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Review Article

Fueling chimeric antigen receptor T cells with cytokines

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Abstract: Chimeric antigen receptor (CAR)-T therapy started a new era of tumor treatment, especially for hematological malignancies. However, many challenges remain, including low-level proliferation and short-term persistence, insufficient CAR T-cell trafficking, suppressive tumor microenvironment (TME), frequent adverse events and the unaffordable manufacturing process. Cytokines are pleiotropic hormones involved in multiple processes of immunity, including activation, expansion, differentiation, and migration of immune cells. Both pre-clinical models and clinical trials showed that armoring CAR-T cells with cytokines strengthened the anti-tumor responses of CAR T cells. This review looked into the key role of cytokines as a promoter of anti-tumor activities of CAR-T cells and consequently a facilitator of clinical translation, mainly, from cytokines of the common γ-chains family, chemokines and chemokine receptors, immunosuppressive molecules and pro-inflammatory cytokines.

Keywords: Chimeric antigen receptor T cells, cytokines, persistence, tumor microenvironment, endogenous immunity

Introduction

Immunotherapy represents an unprecedented breakthrough in the field of cancer treatment [1], and treatment with anti-CD19 chimeric antigen receptor (CAR)-T cells achieved over 90% complete remission [2-6]. However, many challenges remain, including ineffectiveness in solid tumors due to poor expansion, insufficient trafficking and immunosuppressive microenvironment [7], high recurrence rates [5, 8] and frequent adverse events [9], as well as staggering treatment cost [10], etc. Additional strategies are urgently required to optimize CAR-T therapy, including expanding its application, attaining longer-term efficacy and reducing side effects.

Cytokines are pleiotropic hormones, including interleukins (ILs), tumor necrosis factors (TNFs), interferons (IFNs), chemokines, colony-stimulating factors (CSFs), growth factors, among others. They are involved in the activation, proliferation, differentiation, and survival of various immune cells [11]. Due to their ability to regulate immune functions, combine use of CAR-T cells and cytokines could achieve a synergistic effect, which has shown great promise for cancer treatment. This review looked into the application of cytokines in CAR-T treatment from four aspects, i.e., cytokines of the common γ-chains family, chemokines and chemokine receptors, immunosuppressive molecules and pro-inflammatory cytokines.

Cytokines of the common γ-chains family

Proliferation, survival and persistence are the premises for CAR T-cell cytotoxic function to achieve cancer clearance and durable remission. Multiple clinical trials demonstrated that robust expansion [8] and long-time persistence [12] of CAR-T cells resulted in enhanced clinical responses and prolonged survival, while poor *in vivo* proliferation was closely correlated with relapse after transient remissions [13]. Here, we principally discuss the role of common γ-chains (γc)-sharing cytokines in proliferation, survival and persistence of CAR-T cells, *ex vivo* and *in vivo* (**Table 1**).

Ex vivo culture and exogenous administration

Interleukin (IL)-2 is most frequently used for *in vitro* T-cell expansion because of its pivotal role

Table 1. Summary of improving CAR-T efficacy through cytokines of the common γ-chain family

Related cytokines	Application	Mechanism	Diseases	Clinical trials	References
IL-2	Ex vivo culture and administration	Promote T cells expansion	B cell malignancies	NCT00924326	[16-19]
		Promote Treg cells expansion	Ovarian Cancer	NCT00019136	
		AICD	Metastatic Melanoma	NCT04119024	
				NCT03098355	
IL-15, IL-7, IL-21	Ex vivo culture	Preserve memory-enriched phenotype	B cell malignancies	NCT02992834	[21-24]
				NCT02652910	
				NCT04186520	
IL-15	Co-expression	Promote T cells expansion and persistence	B cell malignancies	NCT03721068	[28-31]
		Preserve Tscm phenotype	Glioblastoma		
			Neuroblastoma		
	MbIL15	Preserve Tscm phenotype	B cell malignancies	NCT03579888	[32]
		Continuously activate JAK/STAT5 pathway			
IL-7	IL-7Rα (with exogenous IL-7)	Selective promote CAR-T proliferation	EBV+ lymphoblastoma	-	[34, 35]
			Neuroblastoma		
	C7R	Continuously activate JAK/STAT5 pathway	Metastatic neuroblastoma	NCT04099797	[36, 37]
		Independent on soluble cytokines	Orthotopic glioblastoma	NCT03635632	
			Triple-negative breast cancer		
IL-23	IL-12β p40 subunit (p40-Td cells)	Induce Th17 phenotype	Neuroblastoma	-	[40]
		Autocrine mechanism	Pancreatic cancer		
γc cytokines	28-ΔIL2RBz (YXXQ) CAR-T	Activate JAK/STAT3 and STAT5	B cell malignancies	-	[38]
		In an antigen-dependent manner			

Clinical trial-related information were mainly collected from ClinicalTrials.gov. Abbreviations: CAR, chimeric antigen receptor; IL, interleukin; Treg, regulatory T cells; AICD, activation-induced cell death; Tscm, stem cells memory T; MbIL15, membrane-bound IL-15; EBV, Epstein-Barr virus; JAK/STAT5, Janus kinase/signal transducer and activator of transcription 5; C7R, constitutively active IL-7 cytokine receptor; Th, helper T cells.

