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Antibody-cytokine fusion proteins: Biopharmaceuticals with immunomodulatory properties for cancer therapy

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ABSTRACT

Cytokines have long been used for therapeutic applications in cancer patients. Substantial side effects and unfavorable pharmacokinetics limit their application and may prevent dose escalation to therapeutically active regimens. Antibody-cytokine fusion proteins (often referred to as immunocytokines) may help localize immunomodulatory cytokine payloads to the tumor, thereby activating anticancer immune responses. A variety of formats (e.g., intact IgGs or antibody fragments), molecular targets (e.g., extracellular matrix components and cell membrane antigens) and cytokine payloads have been considered for the development of this novel class of biopharmaceuticals. This review presents the basic concepts on the design and engineering of immunocytokines, reviews their potential limitations, points out emerging opportunities and summarizes key features of preclinical and clinical-stage products.

Contents

1. From cytokines to antibody-cytokine fusion proteins

Harnessing components of the immune system for therapeutic applications ("immunotherapy") is steadily gaining importance for the therapy of cancer, also thanks to the clinical success associated with the use of immune check-point inhibitors [1]. Various approaches to

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modulate the activity of the immune system against neoplastic cells have been considered over the years. Among them, the use of cytokines for therapeutic applications continues to draw attention and R&D investments.

Cytokines are proteins which modulate the activity of immune cells by binding to their cognate receptors and by triggering subsequent signaling events [2]. A number of cytokine products have gained marketing authorization and are routinely used in clinical practice. The most widely used cytokine products include G-CSF (Neupogen®) and GM-CSF (Leukine®) for the treatment of congenital and acquired neutropenia (e.g., induced by radiation), as well as interferon-alpha for the treatment of certain viral conditions (Pegasys®) and interferon-beta for the treatment of multiple sclerosis (Betaseron®, Avonex®, Plegridy®) [3]. However, various forms of interferon-alpha are also used for oncological applications, such as the treatment of hairy cell leukemia, chronic myelogenous leukemia, lymphoma, advanced or metastatic renal cell carcinoma, malignant melanoma and AIDS-related Kaposi's sarcoma (Intron A®, Roferon A®). Despite recent progress with immune check-point inhibitors, interleukin 2 (IL2, Proleukin®) is still used for the treatment of metastatic renal-cell carcinoma and melanoma, as a small portion of treated patients can be cured with this modality [4–6]. Moreover, tumor necrosis factor (TNF, Beromun®) has received marketing authorization for the treatment of unresectable soft tissue sarcoma in combination with melphalan [7,8].

The systemic administration of pro-inflammatory cytokines is often associated with severe dose-limiting toxicities (e.g., flu-like symptoms or vascular leak syndrome that causes hypotension and reduced organ perfusion), which may prevent dose escalation to therapeutically active regimens. These limitations underline the need and opportunity to generate novel cytokine-based products, which retain a potent immunostimulatory activity against tumor cells but display reduced side effects. Striking preclinical results were achieved by the intra- or peritumoral application of cytokines, intratumoral implantation of cytokine-producing cells or cytokine gene transfection of cancer cells before implantation without significant toxicities [9–13]. Although these settings are rarely applicable in the clinic and not suited for treating disseminated tumors, these experiments show that cytokines can mediate cancer cures, provided that sufficiently high concentration of product can be localized within the tumor microenvironment.

Antibodies that are specific to accessible tumor-associated antigens represent ideal “vehicles” for the selective delivery of therapeutic payloads to the tumor environment, helping spare healthy tissues. In principle, the antibody-based targeted delivery of cytokines should lead to a more potent therapeutic benefit with reduced side effects. Indeed, antibody-cytokine fusion proteins (“immunocytokines”) based on certain immunostimulatory payloads have shown impressive activity and selectivity in mouse models of cancer. The very first immunocytokine to enter clinical trials (Hu14.18-IL2) was based on remarkable preclinical findings with the murine analog of the immunocytokine ch.14.18-IL2. Not only was ch14.18-IL2 able to eradicate metastatic melanoma and neuroblastoma in immunocompetent mice, the immunocytokine also induced protective tumor-specific long-term immunity [14,15]. Moreover, in our own experience, the antibody-based delivery of murine interleukin 12 (IL12) to the tumor neovasculature exhibited potent activity in various immunocompetent mouse models of cancer, at a dose which was at least 20-fold lower compared to recombinant murine IL12 used as a “non-targeted” drug [16] (Fig. 1).

Success in immunocytokine development for pharmaceutical applications may depend on several factors. Besides the choice of the cytokine payload and of the antibody target, the engineering of structural features of the fusion protein greatly contributes to pharmacokinetic and pharmacodynamic properties. This review covers basic concepts associated with immunocytokine design and development, surveys preclinical findings for anti-cancer immunocytokines and discusses recent results published for clinical-stage products.

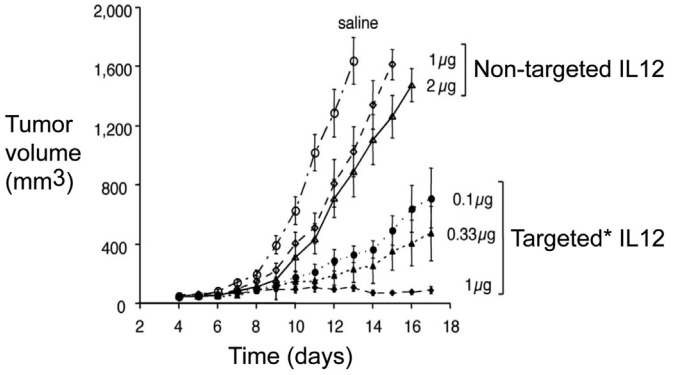


Fig. 1. Immunocompetent mice bearing F9 tumors were treated with non-targeted IL12 or targeted IL12. A clear dose response is visible as well as a markedly better therapeutic benefit of targeted IL12 over untargeted IL12. The indicated IL12-L19 doses are stated as IL-12 equivalents (1 μg IL-12 equivalent corresponds to 1.4 μg IL12-L19). *IL12 was fused to L19, an antibody against the alternatively-spliced domain B of fibronectin. Adapted from Ref. [16].

2. Immunocytokine formats and molecular targets

Various types of antibody formats can be used to design antibody-cytokine fusion proteins (Fig. 2). The antibody moiety can range from a nanobody (MW ~ 14 kDa) to a full-sized immunoglobulin (IgG, MW ~ 150 kDa). The architecture of the antibody portion can have a profound impact on the in vivo targeting performance with differences in blood clearance, extravasation, tissue penetration, diffusion and in vivo binding properties [17].

Immunocytokines based on full-size IgGs are bivalent in nature, leading to a high binding avidity. The extravasation and tumor penetration tend to be rather inefficient for intact immunoglobulins and their derivatives. However, higher tumor uptake compared to small antibody fragments can still be observed [18], resulting from a long circulatory half-life in blood [2]. The fragment crystallizable (Fc) region of an IgG can bind to cognate neonatal Fc receptors (FcRn) on endothelial cells and in the liver, rescuing the antibody product from degradation and thus leading to half-life prolongation [19,20]. In principle, the Fc portion could mediate the delivery of cytokine moieties to non-tumoral cells (e.g., leukocytes) and activate the immune system. Antibody-dependent-cell-mediated cytotoxicity (ADCC) is triggered when natural killer cells (NK cells) or macrophages are recruited to an IgG-coated target cell by interaction of the antibody Fc portion with cognate Fc-gamma receptors [21]. Moreover, the complement cascade may be activated, when C1q recognizes a high-local density of IgG molecules on a cell surface [2]. In order to minimize these potential problems, Roche has adopted a combination of glyco-engineering and amino acid mutagenesis at key positions in order to abrogate the interaction of IgG-based immunocytokines with immune system components [22–25].

Immunocytokines based on small antibody fragments display a rapid clearance from circulation, thus contributing to a more favorable tolerability profile [26]. The smallest antibody format that can be used to generate antibody-cytokine fusion proteins is the monomeric nanobody (i.e., a camel-derived single domain antibody). Even though tissue penetration of small antibody fragments may be increased compared to intact immunoglobulins, rapid clearance and insufficient affinity may lead to a lower tumor uptake compared to full IgG molecules [26]. Favorable tumor-to-organ ratios can be observed with certain immunocytokines that are based on noncovalent single-chain variable fragments and form homodimers (“diabodies”) [27,28]. These bivalent products retain the avidity and long tumor residence time of the parental full IgG counterpart. Good in vivo selectivity profiles (i.e., tumor: organ ratios) have been reported for numerous diabody-based products [27,29].

