

REVIEW

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Advanced cytokine-based immunotherapies: targeted *cis*-delivery strategies for enhanced anti-tumor efficacy and reduced toxicity

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ABSTRACT

First-generation cancer immunotherapies, such as high-dose interleukin-2 (IL-2), have demonstrated clinical efficacy, but are limited by significant systemic toxicities due to their broad expression of cytokine receptors. This has driven the iterative development of targeted cytokine delivery strategies. Early efforts focused on receptor-biased IL-2 variants designed to attenuate or abrogate IL-2 receptor α (IL-2 R α /CD25) binding. Subsequently, the concept of “*cis*-targeting” has emerged as a strategy to deliver cytokines to specific immune cell populations, enhancing anti-tumor responses while mitigating systemic toxicity. This review highlights key common γ -chain cytokines (IL-2, IL-7, IL-15, and IL-21) as well as IL-12, providing an overview of their structures, receptors, as well as their distinct T cell functions. Furthermore, we specifically focus on the current landscape of engineered cytokine variants that facilitate targeted cytokine delivery in *cis* to specific T cells. By successfully restricting cytokine activity to specific T cell populations, *cis*-targeting approaches represent a promising strategy in the field, enabling efficient immunotherapies with improved tolerability and enhanced anti-tumor responses.

ARTICLE HISTORY

Received 29 August 2025

Revised 10 November 2025

Accepted 11 November 2025

KEYWORDS

Immune checkpoint inhibitors; cytokines; Interleukin-2; immunocytokine *cis* targeting

Introduction

The observation that the immune system can mount an anti-tumor response led to the exploration of ways in which this immune response could be leveraged, both endogenously and exogenously. These strategies include methods designed to precisely modulate various stages of T cell priming, activation, differentiation, and migration to the tumor. Immune checkpoint inhibitors (ICIs) are among the most widely used therapies, and act by releasing negative regulation on progenitor exhausted, tumor antigen-specific T cells.^{1–3} These immune checkpoints include programmed cell death protein 1 (PD-1), cytotoxic T-lymphocyte associated protein 4 (CTLA-4), lymphocyte-activation gene 3 (LAG-3), T-cell immunoglobulin and mucin-domain containing-3 (TIM-3) and T cell immunoreceptor with Ig and ITIM domains (TIGIT). All these molecules possess distinct, non-redundant functions, relevant at different stages of T cell activation and exhaustion. Another widely studied strategy is tumor-infiltrating lymphocyte (TIL) therapy, in which autologous tumor-reactive T cells are activated and expanded *ex-vivo* and reinfused into the patient, eliciting a more potent response.⁴ Both ICIs and TIL therapy aim to improve endogenous anti-tumor T cell efficacy and durability by primarily leveraging Signal 1 (antigen recognition) and Signal 2 (co-stimulation).^{2,5}

Beyond these approaches, T cell engineering has made significant progress, broadening both the therapeutic scope as well as the efficacy of such therapies. Cell-based personalized therapies involve the use of genetically modified T cells that possess engineered chimeric antigen receptors (CARs) or T cell receptors (TCRs) to target tumor cells.⁶ CARs are engineered to recognize tumor-associated antigens (TAAs) located at the surface of cancer cells, functioning in a major histocompatibility complex (MHC)-independent manner. This allows for the targeting and killing of cancer cells without TCR-mediated antigen recognition (Signal 1) and CD28 or 4-1BB stimulation (Signal 2), since both functions are integrated into

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the CAR construct.⁷ TCRs are engineered to recognize human leukocyte antigen (HLA)-presented peptides, derived from both surface as well as intracellular antigens, conferring the capacity to recognize a wider range of TAAs.⁸ In addition to cell therapies, T cell engagers (TCEs) act by bridging T cells and tumor cells, thereby enabling the formation of an immunological synapse, ultimately resulting in tumor cell killing.^{9,10} These two strategies bypass the need for MHC-dependent antigen recognition and TCR specificity, resulting in broader therapeutic applicability across different tumor types.¹¹ While TCEs are used successfully for the treatment of hematological malignancies, their efficacy in solid tumors is limited to a few tumor antigens that are not broadly expressed in normal tissues (e.g., DLL3 and STEAP1).^{9,10} Additionally, other limiting factors include tumor heterogeneity and an immunosuppressive tumor microenvironment (TME).¹²

Regardless of the initial mechanism of activation, the sustained expansion, differentiation, and survival of T cells is highly reliant on a third signal (Signal 3). Signal 3 is provided by cytokines, such as interleukin 2 (IL-2), IL-12, interferon γ (IFN γ) and IL-7, which are essential for immune cell function and homeostasis.¹³ The absence of an adequate Signal 3 results in sub-optimal T cell activation and compromised effector functions.¹⁴ The critical role of different cytokines in T cell biology and immune cell function has driven the development of immunotherapies that directly harness cytokine signaling pathways.

The first immunotherapy to be approved by the Food and Drug Administration (FDA) was IL-2 therapy, for the treatment of metastatic melanoma and renal cell carcinoma (RCC).^{15,16} IL-2, originally termed as “T-cell growth factor,” showed promise as an efficient immunotherapy, inducing considerable remission in a cohort of patients. However, despite its ability to boost the anti-tumor immune response, its efficacy was significantly hampered by dose limiting toxicities (DLTs) due to its unintended targeting of endothelial cells, natural killer (NK) cells and immunosuppressive regulatory T cells (Tregs).¹⁷ These considerable limitations prompted research efforts to identify safer ways to administer the cytokine, whilst not compromising its efficacy. In addition to IL-2, the role of other cytokines, such as IL-7, IL-15, IL-12 and IL-21 in modulating the immune response became apparent, with a deepening understanding of their biology and functions.¹⁸

The importance of these cytokines in positively modulating the anti-tumor immune response has catalyzed the development of engineered stroma- and immune-targeting cytokine variants. The latter are designed to have biased-receptor binding and signaling, which, together with the antibody targeted delivery, allow for *cis*-activity (on the same cell), which results in synergies that elicit more robust responses. Importantly, the efficacy of these next-generation cytokines is contingent upon the presence of the target cells, most often CD8⁺ T cells, in the tumor and tumor draining lymph nodes (TdLNs). Therefore, developed molecules aim to provide a dual benefit, by redirecting these cells to the TME and promoting their *in situ* expansion and engagement at the tumor site.^{19–21} In this review, we aim to delineate the importance of these key cytokines in potentiating the anti-tumor response, with a specific focus on their *cis*-delivery to T cells.

The biology of cytokines

Cytokines are cell signaling molecules that are essential mediators of the immune response.²² They are secreted by diverse cell types and possess pleiotropic functions involved in regulating immune homeostasis and response, typically acting in a locally confined manner. Cytokines act by binding to their complementary receptors expressed on different cell types, triggering intracellular signaling cascades that elicit various downstream processes, such as the activation of specific transcription pathways. Cytokines are needed not only to maintain the homeostasis of the immune system, but are also involved in the mediation of an immune response upon pathogen encounter, or during immune surveillance to detect pre-cancerous cells before tumor formation.²³

In this context, the proliferation, differentiation, and survival of T cells is reliant on the presence of certain key cytokines. More specifically, CD8⁺ T cells rely on cytokines such as IL-7, IL-15, IL-2, and IL-21 for their maintenance and function (Figure 1). These cytokines bind to receptors composed of α , β , and γ subunits, all of which share the common γ chain subunit and, along with interleukin-4 (IL-4), and interleukin 9 (IL-9), form the γ chain receptor family. IL-2 and IL-15 also share a common β subunit

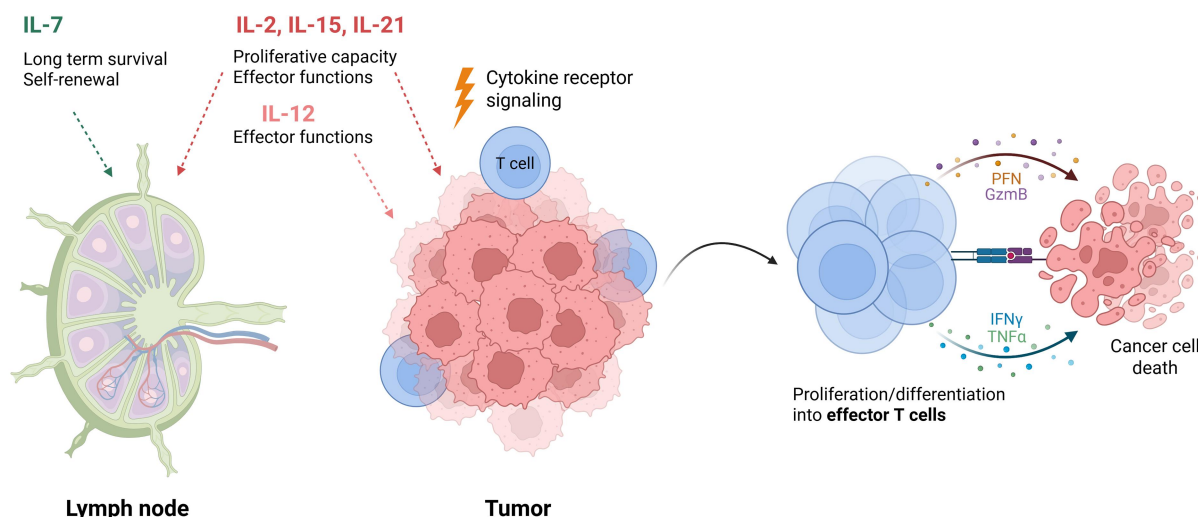


Figure 1. High-level overview of cytokines function. Simplified representation of cytokines primary function with IL-7 playing a key role in long-term survival and self-renewal in lymphoid organs. In a tumor context, IL-2, IL-15, and IL-21 enhance proliferative capacity and effector functions whereas IL-12 primarily drives effector cell functionality. Effector T cells boosted by the action of different cytokines contribute to tumor cell killing. Created with BioRender.com.