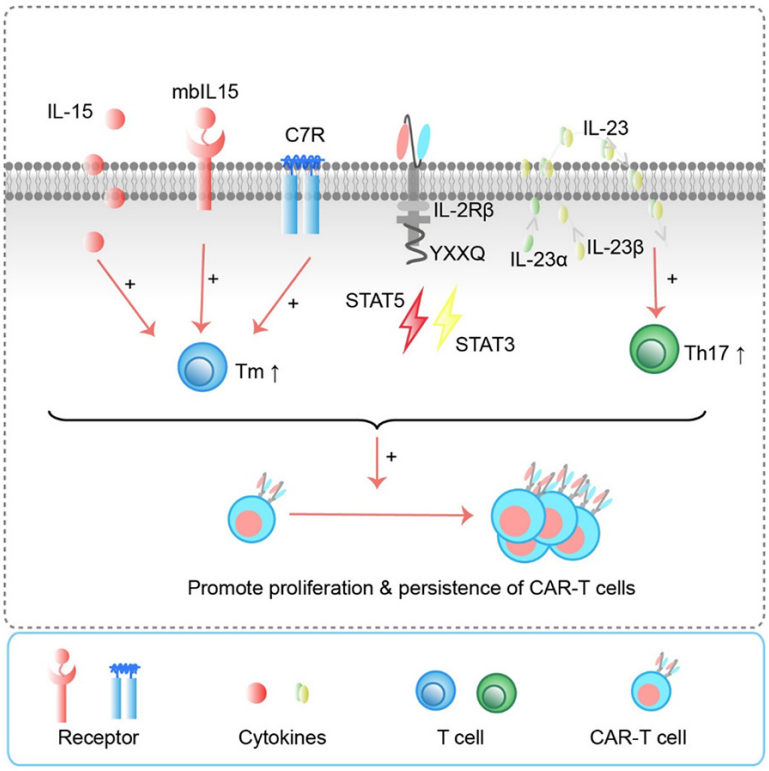

Figure 1. Mechanisms to improve efficacy of CAR-T therapy through cytokines of the common γ-chain family. CAR-T cells co-expressing γc-sharing cytokines (IL-7, IL-15), or directly activating related STAT3 and/or STAT5 pathways (mbIL15, C7R, γc cytokines), exhibit enhanced expansion and persistence. IL-23 does not belong to the common γc family, but it also promotes the proliferation and persistence of lymphocytes through activating STAT3 pathway.

in lymphocyte growth and differentiation, and it is known to promote the expansion of cytotoxic T lymphocytes (CTLs) and the development of terminally-differentiated effector T (Te) cells. However, researches indicated that IL-2 simultaneously elicited clonal growth of regulatory T cells (Treg) [14] and the excessive levels of IL-2 induce apoptosis of Te cells, dubbed activation-induced cell death (AICD) [15]. In addition, a study demonstrated that lower levels of IL-2 worked better on T-cell expansion [16].

Moreover, exogenous administration of cytokines for functional modulation of CAR-T cells also improves the efficacy of immunotherapy [17]. IL-2, as the only γc cytokines approved by FDA, has been extensively investigated in combination with CAR-T therapy for intravenous or subcutaneous administration to treat cancers in initial clinical trials (NCT00924326, NCT00019136, NCT04119024, NCT03098355), and was found to able to promote the expansion of adoptive immune cells *in vivo* [18]. However, the high-dose IL-2 might have systemic toxicity [19]. Subsequently, studies suggested that appropriately reducing the dose of IL-2 [16] and intermittent administration [20] could balance the anti-tumor effects and systemic toxicity.

In fact, apart from IL-2, other cytokines of the common γc family also exhibited essential and distinct functions related to the proliferation, differentiation and persistence of T cells [14]. IL-7, IL-15 and IL-21 facilitated the production of "younger" memory-like lymphocytes such as central memory T (Tcm) cells and stem cells memory T (Tscm) cells, which persisted longer and possessed durable anti-tumor activities *in vivo* [21-25]. What's more, IL-15-mediated CAR-T cells reduced the activation of mTORC1, which held more advantages in survival in the TME [26]. Many pertinent clinical trials aimed to compare IL-2 pre-treated CAR-T cells and IL-7/IL-15 pre-treated CAR-T cells in terms of the safety, efficacy and duration of responses in patients with relapsed and refractory (r/r) CD19+ B cell lymphoma have been registered (NCT02992834, NCT02652910, NCT04186520).

Co-expression in CAR-T cells

In addition to expanding CAR-T cells with supplementing cytokines, expressing favorable cytokines on CAR-T cells also improved proliferation and persistence *in vivo*, and induced less systemic toxicities compared with exogenous administration (**Figure 1**).

IL-15: Unlike IL-2, activated T cells cannot produce IL-15 [27], which prompted researchers to treat tumors by generating CAR-T cells armed with IL-15. Compared with traditional adoptive therapy, IL-15-secreting CAR-T cells could down-regulate the expression of programmed cell death protein 1 (PD1) and achieved 3- to

15-fold more expansion [28], with a higher proportion of Tscm cells in B-cell malignancies, glioblastoma [29] and neuroblastoma [30]. Subsequently, the clinical trial on IL-15-secreting CAR-T cells for r/r neuroblastoma has been registered (NCT03721068). Another study showed that CAR-T cells introduced both IL-15 and IL-21 rather than either of them was capable of enhancing proliferation with more Tscm and Tcm cells populations in hepatocellular carcinoma (HCC) [31].

