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Antibody-cytokine fusion proteins: a novel class of biopharmaceuticals for the therapy of cancer and of chronic inflammation

Patrizia Murer[1], **Dario Neri**[1,*]

[1]Department of Chemistry and Applied Biosciences, Swiss Federal Institute of Technology (ETH Zürich), Vladimir-Prelog-Weg 4, CH-8093 Zurich (Switzerland)

Abstract

Antibody-cytokine fusion proteins represent a novel class of biopharmaceuticals, with the potential to increase the therapeutic index of cytokine payloads and to promote leukocyte infiltration at the site of disease. In this review, we present a survey of immunocytokines that have been used in preclinical models of cancer and in clinical trials. In particular, we highlight how antibody format, choice of target antigen, cytokine engineering as well as combination strategies may have a profound impact on therapeutic performance. Moreover, by using anti-inflammatory cytokines, antibody fusion strategies can conveniently be employed for the treatment of auto-immune and chronic inflammatory conditions.

From cytokines to immunocytokines

Cytokines are small immunoregulatory proteins, which are secreted by leukocytes and, in some cases, by endothelial cells, fibroblasts and stromal cells. Cytokines can induce important functional changes in tissues, such as an increase in vascular permeability or alteration of body temperature. Importantly, these proteins can also have inhibitory or activating effects on immune cells, depending on their features, on their concentration and on the environment in which they act [1]. Considering the important role in many physiological and pathological conditions, cytokines have been considered as products in their own right or as targets for the development of blocking agents.

The antibody-based blockade of pro-inflammatory cytokines [e.g., tumor necrosis factor (TNF), interleukin-2 (IL2), interleukin-12 (IL12), interleukin-17 (IL17)] or their cognate receptors [e.g., the interleukin-6 receptor (IL6R)] has led to the development of successful products for the treatment of chronic inflammatory conditions, such as rheumatoid arthritis, inflammatory bowel diseases and psoriasis. On the other hand, certain recombinant cytokines have received marketing authorization for the treatment of cancer (e.g., IL2, TNF), of viral infections [e.g., Interferon (IFN) α], and for few other biomedical applications.

*Corresponding author: Mailing address: Department of Chemistry and Applied Biosciences, Vladimir-Prelog-Weg 4, CH-8093 Zürich, Switzerland, Tel: +41-44-6337401, neri@pharma.ethz.ch.

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The systemic administration of pro-inflammatory cytokines can lead to severe off-target related adverse effects, which may limit the dose and prevent escalation to therapeutically active regimens. Certain cytokine products (e.g., IL2, TNF, IL12) have exhibited recommended doses in the single-digit milligram range or even below [2–4]. Adverse effects associated with the intravenous administration of pro-inflammatory cytokines may include hypotension, fever, nausea or flu-like symptoms, but cytokines may occasionally also cause serious hematologic, endocrine, autoimmune or neurologic events [5]. In view of these considerations, there is a clear biomedical need for the development of next-generation cytokine products, which are better tolerated and which display a preferential action at the site of disease, helping spare normal tissues.

Antibodies specific to accessible markers, which are over-expressed at the site of disease, may represent ideal “vehicles” for the targeted delivery of therapeutic payloads, including cytokines. In mice, it has been clearly shown that certain tumor-homing antibody-cytokine fusion proteins (“immunocytokines”) can dramatically increase the therapeutic index of the corresponding cytokine payload [6–12]. Similarly, immunocytokines with anti-inflammatory properties, capable of selective accumulation at sites of tissue remodeling, have been considered for the treatment of chronic inflammatory conditions and of endometriosis [13, 14]. In this review, we survey some basic concepts associated with the development of immunocytokine biopharmaceuticals and discuss how engineered cytokine products may look like in the future.

Immunocytokine formats

Antibodies can be used in full immunoglobulin G (IgG) format, in order to exploit the stability and long circulatory half-life of these products. Alternatively, antibody fragments [e.g., single-chain variable fragments (scFvs), diabodies, Fab fragments] may be considered, when a faster blood clearance and a more efficient extravasation is desired [15, 16]. Figure 1 illustrates some of the most popular antibody formats, which have been considered for immunocytokine development. The format may determine different pharmacokinetic and pharmacodynamic properties. These *in vivo* functional aspects may not be evident on the basis of simple *in vitro* assays, since the fusion of cytokine payloads with various types of antibodies often proceeds without loss of biological activity. On the other hand, the residence time of immunocytokines in blood and the ability of the product to extravasate and localize at the site of disease are crucial determinants of pharmaceutical performance [17].

Intact IgG molecules can be fused to cytokines at various sites (N- or C-terminus of heavy or light chain), giving rise to large fusion proteins (e.g., 180 kDa for IL2 fusions). The size and recycling properties mediated by the interaction with the neonatal FcRn receptor may contribute to a longer circulatory half-life *in vivo* [18]. As a potential drawback, a larger molecular size may impair extravasation and penetration into the tumor mass [19]. In addition to the bioactivity of the cytokine moiety, an IgG-based immunocytokine would retain the ability to bind to Fc gamma receptors on leukocytes, with the potential to impair localization at the tumor site and to mediate undesired activation of white blood cells. Changing the amino acid composition, the isotype or the glycans of the Fc portion may reduce these off target effects [20, 21].

Immunocytokines based on small antibody fragments have a much shorter half-life in the circulation, compared to those based on the full IgG format. A rapid blood clearance may lead to a decreased uptake at the site of disease, since extravasation typically represents the rate-limiting step for ligand-based pharmacodelivery applications [15, 22]. Short linkers (less than 11 aminoacids) between the variable heavy (VH) and variable light (VL) chains form non-covalent homodimers (diabodies) [23]. Diabodies and other bivalent formats fused to the cytokine payloads retain a high binding avidity to the cognate antigen, thus potentially leading to a long residence time on the biological target [16, 24, 25]. Multivalent immunocytokines based on antibody fragments have exhibited tumor:organ ratios greater than 10:1 in biodistribution studies performed in mouse models of cancer, 24 hours after intravenous administration [11, 26].

