**Cancer
Immunology
Research**

Antibody–Cytokine Fusions: Versatile Products for the Modulation of Anticancer Immunity

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Abstract

The remarkable clinical success of immune-checkpoint inhibitors for the treatment of a growing number of cancer types has sparked interest in the discovery of novel forms of immunotherapy, which may be used alone or in combination. In this context, cytokine-based therapeutics are well poised to play a role in modern cancer therapy. This article focuses on antibody–cytokine fusion proteins (also called "immunocytokines") as one class of biopharmaceuticals that can substantially improve the therapeutic index and, thus, the applicability of cytokine products. In many preclinical settings, antibodies can be used to preferentially deliver many (but not all) types of cytokines to primary and metastatic tumor lesions. The antibody-based delivery of certain proinflammatory payloads (such as IL2, IL12, and TNF) to the tumor microenvironment can lead to a dramatic potentiation of their anticancer activity. However, although some fusion proteins have advanced to late-stage clinical trials, much work remains to be done in order to fully characterize the mechanism of action and the pharmaceutical potential of immunocytokines in the clinical setting. Various factors contribute to *in vivo* performance, including the target antigen, the antibody properties, the nature of the payload, the format of the fusion protein, the dose, and schedule, as well as their use in combination with other therapeutic modalities. Protein engineering opportunities and insights in cancer immunology are contributing to the development of next-generation immunocytokine products and of novel therapeutic concepts, with the goal to increase antitumor activity and reduce systemic toxicity (a common problem for cytokine-based biopharmaceuticals).

From Cytokines to Immunocytokines

Cytokines are a broad and loosely defined class of relatively small proteins that play a role in regulating the function of the immune system (1). Many cytokines are present in blood at very low concentrations (e.g., single-digit picomolar concentrations for IL6 and IL12), but these values can grow more than 1,000-fold in certain pathologic conditions. Some cytokines stimulate the activity of leukocytes that are important for fighting cancer, and recombinant preparations of these proteins have been considered as biopharmaceutical products. Cytokine-based products that have received marketing approval for cancer therapy include recombinant human IL2 (e.g., Proleukin as a T-cell and NK cell stimulator), IFNα (e.g., Intron A and Roferon-A as proteins with tumor cell biocidal properties and immunostimulatory action), and TNF (e.g., Beromun, a biopharmaceutical that may induce hemorrhagic necrosis of certain tumors and increase inflammation). Cytokines that stimulate the bone marrow to produce granulocytes (e.g., G-CSF and GM-CSF) are routinely used to accelerate white blood cell recovery after bone marrow transplantation or chemotherapy.

Most cytokine products on the market are used at low doses (sometimes less than 1 mg) because these proteins bind to the cognate receptors with low dissociation constants and are, there-fore, very active. Proinflammatory cytokines may cause adverse events (e.g., hypotension and leakage of fluids in the extravascular space, flu-like symptoms, nausea, and vomiting), preventing dose escalation to therapeutically active regimens. It is, therefore, generally accepted that recombinant cytokine products (e.g., IL2) would benefit from an increase in therapeutic index (2). One general avenue for increasing the therapeutic index of cytokines with antitumoral properties consists of the antibody-based delivery of these immunomodulatory payloads to the neoplastic site. The earliest reports on antibody–cytokine fusions include the work of the groups of Reisfeld and Gillies (3–5), Morrison (6–8), and Epstein (9–11). These authors described and characterized antibody fusions with many payloads, including IL2, IL4, IL12, IL21, as well as interferons and members of the TNF superfamily. Those proteins are described in detail in dedicated reviews (12–16) and will not be repeated here. In most cases, antibodies were directed against tumor-associated antigens expressed on the surface of tumor cells. Over the last two decades, our group (in collaboration with Luciano Zardi and with Philogen) has systematically developed a large number of antibody–cytokine fusions directed against components of the modified extracellular matrix of the tumor (12–16). These included the human F8 and L19 antibodies, which are specific to the alternatively spliced EDA and EDB domains of fibronectin, respectively (17, 18). F8 and L19 recognize their cognate antigen with identical affinity in mouse and man. The tumor-targeting performance of these antibodies has been well established in tumor-bearing mice. More than 100 patients with solid tumors (19) or lymphomas (20) have been imaged using radiolabeled preparations of L19, validating the tumor-homing potential of this antibody for clinical applications. Most of the general statements presented in this article stem from work with L19 and F8 derivatives, as these antibodies have been fused to the largest number of cytokine payloads so far (12–16).