Membrane-bound IL-15 (mbIL15), in which, as its name implies, IL-15 is directly bound with IL-15Rα, was able to continuously phosphorylate the signal transducer and activator of transcription (STAT) 5 pathway and yielded pro-survival signals all times. The novel CAR-T cells co-expressing mbIL15 developed long-term persistence independent of CAR signaling [32]. Based on this, Partow Kebriaei *et al* initiated a clinical research (NCT03579888) to ascertain the efficacy and safety of CAR-T cells incorporating mbIL15.

IL-7: IL-7 is also a decisive lymphocyte survival factor [14]. Different from IL-2, Treg cells have no IL-7Rα [33]. Thus, exogenously administered IL-7 could selectively stimulate CAR-T cell proliferation even in the context of fully functional Tregs [34, 35]. Thomas Shum *et al* proposed a novel constitutively active IL-7 cytokine receptor (C7R) [36], which significantly activated STAT5 through IL-7Rα homodimerization independent of cytokine administration or secretion. In many xenograft models [36, 37], less doses of C7R.CAR-T cells persisted longer and exerted more remarkable anticancer properties compared with conventional CAR-T therapy. Subsequently, the same team has also applied for initiation of new clinical trials (NCT04099797, NCT03635632).

γc cytokines: Common γc-sharing cytokines play a critical role in T-cell growth. IL-2, IL-7 and IL-15 tend to induce STAT5 activation, whereas IL-21 preferentially activates STAT3 to stimulate T-cell proliferation [14]. A study innovatively incorporated a truncated IL-2R β-chain (IL-2Rβ) as well as a STAT3 binding motif (YXXQ) into CD19 CAR-T cells. This new-generation CAR-T cells could activate both Janus kinase (JAK)/STAT3 and STAT5 when antigen-stimulated rather than constitutively activated, which had superior expansion, persistence, anti-tumor activities and less side effects such as systemic toxicity [38].

IL-23: IL-23 does not belong to the common γc family, but it also promotes the proliferation and persistence of lymphocytes through activating STAT3 pathway [39]. The IL-23 is composed of two subunits: p19 and p40 [39]. Of interest, T cells increased the expression of p19 subunit without the p40 subunit after TCR engagement. On the basis of this discovery, Xingcong Ma *et al* generated a new CAR-T structure expressing the p40 subunit so that it could form IL-23 upon stimulation, thus facilitating expansion and persistence via autocrine IL-23 signaling without bystander effects in neuroblastoma and pancreatic cancer models [40].

Chemokines and chemokine receptors

To exert their cytotoxic effects, immune cells need to localize at tumor sites. Also, a high density of tumor-infiltrating lymphocytes (TILs) is strongly related to a favorable prognosis of most carcinomas [41]. However, chemokines secreted by tumor cells in the TME "mismatch" the endogenous chemokine receptors expressed on T cells, which poses a dominant obstacle of localization at tumor sites [42]. Therefore, co-expressing appropriate chemokine receptors on CAR-T cells and regulating chemokines secreted by tumors promote the trafficking and infiltration of adoptive immune cells into the tumor bed, thereby enhancing anti-tumor effects (**Figure 2**; **Table 2**).

CCR4/CCR2b

Different tumor malignancies possess distinctly different chemokine profiles. Reed-Stemberg cells in Hodgkin lymphoma (HL) tend to secrete C-C chemokine ligand (CCL) 17 and CCL22, recruiting C-C chemokine receptor (CCR) 4-positive Th2 and Treg cells to form immunosuppressive TME [43, 44]. CD30 CAR-T cells incorporating CCR4 could enhance migration to HL cells, exerting robust anti-tumor effects [45]. Later, clinical trials were initiated to ascertain the effectiveness of CCR4.CAR-T cells in cancer patients (NCT03602157, NCT04153799). Similarly, in neuroblastoma [46] and mesotheliomas [47], CAR-T cells transferring CCR2b also drastically increased TILs by 12.5-fold with enhanced cytotoxic activity.

