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Review Article

Next-generation cytokines for cancer immunotherapy

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Abstract

Most studies focus on the first and second signals of T cell activation. However, the roles of cytokines in immunotherapy are not fully understood, and cytokines have not been widely used in patient care. Clinical application of cytokines is limited due to their short half-life *in vivo*, severe toxicity at therapeutic doses, and overall lack of efficacy. Several modifications have been engineered to extend their half-life and increase tumor targeting, including polyethylene glycol conjugation, fusion to tumor-targeting antibodies, and alteration of cytokine/cell receptor-binding affinity. These modifications demonstrate an improvement in either increased antitumor efficacy or reduced toxicity. However, these cytokine engineering strategies may still be improved further, as each strategy poses advantages and disadvantages in the delicate balance of targeting tumor cells, tumor-infiltrating lymphocytes, and peripheral immune cells. This review focuses on selected cytokines, including interferon-α, interleukin (IL)-2, IL-15, IL-21, and IL-12, in both preclinical studies and clinical applications. We review next-generation designs of these cytokines that improve half-life, tumor targeting, and antitumor efficacy. We also present our perspectives on the development of new strategies to potentiate cytokine-based immunotherapy.

Statement of Significance: Clinical application of cytokines is limited due to their short half-life *in vivo* and severe toxicity at therapeutic doses. Here, we review several modifications of commonly used cytokines that have been engineered to extend their half-life and increase tumor targeting, we summarize their potential issues and newer designs of engineered cytokines.

KEYWORDS: cytokines; immunotherapy; protein engineering; tumor targeting

INTRODUCTION

Cytokines are potent immune-modulating protein molecules that are used in treating cancer [1, 2]. Cytokines stimulate the function, survival, and proliferation of natural killer (NK) and T cells that mediate immune responses against tumors. The discovery of potent antitumor activity of cytokine therapy in animal models has prompted the evaluation of the potential application of some immune molecules for clinical cancer therapy. Such cytokines include interferon (IFN)-α, interleukin (IL)-2, IL-15, IL-21, and IL-12. IL-2 was the first Food and Drug Administration (FDA)-approved cytokine for treating metastatic renal cell cancer and advanced melanoma [3, 4]. IFN-α has been approved for the treatment of several human cancers [5–8]. However, in most patients, systemic administration of cytokines has limited efficacy in clinical trials due to their short half-life and severe adverse effects before reaching therapeutic doses [6, 9–12]. Novel strategies to improve cytokine antitumor effects as monotherapy or combination therapy for both preclinical and clinical applications will be discussed in this review.

Type I IFN-α

Type I IFNs, including IFN-α, IFN-β, IFN-ε, IFN-κ, and IFN-ν, are a family of monomeric cytokines with multiple functions [13]. IFN-α regulates the expression of various genes that modulate tumor cell growth, proliferation, apoptosis, and immune checkpoint-mediated immune suppression [14–18]. Several studies also have shown that the

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Table 1. Engineered IFN-α variants

Drug name	Reference	Drug features	Drug benefits	Clinical phase
Peginterferon alfa-2b	Eggermont *et al*. [24]	PEG conjugated to IFN-α at IFN/IFNR-binding interface	Increases half-life and reduces peripheral toxicity with equivalent antitumor efficacy to IFN-α	III
Anti-CD20/IFN-α	Xuan *et al*. [30]; Liao *et al*. [31]	Anti-CD20 conjugated to mouse IFN-α	Kills lymphomas that express CD20 through adaptive immunity or direct IFN-α-mediated killing	Preclinical
TAK-573	Collins *et al*. [33]	Anti-CD38 conjugated to attenuated IFN-α	Binds to CD38+ cells with high affinity; increases circulating IFN-associated cytokine levels in human patients	I/II
IFN-α/anti-PDL1	Liang *et al*. [37]	Anti-PDL1 conjugated to IFN-α	Induces IFN-mediated upregulation of PDL1, which is then targeted to create feedforward antitumor immunity	Preclinical

type I IFNs play critical roles in tumor control by promoting dendritic cell (DC) cross-priming to (re-) activate T cells [19–21]. IFN-α was FDA approved to treat hematological malignancies and melanoma at high doses [5–8].

Following the clinical success of IFN-α in cancer treatment, multiple strategies have been tested to address the limitations of IFN-α and further improve its clinical efficacy and safety (Table 1). To minimize filtration of IFN-α through the kidney prior to reaching a therapeutic dose, IFN-α requires a longer half-life. One IFN-α variant addresses this issue by conjugating polyethylene glycol (PEG) to IFN-α. PEGylation can cover the domain of IFN-α, which binds to its receptor to minimize peripheral IFN-α activity and uptake into off-target, nontumor tissues. PEGylated IFN-α has a comparable spectrum of biological activity to IFN-α, but with an approximately 10-fold longer plasma half-life, thus allowing for less frequent administration and patient burden. These significant benefits resulting from PEGylation of IFN-α have resulted in its approval as an adjuvant treatment of melanoma [22]. However, the type I IFN receptor is widely distributed on all nucleated cells including those in nontumor tissue, which suggests that PEGylated IFN-α can still induce toxic side effects [23]. Adjuvant therapy with PEGylated IFN-α2b has been associated with severe host toxicity, including fatigue (97 patients, 16%), hepatotoxicity (66, 11%), and depression (39, 6%). In all, 37% of patients discontinued adjuvant therapy because of these adverse toxicities [24]. These studies suggest that lack of tumor-targeted release of PEGylated IFN-α may ultimately limit positive clinical results.