The spatial arrangement of the antibody and cytokine moieties can have a profound influence on the in vivo performance of the

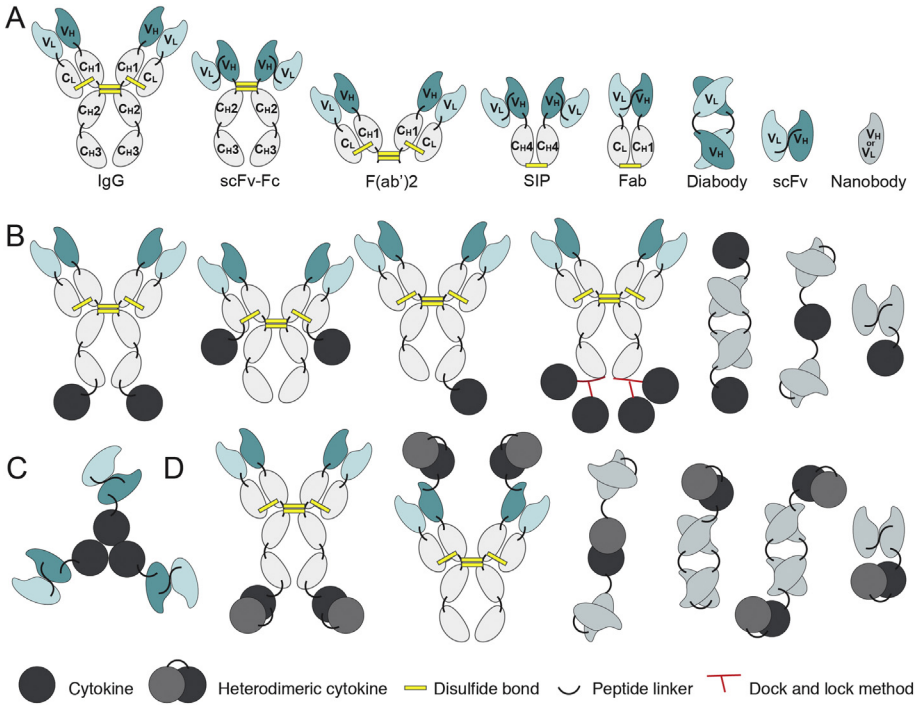


Fig. 2. (A) Schematic representation of antibody formats. VH: heavy chain variable domain. VL: light chain variable domain. C: constant domain of the heavy (C_{H1-4}) and the light chain (C_L). SIP: small immune protein that uses the εCH_4 domain of an IgE antibody. Minibodies (or SIPs) can also be constructed with the CH_3 domain of an IgG. Fab: Fragment antigen binding. scFv: single chain variable fragment. (B) – (D): Common immunocytokine formats used for the generation of monomeric (B), trimeric (C) and heterodimeric (D) cytokines. Cytokine payloads can be fused to practically any site of the antibody moiety (variable or constant domain of heavy or light chain). In certain scFv or diabody fusion structures, the colors for V_H and V_L are not indicates (and depicted in grey), since different V_H-V_L or V_L-V_H arrangements can be considered. The dock and lock method has been described by Rossi and colleagues that features the separate expression of both the antibody domain containing an AD2 peptide and the cytokine fused to a docking and dimerization domain (DDD2) [163,264,265]. When the two proteins are incubated under specific conditions, the DD2 domain binds to AD2 and disulfide bridges are formed which stabilize the construct [163,264,265].

corresponding product. In principle, the cytokine payload can be fused to any site of the antibody. For IgGs, the carboxy-terminus of the heavy chain is the most commonly used position, that typically results in full conservation of cytokine activity [30]. However, other configurations can be considered. For example, the fusion of an IL2 moiety at the C-terminus of the light chain (rather than the heavy chain) was shown to decrease the affinity to the "intermediate-affinity IL2 receptor" (IL2Rβγ) while retaining full affinity for the "high-affinity IL2 receptor" (IL2Rαβγ) [31]. This feature may be advantageous in vivo for retaining full IL2 activity but less toxicity. Heterodimeric payloads, such as IL12, allow for even more flexibility in product design (Fig. 2D).

Immunocytokines should ideally target specific antigens that are abundantly expressed in neoplastic tissues but absent from normal tissue. The tumor microenvironment is a unique niche that emerges during the formation and progression of a tumor. Tissue remodeling and neoangiogenesis can be observed in aggressive malignancies to guarantee an adequate supply of nutrients [32]. Antigens located on new blood vessels or in surrounding extracellular matrix (ECM) structures are particularly attractive for pharmacodelivery applications. The alternatively-spliced extra domains A (EDA) and B (EDB) of fibronectin and the A1 domain of tenascin-C (TnC A1) [32,33] are strongly expressed in the majority of aggressive solid tumors and lymphomas but are absent from normal tissue, except for the female reproductive system during the proliferative phase (i.e., placenta, endometrium and ovarian vessels) [34,35]. Monoclonal antibodies directed against ECM components, such as F8, L19 and F16 [36–42], have shown promising biodistribution profiles in animal models and in patients.

A number of cellular targets have been considered for antibody-based pharmacodelivery activities, including integrins ($\alpha_v\beta_3$), annexin A_1, prostate-specific membrane antigen (PSMA), vascular endothelial growth factors (VEGF) and their receptors, endoglin (CD105), CD44 isoforms and alanyl aminopeptidase (CD13) [33]. The A33 and the carcinoembryonic (CEA) antigen are particularly attractive for the targeting of colorectal cancer [43–47], while carbonic anhydrase IX is one of the best markers of clear-cell renal cell carcinoma [48]. EpCAM, disialoganglioside 2 (GD2) and the fibroblast activation protein (FAP) have been proposed as targets, which are expressed by multiple tumor types [49–51]. Immunocytokine programs against these targets are currently at the preclinical or clinical investigation stage (Table 1).

3. Cytokine payloads for cancer therapy

A large number of cytokines have been tested as immunocytokine payloads during the last few years. A summary of all the immunocytokines that have been investigated in animal tumor models can be found in Table 1. Interleukin-2 (IL2) represents the most commonly studied cytokine payload for anti-cancer applications.

Cytokines are mediators of physiological processes that exert their function in an autocrine or paracrine fashion [2]. Thus, immunocytokines which are specific for tumor associated antigens on the surface of neoplastic cells can bridge tumor cells to certain leukocytes bearing the corresponding cytokine receptor and trigger specific responses. Indeed, an IL2 fusion protein was shown to mediate an immune synapse between a tumor cell and an NK cell that promoted NK cell mediated killing of the tumor cell [52]. Immunocytokines specific for tumor-associated extracellular matrix components were shown to increase the density of leukocytes (especially NK cells and T cells) in the tumor niche [53–56]. Moreover, some cytokines such as IL2 and TNF are able to activate the endothelium at the tumor site thus favoring an increased uptake of therapeutics into the neoplastic tissue [57–59].

Our laboratory has worked with many cytokine payloads and the most promising anticancer results have so far been achieved with products based on IL2, IL12 or TNF. These payloads exhibit different mechanisms of action. Interleukin-2 acts as a mitogen and activator of both NK cell and T cell function [60]. The antibody-based delivery of IL2 to the tumor leads to an increased cellular infiltrate within the neoplastic

Table 1
List of antibody-cytokine fusion proteins and their characterization. [123,125,127,132,133,138,141,149,151,152,155–159,162,164,169,173]

Compound	Format	Antigen	*In vivo* targeting	Tumor Model	Efficacy	Lit.
G-CSF						
GCSF-F8		EDA	+	F9 s.c.	F9 s.c.: I ≈ P	[101]
GM-CSF						
Anti-HER2/*neu* IgG3-GMCSF		HER2/*neu*	+	CT26 s.c., CT26 HER+ s.c.	CT26 HER+: I > NC	[119–122]
CLL1-GMCSF		MHCII	+	ARH-77	n.a.	[76]
L19-GMCSF		EDB	+ (d.d.)	F9 s.c., i.v. C51 s.c., i.v.	F9 s.c.: I > NC, C51 s.c./i.v., F9 i.v.: I > P	[107]
Interleukin 1β						
F8-IL1β, IL1β-F8		EDA	+	F9 s.c.	F9 s.c.: I > NC	[102]
Interleukin 2						
2aG4-IL2		PS	n.a.	4T1 i.v.	Vaccination: I > NC	[123]
Anti-CEA-IL2		CEA	+	MC-38 s.c. MC38-CEA s.c.	MC38-CEA: I > NC	[73]
Anti HER2/*neu* IgG3-IL2		HER2/*neu*	n.a.	CT26-HER2	CT26-HER2: I > NC	[120–122,124–126]
CEA-IL2v		CEA	+	MC38-CEA i.s., PancO2-CEA i.Pc., A549 i.v., Ls174t i.s., N87 s.c., KPL-4 m.f.p.	MC38-CEA i.s., PancO2-CEA i.PC.: I > P A549 i.v., Ls174t i.s., N87 s.c., KPL-4 m.f.p.: combo therapies: I > P	[74,127]
ch14.18-IL2		GD2	+	M21 i.v., i.s., s.c., B16-GD2 i.v., i.s., SK-N-AS i.s., NX2S i.v., s.c., s.m.	M21, i.v., B16-GD2, SK-N-AS, NX2S i.v., s.m., s.c.: I > NC	[14,61,75,128–130]
ch225-IL2		EGF	n.a.	M24met i.s.	M24met i.s.: I > NC	[128,131]
CLL1-IL2		MHCII	+	ARH-77	n.a.	[76]
Di-Leu16-IL2		CD20	n.a.	Daudi i.v.	Daudi i.v.: I > NC	[132]
F8-IL2		EDA	+	Caki-1 s.c., C1498 s.c., NB4 s.c., WM1552/5 s.c., A375M i.v., K1735M2 s.c., F9 s.c., WEHI-163 s.c.	Caki s.c.: I > NC, C1498 s.c.: I i.v. > NC, I i.t. ≈ P, NB4 s.c.: I > NC, WM1552/5 s.c.: I ≈ NC, A375M i.v.: I ≈ NC, K1735M s.c.: I > NC, F9 s.c.: I i.v., i.t. > P, WEHI-163 s.c.: I i.t. ≈ P, LLC s.c.: i.t. I > P,	[55,63,71,77,84,133–135]
F16-IL2		Tnc A1	+	MDA-MB-231 s.c, U87MG s.c., i.c.	MDA-MB-231 s.c.: I > NC U87MG s.c., i.v.: I > P	[78,79]
FAP-IL2v		FAP	n.a.	MC-38-FAP s.c.	MC-38-FAP: I > NC	[127,136,137]