The antibody format and the position of the cytokine payload, as well as the amino acid composition of the linker connecting various protein domains, can substantially influence the *in vivo* performance of fusion proteins, thus offering rich opportunities for protein engineering applications [16, 27]. Design options further increase, when cytokines consisting of multiple subunits (e.g., components of the TNF or of the IL12 superfamily) are considered [7, 17, 26, 28–32] (Figure 2).

Targets for oncological and non-oncological conditions

Ideal target antigens for the development of immunocytokine products should be abundantly expressed at the site of disease and absent in healthy tissues. In tumors and in chronic inflammatory conditions, tissue remodeling and neo-vascularization processes expose antigens, which are otherwise virtually undetectable in healthy organs [33–35]. One example is represented by splice isoforms of fibronectin, a glycoprotein of the extracellular matrix (ECM). The extra-domains A and B (EDA and EDB) of fibronectin are strongly expressed in tumors, at sites of tissue remodeling and during fetal development, but are otherwise not found in normal tissues, exception made for the female reproductive system [36, 37]. Similarly, splice variants of tenascin-C are specifically found in tissues and tumors undergoing neo-angiogenesis, in a process which is regulated by intracellular pH [38]. EDA, EDB and splice variants of tenascin-C represent suitable targets for the delivery of bioactive payloads, including cytokines.

In oncological malignancies molecular targets may include fibroblast activation protein (FAP) [39, 40], cellular antigens (e.g., CEA and PSMA) [41–43] or proteins, which become accessible in necrotic lesions, such as histones [44]. Antibodies which have been extensively characterized in the context of cytokine fusions include F8 (targeting EDA-fibronectin) [45], L19 (targeting EDB-fibronectin) [46], F16 (targeting the A1 domain of tenascin-C) [47], scFv36 (targeting FAP) [40], hu14.18 (targeting the GD2 ganglioside) [48], chCLL-1 (targeting CD20) [49] and anti-HER2/*neu* [50].

In tissues undergoing chronic inflammatory processes, specific cell subtypes that are present at higher local density than in normal tissues, may be targeted (e.g., neutrophils and macrophages) [51]. CD64 (i.e., the high-affinity Fcγ receptor 1) is expressed on the surface of macrophages, monocytes and their progenitor cells. Immunotoxins directed against CD64

were tested in animal models of inflammation [skin, rheumatoid arthritis and ischemia-induced kidney injury] and did not display adverse effects related to the presence of the antigen on other circulating leukocytes [52].

Cytokine payloads

Both for cancer therapy as well as for the treatment of chronic inflammation, several cytokine payloads have been developed and tested in preclinical trials. Pro-inflammatory cytokines such as IL2, TNF, and IL12 have been investigated for tumor therapy, as they have been found to increase and activate the local infiltrate of leukocytes at the tumor site [53–59]. By contrast, immunosuppressive cytokines (e.g., IL10) may be considered as payloads for the treatment of chronic inflammatory conditions [60, 61] or of other diseases (e.g., endometriosis) [62, 63].

The first antibody-cytokine fusions were reported by the groups of Reisfeld/Gillies, Alan Epstein and Sherie Morrison [48, 64, 65]. The authors described the fusions of IgG antibodies with various interleukins, interferons and members of the TNF superfamily [48–50, 64, 66–69]. Our group has mainly focused on the development of antibody-cytokine fusion proteins, which targeted extracellular matrix components (e.g, those based on the F8 and L19 antibodies). Quantitative biodistribution studies using radiolabelled immunocytokine preparations have allowed us to learn some lessons about payloads that can be efficiently delivered to solid tumors, as well as payloads which abrogate the targeting properties of the parental antibody. Payloads can broadly be grouped into five distinct categories in the mouse:

- **i)** Cytokines, which are specifically delivered to the tumor mass, by fusion to the antibody moiety (e.g., IL2, TNF, IL4, IL6, IL10, IFNα) [14, 53, 70–72].
- **ii)** Cytokines, which are specifically delivered to the tumor mass, by fusion to the antibody moiety in a specific format (IL12) [29].
- **iii)** Cytokines which can be efficiently delivered to the tumor site only at higher doses, after saturation of the cognate receptor (e.g., IFN γ, GM-CSF) [73, 74].
- **iv)** Payloads, which are too large or too negatively or positively charged to be able to extravasate and reach the target antigen *in vivo* (e.g., VEGF(120) vs. VEGF(164)) [75].
- **v)** Cytokines, which are heavily glycosylated and are captured by asialoglycoprotein receptor in the liver, which abrogates the tumor-homing performance of the antibody moiety (e.g., B7.2, IL9) [76, 77].

Our group has observed that glycosylation patterns may vary depending on the protein production protocol used (e.g., stable vs. transient gene expression), leading to striking differences in disease-targeting performance [77].

Immunocytokines for cancer therapy

The targeted delivery of cytokines to the tumor aims at inducing a local pro-inflammatory environment, which may activate and recruit immune cells. Here, we briefly present fusion proteins which have shown activity in preclinical models and which have progressed to clinical trials in patients with cancer. A comprehensive list of antibody-cytokine fusions described in the literature can be found in Ref. 78 [78]. Table 1 summarizes the clinical trials performed using immunocytokines for cancer treatment.

IL2-based immunocytokines

Interleukin-2 (IL2) is one of the best-characterized cytokines for tumor therapy applications, since the corresponding recombinant human protein is a product on the market (e.g., Proleukin™). IL2 is constitutively released by several immune cells, upon immune stimulation [1]. It leads to activation, differentiation and maintenance of T cells, that initially express a low-affinity dimeric receptor consisting of β- and γ-chain and (produced after T cell activation) the trimeric high affinity IL2 receptor (IL2R) containing also the α-chain at a later time point [79, 80]. As first immunotherapeutic agent in 1985, high dose IL2 monotherapy (600,000–720,000 IU/kg) led to tumor regression of patients with metastatic renal carcinoma and melanoma and was eventually approved for treatment of these malignancies in 1992 and 1998 respectively [81–83]. Nonetheless, the anticancer activity of untargeted IL2 is insufficient considering the severe adverse effects at these doses (e.g., capillary leak syndrome, hypotension, severe flu-like symptoms) [5, 83].