Various strategies have been developed to increase IFN-α delivery to tumor sites to improve efficacy with reduced toxicity. Fusing therapeutic cytokines to tumor-targeting antibodies or antibody fragments can promote the localization of such treatments within the tumor, thus minimizing cytokine therapy induce toxicity. IFNs have been conjugated to monoclonal antibodies (mAbs) that recognize and bind to tumor-associated proteins such as epidermal growth factor receptor (EGFR), Human Epidermal Growth Factor Receptor 2 (HER2) Erb-B2 Receptor Tyrosine Kinase 2 (ERBB2), cluster of differentiation (CD)20, CD38, CD138, and Vascular Endothelial Growth Factor (VEGF) receptor. These cytokine/antibody fusions may induce direct antiproliferative effects from both the conjugated IFNs and the mAbs [25–29]. A preclinical study discussing the treatment of a B cell lymphoma xenograft model that overexpresses human-CD20 (38C13-hCD20) reported that type I IFN linked to an anti-CD20 antibody increased the antitumor effects of type I IFN by directly killing off IFN-α-sensitive 38C13-hCD20 tumor cells [30]. Because many lymphomas are resistant to direct anti-CD20- and/or IFN-mediated apoptosis, other studies aimed to determine whether targeting tumors with IFN-α could mobilize the adaptive immune system for tumor control. Using a syngeneic anti-CD20- and IFN-α-resistant A20 B cell lymphoma tumor model, one study demonstrated that treating this lymphoma with a conjugated anti-CD20/IFN-α fusion protein abolished B cell lymphoma resistance to anti-CD20 while limiting IFN-associated systemic toxicity in the host. Mechanistically, the anti-CD20/IFN-α fusion employs tumor cells as the dominant antigen presenting cell (APC) for the reactivation of Cytotoxic T lymphocytes (CTLs), which ultimately induces potent antitumor efficacy [31].

Alternatively, conjugation of IFNs muteins with reduced receptor-binding affinity with high-affinity tumor-targeting antibodies can further induce preferential localization of such fusion proteins to the tumor and thus limit their consumption in circulation. Despite its fusion to a tumor-targeting modality, tumor-targeting antibody/wild-type IFN-α fusions can still induce toxicity through ubiquitously expressed IFN-α receptors (IFNAR) on nontumor cells. Furthermore, supposed tumor-associated antigens are not absolutely specific to tumors and can be expressed on nontumor cells, which take up and thus hijack the conjugated immunocytokines. An attenuated form of IFN-α was reported to reduce cell toxicity in the forms of the MLepR-targeting nanobody/IFN [32] and the anti-CD38/IFN-α fusions [27]. The anti-CD38/attenuated IFN-α fusion protein displays a 10 000-fold greater binding-specificity to CD38-positive (tumor) vs. CD38-negative (normal) cells than native IFN-α. In contrast, the corresponding wild-type IFN-α fusion protein showed only a 40-fold greater binding affinity. Therefore, the attenuating mutation in the IFN-α portion of the immunocytokine decreases IFN-α antigen specificity by approximately 250-fold. Treatment of established human multiple myeloma tumor (NCI-H929 xenograft model) bearing mice with CD38-targeting attenuated IFN-α leads to the complete elimination of such tumors. Patients may thus potentially be more safely treated with higher doses of the anti-CD38/attenuated IFN-α than native IFN-α or a "more conventional" IFN-α immunocytokine (anti-CD38/wild-type IFN-α). TAK-573 is a fusion protein of a humanized anti-CD38 IgG4 mAb with two attenuated IFN alpha-2b molecules. Ongoing phase I/II TAK-573-1501 clinical studies in patients with relapsed/refractory multiple myeloma (NCT03215030) indicate that TAK-573-treated patients exhibit an increased type I IFN gene signature and circulating IFN-associated cytokine levels. This preliminary biomarker data indicate that TAK-573 is a pharmacologically active molecule that mediates its effect through IFNAR pathway modulation [33].

IFNs are also the cytokines that most potently induce Programmed death ligand 1 (PD-L1) expression, which subsequently dampens Tc ell responses against the tumor via a negative feedback effect [34–36]. Activation of IFN signaling in the tumor microenvironment (TME) can therefore synergize with PD-L1 blockade therapy against advanced tumors by inducing more robust T cell activation with the absence of PD-L1 inhibition of T cells. Furthermore, the anti-PD-L1 antibody can be conjugated to immunomodulatory molecules to deliver them specifically into tumor tissues with minimal toxicity. Finally, IFN-α-armed anti-PD-L1 creates multiple feedforward responses that increase targeting effects to enhance responses to IFN-α treatment, thereby maximizing antitumor effects [37]. Nevertheless, this immunocytokine, when administered systematically, is still at risk for being taken up by peripheral IFN receptor-expressing cells, and its application might be improved by utilizing a mutant Fc that does not have antibody-dependent cellular cytotoxicity capability or incorporating an IFN activity blocking mask to even further minimize toxicity.

