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Authors

Young, Patricia A
Morrison, Sherie L
Timmerman, John M

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ANTIBODY-CYTOKINE FUSION PROTEINS FOR TREATMENT OF CANCER: ENGINEERING CYTOKINES FOR IMPROVED EFFICACY AND SAFETY

Patricia A. Young[1], **Sherie L. Morrison**[2], and **John M. Timmerman**[1]

[1]Division of Hematology & Oncology, Dept. of Medicine, University of California, Los Angeles (Los Angeles, CA)

[2]Dept. of Microbiology, Immunology, and Molecular Genetics, University of California, Los Angeles (Los Angeles, CA)

Abstract

The true potential of cytokine therapies in cancer treatment is limited by the inability to deliver optimal concentrations into tumor sites due to dose-limiting systemic toxicities. To maximize the efficacy of cytokine therapy, recombinant antibody-cytokine fusion proteins have been constructed by a number of groups to harness the tumor-targeting ability of monoclonal antibodies. The aim is to guide cytokines specifically to tumor sites where they might stimulate more optimal anti-tumor immune responses while avoiding the systemic toxicities of free cytokine therapy. Antibody-cytokine fusion proteins containing IL-2, IL-12, IL-21, TNFα, and interferons α, β and γ have been constructed and have shown anti-tumor activity in pre-clinical and early phase clinical studies. Future priorities for development of this technology include optimization of tumor targeting, bioactivity of the fused cytokine, and choice of appropriate agents for combination therapies. This review is intended to serve as a framework for engineering an ideal antibody-cytokine fusion protein, focusing on previously developed constructs and their clinical trial results.

INTRODUCTION

Cytokines and related signaling molecules are major regulators of the immune system. Released by immune cells, soluble cytokines act over short distances in paracrine and autocrine fashion to orchestrate innate and adaptive immune responses to augment the proliferation, differentiation, effector functions, and survival of leukocytes. Given the ability of the immune system to recognize and destroy cancer cells under appropriate conditions, cytokines have been explored and developed as treatments for cancer. Some success has

Corresponding author: John M. Timmerman, Division of Hematology & Oncology, Department of Medicine, University of California at Los Angeles, Center for Health Sciences, Room 42-121, 10833 LeConte Avenue, Los Angeles, CA 90095-1678, Phone: 310-794-4820, Fax: 310-206-5511, jtimmerman@mednet.ucla.edu.

been achieved most notably with interleukin-2 (IL-2), other members of common gamma chain cytokine family (IL-7, IL-15, IL-21), IL-12, and the interferons (IFNs) [1–3]. However, current therapies using systemic administration of free cytokines have severe limitations, including vascular leak syndrome and systemic inflammation. These toxicities result from large doses of the pro-inflammatory cytokines being absorbed in the peripheral tissues en route to their intended destination in the tumor and its draining lymph nodes. Since free cytokines do not specifically traffic to sites of tumor, efficacy is limited by lack of effective concentrations within tumors. Recently, recombinant antibody technology has allowed the construction of antibody-cytokine fusion proteins (also know as *immunocytokines*). These fusion proteins harness the tumor-targeting ability of monoclonal antibodies to guide cytokines specifically to tumor sites where they might stimulate more optimal anti-tumor immune responses while avoiding the systemic toxicities that limit current cytokine dosing and therefore potential efficacy [4–6]. This review will describe the rationale for and general properties of the antibody-cytokine fusion proteins studied to date, focusing on those that are now moving forward in clinical testing.

CYTOKINE THERAPIES FOR CANCER: PROSPECTS AND LIMITATIONS

Several recombinant cytokines, including IFNα and IL-2, are currently approved for treatment of several specific types of cancer; in addition a number of other cytokines have shown promising results in pre-clinical studies and early phase clinical testing. Table 1 lists the properties of various cytokines under study for cancer therapy, and thus principal candidates for development as antibody fusion proteins.

Interferons

Type I IFNs consist of seven classes with IFNα and IFNβ being the most abundant [7]. Initially described for their anti-viral activities in 1957, type I IFNs are known to possess pleiotropic immunologic activities, including the maturation of dendritic cells, upregulation of class I MHC expression, stimulation of $CD8^+$ cytotoxic T lymphocytes, and activation of natural killer (NK) cells and macrophages, with subsequent release of additional cytokines. Other useful anti-tumor properties of IFNα/β include potent direct anti-proliferative and pro-apoptotic effects, blockade of autocrine growth factor loops, repression of c-myc oncogene expression, downregulation of telomerase activity, inhibition of angiogenesis, and induction of TRAIL-mediated tumor cell apoptosis [8–15]. IFNα was the first cytokine approved for the treatment of a human cancer (hairy cell leukemia) in 1986, and was subsequently found to have some efficacy against other cancers, including melanoma, renal cell carcinoma (RCC), multiple myeloma, chronic lymphocytic leukemia (CLL), chronic myelogenous leukemia (CML), and follicular non-Hodgkin lymphoma (NHL) [3, 16, 17]. However, the clinical effects are modest given the dose-limiting side effects of flu-like symptoms, fatigue, nausea/anorexia, neutropenia, neuropsychiatric symptoms, and injection site reactions. Accordingly, its use in these cancers has been largely supplanted by other therapies. Nonetheless, IFNα has strong antiviral activity *in vivo*, and has been approved, both in native recombinant forms and conjugated to polyethylene glycol (PEG) to increase its *in vivo* half-life for the treatment of viral hepatitis B and C. IFNγ (type II IFN), normally produced by T cells and NK cells, also has potent immunostimulatory effects and direct anti-

proliferative effects against some types of cancer cells *in vitro*, but does not have significant single agent *in vivo* activity against human cancers [15–18].

