Cancer Therapy: Preclinical

Clinical Cancer Research

The Antibody-Based Delivery of Interleukin-12 to the Tumor Neovasculature Eradicates Murine Models of Cancer in Combination with Paclitaxel

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Abstract

Purpose: Interleukin-12 (IL12) is a potent proinflammatory cytokine with antitumor activity. Its heterodimeric nature makes it compatible with a large variety of different immunocytokine formats. Here we report the design, production, and characterization of a novel immunocytokine, based on the fusion of the F8 antibody (specific to the alternatively spliced EDA domain of fibronectin, a marker of tumor neovasculature) with IL12 (termed IL12-F8-F8).

Experimental Design: We developed a novel immunocytokine based on the sequential fusion of interleukin-12 as a single polypeptide with two F8 antibodies in single-chain Fv (scFv) format. The fusion protein was characterized *in vitro*, and its targeting performance was assessed *in vivo*. The immunocytokine antitumor activity was studied as monotherapy as well as in combination therapies in three different murine tumor models. Moreover, depletion experiments and tumor analysis revealed a dominant role of natural killer cells for the mechanism of action.

Results: IL12-F8-F8 can be produced in mammalian cells, yielding a product of good pharmaceutical quality, capable of selective localization on the tumor neovasculature *in vivo*, as judged by quantitative biodistribution analysis with radioiodinated protein preparations. The protein potently inhibited tumor growth in three different immunocompetent syngeneic models of cancer. The treatment was generally well tolerated. Moreover, the IL12-F8-F8 fusion protein could be produced both with murine IL12 (mIL12) and with human IL12 (hIL12).

Conclusions: The potent antitumor activity of mIL12-F8-F8, studied alone or in combination with paclitaxel in different tumor models, paves the way to the clinical development of the fully human immunocytokine. *Clin Cancer Res; 18(15); 4092–103. ©2012 AACR.*

Introduction

Many proinflammatory cytokines have exhibited promising anticancer properties in preclinical experiments, but their administration to patients is often associated with substantial toxicities that hinder an escalation to the dose needed for therapeutic activity. When administered in unmodified form, cytokines do not selectively localize to solid tumors, and striking therapeutic results have often been observed using nonconventional locoregional application modalities (e.g., intratumoral cytokine injections or transfection of tumor cells with cytokine genes) that are usually not directly applicable in the clinical setting (1). A promising avenue to improve the therapeutic index of anticancer cytokines consists in the fusion of cytokines with a suitable antibody serving as a vehicle for targeted delivery to the tumor environment (for reviews, see refs. 1–4). Indeed, a number of antibody–cytokine fusion proteins ("immunocytokines") have been moved to clinical trials using, in most cases, antibodies specific to splice isoforms of fibronectin or of tenascin-C (1, 5). These components of the modified subendothelial tumor extracellular matrix are strongly expressed in the cancer neovasculature and stroma but are virtually undetectable in normal adult tissues (6). Whereas clinical development programs in oncology have so far focused on the proinflammatory cytokines IL2 (interleukin 2), IL12, and TNF as active payloads, several other cytokines have been considered for immunocytokine construction, and their therapeutic activity has been tested in rodent models of cancer (1).

IL12 is a 70-kDa heterodimeric glycosylated cytokine composed by 2 subunits, named p35 and p40, covalently linked by a disulfide bridge (7). IL12 is produced by

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Translational Relevance

The antibody-based targeted delivery of cytokines to the tumor site is an attractive avenue for the improvement of the therapeutic index of proinflammatory cytokines and for the generation of a novel class of potent biopharmaceuticals. So far, only 2 of 8 anticancer immunocytokines that have been tested in clinical trials were based on interleukin-12 (IL12; the other ones were based on IL2 or TNF). However, IL12 possesses a unique potential for immunocytokine development because of its ability to mediate a potent $CD4^+$ T-cell response and because of its benign effect at sites of inflammation, making IL12-based immunocytokines particularly suited for the treatment of polymorbid patients.

In this article, we have continued our long-standing effort toward the identification of suitable formats for IL12-based immunocytokine development, and we have identified a novel format that combines good pharmaceutical properties, easy manufacturability, efficient *in vivo* tumor targeting, and potent anticancer activity. We have previously moved 4 immunocytokines to clinical development programs, and we believe that this novel IL12-based immunocytokine has the quality and the potential to be moved to clinical development programs in oncology. Applications for the treatment of patients with non-Hodgkin lymphoma are particularly attractive, because IL12 has shown activity in this indication and the F8 antibody strongly reacts with the majority of lymphoma types.

antigen-presenting cells, including macrophages, monocytes, neutrophils, and a subset of B cells. This cytokine regulates the balance between Th1 and Th2 responses and is therefore a key regulator of cell-mediated immune responses. In particular, it plays a critical role in the promotion of Th1 responses (8–10) by (i) promoting differentiation of naive T cells into IFNγ-producing Th1 cells (ref. 8; ii) costimulating the maximal secretion of IFNγ by Th1 cells (11) and (iii) stimulating the development of resting memory T cells into IFNγ-producing Th1 cells (9). It seems that a high local concentration of IFNγ at the tumor site, associated with a decrease in Treg cells and with a massive infiltration of $CD4^+$ T cells, is capable of mediating a very potent anticancer action (12, 13).

IL12 also stimulates IFNγ secretion in NK (natural killer) cells, leading to the activation of phagocytic cells and to inflammation. Furthermore, IL12 promotes the differentiation of $CD8^+$ cytotoxic T cells (14) and the reactivation and survival of $CD4^+$ memory T cells (15). Moreover, IL12 enhances the cytotoxic activity of NK cells and of $CD8^+$ cytotoxic T cells (7). Finally, IL12 directly stimulates early hematopoietic progenitor cells and promotes the proliferation and differentiation of bone marrow progenitors through synergy with other hematopoietic growth factors.

IL12 is a key cytokine acting on both innate and adaptive immune system. For this reason, multiple mechanisms have been reported for the antitumor activity of IL12 (16). Following the induction of IFNγ, the production of a cascade of cytokines with proinflammatory, cytotoxic, or cytostatic effects on tumor cells as well as antiangiogenic molecules is observed. Moreover, the IL12-stimulated cytotoxic cells can directly act on cancer or endothelial cells (16).

The antitumor activity of IL12 has been investigated in several preclinical studies. Brunda and colleagues reported impressive therapeutic results in different models of cancer following the intraperitoneal administration of the cytokine, although no cures were achieved (17).

Despite the encouraging preclinical results, the administration of recombinant human IL12 in clinical trials in patients with cancer was associated with limited efficacy and with severe toxicity. The MTD (maximal tolerated doses) for intravenous rhIL12 administration was found to be 500 ng/kg (18). In a phase II clinical trial, a slight change in the administration schedule caused the hospitalization of 12 of 17 patients and the death of 2 of them, with only one partial response (19). Different schedules and administration modalities were used to prevent severe toxicity, but they were associated with a lack of efficacy (refs. 20, 21; exception made for some particular types of cancer, such as cutaneous T-cell lymphoma, AIDS related Kaposi sarcoma, and non-Hodgkin lymphoma, for which partial responses and complete responses could be observed; refs. 22, 23). Recently, the electroporation of *IL12* gene in lesions of patients with metastatic melanoma or of dogs bearing mast cell tumors has been reported. Complete responses were observed (24, 25).

