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Review

PD-1 disrupted CAR-T cells in the treatment of solid tumors: Promises and challenges

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ABSTRACT

Unprecedented efficacy of chimeric antigen receptor (CAR) T cell therapy in the treatment of hematologic malignancies brings new hope for patients with many cancer types including solid tumors. However, the challenges for CAR-T cell therapy in eradicating solid tumors are immense. To overcome these seemingly intractable hurdles, more "powerful" CAR-T cells with enhanced antitumor efficacy are required. Emerging data support that the anti-tumor activity of CAR-T cells can be enhanced significantly without evident toxicity through simultaneous PD-1 disruption by genome editing. This review focuses on the current progress of PD-1 gene disrupted CAR-T cells in cancer therapy. Here we discuss key rationales for this new combination strategy and summarize the available pre-clinical studies. An update is provided on human clinical studies and available registered cancer clinical trials using CAR-T cells with PD-1 disruption. Future prospects and challenges are also discussed.

1. Introduction

Adoptive cell therapy (ACT) and immune checkpoint blockade have radically changed the landscape of cancer treatment and provides new hope and treatment options for cancer patients. Clinically, these immunotherapies have joined the ranks of surgery, radiation, and chemotherapy as a pillar of cancer therapy [1]. Drugs that block the immune regulatory checkpoints, i.e. programed cell death protein 1 (PD-1), PD-ligands (PD-L1, PD-L2), and cytotoxic T cell lymphocyte-associated antigen 4 (CTLA-4), have demonstrated impressive clinical outcomes in a wide spectrum of hematological malignancies and solid tumors, significantly improving overall survival. Indeed, immune checkpoint inhibitors have emerged as frontline treatments for multiple cancers, such as metastatic melanoma and non-small cell lung cancer [2,3]. Adoptive transfer of chimeric antigen receptors (CARs) T cells has also shown remarkable results in the treatment of cancers [4–6]. As a result, the U.S. Food and Drug Administration (FDA) has recently approved the first two cell therapies, based on chimeric antigen receptor (CAR) T cells, for B cell malignancies [7].

Mechanistically these two immune-oncological approaches are very different. Immune checkpoint inhibition is aimed at releasing the brakes that suppress the action of the immune system attack against the tumor cells. Anti-PD-1 and PD-1-ligands, as well as anti-CTLA4 are examples of this type of targeted approach, which are currently being used as single agents or in combination with other therapies. The aim of adoptive transfer of autologous T cells, modified *ex vivo* to express artificial CARs on the cell surface, is to create an immune effector specifically to attack cancer cells using the patient's own immune system [8,9].

Despite CAR-T cell therapy achieving remarkable results in the treatment of hematological malignancies, treatment of solid tumors using CAR-T remains an enormous challenge. The limited efficacy observed is mainly related to poor trafficking, limited persistence and infiltration, and T cell inhibitory activity in the tumor microenvironment (TME) [10–14]. Therefore, it has been proposed that combinational immunotherapy, combining CAR-T and immune checkpoint blockade, may be a more efficacious treatment approach to solid tumors [15–19]. To this end, over 75% of the immunotherapy clinical trials registered at clinicaltrials.gov, combine PD-1/PD-L1 blockade with at least one additional therapy [20].

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Table 1
List of Registered Clinical Trials Using PD-1 Gene Knockout (KO) CAR-T cells/T Cells to Treat Cancer.

NCT Number (Year started)	Location	Status	Therapy	Cancer type targeted	Summary of the Study	Results
03747965 (Nov 2018)	Beijing China	Recruiting	PD-1 KO CAR-T	Adult Solid tumors	A phase 1 open label, single group assignment observing adverse effects and clinical responses of PD-1 knocked out mesothelin-directed CAR-T cells infusion	No
03706326 (Sep 2018)	Guangzhou China	Recruiting	PD-1 KO CAR-T	Advanced oesophageal cancer	A phase 1/2 study aiming to assess the safety and efficacy of PD-1 knocked out engineered anti-MUC1 CAR-T cells in advanced oesophageal cancer.	No
NCT03545815 (Mar 2018)	Beijing China	Recruiting	PD-1 KO CAR-T	Adult Solid tumors	A phase 1 open label, single group assignment investigating adverse events and clinical responses of PD-1 knocked out mesothelin-directed CAR-T cells infusion	No
NCT03525782 (Feb 2018)	Guangzhou China	Recruiting	PD-1 KO CAR-T	Advanced lung cancer	A combined phase 1 and 2 study investigating the safety and efficacy of PD-1 knocked out engineered anti-MUC1 CAR-T cells in advanced non-small cell lung cancer.	Yes [23]
NCT03298828 (Nov 2017)	Chongqing China	Not yet recruiting	PD-1 K CAR-T	Acute leukemia lymphoblastic	A randomised phase 1 study assessing the safety and responses of CD19 CAR and PD-1 Knockout Engineered T cells in acute lymphoblastic leukemia.	No
NCT03525652 (Feb 2018)	Guangzhou China	Recruiting	PD-1 KO T Cells	Advanced Prostate cancer	A randomised phase 1 / 2 study assessing the safety and efficacy of combining PD-1 knockout engineered T cells and therapeutic vaccine in advanced prostate cancer.	No
NCT02793856 (Aug 2016)	Chengdu China	Active	PD-1 KO T cells	Advanced lung cancer	A phase I clinical trial of PD-1 knockout engineered T cells treating patients with advanced non-small cell lung cancer	Yes [116]
NCT03081715 (Mar 2017)	Hangzhou Chian	Recruiting	PD-1 KO T Cells	Esophageal cancer	A phase II trial of PD-1 knockout engineered t cells for the treatment of advanced esophageal cancer	Yes [136]
NCT03399448 (Sep 2018)	Philadelphia USA	Recruiting	TCR/PD-1 KO T Cells	Melanoma, multiple myeloma	A phase 1 trial of autologous T cells engineered to express NY-ESO-1 TCR and CRISPR gene edited to eliminate endogenous TCR and PD-1	No
NCT02867332 (Nov 2016)	Beijing China	Not yet recruiting	PD-1 KO T Cells	Metastatic renal cell carcinoma	A dose-escalation Phase I Trial of PD-1 knockout engineered T cells for the treatment of metastatic renal cell carcinoma	No
NCT03044743 (Apr 2017)	Nanjing China	Recruiting	PD-1-KO-EBV-CTL	EBV associated malignancies	A Phase I/II trial of PD-1 knockout EBV-CTLs for advanced stage EBV associated malignancies, such as advanced stage gastric, nasopharyngeal carcinoma	No
NCT03030001 (Nov 2016)	Beijing China	Not yet recruiting	PD-1 KO T Cells	Refractory prostate cancer	A dose-escalation Phase I Trial of PD-1 knockout engineered T cells for the treatment of castration resistant prostate cancer	No
NCT02863913 (Sep 2016)	Beijing China	Not yet recruiting	PD-1 KO T Cells	Advanced bladder cancer	A phase I trial of PD-1 knockout engineered T cells for the treatment of muscle-invasive bladder cancer	
ChiCTR1800020306 (Dec 2018)	Nanning China	Recruiting	PD-1 KO CAR-T	Refractory B Cell Malignancies	A phase 2 study evaluating the safety and efficacy of PD1 Gene knock-down anti-CD19 CAR-T cells in targeted patients with relapsed/refractory/high risk B cell malignancies	N/A
ChiCTR1800018713 (Oct 2018)	Guangzhou China	Recruiting	PD-1 KO CAR-T	Refractory non Hodgkin lymphoma	A clinical study evaluating the safety and efficacy of PD-1 gene knocked out anti-CD19/CD20/CD22/CD30-CART cells in the relapse/refractory non Hodgkin Lymphoma	N/A
ChiCTR1900022620	Zhengzhou Chian	Recruiting	PD-1 KO CAR-T	Refractory thyroid cancer	A clinical study assessing the safety and efficacy of dPD-1(TSHR + CD19) CART cell in the treatment of refractory thyroid cancer	No
ChiCTR-OIC-17011310 (May 2017)	Hangzhou China	Recruiting	PD-1 KO CAR-T	Non-Hodgkin's lymphoma	A clinical study assessing the safety and efficacy of dPD-1 hCD19CART cells in the treatment of refractory, aggressive non-Hodgkin's lymphoma	No
ChiCTR-OIN-17012136 (Jul 2017)	Hefei China	Recruiting	PD-1 KO T Cells	Advanced cancer	A clinical study evaluating the safety and efficacy of PD-1 KO autologous activated T cells in the treatment of advanced malignant tumors	No
ChiCTR1800016023 (May 2018)	Hangzhou China	Recruiting	PD-1 KO T Cells	Advanced lung cancer	A clinical study examining the application of PD-1 gene deleted T lymphocytes in the treatment of non-small cell lung cancer	No
ChiCTR1900025088 (Aug 10, 2019)	Guangzhou China	Recruiting	PD-1 KO CAR-T cells	Advanced breast cancer	A clinical study for MUC1 positive advanced breast cancer treated with pd-1 knockout MUC1 target chimeric antigen receptor T cells	No

Note: Registered trials listed in the table were from either www.clinicaltrials.gov or www.Chictr.org.cn (Chinese Clinical Trial Registry).