Interleukin-2

IL-2 potently stimulates the immune system through activation of T cells, NK cells, and monocytes. Based on data showing durable responses in a small minority of patients, IL-2 was approved for treatment of metastatic RCC in 1992 and advanced melanoma in 1998. A study in metastatic melanoma patients treated with IL-2 alone versus IL-2 with lymphokine activated killer (LAK) cells showed an overall response rate of 5 in 16 patients (31%) and 6 in 26 (23%), respectively [19]. Furthermore, among 283 patients with metastatic melanoma or metastatic RCC treated with high dose bolus IL-2 at the National Cancer Institute, there was an ORR of 17% (including 7% CR) in metastatic melanoma and 20% (including 7% CR) in metastatic RCC [20]. Similar to IFN, limitations of IL-2 include vascular leak syndrome characterized by hypotension and fevers, and a very short initial serum half-life of 5–7 minutes followed by a secondary clearance of 30–120 minutes [21]. Another drawback for IL-2 is its ability to expand and support the activity of regulatory T cells (Treg) that dampen anti-tumor immunity [22]. Thus, other newer members of the IL-2 family of cytokines have been explored as potential cancer immunotherapies. These “common gamma chain” family cytokines, including IL-7, IL-15 and IL-21, play a major role in lymphocyte homeostasis, providing signals that promote survival and inhibit cell death in T and NK cells, and potentiate effector functions such as perforin/granzyme-mediated target cell killing; all of these properties make them attractive cytokines for cancer immunotherapy [23].

Currently, there are 8 cytokines (IFNα, IFNβ, IFNγ, IL-2, IL-11, G-CSF, GM-CSF, and TNFα) approved for various clinical indications including anticancer treatment, with seven others (IL-7, IL-10, IL-12, IL-15, IL-21, CD40L, and TRAIL) undergoing clinical evaluation [24]. However, despite enormous efforts spanning several decades to develop systemic cytokine therapies for cancer, this approach has severe technical limitations that must be overcome. Foremost is the failure to achieve adequate concentrations of cytokine within the tumor bed following systemic administration. Since injected cytokines generally have half-lives measured in minutes to hours, and receptor expression is distributed throughout the blood and lymphoid compartments, only a tiny fraction of the injected dose reaches the intended target areas within tumors and draining lymph nodes. IFNα, for example, has a half-life of only 5 hours, and pharmacokinetic studies have indicated that only 0.01% of subcutaneously injected IFNα reaches the target tumor site [25]. Dose-limiting systemic toxicities including fever, chills, malaise, hypotension, organ dysfunction, and cytopenias also severely impair the usefulness of traditional systemic cytokine therapies. Thus, the true potential of exogenous cytokines as cancer therapeutics cannot be realized without the development of technologies to safely deliver cytokines to tumor sites in therapeutically effective quantities.

EXPLOITING ANTIBODIES FOR CYTOKINE DELIVERY

Over the last several decades, the development of therapeutic antibodies with their specific tumor-targeting properties has led to a revolution in cancer care. Antibodies against

differentiation antigens such as CD20 on B cell lymphomas (rituximab) or tumor-associated cell surface growth factor receptors such as HER2/neu (trastuzumab) or epidermal growth factor receptor (cetuximab) are among the early successes in monoclonal antibody therapy. These and other useful antibodies rely on three principal mechanisms of action against tumor cells: 1) blockade of growth factor signaling with direct anti-proliferative/pro-apoptotic effects, 2) antibody-dependent cellular cytotoxicity (ADCC) via recruitment of Fc receptor-bearing NK cells and macrophages, and to a lesser extent 3) complement dependent cytotoxicity (CDC) [26]. The capacity of antibodies to bind specifically to tumor-associated cell surface antigens or other targets within the tumor microenvironment can be exploited in cancer treatment by engineering antibodies to act as vehicles for the transport of cytotoxic agents, radioisotopes, or other payloads such as cytokines directly to tumor sites [26]. A variety of chemical conjugation techniques now permit the attachment of powerful chemotherapeutic drugs or radionuclides to antibodies to achieve tumor-specific targeting. Moreover, through recombinant DNA techniques, genes encoding cytokines can be joined to antibody genes to yield antibody-cytokine fusion proteins in which the cytokine domains are linked with either the C-terminus (Fc domain) or N-terminus (variable domain) of an antibody or antibody fragment. When acting as carriers for such payloads, the antibodies utilized need not possess anti-tumor activities themselves. However, if the inherent effector mechanisms of the antibody can be retained and hopefully enhanced, antibody-cytokine fusion proteins would have the potential to be more effective than current antibody therapies because they could act through multiple mechanisms. In this capacity, fusion proteins might bridge innate and adaptive anti-tumor immunity; through activation of ADCC, CDC, acute inflammatory cells, and dendritic cells, tumor antigens released from killed tumor cells could be efficiently presented to host T cells to promote an adaptive immune response.