The systemic administration of untargeted rhIL12 at the MTD leads to an insufficient therapeutic concentration at the tumor site. For this reason, the use of tumor-targeting IL12-based immunocytokines has been proposed by our group and by others. Indeed, a large variety of molecular formats can be considered for IL12-based immunocytokines, because,

i. antibodies can be expressed as full immunoglobulins or as antibody fragments
ii. IL12 can be expressed as a single polypeptide, preserving the functionally relevant intact N-terminus of the p40 subunit in a sequential p40–p35 fusion or by attaching the individual subunits to different recombinant antibody moieties and letting them heterodimerize by disulfide bond formation

Our group has initially obtained promising therapeutic results with a sequential fusion of the p40 and p35 subunits with the tumor-targeting scFv(L19; refs. 26, 27), an antibody fragment specific to the alternatively spliced EDB domain of fibronectin (28). However, the tumor accumulation of this monomeric immunocytokine, as measured by quantitative biodistribution analysis, was less efficient compared with other dimeric or trimeric L19-based immunocytokines (29, 30). For this reason, alternative formats for the fusion of IL12 to tumor-targeting antibodies were tested *in vitro* and *in vivo* (31, 32). These experiments showed that

the fusion of one scFv moiety to each individual subunit of IL12 (i.e., fusion of both p40 and p35 to a scFv moiety) yielded heterodimeric immunocytokines with excellent tumor-targeting properties in biodistribution studies and with potent therapeutic activity. However, the GMP production of this heterodimeric cytokine is challenging because of stability issues for stably transfected cell lines and because of the propensity of the p40 and p35 subunits to homodimerize (32). For this reason, our laboratory has continued to explore alternative formats for IL12-based immunocytokines that would combine good pharmaceutical properties and efficient *in vivo* tumor targeting.

IL12 has also been fused to the C terminus of a humanized antibody (BC1) in IgG format (33), specific to a cryptic epitope on domain 7 of EDB-containing fibronectin (34). The immunocytokine was investigated in a phase I clinical trial. The MTD was found to correspond to 15 μg/kg and one partial response out of 13 treated patients was observed (35).

Here, we present a novel IL12-based immunocytokine format based on the F8 antibody (36), specific to the alternatively spliced EDA domain of fibronectin, a marker of tumor angiogenesis (37). This immunocytokine, termed IL12-F8-F8, combined favorable tumor-targeting properties with excellent biopharmaceutical quality. The therapeutic action of this fusion protein, based on the murine version of IL12, was studied in different tumor models as a single agent, in combination with another immunocytokine (F8-IL2) and with paclitaxel. Furthermore, the IL12-F8-F8 fusion protein was also expressed as fully human immunocytokine, thus paving the way to clinical development programs.

Materials and Methods

Cell lines and mice

For the production of the immunocytokines CHO-S (Chinese Hamster Ovary; Invitrogen) cells in suspension were used. The tumor cell lines used for therapy studies were the murine teratocarcinoma cell line F9 [CRL-1720, American Type Culture Collection (ATCC)], the murine colon carcinoma cell line CT26 (CRL-2638, ATCC), and the murine lymphoma cell line A20 (TIB-208, ATCC; cell culture conditions are described in Supplementary Materials S1.1). Female 129/SvEv mice were obtained from Taconic (Denmark). Female Balb/c mice were obtained from Charles River.

Cloning of fusion proteins

The gene structure for the F8 antibody in diabody format (36) and the isolation of the KSF antibody, specific to hen egg lysozyme (38), have previously been described. For the cloning of F8-based immunocytokines, the gene coding for the F8 diabody was PCR amplified from F8-mIL7 (39). The IL12 *hp40* and *hp35* genes were PCR amplified from the vectors used for F8-mp35/mp40-F8 production, previously reported by our group (32). The corresponding murine *mp40* and *mp35* genes were PCR amplified from the L19-mIL12 clone (27). All immunocytokine genes were PCR assembled and cloned into the mammalian cell expression vector pcDNA3.1(+) (Invitrogen) using a strategy similar to the one used by our laboratory for the preparation of IL7-based immunocytokines (39). More detailed information can be found in the Supplementary Material section (S1.2).

Expression, purification, and characterization of murine IL12 fusion proteins

The immunocytokines F8-hp35/hp40-F8, hIL12-F8-F8, hIL12-F8 diabody, mIL12-F8-F8, and mIL12-KSF-KSF were expressed using transient gene expression as described before (39, 40). For 1 mL of production 1×10^6 CHO-S cells in suspension were centrifuged and resuspended in 0.5 mL ProCHO4 (Lonza). Plasmid DNAs (1.25 μg) were mixed with 150 mmol/L NaCl to reach a final volume of 25 μL, 5 μL of 25-kDa linear polyethylene imine (PEI; 1 mg/mL solution in water at pH 7.0; Polysciences) were mixed with 20 μL of 150 mmol/L NaCl. The PEI/NaCl solution was added to the DNA/NaCl solution and allowed to stand at room temperature for 10 minutes. The solution containing the PEI–DNA complexes was then added to the cells and gently mixed. The transfected cultures were incubated in a shaker incubator at 37°C (40). At 4 hour posttransfection, the transfected culture was diluted with 0.5 mL of PowerCHO-2CD and then incubated at 31°C in a shaker incubator for 6 days. The procedure was scaled up to reach the desired production volume (40).

The fusion proteins were purified from the cell culture medium by protein A affinity chromatography and then dialyzed against PBS. The immunocytokines F8-hup35/hup40-F8, hIL12-F8-F8, and hIL12-F8 diabody were additionally purified on a HiPrep300 (GE Healthcare) after dialysis to isolate the monomeric fraction of the proteins. The size of the fusion proteins was analyzed under reducing and nonreducing conditions by SDS-PAGE and under native conditions by fast protein liquid chromatography gel filtration on a Superdex200 10/300 GL size exclusion column (GE Healthcare). The binding affinity of mIL12-F8-F8 was qualitatively determined by BIAcore on an EDA antigen–coated sensor chip.

Deglycosylation

To deglycosylate purified mIL12-F8-F8 and mIL12-KSF-KSF, 40 μg protein from a 0.4 mg/mL solution in PBS were incubated with 2,500 units PNGase F (NEB) for 20 hours at 37°C.

Stability

The immunocytokines mIL12-F8-F8 and mIL12-KSF-KSF were stored in solution at 37°C for 24, 48, 72, or 96 hours. The stability of fusion proteins was analyzed by SDS-PAGE under nonreducing conditions.

Immunofluorescence analysis on tumor sections

Immunofluorescence staining of cryostat sections (10 μm) of F9 and CT26 tumors was done essentially as described (41); mIL12-F8-F8 respectively mIL12-KSF-KSF were used for staining of EDA. Anti-mouse IL12/IL23 p40

antibody (eBioscience) was used to detect IL12, anti-rat-Alexa594–coupled secondary antibody (Invitrogen) was used for detection. On F9 tumor slides immune infiltrate and blood vessels were stained using as primary antibodies rat anti-CD45 (leukocytes; BD Biosciences), rat anti-CD31 (endothelial cells; BD Biosciences), rat anti-CD4 (CD4 T cells, GK1.5; BioXcell), rat anti-CD8 (CD8 T cells, 2.43; BioXcell), and rabbit anti-Asialo/GM1 (NK cells; Wako Pure Chemical Industries) antibodies. Anti-rat IgG-Alexa-Fluor488, anti-rat IgG-AlexaFluor594, and anti-rabbit IgG-AlexaFluor488 were used as secondary reagents for microscopic detection. Slides were mounted with fluorescent mounting medium (Dako) and analyzed with an Axioskop2 mot plus microscope (Zeiss).