Although anti-PD-1 or PD-L1 monoclonal antibodies (mAbs) are used to block the PD-1/PD-L1 regulatory axis in most clinical studies to-date, cell-intrinsic disruption of immune checkpoints by gene editing in CAR-T cells could be a better way to enhance the antitumor activity of the CAR-T cells. This approach is more likely to elicit a better safety profile than the systemic administration of blocking antibodies [21,22]. The most recent pre-clinical and clinical data demonstrated an improved safety profile and tolerability with enhanced tumor control capability in PD-1 disrupted CAR-T cells compared with the conventional CAR-T [18,23–25]. Potentially, PD-1 knockout (KO) or disruption by precision genome engineering techniques in CAR-T cells could be the next-generation of cell therapies used in solid tumor treatments [18].

The clinical outcome of PD-1KO /disruption in T cells or CAR-T cells by CRISPR (clustered regularly interspaced short palindromic repeats) and CRISPR-associated (Cas) 9 in the treatment of various solid tumors are being actively evaluated in multiple clinical trials. Up to September 26, 2019, the number of registered clinical trials using PD-1 KO/CAR-T cells or PD-1 KO T cells was 20 (details in Table 1), including 4 from our group (NCT03706326, NCT03525782 and NCT03525652 in https://clinicaltrials.gov, and ChiCTR1900025088 from www.chictr.org.cn). Comprehensive reviews discussing immune checkpoint blockades by antibody and CAR-T therapies have been recently published elsewhere [6,7,13,20,26–35]. This article provides the latest progress and available clinical data specifically in PD-1 KO/CAR-T for cancer treatment. We also discuss the major clinical advantages and challenges of CAR-T/PD-1 KO combination immunotherapy.

2. Immunotherapies based on PD-1 inhibition and CAR-T cells

2.1. The PD-1/PDL-1 axis

Ground-breaking science in the 1990′s showed human cells carry specific proteins on their surface to evade attack from the host immune system [36]. Cleverly, to escape attack from the body's immune system, human cells display some of these specific proteins on their cell surface, thereby the immune system (including T cells, also known as T-lymphocytes) recognizes cancer cells as 'self'. To survive cancer cells have evolved multiple immunosuppressive mechanisms to avoid immune detection, thus disarming many immunotherapeutic targets. The fast-track understanding of the cell biology and signalling pathways that cancer cells exploit to avoid immune detection and survive has allowed for the development of new immunotherapies, which are at the leading edge and a mainstay in the war against cancer. One of the key signalling complexes, PD-1 interaction with PDL-1 (also known as B7-H1 or CD274) and/or PDL2 (also known as B7-DC or CD273), is utilized by cancer cells to evade T cell recognition and destruction. Disruption of the PD-1/PD-L1 signalling complex is currently being used in cancer immunotherapy in the clinic. PD-1 is a receptor protein on the surface of T cells that binds to its' ligands PD-L1 [37] or PD-L2 [38] on the surface of host cells as a normal physiological response to prevent cellular damage in response to chronic infection and inflammation [39]. Hence PD-1 is known as a checkpoint molecule that participates in self-recognition of normal cells in the immune surveillance response. One of the duplicitous key mechanisms developed by the cancer cell to avoid detection by T cells surveillance is by turning on the expression of PD-L1 [40] or PDL-2 [41]. PD-L1 binds to PD-1 and serves as an adaptive immune resistance, whereby cancer cells avoiding detection and destruction [39,40]. The expression of PD-1 is commonly found on tumor-infiltrating lymphocytes (TILs), immune cells that penetrate and attack cancer cells compared to normal cells [42]. High expression of PD-1 on TILs was found to be associated with impaired effector function, loss of tumor immune response, and poor prognosis [42]. Equally, high PDL-1 expression on tumor cells was found to be associated with poor tumor response and poor prognosis. In light of these findings, blocking the PD-1/PDL-1 binding was proposed as an important potential target for cancer therapy. There are a number of anti PD-1/PD-L1 blocking mechanisms currently in the clinic, in clinical trials, or in the development phase of discovery, mainly directed at the notoriously resistant, relapsed and metastatic cancers. Therefore, inhibition of the PD-1/PD-L1 axis, either by PD-1 mAbs or PD-1 disruption in T cells, has provided diverse opportunities to enhance antitumor immunity with the potential to produce durable clinical responses [43,44].

2.2. Blocking the PD-1/PD-L1 axis using monoclonal antibodies

In 2008, the first PD-1/PDL-1 blocking strategy, monoclonal antibodies (mAbs), were shown to be safe and well tolerated in a phase 1 clinical trial in patients with advanced hematologic malignancies [48]. Since conception of PD-1/PD-L1/PDL2 clinical trials over 2250 active trials have been initiated (2006- September 2018) [49]. Most clinical trials include combinations of anti-PD-1/PD-L1with other cancer therapies, however, to-date only a few FDA approved combinational therapies are available. These included nivolumab (anti-PD-1) with ipilimumab (anti-CTLA-4), and pembrolizumab (anti-PD-1) with chemotherapy [49], and more recently (2018) Cemipilmab (Libtayo) (anti-PD-1) developed by Regeneron Pharmaceuticals. In addition, three FDA anti-PDL-1 mAbs were approved but for specific cancer types, Atezolizumab (Tecentriq), for urothelial carcinoma [50] and non-small cell lung cancers (NSCLCs) [51,52], Avelumab (Bavencio) for metastatic merkel-cell carcinoma [53]and Durvalumab (Imfinzi) [54,55] for urothelial and unresectable NSCLC).

Advanced lung cancer, melanoma, breast cancer and lymphoma are the most studied anti-PD-1/PD-L1 therapies tested, respectively. One of the early clinical studies (2013) administering lambrolizumab (Anti–PD-1) showed a promising high rate of sustained tumor regression with low grade adverse effects in patients with advanced melanoma [56]. The most extraordinary, efficacious clinical trial benefits to-date are the combination of the dual checkpoint inhibitors, nivolumab with ipilimumab in advanced melanoma (phase 1 dose escalation study: CA209-004; phase II CheckMate 069; phase III CheckMate 067), with a 3-year overall survival (OS) rate of 63% [57,58]. The combinational therapy resulted in a higher rate of survival compared to using monotherapy (nivolumab or ipilimumab), which included patient sub-groups of PD-1 lowly expressing tumors [57,59]. Significant advances in treatment outcomes (OS and PFS) have also been demonstrated in clinical trials for mono- and combined advanced lung cancer patients using FDA approved nivolumab [60–67], pembrolizumab [68–73] and atezolizumab (anti-PD-L1) [51,74–77] and/or conventional chemotherapies [78,79].

Common adverse effects in patients using anti-PD-1 mAbs treatment include, skin rashes, liver, gastrointestinal tract, endocrine systems, nausea, diarrhea, thyroid disorders, as reviewed in [80]. Other, more serious PD-1/PD-L1-mAb associated autoimmune complications include cardiac complications [81,82], neuromuscular disorder [83], and pneumonitis [84], requiring careful monitoring of patients during and post PD-1/PD-L1 [85]. Although anti-PD-1 mAbs treatments for lung cancers have had some positive results there has been considerable serious concerns raised with deleterious side effects and increased risk of mortality in some elderly patients [86–89]. Equally important, continuous treatment of PD-1 mAbs, potentiate a great risk of breaking the patient's immune tolerance and the host immune cells attacking normal cells.