(CD122). The four cytokines (along with IL-7 and IL-21) possess distinct, private α subunits, CD25, CD215, CD127, and IL-21 R, respectively.^{23,24}

The binding of these cytokines to their receptors triggers the recruitment of Janus Kinases (JAK1/3), which phosphorylate and thus activate three main transcriptional pathways: Signal Transducers and Activators of Transcription (STAT),^{1,3,5} Phosphoinositide 3-kinase (PI3K)/AKT, and Mitogen-Activated Protein Kinase (MAPK) (Figure 2A). These pathways are involved in the transcription of genes associated with cell activation, proliferation, differentiation, and survival.²³

In steady state, these cytokines are secreted constitutively at low concentrations by the immune system and contribute to the maintenance of immune homeostasis. During an immune response, these cytokines are secreted at higher levels, and their local secretion and consumption in an autocrine and paracrine manner is critical in eliciting a proficient immune response.²⁵ Therefore, the systemic administration of high-dose cytokines in clinical settings frequently elicits significant toxicities and adverse events.²⁴ These undesired effects occur because cytokines possess common receptor subunits that are widely expressed on different cells, both immune and nonimmune, resulting in on-target, but off-tumor, activation, leading to dose-limiting toxicities. Furthermore, the inherent pleiotropy of these cytokines and their receptors poses an additional challenge to the system. While natural cytokines act locally, providing short-lived cytokines receptor agonism, exogenously administered cytokines can activate target cells constitutively and indirectly activate bystander cells in the TME through the production of endogenous cytokines. These endogenously secreted cytokines can potentially compete with the therapeutic cytokine for receptor binding and initiate unwanted signaling on unintended cell subsets, thus attenuating the effect of the therapy.^{26,27}

Interleukin 2

IL-2 is a key pleiotropic cytokine involved in maintaining immune homeostasis. It is primarily synthesized by activated CD4⁺ T cells, but can also be secreted at lower levels by CD8⁺ T cells, NK cells and activated dendritic cells (DCs).¹⁷ In steady state conditions, IL-2 is needed for the maintenance and function of immunosuppressive naturally occurring Tregs. This specialized subset of CD4⁺ CD25⁺ FOXP3⁺ T cells is involved in maintaining peripheral immune tolerance by regulating immune responses.²⁸ These cells constitutively express the trimeric, high-affinity receptor composed of all 3 subunits (CD25, CD122, and CD132). CD25 is not directly involved in signaling, but acts as an anchor, increasing the affinity of the receptor for IL-2.²⁹ Tregs constitutively express high levels of CD25, providing them with a competitive advantage over other T cell subsets that are CD25^{low} or CD25^{neg} in steady state, in the consumption of low

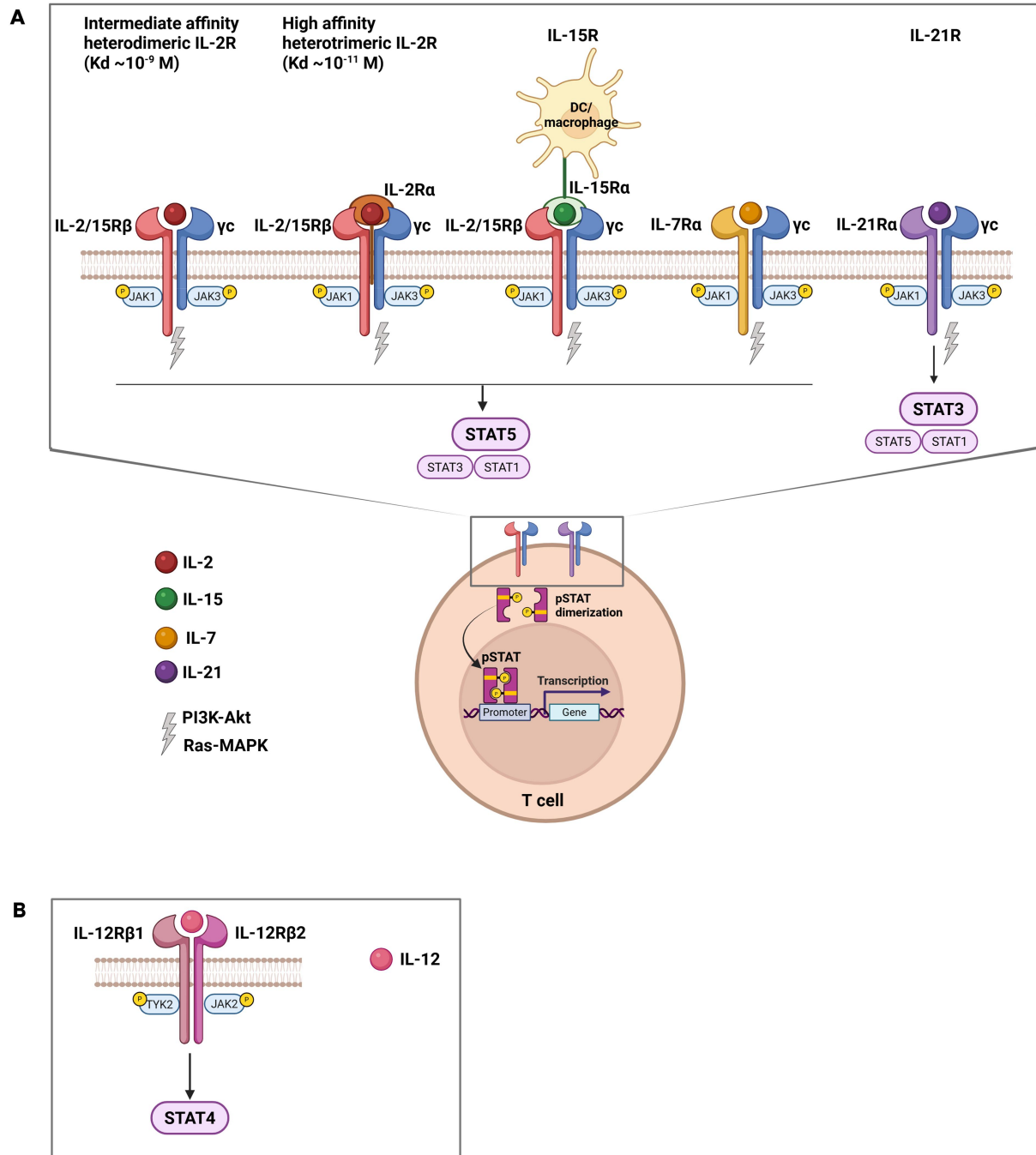


Figure 2. Cytokines receptor composition and downstream signaling. a) Graphical illustration of common γ chain cytokine receptors for IL-2 (intermediate affinity dimeric and high affinity trimeric), IL-15, IL-7 and IL-21. Binding of the cytokine to its specific dimeric or trimeric receptor induces tyrosine phosphorylation by JAK1/3 and STAT1/3/5 recruitment. Phosphorylated STATs dimerize and translocate into the nucleus to activate the transcription of specific target genes. IL-2, IL-15 and IL-7 primarily signal via STAT-5 and IL-21 via STAT-3 as depicted in bold. In addition to the JAK/STAT pathway, common γ chain cytokines downstream signaling also includes the PI3K-Akt and ras-MAPK pathways that contribute to cell survival, proliferation and differentiation. b) Graphical illustration of IL-12 receptor and signaling cascade via TYK2/JAK2 primarily inducing STAT-4 activation. JAK: Janus kinase; STAT: Signal transducer and activator of transcription; PI3K: phosphatidylinositol 3-kinase; Akt: protein kinase B; MAPK: mitogen-activated protein kinase. TYK2: tyrosine kinase 2. Created with BioRender.com.

levels of IL-2. Upon T cell activation, CD8⁺ and CD4⁺ T cells upregulate CD25, leading to the formation of the high affinity trimeric receptor complex. This heterotrimeric IL-2 receptor (IL-2 R) complex enables activated T cells to bind available IL-2, supporting their survival, proliferation, and differentiation in potent effector CD8⁺ T cells in scenarios such as acute infection.³⁰ In the context of prolonged TCR stimulation, such as chronic infection or cancer, ongoing antigen stimulation drives progressive reduction in T cell effector function, a process known as T cell exhaustion,³¹ and generates a pool of PD-1⁺ TCF-1⁺ stem-like CD8⁺ T cells, also known as progenitor exhausted CD8⁺ T cells (T_{PEX}). These T_{PEX} are not terminally exhausted; they retain the expression of the key transcription factor, T cell factor 1 (TCF-1), which maintains their stemness and self-renewal capabilities, while the transient downregulation of TCF-1 drives the acquisition of T cell cytotoxic effector functions.^{32,33} T_{PEX} have been shown to be critical for the clinical efficacy of anti-PD-1 therapy.³⁴

Recent seminal work has uncovered the effect of IL-2 R agonism on T_{PEX}. These findings demonstrated that the delivery of IL-2 to T_{PEX} induces an alternative differentiation path, whereby these cells give rise to a distinct subset of “better effector” cells, as opposed to terminally exhausted cells. This transcriptionally and phenotypically distinct population is highly proliferative and cytotoxic, and shows superior efficacy in chronic infection and tumor models,^{30,35–37} highlighting the importance of IL-2 in sustaining productive T cell responses. The critical role of T_{PEX} in driving an efficacious anti-tumor response is underscored by continuous research of strategies to target this population and give rise to more functional and long-lasting effector T cells for the benefit of patients. Although IL-2 therapy shows durable responses in a subset of patients experiencing tumor remission, its clinical application is limited by moderate to severe systemic toxicities,³⁸ mainly related to the broad expression of IL-2 receptors on various cell types. For instance, the binding of IL-2 to CD25 expressed on endothelial cells^{29,39} contributes to vascular leak syndrome (VLS), a key complication associated with IL-2 therapy, characterized by the increased permeability of blood vessels leading to hypotension, pulmonary edema, and renal failure.⁴⁰ In the context of an efficacious anti-tumor immune response, one of the main unwanted target cells of IL-2 therapy are Tregs, which out-compete other T cells for the consumption of exogenous IL-2, further dampening the anti-tumor response. The activation of effector CD8⁺ T cells by IL-2 therapies might lead to the secretion of endogenous IL-2, which can trigger the expansion of Tregs, limiting the efficacy of the engineered therapy.⁴¹ In addition, IL-2 has unfavorable pharmacokinetics (PK), as it is rapidly metabolized by the kidney, resulting in a very short half-life of minutes. As a result, it needs to be administered frequently, and at high doses, further exacerbating systemic toxicities.¹⁷ These considerable limitations have prompted efforts to find safer ways to administer IL-2 in order to reduce on-target, off-tumor activity by delivering the cytokine to tumor-specific CD8⁺ T cells in a more targeted manner, thus improving the anti-tumor response whilst minimizing the significant side effects associated with the therapy.