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At the preclinical level, the anticancer activity of antibody–cytokine fusions directed against cellular surface antigens or against extracellular matrix components can be of comparable magnitude (21). The biomedical and pharmaceutical potential of the immunocytokine strategy is well illustrated by the following example. When treating immunocompetent mice bearing subcutaneous C51 tumors either with murine IL12 or with the same payload fused to the L19 antibody in single-chain Fv (scFv) format, a dose-dependent therapeutic effect was observed, alongside a >20-fold potentiation of therapeutic activity for the L19 fusion protein (22).

Most observations published indicate that the targeted delivery of immunomodulatory payloads may lead to a potentiation of cytokine activity. A high local concentration of proinflammatory factors may lead to a preferential expansion and activation of NK cells and of tumor-specific T cells, which are found at higher relative frequencies in the neoplastic mass compared with secondary lymphoid organs (23). The surprising observation of a similar therapeutic activity for IL2 fusions based on full immunoglobulins specific to a tumor-associated antigen or to an irrelevant target protein used as negative control has been reported (24). It is possible that the systemic effect of a persistently high concentration of IL2 in blood may drive therapeutic activity in certain animal models of cancer. However, numerous other studies have reported a clear correlation between the use of tumor-specific cytokine fusion proteins and the observation of a therapeutic index gain compared with nontargeted cytokine fusions (3–16).

When small antibody fragments are fused to proinflammatory cytokines, the circulatory half-life is typically short, and the toxicity profile is similar to the one of the unconjugated cytokines. The therapeutic activity of payloads such as IL2, IL12, and TNF increases (at least in mice) as a result of a preferential localization of the fusion protein at the tumor site. More than 30 cytokine payloads have been fused to antibodies, and the resulting products have been characterized, in terms of both pharmacokinetic properties and biological activity in mouse models of disease (reviewed in refs. 12–16).

Which Molecular Formats Can Be Used for the Design of Immunocytokine Products?

Antibody–cytokine fusions can be grouped into two broad categories: those based on immunoglobulins in IgG format and those featuring antibody fragments. Figure 1A shows a schematic domain representation of immunocytokines that have been moved to clinical trials. The first products to be developed industrially feature IgGs bearing a human IL2 moiety at the C-terminus of each heavy chain (3, 12–16). Scientists have developed similar products, bearing only one IL2 moiety, due to an asymmetric arrangement of heavy chains achieved using "knob-into-hole" technology (25). IL12, a heterodimeric cytokine, has been fused as a single polypeptide (with a linker connecting the p40 and p35 subunits) to the extremity of an antibody by others (26, 27). Interestingly, in spite of these IgG derivatives retaining FcRn binding, they typically exhibit a much faster clearance rate compared with intact human immunoglobulins (25–27).

Our group and others have typically preferred to use antibody fragments for the generation of immunocytokines (reviewed in refs. 12–16). Figure 1B illustrates the domain organization of antibody fusions with IL2 (featuring the use of diabody structure) and TNF (featuring the antibody in scFv format), which have been moved to advanced clinical trials for the treatment of patients with cancer (28–34). Biodistribution studies in mouse models of cancer have revealed that tumor:organ ratios greater than 10:1 can be achieved with the best fragment-based immunocytokines 24 hours after intravenous administration (28, 30, 35).

More complex cytokines (e.g., members of the IL12 family) allow various domain arrangements, which may substantially differ in terms of tumor-homing and therapy performance (36, 37). Protein engineering opportunities also exist for members of the TNF superfamily because some of these payloads can efficiently be delivered to tumors, whereas others cannot (38). One group has pioneered the use of TNF-related proteins as single-chain polypeptides, with stabilizing mutations when necessary, leading to a substantial improvement in performance (39).