2.3. Chimeric antigen receptor (CAR) T cell therapy

The CAR-T cell therapeutic approach is a genetically engineered ACT immunotherapy, using the host's immunological defense system to mount an attack on the cancer cell to treat cancer [90]. The engineering of the CAR-T cells is described in detail in the article by Zhang et al [91]. Autologous T cells are collected from the patient and genes are

engineered into the T cells which are then expressed CARs on the surface of the host T-cell. These gene inserts, CARs, are targeted to a specific protein on the surface of the patient's cancer cells. When injected back into the patient, the CAR-T cells multiply, bind to, and kill the tumor cells. In patients with relapsed or refractory B-cell acute lymphocytic leukemia (ALL) CAR-T cell therapy showed promising results with long-term or complete remission in 80–90% of patients [92–95]. Although CAR-T cell therapy has a number of adverse side effects, which can be mild or severe, including cytokine release syndrome (CRS), (low blood pressure, flu-like symptoms, breathing difficulties) and some patients have experienced some neurologic difficulties, these symptoms are usually manageable.

2.4. Summary of CAR-T therapies for cancer

From bench to the clinic, CAR-T cell therapy has passed through four generations since its initial development in 1989 [91]. The 1st generation of CAR-T cell therapies, transfected with single-chain receptors, proved to be very disappointing in clinical trials for different cancer types, with inadequate proliferation, short life-span and inadequate secreted proteins [91]. However, the 2nd generation of CARs using three different receptor types, T-cell antigen receptor, cytokine receptors and co-stimulatory receptors proved to be more efficacious leading to FDA approval for treatment of refractory pre-B cell acute lymphoblastic leukemia and diffuse large B cell lymphomas [6,96–98]. The 3rd generation of CAR-T cell therapies incorporated multiple signaling domains to increase the potency by increasing the killing ability through stronger cytokine manufacture [91]. Although these third generation CAR-T cells were used to treat colon and lymphoma there was little improvement in patient outcome in relation to the 2nd generation with some adverse clinical outcomes [99,100]. The 4th generation of CAR-T cells, named TRUCKS, produce an inducible transgenic 'payload' or protein which is released as an immune TME modifier [101]. These 'TRUCKs' are aimed to attract and shape a favorable TME effectively by delivering chemokines/cytokines to the cancer tissue. More recently the engineering of universal CAR (UniCAR), which are switchable and reprogrammable platforms to recognize more than one epitope are in the pipeline [102,103]. A major stumbling block with CAR-T therapy is, whilst great progress is being made with blood-borne cancers in the clinic [104], the solid tumors are more difficult to penetrate, target and kill individual cancer cells. The development of PD-1KO/with CARs is an interesting approach which is providing some success in pre-clinical trials – the rationale for combining PD-1KO and CARs in T-cell therapies is discussed.

3. Rationale for combining PD-1 knockout (KO) and CAR-T cell therapy to treat solid tumors

Ninety percent of all cancer mortalities results from solid tumors which are notoriously difficult to effectively treat [92,93]. Therefore, the main battlefield for immunotherapy is the treatment of solid tumors. Although the remarkable clinical outcomes of CAR-T cell therapy in hematologic malignancies is driving the development of CAR-T cells to target solid tumors, the challenges ahead are enormous. Currently immune checkpoint inhibitors (ICIs) provides another new treatment option. However, single-agent approaches are effective for only a select subset of patients: therapies like checkpoint inhibitors have proven to be effective in a fraction of the patients treated. For example, although ICIs targeting PD-1 or PD-L1 now present as a standard treatment option for patients with advanced non-small-cell lung cancer (NSCLC) a substantial proportion of patients will not benefit from these treatments [105].

The overall rationale for CAR-T/PD-1 KO combination treatment is to provide an enhanced version of CAR-T with stronger potency to fight against solid tumors. The combination of CAR-T cells with PD-1 blockade through genetic modifications can significantly boost CAR-T anticancer activities with a superior safety profile. The rapid advancements in genome editing techniques, such as CRISPR and Cas9, has enabled the use of genetic approaches to disrupted PD-1 function in CAR-T-cells for cancer treatment.

3.1. Enhanced antitumor activity is required for eradication of solid tumor by CAR-T cells

Despite very promising results observed in patients with relapsed or refractory B-cell acute lymphocytic leukemia (ALL), showing long-term or complete remission in patients (80–90%) [94,95] the therapeutic application of CAR-T cells against solid cancers has limited efficacy due to a number of therapeutic barriers. These barriers include CAR-T cell infiltration, persistence, trafficking, and an unfavorable TME within tumors [106,107]. These factors prevent CAR-T cells to effectively contain solid tumor growth or eradicate tumors (discussed in 6.3. CAR-T/PD-1 cell trafficking into solid tumors). In addition, solid tumors exhibit heterogeneity with regards to intensity and distribution of tumor-associated antigens (TAAs), posing additional challenges for CAR-T-cell therapies [108]. This is a major encumbrance for CAR-T-cell therapy when used as a single agent to treat solid tumors and is often insufficient to mediate clinical tumor regression. To eradicate solid tumors, or to achieve similar efficacy to that observed in homological malignancies, combination of immune-checkpoint inhibitors and CAR-T-cells have to be able to overcome many barriers, as mentioned above, and infiltrate and remain in tumor tissues long enough to kill cancer cells. Conventional CAR-T cells have limited capability to fight solid tumors. Therefore, any strategy that can enhance the potency of CAR-T cells will be of value. The combination of CAR-T with PD-1 blockade is a promising strategy to enhance the antitumor activity of CAR-T cells [45,47].

3.2. Combinational CAR-T and PD-1 blockade leads to enhanced antitumor activity and a better safety profile

A large body of evidence supports that PD-1/PD-L1 interaction of T cells and tumor cells leads to inhibition of T cell effector function [45], coined T cell exhaustion [46]. As such, blocking this interaction with PD-1 or PD-L1 mAbs or genetically engineering PD-1 KOs has the potential to significantly enhance the anti-tumor activity of cytotoxic T cells (CTLs) allowing T cell recognition of cancer cells and facilitating eradication of tumor cells and reducing T cell exhaustion. *In vitro* and *in vivo* PD-1 KO studies demonstrated CTLs were more efficient in killing tumor cells when compared to the normal control CTLs [45,47]. The antitumor activity of PD-1 KO CTLs was verified *in vivo* using a mouse xenograft model [45]. These findings suggest that PD-1/PD-L1 disruption or blockade may be an effective strategy for improving the potency of CAR-T cell therapies, especially in the treatment of solid tumors. Mechanistically, PD-1 KO can enhance CAR-T cell anti-tumor activity via removal of PD-1/PD-L1 and PD-1/PD-L2 signaling in these genetically edited immune T cells. Thus, inhibition of PD-1 expression on T/CAR-T cells provides an opportunity for CAR-T cells to recognize tumor cells [109]. Indeed, combination therapy comprising of CAR-T and PD-1 blockade has been demonstrated to significantly enhance the anti-tumor activity of CAR-T cells. Thus, pre-clinical data using CAR-T in combination with PD-1 supports a promising immunotherapeutic strategy for tumors: enhancing the antitumor efficacy and extending the scope of patient treatment options [110]. Recently, reports have suggested that CRISPR/Cas9-mediated disruption of PD-1 can boost the function of CD19-CAR-T cells [18,111]. A summary of progress on the pre-clinical studies on this combination therapy will be given in the following sections.