Interleukin 15

IL-15 is another important common γ chain cytokine involved in T cell effector function and survival. Although IL-2 and IL-15 share two common subunits, they differ significantly in terms of targeted cells, function, and their ultimate influence on T cell differentiation and persistence. In addition to the γ subunit, the IL-15 receptor (IL-15 R) is composed of the IL-2 R β subunit (CD122) and a private α subunit, CD215, which has a high affinity for IL-15.^{42–44} The binding of IL-15 to its receptor complex induces the same signaling pathways as those induced by IL-2 binding (STAT5, PI3K, and MAPK).⁴⁵ These common pathways indicate several overlapping functions of both cytokines, such as proliferation, survival, and acquisition of effector functions by T cells. Besides their similarities, they also possess very distinct and unique roles in the adaptive immune response. For instance, IL-15 is essential for the survival of CD8⁺ memory T cells,⁴⁶ as well as the maintenance, proliferation, and cytotoxic activity of NK cells.⁴⁷ This is highlighted by studies demonstrating that IL-15-deficient mice lack CD8⁺ CD44^{hi} memory T cells as well as NK cells, resulting in animals more susceptible to pathogens.⁴⁸

One of the main drivers of the differing functions of IL-15 and IL-2 lies in the differential expression of their α subunits. In contrast to CD25, which is mainly expressed on activated T and B cells, CD215 is expressed on activated monocytes and DCs.⁴⁴ Furthermore, unlike conventional cytokines, which are secreted in soluble form, IL-15 is synthesized along with its IL-15 receptor α subunit (IL-15 R α), and is

predominantly membrane bound on the surface of monocytes and DCs.⁴⁹ The dimeric IL-15 $\beta\gamma$ receptors on memory CD8⁺ T cells and NK cells bind in trans to the IL-15/IL-15 R α complex on the surface of myeloid cells, eliciting a downstream signaling pathway.⁵⁰ The Treg-sparing nature of IL-15 is a key therapeutic advance, since conversely to IL-2, Tregs do not possess a competitive advantage for IL-15 over CD8⁺ T cells and NK cells, thereby mitigating inadvertent Treg targeting.⁵¹ This property allows IL-15 to preferentially induce the expansion, survival, and enhanced cytotoxicity of CD8⁺ T cells and NK cells in the tumor. However, IL-15-associated toxicities are a major limitation of its clinical application due to its ability to induce the production of pro-inflammatory cytokines, such as interleukin-1 β (IL-1 β) and tumor necrosis factor α (TNF α).⁵² Preclinical studies demonstrated the potential of IL-15 to induce anti-tumor effects in vivo by boosting cytotoxic T cell effector functions.⁵³ These observations led to the initiation of clinical trials assessing the efficacy of IL-15 as a potential immunotherapy for cancer. Although IL-15 therapy has demonstrated therapeutic activity, severe DLTs as well as its unique binding properties limit its full potential.^{53–55}

Interleukin 21

IL-21 is primarily produced by CD4⁺ T cells (T follicular helper (T_{fh}) and T helper 17 (Th17) cells), natural killer T (NKT) cells as well as by CD8⁺ T cells, albeit at lower levels.⁵⁶ Like the other γ -chain cytokines, IL-21 receptor (IL-21 R) activation induces the recruitment of JAKs and the activation of STAT3 rather than STAT5, PI3K, and MAPK pathways.⁵⁷ IL-21 is known to elicit effects on various immune cells, including B and T cells, NK cells, and even DCs, and plays an important role in inducing the proliferation and differentiation of B, T, and NK cells into their more effector states.⁵⁸ It is known to promote the formation of CD8⁺ stem-like memory T cells (T_{SCM}).⁵⁹ T_{SCM} are typically formed in response to acute infection with the primary role of forming a reservoir of fully functional antigen-specific T cells that upon re-infection are able to differentiate into central memory (T_{CM}) and effector memory cells (T_{EM}).⁶⁰ Although T_{SCM} and T_{PEX} share common characteristics such as self-renewal and multipotency, these two populations arise in different biological contexts and compartments (T_{SCM} upon antigen clearance; T_{PEX} during chronic antigen stimulation^{61,62}), and possess distinct roles. Nonetheless, the observation that T_{PEX} mediate ICB-response⁶³ and that T_{SCM} show superior efficacy in the context of adoptive cell therapies⁶² underscores the importance of both these subsets in inducing strong anti-tumor responses.^{62,64} In addition to its effects on modulating T cell differentiation, IL-21 is also involved in other processes such as apoptosis of DCs and the promotion of antibody production as well as the formation of memory B-cell-like populations.⁶⁵

The versatility of cytokines and their ability to modulate the immune system in various ways is reflected by the fact that, although IL-21 is involved in stimulating the immune response, it is also known to induce inhibitory effects on both lymphoid and myeloid cells. Oftentimes, it does both in tandem, activating some pathways while inhibiting others.⁶⁶ Therefore, although IL-21 has potent immunostimulatory effects on CD8⁺ T cells and NK cells, it is known to inhibit the maturation and activation of DCs, which are necessary for optimal immune responses.⁶⁷ This inhibitory effect is quite critical in the process of T cell priming and activation, making IL-21 a challenging therapeutic option. Based on favorable preclinical studies, clinical trials were initiated to test IL-21 as a potential therapeutic modality, either as a single agent or in combination with other therapies. However, trials have shown that IL-21 therapy is suboptimal in terms of eliciting efficacy as a single agent.²² Furthermore, its multiple and opposing roles demonstrate the need to find ways to direct its activity to specific cell types (such as NK and T cells), and avoid activity on DCs, which would impair the anti-tumor response.

Interleukin 7

IL-7 is known for its indispensable role in the development of the adaptive immune system both in T and B cells.⁶⁸ Pivotal studies showed the importance of IL-7 in the homeostatic survival and formation of naive and memory CD4⁺ and CD8⁺ T cells.^{69,70} Along with its α subunit, CD127, the γ subunit forms a heterodimeric IL-7 receptor (IL-7 R). IL-7 R is expressed on multiple different lymphoid lineage cells, including T cells, pre-B cells, innate lymphoid cells (ILCs), NK cells, monocytes, and macrophages, reflecting the pleiotropic biological roles of IL-7.^{71,72} Like the other common γ -chain cytokines, binding

of IL-7 to its receptor induces the recruitment and activation of JAKs, which in turn promote the common STAT transcriptional pathways (predominantly STAT5, but also STAT1/3), and the PI3K pathway. In addition, the phosphokinase B (PKB) pathway is activated by IL-7 R engagement, involved in B-cell differentiation.⁷³ These pathways promote the transcription of anti-apoptotic factors (such as B-cell lymphoma 2 (Bcl-2)), and the inhibition of pro-apoptotic factors (such as Bcl-2-associated X protein (Bax)).⁷¹ Unlike the other cytokines discussed in this review, IL-7 is mainly produced by non-hematopoietic stromal cells in the bone marrow, as well as thymic and intestinal epithelial cells.⁷⁴ A subset of fibroblastic reticular cells located in the T cell zone of lymph nodes was also identified as a source of IL-7, necessary for the homeostatic proliferation of T cells.⁷⁵ Importantly, IL-7 has been shown to expand naive T cells, resulting in a broadening of the TCR repertoire, which is essential for mounting robust immune responses against novel antigens and heterogeneous tumors.⁷⁶

In addition to its indispensable role in the development and survival of naive T cells, IL-7 is essential for maintaining CD8⁺ memory T cells via STAT5 and, to a lesser extent, STAT3 signaling. Crucially, it dictates the transition of a naive cell into a memory cell, carefully regulating this process to give rise to functional effector cells.^{77,78} Additionally, IL-7's roles in promoting survival and proliferation are key in reversing T-cell exhaustion. This reversal occurs partly by reducing the expression of immune checkpoint receptors, such as PD-1 on T cells,^{79,80} and by modulating the expression of ubiquitin ligases involved in the negative regulation of T-cell activation and transforming growth factor- β signaling.^{80,81} Beyond the T-cell compartment, IL-7 also promotes the survival and development of NK cells, further highlighting its diverse functional roles in eliciting an efficient immune response.⁶⁸ The multi-faceted immunomodulatory effects of IL-7 make it an interesting target in the context of immunotherapy. A promising aspect of clinical trials so far is the fact that IL-7 based therapies are relatively well tolerated in comparison to the other cytokines previously discussed.⁶⁸ In addition, since Tregs express low or absent levels of CD127, IL-7 preferentially confers a competitive advantage to conventional T cells and CD8⁺ T cells over Tregs, a critical characteristic that distinguishes it from the other common γ -chain cytokines.⁸² Specifically, CD127 is expressed at high levels by naive T cells that might represent an important peripheral sink for untargeted IL-7 therapies. Furthermore, CD127 is also expressed on T_{PEX}, which highlights the role of IL-7 in T_{PEX} maintenance.⁶⁸ Therefore, to realize its full therapeutic potential, IL-7 therapies may benefit from being delivered via a more targeted approach.

