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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

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<td><strong>IL-2</strong></td>
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<td>Promote T cells expansion</td>
<td>B cell malignancies</td>
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<td>[16-19]</td>
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<td></td>
<td></td>
<td>Promote Treg cells expansion</td>
<td>Ovarian Cancer</td>
<td>NCT00019136</td>
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<td>AICD</td>
<td>Metastatic Melanoma</td>
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<td>IL-15, IL-7, IL-21</td>
<td>Ex vivo culture</td>
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<td>B cell malignancies</td>
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<td><strong>IL-15</strong></td>
<td>Co-expression</td>
<td>Promote T cells expansion and persistence</td>
<td>B cell malignancies</td>
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<td>Preserve Tscm phenotype</td>
<td>Glioblastoma</td>
<td>NCT03579888</td>
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<td>Preserve Tscm phenotype</td>
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<td>IL-7</td>
<td>IL-7Rα (with exogenous IL-7)</td>
<td>Selective promote CAR-T proliferation</td>
<td>EBV+ lymphoblastoma</td>
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<td></td>
<td>C7R</td>
<td>Continuously activate JAK/STAT5 pathway</td>
<td>Metastatic neuroblastoma</td>
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<td>[36, 37]</td>
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<td>Independent on soluble cytokines</td>
<td>Orthotopic glioblastoma</td>
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<td>IL-12β p40 subunit (p40-Td cells)</td>
<td>Induce Th17 phenotype</td>
<td>Neuroblastoma</td>
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<td>Pancreatic cancer</td>
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<td>γc cytokines</td>
<td>28-ΔIL2RBz (YXXQ) CAR-T</td>
<td>Activate JAK/STAT3 and STAT5</td>
<td>B cell malignancies</td>
<td>-</td>
<td>[38]</td>
</tr>
</tbody>
</table>

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; Mbl15, 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.
Fueling CAR-T cells with Cytokines

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-
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 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-2Rβ-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.
Fueling CAR-T cells with Cytokines

**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].

**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].

**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-CXCR7 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].

**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.

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

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<th>Mechanism</th>
<th>Diseases</th>
<th>Clinical trials</th>
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<td>CCL17/CCL22/CCR4</td>
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<td>Ligands/CXCR1/CXCR2</td>
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<td>[48, 49]</td>
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<tr>
<td></td>
<td>Inhibitor SX-682</td>
<td>Block trafficking of CXCR2+ MDSC</td>
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<tr>
<td>CXCL10/CXCR3</td>
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<td></td>
<td>Increase the secretion of CXCL11</td>
<td>Enhance migration of lymphocytes</td>
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<td>CXCL11</td>
<td>Docetaxel</td>
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<td>Induce more memory cells</td>
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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.
Fueling CAR-T cells with Cytokines

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, downregulating 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-[^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

![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.](image-url)
## Table 3. Summary of targeting immunosuppressive molecules in the TME to promote the CAR-T therapy

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

Clinical trial-related information were mainly collected from ClinicalTrials.gov. Abbreviations: TGFβ, transforming growth factor-β; DnTGFβRII, dominant-negative TGFβ receptor II; Tcm, central memory T cells; Tem, effector memory T cells; ICR, inverted cytokine receptor.
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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 [54, NCT03089203, NCT042272275). 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].

In 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-

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

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

Clinical trial-related information were mainly collected from ClinicalTrials.gov. Abbreviations: TME, tumor microenvironment.
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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 (NCT0-1236573). 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].

**IL-18**

IL-18 is another pro-inflammatory cytokine, possessing many effects comparable to IL-12 [90]. Compared with IL-12, CART 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 CART 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<sup>low</sup> T-bet<sup>high</sup> T-cell phenotype, which exactly fulfills the requirements [92]. Subsequent investigations further verified the safety and robust anti-tumor effects of IL-18-secreting CART therapy in many malignancies [92-94].

**Risks and challenges**

However, the universal use of CART 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 CART 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 CART 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 NFAT<sub>syn</sub> promoter to combine constitutive CAR expression with inducible cytokine secretion through only one vector, thereby simplifying the construct of CART 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 "armed" 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 reinventing into patients, combination use with the aforementioned yc 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 yc 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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