DESIGNING THE IDEAL ANTIBODY-CYTOKINE FUSION PROTEIN

Several key considerations to take into account when constructing optimal fusion proteins are outlined in Table 2. Desirable candidate target antigens would include tumor-associated or lineage-specific antigens with moderate to high-level expression on the surface of tumor cells, but minimal expression on normal tissues to avoid off target effects. In contrast to immunotoxins, which require endocytosis of the antibody-drug conjugate by the tumor cell for intracellular release of the toxin, antibody-cytokine fusion proteins are not required to be internalized after binding their target. Thus the pool of potentially attractive antigen targets might expand to include antigens found specifically within the tumor microenvironment, either on tumor vasculature or released by dead tumor cells (see below). Particularly attractive antibodies might be those with known intrinsic activity against tumor cells (via direct growth inhibition, ADCC, etc), with the added benefit of a targeted cytokine increasing therapeutic efficacy.

The choice of cytokine should be based on its properties as a cancer immunotherapeutic agent (see Table 1). Depending on the biology of the specific cancer being treated, the goal may be to activate T cells, NK cells, or tumor macrophages, or to modulate distinct cell signaling pathways in tumor or stromal cells. Most cytokines chosen for cancer immunotherapy can serve to stimulate T cell immunity, some in part through promoting the maturation of dendritic cells (IFNs, GM-CSF, TNFα). To augment the cytolytic potential of

full-length antibodies, cytokines capable of enhancing ADCC effectors (NK cells, macrophages) may be desired (IFNs, IL-2, IL-12, IL-15, IL-21). IFNs (α, β, γ) are unique among cytokines in their capacity to directly suppress the growth of many types of cancer cells [16, 17]. Among the other cytokines, only IL-21 can directly inhibit the growth of certain B cell malignancies [27]. A few cytokines also have anti-angiogenic activity, including IFNα (through inhibition of VEGF and basic fibroblast growth factor (bFGF)), IFNγ (via induction of interferon-inducible protein (IP-10)), and TNFα, which can directly damage tumor-associated endothelial cells, leading to hemorrhagic necrosis of tumors [3, 15, 28].

Antibody-cytokine fusion proteins have been constructed in a wide variety of structural formats, including full-length antibody, scFv, Fab, minibody, or diabody, having the cytokine moieties joined to the C- or N-terminus of the antibody domains by flexible peptide linkers (reviewed in [24, 29]). A problem that is frequently encountered with fusion proteins is cleavage at the site of fusion. The length and sequence of the linker sequence are crucial for achieving both protein stability (to avoid release of free cytokine into the circulation) and cytokine bioactivity.

The bioactivity of the fused cytokine moiety can vary widely among fusion constructs, dictating pharmacologic properties including tolerability, half-life, and efficacy. The cytokine activity should be compared on a molar basis to free cytokine. Having a fully active and receptor-avid cytokine domain may actually be disadvantageous, as this may impair tumor targeting by competitive binding to receptors on circulating leukocytes, resulting in systemic toxicity. “Attenuation” of the fused cytokine moiety’s bioactivity can be overcome easily by the powerful targeting specificity provided by the antibody (see below). Pre-clinical *in vivo* testing should include syngeneic animal tumor models employing species-appropriate cytokines to reliably assess tumor targeting, toxicity, and efficacy. Such models will also yield pharmacodynamic information valuable for phase I clinical trial designs.

ANTIBODY-CYTOKINE FUSION PROTEINS IN CLINICAL DEVELOPMENT

Therapeutic targets currently under investigation include differentiation antigens, growth factors, angiogenic markers, or stromal and extracellular matrix antigens of the tumor microenvironment. Antibodies to several of these targets fused with various cytokines have been tested in clinical trials. Table 3 summarizes the fusion proteins that have entered or are approaching clinical testing.

IL-2 Fusion Proteins

Hu14.18-IL2—The tumor-associated ganglioside GD2 is an attractive target for immunotherapy given its expression on certain malignancies (neuroblastoma and melanoma) with restricted expression in normal tissues. Murine monoclonal anti-GD2 can effectively lyse a number of human neuroblastoma cell lines *in vitro* through CDC and ADCC and suppress growth of established human neuroblastoma tumors in nude mice, suggesting its potential use for immunotherapy [30]. Given the clinical experience and established immunologic mechanisms of IL-2, this cytokine was chosen for a fusion protein construct.

In established metastatic melanoma xenograft models, animals treated with ch14.18-IL2 (chimeric mouse-human) showed doubled survival times compared to animals treated with a combination of antibody plus IL-2 [31]. These studies also demonstrated localization of the fusion protein to the subcutaneous tumors and to liver and lung metastases. Anti-tumor effects correlated with the level of antigen expression on the cell line panels demonstrating that the tumor must express sufficient levels of the targeted antigen in order to permit a protective response.

