REVIEW

Tumor-Homing Antibody-Cytokine Fusions for Cancer Therapy

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Abstract: Recombinant cytokine products have emerged as a promising avenue in cancer therapy due to their capacity to modulate and enhance the immune response against tumors. However, their clinical application is significantly hindered by systemic toxicities already at low doses, thus preventing escalation to therapeutically active regimens. One promising approach to overcoming these limitations is using antibody-cytokine fusion proteins (also called immunocytokines). These biopharmaceuticals leverage the targeting specificity of antibodies to deliver cytokines directly to the tumor microenvironment, thereby reducing systemic exposure and enhancing the therapeutic index. This review comprehensively examines the development and potential of antibody-cytokine fusion proteins in cancer therapy. It explores the molecular characteristics that influence the performance of these fusion proteins, and it highlights key findings from preclinical and clinical studies, illustrating the potential of immunocytokines to improve treatment outcomes in cancer patients. Recent advancements in the field, such as novel engineering strategies and combination strategies to enhance the efficacy and safety of immunocytokines, are also discussed. These innovations offer new opportunities to optimize this class of biotherapeutics, making them a more viable and effective option for cancer treatment. As the field continues to evolve, understanding the critical factors that influence the performance of immunocytokines will be essential for successfully translating these therapies into clinical practice.

Keywords: antibody-cytokine fusion proteins, cancer therapy, activity on demand, dual-cytokine products

Cytokines and Their Potential for Cancer Therapy

Cytokines are small regulatory proteins, which are crucial for modulating the activity of the immune system. These polypeptides are generally secreted by immune cells such as macrophages, B lymphocytes, and T lymphocytes, as well as stromal cells and endothelial cells under specific conditions. Cytokines primarily act via autocrine and paracrine mechanisms by binding with high specificity and affinity to cognate receptors on target cells. This interaction may trigger signaling cascades that influence immune responses, cell maturation, migration, and death.[1]

In the context of cancer, cytokines may contribute to both tumor suppression and tumor progression. For example, pro-inflammatory cytokines such as interleukin-2 (IL-2) enhance immune surveillance and boost anti-tumor responses by stimulating T-cells and natural killer cells.[2] By contrast, cytokines secreted by tumor cells and stromal cells in the tumor microenvironment (TME), like interleukin-6 (IL-6) and interleukin-10 (IL-10), may drive progression by promoting tumor cell proliferation, survival, angiogenesis, and metastasis.[3] Considering their involvement in numerous physiological and pathological conditions, cytokines have been investigated as standalone therapies and as targets for blocking agents.

Interleukin-2 marked a significant chapter in the history of cancer immunotherapy. Originally identified in the late 1970s for its potent ability to stimulate the growth of T-cells, it was rapidly investigated as a therapeutic agent.[4] IL-2 is naturally produced by activated T cells and promotes the proliferation and activation of T cells and natural killer (NK) cells. The biological activity of IL-2 is mediated through the interaction with its cognate receptor (IL2R), which can be

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expressed as a high-affinity trimeric form composed of α (CD25), β (CD122), and γ (CD132) chains or as a low-affinity dimeric form with only the β and γ chains. The high-affinity IL2R is predominantly expressed on regulatory T cells (Tregs) and activated T cells. By contrast, the low-affinity receptor is typically found on resting T cells and most NK cells.[5,6] FDA approval of recombinant IL2 (Proleukin®) was granted in 1992 for treating metastatic renal cell carcinoma and later extended in 1998 for metastatic melanoma.[7–9] Proleukin is typically administered at 600,000 to 720,000 IU/kg as i.v. bolus every 8 hours for 14 consecutive doses over 5 days. Despite the high-dose treatment, IL-2 therapy has resulted in durable and long-lasting antitumor responses in only a subset of patients, and its broader application is limited by severe side effects such as vascular leak syndrome and cytokine release syndrome.[8,10]

Interferon-alpha (IFNα) is a type I interferon predominantly produced by plasmacytoid dendritic cells and is secreted in response to pathogenic or tumor-derived signals. Its mechanism involves binding to the interferon-alpha/beta receptor (IFNAR), which activates the JAK-STAT signaling pathway.[1] This cascade promotes the phosphorylation of STAT1 and STAT2, leading to the transcription of genes that enhance immune surveillance and exhibit direct antitumor effects by inhibiting tumor cell proliferation and inducing apoptosis.[11,12] Recombinant IFNα (Intron A® and Roferon-A®) has been used to treat several cancers, including melanoma, renal cell carcinoma, and hairy cell leukemia. For example, in melanoma, IFNα is given as an adjuvant therapy at high doses (20 MIU/m²) following tumor resection to reduce the risk of recurrence.[13] Nevertheless, the therapeutic use of IFNα is often limited by significant adverse effects such as flu-like symptoms, fatigue, and bone marrow suppression, which can lead to dose reductions or discontinuation of therapy.

Tumor Necrosis Factor (TNF) is a potent cytokine in the landscape of cancer immunotherapy due to its pro-inflammatory and anticancer properties. Primarily produced by macrophages, TNF induces rapid hemorrhagic necrosis of tumors, primarily by disrupting the vasculature within the tumor microenvironment.[14,15] The biological activity of TNF is mediated through its interaction with two receptors, TNFR1 and TNFR2. The binding activates several signaling pathways, including the NF-κB pathway, which promotes inflammation, immune cell survival, and apoptosis.[1] Recombinant TNF (Beromun®) has found significant use in cancer therapy, particularly in isolated limb perfusion (ILP), a methodology used to treat unresectable sarcomas and melanomas. ILP offers an advantage by avoiding amputation and thus preserving limb function. In this setting, recombinant TNF can be administered at doses of 1–4 mg in combination, for example, with Melphalan.[16] This technique allows for high local concentrations of the drugs without significant systemic exposure. Despite its effectiveness in localized settings, the systemic administration of TNF has been limited due to its toxicity profile. Systemic high doses can cause serious side effects, including septic shock-like syndrome and organ failure.[17]

Colony Stimulating Factors (CSFs) are critical cytokines in managing chemotherapy-induced neutropenia, a common and serious side effect of cancer treatment that increases the risk of infection. Granulocyte Colony Stimulating Factor (G-CSF) and Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF) are the two primary types of CSFs used in clinical settings to accelerate white blood cell recovery, thereby enhancing patients' immune response and improving their ability to continue with chemotherapy. G-CSF (Filgrastim® and its pegylated form Pegfilgrastim®) is typically administered at 5 µg/kg as a subcutaneous injection starting 24 hours after the completion of chemotherapy until neutrophil recovery is achieved. By contrast, pegfilgrastim requires a single dose due to its extended half-life.[18–20] GM-CSF (Sargramostim®) is administered via intravenous infusion at a dose of 250 µg/m² per day, starting within 24 hours post-chemotherapy and continuing until neutrophil recovery.[20] Despite the efficacy of CSFs in managing neutropenia, their use can be associated with side effects such as bone pain, fatigue, fever, and muscle aches. Rare but severe effects include splenomegaly and, in some cases, respiratory complications.

Despite their substantial anti-cancer potential, a rapid blood clearance profile and the ability to induce side effects at very low doses have restricted the clinical use of recombinant cytokines. Unacceptable toxicities often limit the escalation to therapeutically active dose regimens. To mitigate these issues, Nektar Therapeutics and Bristol Myers Squibb developed Bempegaldesleukin, and Synthorx developed THOR-707, both of which are PEGylated forms of IL-2. However, both products encountered setbacks in Phase III trials, failing to demonstrate efficacy.[21,22] In an attempt to improve their therapeutic window, cytokines can be fused to a tumor-targeting antibody (or antibody fragment) which may enable a preferential accumulation at the site of disease. This strategy is described in more detail in the next section.