Which Cytokines Cannot Be Delivered to the Tumor and Why?

The use of the L19 and F8 antibodies, targeting fibronectin splice variants located in the subendothelial aspect of tumor blood vessels (17, 18), has allowed a systematic evaluation of the tumor-targeting performance of dozens of antibody–cytokine fusions in various immunocompetent cancer animal models (reviewed in refs. 12–16). The results of biodistribution studies with radiolabeled protein preparations have allowed grouping of cytokine payloads into three main categories: (i) those efficiently delivered to the tumor at ultra-low doses (e.g., IL2, IL4, IL6, IL9, IL10, IL12, IFNα, and TNF; refs. 12–16); (ii) those trapped by cognate receptors at low doses but can efficiently accumulate in the tumor when the receptor has been saturated (e.g., IFNγ and IL15; refs. 12–16, 40–42); and (iii) those which abrogate the tumor-homing performance of the parental antibody (e.g., payloads which are too large, bear too many positive or negative charges, or are glycosylated with an insufficient number of terminal sialic acid moieties, leading to efficient removal by the asialoglycoprotein receptor). Antibody-based pharmacodelivery problems have been reported for fusions with large splice isoforms of murine vascular endothelial growth factor A (e.g., VEGF-A^{164}), calmodulin, TAT peptides, CD86, as well as IL9 (depending on the glycoforms that were used in biodistribution experiments; refs. 12–16, 36, 43–46).

What Is the Mechanism of Action and Main Side Effects of Anticancer Immunocytokines?

Different payloads display different modes of action, and some cytokines do not display a potent anticancer activity (at least in the mouse and other models tested so far), in spite of an efficient accumulation at the tumor site. For example, IL6 (47) and IL10 (48) can be selectively delivered to solid tumors without mediating a substantial tumor growth retardation. The most striking antitumor activity in mice has been observed with fusion proteins based on murine versions of IL2, IL4, IL12, and TNF (3–16, 22, 23, 28, 30, 34–37, 49). Human IL2 can also be used in mice, as this cytokine cross-reacts with the murine receptor (28). In most cases, products based on these payloads can
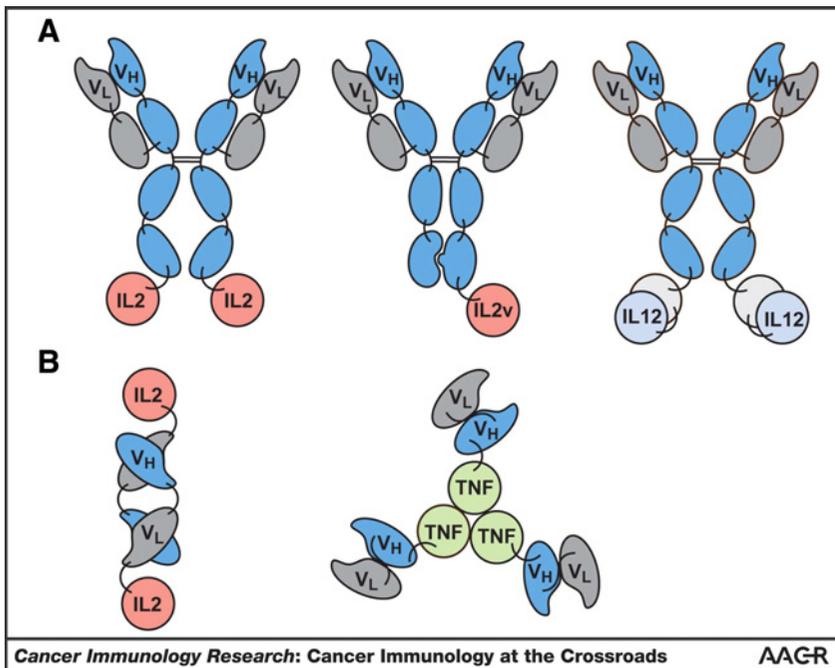
Figure 1.
Formats of immunocytokine products in clinical trials. **A,** IgG-based biopharmaceuticals bearing IL2, an IL2 variant (IL2v) with reduced CD25 binding, or IL12 (in single-chain format) as payloads. In order to achieve the IgG display of a single-cytokine moiety, researchers adopted "knob-into-hole" technology for the heterodimerization of different antibody heavy chains. **B,** Immunocytokines based on antibody fragments (a diabody structure in case of IL2 and scFv fragments for TNF fusions).