Quantitative biodistribution studies

The *in vivo* targeting performance of F8-hp35/hp40-F8, hIL12-F8-F8, hIL12-F8 diabody, mIL12-F8-F8, and mIL12-KSF-KSF was evaluated by biodistribution analysis as described before (39). Radiolabeled F8-hp40/hp35-F8 (17 μg), hIL12-F8-F8 (17 μg), hIL12-F8 diabody (17 μg), mIL12-F8-F8 (7 μg), and mIL12-KSF-KSF (7 μg) were injected into the lateral tail vein of 129/SvEv mice bearing subcutaneous implanted F9 murine teratocarcinoma. Mice were sacrificed 24 hours after injection. Organs were weighed and radioactivity was counted using a Packard Cobra gamma counter. Radioactivity content of representative organs was expressed as the percentage of the injected dose per gram of tissue (%ID/g ± SE).

Blood binding assay

mIL12-F8-F8 was radioiodinated as described over. Blood samples were taken from 3 sacrificed Balb/c mice via cardiac puncture using BD Microtainer LH Tubes (BD). Radioiodinated immunocytokine was mixed to the blood samples to achieve a final concentration of 0.35 μg/100 μL and incubated for 10 minutes. Tubes were centrifuged for 10 minutes at 2,000 × *g*. Plasma and cell fraction were separated, weighed, and radioactivity was counted as described over.

Syngeneic tumor mouse models

Tumor-bearing mice were obtained by subcutaneous injection of F9 teratocarcinoma cells (10^7) in the flank of 12 weeks old female 129/SvEv or subcutaneous injection of CT26 colon carcinoma cells (2×10^6) or A20 lymphoma (6×10^6) in the flank of 12 weeks old female Balb/c. Normally, 4 to 8 days after tumor implantation, when tumors were clearly palpable, mice were randomized in different treatment groups ($n \geq 3$, depending on the number of mice). Therapy was carried out by injection of therapeutic agents (see below) in the tail vein. To assess homogeneity of treatment groups means, medians, 95% confidence intervals for tumor lesions were compared (Supplementary Material S2).

129SvEv mice were injected 4 times, every 48 hours, starting 5 days after tumor implantation, with mIL12-F8-F8 (1.75 μg, corresponding to 1 μg of mIL12 equivalents, $n = 5$), mIL12-KSF-KSF (1.75 μg, corresponding to 1 μg of mIL12 equivalents, $n = 5$) or PBS ($n = 5$).

129SvEv mice were injected 4 times, every 48 hours, starting 8 days after tumor implantation, with mIL12-F8-F8 (6 μg, corresponding to 3.4 μg of mIL12 equivalents, $n = 4$), mIL12-KSF-KSF (6 μg, corresponding to 3.4 μg of mIL12 equivalents, $n = 3$) or PBS ($n = 4$).

129SvEv mice were injected 4 times, every 48 hours, starting 5 days after tumor implantation, with mIL12-F8-F8 (8.75 μg, corresponding to 5 μg of mIL12 equivalents, $n = 5$) intravenous, with mIL12-F8-F8 (8.75 μg, corresponding to 5 μg of mIL12 equivalents, $n = 3$) intratumoral or PBS intravenous ($n = 4$).

129SvEv mice were injected 2 times, every 72 hours, starting 5 days after tumor implantation, with mIL12-F8-F8 (6 μg, $n = 3$), F8-IL2 (20 μg, corresponding to 6.6 μg of IL2 equivalents, $n = 3$), mIL12-F8-F8 (6 μg) in combination with F8-IL2 (20 μg; $n = 4$) or PBS ($n = 4$).

129SvEv mice bearing F9 teratocarcinoma and Balb/c mice bearing CT26 colon carcinoma or A20 lymphoma were injected 2 times, every 96 hours, starting 4 or 8 days after tumor implantation with paclitaxel (10 mg/kg; $n = 4$, $n = 5$) or 4 times, every 48 hours, starting 5 or 9 days after tumor implantation with mIL12-F8-F8 (8.75 μg; $n = 5$, $n = 5$) or with a combination of paclitaxel (10 mg/kg, starting on day 4, every 96 hours, twice) and mIL12-F8-F8 (8.75 μg, starting on day 5, every 48 hours, 4 times; $n = 4$, $n = 5$) or PBS ($n = 4$, $n = 5$).

The mice bearing F9 teratocarcinoma treated with mIL12-F8-F8 and paclitaxel that were cured were rechallenged by injecting 10^7 F9 cells into the opposite flank 17 days after tumor eradication. Mice were monitored daily, tumor volumes were measured daily with a digital caliper and calculated using the formula: volume = length × width^2 × 0.5. Animals were sacrificed when tumor volumes reached 2,000 mm^3.

129SvEv mice were injected 3 times, every 48 hours, starting 6 days after tumor implantation, with mIL12-F8-F8 (8.75 μg, $n = 5$) or PBS ($n = 5$) and sacrificed 48 hours after the last injection. Blood was collected via cardiac puncture and serum was isolated (centrifuged at 4°C, 1,400 × *g*, 15 minutes). Tumors were excised and divided in 2 parts. A part was embedded in cryoembedding medium (ThermoScientific), the second was lysed as described in Supplementary Material and supernatant isolated. All samples were stored at −80°C. Organs (liver, lung, spleen, heart, kidney, and intestine) were excised and stored in formalin.

Experiments were carried out under a project license granted by the Veterinäramt des Kantons Zürich, Switzerland (169/2008).

In vivo depletion of NK cells, CD4, and CD8 T cells

129SvEv mice were injected intraperitoneally with anti-Asialo GM1 (30 μL), anti-CD4 (250 μg), and anti-CD8 (250 μg) antibodies on days 3 (50 μL or 500 μg), 5, 9, and 13 after subcutaneous injection of F9 teratocarcinoma cells (10^7). On day 5 after tumor implantation, mice were grouped ($n =$

5) and injected 3 times, every 48 hours, with 8.75 μg mIL12-F8-F8.

Measurement of IFNγ, IP-10, and MIG in serum and tumors

Cytokine levels in serum and tumor lysate were measured using multiplexing technology by Cytolab (Rairing 66, CH-8108 Dällikon).

Hematoxylin and eosin staining of organs

Organs were collected immediately after sacrifice of mice, stored in formalin solution, and submitted to hematoxylin and eosin staining and pathology analysis, which was carried out by Frimorfo Ltd (Route de l'ancienne papeterie, CH-1723 Marly 1).

Statistical analysis

Data are expressed as the mean (±SE). Differences in tumor volume between therapeutic groups, %ID/g and tumor-to-blood ratio between biodistribution groups were compared using 2-way ANOVA statistical analysis, carried out with Prism software.

Results

Cloning and characterization of hIL12–F8 fusion proteins

In an attempt to develop novel IL12-based immunocytokines which combine efficient *in vivo* tumor targeting with favorable pharmaceutical properties, we compared the fully human immunocytokine named F8-hp35/hp40-F8 previously described by our group (32) with 2 immunocytokines, which featured alternative arrangements of the human IL12 and scFv(F8) moieties and which were produced in mammalian cells (Fig. 1). In one case, human IL-12 was expressed as a single polypeptide at the N-terminal end of a tandem arrangement of 2 units of the scFv(F8) antibody ("hIL12-F8-F8"; Fig. 1B). In a second case, human IL-12 was expressed as a single polypeptide at the N-terminal end of one unit of the scFv(F8) antibody, carrying a 5-amino acid linker between VH and Vl, thus driving the formation of a stable noncovalent homodimeric ("diabody") structure (ref. 42; Fig. 1C). All 3 proteins could be purified to homogeneity and displayed satisfactory purity in SDS-PAGE and in size-exclusion chromatography analysis (Fig. 1D–I).