Common γ chain cytokines: IL-2, IL-15, and IL-21

The common $\boldsymbol{\gamma}$ chain cytokine family exerts numerous functions on T lymphocyte survival, function, and proliferation. This family consists of six members, including IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. Most of these cytokines function through activating janus kinase (JAK)1/JAK3 and the downstream signaling of signal transducer and activator of transcription (STAT)1, STAT3, STAT5, MAPK, and PI3K/also known as Protein Kinase B (AKT).

IL-2. IL-2 was identified as a 15.5 kDa glycoprotein in the 1970s and is mainly produced by activated CD4$^+$ T cells [38, 39]. As a potent inducer of cytotoxic T cells and NK cells, IL-2 was one of the first FDA-approved immunocytokines for metastatic melanoma and renal cell carcinoma [3, 4]. Clinical IL-2 immunotherapy has not been widely applied due to its short *in vivo* half-life, severe toxicity at therapeutic doses, and induction of immunosuppressive responses through regulatory T cell (Treg) expansion [10–12]. Many strategies for addressing these limitations have been implemented, such as fusing the Fc domains of immunoglobulins or PEG molecules to increase half-life, modifying IL-2 function by introducing targeted mutations, fusing IL-2 with antibodies that target the cytokine to the TME, masking IL-2 against Treg binding, and synthesizing tumor-associated protease-activated IL-2 prodrugs (Table 2).

The IL-2 receptor is a heterotrimeric complex formed by three subunits: the IL-2Rα, IL-2Rβ, and IL-2Rγ chains, also known as CD25, CD122, and CD132, respectively. IL-2 exerts stimulatory and regulatory functions by binding to IL-2 receptor subunits. Naïve CD8$^+$ T cells and CD4$^+$/CD8$^+$ memory T cells express a medium-affinity dimeric receptor IL-2Rβ/IL-2Rγ and lack the IL-2Rα chain. When the IL-2Rα chain is also present in the receptor complex, IL-2 is bound with high affinity. IL-2Rα is highly expressed on Treg cells, thus increasing the affinity of IL-2 for Tregs over cytotoxic lymphocytes at lower doses. Therefore, high doses of IL-2 are immunostimulatory, but low doses are immunosuppressive [40–42].

Several second-generation IL-2 variants have been engineered to reduce affinity to IL-2Rα [43, 44] or increase IL-2Rβ affinity. IL-2Rα is highly expressed on pulmonary endothelial cells, a large percentage of Foxp3-negative CD4 T cells in humans, Treg cells, and some innate lymphoid cells. One strategy is by conjugating PEG to IL-2. NKTR-214 is a prodrug of human IL-2 that is PEGylated on the IL-2Rα-binding site, in which six PEG residues are slowly released to make an active and stable form of human IL-2. As the PEG molecules conjugated to IL-2 blocks IL-2 interaction with the IL-2Rα-binding motif, the binding of PEG-IL-2 to Treg cells is inhibited, whereas the binding of IL-2 to receptors on CD8$^+$ T cells is not affected. PEG modulation also increases the half-life of IL-2 [45–47]. NKTR-214 significantly increased the ratio of CD8$^+$ T cell to Treg cells in murine melanoma models and led to improved antitumor efficacy compared with recombinant IL-2 (Aldesleukin). However, NKTR-214 has limited efficacy as a monotherapy in solid tumors. On the other hand, the increased levels of the PD-1 protein on tumor-infiltrating T cells and PD-L1 on cancer cells in patients suggested that combination with immune checkpoint inhibitors might boost the antitumor efficacy of IL-2 variants. Clinically, NKTR-214 combined with nivolumab induced increased systemic and intratumor CD8$^+$ T cell responses with no expansion of Tregs in the tumor and achieved encouraging overall response rates compared with nivolumab alone, according to immunohistochemistry data. Unfortunately, it has been reported that