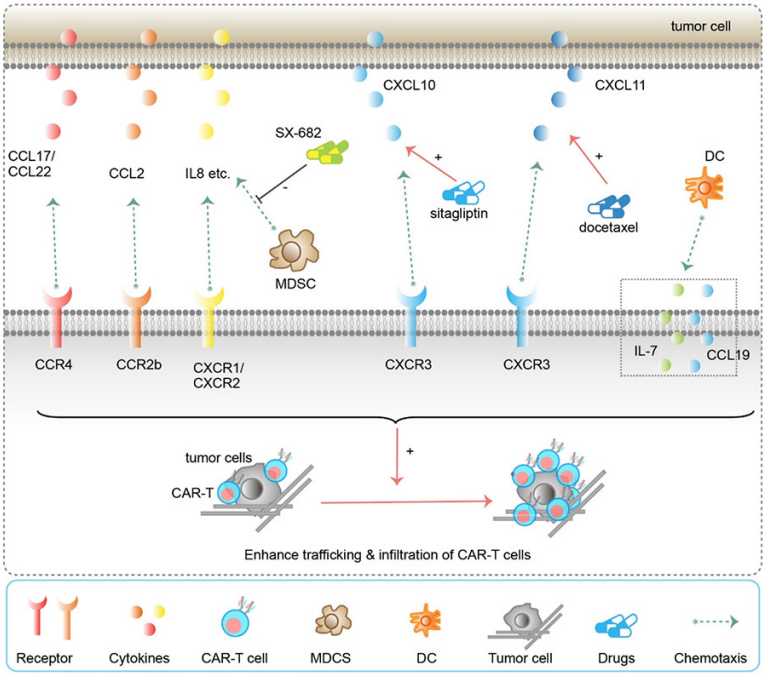

Figure 2. Workflow of using chemokines and chemokine receptors to facilitate CAR T-cell migration and infiltration. On the basis of the chemokines secreted by tumor cells, CAR-T cells are armored with "matched" cytokine receptors including CCR4, CCR2b, CXCR1 and CXCR2 to enhance migration to the tumor bed. Besides, drugs such as SX-682 is able to block the trafficking of CXCR2+ MDSC by downregulating the expression of CXCR1 and CXCR2. Other drugs such as sitagliptin and docetaxel can increase the secretion of CXCL10 and CXCL11 in the TME, respectively, and combine use with CAR-T therapy also dramatically improve the density of TILs. Additionally, CCL19-secreting CAR-T cells can recruit adoptive and endogenous immune cells (T cells and DCs), and further enhance the anti-tumor effects of CAR-T therapy together with aforementioned IL-7.

CXCR1/CXCR2

Recently, researchers took exceptional interest in C-X-C chemokine receptor (CXCR) 1 and CXCR2, whose ligands are predominantly expressed by various tumors to shape hostile TME. Engineering CAR-T cells with CXCR1 or CXCR2 remarkably augmented tumor infiltration. Moreover, this novel CAR-T cells elicited sustained tumor recession and long-lasting immunologic memory in various tumor models such as glioblastoma, ovarian and pancreatic cancers, even in advanced cancers [48, 49]. Similarly, SX-682, the inhibitor of CXCR1 and CXCR2, could block the trafficking of CXCR2+ myeloid-derived suppressor cells (MDSC), subsequently enhancing the infiltration of adoptive and endogenous lymphocytes, producing much stronger anti-tumor responses in HCC [50].

CXCL10/CXCL11

In addition to working on chemokine receptors, fine-tuning ligands expressed by tumors is also a theoretically sensible approach. *In vivo*, C-X-C chemokine ligand (CXCL) 10, CXCL11 and their receptor CXCR3 are vital chemokine pairs that modulate endogenous lymphocyte migration. Anne-Marie Lambeir *et al* demonstrated that dipeptidyl peptidase IV (DPP4 or CD26) is capable of truncating CXCL10 to impair its function [51]. In fact, DPP4 inhibitor, Sitagliptin, coupled with immunotherapy, could enhance the migration of endogenous and adoptive lymphocytes, and producing synergistic anti-tumor effects in animal models [52]. Similarly, docetaxel increases the secretion of CXCL11 through releasing HMGB1, thereby improving the infiltration of endogenous lymphocytes and CAR-T cells and strengthening anti-tumor responses in patients with lung cancer [53].

IL-7 and CCL19

Previous studies demonstrated that T zone fibroblastic reticular cells are implicated in T-cell homeostasis by secreting IL-7 and CCL19 [54, 55]. Keishi Adachi *et al* developed novel CAR-T cells incorporating IL-7 and CCL19 (17X19 CAR-T) to mimic T-zone reticular cells. IL-7 downregulated PD1 expression, promoting CAR T-cell survival and persistence. Besides, CCL19-CCR7 interaction increased tumor infiltration of adoptive and endogenous immune cells (T cells and DCs) [55], thereby improving anti-tumor effects. In the different tumor-bearing mice, 7X19 CAR-T therapies all elicited complete tumor regression and prolonged survival of mice without tumor relapse. Moreover, the 1/4 dose of 7X19 CAR-T cells produced greater or at least comparable anti-tumor efficacy than traditional CAR-T therapy [56]. Numerous cen-

Table 2. Summary of using chemokines and chemokine receptors to facilitate CAR T-cell migration and infiltration

Related cytokines	Application	Mechanism	Diseases	Clinical trials	References
CCL17/CCL22/CCR4	Co-express CCR4	Increase T-cell trafficking and infiltration	Hodgkin lymphoma	NCT03602157	[45]
CCL2/CCR2b	Co-express CCR2b	Increase T-cell trafficking and infiltration	Malignant pleural mesotheliomas	-	[46, 47]
			Neuroblastoma		
CXCL13/CXCR5	Co-express CXCR5	Increase T-cell trafficking and infiltration	Non-small cell lung cancer	NCT04153799	-
Ligands/CXCR1/CXCR2	Co-express CXCR1 or CXCR2	Increase T-cell trafficking and infiltration	Glioblastoma	-	[48, 49]
		Produce long-lasting immunologic memory	Ovarian cancer		
			Pancreatic cancer		
			Hepatocellular carcinoma		
	Inhibitor SX-682	Block trafficking of CXCR2+ MDSC		-	[50]
CXCL10/CXCR3	Inhibitor Sitagliptin	Preserve CXCL10	Hepatocellular carcinoma	NCT02650427	[52]
		Enhance migration of lymphocytes			
CXCL11	Docetaxel	Increase the secretion of CXCL11	Non-small cell lung cancer	-	[53]
		Improve infiltration of lymphocytes			
IL-7+CCL19	Co-expression	Enhance T-cell proliferation and persistence	B-cell lymphoma	NCT03929107	[56]
		Increase T/DC cells trafficking and infiltration	Multiple myeloma	NCT03778346	
		Induce more memory cells	Nectin4+ solid tumor	NCT03932565	