A radioiodinated preparation of the 3 immunocytokines was used for a quantitative biodistribution analysis in immunocompetent mice bearing subcutaneously grafted murine F9 teratocarcinomas (Fig. 1J). Twenty-four hours after intravenous administration, there was no substantial accumulation in the tumor of the diabody-based immunocytokine (light gray). In line with previous reports from our group (31, 32), the heterodimeric F8-hp35/hp40-F8 immunocytokine (black) exhibited a selective accumulation in the tumor, with 10.7% injected dose per gram (%ID/g) in the neoplastic mass. The novel immunocytokine hIL12-F8-F8 (dark gray) showed a lower accumulation in the tumor (6.1%ID/g) compared with F8-hp35/hp40-F8, but increased tumor-to-blood and tumor-to-organ ratios (ranging between 4:1 and 6:1 at 24 hours; for a comprehensive analysis of *P* values, see Supplementary Material S4.1.1).

On the basis of favorable biodistribution results obtained with the fully human IL12-F8-F8 fusion protein, and on the pharmaceutical quality of this single-polypeptide therapeutic protein, we decided to produce its murine counterpart mIL12-F8-F8, containing the murine IL12 moiety, for biodistribution studies and for therapy studies in tumor-bearing mice (hIL12 does not cross-react with the murine receptor).

Cloning and characterization of F8-mIL12 and KSF-mIL12

The chimeric immunocytokine mIL12-F8-F8, containing the single-chain murine IL12 moiety sequentially fused to 2 units of the human scFv(F8), was expressed in CHO cells and purified to homogeneity. In addition, we produced the fusion protein mIL12-KSF-KSF (based on an antibody specific to hen egg lysozyme and thus not reactive with any mouse protein; ref. 38) as negative control for *in vivo* studies (Fig. 2A and B). The correct size of both immunocytokines was confirmed by SDS-PAGE and gel-filtration analysis (Fig. 2C–F). The products were found to be stable upon incubation at 37°C for up to 4 days (Supplementary Fig. S3). A BIAcore analysis revealed that mIL12-F8-F8 bound to the cognate antigen with high functional affinity and slow dissociation kinetics (Fig. 2G). An immunofluorescence analysis revealed that mIL12-F8-F8 strongly reacted with neovascular structures in sections of F9 teratocarcinoma and CT26 colon carcinoma, whereas mIL12-KSF-KSF did not stain the tissue sections (Fig. 2H). The tumor-targeting performance of mIL12-F8-F8 (black) was comparable with the one of the fully human immunocytokine, with 4.3%ID/g in the tumor at 24 hours and a tumor-to-blood ratio of 8:1 (Fig. 2I). As expected, mIL12-KSF-KSF (gray) did not exhibit a selective accumulation in the tumor at the same time point (Fig. 2I; for *P* values, see Supplementary Material S4.1.2).

A radioiodinated preparation of mIL12-F8-F8 was incubated *in vitro* at a concentration of 0.035 μg/mL (i.e., the same concentration used for therapy experiments) with blood freshly obtained from Balb/c mice. After 10 minutes and following centrifugation, more than 80% of the protein could be found in plasma, confirming that the majority of the immunocytokine was not associated with cellular components and was thus available for *in vivo* targeting of the antigen, located in the subendothelial extracellular matrix of tumor blood vessels.

Monotherapy experiments in immunocompetent mice bearing subcutaneous F9 tumors

The therapeutic performance of mIL12-F8-F8 and mIL12-KSF-KSF was tested in immunocompetent mice bearing subcutaneous F9 tumors. With a low dose intravenous injection (1.75 μg immunocytokine corresponding to 1 μg mIL12 equivalents) to mice carrying small tumors (around 50 mm^3), both immunocytokines induced a statistically