From Cytokine to Antibody-Cytokine ("Immunocytokine") Therapeutics

Antibodies which recognize accessible tumor-associated antigens may serve as ideal carriers for the direct delivery of therapeutic cytokines to the tumor microenvironment. Also known as "immunocytokines", these antibody-cytokine fusion proteins represent a novel class of biopharmaceuticals that combine the specificity of antibodies with the potent immunostimulatory effects of cytokines[23–28] (Figure 1). The therapeutic activity of antibody-interleukin-12 (IL12) fusions, specific to splice variants of fibronectin, in immunocompetent mouse models of cancer may represent a simple proof-of-concept experiment to illustrate the potential of tumor-homing immunocytokines. The tumor-homing antibody-IL12 fusion was found to be substantially more active compared to its non-targeted IL12 counterpart, achieving comparable anti-cancer activity with a more than 20-fold dose reduction.[29]

The first step into clinical trials was led by the groups of Stephen D. Gillies and Reisfeld, which developed Hu14.18-IL2. This immunocytokine combines a humanized antibody anti-GD2, a glycolipid expressed on the surface of neuroblastoma and some melanomas, with two molecules of human IL2.[30,31] The antibody portion of Hu14.18-IL2 retains human amino acid sequences within its Fab region, with only the complementarity-determining regions (CDRs) of murine origin.[30–35] In a Phase II clinical trial for metastatic melanoma, Hu14.18-IL2 was administered as an intravenous infusion at 6 mg/m^2/day. The therapeutic outcomes were limited, with only one transient partial response observed among the 14 patients with measurable metastatic melanoma[36] (NCT00109863). Another Phase II clinical trial for Hu14.18-IL2 involved pediatric patients with high-risk neuroblastoma. The immunocytokine was administered at the recommended dose of 12 mg/m^2/day in combination with GM-CSF and isotretinoin. Out of 44 patients assessed for response, 5 achieved complete responses and 2 showed partial responses[37,38] (NCT00082758).
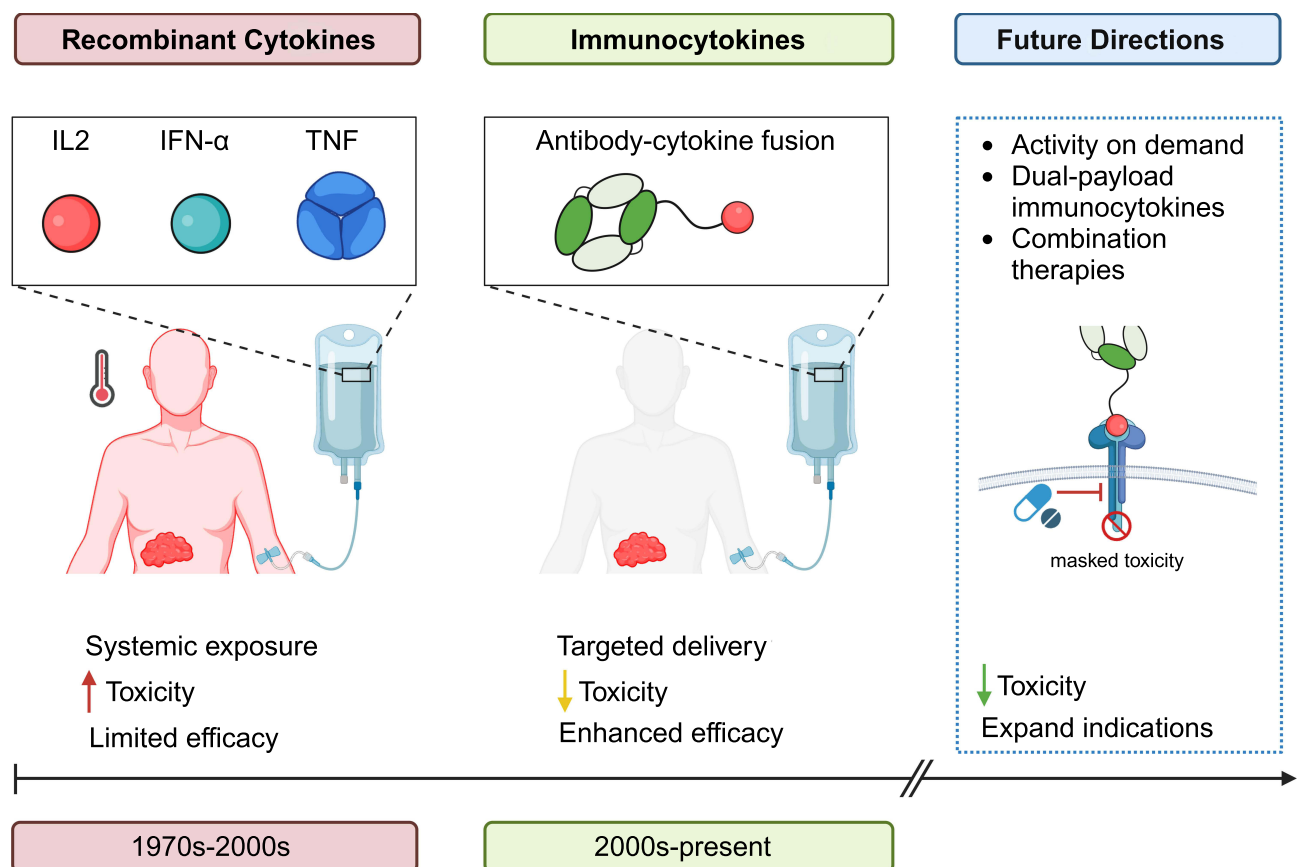

Figure 1 From Cytokines to Immunocytokines as Therapeutic Agents. The systemic administration of recombinant cytokines is associated with high toxicity and limited tumor targeting. Immunocytokines aim for targeted delivery to the tumor site, reduced adverse events, and enhanced therapeutic activity. Innovations such as dual-targeting and activity-on-demand strategies are being explored to further enhance specificity and reduce toxicity.

Meanwhile, Alan Epstein and his team focused on targeting the necrotic core within tumors, an approach they named "tumor necrotic therapy". They developed the NHS76 antibody, identified through phage display techniques using cell extracts from Burkitt's lymphoma Raji cells, and which binds specifically to nucleic acids released in necrotic tumor cells and metastatic sites.[39] Utilizing the humanized NHS76 antibody, Gillies et al developed NHS-IL2LT (Selectikine), an immunocytokine bearing a low-toxicity mutant of IL2 (IL2LT).[40] The IL-2 moiety includes the D20T mutation, which restricts its interaction with the high-affinity IL2R.[40] In the first Phase I study, the maximum tolerated dose was determined to be 0.6 mg/kg, either alone or in combination with cyclophosphamide. Although no objective tumor responses were achieved, long periods of disease stabilization were observed[41] (NCT01032681). In a Phase Ib trial involving metastatic NSCLC patients, NHS-IL2LT was administered alongside local irradiation of a single pulmonary nodule. Among the 13 patients treated, two achieved long-term survival, although no objective responses were observed[42] (NCT00879866). Another immunocytokine targeting necrotic tumor regions is NHS-IL12, which links the humanized NHS76 antibody to interleukin-12 (IL12). During a Phase Ib trial involving patients with advanced solid tumors and metastatic urothelial carcinoma, NHS-IL12 was administered in doses ranging from 4 to 16.8 µg/kg, in combination with avelumab (NCT02994953, NCT04633252). While the treatment was well tolerated across all dose levels and elicited two complete responses in bladder cancer patients during dose escalation, the dose expansion phase failed to meet the efficacy criteria. Similar to NHS-IL2LT, the therapy resulted in disease stabilization rather than tumor eradication.[43]