To reduce the human anti-chimeric antibody response, hu14.18-IL2 was developed and tested in phase II clinical trials. In 39 relapsed/refractory neuroblastoma patients evaluable by [^{123}I] metaiodobenzylguanidine (MIBG) and/or bone marrow histology, there was a 22% CR in patients with non-bulky disease and no response for those with bulky disease. Phase II data in 14 metastatic melanoma patients found 1 of 14 (7.1%) with a partial response (PR) and 4 patients with stable disease (SD), lasting 3–4 months. The study was suspended due to limited availability of hu14.18-IL2 as well as the brief duration of PR and SD [32, 33]. The difference in response between neuroblastoma and melanoma patients is unclear, but perhaps this can be attributed to the level of GD2 expression in these different malignancies. Nonetheless, the toxicities resembled those of high-dose IL-2 therapy, including capillary leak, hypoxia, elevated transaminases, hyperbilirubinemia, hypotension, and renal insufficiency, but were all reversible and tolerable. The development of hu14.18-IL2 demonstrates the need to choose an appropriate target based on tumor specificity and sufficient expression. The preclinical development showed the ability to deliver cytokine to metastatic sites safely, but in the clinical setting showed that efficacy may be limited to non-bulky disease.

DI-Leu16-IL2—Among the various antibody targets, anti-CD20 antibody rituximab (C2B8/Rituxan; Genentech/Biogen-IDEC) has improved treatment outcomes in B cell NHLs [34, 35]. However, some malignancies will not respond or will relapse after rituximab-based therapies. The approach of fusion proteins with anti-CD20 has surfaced with at least four fusion proteins using the CD20 target. CD20 is a promising target antigen because of its specificity for B cell expression, and the fact that it is minimally internalized, downregulated, or shed, thus avoiding potential antigen escape mechanisms.

Gillies *et al* engineered an anti-CD20-IL2 fusion protein (DI-Leu16-IL2) with deimmunized (DI) portions on the variable region as well as the heavy chain. The fusion protein was deimmunized through mutagenesis to remove potential helper T-cell epitopes identified by *in silico* binding to MHC class II molecules. DI-Leu16-IL2 retained full anti-CD20 activity, but more importantly had enhanced ADCC relative to the unfused DI-Leu16 antibody or control anti-CD20 (rituximab). DI-Leu16-IL2 was more effective than a control fusion protein targeted to an antigen with reduced expression or combinations of anti-CD20 antibodies and IL-2 in disseminated xenografts with the human lymphoma cell line Daudi, [36]. These features again illustrate the importance of the expression level of the target antigen on the tumor, the enhanced immune effector functions of the fusion protein, and the superiority of fusion protein over either antibody or cytokine alone.

L19-IL2—Fibronectin is an extracellular matrix component expressed in healthy tissues and body fluids. The fibronectin extradomain B (ED-B) can be inserted into fibronectin under conditions of tissue remodeling and is highly abundant in the vasculature of aggressive solid tumors, but undetectable in healthy adult tissues [29, 37, 38]. L19, a high-affinity antibody that recognizes ED-B, has been shown to localize to tumor blood vessels in animal models and cancer patients, and is thus considered an appropriate target for antibody-based therapies. L19-IL2 fusion protein showed superior anti-lymphoma activity compared to unconjugated IL-2 in xenograft models of NHL. When combined with rituximab, L19-IL2 induced complete regressions of established localized lymphomas and protected from disseminated lymphoma [38].

L19-IL2 has also been evaluated in human solid tumors. Combination of L19-IL2 with dacarabazine in metastatic melanoma induced a 28% objective response among 29 patients [39]. Furthermore, in another phase I study, 51% of patients with solid tumors showed stable disease and among the metastatic RCC patients 83% had stable disease after two cycles of L19-IL2 [40]. This fusion protein demonstrates the possibility of superior efficacy in combination studies of fusion protein, whether this is with chemotherapy or possibly other fusion proteins or immunotherapies.

IL-21 Fusion Proteins

Anti-CD20-IL21—IL-21, a member of the IL-2 cytokine family, regulates T, B, NK, and myeloid cell function, and has potent *in vivo* efficacy in a variety of mouse tumor models [2, 41]. IL-21 activates STAT proteins, leading to proliferation arrest and caspase-dependent apoptosis in a majority of DLBCL cell lines. Sarosiek *et al* showed anti-tumor effects *in vitro* and *in vivo* in IL-21R expressing DLBCL and mantle cell lymphoma (MCL) [41]. In a phase I clinical trial in indolent lymphomas, clinical responses were seen in 42% of 21 patients, suggesting clinical activity of IL-21 and rituximab in combination [27]. Motivated by these preclinical and clinical results in lymphoma, Bhatt *et al* developed anti-CD20-IL21 and showed efficacy in DLBCL and MCL cell lines (even IL-21-resistant cell lines) compared to the individual components [42].

IL-12 Fusion Proteins

BC1-IL12—IL-12 has potential as an anti-tumor cytokine given its stimulatory properties on NK cells and cytotoxic T cells, with potent induction of IFNγ and resulting regulation of Th1:Th2 cell balance towards the Th1 type immune response that generally favors anti-tumor immunity [43]. It has also shown potent anti-tumor effects against MHC-negative cancer cells, likely due to its anti-angiogenic activity, stimulation of nitric oxide by macrophages, and activation of NK cells [1]. The activity of IL-12 is species specific and murine IL-12 (muIL-12) must be used for studies in mice. BC1, like L19, is an antibody that targets tumor-associated vasculature via an ED-B variant of fibronectin and recognizes only the human fibronectin isoform, B-FN. When BC1 fusion to murine IL-12 (huBC1-muIL12) was tested it almost completely eradicated established metastases and inhibited tumor growth in prostate, colon, and epidermoid carcinomas established in SCID mice [44]. Furthermore, a phase I study of BC1 fused to human IL-12 (AS1409) in malignant melanoma and RCC showed stable disease in 6 of 11 patients (46%) with tolerable side

effects. IFN-γ and IP-10 were elevated in the serum in all patients, indicating the anticipated responses to the cytokine [45], suggesting their possible roles in the anti-tumor response.