Interleukin 12

IL-12 is a disulfide-linked heterodimeric cytokine composed of two distinct subunits, the α (p35) and β (p40) chains.⁸³ The genes encoding these subunits are typically expressed on different chromosomes, necessitating their coordinated expression within the same cell for the production of active and functional IL-12.⁸⁴ The biological activity of IL-12 is mediated through its binding to a heterodimeric receptor composed of two subunits, IL-12 receptor β 1 (IL-12 R β 1) and IL-12 receptor β 2 (IL-12 R β 2) (Figure 2B).⁸⁵ The IL-12 R β 1 subunit, encoded by the IL-12B1 gene, is a crucial component in the signaling of both IL-12 and IL-23, mediating high-affinity binding to the β (p40) subunit shared by both cytokines.⁸⁶ The IL-12 R β 2 subunit, while also contributing to IL-12 affinity, possesses a larger cytoplasmic domain that is indispensable for initiating intracellular signal transduction.⁸⁷ Therefore, optimal high-affinity binding of IL-12, coupled with efficient intracellular signaling requires the co-expression of both receptor subunits. Upon IL-12 engagement, the receptor complex initiates the JAK-STAT pathway, specifically activating tyrosine kinase 2 (TYK2) and Janus kinase 2 (JAK2). These kinases subsequently phosphorylate STAT4, leading to the transcriptional activation of associated genes.⁸⁸ IL-12 is primarily produced by antigen-presenting cells (APCs), including monocytes/macrophages, DCs, granulocytes, and B cells. Its secretion is typically induced in response to various stimuli, most notably the recognition of intracellular pathogens, often mediated through Toll-like receptor (TLR) engagement.⁸⁹ IL-12 plays a pivotal role in orchestrating diverse biological functions, critical for host defense and anti-tumor responses. One of its primary functions is the induction of T helper 1 (Th1) cell differentiation and proliferation, promoting naive CD4⁺ T cells to skew to a Th1 phenotype. Th1 cells are indispensable for cell-mediated immunity, which is crucial for the clearance of intracellular pathogens.⁹⁰ Another hallmark function of IL-12 is its potent stimulation of IFN γ production from T and NK cells. Being the most potent activator of NK cells, IL-12 drives robust NK cell

proliferation and significantly enhances its cytotoxic activity against tumor cells. NK cell activation is directly linked to the induction of high levels of IFN γ ,⁹¹ a central cytokine that amplifies the anti-tumor response by enhancing MHC class II (MHCII) expression on APCs, as well as the activation of macrophages.⁹² IL-12 is also a potent activator of CD8⁺ T cells, promoting their proliferation, differentiation, and cytotoxic functions.⁹³ Furthermore, IL-12 contributes significantly to antigen presentation and macrophage activation, directly stimulating DCs to produce additional IL-12, thereby amplifying the immune response and enhancing their antigen-presenting capabilities.

Given its capacity to activate both innate and adaptive immune responses, IL-12 is considered a potent immunotherapeutic agent. Its ability to positively modulate both T cells, as well as NK cells has been leveraged to design IL-12-based therapies. Preclinical studies have validated IL-12's therapeutic potential, both as a monotherapy or in combination with other drugs.^{18,94} The clinical progress of IL-12, initially showing promise, has been slowed due to significant limitations related to systemic toxicity and limited efficacy in Phase 2 studies.⁹⁵ Recently, however, there has been a resurgence in the development of IL-12 therapies, catalyzed by an improved understanding of its biology, coupled with the development of new ways to deliver this cytokine in a more targeted manner. These approaches include fusion constructs, which are designed to deliver IL-12 receptor agonism in a targeted manner by fusing it with ICIs or extracellular proteins/immune targets with the aim of improving delivery and efficacy while mitigating systemic toxicities.⁹²

Overview of immune cell-targeting moieties

The delivery of cytokines in trans to nonimmune cells, such as tumor or stroma antigens, was the first strategy adopted to reduce systemic toxicities, but it is not in scope for this review.

Cis-targeting of IL-2

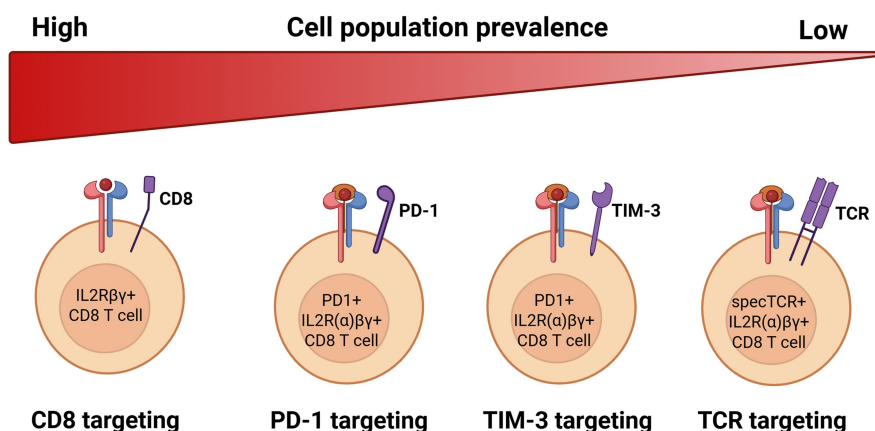
To minimize IL-2-related toxicity, a plethora of assets in the preclinical and clinical space is under development to preferentially deliver IL-2 to CD8⁺ T cells, essential players in anti-tumor immune response. The targeting moieties selected are cell-surface proteins that differ in their prevalence and tumor specificity (Figure 3A). While CD8 is the most broadly expressed antigen used to deliver IL-2 signals, PD-1 is expressed by a subset of CD8⁺ T cells and is a well-characterized receptor that is upregulated on chronically activated antigen-specific T cells. TIM-3 is another immune checkpoint with non-redundant functions, primarily co-expressed with PD-1 on terminally exhausted T cells.⁹⁶ An even more selective strategy is to target TCR families or antigen-specific TCRs to provide IL-2 to a subset of CD8⁺ T cells with low prevalence, but a higher likelihood of being tumor-reactive. In addition to the choice of the target antigen, IL-2 is built into diverse molecular designs in its native form or bearing mutations that bias, abrogate, or enhance binding to CD25 or CD122.

PD-1 mediated delivery

PD-1/PD-L1 blocking agents disrupt the PD-1/PD-L1 signaling pathway in order to enhance immune cell functionality. They are widely used in the treatment of many cancer types and represent a successful class of immunotherapies; however, their clinical efficacy remains limited to certain indications and a subset of patients, while many patients are primary refractory or develop adaptive resistance to ICI therapy.⁹⁷ PD-1 is upregulated on antigen-experienced T cells and most commonly found in settings of chronic antigen stimulation such as chronic infections and cancer. Therefore, PD-1 represents an attractive target to selectively enhance tumor-reactive T cells. Immunocytokines in preclinical development described in conference abstracts are summarized in Table 1 and are out of scope for this review. Here, we focus on the peer-reviewed PD1- targeted IL2 assets illustrated in Figure 3B.

The immunocytokine PD1-IL2v (eciskafusp alfa, Figure 3B, a) comprises a bivalent anti-PD-1 IgG1 containing the PG-LALA (P329G, L234A, L235A) mutation in the Fc-portion fused to an IL-2 variant with abrogated binding to CD25. It was first described in 2022 in a study that identified a novel mechanism of action (MoA) whereby delivering an IL-2 R $\beta\gamma$ biased agonism (IL2v) in *cis* preferentially to PD-1⁺ CD8⁺