The first immunocytokines based on IL2 were developed more than two decades ago, featuring either full immunoglobulins or antibodies in scFv format [48, 84, 85]. A potent anti-cancer activity has been reported for animal models of murine colon carcinoma, human metastatic melanoma, prostate carcinoma and neuroblastoma, using antibodies specific to an epithelial cell adhesion protein (EpCAM G733-2) and the ganglioside GD2 [8, 86–88].

The groups of Reisfeld and Gillies investigated on the ch14.18-IL2 fusion protein, an anti-ganglioside GD2 IgG molecule with IL2 coupled to the C-terminus of the immunoglobulin heavy chain [48]. Strong anti-cancer activity was reported in several murine tumor models [8, 86, 88]. The humanized product Hu14.18-IL2 has been tested in Phase II clinical trials in melanoma and neuroblastoma [89–92]. Recently, Gillies has reported that positioning IL2 at the C-terminus of the light chain may lead to allosteric regulation of cytokine activity upon binding [93]. Additionally, the same research group reduced the toxicity of IL2 by introducing a single amino acid substitution (D20T). This IL2 mutant (IL2LT, standing for “low toxicity IL2”) was fused to the NHS antibody, which recognizes DNA-associated structures in the necrotic core of solid tumors. NHS-IL2LT dramatically decreased the metastasis load in mice injected intravenously with Lewis Lung Carcinoma (LLC) cells [94]. NHS-IL2LT (Selectikine) has been investigated as a single agent in a Phase I [95, 96] trial in patients with advanced solid tumors. Subsequent Phase II clinical studies in advanced melanoma patients have been discontinued [97, 98].

Scientists at Roche have recently described FAP- and CEA-targeted immunocytokines containing an IL2 variant, which does not bind the IL2R in its trimeric form. Three amino

acid mutations were used to abolish CD25 binding in the interleukin-2 moiety [99, 100], in order to decrease an undesired activation of regulatory T cells (Tregs) and of the vascular endothelium [99–103]. The IL2 variant was fused at the C-terminus on the heavy chain of asymmetric IgG molecules, created using knob-in-the-hole technology [104]. In a Phase I clinical trial, CEA-IL2v showed initial evidence of tumor accumulation [100, 105]. The product has recently been tested in combination with atezolizumab in a Phase Ib clinical study (NCT02350673). Similarly, FAP-IL2v is being studied in early-stage clinical trials in combination with atezolizumab (anti PD-L1), trastuzumab (anti-Her2/Neu) or cetuximab (anti-EGFR).

The anti-CD20 immunocytokine Leu16-IL2, developed by Provenance Biopharmaceuticals, induced significantly greater inhibition of tumor growth in lymphoma xenografts, compared to the mixture of the isolated components (i.e., the intact anti-CD20 antibody moiety in IgG format and untargeted IL2) [12]. Even a deglycosylated form of Leu16-IL2 (DI-Leu16-IL2) improved survival of mice bearing Daudi metastases, with little difference compared to the glycosylated antibody, suggesting that ADCC may not play a relevant role in this model. The authors attributed most of the anti-tumoral activity to the targeted delivery of IL2, since a non-tumor specific immunocytokine displayed a substantially lower therapeutic effect [12]. In a Phase I/II clinical study in B cell lymphoma patients, partial responses and complete responses were observed in 5/13 and 3/13 patients, respectively [106].

Our group has mainly focused on the use of antibodies directed against extracellular matrix components (e.g., F8, L19 and F16, binding to alternatively spliced domains of fibronectin and tenascin C respectively) for the targeted delivery of cytokines. The three antibodies have been coupled to IL2 in the non-covalent homodimeric diabody format [11, 107, 108], leading to products with potent anticancer activity [11, 53, 107–114]. In preclinical studies L19-IL2 had shown a significantly stronger tumor growth inhibition, compared to untargeted IL2 [11, 111, 112]. Moreover, treatment with L19-IL2 increased the immune cell infiltrate (NK and T cells) in the neoplastic mass of mice [11, 111]. L19-IL2 (Darleukin) was tested in Phase I clinical studies as monotherapeutic against metastatic renal cell carcinoma (mRCC) and in combination with dacarbazine for the treatment of metastatic melanoma, showing encouraging anti-tumor activity [115, 116]. In an additional Phase II clinical study, the IL2 fusion protein was evaluated alone or in combination with another immunocytokine L19-TNF (Fibromun), with intratumoral injections in melanoma lesions [117, 118]. Based on the promising results, the IL2 conjugate has been brought to Phase III in combination with Fibromun. Additionally, a Phase I/II clinical trial assessing L19-IL2 in combination with Rituximab (anti-CD20) is currently on going in patients with B-cell lymphoma. A strong synergistic effect had been observed in B-cell lymphoma xenografts, driven by the increased number of immune effector cells in the tumor tissue capable of inducing antibody-dependent cell-mediated cytotoxicity (ADCC) [111].

In preclinical studies L19-IL2 has been combined with a small-molecule-drug conjugate (SMDC), selectively homing to tumor cells expressing carbonic anhydrase IX on their surface, delivering an anti-tubulin agent to the neoplastic mass. Combination of the two agents led to cancer cures in two mouse models of cancer, which do not respond to conventional chemotherapy [119]. Besides, when L19-IL2 was systemically administered

after radiotherapy, a significant synergistic anti-tumor effect and a long-lasting immune protection were observed in mice bearing CT26 and C51 tumors [120]. Together, these results indicate that IL2-based immunocytokines may enhance the infiltration of primed immune effector cells in the tumor, after induction of immunogenic cell death.

F16-IL2 (Teleukin), a fusion protein with the same format used for L19-IL2, showed some promising anti-cancer activity in patients with different solid tumors and metastatic breast cancer in combination with Doxorubicin [121], as well as in relapsed acute myeloid leukemia (AML) after allogeneic hematopoietic stem cell transplantation [122]. Phase I clinical studies are now investigating Teleukin in combination with cytarabine or an anti-CD33 antibody.