substantially reduce tumor growth rates upon intravenous administration and tumor clearance can be observed in some (but not all) immunocompetent mouse models.

Depletion experiments have evidenced a crucial role of CD8^{+} T cells and NK cells for IL2- and IL12-based immunocytokines. Tumor-homing fusions based on these payloads mediate a substantial and rapid increase of the density of T and NK cells at the site of disease both in mice (22, 28, 34) and in humans (50). However, it is unclear whether these cells migrate through a barrier of cytokines located at high density in the abluminal aspects of tumor blood vessels or proliferate *in situ*.

The biology of TNF-based immunocytokines differs. The targeted delivery of this payload can trigger a rapid increase in vascular permeability of tumor blood vessels, leading to hemorrhagic necrosis of a vast portion of the neoplastic mass both in mice (30, 31, 35) and in humans (32). In rodents, it has been formally proven that the immune system plays an important role for the eradication of surviving tumor cells, in a process that depends on CD8^{+} T cells and NK cells (23, 51). Interestingly, mice cleared of soft-tissue sarcoma reject subsequent challenges with different tumors from the same mouse strain. In the case of BALB/c tumors, the AH1 peptide (derived from the envelope protein of the murine leukemia virus, endogenous in the mouse genome) represents the dominant tumor rejection antigen (23, 51, 52). A complete understanding of the mechanism of action of anticancer immunocytokines is still missing. It is also reasonable to assume, for example, that some products may play an important role in driving Treg fragility (53).

Proinflammatory immunocytokines are vasoactive and can trigger further cytokine production. The main side effects observed in the clinic are hypotension, flu-like symptoms, nausea, and vomiting. Leukopenia has also been reported for IL12 fusions (54). Dose, schedule, and infusion time strongly affect the tolerability of the product. Although high-dose IL2 regimens feature the administration of up to 800 million international units (IU) of this payload for 1 week (with substantial toxicity; ref. 55), it is common to administer IL2-based immunocytokines at much lower doses (e.g., 20–60 million IU) once a week, with a procedure that can be continued for more than 6 months (25, 29).

Which Combination Opportunities Can Be Considered for Antibody–Cytokine Fusions?

A strong potentiation of certain immunocytokines has been observed with some (but not all) chemotherapeutic agents (30, 31, 37, 40, 42, 51, 56, 57), radiation (58, 59), and immunostimulatory drugs (including immune-checkpoint inhibitors and combinations of immunocytokines; refs. 49, 60–63). Combination studies for immunocytokine products remain an intense area of research, as many factors can influence additive or synergistic benefits. For example, it makes a difference if chemotherapeutic drugs are given *before* or *after* immunocytokines (56). From a theoretical viewpoint, chemotherapy could mediate immunogenic cell death and make tumors more responsive to the action of proinflammatory biopharmaceuticals (64–66). Killed tumor cells release necrotic components that can activate dendritic cells capable of cross-antigen presentation (65), and stressed tumor cells overexpress surface proteins that can be recognized by NK cells (66). However, an excessive dose of cytotoxic agents could be immunosuppressive.

Products based on IL2 and IL12 can substantially increase NK cell density within the tumor mass (50, 57, 67), and, as a consequence, these agents would be expected to potentiate the action of IgG therapeutics, working through antibody–dependent cell cytotoxicity. A potentiation of the anticancer activity of clinical-stage intact antibodies has been reported (67). Similarly, a potentiation of vaccination strategies (23, 68) or of bispecific antibodies (69) could be expected, with a judicious choice of target antigens, cytokine payload, and biopharmaceutical format.