Clinical trial-related information were mainly collected from ClinicalTrials.gov. Abbreviations: CCL, C-C chemokine ligand; CCR, C-C chemokine receptor; CXCL, C-X-C chemokine ligand; CXCR, C-X-C chemokine receptor; MDSC, myeloid-derived suppressor cells; DC, dendritic cells.

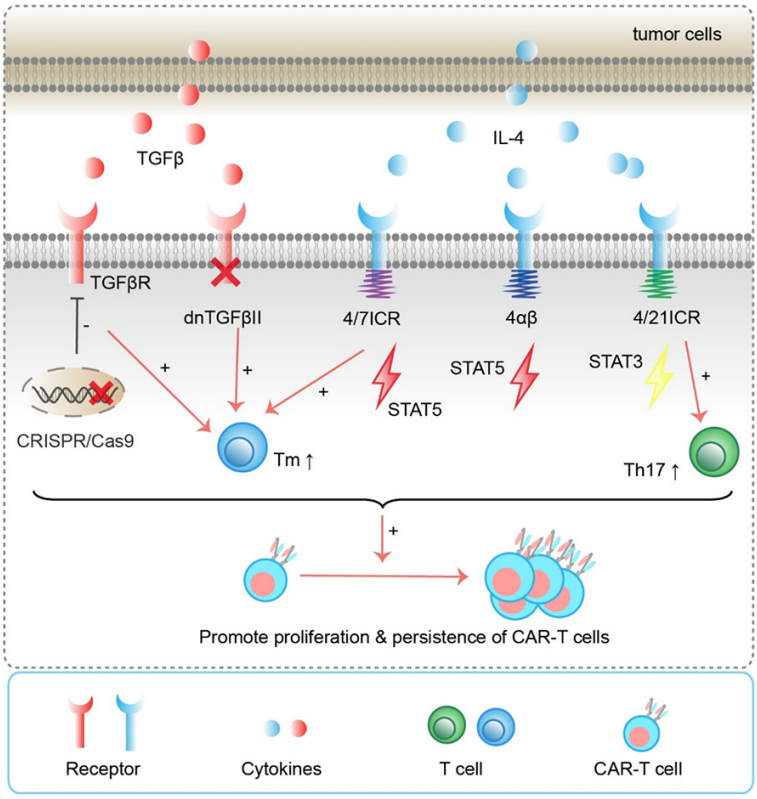

Figure 3. Targeting immunosuppressive molecules in the TME to promote the CAR-T therapy. CAR-T cells equipped with dnTGFβRII or knocking out TGFβRII in CAR-T cells with CRISPR/Cas9 technology strengthened anti-tumor efficacy due to insensitive to TGFβ. Besides, the inhibitory endodomain of IL-4 receptor can be replaced by activator receptor including IL-2/IL-15, IL-7 and IL-21 to transduce pro-survival signals for lymphocytes.

ters have undertaken related clinical trials to assess the safety and effectiveness of 17X19 CAR-T therapy for patients with r/r B cell lymphoma (NCT03929107), multiple myeloma (NCT03778346) and other malignant solid tumors (NCT03932565).

Immunosuppressive molecules

What CAR-T cells confront after trafficking into the tumors is multifarious and intricate inhibitory factors in the TME. The TME consists of tumor cells and various components, including fibroblasts, endothelial cells, mesenchymal cells, suppressive immune cells, along with extracellular matrix and inflammatory mediators they secrete, which not only promote malignant growth of tumors, but also impair anti-tumor immunity in different ways [7]. Soluble inhibitory molecules in the TME, like IL-4, IL-10, transforming growth factor-β (TGFβ) [57] and prostaglandin E2 (PGE2) [58], are one of the main aspects, which can directly promote cancer progression and indirectly forming suppressive milieu, to restrict CAR T-cell proliferation and cytotoxic function. However, modified cytokine receptors, including dominant negative receptor (DNR) and inverted cytokine receptor (ICR), enable CAR-T cells to antagonize aforementioned repressive factors, thus exerting superior anti-tumor effects, the main point of this part (**Figure 3**; **Table 3**).

TGFβ

TGFβ is rich in a variety of carcinomas and favors advanced tumor progression in many ways, including epithelial-to-mesenchymal transition, angiogenesis and metastasis (reviewed in [59]). Besides, TGFβ participates in the formation of the unfavorable TME, which markedly suppresses immune surveillance by inhibiting T-cell proliferation, down-regulating the critical proteins of CTLs (perforin, granzymes and cytotoxins) and generating Tregs (reviewed in [57]).