Antigens localized on neo-vascular structures or within the extracellular matrix (ECM) of tumors are attractive targets for the delivery of therapeutic agents.[44–46] Utilizing encoded combinatorial antibody library technologies, our group developed the F8, L19, and F16 human monoclonal antibodies, which specifically target the alternatively spliced extra domains A (EDA) and B (EDB) of fibronectin and the A1 domain of tenascin-C (TnC A1), respectively.[47–52] Expressed prominently in the angiogenic vasculature and ECM of most aggressive solid tumors and lymphomas, these markers are virtually absent in normal adult tissues, with the sole exception to female reproductive system during the proliferative phase (ie, placenta, endometrium, and some ovarian vessels).[53,54] The biodistribution profiles of radiolabeled preparations of these antibodies, studied in animal models and in cancer patients, have been promising, with a preferential long-lasting accumulation at the site of disease.[48,51,55–59]

The L19 antibody, targeting EDB, has been employed as a carrier for various therapeutic agents, including cytokines, radionuclides, photosensitizers, and coagulation factors.[57,60–64] L19IL2 (Darleukin) utilizes L19 in a diabody format wherein each single-chain variable fragment (scFv) is conjugated to a human IL-2 molecule. L19IL2 has been evaluated in over 200 patients in clinical settings, demonstrating significant clinical benefits with manageable and reversible toxicities. Initial trials investigated its use as a monotherapy in renal cell carcinoma[55] and in combination with gemcitabine for pancreatic cancer (NCT01198522). Further exploration involved combining L19IL2 with dacarbazine (DTIC) in a Phase II clinical trial for advanced metastatic melanoma patients. The trial established a recommended dose of 22.5 million IU per administration. Four patients achieved durable complete responses, and the proportion of patients experiencing partial or complete responses surpassed 34%[65] (NCT02076646).

L19TNF (Fibromun) is a fusion protein that combines L19 in scFv format with human tumor necrosis factor (TNF), exploiting TNF's natural trimerization to yield a homotrimeric molecule. In initial clinical trials, L19TNF demonstrated a favorable safety profile. Doses up to 13 µg/kg were administered systematically in patients with progressive solid tumors over repeated 3-weekly cycles, without identifying a maximum tolerated dose or encountering significant dose-limiting toxicities.[66] Furthermore, in treatments using ILP for locally advanced extremity melanoma, doses of up to 650 µg of L19TNF were well tolerated, with the majority of patients showing objective responses.[67] In two subsequent Phase I clinical trials, L19TNF was administered in combination with doxorubicin across multiple cohorts. Among these, 15 patients with soft tissue sarcoma (STS) demonstrated notable antitumor effects, including one complete remission, one partial remission, and minor tumor shrinkage in seven additional patients. The median overall survival was reported at 14.9 months, which is significant considering the advanced stage of disease in these patients.[68] L19TNF is currently evaluated in a Phase I/II study in combination with lomustine for the treatment of 15 patients with recurrent glioblastoma. Efficacy assessments revealed that 20% of the cohort achieved near-complete responses, while 53% exhibited stable disease. The therapeutic regimen resulted in a median progression-free survival of 4.2 months, with a six-month progression-free survival rate observed in 46% of the patients[69](NCT04573192). Furthermore, L19TNF is investigated

in combination with standard temozolomide chemoradiotherapy for treating newly diagnosed glioblastoma in a Phase I/II study (NCT04443010). The study focuses on determining the optimal dosage for subsequent efficacy studies in combination with chemoradiotherapy.

The co-administration of L19IL2 and L19TNF (Nidlegy™) has also been explored, leveraging the synergistic effects of the different cytokine payloads. In an initial clinical trial involving intralesional administration of melanoma patients, this approach successfully reduced tumor mass in 50% of the participants. Additionally, the combination therapy also elicited an abscopal effect, leading to the regression or complete resolution of distant, untreated lesions, highlighting the potential for broader immune-mediated antitumor activity[66] (NCT02938299).

Building on these promising results, several clinical trials were started to evaluate the efficacy of Nidlegy in different indications, including melanoma and non-melanoma skin cancers. As of today, more than 280 patients have been treated with intratumoral injections of Nidlegy, showing a manageable safety profile with mainly local adverse events. The PIVOTAL trial, evaluating the effect of Nidlegy as a neoadjuvant treatment before surgery versus surgery alone (NCT03567889), met the primary endpoint resulting in a clinically meaningful longer median recurrence-free survival (mRFS) with a reduction of 41% (HR: 059) in the risk of recurrence or death in pre-treated locally advanced melanoma patients[70,71] (NCT03567889, NCT02938299). Nidlegy™ is currently being evaluated for the treatment of various skin cancer types (NCT03567889, NCT06284590, NCT04362722, NCT05329792).

L19IL12 (Dodekin) combines the L19 moiety in tandem-diabody format linked to the human IL12 payload at the N-terminal.[72] The fusion protein is being investigated in a Phase I clinical trial in patients with advanced or metastatic solid tumors who have failed prior treatments with immune checkpoint inhibitors (NCT04471987).

F16 is a fully human monoclonal antibody also developed by phage-display technology, which selectively binds to the A1 domain of tenascin-C, a component highly expressed in the extracellular matrix of solid tumors yet minimally present in normal tissues.[49] This antibody, in its small immunoprotein (SIP) format, has demonstrated a broad capacity to target tenascin-C across a spectrum of malignancies including glioblastoma, lymphoma, and melanoma, underpinning its utility in targeted therapy.[53,58,73–76] Capitalizing on the favorable biodistribution characteristics of F16, its derivative F16-IL2 (Teleukin) has been explored in combination with chemotherapeutic agents such as paclitaxel and doxorubicin for breast cancer, melanoma, and non-small lung cancer (NCT01134250).[77] F16IL2 has been consistently safe and effective when administered weekly at 25 MioIU, demonstrating promising efficacy in combination with paclitaxel for lung cancer and with doxorubicin for various solid and metastatic breast cancers. This regimen has resulted in partial responses and sustained disease control.[77,78]

Trans-Acting Immunocytokines

Trans-acting immunocytokines are a class of therapeutic agents capable of targeting specific structures in the tumor microenvironment, such as components of the extracellular matrix or tumor-associated antigens. However, the cytokine payload acts distally from the site of antibody binding, thereby activating and recruiting immune cells (like T cells and NK cells) that are not the direct target of the antibody component. For example, immunocytokines based on L19 or F8 antibodies specifically target ECM components, localizing their cytokine payloads directly within the tumor milieu. The primary goal is to optimize the therapeutic window of the cytokines by enhancing their concentration at the site of disease while minimizing systemic exposure. Further, these agents exhibit a trans-acting mechanism by recruiting and activating immune cells to disrupt the immunosuppressive conditions that characterize the tumor environment (Figure 2).