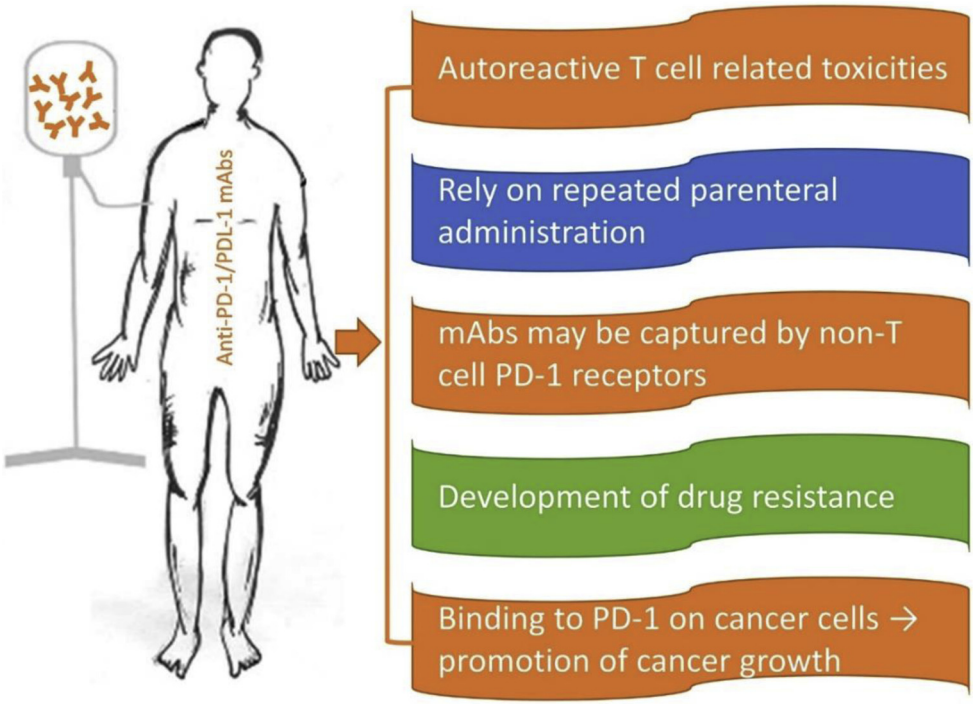

Fig. 1. Adverse effects associated with Anti-PD-1/PDL-1 mAb treatment. PD-1/PD-L1 blockade by monoclonal antibodies has become a viable cancer treatment option. However, systemic administration of PD-1 mAbs comes with a number of adverse effects as listed. Many of these side effects can be averted by PD1 gene knockout/disruption in T cells by gene editing technology.

3.3. Issues surrounding current ICI blockade with systemic use of PD-1 mAbs

As mentioned above, PD-1 and its two natural ligands PD-L1 and PD-L2 are responsible for delivering inhibitory signals that regulate the balance between T cell activation, tolerance, and immunopathology [44]. Inhibition of the PD-1/PD-L1 axis provides diverse opportunities to enhance antitumor immunity with the potential to produce durable clinical responses. Therefore, blocking the PD-1 pathway, either by PD-1 mAbs or PD-1 KO T cells has emerged as a promising strategy for cancer therapy.

Systemic administration of mAbs inhibitors is the most common approach to block the known immune check points [35,112]. However, systemic PD-1 Mab blockade is associated with a number of problems as showed in Fig. 1. Firstly, toxicities due to enhanced activation of autoreactive T cells. Secondly, the blockade efficacy of PD-1 mAbs is short-lived and relies upon repeated administration [47]. Thirdly, anti-PD-1 mAbs can be captured prior to reaching the T cell surface by PD-1 tumor-associated macrophages through their Fc domain, removing their ability to block PD-1 and resulting in suppression of T cell function [113]. Fourthly, innate or development of resistance to checkpoint inhibitors may occur. Therefore, many patients do not experience a complete response upon anti-PD-1 treatment, and some patients are non-responders, highlighting the need to improve our understanding of the molecular and cellular effects of blocking the PD-1/PD-L1 pathway and the mechanism underlying adverse effects [114]. Finally, as reported very recently, in some patients, mAbs binding to the PD-1 receptors on the tumor cells (lung cancer, melanoma, and liver cancer) demonstrated the capacity to promote tumor growth [44,115], an unexpected adverse effect of anti-PD-1 mAb treatment.

As a different technological approach to disrupting the PD-1/PD-L1 interaction, genetic modification, knocking out PD-1 gene in T cells, has some advantages. This has been achieved through CRISPR editing of the PD-1 gene [45]. In a mouse xenograft tumor model, introduction of engineered PD-1/KO cytotoxic T lymphocytes (PD-1/KO/CTLs) repressed tumor growth and increased survival outcome compared to controls [45]. In April 2018, the first reported preliminary phase 1 clinical trials (NCT02793856), using infusion of PD-1 deficient CTLs for non-small-cell lung cancer (NSCLC) showed promising results, with the primary outcome being safety of use [116]. From the 8 patients enrolled in the study, low grade side effects were recorded, mostly fever and hepatic dysfunction. Re-introducing a novel subset of engineered PD-1 deficient TCLs allowed the immune-cells to recognize cancer cells expressing PD-L1/PD-L2 receptors on the membrane surface and provoking an immune-attack. Although to-date, there is not enough evidential studies to support whether PD-1 immuno-editing of host T cells is more beneficial or of equal benefit to treatments with PD-1 mAbs, in the frontier point of view, cell-intrinsic disruption of immune checkpoints by gene targeting in T cells is likely to display a superior safety profile than the systemic administration of blocking antibodies [21].

Compared with combined CAR-T cell and anti-PD-1 mAbs treatment, there are several advantages of using genetically engineered cells, in which PD-1 gene is knocked out or disrupted. Recent studies demonstrated that ablation of Pdcd1 specifically in CAR-T cells might provide a safer way to overcome tumor immunosuppression, particularly when combined with TCR disruption to prevent activation of autoreactive T cells [18,117]. Combinational therapy using PD-1 KO and CAR-T directed to specific cancers may prove to be more efficacious and more tolerated than PD-1 KO or CAR-T individual therapies (refer to section, *PD-1 gene disrupted CAR-T cells).*

3.4. Reliable precision genome editing is possible

The recent rapid advancement in genome editing technology has enabled precision gene editing of T cells. The CRISPR-Cas9 system confers targeted gene editing by small RNAs that guide the Cas9 nuclease to the target site through base pairing [118]. A number of studies have shown that PD-1 gene KO/disruption in human T cells or CAR-T cells using the CRISPR-Cas9 system can be accomplished with high accuracy and success rates [17,18,22,24,119]. The combination of CAR-T cells with PD-1 gene disruption by gene editing techniques has led to observed enhancement of anti-tumor activities in liver cancer [120], and B cell lymphoma [18].

These developments have provided the rationale for combining adaptive cell therapy with PD-1 gene knockout modalities to improve response rates and durability of responses in a variety of cancers [121].

4. Pre-clinical studies on PD-1 disrupted CAR-T cells

Pre-clinical studies directed towards disruption of the PD-1 gene in human primary T cells and CAR-T cells, prior to the introduction of PD-1 KO CAR-T therapies to cancer patients, are reviewed in this section.

4.1. PD-1 gene disrupted T cells

In view of the remarkable clinical results of systemic anti-PD-1 mAb treatment, the PD-1 gene is the most promising target for T cell gene editing. Mechanistically, PD-1 is expressed post T cell activation, and binds to the corresponding ligand PD-L1 or PD-L2 on the surface of the tumor cells, thereby inhibiting the transmission of T cell activation signals [122]. The TME also contributes to promote high expression of PD-1 on the surface of T cells and subsequently impairing their anti-tumor function, which is an important feature of T cell exhaustion [46]. Therefore, disrupting the PD-1 gene in T cells through gene editing technology should provide a promising strategy to overcome the hurdle of immunosuppression in solid tumor treatment.

Although the CRISPR-Cas9 system is currently the most commonly used method to edit genes in primary T cells, previously, zinc finger nuclease (ZFN) and transcriptional activator-like effector nuclease (TALEN) genome editing techniques were used to disrupt the PD-1. In 2015, Beane et al [123] found that in PD-1 KO in tumor infiltrating lymphocytes (TILs) isolated from melanoma patients resulted in an efficiency of 75% using ZFN technology, and the bi-allelic mutation incidence in the range of 40% to 48%, leading to 76% decrease in PD-1 expression on the T cell surface. In this study PD-1 KO did not alter the T cell subsets and proliferation, resulting in a stronger antigen-specific tumor cell killing and cytokine (TNFα, GM-CSF, and IFNγ) release *in vitro*, compared to unmodified TILS. Using TALEN technology, Menger et al. (2016) knocked out the PD-1 gene on tumor antigen-specific T cells isolated from the melanoma and fibrosarcoma mice. They demonstrated that inactivation of the PD-1 gene in melanoma-reactive CD8þ T cells and in fibrosarcoma-reactive polyclonal T cells enhanced the persistence at the tumor site, and controlled the tumor progression compared to the non-engineered T cells [124].