A



B

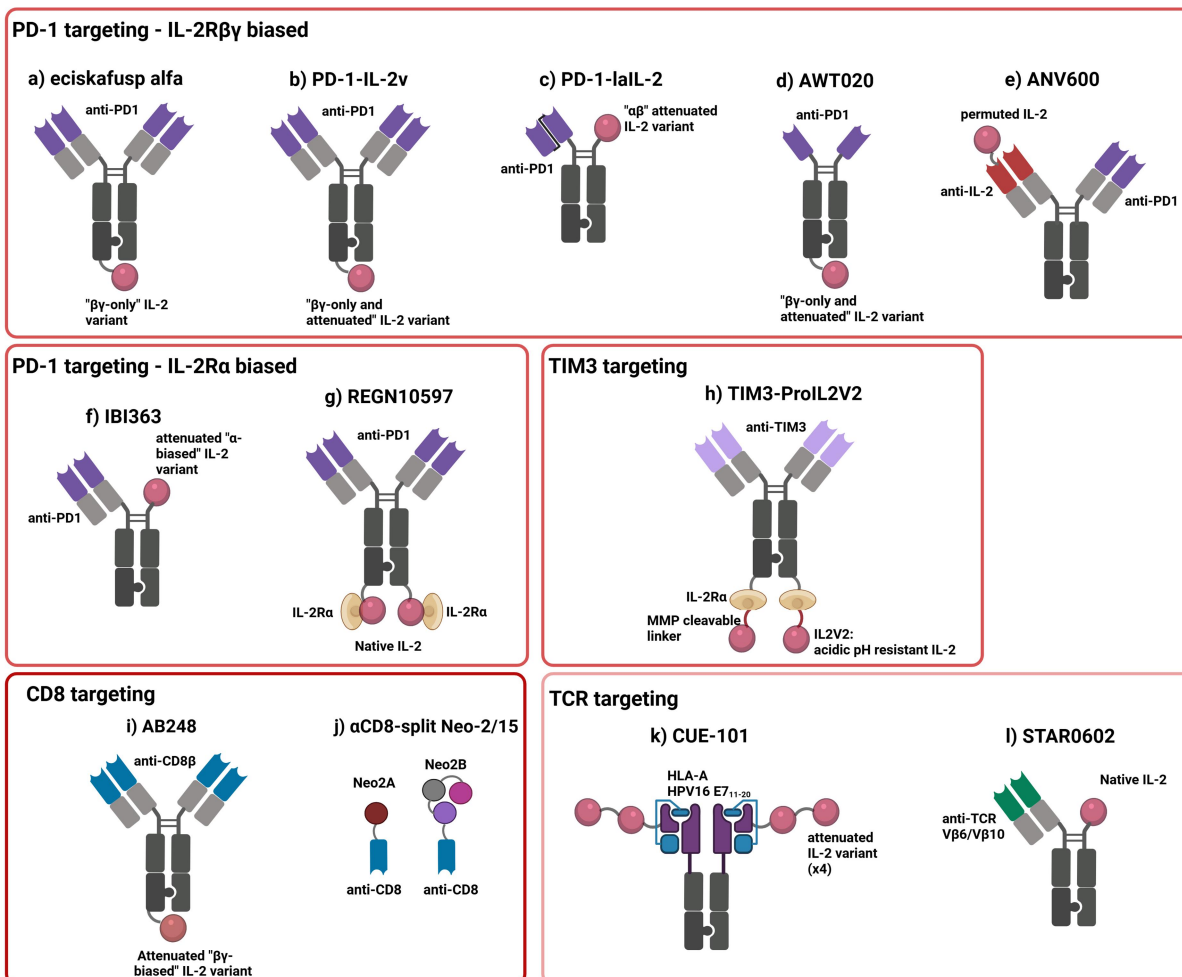


Figure 3. *Cis*-targeting of IL-2 R agonists. A) Delivery of IL-2 signals to different targeting moieties. Cell population prevalence scale illustrates various IL-2 R+ CD8 T cell subsets from most abundant (left) to least abundant (right). Four main targeting moieties are represented to deliver IL-2 R agonism in *Cis*: CD8, PD-1, TIM-3 and TCR. B) Graphical illustration of *cis* targeting IL-2 based designs. Depicted molecules are classified by their targeting moiety and for PD-1, further categorized into IL-2 Rβγ vs IL-2 Rα biased. Each letter refers to a specific molecular design for easier identification in the corresponding text description. a) eciskafusp alfa, b) PD-1-IL2v, c) PD-1-lalIL-2, d) AWT020, e) ANV600, f) IBI363, g) REGN10597, h) TIM3-ProIL2V2, i) AB248, j) αCD8-split Neo-2/15, k) CUE-101, l) STAR0602. Created with BioRender.com.

Table 1. List of additional cis-targeted IL-15, IL-18, IL-21, IL-7 and IL-2 immunocytokines. Agents disclosed in conference abstracts and not in peer-reviewed articles. Each compound is categorized by the target, cytokine, stage of development, and company with a short engineering description.

Target	Cytokine	Agent	Engineering description	Stage	Company
PD-1	IL-2	KY-0118	Bivalent PD-1 blocking antibody fused to IL-2Rbg-biased IL-2 mutein	Phase 1	Novatim Immune Tx
PD-1	IL-2	PTX-912	Bivalent PD-1 blocking antibody fused to conditionally masked IL-2Rbg-biased IL-2 mutein	Phase 1	Proviva Therapeutics, Inc,
PD-1	IL-2	TEV-56278	Bivalent PD-1 non-blocking antibody fused to IL-2Rbg-biased IL-2 mutein	Phase 1	Teva Pharmaceutical Industries
PD-1	IL-2	MDNA113	Bivalent PD-1 blocking antibody fused to β -enhanced IL-2Rbg-biased IL-2 mutein (IL-2 superkine) partially masked by an IL-13 R α 2 selective IL-13 superkine	Preclinical	Medicenna
PD-1	IL-2	MDNA223	Bivalent PD-1 blocking antibody fused to β -enhanced IL-2Rbg-biased IL-2 mutein (IL-2 superkine)	Preclinical	Medicenna
PD-1	IL-2	XTX501	Bivalent PD-1 blocking antibody fused to single domain antibody-based conditionally masked IL-2Rbg-biased IL-2 mutein	Preclinical	Xilio Therapeutics
PD-1	IL-2	MB5029	Bivalent PD-1 blocking antibody fused to IL-2Rbg-biased IL-2 mutein with attenuated γ -binding	Preclinical	MustBio
PD-1	IL-2	MB4	Bispecific PD-1 and VEGF blocking antibodies fused to IL-2Rbg-biased IL-2 mutein	Preclinical	MustBio
PD-1	IL-2	ASKG-812	Bivalent PD-1 blocking antibody fused to masked protease activated engineered IL-2	Preclinical	AskGene Pharma
PD-1	IL-2	MBS309	Bivalent PD-1 blocking antibody fused to IL-2Rbg-biased IL-2 mutein with attenuated IL2Rbg binding	Preclinical	Mabworks Biotech
PD-1	IL-2	BPT958	PD-1 blocking antibody with chemical conjugation to masked protease-cleavable IL-2 payload with intramolecular loop to prevent IL-2Rb binding	Preclinical	Bright Peak Therapeutics
CTLA-4	IL-2	EGL-001	Bivalent Fc-functional CTLA-4 blocking antibody fused to an antagonist IL-2 mutein moiety with biased binding for IL-2R α	Phase 1/2	Egle Therapeutics
LAG-3	IL-2	TNRX-257	Multispecific LAG-3 blocking antibody with LAG-3 conditional partial agonism of IL-2Rbg	Preclinical	Tentarix Bio/collab with Gilead Sciences
LAG-3	IL-2	cLAG3-IL2	Bivalent LAG-3 blocking antibody containing a Dual Binding Antibody for conditional LAG-3 regulated IL-2 delivery	Preclinical	Bonum Therapeutics
LAG-3	IL-2	aLAG3-IL-2c	LAG-3 blocking antibody combined with IL-2c, IL-2 a -2Rbg-biased chimeric molecule containing a fragment from IL-15 with abrogated binding to IL-2R α	Preclinical	Anwita Biosciences
CD8	IL-2	AZD6750	Bivalent CD8 antibody fused to IL-2	Phase 1/2	AstraZeneca
WT1-pHLA	IL-2	CUE-102	HLA-A *02 or HLA-A *24 with WT1-derived peptide epitopes fused to 4 copies of attenuated IL-2	Phase 1	CUE Biopharma
PD-1	IL-15/IL-15Ra	SAR445877	Bivalent PD-1 blocking antibody fused to IL-15/IL15Ra sushi domain complex	Phase 1/2	Sanofi
PD-1	IL-15	ASKG 915	Bivalent PD-1 blocking antibody fused to masked, protease activated IL-15	Phase 1	AskGene Pharma
PD-1	IL-15	AMP-01	Bispecific antibody comprising monovalent PD-1 blocking antibody and monovalent Amplify.R (anti-IL15) antibody to capture endogenous IL-15 in vivo	Preclinical	Reverb Therapeutics
CTLA-4	IL-15/IL-15Ra	JK08	Bivalent CTLA-4 blocking antibody fused to IL-15/IL-15Ra sushi domain	Phase 1/2	Salubris Bio
TIGIT	IL-15	MFA-011	Bivalent TIGIT blocking antibody fused to IL-15	Preclinical	MediMabBio
GITR	IL-15	MFA-021	GITR agonist antibody fused to IL-15	Preclinical	MediMabBio
PD-1	IL-18	BPT567	Bivalent PD-1 blocking antibody fused to IL-18 mutein resistant to IL-18BP	Phase 1/2	Bright Peak Therapeutics
CD8	IL-21	AB821	Bivalent anti-CD8b antibody fused to attenuated IL-21 mutein	Preclinical	AsherBio/Merck
PD-1	IL-7	BICKI [®] IL-7v	Monovalent PD-1 blocking antibody fused to attenuated IL-7 mutein	Preclinical	OSE Immunotherapeutics

T cells triggered the expansion and differentiation of T_{PEX} into “better effectors.” This effector population had a unique transcriptional signature and provided superior efficacy in chronic infection and several tumor models preclinically compared to anti-PD-1 monotherapy or in combination with stroma targeted IL2v, which delivered IL-2 R $\beta\gamma$ biased agonism in trans primarily to bystander CD8⁺ T cells.³⁶ Synergies similar to PD1-IL2v were observed when combining anti-PD-1 and wild type (wt) IL-2, resulting in the expansion and differentiation of T_{PEX} into a highly functional “better effector” population, whose generation relied on CD25 binding.³⁷ The results from preclinical mouse studies in chronic infection and tumor models showed that PD-1 targeting of IL-2 R $\beta\gamma$ agonists in *cis* to CD8 T cells due to anchoring to PD-1 overcome the requirement for CD25 binding and the formation of the high affinity heterotrimeric IL-2 R complex to sustain antigen-specific responses. The functional activity of IL-2 R $\beta\gamma$ biased agonists compared to IL-2 R α biased agonists in the context of PD-1 targeted agents has been reviewed elsewhere.^{21,35} The murine surrogate of eciskafusp alfa demonstrated combinatorial potential with anti-PD-L1 therapy in

a mouse model of de novo pancreatic neuroendocrine cancer since PD-L1 expression was identified as a potential adaptive resistance mechanism to murine PD1-IL2v (muPD1-IL2v) therapy.⁹⁸ Moreover, muPD1-IL2v displayed a profound effect on tumor control and the survival of mice with an orthotopic pancreatic ductal adenocarcinoma (PDAC)⁹⁹ as well as head and neck squamous cell carcinoma (HNSCC) tumor models¹⁰⁰ in combination with radiation therapy. Similarly, in combination with radiotherapy, it inhibited lung cancer growth and remodeled the immune microenvironment.¹⁰¹ PD1-IL2v has also been shown to be a favorable combination partner for tumor vaccination.¹⁰² Recently, using human model systems including lung cancer patient – derived tumor fragments (PDTF), it was demonstrated that PD1-IL2v elicited a multifaceted anti-tumor response, both mediated by CD8⁺ and conventional CD4⁺ T (Tconv) cells.¹⁰³ Given the importance of the *cis*-delivery for the MoA of PD1-IL2v, a mathematical model generated on in vitro and early clinical data was used to define the clinical efficacious dose range at which PD-1 *cis*-binding is maximized.¹⁰⁴ Clinical development of PD1-IL2v has been terminated as part of portfolio prioritization.