The efficacy of trans-acting immunocytokines has been validated across a range of preclinical models. For example, the administration of L19IL12 and F8TNF to mice bearing CT26 tumors induced a targeted expansion of CD8+ T cells within the tumor microenvironment. This response was characterized by an oligoclonal expansion of T cells, predominantly recognizing the AH1 peptide, a derivative of the gp70 protein from the murine leukemia virus naturally expressed in several BALB/c tumor cell lines.[79,80] Treated tumors exhibited a robust infiltration of T cells with a tissue-resident memory phenotype, characterized by the upregulation of activation markers CD69, CD103, and CD49a.[80] Isolated and in vitro-expanded AH1-specific T cells demonstrated targeted cytotoxicity by selectively eliminating antigen-positive tumor cells and producing significant levels of IFNγ and TNFα. Despite effectively inhibiting tumor growth when
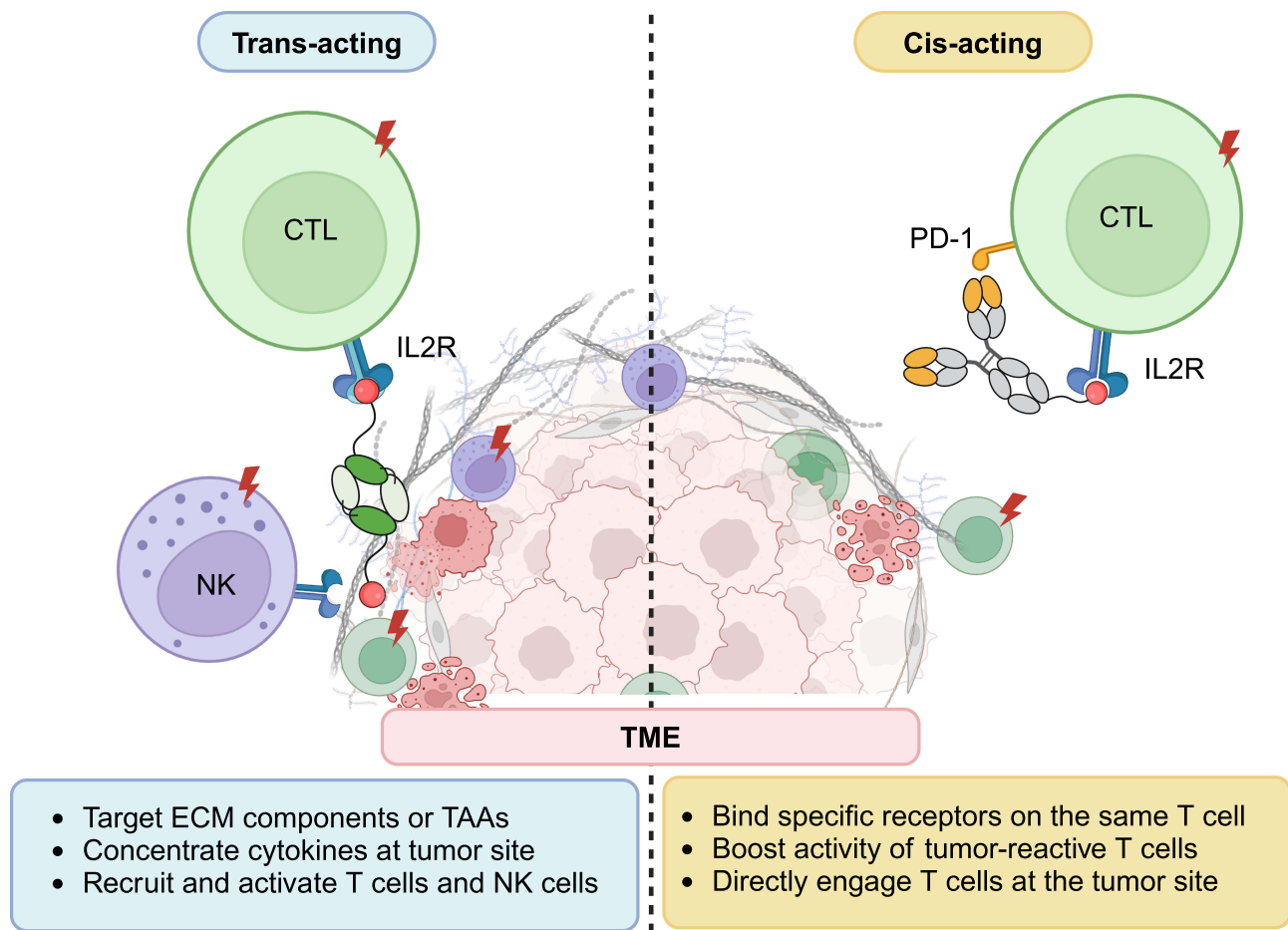
Figure 2 Trans- vs cis-acting immunocytokines. Trans-acting immunocytokines. Cytokines act on immune cells that are recruited to the tumor microenvironment, enhancing systemic immune response. Cis-acting immunocytokines: cytokines directly act on the cells they are targeted to, delivering localized immune activation.

reintroduced into syngeneic cancer models, these T cells failed to achieve complete tumor regression due to suboptimal in vivo persistence.[81]

In preclinical models of glioblastoma, L19TNF and L19IL12 have demonstrated their potential to engage the immune system despite the complex and immunosuppressive microenvironment typical of the malignancy. The immunocytokines promoted a significant infiltration of both CD8+ and CD4+ T cells, as well as NK cells into the tumor stroma.[82] This response was accompanied by a marked increase in pro-inflammatory cytokines, reshaping the tumor microenvironment to favor an immune-mediated attack. Importantly, the study bridged preclinical findings into a clinical context, demonstrating that systemic administration of L19TNF in glioblastoma patients was safe and associated with increased tumor infiltration by T cells and induced observable tumor necrosis.[82]

Trans-acting immunocytokines may help reshape the tumor microenvironment, enhancing localized immune reactions and stimulating broader systemic immune responses. The intralesional application of L19IL2 and L19TNF in melanoma patients serves as a compelling example, where targeted treatment not only addressed the primary tumor but also elicited systemic immune reactions, as evidenced by the abscopal effect observed at distant sites.[66,70]

Cis-Acting Immunocytokines and Attenukines™

In contrast to trans-acting agents, cis-acting immunocytokines direct their therapeutic effects specifically upon the cells they target. The principle of cis-acting immunocytokines is to achieve therapeutic modulation by targeting the cytokine payload to the same immune cell which is recognized by the antibody moiety. While the cytokine payload may also affect

neighboring cells "in-trans", the primary goal is to activate the cells directly targeted by the antibody component (Figure 2).

One of the most important products in this class of biopharmaceuticals is represented by PD1-IL2v (RO7284755). This fusion protein marries an interleukin-2 variant (IL-2v) with an antibody targeting the programmed death-1 (PD-1) receptor. Its design facilitates the simultaneous binding to PD-1 and the IL-2 receptor βγ chains on the same T cell. This dual engagement is proposed to selectively bolster the activity of tumor-reactive T cells expressing PD-1 while avoiding the undesired stimulation of regulatory T cells, which characteristically express CD25.[83,84] Initial in vitro functional assays have substantiated that the PD1-IL2v fusion protein specifically triggers cis signaling in PD-1+ T cells, as T cells with blocked PD-1 receptors did not exhibit STAT5 phosphorylation in the presence of anti-PD1-IL2v.[84] In the RT5 mouse model, PD1-IL2v therapy initiated robust tumor regression. Expansion of tumor-specific CD8+ T cells was observed post-treatment, yet the persistence of these activated T cells diminished over time in relapsed tumors.[83,84] PD1-IL2v is currently being evaluated in a Phase I clinical trial, which explores its safety and efficacy in patients with advanced and/or metastatic solid tumors (NCT04303858). The trial includes an examination of PD1-IL2v both as a monotherapy and in combination with Atezolizumab. Insights from another IL-2v carrying immunocytokine, FAP-IL2v, which was targeting Fibroblast Activating Protein in a different trial (NCT03386721), have led to a strategic shift. Mechanistic insights have emerged suggesting that the engagement of IL-2Rα is indispensable for optimizing the synergistic impact of combined PD-1 and IL-2v therapy.[84,85] Despite the potential shown in preclinical settings, the development of FAP-IL2v has been deprioritized.