TNF-based immunocytokines

TNF-α (TNF) is a homotrimeric pro-inlammatory cytokine mainly secreted by NK cells, macrophages and T cells. When released locally, it acts on endothelial cells triggering the containment of infections, by increasing vasculature permeability, blood clotting, attracting and activating adjacent immune cells [1]. In cancer therapy TNF is able to induce direct apoptosis of certain tumor cells, elicit hemorrhagic necrosis and increase the influx of immune effector cells [123–125]. Systemic administration of recombinant TNF (Beromun™) is approved for tumor size reduction before surgery or for locoregional treatments (i.e., isolated limb perfusion) of patients with non-resectable soft-tissue sarcoma, in combination with melphalan. The clinical application of recombinant TNF is limited, due to the substantial systemic adverse effects (e.g., shock, disseminated intravascular coagulation, organ failure) [126]. TNF has been coupled to various moieties for pharmacodelivery, in order to overcome limiting toxicities [127–130]. TNF-antibody fusion proteins have shown a potent anti-cancer activity in mouse models based on grafted human tumors or on syngeneic tumors [26, 71, 128, 131, 132]. However, cancer cures were only observed in immunocompetent mice bearing TNF-sensitive tumors, indicating that tumor infiltrating leukocytes may play a major role in eradication of TNF treated tumors [10].

L19-TNF is a clinical-stage product composed by the L19 antibody in scFv format, targeting the alternative spliced domain EDB in tumor neovasculature, coupled to TNF, which drives the formation of a homotrimeric structure (Figure 2). L19-TNF preferentially localized to tumor masses in mouse models of cancer, as shown in biodistribution experiments with radiolabeled protein preparations [26]. The immunocytokine showed an increased inhibition of tumor growth compared to untargeted TNF treatment in mouse models of fibrosarcoma and colorectal cancer [10]. Interestingly, most of the subcutaneous tumor mass became necrotic after a single intravenous administration of L19-TNF [26]. A synergistic effect was observed when L19-TNF was combined to mephalan [26]. Combination studies with L19-IL2 and L19-IL12 also showed strong additive antitumoral effects on models of murine teratocarcinoma and neuroblastoma [133, 134]. Phase III clinical studies are currently testing the combination of the fully-human L19-TNF product (Fibromun), administered intravenously, with doxorubicin in soft-tissue sarcoma patients

When used as monotherapeutic agent in a Phase I/II clinical study, L19-TNF did not induce any objective responses [135]. By contrast, combination with melphalan in patients with

advanced extremity melanoma induced 89% objective responses with the agents given through isolated limb perfusion [136]. Immunohistochemical analyses of tumor sections showed accumulation of the L19-TNF at sites of tumor neo-angiogenesis [137]. A potent anticancer activity of L19-TNF was also observed upon intralesional administration in combination with L19-IL2 in patients with inoperable metastatic melanoma [117]. In a Phase II study, 5% complete responses (CR) and 50% partial responses were recorded, with tumor regression visible also in non-injected lesion, suggesting the activation of a protective immunity. The combination is currently tested in a Phase III clinical trial (NCT02938299, EudraCT number 2015-002549-72).

Other antibodies can also be considered for TNF delivery applications. The F8 antibody, specific to EDA fibronectin, was fused to murine TNF in scFv format and used as single agent or in combination with doxorubicin for the treatment of syngeneic murine tumor models of sarcoma. The combination regimen of doxorubicin and F8-TNF led to complete eradication of the tumor and long-lasting immunoprotection [55, 71]. Recent mechanistic studies revealed that the treatment with F8-TNF + doxorubicin increases the number of specific cytotoxic T cells, which recognize a common endogenous retroviral antigen AH1 (derived from an envelope protein of the murine leukemia virus) [55]. Generally, such retroviral sequences have been found also in the genome of vertebrate species and their expression has been associated with autoimmune diseases, chronic infections or cancer [138, 139]. In theory, a TNF-based immunocytokine could cause upregulation of a subset of cytotoxic T cells specific for a retroviral antigen found in human tumors, which would contribute to the antitumoral effect in patients.

Preclinical studies on a murine melanoma model revealed that the therapeutic activity of an anti-melanoma immunoglobulin IgG2a(TA99) was significantly potentiated when combined with the TNF antibody fusion protein TA99-mTNF. The increased density of leukocytes in the subcutaneous melanoma masses of mice treated with the combination treatment favored tumor cell killing through ADCC [140].

IL-12 based immunocytokines

Interleukin-12 (IL12) is a heterodimeric cytokine, composed by the p40 and p35 moieties linked by a disulfide bond, which provides a crucial connection between the innate and the adaptive immune system [141]. IL12 is mainly released by antigen presenting cells (APCs) as a response to infection, inducing the release of IFNγ and the promotion of T_H1 differentiation of CD4+ T cells. IL12 additionally stimulates the proliferation of NK cells, T cells, the MHC1 antigen presentation process, and inhibits Treg activity [142].

The anti-tumor activity of recombinant IL12 has been assessed in mouse models of cancer [143, 144], but showed limited clinical efficacy and high toxicities at doses as low as low as 1 μg/Kg body weight in cancer patients [145]. Two immunocytokines based on IL12 are currently in clinical development, NHS-IL12 and BC1-IL12. NHS-IL12 specifically targets DNA-Histone H1 complex, released upon tumor cell apoptosis [44]. The product, developed by Merck is currently being tested in Phase I trials as monotherapy or in combination with avelumab (anti-PD-L1 antibody) [146, 147].

BC1-IL12 recognizes an epitope on domain 7 of fibronectin, which is cryptic (i.e., not exposed) in normal tissues, but becomes unmasked in the presence of the EDB domain [54]. The fusion protein consists of the full IgG1 linked to the IL12 heterodimer at the C-terminus of the heavy chain. Efficient *in vivo* localization [148] and anti-tumor activity have been confirmed in various mouse models of cancer [54]. When tested on patients with malignant melanoma in a Phase I clinical study, BC1-IL12 led to disease stabilization in 6 out of 11 patients for at least 4 months [149]. The therapeutic immunocytokine was well tolerated and increased IFNγ levels in the serum of all patients.