TNF Fusion Proteins

L19-TNFα—Borsi and colleagues prepared a fusion protein (L19-mTNFα) composed of mouse TNFα and the scFv of L19 specific for ED-B. In a syngeneic murine tumor model using embryonal teratocarcinoma cells, fibrosarcoma cells, and mouse colon adenocarcinoma cells, L19-mTNFα had greater therapeutic activity compared to free mTNFα and control fusion protein. Furthermore, the activity was enhanced by combination with melphalan and the L19-IL2 fusion protein previously discussed [46].

In phase I/II clinical trial with single agent L19-TNFα in metastatic solid tumors, Spitaleri and colleagues treated six cohorts of patients ranging from 1.3–13 μg/kg intravenous on days 1, 3, and 5 in a 3-week schedule. The majority of patients were colorectal cancer patients. No objective tumor responses were detected, although stable disease occurred in 19 of 31 patients. The maximum tolerated dose was not reached, suggesting further studies in combination with chemotherapy [47].

In another phase I/II clinical trial with L19-TNFα plus melphalan-containing isolated limb perfusion (ILP) in locally advanced extremity melanoma, doses of L19-TNFα at 325 μg and 625 μg were given to seven and ten patients, respectively. Although the TNFα-equivalent dose was 3.13 and 6.25% of the approved untargeted TNFα dose of 4000 μg, L19-TNFα ILP induced objective responses of 86 and 89% in the two doses, respectively. At the 650 μg dose, 5 of 10 patients had a CR durable for 12 months; no CRs were seen in the 325 μg group of L19-TNFα [48]. Overall, these results suggest some clinical activity against cancer.

IFNα/β Fusion Proteins

Among cytokines being explored in cancer immunotherapy type I IFNs appear to have a unique and favorable combination of anti-tumor properties, including not only stimulation of T cell immunity, activation of dendritic cells, and enhanced ADCC, but also inhibition of tumor cell growth and angiogenesis (Table 1) [3, 16, 17]. Thus, IFNα/β ranks as a preferred moiety for fusion to tumor-targeting antibodies, and several such fusion proteins are being developed in our laboratories. Our initial studies have focused on targeting the B cell differentiation antigen CD20, which is expressed on most B cell NHLs.

We constructed anti-CD20-IFNα fusion proteins using the variable regions from the rituximab parent antibody 2B8, so that recombinant fusion proteins could be directly compared to this prototypical anti-CD20 antibody [49]. The C-terminal fusion of IFNα to anti-CD20 did not affect the ability of the antibody to bind its antigen. Importantly, murine and human IFNα fusion proteins had reduced IFNα activity *in vitro* compared with native IFNα (<10%), but CD20 targeting permitted efficient anti-proliferative and pro-apoptotic effects against an aggressive rituximab-insensitive human $CD20^+$ murine lymphoma (38C13-huCD20) and a human B-cell lymphoma (Daudi). *In vivo* efficacy was demonstrated against established 38C13-huCD20 tumors grown in syngeneic immunocompetent mice. Optimal tumor eradication required CD20 targeting, with 87% of mice cured of rituximab-

insensitive tumors using a five-dose regimen. No evidence of toxicity was observed. Fusion of the cytokine moiety was required, as co-administering a mixture of free rIFNα and rituximab did not increase survival above controls. Gene knockdown studies revealed that eradication required expression of type I IFN receptors on the tumor cell surface. Anti-CD20-IFNα containing human IFNα also efficiently eradicated large, established Daudi xenograft tumors. More recently, we reported that a tumor with decreased expression of IFN alpha receptors (IFNAR) could be effectively treated with antibody linked to IFNβ, which has a higher affinity for the IFNAR [50].

Based on the above results, a new anti-CD20-hIFNα incorporating the antigen-binding sequences from rituximab and full-length human IgG1/κ constant regions is being developed for the treatment of B cell lymphomas (IGN002) [51]. In pre-clinical studies, the IFNα bioactivity was attenuated to approximately 10–20% of conventional rIFNα, though when targeting $CD20^+$ lymphoma cells, growth inhibitory activity is significantly enhanced over rIFNα. Anti-CD20-hIFNα induced stronger growth inhibition than rituximab against human lymphomas, in association with substantial apoptosis in some cell lines. Anti-CD20-hIFNα retains potent *in vitro* ADCC activity, but surprisingly, has superior CDC compared to rituximab. Moreover, anti-CD20-hIFNα achieved superior *in vivo* efficacy compared to rituximab and non-targeted IFNα fusion protein against human lymphoma xenografts in SCID mice, eradicating 50–88% of established tumors. These results support the further development of anti-CD20-hIFNα for the treatment of B cell NHL, with a phase I first-in-human clinical trial scheduled to begin in 2015 [51].