TGFβ receptor type II (TGFβRII) lacked intracellular domain for signal transduction [60], and thus T cells engineered to express dominant-negative TGFβRII (dnTGFβRII) were insensitive to TGFβ [61]. Multiple centers exhibited that CAR-T cells equipped with dnTGFβRII could promote prostate tumor eradication [62-64]. In addition to dnTGFβRII, utilizing CRISPR/Cas9 technology to knock out endogenous TGFβRII of CAR-T cells also produced comparable anti-tumor effects. Upon re-exposure to the same antigen, the number of Tcm and effector memory T (Tm) cells were sharply elevated, recalling robust and long-term memory responses [65].

Given durable tumor regression in preclinical models, dnTGFβRII was quickly put into clinical practice for the treatment of various tumors (NCT00368082, NCT02065362, NCT008899-

Table 3. Summary of targeting immunosuppressive molecules in the TME to promote the CAR-T therapy

Related cytokines	Application	Mechanism	Diseases	Clinical trials	References
TGFβ	TGFβ antibody	Block TGFβ	Hepatocellular carcinoma	NCT03198546	-
	DnTGFβRII	Insensitive to TGFβ	Melanoma	NCT03089203	[61-64]
			Prostate cancer	NCT04227275	
			EBV+ lymphoma	NCT00368082	
			Nasopharyngeal carcinoma	NCT02065362	
			Her2+ malignancy	NCT00889954	
	Knock out TGFβR2	Insensitive to TGFβ	Lung cancer	-	[65]
		Increase Tcm and Tem phenotype	Pancreatic carcinoma		
IL-4	T4 immunotherapy	Antagonize inhibition of IL-4	Epithelial ovarian cancer	NCT01818323	[68-71]
		Produce crucial growth stimulation	Malignant mesothelioma		
			Head and neck cancer		
	4/7 ICR	Antagonize inhibition of IL-4	Pancreatic cancer	-	[67, 72, 73]
		Preserve Th1 phenotype	Breast cancer		
		Produce long-term persistence	EBV+ lymphoblastoma		
	4/21 ICR	Antagonize inhibition of IL-4	Hepatocellular carcinoma	-	[74]
		Promote Th17-like polarization			

Clinical trial-related information were mainly collected from ClinicalTrials.gov. Abbreviations: TGFβ, transforming growth factor-β; DnTGFβRII, dominant-negative TGFβ recepter II; Tcm, central memory T cells; Tem, effector memory T cells; ICR, inverted cytokine receptor.

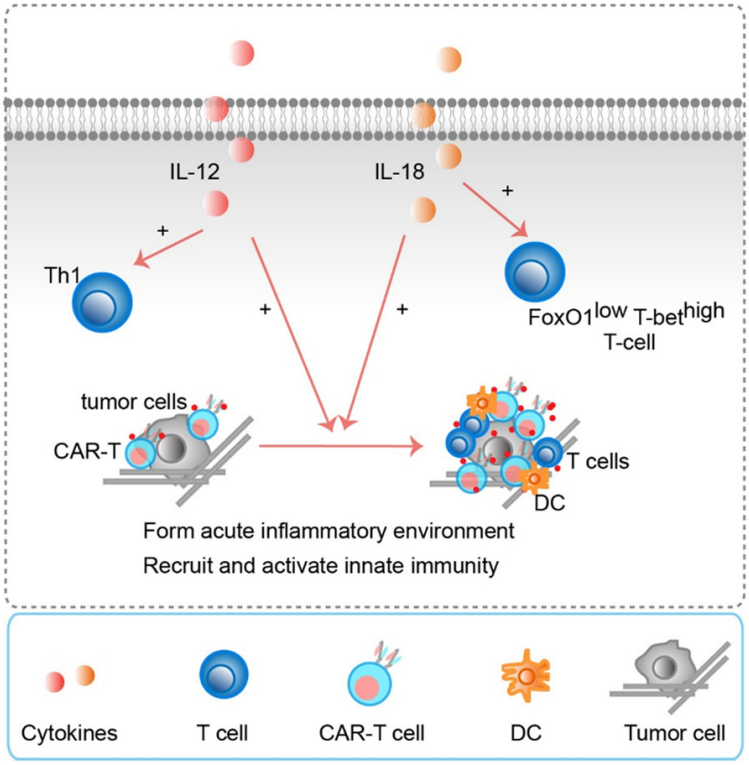

Figure 4. Mechanisms for enhancing CAR-T effects by using pro-inflammatory cytokines. CAR-T cells equipped with pro-inflammatory cytokines like IL-12, IL-18 not only form acute inflammatory microenvironment suitable for immunity, but also reactivate endogenous lymphocytes to exert anti-tumor responses.

54, NCT03089203, NCT04227275). In Hodgkin lymphoma, T cells armed with dnTGFβRII expanded well and persisted up to 4 years after infusion. Four of the seven evaluable patients achieved clinical responses and two patients were in remission for more than four years, including one who achieved only partial response (PR) after conventional CAR-T therapy [66]. Besides, dual-specific CAR-T cells targeting both tumor antigen and soluble TGFβ (GPC3/TGFβ-CART) have also been tried to treat HCC (NCT03198546).