The anti-PD1/IL7v (BICKI®IL7v) represents another example of a cis-acting immunocytokine, merging the specificity of an anti-PD-1 monoclonal antibody with a modified version of interleukin-7 (IL7v). The fusion protein is designed to engage and activate PD-1+ IL-7R+ T cells directly at the tumor site.[86,87] In preclinical settings, anti-PD1/IL7v has demonstrated efficacy in orthotopic hepatocellular carcinoma models, where traditional PD-1 therapies fall short. In combination with sorafenib or oxaliplatin, the immunocytokine achieved complete responses in treatment-resistant models. The treatments led to marked proliferation of PD-1+ CD127+ (IL-7R+) TCF1+ T cells, highlighting anti-PD1/IL7v potential to activate and expand tumor-specific T cell populations effectively.[86,87]

Attenukines represent a different technology of immunocytokines which integrates modified cytokines with tumor-specific antibodies. By selectively activating immune responses directly within the tumor milieu, attenukines leverage the antibody's targeting capability to deliver cytokines that have been molecularly tuned to minimize interaction with non-targeted receptors. The CD38-Attenukine (TAK-573), a fusion of humanized anti-CD38 monoclonal antibody and two molecules of attenuated interferon alpha-2b, is engineered to target CD38+ multiple myeloma cells.[88] This attenukine utilizes a unique epitope, avoiding interference with existing anti-CD38 therapies, and aims to mitigate common side effects of systemic IFNα therapy. The ongoing-phase I/II clinical trial of TAK-573 has shown promising pharmacodynamic effects in patients with relapsed/refractory multiple myeloma[89] (NCT03215030).

The Role of the Antibody Format

Immunocytokines harness the targeting capabilities of antibodies to deliver cytokines directly to tumor sites, enhancing therapeutic efficacy while minimizing systemic toxicity. The choice of antibody format is crucial in determining the pharmacokinetics, biodistribution, and overall therapeutic potential of these fusion proteins (Figure 3).

Molecular size represents a key parameter influencing the pharmacokinetic properties of biopharmaceuticals. Full-length immunoglobulin G (IgG) antibodies comprise both the antigen-binding Fab regions and the Fc region, with a molecular weight of approximately 150 kDa. One primary advantage of this format is the extended half-life of an IgG antibody, which is around 21 days in serum.[90,91] Additionally, the Fc region interacts with the neonatal Fc receptor (FcRn), a process that further prolongs their circulatory half-life by protecting them from lysosomal degradation.[1,92,93] Second, the bivalent nature of IgG antibodies ensures robust binding to target antigens. Their high retention rates, along with an extended half-life, provide IgG antibodies with a prolonged window of activity, enhancing their therapeutic efficacy. Third, IgG antibodies bind to Fc gamma receptors (FcγRs), which are expressed on monocytes, macrophages, and NK cells. However, Fc-mediated immune activation remains a double-edged sword. While it is advantageous for inducing antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) against tumor
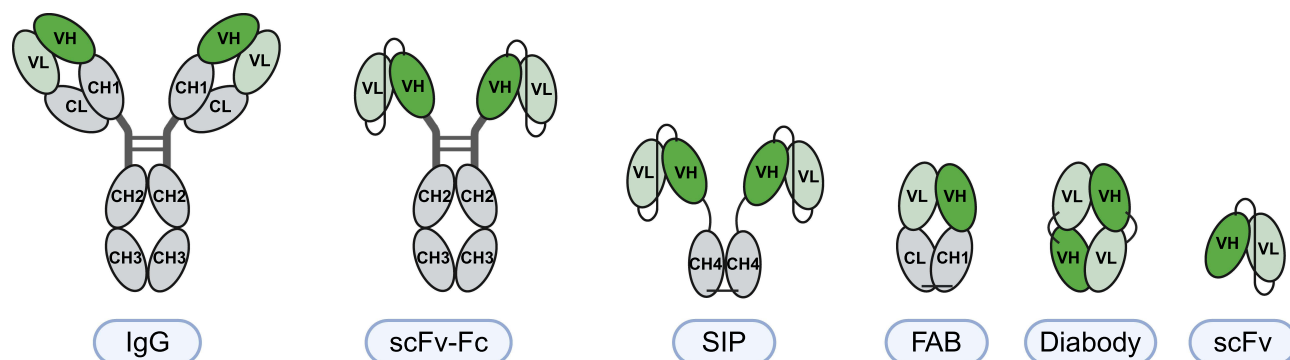
Figure 3 Most commonly used antibody formats for the development of immunocytokines, VH: heavy chain variable domain. VL: light chain variable domain. (**C**) constant domain of the heavy (CH1–4) and the light chain (CL). SIP: small immune protein that uses the εCH4 domain of an IgE antibody to form a covalent dimer. FAB: Fragment antigen binding. Diabody: a dimer composed of two scFv, where the linker between VH and VL is too short to allow intrachain pairing. scFv: single chain variable fragment.

cells, it may also trigger undesired systemic immune responses.[94,95] This, combined with the long half-life and high tissue retention, may lead to higher toxicity in patients. One way to attenuate these effector functions can be through glycoengineering or specific amino acid modifications without compromising the antibody's targeting capabilities.[96,97] The high tumor uptake observed with whole IgG antibodies often exceeds that of smaller antibody fragments. However, this superior tumor localization is frequently offset by suboptimal tumor-to-organ and tumor-to-blood ratios, which can lead to increased off-target effects and systemic toxicity.[50,98,99] To minimize these undesired toxicities, employing antibody fragments as cytokine delivery vehicles could be advantageous.

Antibody fragments represent a diverse group of engineered antibodies that include single-chain variable fragments (scFv), diabodies, Fab fragments, and scFv-Fc, among others. These fragments retain the antigen-binding specificity of full-length antibodies but are smaller in size, ranging from 27 kDa to 60 kDa, which significantly impacts their pharmacokinetics and biodistribution. Due to their smaller size and rapid clearance from circulation, antibody fragments result in lower systemic exposure and thus reduced systemic toxicity compared to full-length antibodies. Additionally, these fragments tend to have better tumor penetration and distribution, enhancing the therapeutic efficacy of the cytokine payload while maintaining a high therapeutic index. For example, the L19 diabody format demonstrated superior biodistribution properties and achieved higher tumor-to-blood ratios compared to full-length IgG in preclinical models, as confirmed by radioiodinated protein preparations and quantitative biodistribution analyses.[50]