Since fusion of IFNα/β to antibodies specific for tumor-associated antigen is expected to increase its therapeutic index and reduce systemic toxicity, this strategy may be valuable in treating a number of IFN-sensitive cancers. Our group has now begun to explore antibody-IFNα/β fusion proteins targeting other cancers, including multiple myeloma (via CD138) [52] and melanoma (via CSPG4). Yang and colleagues have also recently described the treatment of several solid tumors in mice with antibody-IFNβ fusion proteins targeting the epidermal growth factor receptor and Her2/neu [53]. Thus, antibody-targeted type I IFNs appear to be a promising approach to treating multiple types of cancer.

Anti-CD20-tetrameric hIFNα (20-2b-2b)—A similar approach by Rossi *et al* utilizing CD20-targeted tetrameric human IFNα showed efficacy against human lymphoma xenografts. In this approach the “dock and lock” (DNL) method was used to attach four molecules of IFNα2b to the humanized CD20 antibody, veltuzumab. The resulting proteins showed enhanced ADCC compared to veltuzumab but completely lacked CDC. Although promising results were obtained in human lymphoma xenografts, the concern of systemic toxicity of normal tissues could not be evaluated in the xenograft model since mouse immune cells are insensitive to human IFNα2b [54]. This is in contrast to the syngeneic immunocompetent murine model described above in which no toxicity was observed with the mouse IFNα fusion protein [49].

IFNγ Fusion Proteins

IFNγ is produced mainly by T and NK cells and exhibits anti-tumor activity through upregulation of MHC class I and II molecules while stimulating monocytes/macrophages, $CD8^+$ T cells, and NK cells [24]. Clinical trials investigating IFNγ for cancer therapy in combination with chemotherapy have been done in ovarian cancer. Among twenty-eight evaluable patients with advanced ovarian cancer in a phase I/II study, IFNγ (0.1 mg) with carboplatin and paclitaxel for six to nine cycles in first line treatment showed a 71% ORR. There were tolerable grade 1 and 2 adverse effects as expected with IFN (flu-like symptoms), and grade 3/4 neutropenia was seen in 74% [55]. IFNγ fused to L19 antibody has shown an anti-tumor effect in subcutaneous and metastatic murine F9 teratocarcinoma, with enhanced therapeutic efficacy in combination with chemotherapy and L19-IL2 [56].

Fusion Proteins Against Other Targets

Other targets used in previously engineered fusion proteins include tenascin C AI domain, DNA, and HLA-DR. The tenascin C AI target is similar to the L19 target in that tenascin can arise at sites of neo-angiogenesis within tumors. Similar to ED-B, the C domain of tenascin is undetectable in normal tissues but is expressed in the brain and the lung. F16 is a monoclonal antibody to the A1 domain of tenascin C. In the preclinical setting, F16-IL2 (Teleukin) is currently being studied in breast and lung cancers, as well as in acute myelogenous leukemia, and glioblastoma [57–59].

The NHS antibody binds to and targets nucleic acids released into the necrotic core of tumors. DNA-targeting immunocytokines may be useful for treating both localized tumor and residual disease. Phase I clinical data of NHS-IL2LT (Selectikine) in solid tumors had the response expected for targeted IL-2- with strong activation of T cells but only weak NK cell activation. Although no objective tumor responses were observed, there was disease stabilization in some patients [29, 60, 61]. The same target now fused with IL-12, NHS-IL12, is also under investigation but no clinical data in humans has yet been published [62].

Rossi *et al* again used the DNL method to construct C2-2b-2b, a mAb-IFNα comprising tetrameric IFNα2b linked to humanized anti-HLA-DR (hL243). HLA-DR is an attractive target as it is expressed on the cell surface of many hematologic malignancies. C2-2b-2b inhibited various *in vitro* B-cell lymphoma, leukemia, and myeloma cell lines. The fusion protein construct was more effective than CD20-targeted mAb-IFNa or a mixture of parental mAb and IFNα both *in vitro* and *in vivo* against established human lymphoma and myeloma cells. HLA-DR expression/density and sensitivity to IFNα and hL243 affected responsiveness to the fusion protein [63].

Epithelial cell adhesion molecule (EpCAM) is highly expressed on the surface of gastrointestinal, lung, prostate, breast, ovarian and other epithelial origin cancers. Over-expression may lead to disruption of the regulatory balance that facilitates cell proliferation, differentiation, migration and intracellular signaling that controls tumor growth and metastasis. Unfortunately however, the antibody-fusion protein huKS-IL2 targeting the EpCAM antigen did not show benefit in phase I clinical trials [64, 65].

QUESTIONS TO ADDRESS IN DEVELOPING ANTIBODY-CYTOKINE FUSION PROTEIN THERAPY FOR CANCER

Since treatment with free cytokines is limited by the inability to deliver optimal concentrations deep into tumor sites due to dose-limiting systemic toxicity, the true potential of cytokine therapies has certainly not been exploited. Indeed, tumor-targeted cytokines, using antibody domains or other cancer-targeting moieties can be considered to be the ultimate test for cytokines as cancer therapeutics. Since targeted cytokine therapies can activate multiple immune effector cells and mechanisms (T cells, dendritic cells, NK cells, macrophages), they might serve to bridge innate and adaptive anti-tumor immune responses. In translating this technology from the laboratory to the clinic, a number of key questions and challenges remain that we suggest should serve as a framework for future investigations.