Table 2. Engineered IL-2 variants

Drug name	Reference	Drug features	Drug benefits	Clinical phase
NKTR-214	Charych *et al*. [45]	PEG conjugated to IL-2 at IL-2/IL-2Rα-binding interface	Increases half-life, reduces peripheral toxicity, increases affinity towards CD8 T cells	II/III
IL-2 mutein (CD25)	Carmenate *et al*. [51]	Includes mutations that decrease IL-2 affinity for IL-2Rα	Reduces IL-2-mediated toxicity and inhibits metastases	Preclinical
IL-2 superkine	Levin *et al*. [52]	Includes mutations that increase IL-2 affinity for IL-2Rβ	Reduces IL-2-mediated toxicity, more potently inhibits primary tumor growth	Preclinical
NHS-IL2LT (Selectikine)	Gillessen *et al*. [58]	Antinecrotic tumor core conjugated to IL-2 mutein	Exhibits very low toxicity in clinical trials	I/II
CEA-IL2v	Klein *et al*. [59]	IL-2 mutein with decreased affinity for IL-2Rα, conjugated to anti-CEA	Decreased mortality in preclinical tumor models, but was abandoned due to lack of efficacy in clinical trials	II
DI-Leu16-IL2	Lansigan *et al*. [60]	Anti-CD20 conjugated to IL-2	Induces several complete responses in lymphoma patients with few side effects	I/II
SumIL2/Anti-EGFR	Sun *et al*. [61]	Anti-EGFR conjugated to CD8 preferential IL-2 mutein	Inhibits tumor growth, can eliminate metastases when combined with surgery	Preclinical
ALKS 4230	Lopes *et al*. [62]	IL-2Rα conjugated to IL-2	Reduces peripheral toxicity and inhibits melanoma metastases preclinically	Preclinical
IL-2/mAb complexes	Krieg *et al*. [63]	Antibody conjugated to IL-2 at IL-2/IL-2Rα-binding interface	Unable to bind endogenous IL-2Rα, inhibits tumor growth with reduced toxicity	Preclinical
Protease-activated IL-2 prodrug	Hsu *et al*. [67]	CD8 preferential IL-2 mutein masked with tumor protease removable IL-2Rβ	Reduces peripheral toxicity, inhibits tumor growth, can eliminate metastases when combined with surgery	Preclinical
Neo-2/15	Silva *et al*. [85]	Computationally designed IL-2 variant with increased affinity for IL-2Rβ	Mimics natural signaling of IL-2 with reduced peripheral toxicity	I

about seven in 10 humans have raised circulating anti-PEG antibodies. This high prevalence of anti-PEG antibodies may potentially limit the efficacy of NKTR-214 [48]. Lack of tumor-targeted release of PEGylated cytokines could result in toxicity before reaching therapeutic doses [49]. Moreover, most PEGylated proteins are prepared through nonsite-specific PEGylation, which may affect the consistency of manufacture and clinical administration of PEGylated IL-2 [45, 50].

Another approach to shifting IL-2 affinity towards $CD8^+$ T cells is to synthesize IL-2 mutants with different binding properties for the IL-2 subunits. IL-2 exhibits a higher affinity towards Tregs over effector T cells due to the expression of IL-2Rα on Tregs. Therefore, the two major mutagenesis strategies to overcome IL-2-mediated immunosuppression involve decreasing IL-2 affinity for IL-2Rα or increasing IL-2 affinity for IL-2Rβ. One recent IL-2Rα affinity altering mutant has been observed to reduce IL-2-mediated toxicity while reducing metastatic disease. This mutant IL-2 was designed to mutate wild-type IL-2 on residues R38, F42, Y45, and E62. These residues, which all interface with IL-2Rα, were substituted with alanine. Although further in-depth *in vivo* primary tumor studies would provide a more comprehensive assessment of the efficacy of these muteins, this study still demonstrates that reducing IL-2 affinity for IL-2Rα can contribute to increased antitumor efficacy [51]. Other muteins, such as the IL-2 "superkine," include mutants that significantly increase IL-2 affinity for IL-2Rβ [52]. The evolved mutations in the IL-2 superkine elicit potent phosphorylation of STAT5 and vigorous proliferation of T cells irrespective of IL-2Rα expression. Compared with IL-2, the IL-2 superkine induced superior expansion of cytotoxic T cell and NK, leading to improved antitumor responses *in vivo*. The IL-2 superkine also proportionally reduced the expansion of Treg cells. However, increasing binding affinity for IL-2Rβ still activates peripheral NK/T cells, which may lead to increased risk of toxicity at therapeutic doses [53].

To enhance IL-2 delivery to tumor sites, antibody-based tumor-targeted delivery of IL-2 has been attempted by many groups. IL-2 was fused to antibodies against different tumor antigens, such as EpCAM, CD20, ch14.18 (the human-mouse chimeric variant of 14.18) recognizing ganglioside D2 (GD2) and F8, L19, and F16 recognizing tumor angiogenesis markers. These IL-2/antibody fusions significantly improve antitumor effects with low toxicity than either antibody or cytokine monotherapy in both syngeneic mouse models and a human melanoma xenograft model. Therapeutic effects were mediated by both NK cells and CD8$^+$ T cells, and reduced IL-2 retention in peripheral blood led to lower toxicity [54–56]. However, despite their improved tumor-targeting ability, these IL-2/antibody fusions can still lack potent antitumor efficacy [57]. As these fusion proteins still contain native IL-2, they may still exhibit increased binding and activation of Tregs over effector T cells. Multiple groups have thus combined the IL-2/IL-2R mutation and tumor-targeting antibody fusion strategies. Merck KGaA developed NHS-IL2LT (with LT standing for low toxicity), which contains an IL-2 (D20T) variant fused with an antibody (NHS76) that targets the necrotic core of tumors. NHS-IL2LT showed the lowest toxicity in clinical trials among all IL-2 variants to date [58]. Roche Pharma developed CEA-IL2v, which fuses an IL-2 mutein with an antibody that targets carcinoembryonic antigen (CEA). The mutein includes IL-2Rα affinity reducing mutations F42A, Y45A, and L72G. CEA-IL2v also induces significantly decreased mortality in a mouse CEA overexpressing colon cancer model compared with a native IL-2/CEA antibody fusion [59]. However, CEA-IL2v was abandoned due to lack of efficacy as monotherapy and in combination with check-point inhibitors (CPI) in clinical trials. In contrast, DI-Leu16-IL2 (anti-CD20 fused to wild-type IL2), when given subcutaneously, has resulted in several complete responses at doses with little or no side effects in a phase I dose-escalation study [60]. Another IL-2/antibody fusion, termed SumIL2, was invented by incorporating both F42A (an IL-2Rα affinity reducing mutation) and the IL-2 superkine mutations to reduce Treg binding and increase binding CD8+ T cells at the meanwhile [61]. SumIL2 is fused to an EGFR-targeting antibody. This fusion protein induced much more potent antitumor efficacy against murine cancers that overexpressed EGFR than SumIL2 fused to a nonexpressed tumor-associated antigen in preclinical models. This SumIL2/anti-EGFR fusion also preferentially binds to CD8$^+$ T cells instead of Treg cells, resulting in improved tumor control over fusion proteins containing only F42A or IL-2 superkine [61]. However, EGFR is widely expressed in human tissues. Systemic delivery of the SumIL2/anti-EGFR fusion protein may still result in some off-target effects despite its high EGFR tumor-targeting capability.