Which Immunocytokines Are Being Investigated in Trials for Oncological Indications?

The protein formats depicted in Fig. 1 (featuring IL2, IL12, or TNF as payloads) are described thoroughly in the respective studies (12–18) and details will not be repeated here. A fusion protein consisting of the F8 antibody fused to human IL10 has been moved to phase II clinical trials in patients with rheumatoid arthritis and ulcerative colitis, indicating that the immunocytokine concept can also be applied to indications beyond cancer (48).

One issue in the field relates to the format (e.g., full IgGs vs. antibody fragments) and the nature of the payload (e.g., wild-type IL2 vs. mutants with reduced CD25 binding), which should be chosen for product development (16, 25, 28, 29). Clinical trials have not yet provided a definitive answer on this matter. The therapeutic activity of antibody–cytokine fusions is likely to be a composite of systemic effects (which may be greater for cytokine derivatives with long circulatory half-lives) and of immunostimulation at the tumor site (which is likely to correlate with the product uptake in the neoplastic mass). The combination of L19-TNF (given by intravenous infusion) and doxorubicin is being compared with doxorubicin alone in pivotal trials for the treatment of first-line metastatic soft-tissue sarcoma (EudraCT 2016-003239-38 and NCT03420014). The intralesional administration of combination L19-IL2 and L19-TNF as neoadjuvant therapy prior to surgery is being tested in phase III clinical trials for the treatment of fully resectable stage IIIB/C melanoma (NCT02938299 and NCT03567889).

The Future of Immunocytokine Products: Can Systemic Toxicity Be Reduced?

An important (and largely unexplored) area of immunocytokine research relates to the use of cytokine mutants with decreased affinity toward the cognate receptor. The lower biological activity allows the use of higher doses and may provide an avenue to create *in vivo* selectivity. Scientists have described preclinical results using an anti-CD38 antibody fused to a depotentiated version of IFNα (70).

Most large pharmaceutical companies have started immunocytokine programs and, for this reason, the real potential of this class of biopharmaceuticals will become clearer in the next few years. Although immunocytokines may provide a benefit to patients when used as single agent, it is likely that anticancer therapeutic activity will best be displayed when suitable combination strategies are implemented. The development of two products to be used in combination can be cumbersome from a pharmaceutical perspective. For this reason, it would be tempting to incorporate cytokine moieties into other biopharmaceuticals, thus creating multifunctional products. Examples of potency-matched antibody–cytokine–drug conjugates (71) and of dual-cytokine antibody fusions (35, 36, 72, 73) have been reported (Fig. 2), some of which have shown encouraging biodistribution and therapeutic results.

The majority of toxicities of proinflammatory immunocytokines are observed in concomitance with the peak serum concentration of the product. Antibody–cytokine fusions can be more efficacious than the unmodified cytokines because of preferential uptake in the tumor. However, the toxicity profile is often similar to the one of the parental cytokine (in absolute terms) because only a small fraction of the product is taken up by the neoplastic lesion (in the best cases, 0.01%–0.1% injected dose/gram of tumor). In other words, the tumor can be targeted more efficiently compared with normal organs but will not be a "sponge" that absorbs most of the product.