1. Choice of cytokine for antibody fusion proteins?

Given the diverse properties of cytokines, there is no single cytokine that possesses all desirable properties in eliciting anti-tumor immunity. The choice of cytokine to include in an antibody fusion protein depends on the particular goals for the agent (summarized in Table 1). Is the goal to activate and expand T cells (or particular T cell subsets), NK cells, or macrophages? Is the aim to achieve direct inhibition of tumor cell growth? Is the tumor vasculature a preferred target? What cytokine-induced anti-tumor effector mechanisms (T cell cytotoxicity, ADCC, direct effects) are likely to be most effective against the particular cancer being treated? For instance, if the tumor is susceptible to ADCC by the targeting antibody (i.e., lymphoma treated with anti-CD20 mAb), then cytokines capable of augmenting this function are desired. Moreover, combinations of antibody-cytokine fusion proteins are expected to be more effective than single agents, as no one cytokine molecule can fully sculpt the tumor environment to achieve optimal antigen presentation, expansion and activation of effector cell populations.

2. Choice of target antigens for antibody fusion proteins?

Compared to the very large number of cell surface tumor-associated antigens that have been described, to date very few have been explored as targets for antibody-cytokine fusion proteins. While the antigen need not be tumor-specific, broad expression on many normal tissues might not only contribute to toxicity, but might also provide “sinks” that bind fusion protein in peripheral tissues, thus impairing the cytokine’s ability to reach target tumor cells. The target antigen should be expressed at sufficient levels to permit accumulation of cytokine at tumor site. Importantly, antigens expressed by organs that are known to be susceptible to damage by the targeted cytokine should be avoided (i.e. GI tract by IL-12).

3. Does the fusion protein permit safe and efficient delivery of biologically active cytokine to the tumor bed in adequate quantities?

The fusion protein should have an improved therapeutic index over the free cytokine, allowing sufficient doses to be given to achieve the desired effects within the tumor microenvironment without systemic toxicity. To achieve this, it may be required for the cytokine moiety to be “attenuated” to avoid binding to cytokine receptors in the peripheral

tissues before reaching tumor sites via antibody targeting. Once delivered to tumor sites, the cytokine domain(s) must be free to interact with receptors on appropriate cells. For instance, if the ultimate target of the cytokine is T cells, it may be best to avoid antigens that might be engulfed by macrophages upon antibody binding, since this could result in sequestration or destruction of the Ab-fused cytokine.

4. Is the efficacy of the fusion protein truly greater than a combination of the free antibody and cytokine?

For the construction of an antibody-cytokine fusion protein to be worthwhile, its biologic activity and efficacy against tumors should be greater than the sum of its parts, providing supra-additive or synergistic effects compared to co-administered free cytokine plus antibody. Prior to clinical development, it is recommended that the fusion molecule be evaluated in relevant syngeneic immunocompetent animal models using species appropriate cytokines that permit the full engagement of host immune effector mechanisms. If the fusion protein truly targets the cytokine to precisely where it is needed to elicit host anti-tumor immunity, the measured efficacy should far surpass that of equivalent doses of free cytokine plus free antibody.

5. Which agents should antibody-cytokine fusion proteins combine with to further improve anti-tumor effects?

Antibody-targeted cytokine therapy of cancer offers the potential to achieve unprecedented levels of cytokines within tumor sites in a safe and controlled fashion that could make it easy to combine with other therapies. Given the increased safety margin, it is now possible to consider many other rational combination therapies based on antibody-cytokine fusion proteins. These might include mixtures of cytokines that expand the pool (IL-2, IL-7, IL-15) and activate effector functions (IL-12, IL-21) of T cells and NK cells. Fusion proteins might also be valuable in combination with other active immunotherapeutic approaches such as immune checkpoint inhibition [53, 66, 67].

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Table 1

Properties of cytokines under study for cancer immunotherapy

Cytokine	Product Name(s)	Diseases Under Investigation	Stimulation of T cell Immunity	Maturation of Antigen Presenting Cells	Enhanced ADCC	Direct Tumor Cell Growth Inhibition	Inhibition of Angiogenesis	Established Clinical Experience in Cancer	Clinical Status
Interferon-α, Interf eron-β	Roferon A Intron-A, Inferax Pegintron Pegasys	Melanoma, RCC, Lymphoma, HCL, SarcomaHepatit is B/C	+	+	+	+	+	+	Approved
Interf eron-γ	Actimmune	CGD, Malignant osteopetrosis, various cancers	+	+/−	+	+	+	+/−	Approved
IL-2	Proleukin	RCC, Melanoma	+	−	+	−	−	+	Approved
IL-7	CYT107	Breast, Prostate, Melanoma, RCC, Ewing's sarcoma Neuroblastoma	+	−	−	−	−	−	Phase I, II
IL-12	rhIL-12	MF, H&N cancer, Neuroblastoma, B cell lymphoma, Melanoma	+	−	+	−	+/−	+/−	Phase I, II
IL-15	rhIL-15	AML, Melanoma, RCC, Lung, H&N cancer	+	−	+	−	−	−	Phase I, II
IL-18	rhIL-18	Melanoma Lymphoma, Ovarian	+	−	+	−	+/−	−	Phase I, II
IL-21	rhIL-21	RCC, Melanoma, Ovarian, CLL, Lymphoma	+	−	+	+/−	−	+/−	Phase I, II
GM-CSF	Leukine, Leucomax	Neutropenia, Adjuvant for cancer vaccines	+	+	+/−	−	−	+	Approved
TNFα−1a	Beromun	Sarcomas, (limb perfusion)	+/−	+/−	−	−	+	+/−	Approved

