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Antibody-cytokine fusion proteins: applications in cancer therapy

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Abstract

Background—Antibody-cytokine fusion proteins consist of cytokines fused to an antibody to improve antibody-targeted cancer immunotherapy. These molecules have the capacity to enhance the tumoricidal activity of the antibodies and/or activate a secondary antitumor immune response.

Objective—To review the strategies used to develop antibody-cytokine fusion proteins and their *in vitro* and *in vivo* properties, including preclinical and clinical studies focusing on IL-2, IL-12 and GM-CSF.

Methods—Articles were found by searching databases such as PubMed and Clinical Trials of the US National Institutes of Health.

Results/conclusion—Multiple antibody-cytokine fusion proteins have demonstrated significant antitumor activity as direct therapeutics or as adjuvants of cancer vaccines in preclinical studies, paving the way for their clinical evaluation.

Keywords

antibody; antibody–cytokine fusion protein; cancer; cytokine; GM-CSF; IL-2; IL-12; immunotherapy

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1. Introduction

In humans there are five classes of antibodies: IgG (γ), IgA (α), IgD (δ), IgE (ϵ), and IgM (μ), with four subclasses of IgG and two subclasses of IgA. They are classified according to the differences in their heavy chain constant domains and differ in their biological properties. There are two light chain isotypes: kappa (C_K) and lambda (C_λ) that can be found associated with all of the antibody classes. IgG is the most abundant class of antibody in serum [1]. The full IgG antibody has a molecular weight of ~150 kDa. Its general structure is composed of two identical heavy chains and two identical light chains (Figure 1A). Each light chain consists of one variable domain (V_L) and a single constant domain (C_L : κ or λ), while the heavy chain encompasses one variable domain (V_H) and three constant domains (C_{H1} , C_{H2} and C_{H3}). The antigen-binding specificity is provided by three complementary-determining regions (CDRs) of the variable region (F_v) localized at the amino-terminus, while the fragment crystallizable (F_c region) is localized at the carboxy-terminus of the heavy chain (Figure 1A) [1]. The F_c region plays an important role in some biological functions such as antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cell-mediated phagocytosis (ADCP), and complement-dependent cytotoxicity (CDC) [2–4].

Murine monoclonal antibodies used in cancer immunotherapy have been generated by the classical hybridoma technology [5–7]. These murine monoclonal antibodies induce antitumor effects by blocking receptors on the surface of tumor cells, neutralizing soluble ligands and inducing apoptosis in cancer cells [8–10]. However, the F_c regions of these murine monoclonal antibodies have limited ability to bind human effector cells or human complement. Thus, these antibodies have restricted therapeutic potential [8,9]. In addition, since these murine monoclonal antibodies are entirely of mouse origin, they are immunogenic and induce a human anti-mouse antibody (HAMA) response in patients [7]. This response results in the neutralization and faster removal of murine monoclonal antibodies, which further limits their antitumor efficacy. In addition, this HAMA response can also be associated with severe allergic reactions [4,7,11–13]. In order to overcome these problems, chimeric antibodies have been developed using genetic engineering (Figure 1B) [4,7,14]. These antibodies are constructed using expression vectors encoding the constant regions of human antibodies such as the Ig variable heavy chain of a murine monoclonal antibody (mV_H -h[C_{H1} - C_{H2} - C_{H3}]), plus the mouse variable light chain with the human constant light chain (mV_L -h C_L). Because these antibodies contain human F_c regions, they exhibit full biological activity in humans [4,7]. An example is rituximab (Rituxan[®], MabThera[®]), which is approved by the FDA for the treatment of non-Hodgkin's lymphoma (NHL). Rituximab recognizes the CD20 antigen on the surface of malignant B cells and is able to induce ADCC, CDC and apoptosis of these cancer cells [15,16].

Despite the effort to reduce immunogenicity, patients treated with chimeric antibodies may develop a human anti-chimeric antibody (HACA) response to the murine variable regions [4,7]. To decrease the mouse component of chimeric antibodies and further decrease their immunogenicity, humanized antibodies have been developed where only the murine CDR loops are grafted onto the human variable domain framework (CDR-grafting, Figure 1B) [4,17]. An example is trastuzumab (Herceptin[®]) that targets human epidermal growth factor

receptor 2 (HER2)/neuroblastoma-associated gene (*neu*) and has been approved by the FDA as a treatment for breast cancer [18].

Fully human antibodies can be produced to completely eliminate the immunogenicity of antibodies (Figure 1B). In order to produce these human antibodies, the murine immunoglobulin repertoire has been replaced with human variable genes in the murine chromosome of transgenic mice. The XenoMouse™ is a transgenic model that has been used to generate human monoclonal antibodies [7,19]. In this model, the endogenous heavy and light chain loci were disrupted and elements of the human heavy and light chain loci were introduced. The B cells of this transgenic mouse are able to undergo somatic hypermutation and affinity maturation of the human germline antibodies producing polyclonal human antibodies [4,7,19,20]. Through hybridoma technology, these mice can be used to obtain human monoclonal antibodies with high affinity for the desired human antigen [4,7]. The trans-chromosomal (Tc) mice, similar to the XenoMouse™ model, have inactivated endogenous heavy and light chain loci. In addition, large human chromosome fragments were introduced into these mice so that they generate human antibodies [7,21]. Phage display technology can also be used to produce human antibodies [4,7]. The phage display technique consists of human single chain variable region (scFv) genes cloned into a phage display vector [22]. This technology allows screening of the scFv fragments expressed on the surface of the bacteriophage that bind with high affinity to the desired antigen. The selected scFv genes are then cloned into expression vectors to develop fully human antibodies [4,7]. The expression of antibodies in mammalian systems (such as myeloma cell lines) promotes their correct post-translational modification, which increases their biological activity compared with those expressed in non-mammalian expression systems [6].

Due to the domain structure of antibodies, fragments such as Fab, scFv and minibodies can be generated by removing part or all of the Fc constant region. The V_L - V_H (scFv) are joined using a peptide linker, such as $(Gly_4-Ser)_3$ [23,24] to stabilize the proper binding structure in these molecules (Figure 1C). Minibodies are alternative antibody fragments that consist of two scFv regions, a hinge region and the C_H3 domain (Figure 1C) [25,26]. These smaller antibody fragments have faster clearance from the body and better tissue/tumor penetration. Intact antibodies, such as human IgG1, have a half-life of 3 – 4 weeks, while the scFv has a blood clearance of less than 10 h and kidney excretion in 2 – 4 h [4,25]. These features make antibody fragments useful in diagnostic applications and tumor imaging [26,27]. The scFv-Fc format consists of two scFv regions, a hinge region and C_H2 - C_H3 domains that allow it to specifically recognize the antigen and also bind Fc gamma receptors ($Fc\gamma Rs$) (Figure 1C) [4,25,26]. The disadvantage of these fragments (with the exception of scFv-Fc fragment) is that although they retain the capacity for antigen binding, they have lost the ability to induce Fc effector functions including ADCC, which is a relevant antitumoral mechanism of therapeutic antibodies [4,28].

Cytokines such as IL-2, IL-12 and GM-CSF are immunomodulators [29] that have demonstrated antitumor effects and have the ability to enhance the immunogenicity of certain tumor types [30–32]. Given the rapid blood clearance of cytokines and their lack of tumor specificity, systemic high-dose administration is required in order to obtain a

concentration in the tumor microenvironment that is capable of activating an immune response. However, the systemic administration of these cytokines in high doses has frequently been associated with severe toxic side effects in the cardiovascular and respiratory systems including symptoms such as tachycardia, hypotension, respiratory failure, vascular permeability, anemia, fevers and chills [33–35], and in some cases fatal consequences [36]. These side effects depend on the cytokine and its concentration [37,38]. Local injection of cytokines can reduce these side effects. However, since not all tumors are easily accessible for injection due to their size and/or anatomical localization this procedure has limited application. Another problem with local injection is that the cytokine remains at the site of injection for only a short period of time. Targeting cytokines to the tumor microenvironment with an antibody recognizing a tumor-associated antigen (TAA) is an alternative means of achieving locally high concentrations of the cytokine without systemic toxicity [39–41]. The domain conformation of the antibody structure facilitates molecular engineering to construct fusion proteins consisting of cytokines genetically fused to full antibodies or their fragments (Figure 2) [4,40,41]. In addition to concentrating the cytokine in the tumor microenvironment, the antibody–cytokine fusion proteins may also enhance the direct antitumor effect of the antibody without causing the severe toxic side effects of systemic high-dose cytokine administration [40,41]. They are also expected to elicit a secondary (humoral and/or cellular) anti-tumor response with the capacity to eliminate the cancer cells [7,39,40]. Membrane-bound and soluble tumor antigens may be targeted by antibody–cytokine fusion proteins, which may promote their processing and presentation by professional antigen-presenting cells (APC) expressing the specific cytokine receptor, thereby enhancing the secondary antitumor immune response.

There is a broad variety of antibody–cytokine fusion proteins targeting various tumor antigens in which different cytokines have been fused to full-length antibodies or their derivatives. Each cytokine can be fused at the amino- or carboxy-terminus of the antibody depending on the structure of the cytokine and antibody in order to conserve the biological activity of both components. Due to the growing number of antibody derivatives and cytokine combinations that are being generated, the quantity of different antibody–cytokine fusion proteins is very large and it would be difficult to discuss all of them in this work. Therefore, the present review describes the strategies for construction, the properties and advantages of several antibody–cytokine fusion proteins in which antibodies or their derivatives have been genetically fused to the cytokines IL-2, IL-12, or GM-CSF.

2. Antibody–(IL-2) fusion proteins

IL-2 is known to be a potent immunostimulator of the cellular and humoral immune systems with extensive therapeutic potential [42]. This cytokine has diverse stimulatory effects on a variety of immune cells including the activation of natural killer (NK) cells, induction of the lymphokine-activated killer (LAK) cells [43], activation of monocytes [44] and macrophages [42], and the activation of CD8⁺ cytotoxic T lymphocytes (CTLs) [45]. Previous studies have shown that IL-2 can induce T-cell production of IFN- γ [46], a very potent immunomodulator that can inhibit tumor growth by direct induction of apoptosis [47], and through its ability to stimulate CTLs [48], macrophages [49], NK cells [50] and to

increase the expression of the major histocompatibility complex class II molecules (MHC class II) [51].

Since IL-2 administration facilitates ADCC by antibodies, clinical trials for different types of tumors have combined monoclonal antibody therapy with the systemic or subcutaneous use of IL-2 [52,53]. However, in order to maximize the benefits of IL-2 in anticancer immunotherapy, it needs to be administered in high concentrations due to its short half-life and its non-specific tissue localization [54,55]. Still, the toxic side effects on cardiovascular and respiratory systems induced by systemic administration of high doses of IL-2 are problematic [38,56]. Fusion of IL-2 to an antibody specific for a TAA has conferred the ability to concentrate this cytokine in the tumor microenvironment, decreasing its systemic side effects. Biodistribution analysis in different murine models has shown that the tumor-specific antibody–cytokine fusion proteins preferentially accumulate in malignant tissues [57–59]. Table 1 summarizes the antibody–(IL-2) fusion proteins described in this review.

One of the first antibody–(IL-2) fusion proteins reported was developed with specificity for the hapten dansyl (DNS: 5-dimethylamino naphthalene-1-sulfonyl chloride) (Figure 2A (I)). This antibody–cytokine fusion protein consists of the human IL-2 fused to the carboxy-terminus of human IgG3 specific for DNS [60]. Human IgG3 was chosen because it has an extended hinge region, providing spacing and flexibility to facilitate simultaneous antigen and cytokine receptor binding [61,62]. Human IgG3 is also effective in complement activation [63] and binds both human and murine FcγRs [1]. In addition to retaining the ability to bind the antigen, the anti-DNS IgG3–(IL-2) stimulated the proliferation of the IL-2-dependent murine T-cell line CTLL-2 [60]. This antibody–(IL-2) fusion protein exhibited approximately fourfold higher affinity than recombinant human IL-2 (rhIL-2) for the α-subunit of the high-affinity IL-2 receptor, and was significantly more effective in the generation of LAK cell activity compared with IL-2 alone [60]. In addition, the half-life of this antibody–(IL-2) fusion protein injected intraperitoneally in mice is 7 h [64], which is more than one order of magnitude longer than that of free IL-2 [65] but shorter than that of IgG3 alone [64]. A similar increase in half-life of IL-2 fused to an antibody was also described using an anti-Id IgG3–(IL-2) fusion protein, which recognizes the idiotype (Id) of the immunoglobulin expressed on the surface of the malignant murine 38C13 B-cell lymphoma cell line [57]. This antibody–cytokine fusion protein also retains antigen-binding and cytokine activity and was able to target 38C13 subcutaneous tumors in mice. Importantly, using syngeneic (C3H/HeN) mice bearing subcutaneous or intraperitoneal 38C13 tumors, anti-Id IgG3–(IL-2) showed enhanced antitumor activity compared with the combination of antibody and IL-2 administered together [57]. These results suggest the potential use of this approach for the treatment of human B-cell lymphomas.