Antibody fragments can be genetically fused to various cytokines without compromising the payload's bioactivity or the fragment's targeting ability, allowing for a wide range of therapeutic applications and customization. For cytokines like the homotrimeric TNF, the scFv is often the preferred fusion partner to maintain the cytokine's natural oligomeric state. Immunocytokines like F8TNF and L19TNF have demonstrated excellent biodistribution profiles.[59,100,101] Interestingly, fusion proteins of the TNF-superfamily that also form non-covalent homotrimers exhibited biodistribution profiles that were inferior to those of L19TNF and F8TNF.[102] Expressing these fusion proteins as single polypeptides rather than as monomeric units may enhance their biodistribution and stability.[101–103] Glycosylation and the resultant increase in molecular mass can also hinder the effectiveness of tumor targeting in vivo for antibody-fragment-based biopharmaceuticals. Heavily glycosylated payloads, such as murine B7.2, tend to be rapidly cleared via the hepatobiliary route, thus failing to localize efficiently to tumor sites. However, when similar glycosylated payloads are incorporated into an intact IgG, there is a marked improvement in selective tumor uptake.[104–106]

Immunocytokines based on antibody fragments with favorable tumor-targeting profiles, surpassing the efficacy of the parental antibodies, include products based on IL-2, IL-12, GM-CSF, IL-10, and IL-4.[29,47,48,72,107–113] However, certain cytokines like interferon-gamma (IFNγ) may not be ideal partners. Trapping of the cytokine payload by abundant receptors in normal tissues can compromise the targeting performance of these fusion proteins. For instance, antibody-IFNγ fusions were shown to selectively localize to neoplastic lesions only under specific conditions, such as using IFNγ receptor knockout mice or pre-administering a large amount of unlabeled antibody-cytokine fusion protein to wild-type tumor-bearing mice.[114,115]

Overall, antibody fragments are more suitable for the delivery of most cytokine payloads, as they exhibit favorable tumor-to-organ ratios at early time points following intravenous administration, ensuring more precise delivery to tumor sites with reduced off-target effects.

Activity-on-Demand

Upon intravenous injection, conventional cytokine-based pharmaceuticals display full biological activity, which typically persists until the blood concentration levels drop below a certain threshold. Side-effects of pro-inflammatory payloads may be severe, including hypotension, pulmonary edema, flu-like symptoms, nausea and vomit. It would important to devise strategies that retain therapeutic activity while decreasing toxicity, thus leading to an improved therapeutic index.[116] A number of strategies have emerged over the last few years, which can largely be grouped into three main categories [Figure 4].

In a first approach, the cytokine moiety is broken down into smaller components, which may reassemble at the site of disease, upon antibody binding engagement with the cognate antigen. The strategy has been described for the two subunits of interleukin-12,[117] as well as for members of the TNF receptor superfamily.[118]

A number of companies and research groups have explored the possibility of "masking" the cytokine moiety with a binding domain (eg, a peptide ligand), using a linker which could be proteolytically cleaved at the tumor site, thus releasing full biological activity.[119–121] This strategy (which we refer to as "extracellular corks" or "extra-cork technology") may potentially suffer from variable levels of proteolytic activity between animal models and human subjects, or even among cancer patients.
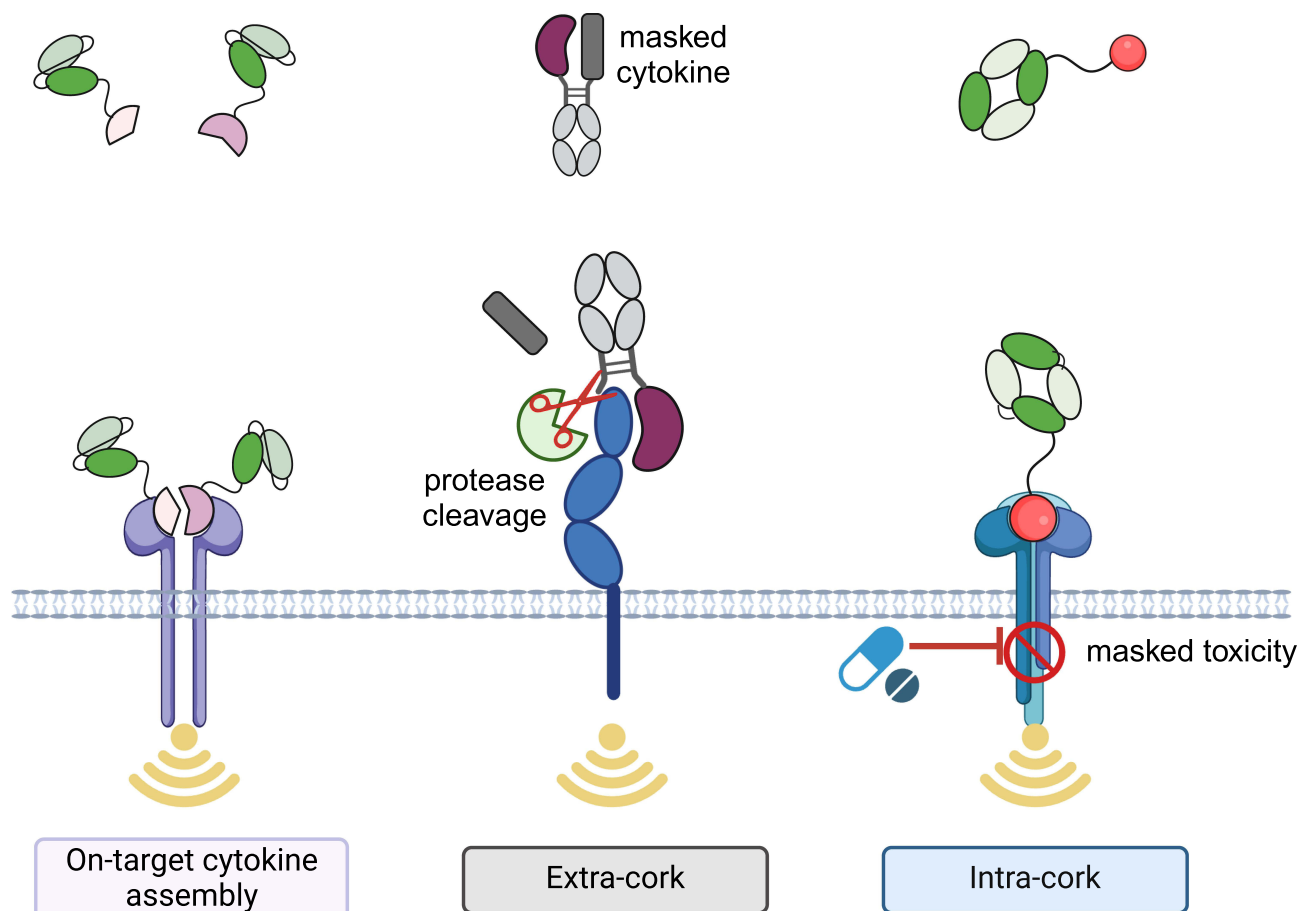

Figure 4 Different strategies for Activity on Demand. On-target cytokine assembly: cytokines assemble and activate directly at the tumor site; Extra-cork: cytokines are activated by external tumor-specific factors; Intra-cork technology: small molecule inhibitors transiently inhibit cytokine signaling in peripheral tissues, enhancing tumor-specific activity.

A temporal shutdown of signaling can also be achieved by the concomitant administration of rapidly-clearing small organic drugs, which selectively block the cytokine's activity, as long as they are present in sufficiently high concentrations in blood ("intra-cork technology"). This strategy is particularly attractive when used in conjunction with antibody-cytokine fusion proteins, which clear rapidly from circulation but exhibit a long residence time in the tumor.[122,123] For example, the co-administration of Ruxolitinib with L19-IL12 allowed to preserve full therapeutic activity in mouse models of cancer while completely abolishing side effects (eg, body weight loss, liver damage) and undesired release of interferon-gamma into peripheral blood.[122]

As most of the above-described strategies have been implemented at the preclinical level, it will be interesting to see how they perform in clinical trials.