Because IL-2 can complex with cell surface IL-2Rα and subsequently bind with high affinity to IL-2R$\beta\gamma$, some groups aim to mask the amino acids on IL-2 that would normally first bind to IL-2Rα. One "natural" mask complex links IL-2 to IL-2Rα via a glycine/serine amino acid linker. In addition to masking IL-2 from endogenous cell surface IL-2Rα, soluble IL2Rα would also stabilize the structure of IL-2 for binding to IL-2Rβ. This approach was used to create the IL-2/IL-2Rα complex denoted ALKS 4230. ALKS 4230 has been observed to reduce peripheral toxicity and inhibit B16 melanoma metastases more effectively than wild-type IL-2 in mice [62]. Another example of a masked IL-2 involves complexing IL-2 to an IL-2 mAb. The investigators specifically used an IL-2 antibody that strongly binds to the IL2Rα-binding region of IL-2, thus creating an IL-2/mAb complex that is unable to bind to endogenous IL2Rα. Similar to the IL-2 superkine, this IL2Rβ preferential IL-2/mAb complex could more potently expand CD8 T cells and inhibit B16 primary tumor growth compared with wild-type IL-2 [63, 64]. In addition to the natural complex, this same group also rearranged the α-helices of IL-2 to graft IL-2 into the mAb. This grafted IL-2/mAb complex even more effectively induces antitumor immunity, expanding more CD8 T cells and fewer Tregs to more potently eliminate 4T1 and B16 metastases [65].

Like the IL-2/mask complexes, a protease-activated IL-2 is also a fusion protein that includes a mask. However, IL-2 is linked to the mask by a protease cleavable linker. The choice of the protease cleavage substrate is sensitive and specific for a protease that is preferentially overexpressed in the tumor. One group developed two protease-activated IL-2 fusion proteins that incorporate IL-2, IL-2Rα, and a cleavable linker specific to either prostate-specific antigen or matrix metalloproteinase (MMP). This fusion protein was able to inhibit peritoneal tumor cell growth in a mouse model [66]. Another group synthesized a protease-activated IL-2 fusion protein that incorporates multiple IL-2 enhancing strategies, including using the SumIL2 mutation to reduce Treg binding, fusion to Fc to increase half-life, and MMP cleavage activation in the tumor to reduce toxicity. This fusion protein reduced pulmonary and liver toxicity than the equivalent "cleaved" SumIL2 without compromising tumor-infiltrating lymphocyte (TIL) expansion and antitumor efficacy [67]. In summary, next-generation IL-2 variants can reduce toxicity while increasing TILs, which allows for more effective control of both primary and metastatic tumors.

IL-15. IL-15 is mainly produced by activated myeloid cells, such as monocytes, macrophages, and DCs [68]. When bound to the transmembrane IL-15Rα, IL-15 is transpresented to NK cells and T cells-expressing IL-2/IL-15Rβ and the common γ chain receptor. Notably, IL-15 is critical for NK cell development and the homeostasis of memory CD8$^+$ T cells [69, 70]. IL-15 also affects other types of immune cells, such as innate lymphoid cells [71], further highlighting its role in potentiating the immune response. Preclinical observations strongly support the antitumor activity of IL-15 mediated by NK cells and T lymphocytes [72, 73]. More importantly, unlike IL-2, IL-15 does not stimulate Tregs since IL-15 does not bind to the IL-2Rα chain, which is required for the formation of the high-affinity receptor complex (IL-2R$\alpha\beta\gamma$) on Tregs to stimulate immunosuppressive signals [74].