FUMK1-IL2		EpCAM	+	MKN-74 s.c.	MKN-74 s.c.: I > NC	[80]
IL2-FuP		EGFR	+	BLM s.c.	BLM s.c.: I > FuP naked	[81]
IL2-MOV19		αFR	+	CT26-αFR i.v., s.c.	CT26-αFR s.c.: I > NC	[82]
KS-IL2		EpCAM	+	CT26-KSA i.s., i.v., s.c., PC-3.MM2 i.v., 4T1-KSA s.c., i.v., LLC-KSA s.c.	CT26.KSA: i.v., i.s., s.c., PC-3.MM2 i.v., 4T1-KSA s.c., LLC-KSA: I > NC	[83,138, 139]
L19-IL2		EDB	+	F9 s.c., C51 s.c., N52 s.c., Ramos s.c., i.v., A20 s.c., DoHH-2 s.c., CT26 s.c., N2A s.c., NIE-115 s.c., K1735M2 s.c., J558L s.c., DanG i.Pc., MiaPaca i.Pc., MiaPaca-A2 i.Pc.,	F9 s.c., C51 s.c., N52 s.c., Ramos, s.c., i.v., Do-HH-2 s.c., CT26 s.c., K1735M2 s.c., J558L s.c, DanG i.Pc., MiaPaca i.Pc.: I > NC, N2A s.c.: I > P, NIE-115 s.c.: I in combo > P	[34,56,84,140–143]
NHS-IL2LT		Histone	+	NX2S i.v., LCC i.v.	NX2S i.v., LLC i.v.: I > NC	[85]
Ta99-IL2		A33	+	B16F10 s.c.	B16F10 s.c.: I > NC	[86]
sm3E-IL2		CEA	+	B16F10 s.c.	B16F10 s.c.: I > NC	[86]
Interleukin 3						
F8-IL3		EDA	+	F9 s.c.	F9 s.c.: I ≈ P	[101]
Interleukin 4						
F8-IL4		EDA	+	F9 s.c., CT26 s.c., A20 s.c.	F9 s.c., CT26 s.c., A20 s.c.: I > NC	[55]
F8-IL4-F8		EDA	+	F9 s.c.	F9 s.c.: I > P	[101]
Interleukin 6						
F8-IL6, IL6-F8		EDA	+	F9 s.c.	F9 s.c.: I > NC	[102]
Interleukin 7						
F8-IL7		EDA	+	F9 s.c.	F9 s.c.: I > NC	[106]
F8-IL7-F8		EDA	+	F9 s.c.	F9 s.c: I > NC	[106]
Interleukin 9						
F8-IL9		EDA	+	n.a.	n.a.	[114]
Interleukin 12						
BC1-IL12		EDB	+	PC3mm2 i.v., s.c., A431 s.c., HT29 s.c.	pC3 i.v., PC3 s.c., HT9 s.c..: I > NC A431 s.c: I ≈ NC	[72,144]
chTNT3-IL12; NHS-IL12		DNA	+	LS147T s.c., DU145 s.c., LLC s.c., MC38 s.c., B16 s.c., MC38/MUC1+ s.c., Panc02/MUC1+ s.c., Renca s.c., Panc02 s.c., MB49 s.c.	DU145 s.c., LLC s.c., MC38 s.c.., B16 s.c.; I > NC, MC38/MUC1+ s.c., Panc02/MUC1+ s.c. , MB49 s.c; I in combo > P, Renca s.c., Panc02 s.c..: I > P	[87,145, 146]

KS-IL12		EpCAM	n.a.	DU145 i.v., CT26-EP21 i.v.	DU145 i.v., CT26-EP21 i.v.: I > NC	[147]
mscIL12-her2.IgG3		Her2/*neu*	n.a.	CT26-HER2 s.c., i.v., CT26 i.v.	CT26-HER2: s.c., i.v, CT26 i.v.: I ≈ NC	[148,149]
IL12-L19(scFv)		EDB	+	F9 s.c.: C51 s.c., i.v.	F9 s.c., C51 s.c.., i.v.: I > NC,	[54,57,88]
IL12-L19(SIP)		EDB	+	F9 s.c.	n.a.	[88]
L19-p35/p40-L19		EDB	+	F9 s.c.	F9 s.c.: I > NC	[88]
F8-p35/p40-F8		EDA	+	F9 s.c.	n.a.	[89]
IL12-F8-F8		EDA	+	F9 s.c., CT26 s.c., A20 s.c.	F9 s.c.: I > NC, CT26 s.c., A20 s.c.: I > P	[71]
IL12-SSI		MSLN	n.a.	NCI-H226 i.p.	NCI-H226 i.p.: I > P	[150]
HRS3scFv-IL12 and HRS3scFv-Fc-IL12		CD30	+	n.a.	n.a.	[90]
Interleukin 13						
F8-IL13		EDA	+	Wehi-164 s.c.	Wehi-164: I > NC	[151]
Interleukin 15						
L19-IL15, IL15-L19		EDB	+	F9 s.c., i.v. C51 s.c., i.v.	F9 s.c.: I > NC, C51 s.c./i.v., F9 i.v.: I > P	[107]
Anti-GD2-RLI (IL15)		GD2	n.a.	EL4 s.c., NXS2 i.v.,	EL4 s.c., NXS2 i.v.: I > NC	[152]
scFv-RD-IL15		FAP	+	B16-FAP i.v.	B16-FAP i.v.: I > NC	[153,154]
Interleukin 17						
F8-IL17/IL17-F8		EDA	+	F9 s.c.	F9 s.c.: I ≈ P	[155]
Interleukin 18						
F8-IL18		EDA	+ (d.d.)	F9 s.c.	F9 s.c.: I ≈ P	[156]
Interleukin 21						
Anti-CD20-IL21		CD20	n.a.	A20-huCD20 s.c.	A20-huCD20 s.c.: I > NC	[157,158]
Interferon α						
F8-IFNα		EDA	+	F9 s.c., Cloudman S91 s.c.	F9 s.c., Cloudman S91 s.c.: I ≈ NC	[159]
CD20IgG3-IFNα		CD20	n.a.	38C13-CD20 s.c., Daudi s.c.	38C13-CD20 s.c., Daudi s.c.: I > NC	[160]
AntiHER2/*neu*-IFNα		Her2/*neu*	n.a.	38C13-HER2+ s.c.	38C13-HER2+ s.c.: I > NC	[160,161]

C2-2b-2b		HLA-DR	n.a.	Daudi i.v., CAG i.v.	Daudi i.v., CAG i.v.: I > NC	[162]
20-2b		CD20	n.a.	Daudi i.v., Raji i.v., NAMALWA i.v.	Daudi i.v., Raji i.v., NAMALWA i.v.: I > NC	[163]
Interferon γ						
L19-IFNγ		EDB	+	F9 s.c., i.v., C51 s.c., i.v., CT26 s.c.	F9 s.c., i.v.: I > NC, C51 s.c., i.v., CT26 s.c.: I ≈ P	[104]
TNT3-IFNγ		DNA	+	LS174T s.c., MAD109 s.c., RENCA i.v.	RENCA: I > NC	[98,164]
TNF						
F8-TNF		EDA	+	WEHI-164 s.c., Sarcoma 180 s.c.	WEHI-164 s.c., Sarcoma 180 s.c.: I > P	[64,116]
FAP-TNF		FAP	n.a.	HT1080-FAP+ s.c.	HT1080-FAP+ s.c.: I > NC	[165]
G250-TNF		CAIX	+	NU-12 s.c., SK-RC17/52 s.c.	SK-RC17/52 s.c.: I > NC	[92]
scFvMEL-TNF		gp240	+	A375 s.c.	A375 s.c.: I > NC	[94,95]
L19-TNF		EDB	+	F9 s.c., WEHI-164 s.c., C51 s.c., N2A s.c., NIE-115 s.c., K1735M2 s.c, J558L s.c.	F9 s.c., WEHI-164 s.c., C51 s.c. K1735M2 s.c., J558L s.c.: I > NC, N2A s.c. I > P, NIE-115 s.c.: I in combo > P	[57,84,96,140–142,166]
MFE23-TNF		CEA	+	LS174T s.c.	n.a.	[97]
TNF-TNT3		DNA	+	LS174T s.c.	n.a.	[98]
TNF-FuP		EGFR	+	BLM s.c.	BLM s.c.: I > NC	[81]
TNF-B1		LeY	n.a.	MCF-7 s.c.	MCF-7 s.c.: I > NC	[167]
ZME/TNF		gp240	+	A375 s.c.	A375 s.c.: I > NC	[99]
heterodimeric cytokines						
KS-IL12/IL2		EpCAM	n.a.	LLC-EpCAM+ i.v., i.t.	LLC-EpCAM+ i.v., i.t.: I > NC	[168]
HRS3scFv-IL12-Fc-IL2		CD30	n.a.	9G10-HRS3A+ s.c.	9G10-HRS3A+ s.c.: I > P	[90]