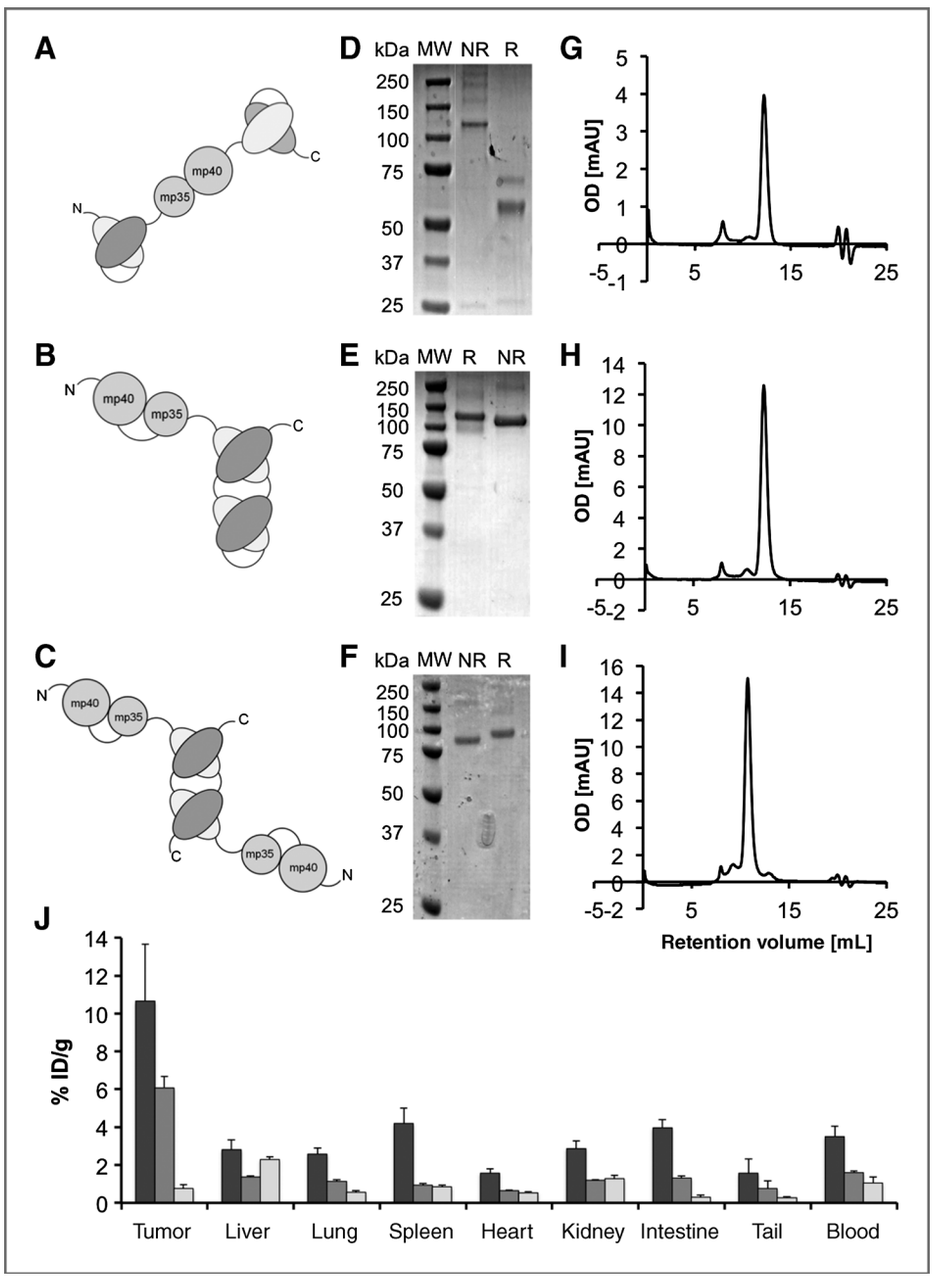

Figure 1. Cloning, expression, and characterization of F8 and human IL12 fusion proteins. Schematic representation of the domain structure of the hIL12-based immunocytokines F8-hup35/hup40-F8 (A), hIL12-F8-F8 (B), and hIL12-F8 diabody (C). Next to the antibody structures, SDS-PAGE analysis (D–F; MW, molecular weights; NR, nonreducing; R, reducing) and gel filtration analysis of purified protein preparations (G–I) are reported. J, comparative biodistribution study of radioiodinated immunocytokines. Immunocompetent 129/SvEv mice bearing syngeneic subcutaneous F9 teratocarcinoma were injected intravenously with 17 μg radiolabeled ^{125}I-F8-hup35/hup40-F8 (black, $n = 5$), ^{125}I-hIL12-F8-F8 (gray, $n = 5$), and ^{125}I-hIL12-F8 diabody (light gray, $n = 5$). Mice were sacrificed after 24 hours. Organs were excised and radioactivity counted, expressing results as percent of injected dose per gram of tissue (%ID/g) ± SE.

significant tumor growth retardation compared with mice that received PBS treatment (for *P* values, see Supplementary Material S4.2.1; Fig. 3A). When a higher dose of immunocytokine was administered (6 μg, corresponding to 3.75 μg mIL12 equivalents) to mice carrying larger tumors (150–250 mm^3), only mIL12-F8-F8 mediated a significant tumor growth retardation (for *P* values, see Supplementary Material S4.2.2; Fig. 3B). IL12 used as single agent displays a modest therapeutic effect, comparable with the one of IL12-based fusion proteins containing antibodies of irrelevant specificity (27). By contrast, the F8 antibody does not mediate any detectable tumor growth inhibition (43). No significant difference in tumor growth retardation was observed when comparing intravenous and intratumoral injections of mIL12-F8-F8 (8.75 μg, corresponding to 5 μg mIL12 equivalents), even though 2 of 3 mice were cured as a result of the intratumoral administration of the immunocytokine (Fig. 3C).

Tumor and serum levels of IFNγ, IP-10, and MIG were upregulated in mice treated with the mIL12-F8-F8 compared with the mice of the saline control group, thus indicating an activation of the IFNγ antiangiogenic pathway (Fig. 3D).

Immunofluorescence analysis of the tumors showed infiltration of the immune system in the tumors treated

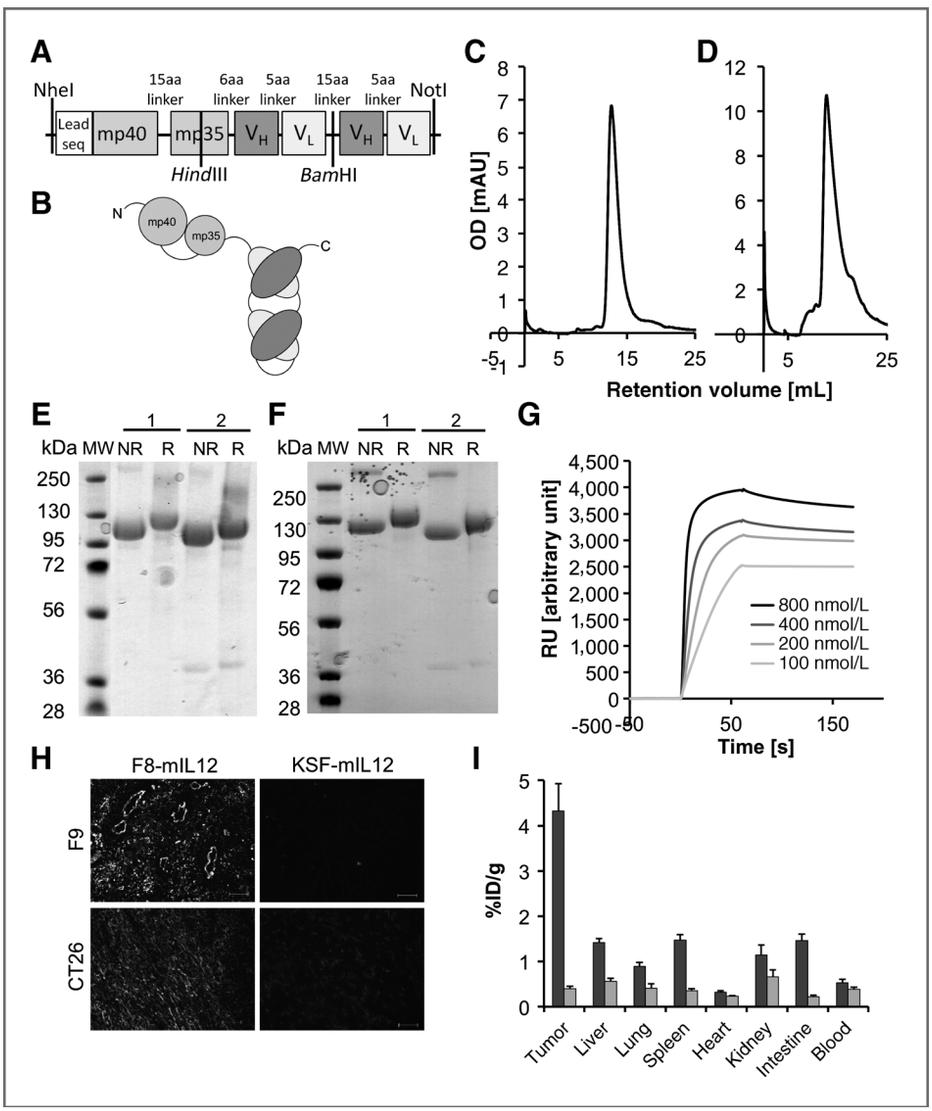

Figure 2. Cloning, expression, and characterization of mIL12-F8-F8 and mIL12-KSF-KSF. A, schematic representation of the cloning strategy of mIL12-F8-F8 and mIL12-KSF-KSF. B, domain assembly of mIL12-F8-F8 and mIL12-KSF-KSF. Gel filtration analysis of purified (C) mIL12-F8-F8 and (D) mIL12-KSF-KSF. The peaks correspond to the monomeric form of the immunocytokines. SDS-PAGE analysis of purified (E) mIL12-F8-F8 and (F) mIL12-KSF-KSF. Lanes in the gels: (1) purified protein, (2) PNGase deglycosylated mIL12-F8-F8 and mIL12-KSF-KSF showed presence of glycosylated monomers. MW, molecular weights; NR, nonreducing; R, reducing. G, BIAcore analysis of mIL12-F8-F8 on EDA-coated microsensor chip. H, immunofluorescence analysis of F9 teratocarcinoma and CT26 colon carcinoma. mIL12-F8-F8 and mIL12-KSF-KSF were used as staining reagents. Scale bars, 100 μm. I, biodistribution study of radioiodinated mIL12-F8-F8 and mIL12-KSF-KSF. Immunocompetent 129/SvEv mice bearing syngenic subcutaneous F9 teratocarcinomas were injected with 7 μg radiolabeled ^{125}I-mIL12-F8-F8 (black, $n = 5$) or with 7 μg radiolabeled ^{125}I-mIL12-F8-F8 (light gray, $n = 5$). Mice were sacrificed after 24 hours. Organs were excised and radioactivity counted, expressing results as percent of injected dose per gram of tissue (%ID/g) ± SE.

with the immunocytokine. In particular, a massive increase in the number of leukocytes and NK cells and a mild increase in the infiltration of CD4 T cells were observed. A heterogeneous result was found for CD8 T cells depending on the tumor studied. Representative pictures are shown in Fig. 3E; all 3 mice are shown in Supplementary Material S5. No particular differences were visible in the blood vessel density (Supplementary Material S5).