The CRISPR-Cas9 system as a gene-editing tool has advanced greatly in recent years. This technology is becoming popular for T cell gene editing, especially for PD-1 gene KO in human primary T cells [45,119,125,126] and CAR-T cells [18,111,127]. Consistently, in all the studies to-date, the antitumor effect of gene modified T cells/ CAR-T cells has been found to be significantly enhanced. Su et al [119] was the first to describe a non-viral mediated approach to KO the PD-1 gene in T cells using electroporation of plasmids encoding sgRNA and Cas9. They demonstrated an efficient disruption of gene PD-1 was achieved by using the sgRNA: Cas9 system in T cells derived from both patients and healthy donors. More importantly, disruption of the PD-1 gene by this method resulted in significant reduction of PD-1 expression in T cells but didn't affect the proliferation of primary human T cells during the prolonged 21 days *in vitro* culture. Gene modification of T cells also led to favorable enhanced cytotoxicity, characterized by the up-regulation IFN-γ production [119]. Using the same CRISPR-Cas9 system to KO PD-1 in T cells, Zhao and colleagues found that introducing PD-1 KO-T cells facilitated apoptosis and enhanced multiple myeloma cell death by 36% compared with control. The enhancement of anti-tumor activity of the PD-1 KO gene edited T cells was verified *in vivo* using a mouse xenograft model. Xenografted mice with PD-1 KO T cells, demonstrated multiple myeloma cell suppression and prolonged survival of the treatment animals compared with the control group. PD-1 KO also increased T cell secretion of TNF-α and IFN-γ by 2.4 and 1.9-fold respectively [45].

A different approach by Ligtenberg and colleagues [128] used a novel self-delivering sdRNA to disrupt PD-1 in healthy donor T cells and TILs from patient with malignant melanoma. Following treating with sdRNA specific for PD-1, extracellular and intracellular PD-1 protein levels in the majority of the human primary T cells were markedly reduced. A significant increase in the capacity of T cells to secrete interferon g (IFN-g) was observed upon polyclonal stimulation. They further demonstrated that after expansion of PD-1 KO, TILs performed with increased functionality against autologous tumors as compared to control T cells. This method of introducing RNAi into T cells to disrupt the expression of PD-1 receptors on the surface of T cells could readily be applied to any ACT protocol to enhance the antitumor functions for solid tumor therapies [128].

These results, summarized above, demonstrate the feasibility of gene editing technology as an approach for efficient PD-1 disruption in T cells, proposing a new strategy for targeting PD-1 or checkpoint inhibition as a clinical application.

4.2. PD-1 gene disrupted CAR-T cells

The success of knocking out the PD-1 gene in primary human T cells using CRISPR/Cas9 raised the prospect of modifying PD-1 in CAR-T cells in order to enhance their anti-tumor activity. As mentioned above, the combination therapy of PD-1 mAbs with ACT is an attractive strategy to break the immunosuppressive TME [16,129], given that systemic administration of therapeutic PD-1 mAbs often cause adverse effects by increasing autoimmune response. To avoid both PD-1-mediated immunosuppression and the adverse effects of checkpoint blockade by antibodies, PD-1 gene disruption in CAR-T cells may be a promising approach. PD-1 KO improved antitumor efficacy of CAR-T in various blood and solid tumor mouse models have been demonstrated through enhancing effector function and survival of T cells in the TME [18,130].

Rupp et al [18] successfully developed a method for combined CRISPR/Cas9-based gene knockout and lentiviral transduction to generate PD-1 gene disrupted human CAR-T cells in one reaction. They routinely obtained a 50% plus success rate in PD-1 gene knockout in anti-CD19 CAR-T cells 48 h post-editing, as confirmed by flow cytometry. In this study, they initially found that the expression of the inhibitory receptor PD-L1 on tumor cells could significantly inhibit the activity of anti-CD19 CAR-T cells, resulting in impaired tumor clearance. This directly confirmed the necessity of removing the PD-1/PDL-1 interaction in CAR-T cells. They further demonstrated that the PD-1 disrupted CAR-T cells significantly enhanced anti-tumor efficacy *in vitro* and *in vivo* (tumor clearance rate of 17% for conventional anti-CD19 CAR-T cells increased to 100% in animals receiving PD-1 edited CAR-T cells at 28 days post tumor implant).

Furthermore, in the study by Hu B et al [17], in one single reaction they successfully used nucleofection to transfect plasmids encoding both CRISPR/Cas9 to disrupt the PD-1 gene and the *piggyBac* transposon system for expressing CD133-specific CAR, in human primary T cells. The efficiency of CRISPR/Cas9-mediated genome editing in CD133-specific CAR-T cells was validated by quantitative PCR and genome sequencing. It was found that between 89.5 and 95% (average 91.5%) of the PD-1 gene sites were disrupted. Their data showed that PD-1 disruption improved the *in vitro* cytotoxicity and the *in vivo* antitumor activity in an orthotopic mouse model of glioma. Importantly, no evident toxicity induced by PD-1-deficient CAR-T cells was observed, reaffirming the safety of PD-1 disrupted CD133-specific CAR-T cells, in addition to the enhanced anti-tumor clinical efficacy. However, cytokine (IFN-c, IL-2, TNF-a, and GM-CSF) secretion was not increased when compared with that of conventional CAR-T cells, making it difficult to explain the improved anti-tumor function of the PD-1 disrupted CAR-T cells.

In order to overcome the suppressive effect of PD-1 on CAR-T cells, Hu et al [24] applied CRISPR/Cas9 system-mediated editing to disrupt PD-1 gene locus in mesothelin-target CAR-T cells. As analyzed by flow cytometry, a 59.2 ± 9.0% (n = 6) reduction in the PD-1 expression was observed in the PD-1 knockout mesothelin-CAR-T cells when compared with the conventional Meso CAR-T cells. Although it had little effect on CAR-T cell proliferation, the reduction significantly

increased cytokine (IFN-γ and IL-2) formation, as measured by intracellular cytokine staining, compared with control CAR-T cells. Stronger *in vitro* cytotoxicity of PD-L1-expressing cancer cells was also evidenced in the PD-1 gene edited CAR-T cells. The *in vivo* anti-tumor effect of PD-1 disrupted CAR-T cells was studied in an orthotopic xenograft mouse model utilizing BT-549 cells stably expressing luciferase. The results demonstrated that PD-1 KO through CRISPR Cas9 enhanced the antitumor activity of CAR-T cells against triple-negative breast cancer (TNBC), presented as a solid tumor. In this model, PD-1 disruption was superior to PD-1 blocking antibody in improving the antitumor effect of CAR-T cells when combined together.

Using the CRISPR/Cas9 gene-editing system, Guo et al [120] disrupted PD-1 gene in the Glypican-3 (GPC3)-targeted CAR-T cells and explored the *in vitro* and *in vivo* antitumor efficacy of PD-1-deficient CAR-T cells against liver cancer, one of the most immunosuppressive cancer types. In their comprehensive study, the upregulation of PD-L1 expression was observed on the hepatocellular carcinoma (HCC) cells exposed to the GPC3-CAR-T cells. Subsequently, they demonstrated that disruption of PD-1 significantly enhanced anti-tumor efficacy of PD-1-deficient CAR-T cells against HCC. Mechanistic studies showed that PD-1 deficient GPC3-CAR-T cells have a greater ability to survive, indicating protection from exhaustion when combating native PD-L1-expressing HCC. Improved *in vivo* persistence and infiltration of PD-1 gene edited CAR-T cells was also observed in the xenograft tumor model. The study shed light on the potential of precision gene editing on the immune -checkpoints to augment the CAR-T cell HCC therapies.