IL2-F8-TNFmut		EDA	+	WEHI-164 s.c., CT26 s.c., C1498 s.c., F9 s.c.	WEHI-164 s.c.: I > NC CT26 s.c., C1498 s.c., F9 s.c.: I > P	[100]
IL4L-Ab-GMCSF and IL4H-Ab-GMCSF		EpCAM	n.a.	n.a.	n.a.	[168]
IL12-IgG3-IL2		Her2/*neu*	n.a.	TUBO s.c.	Vaccine adjuvant: I > P	[122]
IL12-IgG3-GMCSF		Her2/*neu*	n.a.	TUBO s.c.	Vaccine adjuvant: I > P	[122]
Chemokines						
CCL5-F8		EDA	n.a.	n.a.	n.a.	[169]
CCL17-F8		EDA	n.a.	n.a.	n.a.	[169]
CCL19-F8		EDA	+	n.a.	n.a.	[169]
CCL20-F8		EDA	+	n.a.	n.a.	[169]
CCL21-F8		EDA	+	n.a.	n.a.	[169]
CXCL4-F8		EDA	n.a.	n.a.	n.a.	[169]
CXCL9-F8		EDA	n.a.	n.a.	n.a.	[169]
CXCL10-F8		EDA	+	F9 s.c.	F9 s.c.: I > P	[169]
CXCL111-F8		EDA	n.a.	n.a.	n.a.	[169]
F8-ITIP		EDA	n.a.	n.a.	n.a.	[169]
TNF superfamily members						
F8-TRAIL		EDA	+	n.a.	n.a.	[117]
F8-TRAILtrunc		EDA	+	n.a.	n.a.	[117]
F8-CD40L		EDA	+	n.a.	n.a.	[117]
scFv-OX40L		END	n.a.	n.a.	n.a.	[170]
F8-FasL		EDA	+	n.a.	n.a.	[117]
LiGHT-F8		EDA	+	n.a.	n.a.	[117]
scFv-LiGHT		END	n.a.	n.a.	n.a.	[170]
F8-VEGI		EDA	+	n.a.	n.a.	[117]

F8-VEGItrunc		EDA	+	n.a.	n.a.	[117]
F8-LTA		EDA	+	n.a.	n.a.	[117]
scFv36–4-1BBL		FAP	n.a.	n.a.	n.a.	[171]
scFv-4-1BBL		END	+	B16-FAP i.v.	B16-FAP i.v.: in combo I > P	[170]
F8-LTβ		EDA	+	n.a.	n.a.	[117]
F8-LTαI/β2		EDA	+	n.a.	n.a.	[117]
scFv-TNC-GITRL		END	n.a.	n.a.	n.a.	[170]
other payloads						
B7.1Db		FAP	n.a.	B16 i.v	B16 i.v: in combo I > P	[170]
B7.2Db		FAP	+	n.a.	n.a.	[170,172]
F8-B7.2		EDA	+	n.a.	n.a.	[111]
scFv(19)-tTF		EDB	+	C51 s.c., F9 s.c., FE8 s.c.	C51 s.c., F9 s.c., FE8 s.c.: I > NC	[173]
L19- VEGF-A^{164}		EDB	+	n.a.	n.a.	[110]
L19-VEGF-A^{120}		EDB	+	n.a.	n.a.	[110]

Summary of immunocytokines that have been tested in animal models. In vivo targeting was assessed by biodistribution studies. Efficacy was defined as tumor growth retardation ("I > " the immunocytokine performed better, "I ≈" the immunocytokine performed equally) compared with a saline group (P) or negative control group (NC, untargeted cytokine or cytokine fused to an antibody of irrelevant specificity). The format of the immunocytokine is depicted according to the color code of Fig. 2 with the exception of the C_H1-C_H3 and C_HL domains which are depicted in blue. In the structure of dual cytokine fusion proteins, the additional cytokine payload is illustrated with a grey circle. The red domain in the structure of scFv-RD-IL15 illustrates the ILRα domain. Blue shading: immunocytokines in clinical trials.

Abbreviations: +: biodistribution results published, n.a.: biodistribution data not available. i.v.: intravenous, s.c.: subcutaneous, i.s.: intrasplenical, i.Pc.: intrapancreatic, i.c.: intracranial, i.t.: intratumoural, m.f.p: mammary fat pad, s.m.: spontaneous metastasis, I.: Immunocytokine, DD: dose dependence, combo: in combination with a second anti-cancer therapeutic. G-CSF: Granulocyte colony-stimulating factor, GM-CSF: Granulocyte-macrophage colony-stimulating factor; EDA: alternatively-spliced extradomain A of fibronectin, EDB: alternatively-spliced extradomain B of fibronectin, HER2/*neu*: human epidermal growth factor receptor 2, MHCII: major histocompatibility complex class II, PS: phosphatidylserine, CEA: carcinoembryonic antigen, GD2: disialoganglioside, Tnc A1: alternatively spliced A1 domain of tenascin-C, FAP: fibroblast-activating protein, EpCAM: epithelial cell adhesion molecule, EGF: epidermal growth factor, EGFR: epidermal growth factor receptor, αFR: alpha folate receptor, MSLN: mesothelin, HLA-DR: human leukocyte antigen DR, CAIX: carbonic anhydrase IX, LeY: Lewis Y antigen.

mass, thus leading to a gain in therapeutic index [61–63]. The mechanism of action for TNF-based pharmaceuticals is unique, as they can trigger a rapid hemorrhagic necrosis of the tumor mass. At a later stage, tumor reactive CD8+ T cells and NK may facilitate the eradication of minimal residual disease [64]. TNF was originally identified as an endotoxin-induced serum factor that caused the necrosis of certain murine tumors in vivo [65]. Tumor-homing antibody products, such as L19-TNF, can turn a cancer mass into a black scab [64,66]. IL12 is a cytokine which potently activates certain leukocytes (e.g., CD8+ T cells and NK cells), but which also displays anti-angiogenic activity, by upregulation of CXCL10 [67]. IL12 also regulates the balance between type 1 (Th1) and type 2 (Th2) subsets of T helper cells and promotes the differentiation of naïve T cells into IFNγ- producing Th1 cells [68]. High local concentration of IFNγ may promote a tumor infiltration of CD4+ T cells and a decrease in T regulatory (Treg) cells [69–72].

4. Factors influencing the tumor targeting properties of immunocytokine products

In order to learn about factors which influence the tumor targeting properties of antibody fusions, it is convenient to compare a sufficiently large number of different products based on the same antibody moiety. Our laboratory has fused many different cytokine payloads to antibodies of proven in vivo tumor targeting performance (e.g., F8 and L19) and

has characterized the corresponding biodistribution properties in tumor-bearing mice, using radioiodinated protein preparations (Table 1). These studies have shed light on certain parameters which can influence the tumor homing properties of antibody-cytokine fusions. These investigations were facilitated by the fact that antibodies directed against splice variants of fibronectin exhibited similar tumor targeting performance (measured as percent of injected dose per gram of tumor, or as tumor:organ ratios) over a broad range of administered doses (e.g., between 0.1 μg and 150 μg of antibody fusions; Ref. [39], references of Table 1 [16,56,57,63,73–100] and unpublished observations).

The majority of cytokine payloads, when fused to the L19 or to the F8 antibody, can efficiently be delivered to solid tumors in mice, after intravenous administration in the 5–20 μg dose range. In that case, the tumor uptake and tissue selectivity of immunocytokines are typically similar or even better than the ones of the parental antibodies. Cytokine fusions with favorable tumor targeting profiles include products based on IL2, IL3, IL4, IL6, IL10, IL12, IL22, TNF, IFNα and G-CSF [16,55,71,72,77,91,101–103].

Certain payloads, however, exhibited a dose-dependent tumor-targeting performance and other cytokines completely abrogated the tumor homing properties of the parental antibodies. These observations have allowed us to identify molecular parameters which are crucially important for a successful delivery to the tumor. Trapping of the cytokine payload by abundant receptors expressed in normal tissues may compromise the targeting performance of certain fusion proteins. For instance, antibody fusions with IFNγ were shown to selectively localize to neoplastic lesions only if IFNγ receptor knockout mice were used for the experiment, or if a large amount of unlabeled antibody-cytokine fusion protein had been pre-administered to wild-type tumor-bearing mice [104,105]. Similar dose-dependent biodistribution profiles were observed for IL7, IL15 and GM-CSF, suggesting the presence of "titratable" receptors, which could hinder pharmacodelivery applications at low doses [106,107].

The isoelectric point of antibody fusions may also have a negative impact on biodistribution results. For example, the fusion of calmodulin (with high negative charge) or the coupling of Tat peptides (with high positive charge) completely abrogated the tumor homing properties of the parental L19 antibody, even though the products were fully immunoreactive in vitro and well-behaved in biochemical assays (e.g., in SDS-PAGE and gel-filtration analysis) [108,109]. Interestingly, L19 fusions to murine VEGF-120 efficiently targeted tumors, while the larger and positively-charged VEGF-164 payload prevented targeting [110].

