractory (r/r) DLBCL. *J Clin Oncol* 2020;38(15\_suppl):8001.
77. Jaeger U, Worel N, McGuirk JP. et al. Portia: a phase 1b study evaluating safety and efficacy of tisagenlecleucel and pembrolizumab in patients with relapsed/refractory diffuse large B-cell lymphoma. *Blood* 2019;134(Suppl\_1):5325.
78. Jacobson CA, Westin JR, Miklos DB. et al. Abstract CT055: Phase 1/2 primary analysis of ZUMA-6: Axicabtagene ciloleucel (Axi-Cel) in combination With atezolizumab (Atezo) for the treatment of patients (Pts) with refractory diffuse large B cell lymphoma (DLBCL). *Cancer Res* 2020; 80(16 Suppl):CT055 LP-CT055.
79. Hirayama AV, Gauthier J, Hay KA. et al. Efficacy and toxicity of JCAR014 in combination with durvalumab for the treatment of patients with relapsed/refractory aggressive B-Cell non-hodgkin lymphoma. *Blood* 2018;132(Suppl\_1):1680.
80. Chong EA, Melenhorst JJ, Lacey SF. et al. PD-1 blockade modulates chimeric antigen receptor (CAR)-modified T cells: refueling the CAR. *Blood* 2017;129:1039–41.
81. Hill BT, Roberts ZJ, Xue A. et al. Rapid tumor regression from PD-1 inhibition after anti-CD19 chimeric antigen receptor T-cell therapy in refractory diffuse large B-cell lymphoma. *Bone Marrow Transplant* 2020;55:1184–7.
82. Li AM, Hucks GE, Dinofia AM. et al. Checkpoint inhibitors augment CD19-directed chimeric antigen receptor (CAR) T cell therapy in relapsed B-Cell acute lymphoblastic leukemia. *Blood*. 2018;132(Suppl\_1):556–556.
83. Jacobson CA, Locke FL, Miklos DB. et al. End of phase 1 results from zuma-6: axicabtagene ciloleucel (Axi-Cel) in combination with atezolizumab for the treatment of patients with refractory



- diffuse large B cell lymphoma. *Biol Blood Marrow Transplant* 2019;25(3):S173.
84. Nowicki TS, Berent-Maoz B, Cheung-Lau G. et al. A Pilot trial of the combination of transgenic NY-ESO-1-reactive adoptive cellular therapy with dendritic cell vaccination with or without ipilimumab. *Clin Cancer Res* 2019;25:2096–108.
  85. Martinez M, Kim S, St Jean N. et al. Addition of anti-TIM3 or anti-TIGIT antibodies to anti-PD1 blockade augments human T cell adoptive cell transfer. *Oncoimmunology* 2021;10:1873607.
  86. Sharma P, Allison JP. The future of immune checkpoint therapy. *Science* 2015;348:56–61.
  87. Cherkassky L, Morello A, Villena-Vargas J. et al. Human CAR T cells with cell-intrinsic PD-1 checkpoint blockade resist tumor-mediated inhibition. *J Clin Invest* 2016;126:3130–44.
  88. Chen T, Yuan Y, Huang L. et al. Dominant-negative PD1-armed CART cells induce remission in refractory diffuse large B-cell lymphoma (DLBCL) patients. *J Clin Oncol.* 2019;37(15\_suppl):e19028–e19028.
  89. Liu X, Zhang Y, Li K. et al. A novel dominant-negative PD-1 armored anti-CD19 CAR T cell is safe and effective against refractory/relapsed B cell lymphoma. *Transl Oncol* 2021;14:101085.
  90. Prosser ME, Brown CE, Shami AF. et al. Tumor PD-L1 co-stimulates primary human CD8(+) cytotoxic T cells modified to express a PD1:CD28 chimeric receptor. *Mol Immunol* 2012;51:263–72.
  91. Ankri C, Shamalov K, Horovitz-Fried M. et al. Human T cells engineered to express a programmed death 1/28 costimulatory retargeting molecule display enhanced antitumor activity. *J Immunol* 2013;191:4121–9.
  92. Liu X, Ranganathan R, Jiang S. et al. A chimeric switch-receptor targeting PD1 augments the efficacy of second-generation CAR T cells in advanced solid tumors. *Cancer Res* 2016;76:1578–90.