From the histologic analysis of the organs of the mice treated with mIL12-F8-F8 or of the control group, no pathologic findings were seen in heart, kidney, lung, spleen, and intestine. A slight mononuclear cell infiltration and some necrotic cells were observed in the liver of the mice treated with the immunocytokine (data not shown).

Combination therapy in immunocompetent mice

As combinations of different immunocytokines have previously shown the ability to completely eradicate tumors in rodents (26, 29), we tested the therapeutic activity of mIL12-F8-F8 (6 μg) in combination with F8-IL2 (20 μg; ref. 44; Fig. 4). F9 tumor–bearing mice were injected twice (days 5 and 8). Substantial toxicity was observed after the second injection in the combination group (lethargy, ruffled fur, body weight loss around 10%), indicating an additive toxicity of the 2 immunocytokines. Both immunocytokines (alone or in combination) significantly reduced tumor growth rate compared with the PBS group (for *P* values, see Supplementary Material S4.2.3), but the difference between the therapeutic performance of F8-IL2 alone and the same product in combination with mIL12-F8-F8 was not statistically significant.

The therapeutic performance of mIL12-F8-F8 was also tested in combination with paclitaxel, as we have frequently observed that this cytotoxic agent strongly potentiates the action of other immunocytokines (39, 43, 44). Both mIL12-F8-F8 used as a single agent and the combination of

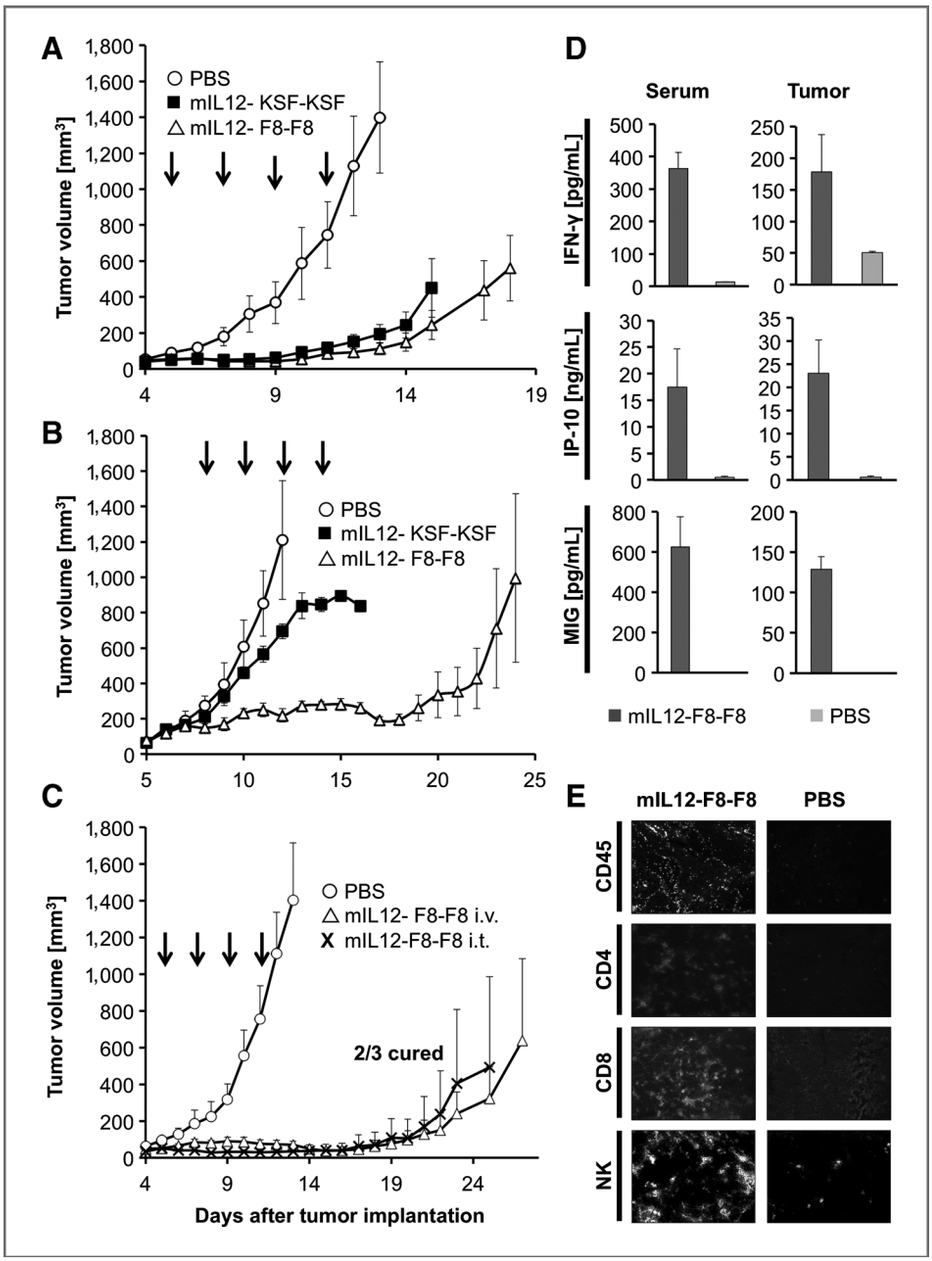

Figure 3. Therapeutic activity of mIL12-F8-F8 alone in immunocompetent 129/SvEv mice bearing syngeneic subcutaneous F9 teratocarcinoma. A, tumor-bearing mice were treated intravenously with mIL12-F8-F8 (1.75 μg, corresponding to 1 μg of mIL12 equivalents, $n = 5$, Δ), mIL12-KSF-KSF (1.75 μg, corresponding to 1 μg of mIL12 equivalents, $n = 5$, ■) or PBS ($n = 5$, ○). Treatment was carried out on days 5, 7, 9, and 11 after tumor implantation (black arrows). B, tumor-bearing mice were treated intravenously with mIL12-F8-F8 (6 μg, corresponding to 3.4 μg of mIL12 equivalents, $n = 4$, Δ), mIL12-KSF-KSF (6 μg, corresponding to 3.4 μg of mIL12 equivalents, $n = 3$, ■) or PBS ($n = 4$, ○). Treatment was carried out on days 8, 10, 12, and 14 after tumor implantation (black arrows). C, tumor-bearing mice were treated with mIL12-F8-F8 (8.75 μg, corresponding to 5 μg of mIL12 equivalents, $n = 5$, Δ) intravenously, mIL12-F8-F8 (8.75 μg, $n = 3$, **X**) intratumoraly, or PBS ($n = 4$, ○). Treatment was carried out on day 5, 7, 9, and 11 after tumor implantation (black arrows). Two mice out of 3 treated with intratumoral injections were cured. Data represent mean tumor volumes ± SE. Tumor growth curves were stopped when the first tumor per group reached 2,000 mm^3. D, tumor-bearing mice were treated intravenously with mIL12-F8-F8 (8.75 μg, corresponding to 5 μg of mIL12 equivalents, $n = 5$) or PBS. Treatment was carried out on days 6, 8, and 10. Animals were sacrificed on day 12, blood was collected and tumor was partially lysed and partially frozen. Serum and supernatant of tumor lysate were analyzed for IFNγ, IP-10, and MIG expression levels. E, cryostat tumor sections were analyzed in an immunofluorescence procedure using anti-CD45, anti-CD4, anti-CD8, and anti-Asialo GM1 as staining reagents. i.t., intratumoral.