Two antibody–cytokine fusion proteins were constructed that are also specific for the Id antigen, mouse/human chimeric anti-Id IgG1–(mIL-2) [chS5A8–(IL-2)] and scFvS5A8–(IL-2) [66]. Both antibody–(IL-2) fusion proteins retain IL-2 biological activity and the capacity to increase cell-mediated cytotoxicity of 38C13 cells, *in vitro* [66]. The chS5A8–(IL-2) but not scFvS5A8–(IL-2) fusion protein induced *in vivo* tumor eradication in 38C13-tumor bearing mice. These results may be explained by the fact that the antigen-binding ability of scFvS5A8–(IL-2) was 30–40-fold lower than that of chS5A8–(IL-2). Additionally,

scFvS5A8-(IL-2) had a short life and was eliminated 20-fold faster than chS5A8-(IL-2). Finally, scFvS5A8-(IL-2) lacks of the Fc region that is necessary to induce ADCC-mediated cell lysis, which may be an important mechanism involved in the antitumor activity of chS5A8-(IL-2) in this model [66].

HER2/*neu* (erbB2) is an orphan receptor of the EGF receptor family (type 2) that like the other members of this family has receptor kinase activity. It is overexpressed in ~ 20 – 30% of human breast and ovarian cancers and its overexpression is associated with poor prognosis [67,68]. The anti-HER2/*neu* IgG3-(IL-2) (Figure 2A (I)) was developed by the fusion of the amino-terminus of human IL-2 to the carboxy-terminus of human IgG3 [69]. Human IL-2 was used because it retains full bioactivity in mice [69]. The antibody-(IL-2) fusion protein was constructed using human IgG3 and the variable regions of the humanized anti-HER2 /*neu* antibody trastuzumab [69]. This antibody-(IL-2) fusion protein retained the binding specificity of the antibody and cytokine biological activity similar to that of rhIL-2 [69]. We have also observed that anti-HER2/*neu* IgG3-(IL-2) retains the heparin-binding activity of IL-2 [70], a property that allows the binding of this cytokine to glycosaminoglycans present in the surface of cells [71] and that may play a role in the control of T-cell responses by IL-2 [72]. The authors explored the *in vivo* efficacy of anti-HER2 /*neu* IgG3-(IL-2) as a direct antitumor agent in a murine colon carcinoma cell line expressing human HER2/*neu* (CT26-HER2/*neu*), which was injected subcutaneously into immunocompetent syngeneic BALB/c mice. Treatment with this antibody–cytokine fusion protein resulted in significant retardation in the growth of CT26-HER2 /*neu* tumors under conditions in which the antibody alone (anti-HER2 /*neu* IgG3) failed to confer protection (Figure 3) [69]. Our results demonstrate that the anti-HER2/*neu* IgG3-(IL-2) fusion protein is effective in eliciting protection as a direct therapeutic agent against a tumor model expressing HER2/*neu* [69]. In section 5 we describe in detail the use of anti-HER2/*neu* IgG3-(IL-2) as an adjuvant in the context of a prophylactic protein vaccination against HER2/*neu* expressing tumors.

Another antibody–cytokine fusion protein, anti-erbB2 scFv-Fc-(IL-2) (Figure 2A (III)) has been developed. This antibody–cytokine fusion protein was designed in the scFv-Fc antibody format that consists of human IL-2 fused to the carboxy-terminus of human IgG1 C_H2-C_H3 domains linked to a scFv specific for HER2/*neu* [73]. This antibody–cytokine fusion protein retained antigen binding, and retained the biological activity of the cytokine, which was evaluated by the induction of proliferation of CTLL-2 T-cell line [73]. Recent studies have shown that anti-erbB2 scFv-Fc-(IL-2) improved the ability of IL-2 to mediate ADCC activity *in vitro* [74]. BALB/c athymic nude mice received subcutaneous injections of the human ovarian carcinoma cell line SKOV-3, which expresses high levels of HER2/*neu*. These mice treated intravenously with the antibody–cytokine fusion protein plus human LAK cells showed a reduction of tumor growth in comparison with treatment with rIL-2 plus LAK cells [74]. These results demonstrate the antitumor activity of this antibody–cytokine fusion protein in xenograft models.

Fibronectin has different isoforms due to alternative splicing. The B-FN isoform of fibronectin contains the ED-B (‘extra domain’) and is only found in fetal and malignant tissues [75]. ED-B is a marker of angiogenesis in the tumor microenvironment that is

recognized by the human antibody L19 [75,76]. Human IL-2 was fused to the carboxy-terminus of scFvL19 [L19-(IL-2)] (Figure 2A (IV)), and the antibody–cytokine fusion protein retains both the biological activity of the cytokine and the specificity of the antibody [77]. *In vivo* studies demonstrated that this antibody–cytokine fusion protein concentrates IL-2 in the tumor vasculature, enhancing the antitumor activity of the cytokine and decreasing tumor growth compared with a non-specific antibody–cytokine fusion protein, D1.3-(IL-2), in athymic nude mice challenged with subcutaneous injections of mouse F9 teratocarcinoma, C51 mouse colon carcinoma and N592 human small cell lung cancer (SCLC) cell lines [77]. Mice treated with L19-(IL-2) showed an increase in the number of T cells, macrophages and NK cells plus IFN- γ accumulation in the tumor microenvironment [77]. Due to the fact that human and mouse ED-B share 100% homology, the efficacy of L19-(IL-2) as an antitumor treatment for solid cancers was evaluated in five cohorts of cancer patients with refractory/recurrent solid tumors [78]. The maximum tolerated dose was 22.5 million units IL-2 equivalent dose in the 18 patients enrolled. This antibody–cytokine fusion protein has drug-related limiting toxicities, such as hypotension and acute renal failure, which were reversible. Five patients demonstrated stable disease and the remaining patients progressed. In addition, a discrete IL-2-related transient activation of T-cell subsets was observed [78].

Another antigen targeted for antibody–cytokine fusion protein-mediated therapy is mucin-1 (MUC-1), a transmembrane mucin expressed on the surface of normal glandular epithelial cells [79] and overexpressed in the surface of epithelial glandular cancer cells (adenocarcinoma) [79,80]. The C595 mouse monoclonal antibody that recognizes the peptide RPAP in the sequence of the human MUC-1 protein core was used to generate a scFv that retained binding specificity [59]. This C595scFv was genetically fused to the amino-terminus of the hinge-Fc region of human IgG1 (scFv-Fc), while IL-2 was fused to the carboxy-terminus of this Fc, generating the mouse/human C595 scFv-Fc-(IL-2) (Figure 2A (III)). This fusion protein retained the ability to bind MUC-1-positive T-47D (human mammary carcinoma) cells and also retains IL-2 bioactivity as evidenced by the induction of proliferation of activated CD25⁺ lymphocytes and the activation of NK cells resulting in the destruction of MUC-1-positive tumor cells *in vitro* [59].

CD30, an antigen expressed on the surface of Hodgkin's lymphoma cells [81], has also been explored as a target of an antibody–(IL-2) fusion protein [82]. This antibody–(IL-2) fusion protein consists of an anti-CD30 scFv antibody fragment derived from the anti-CD30 monoclonal antibody HRS3 fused to amino-terminus of the human IgG1 hinge-Fc region in which human IL-2 was fused at the carboxy-terminus of the C_H3 domain [HRS3scFv-Fc-(IL-2)] (Figure 2A (III)). This antibody–(IL-2) fusion protein retains the specificity for the CD30 antigen and the immunostimulatory function of IL-2 including the activation of T and resting NK cells [82]. HRS3scFv-Fc-(IL-2) was able to induce IFN- γ secretion in SCID mice that had received subcutaneous injections of L540, a human CD30⁺ non-Hodgkin's lymphoma cell line, which is important for the induction of an efficient antitumor response *in vivo* and the shift of a T-helper type 2 (T_H2) immune response in lymphoma to a T_H1 response [82]. These results support the concept that the HRS3-scFv-Fc-(IL-2) fusion protein could be valuable for the specific immunotherapy of Hodgkin's lymphoma.

CD20 is an important marker of NHL that is used as a target for immunotherapy [83,84]. This antigen has been used to generate an antibody–cytokine fusion protein using the murine antibody Leu16 (anti-CD20). The potential helper T-cell epitopes were removed from the variable regions of antibody Leu16 (a process called deimmunization), which was then humanized by combining these variable regions with the human light and heavy chain constant regions [85]. Human IL-2 was fused to the heavy chain, resulting in the humanized antibody fusion protein DI-Leu16-(IL-2) (Figure 2A (I)) [85], which retains antigen recognition and IL-2 activity. This antibody–(IL-2) fusion protein induced apoptosis of CD20⁺ Daudi cells (a human lymphoma cell line) and retained the ability to bind the Fcγ R required for the induction of ADCC activity mediated by human peripheral blood mononuclear cells (PBMC). The humanized DI-Leu16-(IL-2) was effective for the treatment of disseminated lymphoma for more than 54 days through the induction of an antitumor immune response in SCID mice receiving intravenous injections of human CD20⁺ Daudi Burkitt lymphoma cells, and treated with the antibody–cytokine fusion protein and human PBMC. The effector mechanisms induced in this model remain unknown. However, this treatment showed an increase in antitumor activity of Leu16-(IL-2) compared with rituximab plus free rhIL-2, which could be, at least in part, due to ADCC activity [85].

Human MHC class II molecules are present in a high percentage of B-cell NHL and chronic lymphocytic leukemia. These molecules are targeted by the murine antibody Lym-2 [86], which was used to generate the mouse/human chimeric antibody chCLL-1. The chCLL-1-(IL-2) fusion protein (Figure 2A-(I)) was then constructed with human IL-2 fused to the carboxy-terminus of this chimeric antibody [58]. This antibody–(IL-2) fusion protein retained IL-2 bioactivity as evidenced by the induction of proliferation of CTLL-2 cells, and also demonstrated ADCC *in vitro*, using human mononuclear cells as effector cells [58]. Biodistribution studies showed that chCLL-1-(IL-2) also retains antigen binding in ARH-77 B-cell malignant tumor-bearing nude mice [58].

The antibody–cytokine fusion protein (IL-2)-183B2scFv (Figure 2A (IV)) specific for OC183B2, a human ovarian-carcinoma-associated antigen, was developed by fusing IL-2 to the scFv derived from the murine antibody 183B2 (COC183B2) [87]. This (IL-2)-183B2scFv was able to induce the proliferation of CTLL-2 cells and retained the ability to recognize the antigen OC183B2 expressed on the SKOV3 human ovarian carcinoma cell line [87].