PD-1-IL-2 v (Figure 3B, b) is a bivalent PD-1/PD-L1 blocking antibody fused to a “βγ only” IL-2 agonist. Structural shuffling of α-helices in IL-2 were introduced to select a mutated cytokine with a conformational change that almost abrogates binding to CD25 and IL2Rβ alone but is able to activate the dimeric IL2Rβγ receptor.¹⁰⁵ Another PD1-targeted IL-2 R agonist, PD1-laIL2 (Figure 3B,c) is a low affinity (la) IL-2 variant that exhibits reduced binding to CD25 and IL2Rβ.¹⁰⁶ PD1-laIL2 showed superior efficacy compared to anti-PD1 treatment in a variety of preclinical tumor models and primarily expanded and restored the effector functions of PD-1⁺TIM-3⁺ CD8⁺ T cells.¹⁰⁶ AWT020 (Figure 3B,d) is a fusion protein composed of bivalent anti-PD1 VHH, also called nanobody, and IL-2c, an engineered cytokine with abolished binding to CD25 and attenuated affinity to IL-2 Rβγ.¹⁰⁷ IL-2c contains 3 mutations, F42A, Y45A and L72G to abolish binding to CD25 and an affinity to IL-2 Rβγ reduced by >1,000 fold compared to rhIL2 in order to preferentially deliver IL-2 signals via high-affinity PD-1 binding.¹⁰⁷ The murine surrogate of AWT020, mAWT020 demonstrated CD8⁺ T cell-dependent efficacy in preclinical tumor models and minimal activity on NK cells compared to mPD1-IL-2x compound without affinity detuning, which translated into an improved tolerability profile.¹⁰⁷ AWT020 is currently being evaluated in a Phase 1 study in patients with advanced cancer (NCT06092580). ANV600 (Figure 3B,e) is also a PD-1 targeted IL-2 Rβγ agonist that contains a non-blocking PD-1 targeting antibody that does not compete with PD-1 checkpoint inhibitors, to enable combination with PD-1 standard-of-care therapies. The binding to CD25 is prevented via fusion of an IL-2 moiety circularly permuted and fused to an anti-IL-2 antibody, thereby generating an IL-2 Rβγ-biased agonist.¹⁰⁸ In preclinical experiments, it selectively expanded tumor antigen-specific T cells and potentiated ICI therapy in multiple syngeneic models in huPD-1 transgenic mice.¹⁰⁹ The combinatorial strategy of delivering IL-2 signals to PD-1⁺ cells via ANV600 together with approved checkpoint inhibitors may facilitate dose-selection and -optimization to meet the different PK/pharmacodynamics (PD) relationships between agonists and antagonists. The safety, tolerability, PK/PD and efficacy of ANV600 are being investigated in a Phase 1/2 study in patients with advanced solid tumors (NCT06470763).

In addition to the five aforementioned IL-2 Rβγ biased assets, two PD-1-targeted compounds utilize CD25-biased strategies in an attempt to further enrich the selectivity toward tumor-reactive CD8 T cells. IBI363 (Figure 3B, f) is composed of a monovalent anti-PD-1 antigen-binding fragment (Fab) with an N-terminal fusion of an attenuated, IL2Rα biased IL-2 variant. The molecule was designed based on the preclinical findings that CD25 engagement in tumor-specific T cells leads to an autocrine IL-2 signaling that promotes potent anti-tumor activity.¹¹⁰ IBI363 is currently being studied in Phase 1/2 and pivotal clinical trials in patients with advanced solid tumors and shows a promising efficacy/tolerability profile. It received two Fast Track designations (FTD) from the FDA for the treatment of melanoma and non-small cell lung cancer (NSCLC). Monotherapy of IBI363 against pembrolizumab is being evaluated in a randomized, multicenter pivotal trial in patients with unresectable, locally advanced mucosal or acral melanoma (NCT06797297). IBI363 recently showed promising efficacy signals in checkpoint inhibitor (CPI)-refractory NSCLC patients (NCT05460767)¹¹¹ and in immune cold colorectal tumors in combination with bevacizumab, particularly in patients without liver metastases (NCT05460767),¹¹² making it the most advanced PD-1 *cis*-targeted immunocytokine in the clinic. REGN10597 (Figure 3B,g) also named PD1-IL2Ra-IL2, is a bivalent PD-1-targeted, CD25 receptor-masked IL-2 wt immunocytokine that preferentially delivers IL-2 signals to cells that co-express PD-1 and CD25 compared to unmasked controls.¹¹³

REGN10597 is a symmetrical molecule that contains two masked native IL-2 that retain the ability to signal primarily on PD-1⁺CD25⁺ cells. In syngeneic tumor models in human PD-1 knock-in mice, REGN10597 demonstrated strong efficacy and a favorable tolerability profile shown via mild body weight loss and pulmonary weight measurements compared to unmasked IL-2.¹¹³ The PK, safety, tolerability, and efficacy of REGN10597 are currently being investigated in a Phase 1/2a first-in-human trial in patients with advanced solid tumors (NCT06413680).

In addition to the cytokine component, the PD1-IL2 molecules described here also differentiate by their Fc region, and their PD-1 binding component (i.e., Fab, scFv, VHH), monovalent vs bivalent with different affinities and avidities. It would be interesting and important to decipher how much PD-1/PD-L1 blocking and strength of the delivery of IL-2 R agonism to PD-1⁺ cells are needed for an optimal targeting and expansion of T_{pex} to provide deeper and more durable anti-tumor responses.

TIM-3 mediated delivery

In addition to PD-1, other immune checkpoints have been identified and found upregulated on TILs and could therefore serve as attractive targeting moieties, such as LAG-3, TIGIT, and TIM-3. Unlike CTLA-4 and PD-1, there are currently no approved therapies blocking the TIM-3 pathway, but several compounds are in clinical trials. TIM-3 expression is not found on T_{PEX} and marks more terminally dysfunctional CD8⁺ T cells.⁹⁶ TIM-3-ProIL2V2 (Figure 3B,h) is a fusion protein composed of bivalent TIM-3 targeting with a conditionally activated cytokine mutein.¹¹⁴ IL2V2 was engineered for increased binding to IL-2 R β at acidic pH as low pH typically found in the tumor microenvironment was recently shown to affect IL-2 activity.^{115,116} The functionality of IL2V2 was concealed by C-terminal fusion to IL2R α via a metalloproteinase (MMP)-cleavable linker to prevent systemic activity. TIM-3 targeting favors accumulation to the tumor site, which then triggers IL2V2 release via MMP-mediated cleavage. Interestingly, this molecular design enabled IL2V2 to activate not only exhausted TIM-3⁺ T cells, but also PD-1⁺TIM-3⁻ CD8⁺ T cells, which contribute significantly to anti-tumor immunity. The efficacy of TIM-3-ProIL2V2 observed in the MC38 mouse tumor model was dependent on CD8⁺ T cells and IFN- γ . TIM-3-ProIL2V2 also demonstrated anti-tumor efficacy in the B16F10 model in combination with anti-PDL1.¹¹⁴

CD8-mediated delivery

The two concepts described in this section are illustrated in Figure 3B. AB248 (CD8-IL2, Figure 3B, i) is composed of a bivalent anti-CD8 β IgG1 with a C-terminal fusion of a mutated IL-2 with abrogated binding to CD25 and reduced affinity to IL-2 R β γ .¹¹⁷ In vitro pSTAT-5 assays with human peripheral blood mononuclear cells (PBMCs) demonstrated a strong selectivity of AB248 for CD8⁺ T cells over other IL-2 R⁺ cell types, such as Tregs and NK cells, with over 500-fold higher potency calculated based on effective concentration 50 (EC50) values.¹¹⁷ The murine surrogate of AB248 (CD8-mIL2) showed potent efficacy in multiple tumor models and, in cynomolgus monkeys, administration of AB248 at dose levels of up to 1 mg/kg led to Ki67 induction in CD8⁺ T cells and a preferential expansion of CD8⁺ T cells over NK cells.¹¹⁷ The potential of AB248 to treat human tumors was studied using patient-derived tumor fragments.¹⁹ Compared to PD-1 blockade, AB248 demonstrated superior reinvigoration of dysfunctional T cells that acquired features of polyfunctionality (e.g., release of soluble Granzyme B, TNF α , and IFN γ), as well as T cell activation in tumors resistant to PD-1 blockade.¹⁹ Interestingly, AB248 required concomitant TCR engagement for full effector functionality. In addition, AB248 represents an attractive option for the treatment of infectious diseases, such as Hepatitis B virus.¹¹⁸ AB248 is currently being investigated in a Phase 1a/b clinical trial as monotherapy or in combination with pembrolizumab (NCT05653882). The potential use of AB248 together with tarlatamab, a DLL3-specific TCE, will also be evaluated in patients with advanced stage small cell lung cancer.

Unlike the other approaches based on native or mutated version of the cytokine, the IL-2 mimetic Neoleukin-2/15 (Neo-2/15) was generated by de novo computational protein design and recapitulates the functions of IL-2 and IL-15 by activating IL-2 R β and IL-2 R γ , without relying on CD25 and CD215 receptors.¹¹⁹ This novel strategy was further developed to mitigate the dose-limiting toxicities of cytokines by separating Neo-2/15 into two components that require colocalization to achieve

conditional activation. This allows for independent targeting to cells expressing one or two specific surface markers such as epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor (HER) for *trans*-activation, or CD8 for *cis* activation of immune cells.¹²⁰ Importantly, activation of the split Neo-2/15 is dependent on the sufficient expression of surface markers on target cells¹²⁰ and may therefore not be suitable for targeting antigens expressed at a low or moderate density. In a syngeneic mouse melanoma model, treatment with α CD8 split Neo-2/15 (Figure 3B, j) led to delayed tumor growth and improved survival compared to mice treated with untargeted split Neo-2/15.