A major challenge to clinical application of IL-15 is its short half-life and insufficient effectiveness *in vivo* [75, 76]. Preclinical studies indicate that the IL-15/IL-15Rα dimer, rather than the IL-15 monomer, is more bioactive when transpresented to NK and CD8$^+$ memory T cells [77–79]. Fusion protein RLI, which consists of IL-15 linked to the cytokine-binding (sushi) domain of IL-15Rα, displayed super-agonistic activity towards the IL-15Rβ/γ complex and exerted potent antitumor properties *in vivo* [80]. ALT-803, another variant of IL-15 that encompasses human IL-15 covalently linked to the sushi domain of human IL-15Rα fused to an IgG1 Fc domain, has been tested in clinical trials. In the dose-escalation phase I clinical study with this recombinant protein, 33 patients with hematological cancer received ALT-803. In all, 19% of patients met the criteria for clinical benefit [81]. However, ALT-803 did not induce significantly better patient outcomes than DI-Leu16-IL2 [60]. Another clinical trial involves combining ALT-803 therapy with anti-PD-1 therapy in 21 patients with metastatic Non-small-cell lung carcinoma (NSCLC). In all, 29% of patients achieved an objective response with a tolerable safety profile [82]. However, the response rate has no advantage compared with the historical response rates of immune checkpoint blockade (ICB) alone [83]. All patients showed increased circulating NK and CD8$^+$ T cells in the above clinical trials, indicating that IL-15-associated toxicity may exist at certain high doses [84].

In order to reduce IL-15/IL-15Rα dimer toxicity, several novel IL-15 variants have been developed and tested in preclinical studies. For example, Neo-2/15 is a computationally designed *de novo* protein mimic of IL-2 and IL-15 that binds to human and mouse IL-2R$\beta\gamma$ chains but not to IL-2Rα or IL-15Rα. Neo-2/15 can mimic the natural signaling function but does not carry the adverse effects induced by IL-2Rα or IL-15Rα binding. Neo-2/15 has been demonstrated to exhibit potent therapeutic activity with reduced toxicity in murine tumors [85]. Other clinical studies involving IL-15 proteins combined with other immunotherapies are ongoing, such as recombinant IL-15 with both anti-CTLA-4 and anti-PD-1 therapy, with a CD40 agonist, and with mAbs including anti-CD52 and anti-CD20 antibodies [71, 86].

IL-21. IL-21 is a 4α-helix bundle cytokine produced by follicular helper T (Tfh) cells, T helper 17 (Th17) cells, and natural killer T (NKT) cells. IL-21 signals via heterodimerization with the IL-21 receptor (IL-21R) [87, 88] and the common cytokine receptor γ-chain. Functional IL-21R is broadly expressed in hematopoietic cells, including T and B lymphocytes, NK cells, and myeloid cells [89]. IL-21 exerts pleiotropic functions by supporting the differentiation of CD4$^+$ Th17 [90–92] and Tfh cells [93], facilitating the maturation and enhancing the cytotoxicity of CD8$^+$ T cells and NK cells, and promoting the differentiation of memory CD8$^+$ T cells [88, 94–96]. In addition, IL-21, in contrast to IL-2, blunts Treg expansion by suppressing Foxp3 expression and favors the enrichment of antigen-stimulated CD8$^+$ T cells [97].

Clinical phase I/II studies have shown that nontumor targeting free IL-21 induces objective responses or disease stabilization in a fraction of metastatic melanoma and renal cancer patients [98–101]. However, systemic IL-21 therapy fails to achieve adequate concentrations in the TME to activate TILs due to its short half-life and peripheral consumption, which necessitates a high dose that induces significant toxicity. Local intratumoral administration of recombinant IL-21 is difficult to administer for most patients. Low patient responses to IL-21 limit its further administration in the clinic [102]. To address this limitation, immunocytokine fusion proteins have been generated. By conjugating IL-21 to tumor or T cell-targeting antibodies, IL-21 can be delivered directly to markers, such as PD-1 on T cells, which are more highly expressed in the tumor. Such conjugations can counterbalance the high affinity of a cytokine to ubiquitously expressed cytokine receptor on immune cells in nontumor tissues and therefore decrease toxicity [103]. An anti-PD-1/IL-21 mutant fusion protein, which incorporates a highly attenuated IL-21 mutein (R9E: R76A) fused to a PD-1 antibody, was designed to restrict cytokine activity to the targeted PD-1$^+$ cells and exhibits an improved *in vivo* half-life [101]. This anti-PD-1/IL-21 fusion exhibits antitumor efficacy in a humanized mouse model that is refractory to anti-PD-1 monotherapy. Alternatively, it has also been shown that anti-CD20 and anti-EGFR fusions to IL-21 prolong the half-life of IL-21 and enhance its antitumor efficacy in non-Hodgkin lymphoma and colon adenocarcinoma models [104, 105]. Compared with free IL-21, systemic administration of anti-EGFR/IL-21 improves safety by enabling targeted delivery to EGFR positive tumor tissue. This anti-EGFR/IL-21 fusion protein can also synergistically combine with ICB therapy. ICB can "release the brakes" of host immune responses, but may have a limited effect on the rapid expansion of functional TILs. Providing T cell growth factors, for example, tumor-targeting IL-21, may more directly expand TILs. Mechanistically, anti-EGFR/IL-21 can increase antigen-specific CD8$^+$ T function and proliferation, especially the PD-1^{int}Tim-3$^-$ population. In preclinical models, combining anti-EGFR/IL-21 therapy with ICB therapy achieved significantly increased antitumor effects and decreased mortality compared with any single treatment.