The disialoganglioside (GD2) is a sialic-glycolipid expressed on the surface of the cells and overexpressed in melanoma, neuroblastoma, glioblastoma and small-cell lung carcinoma [88]. The murine monoclonal antibody 14.18 has the ability to recognize the GD2 antigen, and has been used to develop chimeric [89] and humanized antibody–cytokine fusion proteins [90]. Ch14.18-(IL-2) (Figure 2A (I)) was developed by fusing human IL-2 to the carboxy-terminus of the mouse/human chimeric antibody ch14.18. This antibody–cytokine fusion protein increased the half-life of IL-2 to 4.1 h, which is significantly higher than rhIL-2 injected intra venously into BALB/c mice [89]. In order to test the anti tumor activity *in vivo* of this antibody–(IL-2) fusion protein, B16 melanoma cells (GD2⁺) were injected intravenously to induce lung metastases or intrasplenically to induce hepatic metastases in syngeneic C57BL/6 *beige/beige* or C57BL/6 *scid/scid* mice [91]. The results showed that

intravenous administration of ch14.18-(IL-2), one week after tumor challenge, induced complete eradication of pulmonary and hepatic metastases, which was not observed with IL-2 alone or with an irrelevant antibody. The antitumor activity of this antibody-(IL-2) fusion protein, which was long-lived and transferable in melanoma models is mediated by CD8⁺ T cells [91–93]. Using the model NXS2, a murine neuroblastoma cell line that expresses human GD2, ch14.18-(IL-2) was able to induce a NK-cell-mediated antitumor response that eradicates the stable bone marrow and liver metastases in a syngeneic model [94]. This antibody-(IL-2) fusion protein was humanized to generate the hu14.18-(IL-2), an IgG1 recognizing GD2 genetically fused to human IL-2 (Figure 2A (I)) [90]. Hu14.18-(IL-2) or EMD 273063 (EMD Pharmaceuticals Inc) has been evaluated in clinical trials for its safety and antitumor activity. In a Phase I clinical trial conducted in adult melanoma patients [95], EMD 273063 showed stable disease in 58% of the 33 treated patients after the first course and 24% of the patients at the end of the second course [96]. EMD 273063 was administered by a daily 4-h intravenous infusion over 3 days during the first week of two courses. Treatment elicited immunologic activity, including lymphocytosis, an increase in NK-activity, NK cell numbers, and increased ADCC activity mediated by PBMC obtained from patients treated 1 day prior to PBMC isolation [96]. The maximum tolerated dose was 7.5 mg/m² per dose and the mean half-life was 3.7 h. Although anti-idiotypic antibodies against EMD 273063 were detected in the serum of some patients, the treatment was generally well tolerated with no report of severe adverse events. A second Phase I clinical trial conducted in 27 pediatric patients with neuro-blastoma or melanoma, treated with EMD 273063 by a 4-h intra venous infusion for 3 consecutively days, showed no measurable complete or partial responses. However, three patients showed evidence of antitumor activity measured by bone marrow evaluation [95]. Treatment elicited immunologic activity, including lymphocytosis and elevated levels of soluble IL-2 receptor α [90,95]. The maximum tolerated dose was 12 mg/m² /dose and the mean half-life was 3.1 h. Overall, the treatment was well tolerated with pediatric patients presenting reversible toxicities such as hypotension, allergic reactions, pain, fever and hematologic toxicity. Phase II studies are currently planned in pediatric patients with neuroblastoma and melanoma [95].

The epithelial cell adhesion molecule EpCAM (KSA or KS1/4) is a type I transmembrane glycoprotein that is overexpressed in human epithelial carcinomas including prostate [97], head and neck [98], colorectal [99], breast [100] and hepatic carcinoma [101]. Human IL-2 was fused to the carboxy-terminus of humanized anti-EpCAM (KS1/4) (Figure 2A (I)) [102]. This huKS-(IL-2) was tested in a syngeneic model where murine colon cancer cells that express human KSA (CT26-KSA) were injected into the spleens of mice to induce hepatic metastases or into the tail vein to induce pulmonary metastases [102]. This antibody-(IL-2) fusion protein concentrated IL-2 in the tumor microenvironment, generating an anticancer response mediated by CD8⁺ T cells that was able to eliminate the established metastases [102,103]. Other results obtained with the CT26-Ep21.6 model (CT26 cells that express KSA and have low MHC class I expression) demonstrated that the level of the expression of MHC class I modulates the type of the immune response generated by huKS-(IL-2). Low expression of MHC class I favors an immune response mediated by NK cells [104]. HuKS1/4-(IL-2) [also known as huKS-(IL-2)] has been used in combination with CXCL9 (a chemokine) gene therapy [105] or with drugs such as cyclophosphamide [106].

These combinations have demonstrated that it is possible to improve antibody–cytokine fusion protein mediated therapy by using them in combination with other cancer therapies. HuKS-(IL-2) (huKS1/4-(IL-2): EMD 273066) has been evaluated in Phase I clinical trials conducted in patients with androgen-independent prostate cancer treated with a continuous 4-h intravenous infusion at the dose determined by an escalation protocol [107]. EMD 273066 demonstrated immunological activity, as measured by the increase in lymphocyte counts, NK-activity, NK cell numbers and ADCC activity [107]. The maximum tolerated dose was 6.4 mg/m²/dose and the mean half-life was dose-independent, in the range of 4.0 – 6.7 h. Patients treated with this antibody–cytokine fusion protein experienced toxic side effects associated with the toxic effects of IL-2 such as rigors, chills and anemia [107]. Additionally these patients showed titers of anti-antibody–(IL-2) fusion protein, but no hypersensitivity reactions were observed. In general, EMD 273066 was well tolerated without any further effects beyond those expected for high dose IL-2 administration alone [107]. EMD 273066 has also demonstrated high specificity for EpCAM in biopsies from women with ovarian cancer [108]. In addition to this characteristic, EMD 273066 significantly increased the expression of the activation marker CD69 on peritoneal effector cells (CD45⁺) isolated from patients with ovarian carcinoma. EMD 273066 can enhance cell lysis, in part by its ability to induce ADCC by effector cells derived from the peritoneal cavity. These data suggest that EMD 273066 has a potential immunotherapeutic role in the treatment of ovarian cancer [108].

3. Antibody–(IL-12) fusion proteins

IL-12 is a heterodimeric cytokine composed of the p40 and p35 subunits encoded by two separate genes, IL-12A (p35) and IL-12B (p40). Only the heterodimer is active and functional [109]. IL-12 is a promising cytokine with the potential to activate an effective immunological response against cancer cells [110]. IL-12 promotes cell-mediated immunity by inducing IFN- γ -mediated naive CD4⁺ T-cell differentiation into T_H1 cells [111], enhancing the cytotoxicity of NK cells and increasing cytotoxic T lymphocyte (CTL)-response [112]. It promotes the secretion of IFN- γ by T and NK cells, which can retard tumor growth [113] and upregulate MHC class I expression [114]. IL-12 also has antiangiogenic activity by increasing the production of IFN- γ , which induces the upregulation of IFN- γ -inducible protein 10 (IP-10) (CXCL10) and monokine induced by IFN- γ (MIG) chemokines [115,116]. These chemokines inhibit endothelial cell chemotaxis and block their differentiation into tube-like structures [117]. Due to its ability to induce a cellular immune response, IL-12 has been used in cancer immunotherapy. However, systemic injection of IL-12 in patients has been associated with severe toxicities, which in some cases were fatal [36]. Table 2 summarizes the antibody–(IL-12) fusion proteins described in this review.

The HER2/*neu* antigen, an EGF family member, is overexpressed in a subset of human breast and ovarian cancers [67,118] and has been targeted by an antibody–(IL-12) fusion protein. Because human IL-12 is not active in mice, murine IL-12 as a single chain version (mscIL-12: p40.L.Dp35) with the two subunits joined with a (Gly₄Ser)₃ flexible linker, was fused to a humanized IgG3 antibody containing the variable regions of the recombinant antibody trastuzumab [119]. Therefore, mscIL-12 was fused to the amino-terminus of the

anti-HER2 /*neu* IgG3 [119] to avoid blocking the folding of the amino-terminus of the p40 subunit and the subsequent decrease of its activity [120]. Another flexible linker (Gly₄Ser)₃ was added between mscIL-12 and the amino-terminus of the heavy chain of the antibody to facilitate correct folding of the antibody and antigen binding [119]. *In vitro* assays demonstrated that this anti-HER2/*neu* (IL-12)-IgG3 (Figure 2A (II)) retained the capacity to induce proliferation of phytohemagglutinin (PHA)-activated PBMC in a dose-dependent manner similar to mscIL-12 alone [119]. In addition, since IL-12 is a heparin binding protein [121,122], we studied this activity *in vitro* and demonstrated that anti-HER2/*neu* (IL-12)-IgG3 retains the heparin-binding activity of IL-12 [70], including the ability to induce IFN- γ secretion from NK-susceptible lymphoma KY-1 cells [70]. The (IL-12)-antibody fusion protein also retained the ability to bind the human HER2/*neu* antigen expressed in the murine colon cancer cell line, CT26-HER2/*neu* [70]. *In vivo* studies in a direct therapeutic setting showed that anti-HER2/*neu* (IL-12)-IgG3 injected intravenously into immuno-competent mice challenged subcutaneously with CT26-HER2/*neu* cells significantly retarded tumor growth in comparison with the antibody alone [119]. Recombination activating gene 2 (Rag2) knockout and SCID-beige mice bearing subcutaneous CT26-HER2/*neu* tumors treated with anti-HER2/*neu* (IL-12)-IgG3 showed that T cells and NK cells are involved in this anti-tumor activity [123]. In addition to promoting the isotype switch of the antibody response to T_H1 (IgG2a) in mice, anti-HER2/*neu* (IL-12)-IgG3 induced the generation of NK1.1⁺ cells and also demonstrated antiangiogenic activity [123].

In other studies, mice challenged intraperitoneally with epithelial syngeneic cancer models expressing human HER2/*neu* D2F2/E2 (mammary cancer cells, in a syngeneic BALB/c model), CT26-HER2/*neu* (colon cancer cells in a syngeneic BALB/c model), or MC38-HER2/*neu* (colon cancer cells in a syngeneic C57BL/6 model), and treated intraperitoneally with the anti-HER2/*neu* (IL-12)-antibody fusion protein, showed significant levels of protection in all tumor models [70]. Moreover, long-term survivors among mice challenged intraperitoneally with D2F2/E2 tumors retained significant protection after subcutaneous rechallenge with the same cell line more than 4 months later, and subsequent subcutaneous challenge with the parental cell line D2F2 not expressing human HER2/*neu* [70,124]. These results suggest that under these conditions, treatment with anti-HER2/*neu* (IL-12)-IgG3 could elicit a systemic long-term immune response and that this response could be expanded to antigens other than the targeted antigen [70,124]. In Section 5 the use of anti-HER2/*neu* (IL-12)-IgG3 in the context of prophylactic cancer vaccination is discussed.

To test the effect of antibody-(IL-12) fusion proteins on angiogenesis, the L19-(mIL-12) fusion protein was developed using murine IL-12 and the scFvL19 derived from the human antibody L19 [125,126] that recognizes the ED-B domain of human fibronectin, a marker of angiogenesis that is secreted by tumor and endothelial cells [75,76]. (IL-12)-scFvL19 (Figure 2A (IV)) retained the *in vitro* biological activity of IL-12 and the specificity of L19 [126]. *In vivo* studies demonstrated the ability of (IL-12)-scFvL19 to decrease tumor growth of the syngeneic C51 colon carcinoma in BALB/c mice or the syngeneic F9 teratocarcinoma in 129Sv mice. In addition to inducing IFN- γ secretion, (IL-12)-scFvL19 increased the antibody response in these mice [126]. In order to increase the antitumor activity of this

antibody–(IL-12) fusion protein, different strategies have been designed. The p40-scFvL19/scFvL19-p35 fusion protein (Figure 2A (V)) exploited the heterodimeric nature of IL-12 to display two scFv-L19 moieties onto mIL-12 [127]. The p40 subunit was fused to the amino-terminus of one scFv-L19, while the p35 subunit was fused to the carboxy-terminus of another scFv-L19. *In vivo* biodistribution assays for this antibody–(IL-12) fusion protein in immunocompetent mice bearing syngeneic F9 murine teratocarcinomas showed that p40-scFvL19/scFvL19-p35 is localized early in the tumor microenvironment. This p40-scFvL19/scFvL19-p35 retains IL-12 biological activity, which was similar to that of recombinant murine IL-12 (rmIL-12) but without side effects. The p40-scFvL19/scFvL19-p35 also demonstrated potent anti-tumor activity [127]. A single injection of this antibody–(IL-12) fusion protein into mice bearing the F9 murine terato-carcinoma resulted in substantial growth retardation and decreased tumor volume. Clinical trials to determine the safety and antitumor efficacy of these antibody–cytokine fusion proteins are being planned [127].