The heterodimeric nature of the IL12 and of the resulting fusion proteins allows the implementation of various types of immunocytokine formats (Figure 2) [7, 17, 29, 32], which however may represent a challenge for successful protein production. In the formats studied so far, the p35 and p40 subunits were typically connected by a peptide linker. Halin et al. linked the p40 domain of IL-12 to the N terminus of its p35 domain by a 15-amino acid linker and fused it by a 6-amino acid linker to the N terminus of the L19 antibody in the scFv format [7]. The fusion protein was compared to untargeted IL12 in therapy studies on a mouse model of lung metastasis and two subcutaneous tumor models. IL12-L19 displayed much stronger inhibition of tumor progression compared to the untargeted control and increased leukocyte infiltrate into the tumor masses. The therapeutic effect of IL12-L19 could be improved by combination with L19-TNF [134]. Other formats were investigated with the aim to increase tumor:organ ratios. IL12-SIP(L19) featuring a C-terminal CH4 domain of human IgE in order to favor the homodimerization of the fusion protein, accumulated mainly in the liver and exhibited modest tumor uptake compared to a heterotrimeric fusion protein having p40 linked to scFv(L19) forming a non covalent dimer with scFv(L19)-p35. This fusion protein showed an efficient tumor uptake in F9 teratocarcinoma mouse tumor models, with tumor:organ ratios of 10:1–20:1, 24 hours after intravenous administration [29].

The F8 antibody, specific to EDA fibronectin, was fused to murine IL12 in different formats [17, 32] and tested both *in vitro* and *in vivo*. The most promising version (named IL12-F8-F8) featured the IL12 moiety fused to the N-terminus of a “tandem” diabody fusion of two F8 moieties. IL12-F8-F8 efficiently inhibited tumor growth in three different immunocompetent syngeneic models of cancer. The effect was strongly potentiated by combination with paclitaxel [17].

IL12 has also been conjugated to a CD30 targeting antibody in the HSR(scFV)-IL12 construct. Binding and killing of CD30 positive cells, as well as increased IFNγ release by T cells were confirmed *in vitro* [150].

Other cytokine payloads

Other immunocytokines have been tested mainly in preclinical studies, but did not exhibit successful accumulation in the tumor tissue, had insufficient therapeutic effect and/or induced high transient body weight loss in mice. (e.g., F8-IL1β [72], F8-IL6 [72], F8-IL7-F8 [151], F8-IL17 [152]). Our group systematically explored the targeted delivery of cytokines from the TNF superfamily (e.g., CD40L, FasL, TRAIL, VEGI, LiGHT) in biodistribution studies. A lower accumulation (compared to TNF fusions) was observed in

the tumor mass of various murine cancer models [153]. Recent work of the group of Roland Kontermann showed that better tumor homing properties might be achieved if the TNF superfamily cytokines are expressed as single chain polypeptides [30, 154] (Figure 2). Roche developed an anti-FAP-mu4-1BBL conjugate based on the murine single chain 4-1BBL able to potentiate the anti-tumor activity of anti-PD-L1 and Trastuzumab (anti-HER2) in different mouse models of cancer [155]. The humanized anti-FAP-4-1BBL is currently tested in Phase I clinical trials [156].

IL15 is a pro-inflammatory cytokine that features certain functional redundancies with IL2, as both cytokines signal through the beta and gamma subunits of the same receptor. A fusion protein consisting of a FAP binding moiety, IL15 and one domain of the IL15 receptor (IL15Rα), was generated by the group of Dafne Müller. The antibody-IL15/IL15Rα fusion protein was able to specifically activate intermediate affinity receptor expressing NK and T cells. Stronger tumor growth inhibition was observed in murine B16 metastasis model, compared to the untargeted and to the IL15Rα missing forms [157].

Granulocyte/macrophage-colony stimulating factor (GM-CSF) was coupled to antibodies in IgG, diabody or scFv format, targeting various tumor-associated antigens (e.g., HER2/Neu, CEA, EDB) [50, 74, 158]. Targeted delivery of GM-CSF to the tumors showed enhanced antitumor activity [50, 74], as well as increased antigen processing and presentation [158, 159].

Finally, it is conceivable to fuse two cytokines the same antibody, in order to exploit a synergistic benefit by the simultaneous presence of the two payloads at the site of disease. Dual-cytokine antibody fusions have been described for IL2 and IL12 [160–162], IL12 and GM-CSF [161], TNF and IL2 [57], as well as IL15 and 4-1BBL [40]. The group of Roland Kontermann has introduced the concept of Duokines, dual-acting cytokine fusion proteins generated by connecting two TNF superfamily members (combinations 4-1BBL, OX40L and CD27L) by a flexible peptide linker in different conformations [163].

Immunocytokines for the therapy of chronic inflammatory conditions

The *in vivo* activity of cytokines and of their derivatives depends on the immunological environment and on the concentration of the cytokine at the site of disease. Certain payloads may display a pro- or anti-inflammatory action depending on the immunological context [164–166]. For example, IL12 caused disease worsening and increased TNF production in the collagen-induced model of arthritis, when the product was given at low doses (5ng/day). By contrast, higher doses of (500ng/day) suppressed disease development inducing production of the anti-inflammatory cytokine IL10 and corticosterone [167]. More examples, reviewed by Cavaillon JM (e.g., IL6, IL4, IL10, TGFβ), indicate the dependence on the responding cell, the timing and even the experimental model [165].

Even though research has mainly focused on immunocytokines directed against oncological targets, potential applications in chronic inflammatory diseases have recently gained momentum. One product based on IL10 (F8-IL10) is currently being investigated in Phase II

clinical trials in patients with rheumatoid arthritis or ulterative colitis, while other products have shown promising signs of efficacy in animal models [14, 168].

IL10 is a homodimeric, pleiotropic cytokine, with a potential to display an immunosuppressive activity at sites of inflammation, for example by reducing antigen presentation and the release of pro-inflammatory mediators [169]. Recombinant IL10 has been tested in various mouse models of inflammation showing encouraging results [60, 61]. Initial clinical studies have shown no significant differences in remission rates or disease improvements were reported [170, 171]. However, a pegylated version of murine IL10 induced rejection of solid and metastasizing tumors in mice by induction of cytotoxic T cells [172, 173]. In clinical trials the recombinant pegylated interleukin-10 (PEG-rIL-10) increased the density of activated intratumoral cytotoxic T cells in patients [174]. PEG-rIL-10 is currently being investigated in combination with immunecheckpoint inhibitors [anti-PD-1 antibodies Nivolumab (NCT03382912) and Permbrolizumab (NCT03382899)] or with chemotherapy (NCT02923921).