Recently, Liu et al [131] developed new methods to carry out multiplex gene editing of *TRAC*, *B2M,* and *PD-1* in CAR-T cells based on the CRISPR-Cas9 system. In the PD-1 KO CART cells, the PD-1 expression on the CAR-T cell surface was significantly decreased while the proliferation and immune phenotype were not altered. The gene edited CAR-T cells demonstrated a stronger anti-tumor effect both *in vivo* and *in vitro*. Their studies illustrated, the advantage of using the CRISPR-Cas9 platform. Multiple genes could be knocked out simultaneously and, as such, enabling a multiplex gene editing technique readily applicable to CAR-T cells, resulting in a promising immunotherapy product for cancer treatment [111,131–133].

The advantage of combining PD-1 disruption with CAR-T cell therapy has been highlighted in studies described above. The results thus far have been very promising and have led to an increasing number of clinical studies. However, in some studies [134] PD-1 KO T cells presented some problems. For example, although PD-1 KO showed favorable enhanced short-term proliferation and cytotoxic effects, PD-1 gene edited T cell were susceptible to T cell exhaustion and lacked long-term durability. Therefore, further well-designed follow-up studies and clinical validation are needed to fully understand the therapeutic potential of PD-1 gene knockout or disruption [134].

5. Data from human clinical studies and ongoing clinical trials

Pre-clinical studies, as outlined above, have demonstrated that recent advances in gene editing technology has enable us to precisely and intrinsically modify the immune check-point PD-1 gene on the CAR-T cells to enhance antitumor activity. The strategy of using PD-1 KO CAR-T cells to treat cancer has clear advantages. It would prevent potential toxicity associated with systemic anti-PD-1 or anti-PD-L1 administration (e.g. opportunistic autoimmunity) while avoiding interfere with normal homeostatic functions of these ICP molecules within the body. The full potential of this approach would need to be explored and validated through rigorous clinical trials.

Although clinical trials evaluating the safety and efficacy of infusing autologous $CD4^+$ T cells with CCR5 gene KO in patients with chronic HIV infection commenced a decade ago (2009) (NCT00842634, NCT01044654), the first case of CRISPR gene editing tested in cancer patients did not begin until 2016 [135]. The first clinical trial of CRISPR/ Cas9 uses CRISPR/Cas9 mediated PD-1 KO T cells in patients with lung cancer [135]. Subsequently, similar clinical trials with PD-1-knockout autologous T cells are underway for prostate cancer (NCT02867345), bladder cancer (NCT02863913) and renal cell carcinoma (NCT02867332). Now clinical trials using PD-1 deficient CAR-T cells are being conducted for lung cancer (NCT03525782), Acute Lymphoblastic Leukemia (ALL), Burkitt Lymphoma (NCT03298828), oesophageal cancer (NCT03706326), prostate cancer (NCT03525652), refractory B cell malignancies (ChiCTR1800020306), and various solid tumors (NCT03747965, NCT03545815). The safety and efficacy of PD-1 KO modified T cells and PD-1 KO CAR-T cells in the treatment of cancer patients are being actively evaluated.

All clinical trials registered in Clinicaltrial.gov and www.Chictr.org.cn, using PD-1 knockout primary T cells and CAR-T cells for cancer treatment are summarized in Table 1.

At present, most of the above clinical trials have not reported their findings to-date. Among all of these registered trials, only three of them have reported preliminary findings. Jin et al [136] investigated the safety and activity of PD-1 KO primary T cells in patients with advanced esophageal squamous cell carcinoma (ESCC, n = 21). After PD-1 was knocked out by CRISPR)/Cas9 system, T lymphocytes were expanded and reinfused back to the patients. In this study, they found that the most common adverse events were transient fever (7 patients, the highest was 39.1℃) and chills (3 patients) and moderate skin rash (1 patient), among the 21 treated ESCC patients, with no grade 3 or 4 adverse events observed. Only limited efficacy data was made available. Disease control rate and median overall survival was reported to be 35% (6/17) and 127 days, respectively. They also showed that the PD-1 knockout T cells infiltrated into and persisted for a durable time in ESCC that responded to therapy. Lu and colleagues [116] conducted the first-in-man clinical trial (NCT02793856) to assess the safety of CRISPR/Cas9-mediated knockout of PD-1 gene in autologous T lymphocytes in patients with advanced non-small cell lung cancer (NSCLC). They used an escalating dosage scheme on 11 patients. From the data collected from 8 patients, who received 16 cycles of PD-1 KO T cells infusions, they found that the most common adverse effects were acute fever and hepatic dysfunction (both 15.4%). No DLT and 3–5 grade adverse effects were noticed, again reassuring the safety of the PD-1 KO T cells in patients. Using the next generation sequencing, they observed evidence indicating the existence of potential responsive T cell clones in patient peripheral bloods during the cell therapy. Our updated observations from 8 enrolled patients with advanced NSCLC treated with PD-1 deficient MUC1 targeted CAR-T cells, showed low rates of adverse events during the MUC1-CAR + /PD-1-KO therapy, reported at the 2018 ESMO Immuno-oncology Congress. No grade 3–5 adverse effects were observed, indicating excellent tolerability of the PD-1 KO product. Preliminary data supported a moderate treatment response in patients during the low dose regimen. Complete efficacy data are still being collected.

All these available human clinical data currently support that cancer treatment with PD-1 KO T cells or PD-1 deficient CAR-T cells are safe, with no major safety concerns detailed to-date. Further clinical studies are required to ascertain the safety and efficacy of the CRISPR/Cas9-mediated PD-1 KO in primary T cells and CAR-T cells, as no safety profile can be estimated using computer programs with clinical trials. We await updated results from more clinical studies.

6. Challenges

While the advantages and importance for combinational PD-1 disruption and CAR-T cells are showing promise, there are still many technological challenges for PD-1 disrupted CAR-T cells, especially for solid tumors where the TME is unfavorable for conventional CAR-T cell therapy. Both CAR-T cells for the treatment of solid tumor and CRISPR/Cas9 for genome editing for primary T cell are still in their infancy.

6.1. Challenges in CAR-T cell design and therapy

Effective CAR-T cell-based therapy need to address five major classes of functional challenges [14]. Firstly, trafficking to the site of the tumor is a significant issue for solid tumors treatment. It appears that relatively little work has been done to design strategies for improving trafficking. Secondly, tumor target recognition of CAR-T cells is critical for the killing of tumor cells. Finding more efficient targets or combined targets are needed address this issue. Thirdly, the proliferation and persistence of CAR-T cells influence their clinic efficacy. Thus, increase of proliferation of healthy CAR-T cells is a major focus area of current research. Fourthly, overcoming the TME since many solid tumors have an immunosuppressive TME. Previous work has validated that the immunosuppressive TME is an important factor limiting the wider applications of CAR-T cell therapy. PD-1 deficient CAR-T is an effective strategy to overcome the suppressive TME. Finally, the stringency of the manufacture of the CAR-T cell product is critical for this type of personalized treatment. Uncontrolled CAR-T cell response has typically been shown to lead to severe toxicity and adverse side effects [137]. A recent report tracked a patient's resistance to CAR-T therapy and subsequent relapse to a single anti-CD19 CAR genetically engineered leukemic B-cell introduced during T cell production [138]. However, this incidence is believed to be a rare event highlighting the need for stringent Good Manufacturing Practice (GMP). In the case of CAR-T cell preparation for solid tumors the incidence of this type of error is hardly applicable.

6.2. Technical challenges of CRISPR-Cas9 to clinical translation

Genome editing with CRISPR-Cas9 holds immense therapeutic potential for improving T cell-based immunotherapy. However, T cell therapy based on genome editing, still faces some challenges for complete clinical applications [139,140]. The issue of priority is the safety of genetically engineered T cells. In view of this, some studies have attempted to increase the specificity of gene editing with minimal off-target effects, but the degree of accuracy still needs to be determined for specific clinical applications. Another challenge is the fitness of edited cells and the therapeutic threshold of editing. If the edited cells possess a greater capability to proliferate or show greater adaptability than the unedited cells, this will facilitate the edited product to reach the therapeutic threshold required for successful treatment outcomes. In addition, it is not clear how the autoimmune system will response to genetically engineered cells. Extensive research and encouraging results from the use of universal CAR-T therapy in clinical applications have shown that T cell therapy with CRISPR-Cas9 technology will increase the efficacy, with a broader spectrum of patient-targeted treatment. Therefore, although with some challenges, we envision continued advancement of CRISPR-Cas9 technology will lead to more specific cancer therapeutic applications in the future.