Human fibronectin has also been targeted by the antibody BC1, which recognizes an epitope adjacent to the ED-B domain. The region of human fibronectin recognized by BC1 is not homologous to mouse fibronectin therefore this antibody only targets human fibronectin unlike L19, which targets both mouse and human fibronectin. [128]. The variable regions of the antibody have been humanized by CDR grafting, generating the IgG1 antibody huBC-1 [129]. The p35 subunit of human IL-12 was fused to the carboxy-terminus of the antibody to generate huBC1-(IL-12) (Figure 2A (I)). The huBC1-(IL-12) fusion protein with the p35 subunit was expressed in murine myeloma cells (NS/0) that already expressed high levels of the p40 subunit of human IL-12 [129]. HuBC1-(IL-12) demonstrated 10-fold lower IL-12 activity compared with rmIL-12 as determined by the level of IFN- γ secreted by PBMC. Xenograft models are limited in SCID mice because the binding specificity of the BC1 antibody is limited to human fibronectin that is secreted by the human cancer cells and not the fibronectin that is present in the murine stroma. Despite this fact, intravenous injection of the antibody–cytokine fusion protein with murine IL-12 [huBC1-(mIL-12)] produced a decrease in lung metastases in SCID mice challenged subcutaneously with human PC3mm2 prostate carcinoma cells, A431 human skin cancer cells, or HT29 human colon cancer cells [129]. Although SCID mice lack T and B cells, one cycle of treatment with huBC1-(mIL-12) was effective against the tumor cells in the different xenogeneic models. Interestingly, the antitumor activity of huBC1-(mIL-12) was greater in comparison with the combination of huBC1 and rmIL-12, or rmIL-12 alone, suggesting that the physical linkage of the antibody to the cytokine is important for improving the antitumor activity by depositing the cytokine in the extracellular matrix (ECM) of the tumor microenvironment [129].

CD30 antigen expression in lymphoma has led to the development of an anti-CD30 antibody–(IL-12) fusion protein to enhance the antitumor response against human Hodgkin's lymphoma cells [130]. The anti-CD30 HRS3scFv, previously used to develop an antibody–(IL-2) fusion protein [82], was also used to develop HRS3scFv-hi-(IL-12) (Figure 2A (IV)). The amino-terminus of the murine p40 IL-12 subunit was fused to the murine IL-12 p35 subunit, which was then fused to the HRS3scFv using the sequence of amino acids that correspond to the human IgG1 hinge region. This antibody–(IL-12) fusion protein

binds to CD30⁺ L540 tumor cells (human Hodgkin's lymphoma) and retains its ability to induce IFN- γ secretion by activated T cells and resting NK cells. In addition, HRS3scFv-hi- (IL-12) promoted L1236 lymphoma cell lysis by NK cells *in vitro* [130].

Human IL-12 has also been fused to the variable region of the T84.66 murine antibody specific for the carcinoembryonic antigen (CEA), generating the fusion protein aCEA-(hIL-12) [131]. CEA is a tumor marker, especially for cancers of the gastrointestinal tract [132]. This antibody–cytokine fusion protein was generated by fusing the p40 subunit of human IL-12 to the amino-terminus of V_L domain of the monoclonal antibody T84.66, while the p35 domain was linked to the carboxy-terminus of the T84.66 V_H domain (Figure 2A (VI)) [131]. Additive-induced stepwise dialysis (AISD) was used to induce the functional folding of IL-12 in the fusion protein. The aCEA-(hIL-12) fusion protein induced the *in vitro* proliferation of PHA-PBMC. This antibody– (IL-12) fusion protein also retains the antigen binding. The aCEA-(hIL-12) fusion protein was able to induce LAK cells to inhibit the *in vitro* growth of TFK-1 cells (human bile duct carcinoma) [131]. The strategy used for refolding the aCEA-(hIL-12) could be applicable to the construction of different heterodimeric proteins, increasing the stabilization of the proteins as antibody–cytokine fusion proteins [131].

4. Antibody–(GM-CSF) fusion proteins

GM-CSF is a potent immunostimulatory cytokine with multiple functions including the stimulation of hematopoietic stem cells to generate granulocytes (neutrophils, eosinophils, and basophils) and monocytes. It also upregulates the expression of MHC class II molecules and increase the phagocytic activity and antigen-presenting abilities of APC [133,134]. These properties make GM-CSF a potent adjuvant in cancer immunotherapy [135,136]. In addition to their ability to recruit and activate dendritic cells (DC) and NK-T cells (natural killer T cells: cells that express an $\alpha\beta$ T-cell receptor [TCR] and cell surface molecules of NK cells), GM-CSF-transfected tumor cells induce the production of cytokines such as IL-12, which are required for CD4⁺ T cell activation that promotes a cellular immune response and antibody production [134]. The anti-tumor activity of GM-CSF has been demonstrated by its ability to induce tumor necrosis and rejection of tumors in mice vaccinated with irradiated GM-CSF-transfected tumor cells [135]. Subcutaneous GM-CSF administration has been associated with mild side effects including skin reaction at the site of injection, fever, bone pain and nausea [137], as well as severe side effects, including a fatality [138]. Systemic GM-CSF administration in melanoma patients has shown the ability of GM-CSF to enhance the immune response to melanoma-associated peptides inducing tumor rejection [139]. However, dose-dependent toxic side effects such as myalgia, fever, fluid retention and serosal effusion limit its use by systemic administration [140]. In order to decrease these side effects and enhance its antitumor activity, GM-CSF has been fused to antibodies against different targets. Table 3 summarizes the antibody–(GM-CSF) fusion proteins described in this review.

The transferrin receptor (TfR) is involved in the uptake of iron, which is necessary for cell proliferation, and is overexpressed in several types of malignancies [141]. Murine GM-CSF was fused to the carboxy-terminus of the ch17217 rat/mouse chimeric anti-mouse TfR

antibody (Figure 2A-(I)) [142]. In addition to retaining the antibody and GM-CSF activities, ch17217-(GM-CSF) demonstrated antitumor activity by decreasing the growth of murine hepatic metastases of neuroblastoma NXS2 cells and pulmonary metastases of murine colon carcinoma CT26 cells in syngeneic A/J and BALB/c mice, respectively [142]. This antibody–cytokine fusion protein can potentially be used against different tumor types due to TfR overexpression in a wide variety of cancer malignances.

Another targeted fusion protein is ch14.18-(GM-CSF) that consists of the mouse/human chimeric IgG1 antiganglioside GD2 antibody 14.18 fused to GM-CSF (Figure 2A (I)) [143]. This antibody–cytokine fusion protein retains the ability to induce ADCC and CDC activity [143]. The fusion of GM-CSF to the antibody increased its cytotoxicity against NMB7 neuroblastoma cells by granulocytes isolated from neuroblastoma patients, and increased ADCC-mediated by mononuclear cells from the same patients, in comparison with ch14.18 alone [143]. The humanized version, hu14.18-(GM-CSF), showed an increase in ADCC-mediated activity by polymorphonuclear leukocytes against neuroblastoma cells associated with FcγRII and macrophage antigen-1 (Mac-1)-dependent (a major polymorphonuclear β-integrin receptor) enhanced adhesion and degranulation, compared with antibody or GM-CSF alone [144].

In order to study the efficacy of antibody–(GM-CSF) fusion protein in NHL, human GM-CSF was fused to carboxy-terminus of chCLL-1, a mouse/human chimeric IgG1 with the variable region of the murine antibody Lym-2 [chCLL-1-(GM-CSF)] (Figure 2A (I)) [58]. This antibody is specific for a human MHC class II molecules, which is present in a high percentage of B-cell NHL and chronic lymphocyte leukemia [86]. This antibody–cytokine fusion protein retains GM-CSF bioactivity, evaluated by its ability to induce the formation of hematopoietic progenitor colonies from bone marrow mononuclear cells. This antibody–(GM-CSF) fusion protein also retains the ability to bind the antigens on the surface of cancer cells that express MHC class II. It also demonstrated ADCC *in vitro*, using human mononuclear cells as effectors. Biodistribution studies in xenograft models showed that chCLL-1-(GM-CSF) could specifically target ARH-77 [58], a human malignant B-cell line. However, since human GM-CSF does not exhibit bioactivity in mice [140], it was not possible to fully test the efficacy of this antibody–cytokine fusion protein in the mouse models used in this work [58].

Antibody–cytokine fusion proteins based on the human antibody L19, which targets the ED-B isoform of the fibronectin, have been shown to have therapeutic effects, providing strong support for their continued development. L19-(GM-CSF) was constructed in the diabody format (two non-covalently associated scFvs) by fusing mGM-CSF to the carboxy-terminus of each scFv, which improves the pharmacokinetic properties and the avidity of the antibody–cytokine fusion protein due to its bivalent nature [25,27,145]. This antibody–(mGM-CSF) fusion protein has good tumor organ distribution and dramatically reduced the tumor growth in 129SvEv mice injected subcutaneously with F9 murine teratocarcinoma or C51 murine colon adenocarcinoma cells. Additionally, this antibody–(mGM-CSF) fusion protein showed an antimetastatic effect in syngeneic F9 and C51 tumor-bearing mice. The depletion of CD4⁺ and CD8⁺ T cells demonstrated the importance of CD8⁺ T cells in the antitumor activity of L19-(GM-CSF) [145].

We have also constructed an antibody–(GM-CSF) fusion protein (Figure 2A (I)) consisting of mGM-CSF genetically fused to the carboxy-terminus of a human IgG3 with the variable regions of the humanized anti-HER2/*neu* antibody trastuzumab [146]. Because human GM-CSF is not active in mice [140], we used murine GM-CSF to construct this antibody–cytokine fusion protein, which retains the ability to bind HER2/*neu*, and also retains the cytokine activity supporting the growth of a GM-CSF-dependent murine myeloid cell line FDC-P1. In addition, the anti-HER2/*neu* IgG3-(GM-CSF) protein was capable of activating J774.2 murine macrophage cells enhancing their direct cytotoxic activity and also enhancing antibody-dependent macrophage-mediated tumor cell lysis. Moreover, anti-HER2/*neu* IgG3-(GM-CSF) was able to target the HER2/*neu*-expressing murine tumor CT26-HER2/*neu* and enhance both T_H1- and T_H2-mediated anti-HER2/*neu* immune responses. Importantly, treatment with this antibody–cytokine fusion protein resulted in significant retardation in the growth of subcutaneous tumors under conditions in which the antibody alone failed to confer protection [146]. These results suggest that the anti-HER2/*neu* IgG3-(GM-CSF) fusion protein will be useful in the treatment of human HER2/*neu*-expressing tumors.

5. Antibody–cytokine fusion proteins as adjuvants of antitumor response

The immune-stimulatory effect of IL-2 as an adjuvant has been used to increase the immune response to antigens in animal models [147–149] and in patients with renal cell carcinoma and melanoma [150,151]. In order to enhance the immunogenicity of soluble antigens, bovine serum albumin (BSA) complexed to the hapten DNS in combination with anti-DNS IgG3-(IL-2) [60] (previously described in this review), was injected intraperitoneally into BALB/c mice [64]. The humoral response in mice receiving BSA-DNS plus anti-DNS IgG3-(IL-2) increased compared with the treatment with BSA-DNS plus anti-DNS-IgG3 or BSA-DNS plus anti-Id IgG3-(IL-2). The use of a non-DNS-specific antibody control (anti-Id IgG3-(IL-2)) confirmed that the physical association between the antigen and the specific antibody–cytokine fusion protein is required to improve the immune response [64]. Antibodies elicited in mice treated with the combination of BSA-DNS plus anti-DNS IgG3-(IL-2) were able to recognize unconjugated BSA, suggesting a stimulator effect of IL-2 in the antibody–cytokine fusion protein against soluble antigens [64]. These results suggest that this adjuvant activity of antibody–cytokine fusion proteins can be harnessed against other targets, including tumor antigens.

The HER2/*neu* oncoprotein and especially its secreted extracellular domain (ECD^{HER2}) [152] found in the serum of patients with breast cancer [153] are related to their short survival. Inducing an immune response against self-antigens (such as HER2/*neu*) in humans or some animals is difficult, however the immunization of rats with rat HER2/*neu*-derived peptides but not the full protein, can elicit an immune response [154]. We have tested the capacity of our previously mentioned antibody–cytokine fusion proteins anti-HER2/*neu* IgG3-(IL-2), (IL-12)-IgG3 and IgG3-(GM-CSF) as enhancers of the immunogenicity of the HER2/*neu* protein in a prophylactic vaccination setting (Figure 4A) [155]. Subcutaneous injection of human ECD^{HER2} complexed to IgG3-(IL-2), (IL-12)-IgG3, or IgG3-(GM-CSF) induced long-term antitumor protection against TUBO cells, a BALB/c syngeneic mammary tumor cell model expressing rat HER2/*neu*. Thirty six days after tumor challenge, 17 out of 23 mice treated with the complexes (antibody–cytokine fusion protein-ECD^{HER2}) were still

alive compared with only 1 out of 24 mice treated with PBS, ECD^{HER2} or ECD^{HER2} plus anti-HER2/*neu* IgG3 remaining alive 5 weeks after tumor challenge (Figure 4B, C) [155].