Glycosylation and excessive molecular mass may also prevent efficient tumor targeting in vivo. Certain heavily glycosylated payloads (e.g., murine B7.2) were rapidly cleared via the hepatobiliary route and did not efficiently localized to tumors [111]. Interestingly, when similar payloads were fused to an antibody in IgG format, selective tumor uptake and tumor growth retardation was observed [112,113]. L19-IL12 and L19-TNF efficiently localized to solid tumors in mice. However, when murine IL12 and TNF were simultaneously fused to the L19 antibody, the resulting fusion protein (with a molecular mass of approximately 120 kDa) had poor biodistribution properties, while being completely immunoreactive in in vitro assays [57]. Additionally, protein production methods can influence antibody glycosylation and, as a consequence, biodistribution results. For example, production of the F8-IL9 fusion protein with different set-ups (transient gene expression or stable gene expression) reproducibly led to protein preparations of similar biochemical properties (SDS-PAGE, gel filtration, BIAcore analysis) but completely different tumor homing performances. Subtle differences in glycosylation patterns had a major impact on the biodistribution profile [114] and preparations with higher proportions of terminal sialic acid residues tended to perform better.

Biodistribution studies, performed with fusions of the F8 antibody to various members of the TNF superfamily, have surprisingly revealed big differences in pharmacokinetic and tumor uptake properties, even if the products had similar molecular formats and biochemical properties. TNF and proteins of the TNF superfamily are non-covalent homotrimers, which may, however, differ in terms of thermodynamic stability [115]. TNF is a potently vasoactive payload, which exhibited excellent biodistribution profiles, when fused either to L19 or to F8 [57,96,116]. However, antibody fusions with CD40L, FasL, TRAIL, truncated versions of TRAIL, VEGI, truncated versions of VEGL, LIGHT and various lymphotoxin combinations exhibited biodistribution results which were, to a varying extent, worse compared to the ones of L19-TNF and F8-TNF [117]. Recent research results of Roland Kontermann and collaborators have shown that members of the TNF superfamily may benefit from being expressed as a single polypeptide, connecting the three monomeric units, instead of being expressed as monomeric units that form non-covalent homotrimers [115,118].

5. Immunocytokines with promising preclinical results

A large number of immunocytokines has been investigated in preclinical mouse models of cancer. Table 1 presents a summary of fusion proteins which have been proposed for therapeutic applications. For many of them, quantitative biodistribution studies and therapy results in tumor-bearing mice have been reported. The table also displays the molecular arrangement of the antibodies (or antibody fragments) and cytokine payloads.

The most promising therapy results have so far been achieved with immunocytokine products based on IL2, IL12 or TNF as payloads [14,16,27,56,57,61,63,64,72–75,77–83,85–90,92, 94,95,97,99,100, 116, 122,124,129–131,136,137,139,147,148,150,165,167,168,174–180]. Not surprisingly, these cytokines are not only important anti-cancer weapons from an immunological viewpoint, but also exhibit favorable tumor homing properties in biodistribution studies [16,56,57,63, 73–100], when fused to suitable antibodies. Some fusion proteins based on IL2, IL12 or TNF have progressed to clinical trials, as described in chapter 8.

Fig. 1 illustrates the benefit which can be achieved by the targeted delivery of a cytokine payload to the tumor site. In this case, murine IL12 displayed a much more potent anti-cancer activity in immunocompetent mice bearing F9 murine tumors when fused to the L19 antibody, compared to the non-targeted recombinant murine IL12 counterpart [16]. Therapeutic activity was clearly dose-dependent.

The groups of Reisfeld and Gillies have previously reported that IgG fusions, featuring IL2 as a payload, exhibited strong anti-cancer activity in various mouse models of cancer, including metastatic melanoma, neuroblastoma, prostate carcinoma, colon adenocarcinoma, non-small cell lung carcinoma and lymphoma [14,15,22,52,61,75,85,128–130, 139,175,176,181,182]. The fusion proteins were typically not able to cure tumor-bearing mice, but durable complete responses were reported for melanoma, neuroblastoma and lymphoma bearing mice [14,75,128–130,176].

The groups of Morrison and Penichet showed that anti-HER-2/*neu* antibody fusion proteins based on IL2 [120–122,124,126,183], IL12 [148,184] and GM-CSF [119–122], IFNα[160,161] can elicit potent anti-tumor responses against anti-HER-2/*neu* or CEA expressing murine tumors or lymphomas. Immunocytokines based on IL2 or GM-CSF were not only potent in inhibiting tumor growth, but also in preventing tumor growth in mice when used in combination with vaccines [120,122].

Dafne Müller and colleagues have extensively studied antibody-cytokine fusion proteins that are composed of antibodies in the diabody or the scFv format linked to members of the TNF superfamily [170–172]. Recently, the group of Müller has described an antibody fusion protein based on an antibody moiety targeting the fibroblast activation protein, interleukin 15 (IL15) and a fragment of the IL-15Rα chain. This IL15 fusion protein should mimic physiologic *trans*-presentation of the cytokine payload [153]. IL15 normally acts in a membrane-bound form bound to the IL-15Rα expressed on monocytes and dendritic cells

[185]. IL15/IL15Rα can then bind to the heterodimeric intermediate affinity receptor IL2/IL15Rβγ on NK or CD8+ T cells and trigger subsequent signaling events [185]. The domain of IL15Rα involved in the binding of IL15 has been identified [186]. The Müller group reported the successful generation of a fusion protein that consists of the IL15 and an extended IL15Rα domain which displayed a superior antitumor activity compared to the untargeted or the receptor domain missing forms [153]. The group also described a trifunctional fusion protein consisting of a tumor homing antibody, IL15 linked to the IL15Rα fragment and the extracellular domain of 4-1BBL [154]. This trifunctional fusion protein was even more potent in a mouse model of melanoma than the corresponding bifunctional molecules [154].

The fusion proteins L19-IL2 and F8-IL2 have exhibited favorable biodistribution results in tumor-bearing mice and a potent tumor growth inhibitory activity in various models of cancer [34,55,56,63,77,84,140]. Single-agent cancer cures were rare, but certain models (e.g., BALB/c mice bearing CT26 colon carcinoma or C57BL/6 mice bearing TIB49 acute myeloid leukemia) typically responded better than other models (e.g., C57BL/6 mice bearing Lewis Lung Carcinoma), for reasons that are still not fully understood [63,140].

Interleukin-12 is a promising payload, as it can activate NK cells and CD8+ T cells, while also favoring a Th1 polarization of CD4+ T cell response [67,68]. Both tumor homing properties and therapeutic performance crucially depend on the molecular arrangement chosen for the antibody moiety and the heterodimeric IL12 payload [88]. A format based on a single polypeptide, connecting the two IL12 subunits with the F8 antibody in single-chain diabody format, is easier to express compared to other molecular arrangements [71,88,89] and performs well in vivo. This format may thus represent the best candidate for future clinical development programs.

TNF is a homotrimeric cytokine, which can conveniently be fused to antibodies in scFv format, leading to a stable non-covalent homotrimeric product [58,91,96,97,187]. In our experience, murine TNF was the only cytokine payload (among many that we have tested) capable of promoting a rapid hemorrhagic necrosis of the tumor mass in preclinical models [64]. Neoplastic lesions can be converted into necrotic scabs within few hours and this process correlates with therapeutic performance [64]. In most cases, TNF-based immunocytokines cannot cure tumor-bearing mice when used as single agents, since a rim of residual tumor cells survives and may eventually regrow. However, TNF-based products may be ideally suited for debulking strategies or as combination partners with other modalities, as discussed in a later chapter.

6. Opportunities for combination therapy

Immunocytokines based on pro-inflammatory cytokines may be ideal combination partners for various anti-cancer therapeutic agents. Besides boosting the immune system, pro-inflammatory cytokines can activate the endothelium and increase vascular permeability [57–59], thus favoring the accumulation of other drugs at the tumor site. Various combination partners have been tested in preclinical experiments, including (cytotoxic) drugs [63,77–79,96,116,134,145], small molecule drug conjugates (SMDC) [188], intact antibodies [34,74,86,145], bispecific antibodies [170,172], radiation [145,189–191], radiofrequency ablation [181], immune check-point inhibitors [57,100,137,146,192], antibody-drug conjugates (ADC) [177,193], vaccination strategies [120,122] and even other immunocytokine products [55,57,71,74,90,100,122,135,140,154,168].

Tumor surgery and conventional chemotherapy are often the first-line treatment given to cancer patients. However, chemotherapy often is accompanied by serious side effects (e.g., nausea, myelotoxicity) that prevents dose escalation to therapeutically active regimens. Immunocytokines appear to synergize well with some, but not all, cytotoxic drugs. In addition to increasing drug uptake within the tumor mass [59,96], certain immunocytokines can exploit the immune reaction against tumor cells, initially promoted by drug-related immunogenic cell death [134]. In addition to the beneficial effects on tumor immunogenicity, certain drugs could in principle also antagonize the effects of immunotherapy (especially when used at high doses), as cytotoxic agents may be myelotoxic and kill the very same leukocytes needed to fight cancer. In this context, dose and schedule appear to be crucially important. For example, melanoma cures were observed when an IL2-based product was administered *after* paclitaxel chemotherapy in a mouse model of the disease, while the reverse schedule did not exhibit any additive benefit [134].