Dual Cytokine Products

The immune system often deploys multiple signals to boost a response against tumors or pathogens. In particular, the simultaneous concentration at the tumor site of multiple cytokine combinations holds great promise in promoting a more potent anti-cancer activity[62,79,116,124] and facilitating the expansion of tumor-specific T cell populations. This potential has led to the testing of antibody-cytokine fusion products at the preclinical and clinical levels.[36,37,43,55,66–70,72,78,125–127] For example, the combination of L19IL2 and L19TNF has recently completed Phase III clinical trials for the treatment of stage IIIB and C melanoma patients[70] (NCT 03567889, NCT02938299) and is currently being investigated in Phase II clinical studies in patients with high-risk non-melanoma skin cancer (NCT05329792, NCT04362722).

However, it is important to acknowledge that the industrial development of combination products may have its challenges. As individual dose escalation studies would need to be initialized, the potential for duplication of production costs and regulatory activities could pose significant hurdles.[128–131]

Fusing two synergistic cytokines into the same antibody moiety could simplify clinical development, enabling the simultaneous delivery of two payloads at the disease site. Our team and others have extensively described this strategy named "dual cytokine antibody fusion proteins"[124,127,132–139] [Figure 5].
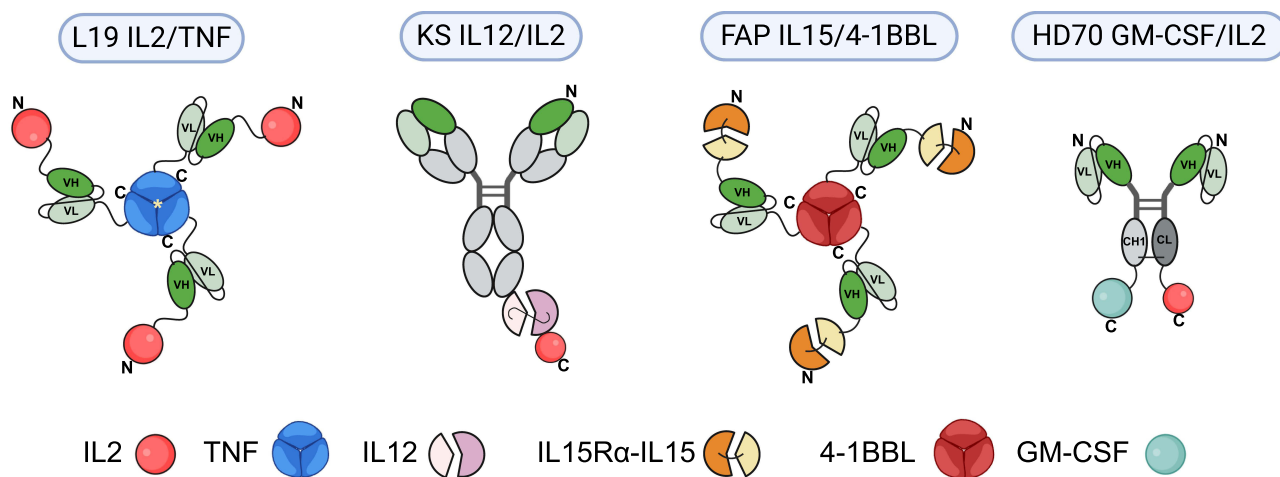

Figure 5 Examples of "dual-cytokine" antibody-fusion proteins. From left to right: L19 IL2/TNF: potency-matched dual-cytokine antibody fusion protein targeting a spliced variant of fibronectin and featuring IL2 and TNF;[124] The IL2 moiety is attached to the N-terminus of the scFv through a polypeptide linker, while the TNF payload is linked to the C-terminus of the scFv via another polypeptide linker. The TNF moiety facilitates the formation of a non-covalent trimer. KS IL12/IL2: anti-EpCAM antibody-cytokine fusion protein engineered with IL12 and IL2;[132] The p35 subunit of IL-12 is fused to the C-terminus of the IgG heavy chain, with the p40 subunit of IL-12 connected to p35 via a polypeptide linker. Additionally, IL-2 is fused to the C-terminus of the p40 subunit. FAP IL15/4-1BBL: Fibroblast Activating Protein (FAP) – targeting antibody fragment fused to both 4–1BBL and IL15;[134] IL15Rα is fused to IL15 via a polypeptide linker, the IL15 moiety is attached to the N-terminus of the scFv through a polypeptide linker, while the 4–1BBL payload is linked to the C-terminus of the scFv via another polypeptide linker. The 4–1BBL moiety facilitates the formation of a non-covalent trimer. HD70 GM-CSF/IL2: anti-EpCAM antibody-cytokine fusion protein featuring GM-CSF and IL2.[136] The fusion protein is based on a heterodimerized core structure formed by CH1 and CL domains (heterominibody) with C-terminally fused cytokines and N-terminally fused scFv.
Abbreviations: VH, heavy chain variable domain; VL, light chain variable domain; CH1, constant domain 1 of the heavy chain; CL, constant domain of the light chain (CL); N, N-terminus, C, C-terminus.

One of the first examples of this approach was a fusion protein featuring IL12 and IL2.[132] The molecule (KS-IL12/IL2) was able to induce tumor regression when injected intratumorally into LLC tumors expressing human EpCAM. Similarly, the targeted delivery of IL15 and 4–1BBL showed encouraging therapeutic efficacy in a mouse lung metastases model.[134]

Dual cytokine fusion proteins have been investigated at the preclinical level for the treatment of hematologic malignancies.[135,139] A bivalent anti-CD30 antibody fragment fused with IL12 and IL2 inhibited tumor growth in mice transplanted with hybridoma cells.[135] Our group has previously described a promising dual-cytokine antibody-fusion protein for the treatment of multiple myeloma (MM).[140] The prototype combined an anti-CD38 antibody with IL2 and TRAIL, two cytokines that exert their biological activity at similar molar concentrations. The product induced apoptosis in MM and lymphoma cell lines and selectively killed plasma cells isolated from patients in vitro.

Schanzer et al described a dual fusion protein incorporating IL2 and GM-CSF in a heterominibody specific to EpCAM. However, the single cytokine fusion proteins showed similar antitumor activity as the dual cytokine fusion protein.[136]

Despite numerous prototypes exploring multiple cytokine payloads at the preclinical level, this class of biotherapeutics has yet to advance into clinical investigation. The excessive molecular weight of these pharmaceuticals might hinder their pharmacokinetic profiles and tumor-targeting abilities.[133] Moreover, the fusion of two cytokine payloads with different biological activities could potentially compromise the therapeutic efficacy of the fusion protein. For example, MTD of IL12 and TNF in cancer patients has been reported in the µg range,[15,140,141] while IL2 and IL4 have been administered at the mg scale.[142–144]

Our team has introduced the concept of "potency-matched" antibody-cytokine fusion proteins, wherein a single amino acid substitution is employed to attenuate the potency of the most active cytokine. We first described a molecule featuring IL-2 and a human TNF variant (R108A mutant) fused to the F8 antibody, which targets the alternatively spliced extra domain A of fibronectin (EDA). Remarkably, this fusion protein induced cancer cures in immunocompetent tumor-bearing mice and showed a superior safety profile compared to a similar fusion protein based on wild-type cytokine payloads.[124]

Combination Opportunities

Immunocytokines featuring pro-inflammatory payloads may be suitable for combination studies with several anti-cancer therapeutic agents as they can mediate the activation of the immune system and increase vascular permeability, thus enhancing drugs' uptake at the tumor site.