In mice, the presence of T_H1 cytokines in CD4⁺ T cells stimulated with antigen can shift the isotype of the antibody produced by B cells to IgG2a, while T_H2 cytokines shift the isotype towards IgG1 [156]. Analysis of vaccinated mice showed that anti-HER2/*neu* antibody–cytokine fusion proteins complexed with ECD^{HER2} were able to induce different humoral responses to the antigen, depending on the cytokine motif induced by the complex. The treatment of mice with anti-HER2/*neu* IgG3-(GM-CSF) or anti-HER2/*neu* IgG3-(IL-2) plus ECD^{HER2} resulted in high titers of anti-ECD^{HER2} IgG1 (T_H2 response), as well as significant levels of IgG2a (T_H1 response) [155]. In contrast, ECD^{HER2} plus anti-HER2/*neu* (IL-12)-IgG3 vaccinated mice exhibited a significant increase in IgG2a titers (T_H1 response), with low IgG1 titers (T_H2 response) compared with the other treatments. In addition, sera from mice vaccinated with the antibody–cytokine fusion proteins complexed to ECD^{HER2} has been effective in generating an antiproliferative response against SK-BR-3 cells [155], a human breast cancer cell line overexpressing HER2/*neu* (Herceptin like activity) [157]. The passive transfer of immune sera from mice vaccinated with the antibody–cytokine fusion proteins complexed to ECD^{HER2} induced a significant retardation of tumor growth in mice challenged with TUBO cells [155]. We also demonstrated that a physical association between ECD^{HER2} plus anti-HER2/*neu* IgG3-(IL-2) or anti-HER2/*neu* IgG3-(GM-CSF) was required to elicit the most effective antitumor immune response against TUBO cells [158]. In addition, depletion studies demonstrated that CD4⁺ T cells and NK cells play important roles as effector cells in preventing tumor growth in mice vaccinated with ECD^{HER2} plus anti-HER2/*neu* IgG3-(IL-2) while CD8⁺ cells seem to play only a minor role (if any). Depletion of CD4⁺ or CD8⁺ T cells resulted in only partial loss of protection in the case of mice vaccinated with ECD^{HER2} plus anti-HER2/*neu* IgG3-(GM-CSF) while removal of NK cells has no effect on protective immunity [158]. Using B-cell-deficient mice, we demonstrated that B cells play a crucial role in the antitumor immune response induced by vaccination with ECD^{HER2} plus anti-HER2/*neu* IgG3-(IL-2) or ECD^{HER2} plus anti-HER2/*neu* IgG3-(GM-CSF) [158].

The low immunogenicity of ECD^{HER2} has been explained by the fact that although this molecule is readily internalized by DC, it is poorly processed due to its high level of glycosylation, which results in early endosomal retention and recycling back to the cell surface [159]. We have shown that anti-HER2/*neu* IgG3-(IL-2) and anti-HER2/*neu* IgG3-(GM-CSF) promote ECD^{HER2} processing and presentation by DC [160], which is consistent with the observation that physical association between the antibody–cytokine fusion protein and the antigen ECD^{HER2} was required to elicit the most effective antitumor activity [158]. Because a readout system using ECD^{HER2}-specific T cells was not available to us, ovalbumin fused to ECD^{HER2} (OVA-ECD^{HER2}) was used to study the antigen processing and presentation of complexes of OVA-ECD^{HER2} and anti-HER2/*neu* IgG3-cytokine fusion proteins in bone marrow-derived dendritic cells (BMDC) [160]. The authors have confirmed that anti-HER2/*neu* IgG3-(IL-2) or anti-HER2/*neu* IgG3-(GM-CSF) complexed to OVA-ECD^{HER2} promoted efficient antigen presentation by BMDC, as demonstrated by an increase in IL-2 production as a consequence of OVA-specific T-cell activation. This effect

was not observed with the combination of OVA-ECD^{HER2} with the antibody alone, or plus the recombinant cytokines, confirming that the IL-2 and GM-CSF need to be attached to the anti-HER2/*neu* IgG3 to induce effective antigen presentation [160].

In order to evaluate the trafficking of ECD^{HER2} to antigen-processing compartments in murine BMDC, Alexaflour 488-labeled ECD^{HER2} was used for confocal microscopy analysis. After 90 min of incubation to allow the antigen internalization, 48% co-localization of the ECD^{HER2} antigen with TfR, an early endosome marker, and only 8% co-localization of the antigen with the lysosome-associated membrane glycoprotein 1 (LAMP-1) marker for lysosomal compartments were observed, demonstrating that ECD^{HER2} is retained in early endosomes [160]. The authors have also found that, in contrast with the OVA antigen alone, OVA-ECD^{HER2} does not efficiently traffic to the lysosomal compartments of BMDC [160]. These results were consistent with observations obtained using human DC [159]. In contrast, after a 60-min incubation of BMDC with ECD^{HER2} complexed to anti-HER2/*neu* IgG3-(IL-2), 69% of co-localization was with LAMP-1, and 32% of co-localization was with H2-DM (equivalent to human HLA-DM; a marker of class II compartments [161]) indicated that ECD^{HER2} complexed with anti-HER2/*neu* IgG3-(IL-2) is redirected to antigen processing compartments [160]. These studies demonstrate that the complex ECD^{HER2} with anti-HER2/*neu* IgG3-(IL-2) promotes the uptake and presentation of the specific antigen by BMDC to T cells. This altered trafficking was not observed with the complex of anti-HER2/*neu* IgG3 and ECD^{HER2} [160]. These observations are in agreement with the necessity of a physical linkage between the antibody–cytokine fusion protein complexed to the antigen for the alteration in trafficking and antigen presentation through BMDC, and suggest that IL-2 receptors play a critical role in the activation of the immune response, as we propose in the model shown in Figure 5. Although ECD^{HER2} uptake in the anti-HER2/*neu* IgG3-(GM-CSF) complex is rapidly decreased and lower than that of the complex with anti-HER2/*neu* IgG3-(IL-2) and ECD^{HER2} [160], T-cell activation assays suggest that a relatively small amount of the internalized ECD^{HER2} complexed with anti-HER2/*neu* IgG3-(GM-CSF) is required for antigen processing and presentation by BMDC to induce efficient T-cell activation [160].

6. The use of combinations of cytokines in the context of antibody–cytokine fusion proteins: bi-functional antibody–cytokine fusion proteins and combination of mono-functional antibody–cytokine fusion proteins

Several reports have shown an advantage in using combinations of cytokines, compared with their single use, for orchestrating an effective anticancer immune response. Combinations of IL-2 and IL-12 [162], IL-2 and IL-4 [163], GM-CSF and IL-4 [164] or GM-CSF and IL-12 [165] have shown a significant enhanced antitumor activity in preclinical models. Therefore, in recent years several groups have begun to study the potential synergistic activity of the combination of two cytokines fused to the same antibody molecule [bi-functional-antibody–cytokine fusion proteins (bi-antibody–cytokine fusion proteins)] (Figure 2B) or as a combination of mono-functional antibody–cytokine fusion proteins (mono-antibody–cytokine fusion proteins) with single cytokines, to enhance the

immune response in preclinical cancer settings [124,125,166–169]. Table 4 summarizes the bi-antibody–cytokine fusion proteins described in this review.

To explore the potential of bi-antibody–cytokine fusion proteins as cancer therapies, the murine cytokines IL-12 and IL-2 were fused to the same humanized anti-EpCAM IgG1 [166]. These bi-antibody–cytokine fusion proteins target the epithelial cell adhesion molecule known as EpCAM, KSA, or KS1/4, which is overexpressed in human epithelial carcinomas [97–100]. Different antibody–cytokine fusion protein configurations were designed against EpCAM. Two of them were constructed in which IL-12 was fused to the carboxy-terminus of the Fc region and IL-2 was fused in tandem to the carboxy-terminus of the IL-12 p40 subunit, where p35 and p45 heterodimeric structure was stabilized either by disulfide bond [IgG1-(p35)-(p40-IL-2)] (Figure 2B (III)) or by a flexible linker (IgG1-p35-linker-p40-(IL-2)) (Figure 2B (III)). In the other two configurations, IL-2 was fused to the carboxy-terminus of the heavy chain and scIL-12 was fused either to the amino-terminus of the heavy chain [(p35-linker-p40)-linker-IgG1-(IL-2)] (Figure 2B (I)) or to the amino-terminus of the light chain [p40-linker-p35-kappa + IgG1-(IL-2)] (Figure 2B (II)) [166]. All of these bi-antibody–cytokine fusion proteins showed effective antigen binding and cytokine activity but this activity was lower than that of free cytokines. Importantly, these bi-antibody–cytokine fusion proteins exhibited synergistic activity to induce IFN- γ secretion by PHA-activated PBMC *in vitro*, similar to the synergistic activity observed with an equimolar combination of rhIL-12 and rhIL-2 [166]. Finally, *in vivo* studies carried out in immunocompetent mice in a subcutaneous syngeneic Lewis lung carcinoma tumor model that expresses human EpCAM (LLC-EpCAM), showed that the treatment with KS-(IL-12/IL-2) in the tandem configuration (Figure 2B (III)) exhibits significant antitumor activity, similar to that observed when the combination of KS-(IL-2) (Figure 2A (I)) and KS-(IL-12) (Figure 2A (I)) was used, although no animal showed complete tumor regression [166].

The therapeutic potential of the combination of IL-2 and GM-CSF was investigated in the context of a different bi-antibody–cytokine fusion protein against human EpCAM [168,169]. Different fusion proteins were constructed as a heterominibody in which GM-CSF was fused to the carboxy-terminus of the C_H1 domain of human IgG1, while IL-2 was fused to the carboxy-terminus of the C_L domain (kappa, C_K), plus a scFv fused in the amino-terminus of each constant region. Murine IL-2 and GM-CSF fused to the scFv from the murine anti-EpCAM antibody 3B10 (mDCH) [169], or human IL-2 and GM-CSF fused to the human anti-EpCAM antibody HD70 (DCH) [168] were further fused to the C_H1 or C_L regions through the extended flexible IgG3 hinge region [scFv-hinge-C_H1-(GM-CSF)/scFv-hinge-C_K-(IL-2)] (Figure 2B (V)). These bi-antibody–cytokine fusion proteins exhibited the ability to bind the targeted antigen and retained the biological activity of murine IL-2 and GM-CSF to induce CTLL-2 and FDC-P1 cell proliferation respectively [168,169]. DCH induced human PBMC and T-cell-mediated cytotoxicity of Kato III cells (from a human gastric carcinoma that over-express the EpCAM antigen) [168]. The mDCH-3B10 fusion protein, tested by a systemic treatment with the bi-antibody–cytokine fusion protein, induced significant inhibition of pulmonary metastases of mouse colon carcinoma CT26 expressing human KSA (CT26-KSA) cells in immunocompetent BALB/c mice [169]. Mono-antibody–cytokine fusion proteins fused either to GM-CSF or IL-2 showed the same level of

protection as that of the bi-antibody–cytokine fusion protein, which failed to confer synergistic protection by the combination of GM-CSF and IL-2, fused to the same antibody–cytokine fusion protein [168,169].

Another bi-antibody–cytokine fusion protein combined the murine cytokines IL-12 and TNF- α fused to the human scFv of the antibody L19, which is specific for the ED-B domain of the B-FN isoform of fibronectin. The ED-B domains of mice and humans are 100% homologous and thus antibody L19 targets both mouse and human B-FN. This bi-antibody–cytokine fusion protein was constructed in the sequence [(p40-linker-p35)-linker-scFv-linker-(TNF- α)], where IL-12 was fused to the amino-terminus of the L19 scFv with one TNF- α monomer fused to the carboxy-terminus of the (IL-12)-scFv (Figure 2B (IV)). This bi-antibody–cytokine fusion protein was active *in vitro* but it failed to localize in the tumors and exhibited limited therapeutic activity in immunocompetent 129sV mice bearing a subcutaneous syngeneic F9 teratocarcinoma [125]. This is most probably due to the fact that TNF- α naturally forms a non-covalent trimer and thus, this bi-antibody–cytokine fusion protein resulted in a large protein complex consisting of three bi-antibody–cytokine fusion proteins non-covalently linked through the three TNF- α monomers. In contrast, systemic administration of the combination of (IL-12)-L19 and L19-(TNF- α) but not the bi-antibody–cytokine fusion protein, was able to target the tumor and produce a pattern of total histologic tumor regression in F9 murine teratocarcinoma-bearing mice, due to the presence of lymphocytes, LAK cells, NK cells and macrophages [125]. In addition, the combination of these mono-antibody–cytokine fusion proteins exhibited synergistic antitumor activity and could more effectively eliminate the tumor than the single use of the antibody–cytokine fusion proteins or the bi-antibody–cytokine fusion protein, suggesting that the combination of these two cytokines in different antibody–cytokine fusion proteins may be advantageous for eliciting an effective local antitumor activity [125].