In the past, effective genome engineering of primary T cell was unachievable due to low transfection efficiencies. But recent advances in electroporation tools as well as T cell activation methods allow highly efficient gene editing in primary T cells. However, there are still challenges remaining in current T cell engineering methodology. First, CRISPR/ Cas9-mediated gene-editing can induce apoptosis and growth retardation in engineered T cells, partially attributable to p53-mediated DNA damage and type I interferon responses [130,141,142]. Although PD-1 KO showed favorable enhanced short-term proliferation and cytotoxic effects, PD-1 gene edited T cell were still shown to be susceptible to T cell exhaustion and lacked long-term durability. Therefore, further well-designed follow-up studies and clinical validation are needed to fully understand the therapeutic potential of PD-1 gene knockout or disruption [134].

6.3. CAR-T/PD-1 cell trafficking into solid tumors

The TME provides a particularly hostile suppressive environment for the immune response team, by providing a physical barrier and secreting suppressive cytokines, reviewed in [143]. A number of strategies have been designed to overcome the major hurdle of infiltration and survival of CAR-T cells in solid tumors, from injection of CAR-T cells directly into the tumor site to engineering the T cells to stimulate anti-tumor properties, reviewed in [144]. To enhance infiltration into the solid tumor one approach was to engineer a heparinase enzyme into the CAR-T cell enabling the degradation of the rich stromal- extracellular matrix of the tumor [145]. As newly formed vasculature is a significant feature of solid tumors, CAR-T cell targeting or 'homing-in' on receptors on newly formed blood vessels, such as the vascular endothelial growth factor receptor-2 (VEGFR2) [146], integrin alpha v beta 3 [147], or perturbation of vascular endothelin-B-receptor (ET_BR - decrease) and endothelin-A-receptor (ER_AR - increase) [148], have demonstrated promising results in pre-clinical experimentation. Importantly, using checkpoint inhibitors, like PD-1 mAbs and PD-1 disruption helps T cell activation and tumor death, with even greater efficacy when combined with enhancer effector molecules such as the A2AR antagonist (blocking the adenosine immunosuppressive pathway), granzyme B and interferons, reviewed in [144]. The highly metabolic, hypoxic, conditions of the solid tumor also contributes to the unfriendly immune unresponsiveness of CAR-T cell therapies. The dependence on glycolysis by the highly proliferative tumor cells leads to excess production of lactic acid reducing the availability of essential nutrients (glucose and amino acids) [149,150]. The accumulation of metabolic waste by-products from the cancer cell are detrimental to T cell function [151], thus potentially impairing CAR-T cell therapeutic action. PD-L1 expression on the tumor cell not only acts as a handshake to evade T cell recognition, it is involved in many activities to protect the cancer cell from immune attack and destruction including PD-L1 upregulation of glycolysis genes and increased glycolysis [152]. Tentatively, by disrupting the PD-1/PD-L1 signaling there is a distinct possibility of change of PD-L1 metabolic function. Linking CAR-T/PD-1 cell immune-therapy with metabolic reprogramming of the cancer cells targets two of the major hallmarks of cancer (disruption of the immune system and metabolism).

Poor infiltration of T-cells into solid tumors has been a major stumbling block for CAR-T cells therapies. Recent development in this area comes from the preclinical use of a penetrating peptide iRGD in synergy with PD-1/KO to provide a superior therapeutic outcome, as evaluated recently for gastric cancers [153].

6.4. Controlling potential enhanced auto-immune response

Continuous development of CAR-T, checkpoint inhibition technology is paramount to addressing many concerns around efficacy, safety of use and ethical concerns raised. The CAR-T cells can multiply in the host and, as reported by Kenderian's group, they have the capacity to differentiate into memory cells remaining up to 4 years in some patients [154]. This has a double-edged effect, the engineered CAR/PD-1KO T-cells may have a long-term positive effect in combating specific tumor cells, conversely, presence of these manufactured T-cells may promote or enhance an auto-immune response. To overcome some of the problems of short- and long- term, known and unknown adverse side effects of CAR-T-cell therapy is the introduction of suicide genes. Manufacture of CAR-T cells with 'off-switches' can limit the time span of CAR-T-cell survival and also reduce the severity of side effects such as CRS. To this effect, technology such as precision engineering of the T cells by the insertion of inducible suicide genes such as caspase9 (iC9) [155] or co-expression of CAR-T and suicide genes [156] are under development.

To combat the problem of on- or off- target effects, these can potentially be minimized by simultaneously using two tandem CAR tumor

antigens, or one tumor antigen one normal antigen which inhibits the activity of the tumor antigen, described in [30]. The possibility of unwanted mutations through CRISPR integration is also being addressed through designed RNA-guided recombinase-Cas9 fusion technology to target genomic DNA deletion and integration and bypass the host T-cell endogenous DNA repair mechanism [157].

6.5. Reducing manufacturing costs and broader application

Major challenges of translating CAR-T-based therapies into the clinic are the time of manufacture and the high costs involved. Currently, the individualization of CAR-T treatment and the cost of biomanufacturing technology of CAR-T/PD-1 is prohibitive for large scale clinical application. In recent years, the biomanufacturing technology (such as manufacturing process automation) of CAR-T based therapeutic products has been significantly shortened from two weeks to one day [158,159]. This technological advancement provides a greater window of survival opportunity, offering rapid more cost-effective treatment to patients with advanced cancer. Individual patient expansion of autologous CAR/PD-1KO T-cells are expensive to manufacture and demands high quality control (GMP facilities) and are time consuming. Also, to reduce cost and increase manufacturing productivity, alternative simple and affordable alternatives for large scale CAR-T manufacture using non-viral gene transfer methods such as the Sleeping Beauty [30,160–162] and PiggyBac transposon/transposase [163] are under consideration.

6.6. Using allogeneic ‘off the shelf” CAR-T/PD-1KO cells

The use of allogeneic, universal CAR-T ‘off-the-shelf’ based therapies are also being actively researched, although the safety of using donor (graft) versus host (GVHD) T cells is challenging, reviewed in [164]. *In vitro* and *in vivo* experimentation of allogeneic CAR-T PD-1 cells deficient in PD-1, human leukocyte antigen (HLA) class 1 molecule and TCR (using multiplex genome editing) to reduce alloreactivity (the risk of immune rejection by the host or GVHD) may be an alternative 'off-the-shelf' strategy to reduce costs, time of production-to-clinical application [133]. Also, the combination of TCR and CD52, down-regulation of CD3/TCRαβ, has demonstrated promise in CAR-T cell production [165]. To increase flexibility in antigen recognition a split universal and programmable (SUPRA) CAR system has been developed, which incorporates a dissociable antigen recognition motif targeting multiple antigens [165]. More recently, Lee and colleagues recently published a method to expand clinical-grade allogeneic T cells to be used as an ‘off-the-shelf” ACT to target different cancer types [166]. In this report, these ‘of-the-shelf’ engineered cells in pre-clinical models demonstrate no apparent off-tumor toxicity, does not induce host-versus-graft reaction and can be cryopreserved, ready for use.

6.7. Ethical concerns with CAR-T and PD-1 therapy

There are a few generic ethical concerns for genetically manipulated gene-transfer technology still to be resolved [167]. To-date, uncertainties still exist as to how exactly the autoimmune system will respond to the genetically engineered CAR-T cells, as mentioned above. The most conspicuous ethical challenge facing clinical application of genetic-based therapies, such as PD-1 KO/CAR-T cell, is the long- and short- term associated risks for the patient, which are currently too early to assess due to newness of the technology [167]. Evaluation of the present risk is questionable, mainly because the early dramatic efficacy of CAR-T cell treatments has not been fully assessed against safety risks. Increased quality of life in patients receiving CAR-T therapy with no other viable treatment options often delays relapse onset with development of resistant cancer cells within 6 months of treatment [168].