The administration of pro-inflammatory cytokines activates the endothelium (potentially leading to hypotension, which may be dose-limiting) and triggers flu-like symptoms [4,59]. These side effects often do not overlap with those of chemotherapy (e.g., myelotoxicity) and, for these reasons, it is often possible to combine the two regimens at the recommended dose, without a forced reduction in the administered quantities. Another strategy is to use antibody-drug conjugates (ADC) which can deliver highly potent cytotoxic drugs to the tumor site sparing normal tissues. In an immunocompetent mouse model of AML, the combination of an ADC and an immunocytokine based on IL2 achieved significantly better results than the ADC or the immunocytokine alone [177]. A trifunctional antibody-drug-cytokine conjugate (consisting of IL2 and a maytansinoid DM1 microtubular inhibitor fused to an antibody moiety) showed selective tumor-homing performance and potent anticancer activity in two mouse models, further encouraging this combinatorial approach [193]. Small molecule-drug conjugates may offer additional benefits compared to ADCs in terms of rapid diffusion into the tumor [194], immunogenicity [195] and lower cost-of-goods [196]. The activity of a non-internalizing small molecule-drug conjugate was recently shown to be enhanced in combination with L19-IL2 [188].

The combination of intact IgGs and immunocytokines has also shown promising preclinical results [34,74,86,145]. Products that mediate an increased density and activity of NK cells within the tumor mass are likely to potentiate antibody-dependent cell cytotoxicity (ADCC) [34,74].

There is a considerable therapeutic potential associated with the combination of pairs of judiciously chosen immunocytokine products. Immune reactions are often boosted by the simultaneous presence of two or more stimuli [2]. A synergistic effect has been reported for the combined use of L19-IL2 and L19-TNF, both in a preclinical and clinical setting [84,135,140,142,197,198]. In a mouse model of neuroblastoma, treatment with the combination of L19-IL2 and L19-TNF resulted in a 70% complete cure rate compared to the 30% cure rates observed for the respective monotherapies [198]. Moreover, total splenocytes from cured mice were able to fully protect naïve mice against a homologous tumor mediated predominantly by CD8+ T cells, thus suggesting a vaccination effect. Also in a myeloma model, the combined administration of L19-IL2/ L19-TNF was significantly better than the monotherapies [142]. In a different syngeneic immunocompetent mouse model of cancer, a single intratumoral injection of L19-IL2 combined with L19-TNF resulted in complete remission whereas the two components administered separately did not lead to cures [140]. These results were confirmed in two additional mouse models of cancer in an independent study (K1735 M2 melanoma, WEHI-163 sarcoma) [84]. Other examples of immunocytokine combinations leading to potent anticancer activity include products based on the pairs IL2/IL12 [90,122,168], IL12/TNF [57] and IL4/IL12 [55]. The latter combination was surprising considering that IL4 and IL12 are technically antagonistic in polarizing different fates of CD4+ T cell development [2].

The combination of multiple immunocytokine products is attractive from an immunological perspective, but difficult to implement in pharmaceutical development, as it requires more than one product and involves double the amount of studies and regulatory approvals. For this reason, it may be attractive to generate a novel class of biopharmaceutical agent featuring multiple cytokine payloads. This approach would

lead to single products for industrial development (thus reducing development time and costs), but presents certain challenges. Not all payloads can be combined into the same molecular entity whilst retaining an adequate tumor targeting performance [57]. Moreover, different cytokine payloads may be active at different doses.

Gillies et al. have generated a series of dual cytokine fusion proteins which are composed of IL2, IL12 and the anti-EpCAM antibody (KS-1/4) [168]. Cytokine activity was preserved in those constructs where the cytokines were fused to the C-terminus of the heavy or light chain domain, or where one cytokine was linked to the C-terminus of the heavy chain while the other payload was linked to the N-terminus of the heavy or light chain variable region [168]. These KS-IL2/ IL12 fusion proteins showed remarkable anti-tumor activity in a mouse model of Lewis Lung carcinoma [168].

Our group has recently reported the successful generation of a "potency-matched" dual cytokine fusion protein [100]. This novel fusion protein consists of the tumor targeting antibody F8 linked to IL2 and TNF [100]. A single point mutation of TNF was enough to match its potency with IL2 (IL2-F8-TNF^{mut}) [100]. IL2-F8-TNF^{mut} eradicated soft tissue sarcomas that are typically not responsive to the individual cytokine fusion proteins or the standard treatment with doxorubicin [100]. Also, in other mouse models of cancer (CT26, C1498, F9), the dual cytokine fusion protein mediated a potent therapeutic activity [100].

Antibodies against immune check-point inhibitors (such as the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), programmed cell death 1 (PD-1) or programmed cell death ligand 1 (PD-L1)) are rapidly gaining importance for the treatment of various forms of cancer [199]. Marketed products include ipilimumab (directed against CTLA-4), nivolumab and pembrolizumab (directed against PD-1) and atezolizumab, durvalumab and avelumab (all directed against PD-L1). Ipilimumab was the first immune check-point inhibitor that was approved by the FDA. The CTLA-4 blocker is approved for the treatment of melanoma and is undergoing numerous clinical trials for including among others: the treatment of non-small cell lung carcinoma (NSLC), small cell lung cancer, bladder cancer and prostate cancer. Checkpoint inhibitors provide a clear benefit to a proportion of treated patients, but cancer cures are still rare for many indications. It has been argued (and shown preclinically) that certain cytokine combinations (e.g., featuring the use of PEGylated products [200,201] or antibody fusions [142]) may turn "cold" tumors "hot" and may be used to potentiate immune-oncology drugs. L19-IL2 has been shown to effectively synergize with CTLA-4 blockade in mice with CT26 colon carcinoma [62]. Cured mice were able to reject subsequent rechallenge with the same tumor model which means that a protective long-term immunity was achieved. These experiments provide a first rationale for the clinical use of targeted cytokines with immune check-point inhibitors.

7. Immunocytokines in clinical development

Many immunocytokines have been studied in mouse models of cancer, but relatively few products have entered clinical trials for oncological applications. Those products feature IL2, TNF or IL12 as immunomodulatory payloads. A list of clinical-stage antibody-cytokine fusions is shown in Table 2. Selected examples are described more closely in this section.

The only TNF-based immunocytokine in clinical development is L19-TNF (Fibromun). Fibromun is composed of the L19 antibody in scFv format specific for the EDB domain of fibronectin and human TNF [96,166]. TNF naturally forms a stable homotrimer and hence L19-TNF arranges into a trivalent antibody-cytokine fusion protein. Murine TNF fused to L19 has shown remarkable tumor targeting in vivo in preclinical experiments in mice with a tumor to blood ration of 100:1 (24 h postinjection) and superior antitumor effects relative to untargeted TNF in various mouse models of cancer [57,84,96,140,166,198]. Fibromun is currently being studied in Phase III clinical trials for the *i.v.* treatment of patients with metastatic soft-tissue sarcoma in combination with doxorubicin (EudraCT number 2016–003239-38). Moreover, the product is being tested in a Phase III clinical trial in combination with L19-IL2 (Darleukin) for the intralesional administration to patients with fully-resectable stage IIIB/C melanoma (clinicaltrial.gov identifier NCT02938299, EudraCT number 2015–002549-72) [216,217].

When used as monotherapy in a Phase I/II clinical study, L19-TNF was well tolerated as up to 1 mg doses per patient and the maximum tolerated dose (MTD) was not reached [7]. Tumor stabilizations were recorded but no objective tumor responses were observed [7]. The same product, when used in isolated limb perfusion in combination with melphalan in patients with locally advanced extremity melanoma, induced objective responses in 89% of the patients [232] at a dose which was more than ten times lower compared to the one of recombinant TNF used for similar procedures [233]. An ex vivo immunohistochemical analysis of tumor lesions confirmed a preferential localization of L19-TNF to the tumor neovasculature [232].

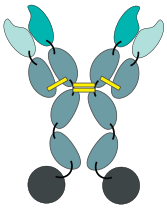
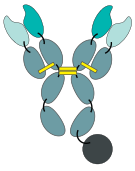
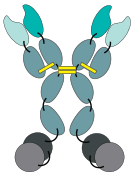
The clinical development of L19-TNF in combination with doxorubicin for the treatment of soft-tissue sarcoma is motivated by the high sensitivity of sarcoma lesions to TNF [234] and by the observation of cancer cures in immunocompetent mouse models of the disease [64,116]. Interestingly, mechanistic studies in mice revealed the upregulation of cytotoxic CD8+ T cell specific for a common endogenous retroviral antigen AH1 (derived from the gp70 envelope protein of the murine leukemia virus) upon treatment with F8-TNF and doxorubicin [64]. Such retroviral sequences have been found in the genome of all vertebrate species and their expression has been associated with autoimmune diseases and chronic infection [235–237] as well as with cancer [238,239]. Furthermore, cytolytic CD8+ T cells specific for retroviral antigens, which potently lysed melanoma cells, have already been detected in patients [240]. These facts raise the question if, similar to the mouse model, specific CD8+ T cells against retroviral antigens could also be increased in patients after L19-TNF treatment, which could then contribute to tumor eradication.