The combination of antibody–(IL-2) and antibody–(IL-12) fusion proteins fused to antibodies targeting CD30 was evaluated as a potential therapeutic for Hodgkin's lymphoma [170]. An antibody–(IL-2) fusion protein was generated by the fusion of murine anti-CD30 scFv from the clone HRS3 to the Fc region of human IgG1 (C_H2-C_H3 domains), and subsequently fused either to human IL-2 [HRS3-scFv-Fc-(IL-2)] (Figure 2A (III)) or to murine scIL-12 [HRS3-scFv-Fc-(IL-12)] (Figure 2A (III)). The antibody–cytokine fusion proteins bound specifically to CD30, and their cytokine moieties exhibited biological activity at the same level as their recombinant IL-2 and IL-12 counterparts *in vitro* [170]. The activation of resting NK cells was increased by co-incubation with HRS3-scFv-Fc-(IL-2) plus HRS3-scFv-Fc-(IL-12) [170]. In addition, the combination enhanced the cytotoxicity of CD30⁺ tumor cells and IFN- γ secretion by NK cells, suggesting that this approach may be suitable for the immunotherapy of Hodgkin's lymphoma [170].

We have developed two anti-HER2/*neu* bi-antibody–cytokine fusion proteins. They consist of the variable region of the humanized antibody trastuzumab and the constant region of human IgG3 in which mscIL-12 was genetically fused through a flexible linker (Gly₄-Ser)₃ to the amino-terminus of the V_H, and human IL-2 or murine GM-CSF was fused at the C_{H3} domain of the same antibody resulting in anti-HER2/*neu* (IL-12)-IgG3-(IL-2) and anti-HER2/*neu* (IL-12)-IgG3-(GM-CSF) respectively (Figure 2B (I)) [70]. Both bi-antibody–

cytokine fusion proteins retained their ability to bind the antigen (ECD^{HER2}) as well as the biological activity of the cytokines. The *in vivo* protection elicited by the treatment with the either combination of mono-antibody–cytokine fusion proteins or bi-antibody–cytokine fusion proteins targeting HER2/*neu* expressed on tumor cells was explored in two different tumors settings: i) as adjuvants of human ECD^{HER2} protein in a prophylactic vaccination setting against TUBO, a BALB/c syngeneic mammary tumor model expressing rat HER2/*neu* [167]; and ii) as direct therapeutics in local treatment of syngeneic mammary and colon cancer models expressing human HER2/*neu* in the peritoneal cavity [70]. In the prophylactic vaccination setting, mice were immunized with complexes consisting of human ECD^{HER2} protein and the combination of cytokines GM-CSF and IL-12, or IL-2 and IL-12 as either bi-antibody–cytokine fusion proteins or two single antibody–cytokine fusion proteins playing the role of adjuvants, and subsequently challenged with TUBO cells [167]. Vaccination with ECD^{HER2} plus the anti-HER2/*neu* bi-antibody–cytokine fusion proteins-(IL-2/IL-12) or anti-HER2/*neu* bi-antibody–cytokine fusion proteins-(GM-CSF/IL-12) activated both T_H1 (IgG2a) and T_H2 (IgG1) arms of the immune system more than ECD^{HER2} complexed to mono-antibody–cytokine fusion proteins of IL-2, GM-CSF or IL-12. In addition, vaccination with the combination of cytokines, either as bi-antibody–cytokine fusion proteins or as two mono-antibody–cytokine fusion proteins, resulted in 59% of mice being long-term survivors (19 of 32 mice after 250 days), compared with 29% of mice vaccinated with a single antibody–cytokine fusion protein (7 of 24 mice after 250 days), or 0% of mice vaccinated with IgG3 complexed with ECD^{HER2} or ECD^{HER2} alone (0 of 8 mice after 250 days) [167]. The overall increase in the number of long-term survivors using bi-antibody–cytokine fusion proteins or the combination of mono-antibody–cytokine fusion proteins suggests that there is an advantage over their single use. In addition, it may be an advantage that one antibody–cytokine fusion protein (bi-functional) alone can achieve the effect of the combination of two different antibody–cytokine fusion proteins (mono-functional), since reduced development and production costs may result [167].

The GM-CSF/IL-12 and IL-2/IL-12 antibody–cytokine fusion proteins combinations were also studied in the context of a direct therapy against human HER2/*neu* expressing malignancies [70]. In these studies, mice were challenged intraperitoneally with different epithelial syngeneic cancer models expressing human HER2/*neu* (CT26-HER2/*neu* and D2F2/E2 syngeneic to BALB/c mice, and MC38-HER2/*neu* syngeneic to C57BL/6 mice) and treated with anti-HER2/*neu* (IL-12)-IgG3, anti-HER2/*neu* (IL-12)-IgG3-(GM-CSF) or anti-HER2/*neu* (IL-12)-IgG3-(IL-2) [70]. The anti-HER2/*neu* (IL-12)-IgG3 fusion protein showed similar levels of protection in all tumor models but the bi-antibody–cytokine fusion proteins exhibited differential protection, where in mice treated with D2F2/E2, the survival was similar to that for anti-HER2/*neu* (IL-12)-IgG3 but slightly reduced for MC38-HER2/*neu*, and significantly reduced for CT26-HER2/*neu* challenges [70]. However, the combination of anti-HER2/*neu* (IL-12)-IgG3 plus anti-HER2/*neu* IgG3-(GM-CSF) achieved the most significant protection, with 100% long-term survivors in mice challenged intraperitoneally with D2F2/E2 cells and 75% in those challenged with CT26-HER2/*neu* cells [70]. This protection was retained after subcutaneous rechallenge with the same cell line more than 4 months later, demonstrating that the immune response was long-term. In addition, a later challenge of D2F2/E2 survivors with the parental cell line D2F2 not

expressing human HER2/*neu* showed 100% survival, suggesting the generation of immunity against antigens different from that one originally targeted [70,124]. The D2F2 mammary cancer long-term survivors treated with anti-HER2/*neu* (IL-12)-IgG3 plus anti-HER2/*neu* IgG3-(GM-CSF) were subsequently challenged subcutaneously with the CT26-HER2/*neu* (10 months after the initial challenge) and the parental CT26 cell line (15 months after the initial challenge), in both cases showing lower but significant protection [124]. These observations illustrate the potential of targeted immunotherapies for the generation of broad long-term immunological memory [124]. Our results suggest that this approach may prevent the occurrence of metachronous cancer, a condition in which a patient exhibits a subsequent primary cancer with a behavior and histology different from that of the original tumor [171,172]. These events are becoming more frequent since improvement in the therapy of cancer has resulted in extended life expectancy of patients.

7. Expert opinion

The development of monoclonal antibody technology has enabled the generation of therapeutic antibodies with the ability to selectively target antigens expressed on the surface of cancer cells, making them a fundamental tool for the immunotherapy of cancer. In order to decrease their immunogenicity and enhance their anticancer effects in patients, these antibodies have evolved from murine monoclonal antibodies to chimeric, humanized, and fully human antibodies. Today, there are several recombinant antibodies that have been approved by the FDA for the treatment of cancer.

The versatility of the domain structure of antibodies together with advances in genetic engineering and expression systems have led to the development of antibodies fused to immunostimulatory cytokines. These antibody–cytokine fusion proteins have novel properties and include antibodies with specificity for tumor antigens fused to cytokines such as IL-2, IL-12 and GM-CSF. They successfully combine the properties of the antibodies with those of the cytokines. The increased half-life of the fused cytokine and the antibody specificity for the tumor allows the localization of the immunostimulatory cytokine in the tumor microenvironment without the need for high-dose systemic injection of the cytokine, which is associated with severe side effects. Approaches based on antibody–cytokine fusion proteins have two important goals: to enhance the antitumor activity of the antibody and to elicit a secondary humoral and/or cell-mediated immune response that can potentially induce the complete elimination of primary and/or metastatic tumors. In addition, these novel molecules can be used as direct antitumor agents and as adjuvants of cancer vaccines. Importantly, these strategies are not mutually exclusive.

Antibody based therapeutics are expensive to develop and manufacture, and frequently require large doses to reach the therapeutic threshold. In addition, the royalty payments for the use of intellectual property that are frequently necessary to allow the generation, optimization and production of antibodies multiply their cost. Since the earliest antibody–cytokine fusion proteins were developed in the 1990s, it is to be expected that it will take time and resources until their safety and efficacy has been properly evaluated in a clinical setting. However, the number of antibody–cytokine fusion proteins has been increasing and the field is mature enough to start moving from the ‘bench to the bedside’. In fact, a few

antibody–cytokine fusion proteins have moved into clinical trials. These clinical trials have been led by the antibody–(IL-2) fusion proteins, which have been the most studied in preclinical settings and contain the cytokine IL-2, a potent immunostimulator with a long record of effective antitumor activity in patients with melanoma and renal cell carcinoma.

Further advances in the knowledge of the tumor-host interaction and the mechanisms involved in tumor tolerance will help to identify optimal immunostimulators and more specific TAAs. The possibility of engineering multiple cytokines into one antibody, or of combining different antibody–cytokine fusion proteins specific for the same or different TAAs promise, in principle, a new age of immunotherapy in which it will be possible to ‘fine tune’ an optimal orchestration of the immune response against the tumor and eventually elicit permanent antitumor immunity. These strategies, in rational combination with other antitumor agents, are expected to increase the chances of curing patients with cancer.

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Declaration of interest

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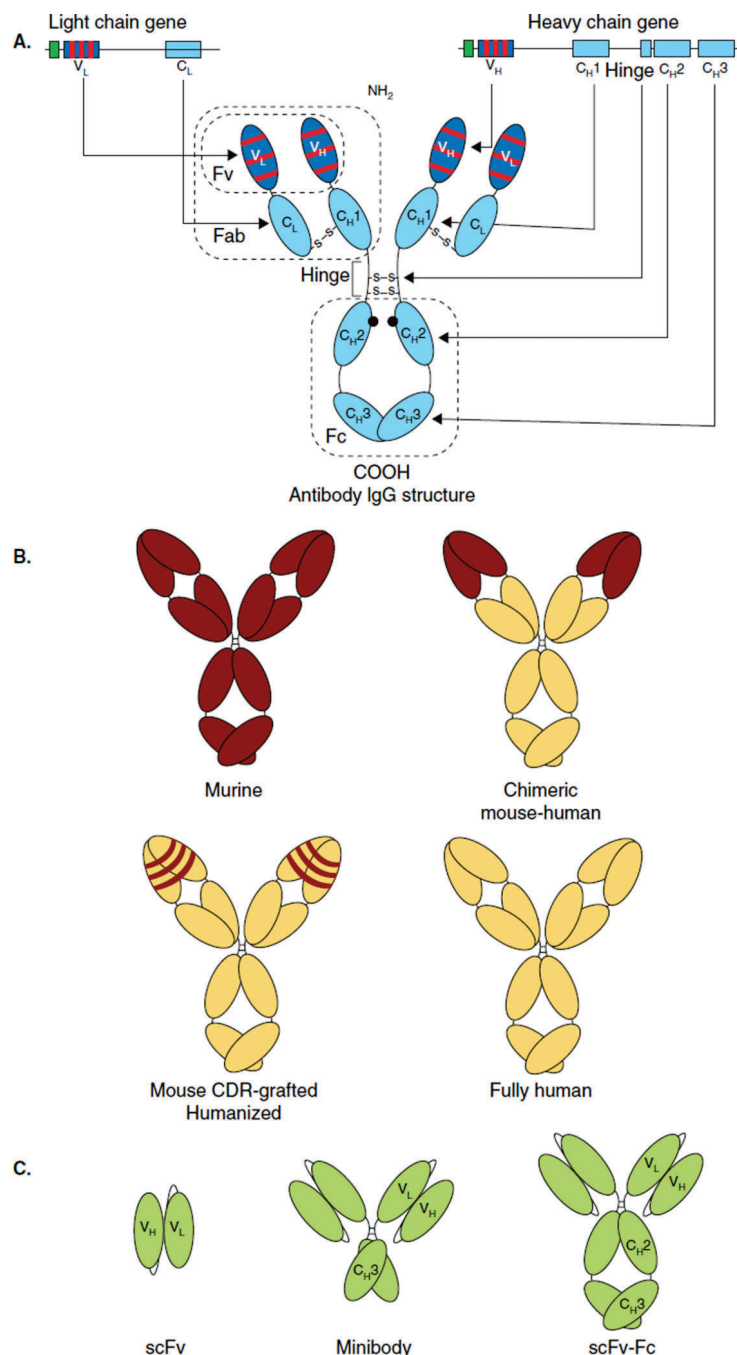


Figure 1. Schematic representation of an IgG molecule and its genetic engineering evolution
A. The general structure of an IgG antibody. In the top, the gene of IgG consists of exons represented by boxes and introns represented by the central line. The green boxes indicate the signal peptide that is required for antibody secretion. The CDR frameworks that encode the hypervariable regions are represented in wide red lines within the variable regions of both the heavy and light chains (boxes in dark blue). These hypervariable regions are localized in the variable regions (Fv), which consist of (V_L - V_H)₂ in the amino-terminus of the protein. The constant region includes the domains C_L (in the light chain) and C_{H1} , C_{H2}

and C_H3 (in the heavy chain). The light and heavy chains are linked by a disulfide bond. Fab₂ and Fc are linked by the hinge region that confers flexibility to the molecule. Human IgG has one glycosylation site (black circle) in each C_H2 domain. **B.** Antibody evolution: murine (brown), chimeric (murine variable regions and human constant regions), humanized antibodies [murine CDRs (brown lines)] and fully human antibodies (light brown). **C.** Different fragments of an antibody can be generated by genetic engineering. Single chain Fv (scFv) fragments consist of V_L and V_H domains linked by a peptide linker. A minibody consists of two scFv domains linked to C_H3 domains by the hinge. The scFv-Fc format consists of two scFv regions linked to the hinge region followed by C_H2-C_H3 domain.