IL-12. IL-12 is a heterodimer composed of two independent subunits linked together by disulfide bonds: α (IL-12p35) and β (IL-12p40). IL-12 is mainly produced by activated APCs such as DCs, macrophages, monocytes, and B cells. IL-12 binds to the IL-12 receptor (IL-12R), which is expressed as a high-affinity heterodimer of IL-12Rβ1 and IL-12Rβ2 subunits, on T cells and NK cells. The β1 subunit is constitutively expressed on immune cells, whereas expression of the β2 subunit is upregulated in T cells and NK cells upon activation [106]. In preclinical studies, systemic administration of recombinant mouse IL-12 elicited potent antitumor effects in various mouse models. IL-12 stimulates the effector functions of activated T cells and NK cells via induction of cytotoxic enzymes and cytokines, such as IFN-gamma (IFN-γ), which are required for potent antitumor immunity [107, 108].

Table 3. Engineered IL-12 variants

Drug name	Reference	Drug features	Drug benefits	Clinical phase
IL12-GEMy	Kaczanowska *et al.* [119]	Genetically engineered myeloid cells that express IL-12	Reverses immune suppression in the premetastatic niche, reduces primary and metastatic tumor burden through T and NK cell activation	Preclinical
CAR-T-expressing IL-12	Zhang *et al.* [122]	Tumor-infiltrating lymphocytes engineered to secrete IL-12	Induces objective responses in metastatic melanoma patients, but induces toxicity	I/II
CBD-IL-12	Mansurov *et al.* [123]	Collagen-binding domain conjugated to IL-12	Localizes to exposed collagen on tumor vasculature, induces sustained levels of IFN-γ in TME, reduces toxicity	Preclinical
NHS-IL-12	Xu *et al.* [126]	DNA/DNA–histone complex antibody conjugated to IL-12	Combines with anti-PDL1 to potently expand and activate T cells in tumor tissue, expands TCR diversity of tumor-infiltrating T cells in human patients	I/II
IL-12 mutein partial agonists	Glassman *et al.* [128]	Includes mutations that decrease IL-12 affinity for IL-12Rβ1	Preferentially supports CD8 T cell function over NK cells, producing potent antitumor immunity with reduced toxicity	Preclinical

However, systemic delivery of IL-12 in the clinic at therapeutic doses has been limited due to its short half-life *in vivo*, severe side effects, and lack of tumor targeting [109, 110]. In clinical studies, antitumor effects of IL-12 have been evaluated in numerous malignancies such as cutaneous T cell lymphoma, acquired immunodeficiency syndrome (AIDS)-related Kaposi sarcoma, and non-Hodgkin's lymphoma. However, frequent systemic administrations of IL-12 have been associated with severe host toxicity through the undesirable release of proinflammatory cytokines from peripheral T and NK cells. Therefore, various strategies have been implemented to improve efficacy with reduced toxicity (Table 3). In current clinical trials, intratumoral delivery of IL-12 via plasmid or virus vectors has been observed to generate systemic adaptive immune responses, activate or reactivate tumor-infiltrating CD8^{+} T cells, and control both primary tumors and metastases [111, 112].

Many preclinical studies have used IL-12-transduced cells for local cancer therapy. DCs [113–115] and macrophages [116–118] have been engineered to express IL-12. Transduction of these APCs increases their ability to induce robust antitumor immune responses. In a recent study, genetically engineered myeloid cells (GEMys)-expressing IL-12 (IL12-GEMy) successfully reversed immunosuppression in the premetastatic niche and reduced metastatic and primary tumor burden through T and NK cell activation [119]. Adoptive T cell therapy has potent efficacy against hematologic malignancies, but has yet to achieve clinical benefit against solid tumors. Engineering Chimeric antigen receptor T (CAR-T) cells to express IL-12 using an Nuclear factor of activated T-cells (NFAT)-responsive promoter is under investigation to improve antitumor efficacy against solid tumors [120, 121]. Etxeberria *et al.* [122] also engineered tumor-specific CD8^{+} T cells that transiently express IL-12. Preclinically, when injected intratumorally but not intravenously, these engineered T cells induced to complete rejections not only of the injected lesion but also of distant concomitant tumors [123]. Further improvement of this strategy is necessary to achieve longer-lasting tumor control and systemic administration to avoid the difficulties involved with local intratumoral injection.

In addition, tumor-targeting antibody fragment/IL-12/Fc fusions have been synthesized to increase IL-12 half-life and delivery to tumor sites. Indeed, tumor-targeting antibody/IL-12 fusion proteins selectively accumulate in tumors following intravenous administration in multiple murine tumor models [124, 125]. Mechanistically, the fusion protein alone can stimulate effective CD8^{+} T cell-mediated antitumor immune responses, activate NK cells, and induce IFN-γ production and STAT4 phosphorylation [124, 125]. More importantly, reducing IL-12 retention in peripheral tissues and blood leads to less adverse weight loss. Finally, the fusion protein controls both primary and metastatic tumor growth more effectively than either antibody or cytokine monotherapy.