IL-4

IL-4 is also a soluble inhibitory molecule commonly found in various cancer milieu. It can promote expansion and invasion of cancer cells, support M2 macrophage differentiation and upregulate secretion of Th2-polarized cytokines, thereby promoting tumor proliferation and inhibiting immune cells function [67].

Inverted cytokine receptor (ICR) that enables suppressive factors to deliver activation signals also shows good prospect of application. The early attempt was "T4 immunotherapy", in which IL-4Rα was fused with endodomain β-chain shared by IL-2R and IL-15R (4αβ), thereby allowing IL-4-mediated CAR T-cell proliferation rather than inhibition [68]. Introducing 4αβ to CAR-T cells had great survival advantages in the IL-4-rich TME with robust anti-tumor effects on epithelial ovarian cancer and malignant mesothelioma [69, 70]. And a study has already been registered as a clinical trial for head and neck cancer (NCT01818323) [71]. Apart from 4αβ, IL-4Rα can also link to the IL-7R intracellular segment (4/7 ICR). Researches showed that CAR-T cells incorporating 4/7 ICR retained Th1 phenotype with long-term persistence and durable tumor control in pre-clinical models of lymphoblastoma [67], pancreatic [72] and breast cancers [73]. 4/21 ICR worked in a similar way, but 4/21 ICR induced STAT3 phosphorylation and promoted Th17-like polarization [74], which is a less differentiated subset, and is closely correlated with anti-tumor responses involving CAR-T therapy [75].

Pro-inflammatory cytokines

Given their ability of directed migration to tumor sites, CAR-T cells can serve as a targeting vehicle to transfer immune-modulating transgenic protein ("payload") and discharge it upon CAR engagement with tumor antigens. The most common and powerful payloads are versatile pro-inflammatory cytokines, which can recruit endogenous immune cells to elicit anti-tumor attack against the cancer cells invisible to CAR-T cells (**Figure 4**; **Table 4**). This strategy is represented by T cells redirected for universal cytokine killing (TRUCK) [76, 77]. Also, TRUCK cells are locally delivered, thereby overcoming the systemic toxicity of exogenous administra-

Table 4. Summary of enhancing CAR-T effects by using pro-inflammatory cytokines

Related cytokines	Application	Mechanism	Diseases	Clinical trials	References
IL-12	Co-expression	Enhance T-cell omnifarious performance	Hodgkin lymphoma	NCT01236573	[79-82, 84, 87-89]
		Trigger programmatic changes in the TME	Metastatic melanoma	NCT02498912	
		Recruit and activate endogenous immunity	Ovarian cancer	NCT03542799	
			Colorectal tumor	NCT01457131	
			Hepatocellular carcinoma		
			NY-ESO-1+ metastatic cancer		
IL-18	Co-expression	Augment T-cell proliferation and persistence	CD19+ malignancies	-	[90-94]
		Transform Th1 phenotype	Pancreatic tumor		
		Regulate innate immune cells and TME	Lung tumor		
		Induce FoxO1^{low} T-bet^{high} T-cell phenotype	Ovarian cancer		
			Breast cancer		

Clinical trial-related information were mainly collected from ClinicalTrials.gov. Abbreviations: TME, tumor microenvironme.

tion, and release cytokines upon CAR stimulation, avoiding repetitive drug application [78]. Clinical trials showed remarkable efficacy and great prospects of future application. The expression of cytokines is either constitutive or inducible according to distinct promoters.

IL-12

IL-12 is the paradigmatic and impressive cytokine for "TRUCKs" (reviewed in [77]), which promotes tumor recession by at least three mechanisms, including prolonging CAR-T cells survival and persistence [79, 80], inverting inhibitory or chronic inflammatory TME into acute inflammation suitable for immunity [80-83], recruiting and activating innate immune cells to cause a second wave of anti-tumor responses [84-86].

The first application chose EBV-specific CTLs for the treatment of HL. The armored CTLs secreting IL-12 were insensitive to the suppressive environment, persisting longer and mounting stronger anti-tumor responses [82]. Later pre-clinical models of ovarian cancer [79, 80], colorectal tumor [84], HCC [81] and so on [87], whether immunodeficiency or immunocompetence, whether constitutive or inducible expression, IL-12 secreting CAR-T cells could produce sustained tumor eradication. Consequently, the first-in-human clinical trial was initially conducted in metastatic melanoma (NCT01236573). Results showed that TILs transduced with IL-12 achieved comparable effects at a dose 10- to 100-fold lower than conventional TILs. Even though therapeutic effects were transient owing to the short persistence of TILs *in vivo* and unexpected toxicity occurred when given at higher doses, this innovative method still showed a promise of future application [88]. To date, more and more centers have registered clinical trials aimed to evaluate the safety and effectiveness of CAR-T cells co-expressing IL-12 (NCT02498912, NCT03542799, NCT01457131) [89].

IL-18

IL-18 is another pro-inflammatory cytokine, possessing many effects comparable to IL-12 [90]. Compared with IL-12, CAR-T therapy integrating IL-18 can reduce oedema-like toxicity due to lower level of IFN-γ and TNFα, and increase TILs by decreasing macrophage recruitment, thus producing safer and higher anti-tumor activities [91]. It is known that CAR-T cells with acute inflammatory and less differentiated signatures are believed to be superior in triggering long-time anti-tumor responses. And IL-18 can induce $FoxO1^{low}$ $T\text{-}bet^{high}$ T-cell phenotype, which exactly fulfills the requirements [92]. Subsequent investigations further verified the safety and robust anti-tumor effects of IL-18-secreting CAR-T therapy in many malignancies [92-94].