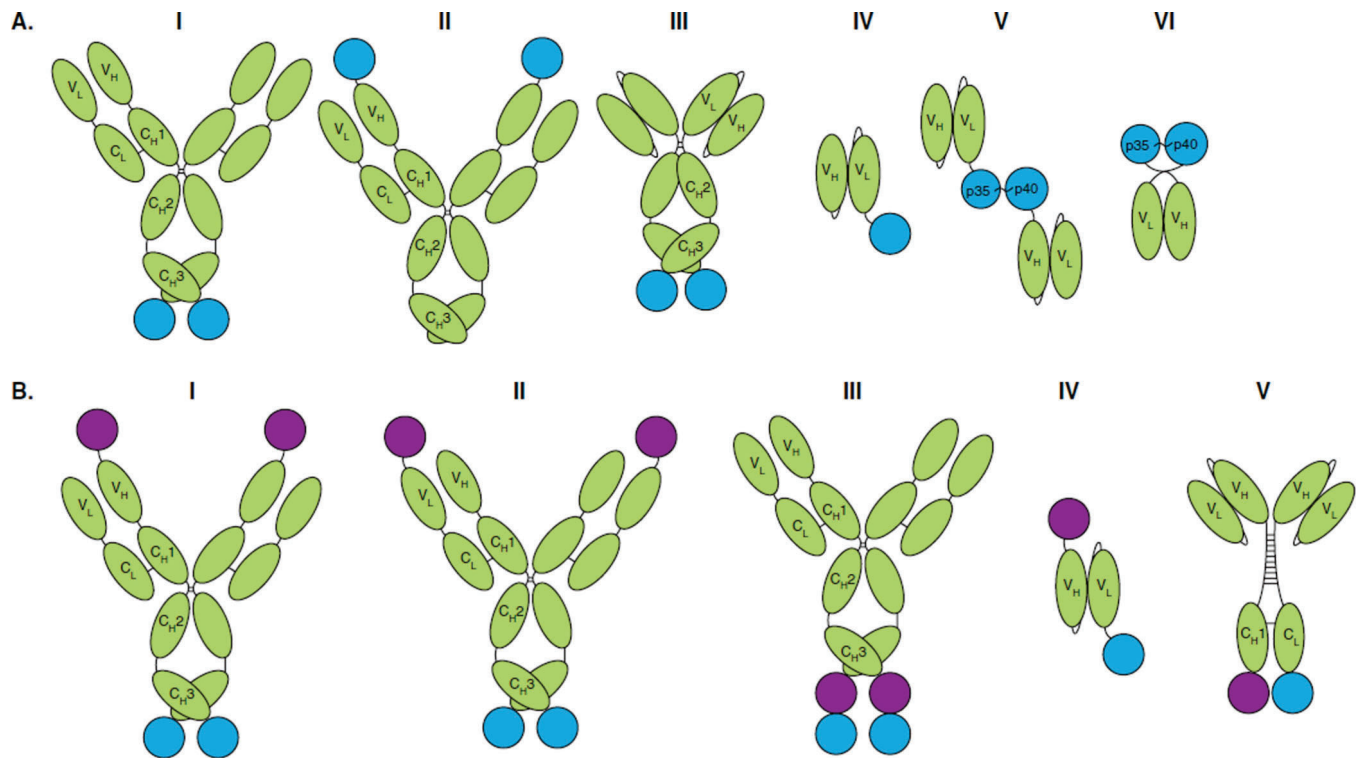


Figure 2. Schematic representation of antibody-cytokine fusion proteins and bi-antibody-cytokine fusion proteins

Cytokines can be fused to different fragments of the antibody to generate an antibody-cytokine fusion protein. **A.** A cytokine (blue circle) can be fused to the carboxy-(I) or amino-terminus (II) of the full antibody or to different antibody fragments (III-VI) to generate an antibody-cytokine fusion protein. **B.** Two different cytokines (blue and purple circles) can be fused in a single (I-II and IV-V) or in tandem format (III) at the ends of the same antibody or fragment. The domain structure of the antibodies facilitates their use to develop bi-functional antibody-cytokine fusion proteins (bi-antibody-cytokine fusion proteins) that can consist in cytokines fused to amino-terminus of the heavy (I) or light chain (II) and the second cytokine fused to the carboxy-terminus of the heavy chain. The dual cytokine heteromimibody (DCH) (V), consists of a cytokine fused to the C_H1 domain, the second cytokine fused to the C_L domain linked both to two scFv by IgG3 hinge region.

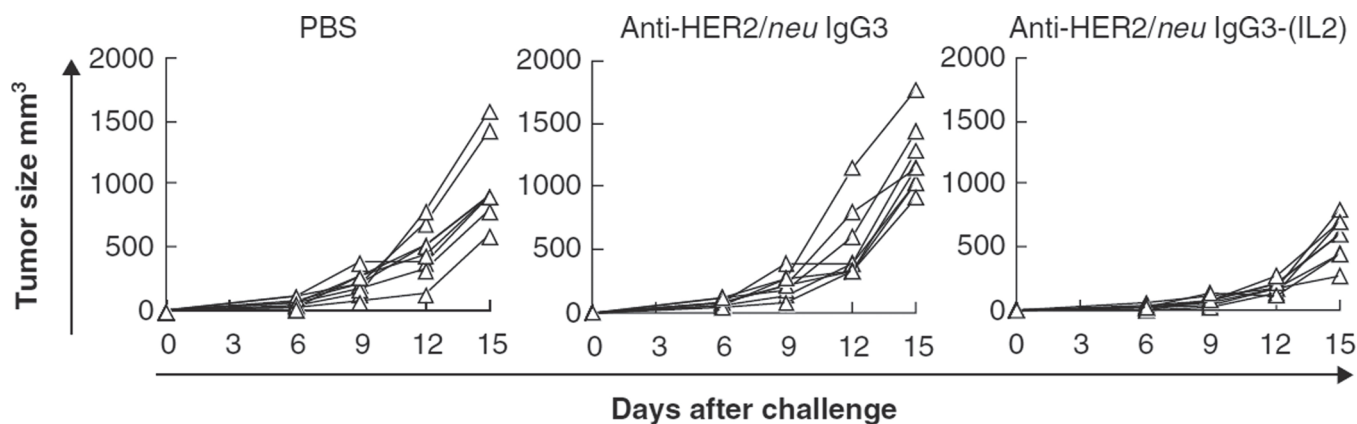


Figure 3. Antitumor activity of anti-HER2/neu IgG3-(IL-2)

10^6 CT26-HER2/*neu* cells were injected subcutaneously into the right flank of female BALB/c mice. The following day groups of 8 mice received 5 daily intravenous injections of PBS containing 20 μ g of anti-HER2/*neu* IgG3-(IL-2), the equivalent molar amount of anti-HER2/*neu* IgG3 or nothing. Tumor growth was measured with a caliper every 3 days until day 15. Tumor volume was calculated for each mouse of each treatment group, PBS (left), anti-HER2/*neu* IgG3 (center), and anti-HER2/*neu* IgG3-(IL-2) (right). Reprinted from Human Antibodies, 10, Penichet ML, Dela Cruz JS, Shin S et al, A recombinant IgG3-(IL-2) fusion protein for the treatment of human HER2/*neu* expressing tumors, 43–49., Copyright (2001), with permission from IOS press.

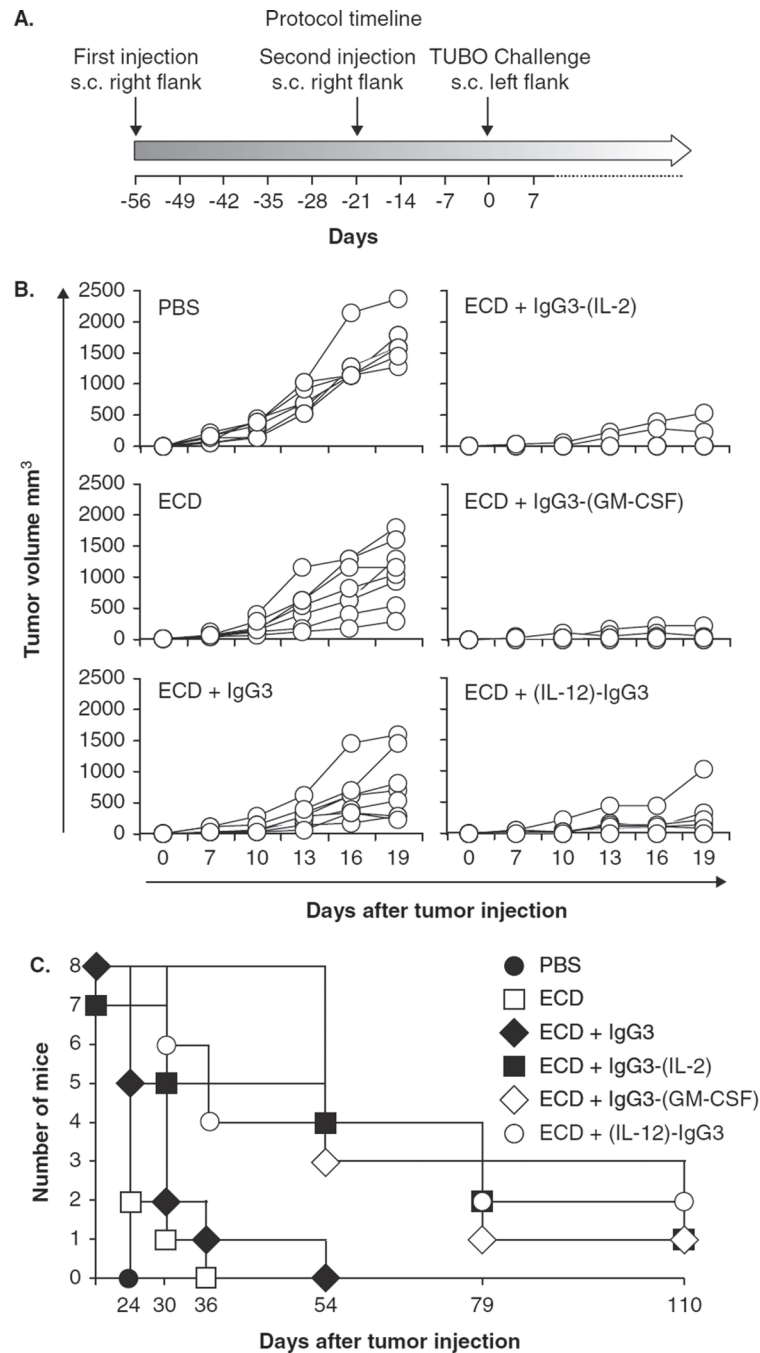


Figure 4. Tumor growth in vaccinated mice challenged with TUBO cells
 (A) Time course schedule of the vaccination procedure and the tumor challenge. Groups of 8 female BALB/c mice were injected subcutaneously in the right flank 56 and 21 days before the tumor challenge with either PBS alone, 8 μ g ECD^{HER2} alone (ECD), 8 μ g ECD^{HER2} plus 14 μ g anti-HER2/*neu* IgG3 (IgG3), or equivalent molar quantities of ECD^{HER2} plus anti-HER2/*neu* IgG3-(GM-CSF) [IgG3-(GM-CSF)], ECD^{HER2} plus anti-HER2/*neu* IgG3-(IL-2) [IgG3-(IL-2)] or ECD^{HER2} plus anti-HER2/*neu* (IL-12)-IgG3 [(IL-12)-IgG3]. Previously ECD^{HER2} plus the respective antibodies were incubated for 2 h at 37°C to allow complexes

to form. Fifty six days after the first injection (day 0), 10^6 TUBO cells were injected subcutaneously in the left flank. Mice with tumors exceeding 1.5 cm in diameter at the time of inspection were euthanized and considered to have not survived the tumor challenge. (B) Individual tumor sizes were measured using a caliper, starting on day 7 and every 3 days until day 19. (C) Survival Kaplan-Meier curve. Adapted from Vaccine, 21, Dela Cruz JS, Lau SY, Ramirez EM et al, Protein vaccination with the HER2/*neu* extracellular domain plus anti-HER2/*neu* antibody–cytokine fusion proteins induces a protective anti-HER2/*neu* immune response in mice, 1317–1326., Copyright (2003), with permission from Elsevier Science Ltd.