Although the use of cytokine-based therapeutics has traditionally been limited by systemic toxicity, it may be possible to circumvent this problem in a number of ways. On the one hand, tumor-targeting antibody–cytokine fusions may allow for administering less product, thus improving tolerability. A second aspect relates to the nonlinearity of cytokine-mediated toxicity and to the possibility of progressively increasing payload concentration in the tumor. At the clinical level, undesired side effects are often seen when the peak concentration of cytokine activity grows above a certain threshold. In most cases, adverse events disappear when cytokines are cleared from circulation. It should be possible to implement repeated administrations of small doses of antibody–cytokine products (or use very slow infusion procedures) to progressively build up cytokine concentrations within the neoplastic mass, while
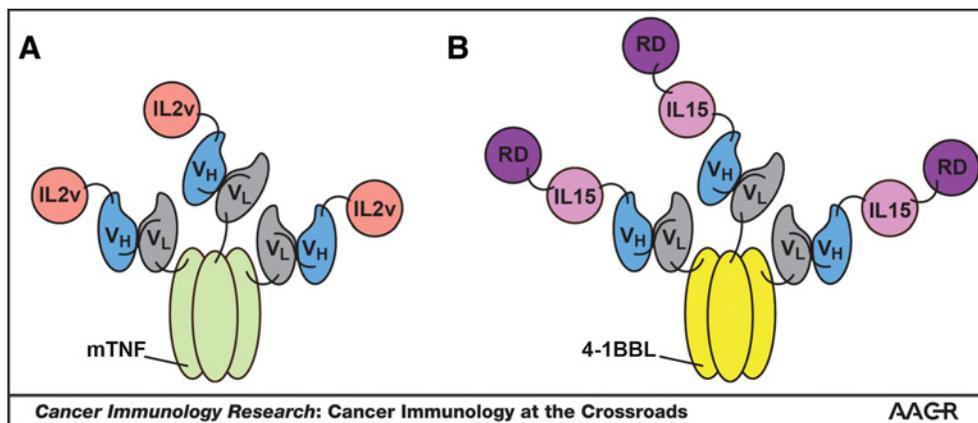

Figure 2.
Domain arrangement for dual-cytokine fusion proteins. These homotrimeric fusion proteins feature antibodies in scFv fused to members of the TNF superfamily and to other immunomodulatory payloads. **A,** Dual-cytokine fusion protein based on IL2 and murine (m) TNF. **B,** Fusion protein featuring 4-1BBL, IL15, and the alpha-subunit of the IL15 receptor (RD). The VH and VL domains of antibodies in scFv format are indicated.

keeping blood concentrations sufficiently low. In this context, the use of antibodies with efficient tumor-homing properties and the targeting of stable antigens that do not internalize (e.g., extracellular matrix components) should facilitate the creation, over time, of a concentration differential between the cancer site and normal organs. In the clinical trial NCT02076646, the administration of L19-IL2 with a 3-hour infusion procedure allowed for increasing the maximal tolerated dose (MTD) of the product by at least 3-fold, compared with the MTD which had previously been reported for a 1-hour infusion procedure (29, 74). The use of very slow infusion procedures for cancer immunotherapy is not uncommon. For example, blinatumomab was found to be efficacious when given as a 30-day infusion but not as bolus injection (75).

Future research programs will likely aim at the generation of immunocytokine products with "activity-on-demand," which may display their therapeutic potential at the tumor site, while sparing normal organs. We have described "split cytokine fusions," a biopharmaceutical strategy that may be particularly suitable for immunomodulatory payloads consisting of multiple subunits (76). In principle, it should be possible to sequentially deliver cytokine subunits to the site of disease, allowing a reconstitution of biological activity within the tumor mass. These approaches, however, may be challenging if individual cytokine subunits retain potent biological activity (77). Selectivity may also be created if antigen binding by immunocytokine products would allosterically enhance biological activity. Gillies and collaborators have reported that the

fusion of IL2 at the light chain C-terminus of antibodies in IgG format with judiciously chosen linkers could lead to a product activation upon antigen binding, possibly as a result of steric hindrance and conformational changes of the hinge region (77). Alternatively, the transient inhibition of immunocytokines with kinetically tuned blocking moieties, which progressively dissociate and clear from blood, may represent an avenue for building up activity at the tumor site, while decreasing systemic toxicity.

In summary, immunocytokines are modular biopharmaceutical agents, which have shown exceptional activity in preclinical models of cancer but whose pharmaceutical potential is still largely unexplored. The opportunities provided by protein engineering technologies and by the implementation of judicious combination modalities suggest that antibody–cytokine fusions will play an important role for the immunotherapy of cancer in a not too distant future.

Disclosure of Potential Conflicts of Interest

D. Neri is a board member for and has ownership interest in Philogen.

Acknowledgments

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