The antibody-cytokine fusion L19-IL10 selectively localized to sites of inflammation in mouse models [13]. L19-IL10 features the L19 antibody (specific to EDB fibronectin) in diabody format, fused to human IL10. In the mouse model of collagen induced arthritis (CIA), L19-IL10 treatment induced a significant inhibition of disease progression, compared to control mice treated with saline or with an IL10 fusion based on an antibody of irrelevant specificity [13]. Similarly, fusion of IL10 with the F8 antibody (specific to EDA fibronectin) [175] showed disease targeting and an encouraging activity in the murine CIA model [175, 176], both, alone or in combination with methotrexate or a TNF inhibitor. The product was also active in a mouse model of endometriosis [63]. The fully human F8-IL10 fusion protein (Dekavil) was tested in a Phase Ib clinical trial against rheumatoid arthritis in combination with methotrexate (NCT02076659). 15 out of 23 treated patients experienced therapeutic benefit, including 2 long-lasting remissions [177] and is now being investigated in multiple Phase II clinical trials (NCT02270632, EudraCT number 2013-005418-37). Additionally, F8-IL10 is tested in a Phase II clinical trial as add-on therapy to infliximab in patients with ulcerative colitis (EudraCT number 2017-002108-28).

In another approach, viral IL10, which is considered less immunostimulatory, was fused to an antibody fragment targeting ROS-CII (1-11E). Treatment of arthritic mice with 1-11E/IL10 inhibited disease progression and lowered pro-inflammatory cytokines serum levels [178, 179].

Immunomodulatory products can also be produced by antibody fusion with IL4, a so-called “prototypic immunoregulatory cytokine” [180], able to interact with various target cells. IL4 production is limited to a small subset of hematopoietic cells and the effects are mediated by a high affinity heterodimeric IL4 receptor. Recombinant IL4 reduced disease progression in preclinical models of rheumatoid arthritis [181], but failed to reproduce these results in human clinical studies[182]. By contrast, recombinant IL4 switched the immune environment to a T_H2 pattern and markedly improved conditions in patients with moderate-to-severe T_H1 mediated psoriasis [182]. The antibody-based delivery of IL4 at the site of chronic inflammation is thought to generate a more specific and focused immunomodulatory

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action. The F8-IL4 fusion protein (Tetravil), based on the EDA targeting antibody in diabody format, selectively accumulated in the proximity of newly formed blood vessels in arthritic paws and toes of mice with CIA. Therapy studies in CIA mice induced 100% complete remission of the disease when F8-IL4 was given together with dexamethasone [14]. The combination therapy rapidly resolved the inflammatory process in the paws, by recruiting TH2 cells and Tregs and by normalizing the concentration of pro-inflammatory cytokines [14, 168, 183]. In addition, treatment with F8-IL4 significantly inhibited endometriotic lesion growth in a mouse model, compared to untargeted IL4, which did not exhibit *in vivo* activity [62]. The therapeutic effect of F8-IL4 was also observed in imiquimod-induced and contact-hypersensitivity-induced mouse models of skin inflammation [184]. Motivated by these encouraging data, F8-IL4 is currently being considered for clinical testing for the treatment of rheumatoid arthritis and endometriosis [185].

Conclusions

A large variety of immunocytokines has been generated for multiple therapeutic applications in the past two decades. Even though only a few of those proceeded to clinical stage so far, the potential of antibody-cytokine fusion proteins is enormous. Immunocytokines may synergize with several combination partners, such as cytotoxic drugs [26, 44, 53, 71, 107–109, 186], radiation [44, 96, 120, 187], monoclonal antibodies [44, 99, 111], SMDCs [119], antibody drug conjugates [188, 189], cancer vaccines [159, 161], immunecheckpoint inhibitors [39, 56, 57, 134, 190, 191], bispecific antibodies [192–194], and other immunocytokines[17, 57, 114, 134, 160–162, 195, 196]. Over the next few years, the outcome of on-going clinical trials will shed light on the therapeutic benefit that can be achieved, while new protein engineering approaches will contribute to the development of second-generation products.

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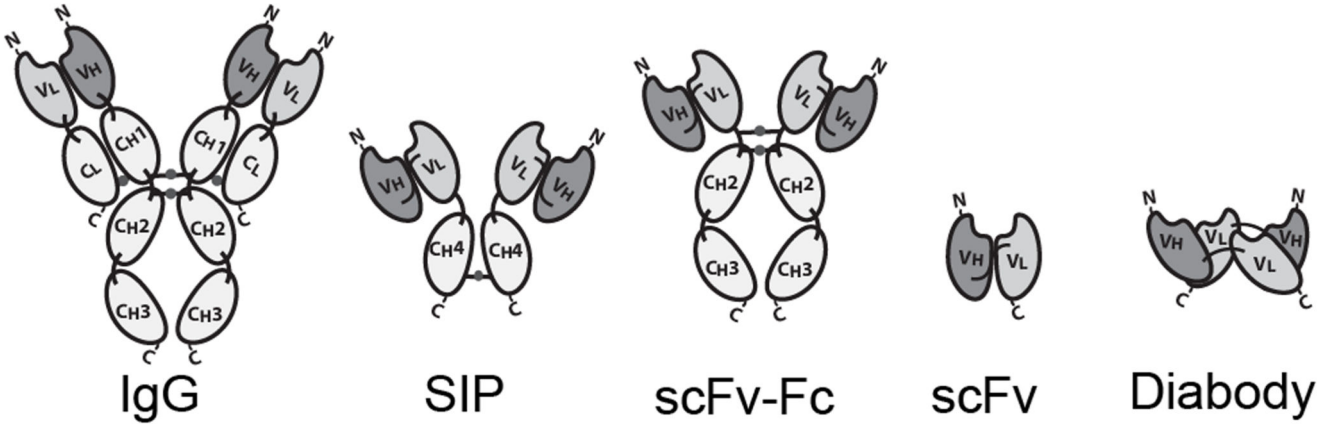

Figure 1.