mIL12-F8-F8 and paclitaxel exhibited an additive therapeutic effect in mice bearing F9 tumors (Fig. 5A). Although paclitaxel had only a small tumor growth retardation effect, in spite of being used at high dose (10 mg/kg), the use of mIL12-F8-F8 at a dose of 8.75 μg (4 injections) led to a long-lasting tumor growth control (for *P* values, see

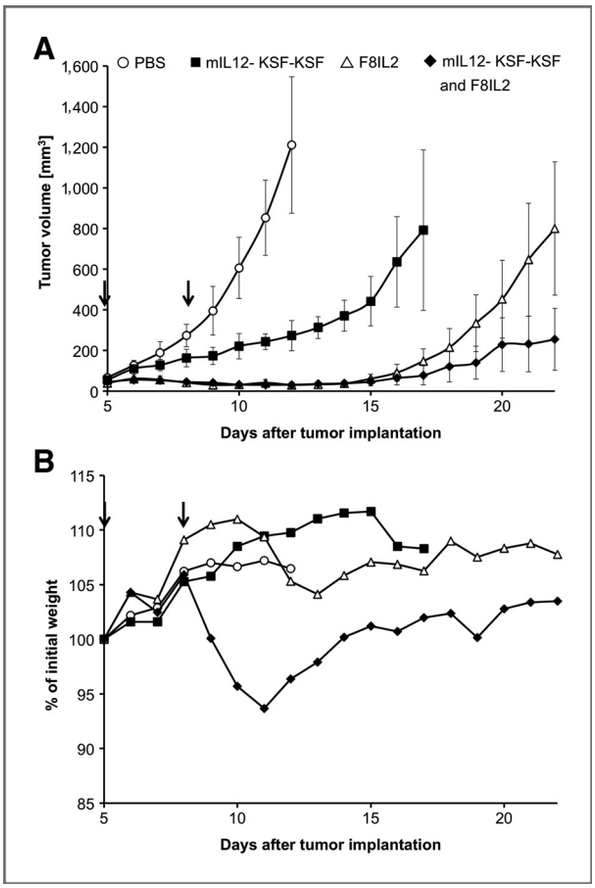

Figure 4. Therapeutic activity of mIL12-F8-F8 in combination with F8-IL2 in immunocompetent 129/SvEv mice bearing syngenic subcutaneous F9 teratocarcinoma. A, tumor-bearing mice were treated intravenously with mIL12-F8-F8 (6 μg, corresponding to 3.4 μg of mIL12 equivalents, $n = 3$, ■), F8-IL2 (20 μg, corresponding to 6.6 μg of IL2 equivalents, $n = 3$, Δ), mIL12-F8-F8 (6 μg) in combination with F8IL2 (20 μg; $n = 4$, ◆), or PBS ($n = 4$, ○). Treatment was carried out on days 5 and 8 after tumor implantation (black arrows), then the treatment was stopped due to toxicity issues. Data represent mean tumor volumes ± SE. Tumor growth curves were stopped when the first tumor per group reached 2,000 mm^3. B, weight monitoring depicted as percentage of the initial weight. The combination group showed a transient weight reduction and signs of toxicity from days 9 to 11.

Supplementary Material S4.2.4). Although eventually tumors grew in all mice treated with mIL12-F8-F8, 2 of 4 mice, which had received the combination treatment, were cured and were later submitted to a rechallenge with 10^7 F9 tumor cells. Unfortunately, both mice developed tumors after the rechallenge at the new cell injection site, indicating that a protective immunity had not been established.

Paclitaxel injections in the combination group caused a transient body weight loss, but mice recovered after administration of mIL12-F8-F8 (Supplementary Material S6.1).

A similar tumor therapy experiments was carried out in Balb/c mice bearing murine CT26 tumors (Fig. 5B) or A20 lymphomas (Fig. 5C). In the CT26 model, 2 of 5 mice in the combination group were cured. No significative tumor growth retardation could be observed between the mIL12-F8-F8 and the combination group (for *P* values, see Supplementary Material S4.2.5). When the same treatment schedule reported above was administered to mice bearing A20 lymphomas, a remission of established tumors was observed. Specifically, 5 of 5 mice in both the mIL12-F8-F8 and in the combination arm experienced a complete regression that was still on going at day 32 when mice were sacrificed and the experiment ended.

In vivo depletion

The selective depletion of cells of the immune system revealed that, at least for the F9 teratocarcinoma model, the reported antitumor effect was exerted predominantly by the NK cells (Fig. 6; for *P* values see Supplementary Material S4.2.7). In fact, when antibodies depleting the CD4 and CD8 compartment were administered, no significant therapeutic difference could be observed compared with non-depleted mice. On the contrary, when NK cells were depleted with an anti-Asialo GM1 antibody, a substantially lower response to the treatment with mIL12-F8-F8 was recorded.

Discussion

There is a growing interest in the use of immunocytokines as a promising type of "armed" antibodies. Compared with other forms of antibody derivatives (e.g., antibody–drug conjugates or radiolabeled antibodies; refs. 45–47), immunocytokines are easier to develop in clinical trials when the corresponding cytokine has already been studied and may represent "biosuperior" versions of previously used biopharmaceuticals (1, 48). Importantly, unlike antibody–drug conjugates or radiolabeled antibodies, immunocytokines typically spare the organs that mediate the clearance of the product from circulation. The limiting toxicities associated with proinflammatory cytokines are hypotension and flue-like symptoms, which can be manageable and which are orthogonal to the side effects of most cytotoxic drugs, thus favoring combination studies in which both agents are used at the recommended dose (49).

The ability of immunocytokines to selectively localize at the tumor site is crucial for displaying superiority compared with the nontargeted version of the cytokine. Indeed, in mouse models of cancer, the antibody-mediated targeted delivery of anticancer cytokines allows to achieve comparable therapeutic effects by administering 20-fold lower concentrations (or more) of the immunocytokine, compared with the parental recombinant cytokine (27, 29, 30). In the case of IL12-based immunocytokines, the best tumor-targeting results were so far obtained with a heterodimeric format (31, 32), which however complicated GMP manufacture procedures and pharmaceutical analytics. The novel format described in this article combines good pharmaceutical quality, easy manufacturability, efficient *in vivo* tumor targeting, and potent anticancer activity. It is thus ideally suited for pharmaceutical development.