When used in combination with L19-IL2, the intralesional administration of L19-TNF showed potent therapeutic activity [197]. A potent anticancer activity was seen not only in injected lesions, but also in a high proportions of non-injected lesions, suggesting that the product may be able to induce a systemic anti-cancer protective immunity [197].

DI-Leu16-IL2 (an immunocytokine specific to the CD20 antigen expressed on B cells and on certain B cell malignancies) showed remarkable superior anticancer activity compared to 25-fold higher doses of an anti CD20 immunocytokine combined with untargeted IL2 in mouse model of human B lymphoma [176]. When tested in B cell lymphoma patients, 5 out of 13 patients showed a partial response (PR, 2/13) or a complete response (CR, 3/13) [210]. Hu14.18-IL2, a product with similar molecular format (IgG-IL2 fusion) but specific to GD2 (a disialoganglioside which is abundantly expressed on tumors of neuroectodermal origin and normally found only in the cerebellum and peripheral nerves), was studied in a Phase II clinical trial for children with relapsed or refractory neuroblastoma. No responses were detected in 13 children with bulky disease, but 5 out of 23 children with less prominent but still evaluable disease showed a CR [202].

Roche has focused on antibody-IL2 fusions based on intact IgG formats, but the company has preferred to use mutated versions of IL2, which display reduced binding affinity to the alpha subunit of the IL2 receptor (CD25) [74,136,137,241]. This choice was motivated by the fact that CD25 is highly expressed on regulatory T cells (T_{reg}). Preferential binding of IL2 fusions to those cells could result in immunosuppressive effects, as suggested by the clinical experience with low-dose IL2 in patients with graft-vs-host responses [242]. In order to generate products with a single cytokine payload, researchers at Roche employed "knob-into-hole" technology [243], favoring heterodimer formation between one antibody heavy chain devoid of IL2 and a second antibody heavy chain, fused to the cytokine payload (Table 2). Two products, targeting the CEA in colorectal cancer (CEA-IL2v) or FAP (FAP-IL2v) in various types of malignancies, are currently being investigated in clinical trials,

Table 2
List of immunocytokines in clinical trials.

Compound	Generic Name	Antibody format	Antigen	Indications	Clinical trial identifier	Company	Literature
Interleukin 2							
Hu14.18-IL2	EMD 273063; APN301		GD2	Neuroblastoma, malignant melanoma	Phase I: NCT03209869, NCT00003750 Phase II: NCT00590824, NCT00109863, NCT00082758, NCT01334515	Merck KGaA	[202–204]
NHS-IL2LT	Selectikine; EMD 521873; MSB0010445		DNA/ Histone complex	Lung cancer, NSCL carcinoma, Non-Hodgkin lymphoma, melanoma	Phase I: NCT00879866, NCT01032681 Phase II: NCT01973608	Merck KGaA	[189,205]
huKS-IL2	Tucotuzumab celmoleukin; EMD 273066		EpCAM	Ovarian cancer, colorectal cancer, NSCL carcinoma, prostate cancer	Phase I: NCT00132522, NCT00016237	Merck KGaA	[206–209]
anti-CD20-IL2	DI-Leu16-IL2		CD20	Lymphoma	Phase I: NCT00720135 Phase I/II: NCT01874288 NCT02151903	Alopexx Oncology, LLC	[210–212]
L19-IL2	Darleukin		EDB	Solid tumors, lymphoma, melanoma, pancreas cancer, DLBCL	Phase I: NCT02086721, NCT01198522 Phase I/II: NCT02957019, NCT02076646, NCT01058538 Phase II: NCT02076633, NCT02735850, NCT01253096, NCT01055522 Phase III: NCT02938299	Philogen	[142,197,213–218]
F16-IL2	Teleukin		Tnc A1	AML, lung cancer	Phase I: NCT02957032, NCT03207191 Phase I/II: NCT01131364, NCT01134250 Phase II: NCT02054884	Philogen	[63,219–222]
anti-CEA-IL2v	Cergutuzumab amunaleukin; RO6895882		CEA	Solid CEA+ cancers	Phase I: NCT02004106 NCT02350673	Roche Glycart	[223–227]
anti-FAP-IL2v	RO6874281; RG7461		FAP	Renal cell carcinoma, solid tumors, breast cancer, cancer of head and neck	Phase I: NCT03063762, NCT02627274 Phase II: NCT03386721	Roche Glycart	[224]
Interleukin 12							
NHS-IL12	M-9241		DNA/ Histone complex	Advanced solid tumors, malignant, epithelial tumors, malignant mesenchymal tumors	Phase I: NCT02994953, NCT01417546	Merck KGaA	[228]
BC1-IL12	AS1409		FN D7	Metastatic melanoma, metastatic renal cell carcinoma	Phase I: NCT00625768	Antisoma	[72,229–231]
TNF							
L19-TNF	Fibromun		EDB	Malignant melanoma, unresectable or metastatic soft tissue sarcoma	Phase II: NCT02076633, NCT03420014 Phase III: NCT02938299	Philogen	[7,8,197,216,217]

Clinical stage products. The format of the immunocytokine is depicted according to the color code of Fig. 2 with the exception of the C_H1-C_H3 and C_HL domains which are depicted in blue. The indications for each biopharmaceutical are given as well as the clinicialtrial.gov identifier number.
Abbreviations: GD2: disialoganglioside, EpCAM: epithelial cell adhesion molecule, EDB of fibronectin: alternatively-spliced extradomain B of fibronectin, Tnc A1: alternatively-spliced A1 domain of tenascin-C, CEA: carcinoembryonic antigen, FAP: fibroblast-activating protein, FN D7: domain 7 of fibronectin.

alone or in combination with atezolizumab (anti PD-L1 antibody, clinicaltrial.gov identifier: NCT02350673, NCT03063762, NCT03386721), trastuzumab (anti HER-2 antibody, clinicaltrial.gov identifier: NCT02627274), or cetuximab (anti EGFR antibody, clinicaltrial.gov identifier: NCT02627274).

Merck KGaA has also developed a fusion protein (NHS-IL2LT) in IgG format, featuring two mutant IL2 payloads at the C-terminal end. A single mutation (D20T) in the IL2 domain was introduced in order to decrease vascular toxicity by eliminating the toxin motif that binds endothelial cells [85]. Interestingly, this mutation also increased the specificity for activating the high-affinity IL2 receptor thus preferentially binding to activated T cells [85,244]. However, Tregs (which bear the high-affinity receptor) are also activated and thus the potential problem of stimulating more Tregs than antitumor effector cells remains. NHS-IL2LT binds to DNA-histone complexes, which become exposed and accessible in necrotic tissues (a characteristic feature of many rapidly-growing solid tumors) [85,245]. In a Phase I clinical trial with patients bearing localized or metastatic refractory solid tumors, NHS-IL2LT mediated a prolonged disease stabilization in a proportion of patients, but no objective tumor responses were reported [189].

L19-IL2 is an antibody-IL2 fusion in diabody format, which has been studied in various clinical trials, both as monotherapy or in combination with other modalities. The tumor-homing properties of the fusion protein were characterized by quantitative biodistribution studies with radiolabeled protein preparations [56]. Moreover, the parental L19 antibody has been used to image >100 patients with different types of malignancies [38,42,246]. In preclinical studies, L19-IL2 strongly reduced tumor growth in various mouse models of cancer [56,84,143,192,198], but was rarely able to completely eradicate cancer when used as single agent. A microscopic analysis of tumor lesions revealed a rich infiltrate of NK cells and T cells within the neoplastic mass, which was clearly different from the one observed with recombinant IL2 or with IL2 fusions with antibodies of irrelevant specificity in the mouse [56]. In a monotherapy study in patients with renal cell carcinoma, stable disease was observed in 83% patients after 2 cycles (total 6 infusions) of L19-IL2 [213]. Treatment was well tolerated with manageable toxicities that resolved within hours or days after L19-IL2 administration.

L19-IL2 has also been used in combination with other modalities, including L19-TNF (as described above). The combination of L19-IL2 with dacarbazine has led to encouraging results for the treatment of patients with stage IV melanoma [214]. On the basis of those results, a controlled Phase II trial has been initiated (EudraCT number 2012–004495-19). Preclinical findings have shown that L19-IL2 was able to potentiate the therapeutic activity of external beam radiation, promoting an abscopal effect [190]. A clinical study, featuring the administration of L19-IL2 after stereotactive ablative radiotherapy, has recently begun, in the frame of the European Union IMMUNOSABR project. A potent synergistic effect has also been observed when combining L19-IL2 with the anti-CD20 antibody rituximab [34]. A high local concentration of IL2 within the tumor mass promotes the influx and activation of certain leukocytes, including NK cells (which are crucially important for antibody-dependent cell cytotoxicity). A Phase Ib clinical trial (clinicaltrial.gov identifier NCT02957019), featuring the administration of L19-IL2 and rituximab to patients with relapsed or refractory diffuse large B-cell lymphoma, has recently started.