Schematic illustration of different antibody formats suitable for antibody-cytokine fusion proteins. From the left: full immunoglobulin (IgG), Small immunoprotein (SIP), scFv-FC, scFv, Diabody. N = N-terminus, C = C-terminus.

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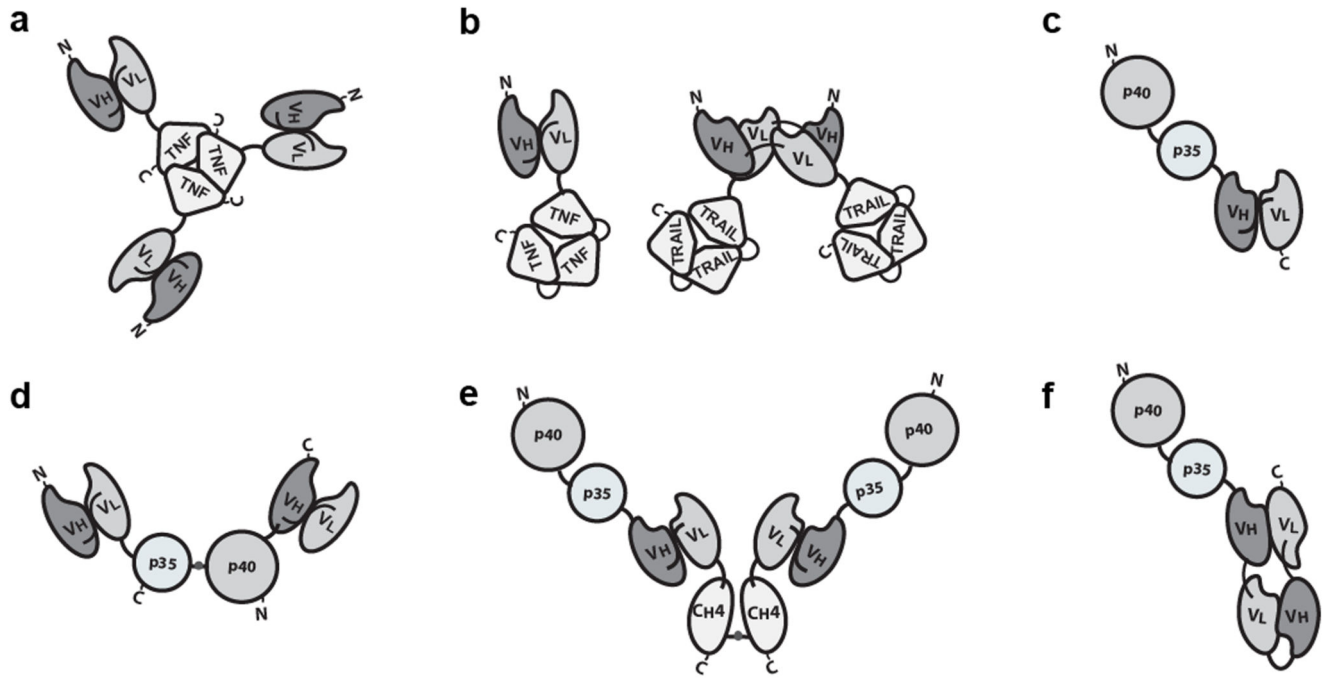

Figure 2.

Examples of immunocytokines built with multiple subunits. **a)** Homotrimeric scFv-TNF [26, 71]. **b)** Left: scFv fused to single chain TNF (scTNF). Right: Homodimeric immunocytokine formed by two diabody-scTRAIL subunits. Other TNF superfamily cytokines can be engineered with the same approach [28, 154]. **c)** The C-terminus of the p40 is connected to the N-Terminus of the p35 subunits of IL12 15 amino acid linker. The recombinant cytokine is linked to the N-terminus of a scFv [7]. **d)** IL12-based immunocytokine consisting of two polypeptides, scFv-p35 and p40-scFv, joined by a disulfide bond between the two cytokine subunits[32]. **e)** IL12-SIP with a C-terminal CH_4 domain of human IgE, wich favor homodimerisation. **f)** The p40 and p35 subunits of IL12 sequentially fused to a “forced” diabody or tandem diabody [17].

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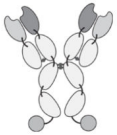
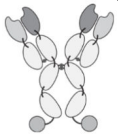
Table 1

List of immunocytokines in clinical trials. NK: Natural Killer; NSCLC: Non-Small-cell Lung Carcinoma; EDB: extra domain B of Fibronectin; CEA: Carcinoembryonic Antigen; FAP: Fibroblast Activation Protein; PK: Pharmacokinetic; PD: Pharmacodynamic; RCC: Renal Cell Carcinoma; MTD: Maximum Tolerated Dose;

Antibody	Antigen/Format	Clinical Trials		Indication	Status	
Inteleukin-2						
Hu14.18-IL2 / EMD 273063; APN301	GD2-ganglioside 	NCT00003750	Phase I: Efficacy study	Children with refractory or recurrent Neuroblastoma, or other Solid Tumors	Completed	[197]
		NCT00109863	Phase II: Efficacy study	Melanoma	Completed	
		NCT00082758	Phase II: Efficacy study	Neuroblastoma	Completed	[91]
		NCT01334515	Phase II: Combination with GM-CSF and isotretinoin	Neuroblastoma	Completed	[92]
		NCT00590824	Phase II: Antitumor activity in minimal residual disease setting	Melanoma	Active, not recruiting	[90]
		NCT03209869	Phase I: Combination with expanded haploidentical NK cells	Neuroblastoma	Recruiting	
NHS-IL2LT / Selectikine; EMD 521873; MSB0010445	DNA/Histone complex 	NCT00879866	Phase I: Dose-escalation combined with local irradiation	Lung Cancer, NSCLC	Completed	[96]
		NCT01032681	Phase I: Safety and tolerability	Advanced Solid tumors or Solid B-cell non-Hodgkin lymphoma	Completed	[95]
		NCT01973608	Phase II: Dose-escalation combined with stereotactic body radiation therapy	Melanoma	Terminated	[98]
DI-Leu16-IL2	CD20 	NCT00720135	Phase I: Dose-escalation (i.v.) following Rituximab blood B cell depletion	B-cell non-Hodgkin lymphoma	Completed	
		NCT01874288	Phase I/II: Dose-escalation (s.c) following Rituximab blood B cell depletion	B-cell non-Hodgkin lymphoma	Completed	[106, 198, 199]
		NCT02151903	Phase I/II: Extension study	B-cell non-Hodgkin lymphoma	Completed	
L19-IL2 / Darleukin	EDB 	NCT01198522	Phase I: Safety and dose-escalation in combination with Gemcitabine	Pancreatic cancer	Terminated	
		NCT01058538	Phase I/II: Dose-escalation and extension study on RCC	Advanced Solid Tumors	Completed	[116]
		NCT02957019	Phase I/II: Dose-finding combined with Rituximab and activity evaluation	Diffuse Large B-cell Lymphoma	Recruiting	