Various combinations have been investigated at the preclinical level, including intact antibodies,[54,145–149] immune check-point inhibitors,[150–153] bispecific antibodies,[154,155] other immunocytokines,[107,115,124,126,127,132,134,139,150,156,157] antibody-drug conjugates (ADCs),[158,159] cytotoxic drugs,[76–79,100,160–163] small molecule drug conjugates (SMDCs)[164–167] and radiation.[42,57,167–176]

Surgery and conventional chemotherapy are usually the first-line treatment available to cancer patients. Unfortunately, chemotherapy may induce severe side effects, preventing dose escalation to therapeutically active regimens. Immunocytokines can synergize with cytotoxic drugs by promoting drug uptake at the neoplastic lesion. However, these drugs could, in principle, also kill tumor-infiltrating leukocytes (TILs) needed to eradicate tumors. It has been shown that the schedule in which immunocytokines and cytotoxic drugs are administered may dramatically change the therapy outcome. For example, our group showed that tumor remission in immunocompetent mouse models of melanoma was observed when paclitaxel was administered after targeted IL2.[163,177] More recently, we have shown that the combination of targeted TNF with lomustine could cure mice bearing orthoptic gliomas and showed objective responses in recurrent glioma patients[178] (NCT04573192).

Another strategy to deliver cytotoxic drugs to the tumor site is to use ADCs or SMDCs against tumor-associated antigens, thus sparing healthy organs. The combination of an immunocytokine featuring IL2 and an ADC showed superior anticancer activity compared to both agents when used as monotherapy.[158] It has been shown that SMDCs may be superior compared to ADCs in terms of tumor-tissue penetration, immunogenicity and lower cost-of-goods.[179–182] Recently, we have shown that non-internalizing SMDCs could efficiently synergize with L19-IL2.[164,166,167,183]

Immune check-point inhibitors have revolutionized the field of cancer therapy, and several products (against, for example, CTLA-4, PD1 or PD-L1) have gained marketing authorization for the treatment of several malignancies.[184,185] Immunocytokines have been shown to efficiently potentiate the activity of immune check-point inhibitors in several

immunocompetent mouse models of cancer by turning "cold" tumors "hot".[84,125,138,150,152,153,165,184–189] For example, the combination of targeted-IL12 with an anti-PD1 antibody induced a complete response in all treated mice in a tumor model that usually does not respond to anti-PD1 treatment.[125]

As described in the previous chapter, combining multiple immunocytokine products featuring synergistic cytokine payloads offers an elegant strategy to increase their therapeutic index. At the preclinical level, several immunocytokine combinations have been investigated.[132,133,137,150,155,190] However, only a few products entered clinical trials.[37,70,71]

Future Trends

The continuous evolution of immunocytokines is imperative for enhancing their clinical utility, primarily by improving their safety profiles. Historically, the administration of cytokines such as IL2 has been associated with severe side effects, including vascular leak syndrome and cytokine release syndrome.[7,8] Modern immunocytokine design focuses on maintaining therapeutic efficacy while minimizing these adverse events.[25] In the clinical setting, prolonged infusion times have improved the safety profile of immunocytokines. This approach allows for the accumulation of therapeutic levels within the tumor while maintaining low blood levels, thereby reducing the risk of adverse events.[116,191]

Advancements in protein engineering have given rise to attenuated cytokine variants with markedly reduced toxicity. An example is the development of attenukines, immunocytokines engineered with modified receptor-binding affinities.[89] These molecules are designed to confine immune activation to the tumor microenvironment, thereby minimizing systemic exposure. Another approach involves protease-activable cytokines, which are activated by tumor-specific proteases.[120] Similarly, chemically controlled cytokines are engineered to respond to specific chemical signals, allowing temporal and spatial control over immune activation.[119] Intra-Cork technology offers a novel and straightforward strategy to improve the safety profile of antibody-cytokine fusions. By employing pathway-selective small-molecule inhibitors, this approach transiently inhibits cytokine signalling in peripheral tissues, thereby reducing systemic toxicity. This method ensures that cytokine activity is localized at the tumor site, thereby enhancing therapeutic efficacy. For instance, the combination of L19-IL12 with the JAK2 inhibitor Ruxolitinib effectively abrogates cytokine-driven toxicity without compromising anti-tumor effects.[122]

Exploring synergies between immunocytokines and other therapeutic modalities such as immune checkpoint inhibitors and radiotherapy holds significant promise. The combination of these therapies has the potential to amplify anti-tumor responses through complementary mechanisms of action. The integration of immunocytokines with ICIs, such as anti-PD1, can enhance immune activation by simultaneously stimulating effector T cells and relieving inhibitory checkpoints. This synergy can result in a more robust and sustained immune attack of tumor cells. Preclinical and clinical studies have shown that immunocytokines, by enhancing T cell infiltration and activation within the tumor microenvironment, can potentiate the efficacy of ICIs, leading to improved outcome.[150–153] Combining immunocytokines with radiotherapy offers another promising strategy. Radiotherapy can induce immunogenic cell death and enhance the presentation of tumor antigens, thereby creating an environment prone to immune activation. Immunocytokines may further boost this effect by promoting the recruitment and activation of immune cells.[167,168] Co-administration of different immunocytokines can also be a powerful approach. For example, the combination of L19IL2 and L19TNF has shown remarkable anti-tumor activity by leveraging the complementary mechanisms of IL2's immune activation and TNF's direct cytotoxic effects on tumor vasculature.[70] Such combinations can enhance therapeutic outcomes by simultaneously targeting multiple pathways involved in tumor growth and immune evasion.

Fusing two synergistic cytokines into the same antibody moiety can simplify clinical development, enabling the simultaneous delivery of two payloads at the disease site. Our group and others have described this strategy named "dual cytokine antibody fusion proteins".[124,132,136] Examples of such immunocytokines include KS-IL12/IL2 and IL15/4-1BBL, both demonstrating potent anti-tumor activity in preclinical models.[132,134] Our development of "potency-matched" fusion proteins, such as the IL2 and TNF variant fused to F8/L19, represents a significant advancement. By employing single amino acid substitutions to attenuate cytokine potency, we ensure robust anti-tumor responses while maintaining an optimal safety profile.[124]

Antibody-cytokine fusion proteins represent a promising class of therapeutic agents. However, their cost-effectiveness remains a critical consideration. Despite their innovative approach, the development and production of immunocytokines

involve complex and expensive processes, including cell-line development activities, up- and down-stream process optimization, manufacturing activities and clinical operations. This complexity may translate into high costs for patients. Nevertheless, their ability to provide targeted treatment and potentially reduce the need for additional therapies or interventions may offset their higher initial costs. Effective cost management and careful assessment of their long-term benefits versus traditional therapies are essential to fully evaluate their economic viability and overall impact on patient care.

The evolution of immunocytokine therapy through protein engineering and innovative combinations opens new opportunities. By addressing the challenges of toxicity and optimizing multi-cytokine strategies, these approaches promise to significantly improve clinical outcomes for cancer patients.

Disclosure

Dario Neri is a co-founder and shareholder of Philogen (www.philogen.com), a Swiss-Italian Biotech company that operates in the field of ligand-based pharmacodelivery. Eleonora Prodi and Roberto De Luca are employees of Philochem AG, a daughter company of Philogen acting as discovery unit of the group. The authors report no other conflicts of interest in this work.

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