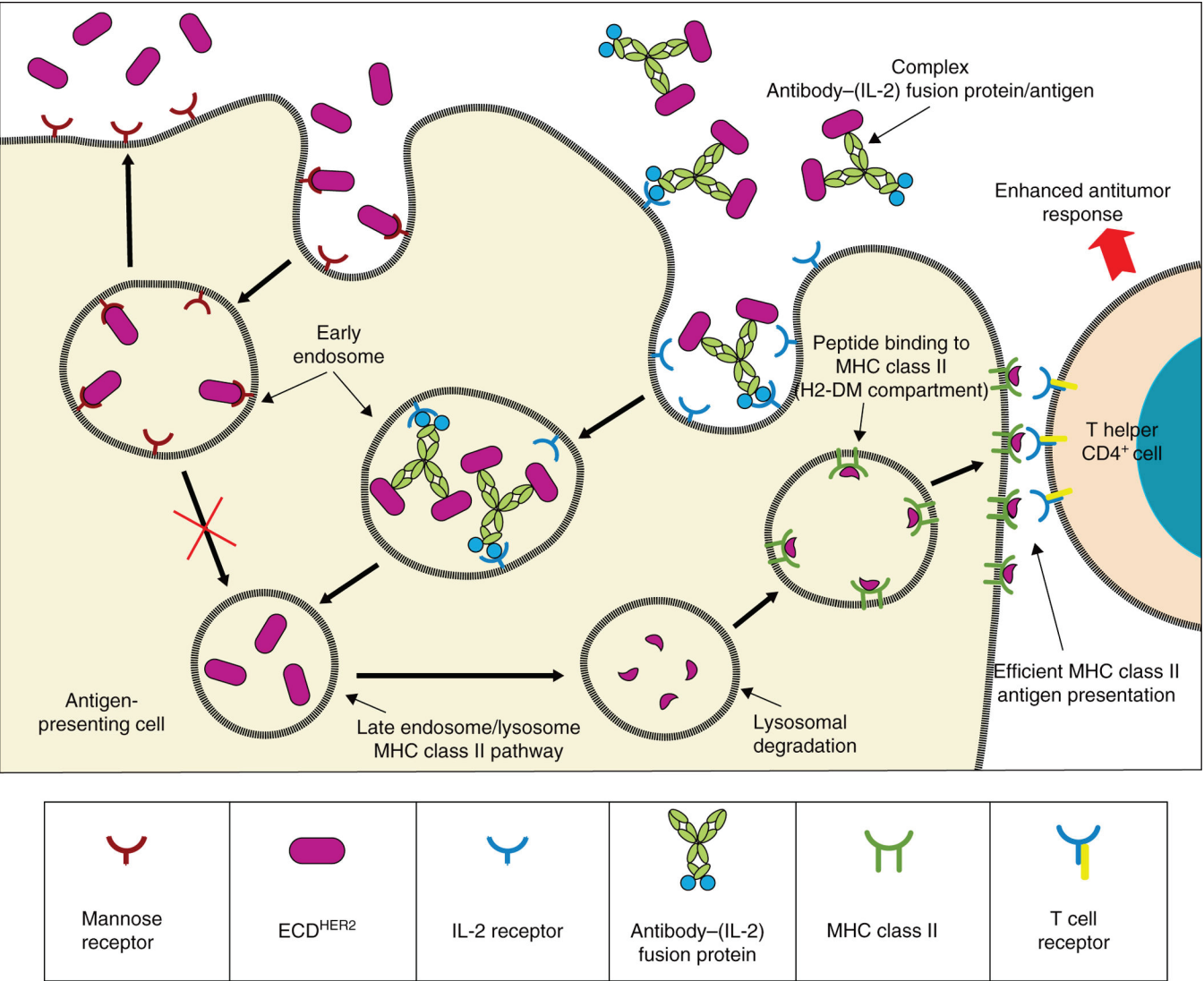


Figure 5. Schematic model of the mechanism involved in the antitumor effect of vaccination with complexes consisting of ECD^{HER2} and anti-HER2/neu IgG3-(IL-2) in DC
Anti-HER2/neu IgG3-(IL-2) can bind HER2/neu expressed as soluble antigen ECD^{HER2}. This soluble antigen is poorly processed because of its high level of glycosylation that results in its binding to the mannose receptor, early endosomal retention, and recycling back to the cell surface. However, the interaction between the complex of ECD^{HER2} plus anti-HER2/neu IgG3-(IL-2) with IL-2 receptor (IL-2R) expressed on the cell surface induces ECD^{HER2} internalization and trafficking to the late endosome/lysosome MHC class II pathway facilitating the processing and presentation to CD4⁺ T cells via MHC class II molecules in order to stimulate the immune response against HER2/neu positive tumor cells.

Table 1

Antibody-(IL-2) fusion proteins.

Antibody fusion protein	Targeted antigen	Tumor *	Status	Ref.
CD95 scFv-Fc-(IL-2)	MUC-1	Mammary cancer	<i>In vitro</i>	[59]
ch14.18-(IL-2)	GD2	Neuroblastoma and melanoma	Animal models	[89,91–94]
hu14.18-(IL-2) (EMD 273063)	GD2	Neuroblastoma and melanoma	Clinical trials	[90,95,96]
HRS3 scFv-Fc-(IL-2)	CD30	Hodgkin's lymphoma	Animal models	[82,170]
huKS1/4-(IL-2)	EpCAM	Colon cancer	Animal models	[102–106]
huKS-(IL-2) (EMD 273066)	EpCAM	Ovarian and prostate cancer	Clinical trials	[107,108]
(IL-2)-183B2scFv	OC183B2	Ovarian cancer	<i>In vitro</i>	[87]
DI-Leu16-(IL-2)	CD20	NHL	Animal models	[85]
anti-HER2/ <i>neu</i> IgG3-(IL-2)	HER2/ <i>neu</i>	Mammary and colon cancer	Animal models	[69,70,124,155,158,160,167]
anti-erbB2scFv-Fc-(IL-2)	HER2/ <i>neu</i>	Ovarian cancer	Animal models	[73,74]
L19-(IL-2)	ED-B	Colon cancer, teratocarcinoma, small-cell-lung cancer and other solid tumors	Clinical trials	[77,78]
anti-Id IgG3-(IL-2)	Id	B-cell lymphoma	Animal models	[57]
chS5A8-(IL-2)	Id	B-cell lymphoma	Animal models	[66]
scFv S5A8-(IL-2)	Id	B-cell lymphoma	Animal models	[66]
chCLL-1-(IL-2)	MHC-II	NHL	Animal models	[58]

* Tumor targeted by the antibody–cytokine fusion protein in animal models or in patients as described in the given references.

CD20: Cluster of differentiation 20; CD30: Cluster of differentiation 30; ED-B: Extra domain-B of the B-FN isoform of human fibronectin; EpCAM: Epithelial cell adhesion molecule; GD2: Disialoganglioside; HER2/*neu* (also known as erbB-2): Human EGF receptor 2; Id: Idiotype; MHC-II: Major histocompatibility complex class II; MUC-1: Mucin-1; NHL: Non-Hodgkin's lymphoma; OC183B2: Ovarian carcinoma-associated antigen; scFv: Single-chain Fv.

Table 2

Antibody–(IL-12) fusion proteins.

Antibody fusion protein	Targeted antigen	Tumor *	Status	Ref.
anti-HER2/ <i>neu</i> (IL-12)-IgG3	HER2/ <i>neu</i>	Mammary and colon cancer	Animal models	[70,119,123,124,155,167]
(IL-12)-L19	ED-B	Colon cancer and teratocarcinoma	Animal models	[125–127]
p40-scFvL19/scFvL19-p35	ED-B	Teratocarcinoma	Animal models	[127]
HRS3-scFv-hi-(IL-12)	CD30	Hodgkin's lymphoma	<i>In vitro</i>	[130]
HRS3-scFv-Fc-(IL-12)	CD30	Hodgkin's lymphoma	<i>In vitro</i>	[170]
aCEA-(IL-12)	CEA	Bile duct carcinoma	<i>In vitro</i>	[131]
huBC1-(IL-12)	Fibronectin	Prostate, colon and skin cancer	Animal models	[129]

* Tumor targeted by the antibody–cytokine fusion protein in animal models as described in the given references.

CD30: Cluster of differentiation 30; CEA: Carcinoembryonic antigen; ED-B: Extra domain-B of the B-FN isoform of human fibronectin; HER2/*neu* (also known as erbB-2): Human EGF receptor 2; scFv: Single-chain Fv.

Table 3

Antibody–(GM-CSF) fusion proteins.

Antibody fusion protein	Targeted antigen	Tumor *	Status	Ref.
chCLL-1-(GM-CSF)	MHC-II	Malignant B-cells	Animal models	[58]
anti-HER2/ <i>neu</i> IgG3-(GM-CSF)	HER2/ <i>neu</i>	Mammary and colon cancer	Animal models	[70,146,155,158,160,167]
ch17217-(GM-CSF)	TfR	Neuroblastoma and colon cancer	Animal models	[142]
ch14.18-(GM-CSF)	GD2	Neuroblastoma	Animal models	[143]
hu14.18-(GM-CSF)	GD2	Neuroblastoma	Animal models	[144]
L19-(GM-CSF)	ED-B	Colon cancer and teratocarcinoma	Animal models	[145]

* Tumor targeted by the antibody–cytokine fusion protein in animal models as described in the given references.

ED-B: Extra domain-B of the B-FN isoform of human fibronectin; GD2: Disialoganglioside; HER2/*neu* (also known as erbB-2): Human EGF receptor 2; MHC-II: Major histocompatibility complex class II; scFv: Single-chain Fv; TfR: Transferrin receptor.

Table 4

Bi-functional antibody–cytokine fusion proteins.

Antibody fusion protein	Targeted antigen	Tumor *	Status	Ref.
anti-EpCAM (mGM-CSF/IL-2)	EpCAM	Colon cancer	Animal models	[169]
anti-EpCAM (hGM-CSF/IL-2)	EpCAM	Gastric cancer	<i>In vitro</i>	[168]
anti-HER2 / <i>neu</i> (IL-12)-IgG3-(IL-2)	HER2/ <i>neu</i>	Mammary and colon cancer	Animal models	[70,124,167]
anti-HER2 / <i>neu</i> (IL-12)-IgG3-(GM-CSF)	HER2/ <i>neu</i>	Mammary and colon cancer	Animal models	[70,124,167]
(IL-12)-L19-(TNF- α)	ED-B	Teratocarcinoma	Animal models	[125]
KS-(IL-12/IL-2)	EpCAM	Lewis lung cancer	Animal models	[166]

* Tumor targeted by the antibody–cytokine fusion protein in animal models as described in the given references.

ED-B: Extra domain-B of the B-FN isoform of human fibronectin; EpCAM: Epithelial cell adhesion molecule; HER2/*neu* (also known as erbB-2): Human EGF receptor 2; scFv: Single chain Fv.