A variety of cytokines, including IL-12 immunocytokines, are also administrated in combination with ICB therapy. Because simply removing suppression by ICB may not restore sufficient immunity against tumors, additional T cell cytokines may be necessarily to expand effector TILs and sustain antitumor immunity. For example, the administration of NHS-muIL-12, which incorporates an antibody (NHS76) recognizing DNA/DNA–histone complexes fused with two molecules of murine IL-12, with avelumab (an anti-PDL1 antibody) expanded CD8^{+} T cells and enhanced T cell activation in tumor tissue more than either agent alone [126]. Furthermore, these combined treatments increased proliferation of cytotoxic NK and CD8+ T cells, T-bet expression, plasma cytokine levels, and messenger RNA levels of innate and adaptive immune genes [127]. Preliminary clinical trials report increased NK cell frequencies in the peripheral blood and broadened T-cell receptor (TCR) diversity of tumor-infiltrating T cells in patients receiving NHS-IL-12. In addition, five out of 59 patients experienced stable disease conditions, and a dose-tolerated safety profile was also reported [128].

Nevertheless, such immunocytokine fusions retain complete cytokine activity in circulation, allowing them to interact with circulating lymphocytes and induce cytokine-mediated toxicities. In another recent study, a set of IL-12 mutations were screened out to preferentially activate CD8+ T cells but not NK cells [129]. Based on critical differences in IL-12Rβ1 expression between activated CD8+ T cells and NK cells, Glassman *et al.* [129] introduced a series of alanine mutations in murine p40 to reduce the affinity to IL-12Rβ1. These IL-12 partial agonists preferentially support T cell function and preserve IFN-γ induction by CD8+ T cells while impairing cytokine production from NK cells *in vitro*. Finally, the IL-12 partial agonists produced potent antitumor immunity with reduced toxicity relative to IL-12. Further investigation of the potential immunogenicity of a nonendogenous mutation may assist in the evaluation of the therapeutic potential of this IL-12 variant. Taken together, systemic administration of IL-12 is still challenging in the clinic because of many unwanted adverse effects.

Next-generation pro-cytokines in cancer immunotherapy

All of the modified cytokines mentioned above are innovatively designed to reduce toxicity and improve the antitumor efficacy of systemically delivered cytokine drugs. Many biotechnology and pharmaceutical companies have conducted multiple clinical trials for cancer therapies with these modified cytokines [49, 58, 60, 130]. Balancing maximum cytokine activity and minimum peripheral toxicity is crucial for clinical cytokine immunotherapy. Tumor-targeted therapy has been explored to solve this problem, but the potential expression of the targeted antigens in normal tissue, even at low levels, often results in off-target effects. In addition, immunogenicity is a general issue for all muteins and conjugated nonendogenous proteins. Promising next-generation cytokine prodrugs (“pro-cytokines” [67]) may overcome the above obstacles in multiple mouse tumor models. Ideal pro-cytokines mask cytokine activity with blocking polypeptides, such as natural cytokine receptor-binding domains, to achieve minimal toxicity. Using natural cytokine receptors minimizes immunogenicity to the host without compromising tight control over the reduction in peripheral cytokine activity. More extended receptor domains potentially block cytokines more effectively but might reduce efficacy, whereas shorter domains may enhance efficacy but increase toxicity. Moreover, different receptor domains can be linked to cytokine subunits with a specific peptide sequence selectively cleavable by proteases that are overexpressed in the TME. This strategy masks the toxic activity of the cytokine until it enters tumor tissue, thus providing effective “passive” tumor targeting. Although tumors may enter a dormant phase, metastasis is always accompanied by the production of various tumor-specific proteases, which may indicate increased activation of protease activated cytokines in more advanced cancers [131]. In addition, different types of tumors may contain distinguished proteases, which can be evaluated for use in pro-cytokine engineering and design. Therefore, personalized pro-cytokines may potentially significantly improve patient outcomes. This concept is similar to the technology of antibody-directed enzyme prodrug therapy (ADEPT). However, in ADEPT designs, either extraordinarily high or specific expression of tumor antigens is required. In contrast, pro-cytokines target membrane-type proteases, for example, MMP 14, a membrane-bound MMP expressed on tumor cells and inflammatory cells, is greatly enriched inside the TME of almost all tumors [132, 133]. The membrane-bound property of such proteases also decreases the potential leakage of pro-cytokines out of tumor tissue after entering the TME. These tumor-specific membrane-bound MMPs therefore increase antitumor efficacy and tumor targeting of pro-cytokines. Moreover, the pro-cytokine concept is highly flexible and easily applicable to different cytokines and fusion proteins. Altogether, multiple approaches to reducing toxicity without compromising antitumor efficacy generate a diverse portfolio of therapeutic modalities, holding considerable promise for translation into effective antitumor immunotherapy.

DATA AVAILABILITY STATEMENT

All data included in this review are available upon request by contact with the corresponding author.

CONFLICT OF INTEREST STATEMENT

The authors declared that they have no conflicts of interest to this work.

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