F16-IL2 is a fusion protein with a format, which is similar to the one of L19-IL2. The immunocytokine consists of a diabody fused to two IL2 moieties which targets the alternatively spliced A1 domain of tenascin-C [78]. This antigen is overexpressed in many cancer types but virtually undetectable in normal tissue [247,248]. The F16 antibody selectively accumulates at neovascular tumor sites in animal models and most human breast, lung, and head/neck cancers [78,79,247–249]. In a xenograft model of human breast cancer in mice, F16-IL2 showed potent therapeutic activity alone as well as in combination with doxorubicin and paclitaxel [78]. In combination with temozolomide, F16-IL2 exhibited a strong antitumor activity in subcutaneous and intracranial glioblastoma xenografts [79]. F16-IL2 is now extensively being studied in several clinical trials. F16-IL2 is being investigated in combination with doxorubicin (clinicaltrial.gov identifier NCT01131364) or paclitaxel (clinicaltrial.gov identifier NCT02054884, NCT01134250, EudraCT number 2012–004018-33) in patients with solid tumors or metastatic breast cancer [219,221,222], with low dose cytarabine (clinicaltrial.gov identifier NCT02957032) or with BI 836858 (an anti CD33 antibody, clinicaltrial.gov identifier NCT03207191) in AML [63,220] and with nivolumab (anti PD-1 inhibitor) [218] in non-small cell lung carcinoma patients.

The only two products in clinical trials based on IL-12 are NHS-IL12 and BC1-IL12. NHS-IL12 [145,146] is specific for DNA-histone H1 complex exposed in necrotic tumors. In a preclinical experiment, NHS-IL12 was able to achieve a partial response in 2 out of 11 canines with spontaneously occurring solid tumors by a single injection [228]. In order to determine the maximum tolerated dose in humans, NHS-IL12 is now being investigated in Phase I clinical trials, alone (clinicaltrial.gov identifier NCT01417546) or in combination with the immune check-point inhibitor avelumab (clinical trial identifier NCT02994953) [228].

BC1-IL12 targets a cryptic epitope on domain 7 of fibronectin, which becomes exposed in the presence of the alternatively-spliced EDB domain [72,248,250]. The BC1 antibody is a humanized version of the parental murine BC1 product that has been used for immunoscintigraphy to image tumors in glioblastoma patients [231]. The tumor-homing properties of the humanized BC1 antibody in full IgG format, linked to the dimeric IL12 at the C-terminus of the heavy chain, have been characterized in tumor-bearing mice, using quantitative biodistribution studies [144]. In a Phase I clinical trial, BC1-IL12 induced a disease stabilization for at least 4 months in 6 out of 11 patients with malignant melanoma [229]. Only one patient had achieved a sustained partial response 17 months later [229]. The product was well tolerated [229], with pyrexia (85%), fatigue (92%), chills (62%), nausea (50%), vomiting (62%) and transient liver function abnormalities (77%) as main toxicities being observed in the majority of patients. IFN-γ and CXCL10 concentrations were elevated in the serum of all patients, thus indicating a cell-mediated immune response, which is in line with previous preclinical observations [168].

8. Potential drawbacks of immunocytokines and possible strategies to minimize them

Immunocytokines are promising biopharmaceuticals but, like cytokine products, can cause side effects in patients. These side effects may vary, to a certain extent, from cytokine to cytokine, due to differences in mode of action. Pro-inflammatory agents are typically active on the vasculature and it is not surprising that hypotension was reported as one of the limiting toxicities for clinical-stage antibody-cytokine fusions [202]. Similarly, cytokine payloads may cause flu-like symptoms, nausea and vomit [205,229,251].

There are substantial differences in the way recombinant cytokines were used and current procedures for antibody-based fusions. For example, the high-dose IL2 regimen of Rosenberg and colleagues features the administration of up to 800 million international units (Mio IU) of IL2 in one week which is repeated after one week of rest [252,253]. By contrast, various antibody-IL2 fusions have been administered at weekly doses in the 67.5–110-Mio IU/m^2 range for >6 months [202,213].

The side effects of immunocytokines are influenced not only by the pharmacokinetic properties of the product, but also by the administration schedule and by the animal species. For example, different mouse strains exhibit variations in the maximal tolerated dose. In general, side effects are associated with peak concentrations of the product in blood. It is therefore tempting to hypothesize that immunocytokines may display a non-linear toxicity profile and that the products may be better tolerated when administered with slow infusion procedures. There is initial clinical evidence in support of this hypothesis. For

example, the maximal tolerated dose (MTD) of L19-IL2 was found to be 22.5 Million International Units of IL2 equivalents in clinical trials with 1 h infusion regimens [213], while three-fold higher doses could be safely administered to patients, using a 3-h infusion protocol (unpublished results).

Immunocytokine products based on antibody fragments (e.g., L19-IL2, L19-TNF, F16-IL2) were found to be not immunogenic in clinical trials [213,214,219,254]. Interestingly, a small set of patients who developed a low human antibody titer against the fusion proteins subsequently lost this reactivity upon continuation of the treatment at later cycles, possibly pointing to an induction of tolerance [254]. These findings are robust, as they were confirmed both by sandwich ELISA and by BIAcore. A similar immunogenicity study has been performed on hu14.18-IL2, an immunocytokine product based in IgG format. In this case, anti-immuncytokine antibodies were found in most patients, but they did not induce allergic reactions or increase toxicity [255].

Looking at the future, strategies that provide "activity-on-demand" for immunocytokine products may be particularly attractive. Ideally, antibody fusions would gain activity at the site of disease, upon antigen binding. Strategies that have been proposed include the use of "split cytokine" payloads [256] and allosteric modulation of cytokine activity upon antigen binding [31]. It is also possible that combination treatments may allow the use of immunocytokine products at doses which are well below the MTD.

9. Emerging trends and open questions

Although antibody-cytokine fusions have now been investigated for >20 years, the field has only recently gained clinical momentum, also thanks to the growing interest in cancer immunotherapy. It is clearly established that, at least at the preclinical level, the antibody-based delivery of suitable payloads (e.g., IL2, IL4, IL12, TNF) may promote a potent anticancer activity and an influx of leukocytes into the tumor mass. In most studies, increased therapeutic activity correlated with a preferential product uptake within the tumor mass. However, the group of Dane Wittrup has reported results with full immunoglobulins fused to IL2, which were independent of the tumor homing properties of the antibody [86]. Future research activities will certainly focus on the preferred immunocytokine format (e.g., intact IgGs vs. antibody fragments) and on the exploration of novel combination strategies.

Even the best antibody-cytokine fusions display certain side effects (both preclinically and clinically) which are comparable to the ones of the non-targeted recombinant cytokine products. This feature may be counterintuitive but is easy to understand, if we consider that only a small portion of any antibody product reaches the tumor mass in vivo. Tumor:organ ratios of 10:1 and better may be achieved 24 h after intravenous administration [100], but the absolute quantity of intact or armed antibodies within the neoplastic mass tends to be low [42]. Pro-inflammatory payloads tend to cause hypotension, nausea and flu-like symptoms as their main side-effects. Since these adverse events manifest themselves when cytokine concentrations in blood are high, there may be opportunities for clinical improvement associated with a slow drug administration (e.g., continuous infusion or subcutaneous injection). Alternatively, molecular strategies aimed at promoting "activity on demand" (e.g., preferential activity at the site of disease) should be considered.

Steve Gillies described an approach to reduce toxicity and enhance anti-tumor activity by simply moving the IL2 moiety from the C-terminus of the heavy chain to the C-terminus of the light chain which changed the activity of IL2 [31]. In fact, this structural modification preserves the interaction with the high affinity IL2 receptor, but decreases the interaction with the intermediate affinity IL2 receptor [31].

An alternative approach to prevent the stimulation of Tregs and reduce vascular toxicity of IL2, was recently described by the group of Onur Boyman [257]. A monoclonal antibody to human IL-2 (called NARA1) acts as a CD25 mimic, thus masking and preventing the interaction of IL2 with CD25. This mimobody was shown to preferentially stimulate CD8+ T cells over Tregs and displayed potent anticancer activity [257].

Our group has reported the successful generation of split cytokine antibody fusion proteins in order to localize cytokine activity only to the tumor site [256]. For this, the IL12 subunits, p40 and p35, were both seperately fused to tumor homing antibodies. Upon simultaneous *i.v.* administration, these subunits are thought to assemble only at the tumor site, generating the reconstituted IL12 with full immunomodulatory potential.

From our perspective, the combination of immunocytokines appears to be one of the most promising avenues to modulate the activity of the immune system at the site of disease. Until now, these explorations have been empirical in nature, screening product combinations in tumor-bearing mice. In the future, it would be conceivable to systematically probe combination partners and doses on isolated leukocytes (e.g., exhausted tumor-specific T cells), in order to discover whether certain product combinations can promote the desired changes in phenotype and activity. After having firmly established the synergistic action of two (or more) payloads, the development of "potency-matched" multiple cytokine products may be considered.

Different groups have so far focused on tumor-associated antigens expressed either on the surface of tumor cells or in the extracellular matrix. A comparative evaluation of similar products, targeting different antigen classes, may shed light on the preferred strategy for product development. These experiments are currently on-going in our laboratory.

Finally, it should be mentioned that certain immunocytokine products have additionally emerged to display beneficial effects in non-oncological diseases, such as chronic inflammatory conditions (rheumatoid arthritis) or endometriosis [258–263]. More preclinical and clinical studies are warranted, in order to identify the therapeutic potential of antibody-cytokine fusions beyond oncology.

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Disclosure of potential conflicts of interest

D.N. is co-founder, shareholder and board member of Philogen (www.philogen.com), a Swiss-Italian biotech company which develops novel immunocytokine products.

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