Although the two concepts reviewed in this section share common attributes, e.g., they target CD8 and are biased toward IL-2 R $\beta\gamma$ binding, there are also considerable differences and unique features in their molecular designs, in particular, the level of cytokine attenuation, the split computationally generated cytokine mimetic component vs mutated IL-2, as well as the presence of an Fc region for half-life extension and the choice of the anti-CD8 binder via Fab or VHH. It would be interesting to understand the contribution of individual components to the overall efficacy, tolerability, and immunogenicity profiles.

TCR-mediated delivery

To enhance selectivity, novel approaches aim at delivering IL-2 directly to tumor-antigen specific CD8⁺ T cells or a pre-defined TCR (Figure 3B). CUE-101 (Figure 3B, k) is the first Immuno-STATTM (Selective Targeting and Alteration of T cells) platform that uses TCR engagement via HLA-A *0201-peptide complex. CUE-101 is composed of 2 MHC-peptide complexes, HLA-A *0201 loaded with a peptide epitope derived from the human papilloma virus (HPV)16 E7 protein and four attenuated IL-2 molecules to activate HPV16⁺ E7₁₁₋₂₀ specific CD8⁺ T cells for the treatment of HPV16-driven cancer types.¹²¹ Currently, CUE-101 is being evaluated in a Phase 1 dose escalation and expansion study as a monotherapy or in combination with pembrolizumab in patients with HPV⁺ recurrent/metastatic HNSCC (NCT03978689). While this approach selectively enhances tumor-specific T cell responses, it is also limited to patients carrying the HLA-A *0201 haplotype whose prevalence varies across ethnic groups. Another approach is exemplified by STAR0602 (Figure 3B, l), also known as invikafusp alfa, a bifunctional antibody that monovalently targets germline V β 6 and V β 10 TCRs fused at the N-terminal with native IL-2 to selectively deliver IL-2 R agonism to a common tumor-specific T cell subset detected across many cancer types.¹²² STAR0602 expanded V β 6⁺ T cells in non-human primates as well as HPV-specific T cells in an in vitro human tumor organoid model.¹²² mSTAR1302, the murine surrogate of STAR0602, demonstrated pronounced and durable efficacy in preclinical mouse tumor models.¹²² STAR0602 is currently being tested in a Phase 1/2 study in patients with advanced solid tumors that are immunogenic, such as tumors with a high mutational burden (TMB-H), microsatellite instability (MSI-H)/DNA mismatch repair (dMMR) or virally associated cancers (NCT05592626). It is of particular relevance to investigate whether preventing systemic activation and expansion of non-tumor specific CD8⁺ T cell populations may alleviate IL-2-related toxicities and improve the safety profile in the clinical setting while maintaining efficacy via preferential *cis* targeting.

Targeting of other cytokines: IL-15, IL-21 and IL-12

IL-15

PF-07209960 (Figure 4A, a), also referred to as anti-PD1-IL15m, is a fusion protein comprising a potency-reduced IL-15 mutein conjugated to a bivalent high affinity anti-PD1 antibody at the C-terminal.¹²³ Five mutations were introduced in wt IL-15 to abrogate binding to IL15R α and reduce the affinity to IL2R $\beta\gamma$ to minimize binding to NK cells. The anti-PD1-IL15m construct demonstrated the capacity to elicit proliferation, activation, and cytotoxicity preferentially in PD-1⁺ TILs, including both CD4⁺ and CD8⁺ subsets. In MC38 and B16F10 murine tumor models, the efficacy of a surrogate molecule was dose-dependent and relying on intra-tumoral CD8⁺ T cells, while NK cells were dispensable for anti-tumor effects.¹²³ PF-07209960 was tested in a PK/PD and good laboratory practice (GLP) toxicology study, which determined the highest non-severely toxic dose at 0.3 mg/kg/dose.¹²⁴ The results of PF-07209960 Phase 1 clinical trial highlighted a high anti-drug antibody (ADA) incidence against the PD-1 binder, as well as both IL-15

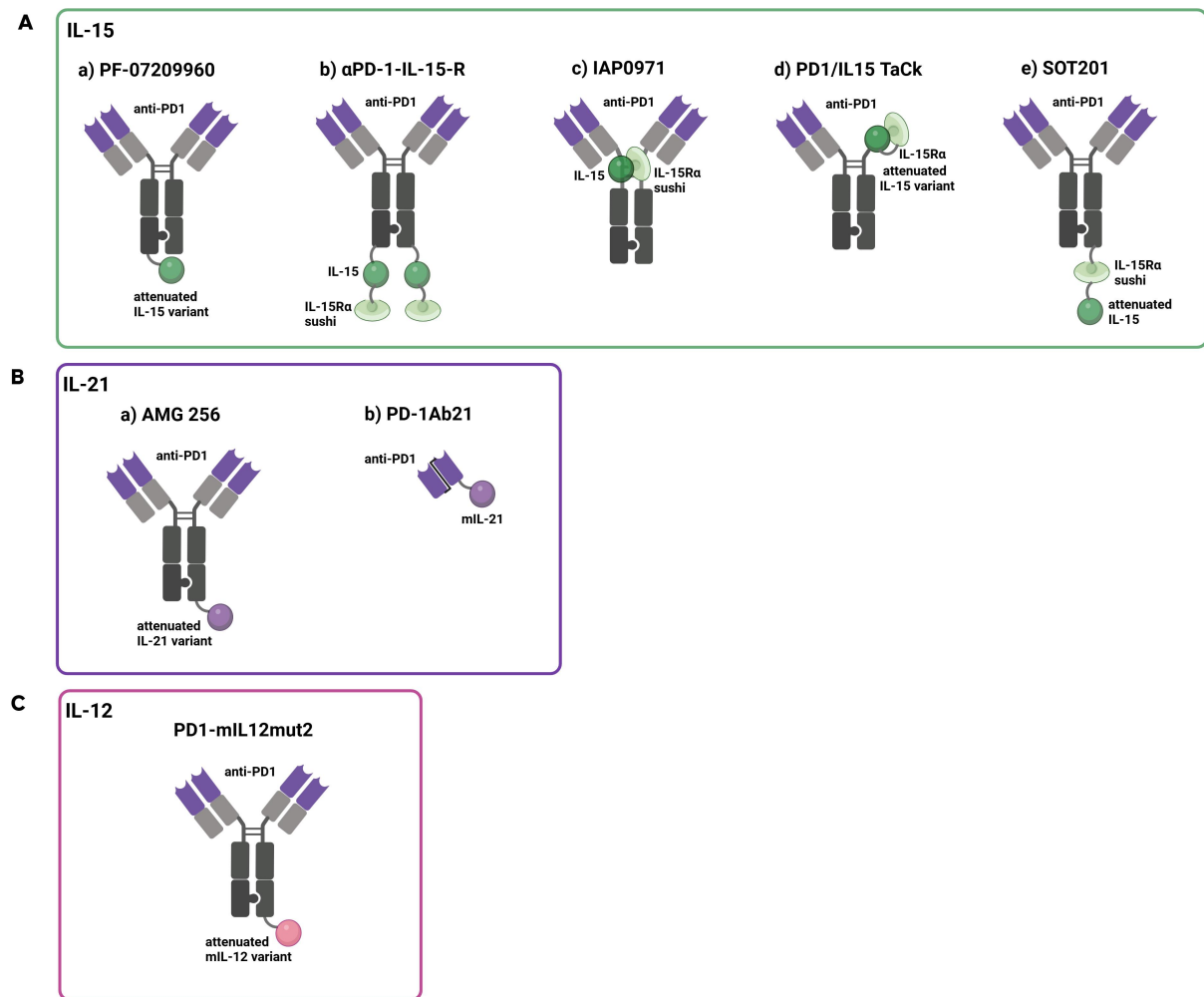


Figure 4. *Cis*-targeting of other cytokines: IL-15 R, IL-21 R, IL-12 R agonists. Graphical illustration of A) IL-15 compounds. a) PF-07209960, b) α PD-1-IL-15-R, c) IAP0971, d) PD1/IL15 TaCk, e) SOT201. B) IL-21 compounds, a) amg 256, b) PD-1Ab21 and C) IL-12 compound, PD1-mIL12mut2. Created with BioRender.com.

mutein and endogenous wt IL-15, impacting exposure starting at cycle two and hampering the evaluation of PK/PD properties as well as safety and potentially efficacy.¹²⁵

α PD-1-IL-15-R (Figure 4A, b) is a fusion protein consisting of a bivalent anti-PD1 antibody conjugated to two IL15-IL15Ra complexes via an Fc linker, designed to restrict IL-15 localization to PD-1 expressing cells.¹²⁶ The concealing of IL-15 by the Fc region of anti-PD1 significantly diminished the ability of IL-15 to activate NK cells and mitigated associated toxicity. *Cis* delivery to PD-1 restored the activity of IL-15 on tumor-specific CD8⁺ T cells, resulting in efficacy in several murine models of cancer.¹²⁶

IAP0971 (Figure 4A, c) is a fusion protein incorporating a bivalent anti-PD1 antibody conjugated to a single wt IL15-IL15Ra complex via the hinge region.¹²⁷ In vitro activity, in vivo efficacy in a mouse tumor model, as well as PK and safety studies in NHP, demonstrated a potent anti-tumor activity and acceptable tolerability profile, respectively, despite a relatively short half-life.¹²⁷ IAP0971 is currently being evaluated in a Phase 1/2 clinical trial in patients with advanced tumors for tolerability, safety, tolerability, and preliminary signs of efficacy (NCT05396391).