  93. Liu H, Lei W, Zhang C. et al. CD19-specific CAR T cells that express a PD-1/CD28 chimeric switch-receptor are effective in patients with PD-L1-positive B-cell lymphoma. *Clin Cancer Res* 2021;27:473–84.
  94. Shin JH, Park HB, Oh YM. et al. Positive conversion of negative signaling of CTLA4 potentiates antitumor efficacy of adoptive T-cell therapy in murine tumor models. *Blood* 2012;119:5678–87.
  95. Hoogi S, Eisenberg V, Mayer S. et al. A TIGIT-based chimeric co-stimulatory switch receptor improves T-cell anti-tumor function. *J Immunother Cancer* 2019;7:243.
  96. Giuffrida L, Sek K, Henderson MA. et al. CRISPR/Cas9 mediated deletion of the adenosine A2A receptor enhances CAR T cell efficacy. *Nat Commun* 2021;12:3236.
  97. Smith I, Greenside PG, Natoli T. et al. Evaluation of RNAi and CRISPR technologies by large-scale gene expression profiling in the connectivity map. *Plos Biol* 2017;15:e2003213.
  98. Su SW, Lu ZK, Gou SC. et al. Controllable vacuum-induced diffraction of matter-wave superradiance using an all-optical dispersive cavity. *Sci Rep* 2016;6:35402.
  99. Guo X, Jiang H, Shi B. et al. Disruption of PD-1 enhanced the anti-tumor activity of chimeric antigen receptor T cells against hepatocellular carcinoma. *Front Pharmacol.* 2018;9:1–15.
  100. Hu W, Zi Z, Jin Y, et al. CRISPR/Cas9-mediated PD-1 disruption enhances human mesothelin-targeted CAR T cell effector functions. *Cancer Immunol Immunother* 2019;68:365–77.
  101. Rupp LJ, Schumann K, Roybal KT. et al. CRISPR/Cas9-mediated PD-1 disruption enhances anti-tumor efficacy of human chimeric antigen receptor T cells. *Sci Rep* 2017;7:737.
  102. Zhang Y, Zhang X, Cheng C. et al. CRISPR-Cas9 mediated LAG-3 disruption in CAR-T cells. *Front Med* 2017;11:554–62.
  103. Iwamura K, Kato T, Miyahara Y. et al. siRNA-mediated silencing of PD-1 ligands enhances tumor-specific human T-cell effector functions. *Gene Ther* 2012;19:959–66.
  104. Condomines M, Arnason J, Benjamin R. et al. Tumor-targeted human t cells expressing cd28-based chimeric antigen receptors circumvent CTLA-4 inhibition. *Plos One* 2015;10:e0130518.
  105. Suarez ER, Chang de K, Sun J. et al. Chimeric antigen receptor T cells secreting anti-PD-L1 antibodies more effectively regress renal cell carcinoma in a humanized mouse model. *Oncotarget* 2016;7:34341–55.
  106. Li S, Siriwon N, Zhang X. et al. Enhanced cancer immunotherapy by chimeric antigen receptor-modified T cells engineered to secrete checkpoint inhibitors. *Clin Cancer Res* 2017;23:6982–92.
  107. Rafiq S, Yeku OO, Jackson HJ. et al. Targeted delivery of a PD-1-blocking scFv by CAR-T cells enhances anti-tumor efficacy in vivo. *Nat Biotechnol* 2018;36:847–56.
  108. Qin L, Zhao R, Chen D. et al. Chimeric antigen receptor T cells targeting PD-L1 suppress tumor growth. *Biomark Res* 2020; 8:19.
  109. Xie J, Zhou Z, Jiao S, Li X. Construction of an anti-programmed death-ligand 1 chimeric antigen receptor and determination of its antitumor function with transduced cells. *Oncol Lett* 2018; 16:157–66.
  110. Yang CY, Fan MH, Miao CH. et al. Engineering chimeric antigen receptor T cells against immune checkpoint inhibitors PD-1/PD-L1 for treating pancreatic cancer. *Mol Ther Oncolytics* 2020;17:571–85.
  111. Anna F, Bole-Richard E, Lemaoult J. et al. First immunotherapeutic CAR-T cells against the immune checkpoint protein HLA-G. *J Immunother Cancer.* 2021;9(3):e001998.
  112. Lee WS, Ye Z, Cheung AMS. et al. Effective killing of acute myeloid leukemia by TIM-3 targeted chimeric antigen receptor T cells. *Mol Cancer Ther* 2021;20:1702–12.