ADCC, antibody dependent cellular cytotoxicity; AML, acute myelogenous leukemia; CGD, chronic granulomatous disease; CLL, chronic lymphocytic leukemia,; GM-CSF, granulocyte-macrophage colony stimulating factor; H&N, head and neck cancer; HCL, hairy cell leukemia,; MF, mycosis fungoides; RCC, renal cell carcinoma

Table 2

Considerations in the design and development of antibody-cytokine fusion proteins

Choice of target antigen	• Tumor-associated antigens with minimal expression by normal tissues. • Cell surface antigens: internalization after binding not required. • Tumor microenvironment-associated antigens acceptable. • Not shed or found circulating in significant amounts.
Choice of cytokine	• What are the goals of targeting the cytokine directly to tumor sites? • Which cell types are activated by the cytokine (T, NK, macrophages)? • What signaling pathways are activated/inhibited by the cytokine? • Does the cytokine have direct effects on tumor vs. normal cells?
Structural format of fusion protein	• Full-length antibody, scFv, Fab, minibody, diabody, or other
Linker	• Resistant to cleavage to avoid release of cytokine into circulation.
Bioactivity of fused cytokine moiety	• Fully intact binding affinity and bioactivity of native cytokine versus attenuated to avoid off-target systemic toxicity. • Quantitiated by molar comparison to free recombinant cytokine.
Pharmacokinetics & pharmacodynamics	• Sufficiently long half life to permit tumor-specific targeting, with ability to accumulate at tumor sites and induce desired immunologic effects.
Preclinical efficacy assessment	• Testing in relevant *in vivo* tumor models with species-appropriate cytokines to assess for immunologic toxicity in addition to anti-tumor efficacy.
Dosing and schedule	• Based on tolerability and pharmacodynamic effects achieved within the tumor microenvironment and host immune effector cell populations.

Table 3

Antibody-cytokine fusion proteins in clinical development

Fusion Protein	Company	Antigen	Malignancies Under Investigation	Structure	Clinical Stage	Clinical Trial Identifiers	References
Hu14.18-IL2, (EMD273063, APN301)	Merck KGaA	GD2	Neuroblastoma, Melanoma	IgG	Phase II	NCT00590824 NCT00082758 NCT00109863 NCT00003750	[30–33]
DI-Leu16-IL2	Alopexx Oncology	CD20	B cell NHL	IgG	Phase I/II	* NCT01874288 NCT00720135	[36]
Anti-CD20 tetrameric hIFNα (20-2b-2b, 20-2b)	Immunome dics	CD20	B cell NHL	IgG	Preclinical	N/A	[54]
Anti-CD20-IFNα, (IGN002)	Immungene	CD20	B cell NHL	IgG	Pre-phase I	N/A	[49,51]
Anti-CD20-IL-21	none	CD20	B cell NHL	IgG	Preclinical	N/A	[42]
Anti-HLA-DR-tetrameric IFNα-2b, (C2-2b-2b)	Immunome dics	HLA-DR	Hematopoietic cancers, B cell lymphoma/leukemia, Multiple myeloma	IgG	Preclinical	N/A	[63]
NHS-IL2LT, (EMD 521873, Selectikine)	Merck KGaA	DNA	Solid tumors, NHL, NSCLC	IgG	Phase I	NCT01032681 NCT00879866	[60,61]
NHS-IL12	Merck KGaA	DNA	RCC, Kaposi sarcoma, T-cell lymphoma, NHL	IgG	Phase I	* NCT01417546	[62]
F16-IL2	Philogen	A1 domain of Tenascin C	Breast, Merkel cell carcinoma, glioblastoma, AML, solid tumors	Diabody	Phase Ib/II	* NCT01134250 * NCT02054884 NCT01131364 * NCT02076620	[57–59]
L19-IL2	Philogen	ED-B Fibronectin	Melanoma, solid tumors, pancreatic cancer, RCC	Diabody	Phase II	NCT01055522 * NCT01253096 * NCT02076633 NCT01253096 NCT01058538 * NCT01198522	[37–40]

Fusion Protein	Company	Antigen	Malignancies Under Investigation	Structure	Clinical Stage	Clinical Trial Identifiers	References
L19-TNFα (Fibromun)	Philogen	ED-B Fibronectin	Melanoma, solid tumors, colorectal, melanoma, isolated limb perfusion	Diabody	Phase I/II	* NCT02076620	[46–48]
						* NCT02076633	
						NCT01253837	
						NCT01213732	
huBC1-IL12, (AS1409)	Antisoma	ED-B Fibronectin	RCC, melanoma	IgG	Phase I/II	NCT00625768	[44,45]
huKS-IL2, (EMD273066)	Seono	EpCAM	Ovarian, prostate, colorectal, NSCLC	IgG	Phase I/II	NCT00132522	[64,65]
						NCT00408967	
						NCT00016237	

AML, acute myelogenous leukemia; EDB, extradomain B of fibronectin; NHL, non-Hodgkin's lymphoma; NSCLC, non-small cell lung cancer; RCC, renal cell carcinoma

N/A, not available

* active clinical trial