PD1/IL15 TaCk (Figure 4A, d) is a targeted cytokine (TaCk) that combines a monovalent non-blocking anti-PD1 with a single chain IL-15 R α -IL15 complex, a sushi domain of IL-15 R α fused to a variant of human IL-15 engineered to reduce the binding to IL-15 R $\beta\gamma$ allowing for *cis*-delivery. This design enhances CD4⁺ and CD8⁺ T cell function while sparing NK cells and minimizing systemic toxicities by preferentially delivering IL-15 signals to PD-1⁺ cells.¹²⁸ As PD1/IL15 TaCk is not mouse cross-reactive, the PK/PD relationship and translational work informing on the selection of first-in-human dose was evaluated in

cynomolgus monkeys.¹²⁸ Another PD-1-targeted IL-15 immunocytokine, SOT201 (Figure 4A, e), a bivalent anti-PD1 antibody fused to RLI-15 (IL-15/IL-15 R α sushi complex) bearing N65A mutation, was recently described.¹²⁹ The mutation was introduced to lower the cytokine activity in the absence of PD-1, thereby increasing the *cis* activity targeting window. The murine surrogate's functional profile and efficacy were directly compared to that of mPD1-IL2v to elucidate potential differences between IL-15 and IL-2 and, more specifically, the impact of IL-15 attenuation. In the MC38 tumor model, mSOT201 was tolerated at a higher dose than mPD1-IL2v (5 vs 0.5 mg/kg) and showed longer half-life due to lower IL-2 R target-mediated drug disposition and internalization.¹²⁹ The safety, tolerability, and preliminary efficacy of SOT201 are being investigated in a Phase 1 study for patients with advanced unresectable or metastatic solid tumors (NCT06163391).

IL-21

AMG 256 (Figure 4B, a) is a fusion protein composed of a bivalent PD1-blocking antibody conjugated to a potency-attenuated IL-21 mutein variant.¹³⁰ The IL-21 R9E:R76A variant stems from structure-guided engineering and incorporates two mutations that extensively attenuated IL-21 potency to promote *cis* targeting of PD-1⁺ cells while minimizing the inhibitory effect of IL-21 on dendritic cells. This construct enhances cytotoxicity and effector cytokine production in antigen-specific (cytomegalovirus (CMV)) CD8⁺ T cells and demonstrates superior tumor control in a tumor model with PD1-IL-21mut compared to anti-PD1 monotherapy.¹³⁰ A Phase 1 study was conducted to evaluate the safety, tolerability, and early signs of efficacy of AMG 256 in patients with advanced solid tumors (NCT04362748). Translational work between non-human primates and first-in-human studies highlighted the contribution of IL-21 mutein in eliciting immunogenicity with significantly higher ADA rate and titers than with anti-PD1 monotherapy.¹³¹ Another preclinically explored approach is PD-1Ab21 (Figure 4B, b), a noncovalent homodimeric form ('diabody') of anti-PD-1 single chain variable fragment (scFv) fused to murine IL-21, which induced a robust tumor growth inhibition in CT26, MC38, and B16-OVA murine tumor models via tumor-specific CD8⁺ T cells expansion and memory formation.¹³²

IL-12

IL-12 is a potent anti-tumor cytokine whose full therapeutic potential has been hindered by its off-tumor toxicity. There are considerable efforts to design novel compounds with targeted delivery of IL-12. In this context, PD1-mIL12mut2 (Figure 4C) was recently described as an immunocytokine composed of a bivalent anti-PD1 antibody fused at the C-terminus to a murine low affinity IL-12 mutant-2.¹³³ *Cis*-targeting of intra-tumoral CD8⁺ T cells combined with reduced potency of IL-12 minimized activity on NK cells which led to improved tolerability in mice. Interestingly, in the MC38 tumor model, intra-tumoral administration of PD1-mIL12mut2 in one flank led to tumor control not only locally, but also of the distant tumor implanted on the opposite flank, indicative of an abscopal effect.¹³³

Pharmacological and immunological considerations

Despite the enhanced targeted activity and efficacy of these new therapeutic modalities, their clinical translation faces critical pharmacological hurdles related to developability, toxicity, and immunogenicity.^{134,135} From a PK perspective, the structure, size, modality, and relative affinities of the construct affect their availability in the TME, and ultimately their performance.^{136–138} While the antibody's Fc region is designed to extend the cytokines' half-lives, initial target-mediated tumor accumulation is often followed by internalization and degradation, processes that can affect clearance and exposure, potentially prompting for a more frequent dosing regimen.¹³⁹

Additionally, the molecular engineering of these agents, such as cytokine muteins, linkers, or specific antibody fragments, can be identified by the immune system as foreign antigens. These can in turn elicit an adaptive immune response, inducing the formation of ADAs, which may accelerate systemic clearance of the drug and abrogate therapeutic benefit, leading to exposure loss and contributing to treatment failure.^{125,140,141} Preclinical studies in human IgG transgenic mice have shown that IL-2 based

cytokines, by virtue of binding to CD4⁺ T helper cells, pose a higher risk of immunogenicity.¹⁴¹ In addition, PD-1 receptors are also expressed by follicular T helper cells (Tfh), making this CD4⁺ T cell subset a target of anti-PD-1-based immune-cytokines in secondary lymphoid organs.¹⁴² This could lead to Tfh expansion and promotion of their effector functions aimed at supporting B cell class switch and the development of antibody-producing plasma cells, explaining the significant increase in ADAs.¹⁴³ An unfavorable immunogenicity profile may have profound implications for exposure and efficacy. However, critical safety aspects must also be considered when developing cytokine-based therapies, particularly the potentially detrimental cross-reactivity of ADAs toward endogenous cytokines.¹⁴⁴ Furthermore, as addressed earlier, the administered cytokine-based therapy contends with the already present cytokine milieu of the TME, creating a complex interplay and competition between endogenous and exogenous cytokines as well as their pleiotropic targets.^{26,27} Addressing these multi-faceted challenges through optimized protein engineering is essential to realizing their full potential and minimizing adverse effects.

Conclusion and future outlook

Key common γ -chain cytokines and IL-12 are fundamental modulators of T cell-mediated immune responses, exhibiting pleiotropic functions. Clinical application of cytokine therapies is severely limited by significant toxicities and broad on-target, off-tumor activity. This critical challenge has driven the iterative development of novel engineered cytokines, culminating with the invention of *cis*-targeting strategies to enable selective cytokine delivery to specific immune cells. Successfully restricting cytokine delivery and activity to specific cell subsets results in anti-tumor efficacy, while mitigating systemic toxicities. This finding highlights the importance of *cis*-targeting as a crucial advancement in the field of cytokine-based immunotherapy. A main focus of this review was on PD-1-targeted IL-2 R agonists. However, it will be interesting to compare their functionality profile to potency attenuated IL-7 R agonists such as BICKI@IL-7 v that specifically aims at expanding T_{PEX} cells for long-term anti-tumor immunity.¹⁴⁵

While the majority of known immunocytokines are targeted to PD-1, there are also a number of emerging assets that use alternative checkpoint molecules, such as CTLA-4, TIGIT, and LAG-3 as targets (Table 1). LAG-3 is a non-redundant immune checkpoint that complements PD-1 in imprinting an exhaustion program on tumor-specific T cells. In contrast to TIM-3, which is expressed by terminally exhausted TILs, LAG-3 is co-expressed with PD-1 on T_{PEX}, preventing their expansion, differentiation, and acquisition of effector functions against the tumor.¹⁴⁶ Therefore, LAG-3 blockade could lead to T_{PEX} expansion and differentiation into cytotoxic CD8 TILs, resulting in tumor killing. There are several LAG-3-targeted IL-2 R agonists in preclinical development, including cLAG3-IL2, which uses a proprietary platform based on Dual Binding Antibody (DBA) to conditionally activate IL-2 R signaling on LAG-3-expressing TILs.¹⁴⁷ One potential limitation of the strategy to deliver IL-2 v to LAG-3⁺ TILs is represented by Tregs. These, as opposed to CD8 and conventional CD4 TILs, constitutively express high levels of LAG-3, both in the tumor and in the periphery, and therefore might represent an important sink for the cLAG3-IL2. In addition, it has been shown that LAG-3 limits Tregs proliferation and suppressive function.¹⁴⁸ Therefore, blocking LAG-3 on Tregs while delivering a potent IL-2 R agonism might detrimentally increase their proliferation and suppressive function, potentially off-setting the benefit of targeting tumor-specific CD8 TILs.

Further evolution of targeted cytokine delivery toward conditionally active and tumor tropic drugs might confer an improved safety profile by being “masked” to non-target (T) cells, thereby mediating fewer systemic side effects and reducing immunogenicity. However, two important questions remain: 1) Will such a favorable safety and immunogenicity profile come at the expense of efficacy, due to the exclusion of important immunological compartments like TdLNs; and 2) How will these approaches affect the immunogenicity profile?

The field is further diversifying with advanced therapeutic modalities, including bispecific antibodies (BsAbs) and cytokine/antibody complexes. Engineered BsAbs are designed to directly agonize cytokine receptors by co-targeting receptor subunits, mimicking the activating signal of a cytokine like IL-2, by physically bridging their receptor subunits on the immune cell surface.¹⁴⁹ In contrast, cytokine/antibody complexes and immunocytokines leverage the antibody component to selectively guide the cytokine to

specific receptor- expressing cells.^{36,150} Additionally, the concept of cytokine *cis*-targeting is being explored in the context of chimeric antigen receptor (CAR) T cell therapies to enhance anti-tumor activity.^{151,152}

Ultimately, the successful development of *cis*-targeted cytokine therapies will depend on the ability to engineer constructs with conditional activity at the tumor site to mitigate DLTs, while maintaining durable and complete anti-tumor responses with minimal immunogenicity to preserve the therapy's long-term efficacy.

Disclosure statement

LP is employed at the Roche Innovation Center Zürich, Switzerland. LP declares ownership of Roche stock. AM is employed at the Roche Innovation Center Zürich, Switzerland. LCD is employed at the Roche Innovation Center Zürich, Switzerland. CK is a previous employee of Roche and Curie.Bio and declares stock ownership and patents/royalties with Roche.

Funding

The author(s) declare that no financial support was received for the research and/or publication of this article.

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