Risks and challenges

However, the universal use of CAR-T cells armed with cytokines still has a long way to go even if the impressive results indicate a high potential for future application. The production of novel CAR-T cells entails introduction of two vector constructs for CAR expression and cytokine secretion, which might increase the risk of insertional mutagenesis and integration of viral vectors [95]. And two products, the CAR and cytokines, further complicate the definition of cellular kinetics and clinical pharmacology [96]. Besides, transgenic CAR-T cells might aggravate adverse effects of cellular therapy such as cytokine release syndrome (CRS) and "on target off tumor" toxicity, because of the additional release of pro-inflammatory cytokines, resulting in more severe tissue damage and local reaction. Another safety concern is the background cytokines release independent of CAR stimulation due to the "leakage" of promoters. Currently, the nuclear factor of activated T-cell (NFAT) promoter is mostly applied for inducible cytokines secretion, which is also triggered by normal TCR engagement, and exceeding physiological levels may produce "off target off tumor" toxicity in healthy tissues [88]. What's more, constitutive expression of pro-survival cytokines or continuous activation of signaling pathways may induce autonomous proliferation, and the risk of malignant transformation cannot be entirely ruled out [97].

To overcome these obstacles, a variety of strategies for improving safety have been proposed. One study worked out an "all-in-one" vector system, which utilized a modified NFATsyn promoter to combine constitutive CAR expression with inducible cytokine secretion through only one vector, thereby simplifying the construct of CAR-T and reducing insertional mutagenesis [95]. Regulatable switches are appropriate

alternatives to reduce the toxicity of excessive release of cytokines and potential autonomous proliferation, including suicide genes, synNotch receptors, switch CAR, among others (reviewed in [98]). Considering the background expression of cytokines, a well-designed dose-escalation plan is required in the clinical trials for determining the maximum tolerated dose (MTD), whereas exploring strictly CAR-dependent promoters shows more advantages in the long run.

Conclusions and perspective

Use of CAR-T cells has revolutionized cancer treatment, but tapping the full capacity of CAR-T therapy requires further scientific exploration [99]. Arming CAR-T cells with diverse cytokines can enhance CAR T-cell proliferation and persistence, improve trafficking and infiltration of CAR-T cells, antagonize inhibitory molecules in the TME and reactivate innate immunity, thus strengthening anti-tumor responses and inducing long-term cancer regression. Besides, preclinical models and trials with "armored" CAR-T therapy also exhibited impressive effects. CAR-T cells incorporating cytokines not only achieved comparable anti-tumor results with 10- to 100-fold lower doses [88], but also expanded well and persisted for over four years after infusion [66]. Moreover, TRUCK cells can reactivate endogenous immunity, thus initiating a second attack toward those antigen-loss tumor cells which might lead to antigen escape.

Identifying appropriate cytokines remains a challenge, and the key factor is the specific clinical response of different tumors. If the CAR expression is rarely detected within a few weeks after reinfusing into patients, combination use with the aforementioned γc cytokines should be considered [100]. In dealing with "cold" tumors, where immune cells have no or little infiltration [41], co-expression of tumor-related chemokine receptors or modification of inhibitory receptors are powerful tools to improve prognosis. Also, the high frequency of targeted antigen-negative relapse [101] suggests that the therapy should be used in combination with IL-12 or IL-18 to mobilize endogenous immune cells. Definitively, high-throughput proteomics of serum from patients or cytokine screen for specific phenotype CAR-T cells is critical to the discovery of novel and effective cytokines [92]. Additionally, cytokines with different functions can work synergistically. For example, CAR-T cells integrating IL-15 and IL-21 exhibited enhanced proliferative capacity, with more Tscm and Tcm cells populations [31]. 7X19 CAR-T cells co-expressing two functional cytokines (IL-7 and CCL19) can enhance proliferation and infiltration and recruit endogenous immune cells, and are a promising alternative [56]. Recent study showed that 7X21 CAR-T cells also had similar synergistic effects [102]. However, not all these cytokines are effective in all kinds of cancers due to tumor heterogeneity. It is critical to determine the appropriate cytokines or cytokine receptors in CAR-T therapy on the basis of the signature of cytokines secreted by distinct tumors. The receptors include "matched" chemokine receptors to enhance migration and modified receptors, such as ICR or DNR, to antagonize immunosuppressive molecules. Other survival-promoting cytokines target CAR-T cells themselves, and can be applied to various tumors to improve CAR-T efficacy. As mentioned above, co-expression of certain γc cytokines could enhance the expansion and persistence of CAR-T cells *in vivo* by activating JAK/STAT3 or STAT5 pathway regardless of tumor types, thus improving the efficacy of CAR-T therapy.

This review outlines CAR-T therapy coupled with various cytokines, which exerts promising anti-tumor effects and potential for clinical application even if some challenges remain. Undoubtedly, further researches will further improve the efficacy and safety of CAR-T cell therapy.

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Disclosure of conflict of interest

None.

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