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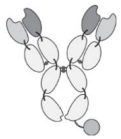
Antibody	Antigen/Format	Clinical Trials		Indication	Status	
		NCT01055522	Phase II: Dose finding (i.v.) in combination with Dacarbazine and efficacy study	Metastatic melanoma	Terminated	[115]
		NCT02076646	Phase I/II: Dose-finding (i.v.) in combination with Dacarbazine and efficacy study	Metastatic melanoma	Completed	
		NCT01253096	Phase II: Safety and efficacy study (i.t.)	Metastatic melanoma	Completed	[118, 200, 201]
		NCT02086721	Phase I: Dose-escalation (i.v.) combined with Stereotactic Ablative Body Radiotherapy	Oligometastatic Solid Tumors	Completed	[120]
		NCT03705403	Phase II: Therapeutic efficacy combined with Stereotactic Ablative Body Radiotherapy	Metastatic Non-small cell lung cancer	Not yet recruiting	
F16-IL2 / Teleukin	Tenascin C 	NCT01131364	Phase I/II: Dose-escalation combined with Doxorubicin and prospective therapy study	Solid tumors, Breast Cancer	Completed	[121]
		NCT01134250	Phase I/II: Dose-escalation combined with Paclitaxel and prospective therapy study	Solid Tumour, Breast Cancer, Metastatic melanoma, NSCLC	Completed	[202, 203]
		NCT02054884	Phase II: Therapeutic Efficacy combined with Paclitaxel	Merkel Cell Carcinoma	Terminated (lack of enrollment)	
		NCT02957032	Phase I: Dose-escalation combined with Cytarabine	Acute Myeloid Leukemia	Recruiting	
		NCT03207191	Phase I: Dose-escalation study in combination with an anti-CD33 antibody after allogeneic hematopoietic stem cell transplantation	Acute Myeloid Leukemia	Recruiting	[53, 122]
anti-CEA-IL2v / Cergutuzumab amunaleukin	CEA 	NCT02004106	Phase I: Dose-escalation study	CEA-positive solid tumors	Completed	[99, 105, 204]
		NCT02350673	Phase I: Dose-escalation study and extension combined with Atezolizumab	CEA-positive solid tumors	Recruiting	
anti-FAP-IL2v / RO6874281	FAP 	NCT03063762	Phase I: Safety, Tolerability, PK, PD and therapeutic activity study combined with Atezolizumab with/without Bevacizumab	RCC	Recruiting	
		NCT02627274	Phase I: Dose-escalation as single agent or combined with Trastuzumab or Cetuximab	Solid Tumor, Breast Cancer, Head and Neck Cancer	Recruiting	[205]
		NCT03386721	Phase II: Therapeutic efficacy in combination with Atezolizumab	Solid and/or metastatic tumors	Recruiting	

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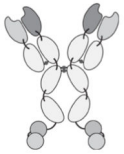
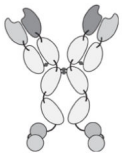
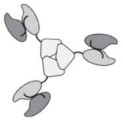
Antibody	Antigen/Format	Clinical Trials		Indication	Status	
huKS-IL2 / EMD273066	EpCAM 	NCT00132522	Phase I: Safety and Tolerability study in combination with Cyclophosphamide	Ovarian Cancer, Colorectal Cancer, NSCLC, Prostate Cancer	Completed	[206, 207]
Interleukin-12						
NHS-IL12 / M-9241	DNA/Histone complex 	NCT01417546	Phase I: Dose-escalation study	Solid tumors	Recruiting	[146]
		NCT02994953	Phase I: Dose-escalation and extension study combined with Avelumab	Solid tumors	Recruiting	[147]
BC1-IL12 / AS1409	D7 	NCT00625768	Phase I: Safety, tolerability and MTD determination	Metastatic RCC, Malignant Melanoma	Completed	[149, 208]
TNF superfamily members						
L19-TNF / Fibromun	EDB 		Phase I/II: safety and first clinical activity study	Metastatic solid cancer	Completed	[135]
			Phase I: Safety and Clinical Activity as isolated limb perfusion combined with melphalan	Metastatic melanoma	Completed	[136]
		NCT03420014	Phase II: Therapeutic efficacy combined with Doxorubicin	Soft Tissue Sarcoma	Recruiting	
		EudraCR number: 2016-003239-38	Phase III: Efficacy of the combination with Doxorubicin against Doxorubicin monotherapy	Advanced or metastatic soft tissue sarcoma	Recruiting	
		NCT03779230	Phase I/II: Dose-finding, safety and therapeutic activity study.	Glioma of the Brain	Recruiting	

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Antibody	Antigen/Format	Clinical Trials		Indication	Status	
FAP-4-1BBL / RG7827	FAP 		Phase I: Single agent dose-escalation and combination with Atezolizumab	Solid Tumors		[155, 156, 209]
Combinations						
L19-IL2 + L19-TNF / Daromun	EDB 	NCT02076633	Phase II: Safety and efficacy of intratumoral administration	Malignant Melanoma	Completed	[117, 210]
		NCT03567889	Phase III: Efficacy as neoadjuvant intratumoral treatment followed by surgery	Melanoma Stage IIIB/C	Recruiting	
		NCT02938299	Phase III: Pivotal study as neoadjuvant intratumoral treatment	Malignant Melanoma	Recruiting	