Ethical concerns on the credibility of gene-transfer has also been raised in the context of conflict of interest between the pharmaceutical companies, research institutes and investors [169]. Further, the issue of publication bias has been raised questioning the credibility of pre-clinical studies used as evidence for the basic move from laboratory into the first in-human Phase 1 gene editing cancer trials [169]. In summary, the advantages of immune-based therapies for advanced cancers, where little hope exists, out-ways the currently known adverse effects. Reference to acute pharmacovigilance to avert immediate acute toxicities, and undefined long-term impacts continues to be important for the assessment of clinical outcomes of the new treatments.

7. Concluding remarks and future direction

It is exciting to witness the speedy advancement of CAR-T cell therapies, ICI therapies and gene editing technology such as CRISPR-Cas9 in combination therapies to combat solid tumor as well as hematological-based tumors. Combining PD-1 blockade and CART into a single therapy is a promising strategy and has the potential to be a more effective treatment for solid tumors. Although it is in its infancy, considerable pre-clinical evidence supports that PD-1 gene disrupted CAR-T cells significantly improves the antitumor activity and safety profile, while the features of T cell proliferation and subsets are retained. The most encouraging phenomena is a significant enhancement in the *in vitro* and *in vivo* antitumor activities of CAR-T cells with disrupted PD-1 gene, which have been consistently observed in all pre-clinical studies. The enhanced antitumor activity is closely associated with the interrupted PD-1 expression on CAR-T cells, reaffirming the importance of blocking PD-1 axis using this new approach. Other mechanism underlying the enhanced antitumor property of PD-1 deficient CAR-T cells may be related to increased secretion of cytokines such as IFN-γ and IL-2 (Fig. 2). Despite some technical challenges and some ethical concerns, as discussed, a number of clinical trials are under way using PD-1 knockout CAR-T cells to treat various solid tumors and refractory B cell malignancies. Promisingly, the use of PD-1 knockout/disrupted CAR-T cells in the treatment of cancer does not appear to increase the toxicity profile as observed in the combination therapy using PD-1 blocking mAbs and CAR-T cells. All available data to-date supports that combination of CAR-T cells with the disruption of endogenous inhibitory immune checkpoint PD-1 by CRISPR-Cas9 technology represents a promising immunotherapeutic modality for cancer treatment. The full clinical potential of this new approach for the treatment of solid tumor is too early to appreciate until more clinical trials are completed and data assessed.

The strategy of using PD-1 gene knockout/disruption CAR-T cells to treat solid tumors mainly relies on CAR-T cell, ICI and CRISPR-Cas9 technology. Future in-depth investigation into these three areas would promote the development of intrinsic PD-1 blockade incorporation into CAR-T cells as a single therapy. The strategy of blocking PD-1 by disrupting the gene within the T cells of the patient has to date been demonstrated to be safe with a decrease in the immune-related adverse effects associated with systemic checkpoint blockade [34]. Indeed, all available pre-clinical and clinical data confirm that the safety profiles of PD-1 KO are promising, providing a solid foundation to move this approach forward. Future direction, we envisage continued advancement of a more user friendly, precision CRISPR-Cas9 engineered technology playing an important role in ADT cell cancer immunotherapy in the near future, despite presenting some challenges [140]. New breakthrough in CAR-T cell therapy research such as discovery of novel targets will evolve and advance CAR-T technology. For example, very recently Du and colleagues developed CAR-T cells targeting B7-H3. The anti-tumor activity (both *in vitro* and *in vivo*) of the new anti-B7-H3/CAR-T cells is significantly enhanced without detectable side effects [170]. With optimal CAR targets, the anti-tumor activity of CAR-T cell combined with intrinsic PD-1 inhibition would further enhance treatment efficacy. Barriers posed by the TME are slowly being removed, CAR-T/PD-1 cells engineered with inbuilt safety mechanisms (suicide

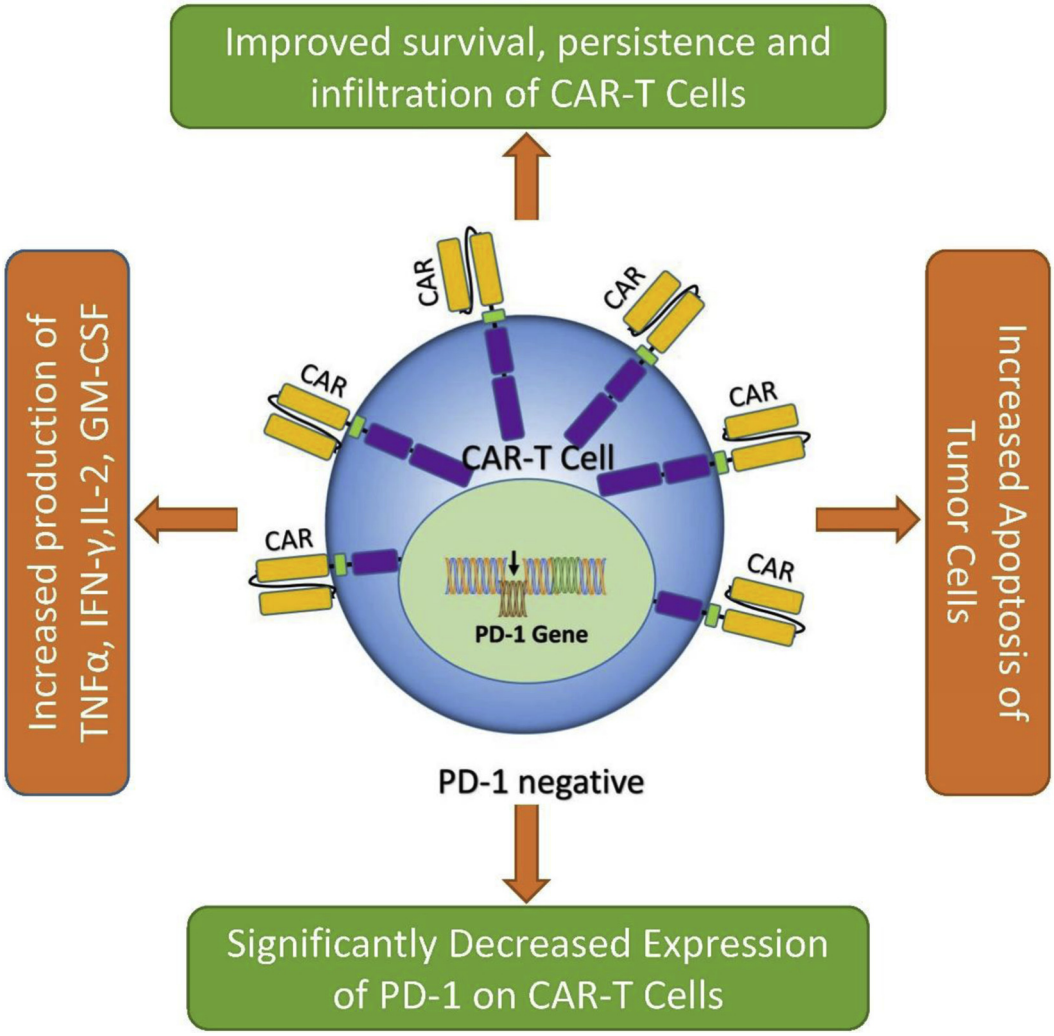

Fig. 2. Mechanisms underlying enhanced antitumor activity of PD-1 disrupted CAR-T cells. All pre-clinical studies have shown that CRISPR-Cas9 system mediated disruption of PD-1 in CAR-T cells prevent PD-1 expression and significantly improve the anti-tumor efficacy of CAR-T cells. Detailed mechanisms are not fully understood, but possible mechanisms of action are presented in this diagram.

genes), improved specificity, reduced side effects, and improved efficacy are also steps forward for solid tumor eradication.

Lastly, further understanding of solid tumor biology within the TME, together with speedy genome editing technological advances in PD-1 disrupted CAR-T cell biology to overcome multiple tumor eradication barriers, will lead to enhanced antitumor efficacy of immune-based therapies. The biomanufacturing technology (such as manufacturing process automation) of CAR-T based therapeutic products is evolving to meet the demanding clinical needs. The manufacturing time of CAR-T cell products can be significantly shortened to one day. We await the development of next generation allogeneic CAR /PD-1KO T cells as a safe, personalized and economical therapy for all solid tumors.

Data availability

The data used to support the findings of this study are included within the article.

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Declaration of Competing Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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