When comparing different formats of F8- and IL12-based immunocytokines, we found that the IL12-F8 diabody format did not localize at the tumor site, in spite of being

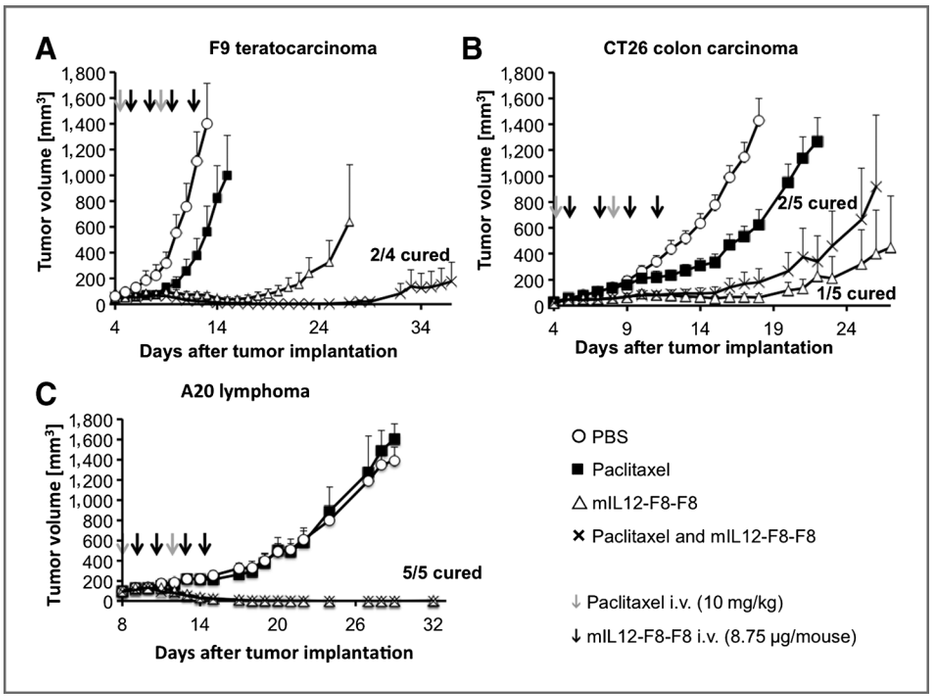

Figure 5. Therapeutic activity of mIL12-F8-F8 in combination with paclitaxel in immunocompetent mice bearing syngenic subcutaneous murine tumor models. Tumor-bearing mice were treated intravenously with mIL12-F8-F8 (8.75 μg, corresponding to 5 μg of mIL12 equivalents, $n = 5$, Δ), paclitaxel (10 mg/kg, $n = 4$, ■), mIL12-F8-F8 (8.75 μg) in combination with paclitaxel (10 mg/kg; $n = 4$, **X**), or PBS ($n = 4$, ○). Data represent mean tumor volumes ± SE. Tumor growth curves were stopped when the first tumor per group reached 2,000 mm^3. A, in the F9 teratocarinoma model (129SvEv mice) paclitaxel was administered on days 4 and 8 (gray arrows), mIL12-F8-F8 on days 5, 7, 9, and 11 (black arrows). Two mice of 4 of the combination group were cured. B, in the CT26 colon carcinoma model (Balb/c mice) paclitaxel was administered on days 4 and 8 (gray arrows), mIL12-F8-F8 on days 5, 7, 9, and 11 (black arrows). Two mice of 5 of the combination group and one mouse of 5 of the mIL12-F8-F8 group were cured. C, in the A20 lymphoma model (Balb/c mice), paclitaxel was administered on days 8 and 12 (gray arrows), mIL12-F8-F8 on days 9, 11, 13, and 15 (black arrows). Five mice of 5 of the combination group and the mIL12-F8-F8 group were cured.

fully immunoreactive *in vitro* and well behaved in biochemical tests (e.g., gel-filtration analysis; Fig. 1C, F, I, and J). These results are not completely surprising, as we have previously reported that IL12-based immunocytokines larger than 150 kDa abrogate the tumor-targeting properties of the parental antibody (26, 31). It is well known that IgG-based pharmaceuticals extravasate slowly and that IgMs are even less efficient in their extravasation properties. There is thus an interest to learn whether there is an upper limit in molecular weight for the development of tumor-targeting immunocytokines, which retain the tumor-targeting performance of the parental antibody. To that end, quantitative biodistribution assays, as described in this article, are absolutely indispensible.

Our laboratory has tested a large variety of different immunocytokines for cancer treatment (1), and IL12 stands up as one of the most promising candidates for product development, also thanks to the promising preclinical data obtained in murine tumor models of cancer which cannot be cured by conventional chemotherapy (26, 27, 29–31, 44).

IL12 not only directly enhances cytotoxic cell activity but also stimulates IFNγ production through several pathways. IFNγ was shown to be crucial for the eradication of tumors by $CD4^+$ T cells, with a mechanism that may involve a direct

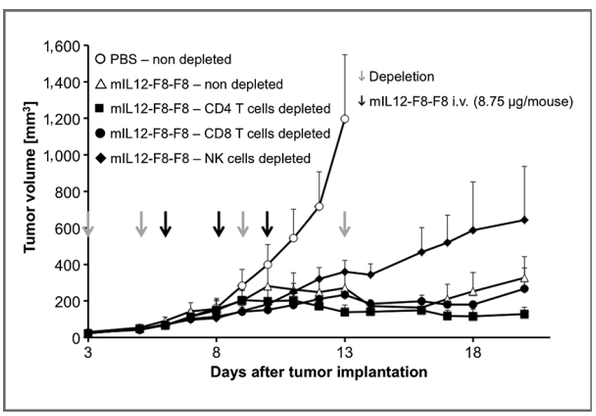

Figure 6. *In vivo* depletion of CD4 T cells, CD8 T cells, and NK cells. F9 teratocarcinoma–bearing mice were treated with mIL12-F8-F8 (i.v., 8.75 μg, corresponding to 5 μg of mIL12 equivalents, $n = 5$, Δ), mIL12-F8-F8 (i.v., 8.75 μg) and anti-CD4 Ab (i.p.; $n = 5$, ■), mIL12-F8-F8 (i.v., 8.75 μg) and anti-CD8 Ab (i.p.; $n = 5$, •), mIL12-F8-F8 (i.v., 8.75 μg) and anti-Asialo GM1 Ab (i.p.; $n = 5$, ◆), or PBS ($n = 4$, ○). Depletion antibodies were administered on days 3, 5, 9, and 13 (gray arrows), mIL12-F8-F8 on days 6, 8, and 12 (black arrows). Data represent mean tumor volumes ± SE. Tumor growth curves were stopped when the first tumor per group reached 2,000 mm^3.

cytotoxic activity on tumor cells and an enhanced expression of MHC class II molecules (12). Moreover, the production of the antiangiogenic chemokine IP-10 (IFN-γ inducible protein 10, CXCL10), capable of inducing tumor growth retardation, is also stimulated by IL12.

Although immunocytokines based on IL2 or TNF transiently worsened inflammatory conditions in animal models of psoriasis, arthritis, and endometriosis (but, importantly, not in animal models of atherosclerosis), the targeted delivery of IL12 does not seem to have a negative impact on inflammation in mouse models (for a review and the corresponding literature; see ref. 1).

The combination of IL2- and TNF-based immunocytokines has previously been reported to eradicate tumors, which cannot be cured by the action of IL2 or TNF alone (29). We have also previously reported on the synergistic action of IL12- and TNF-based immunocytokines. In this study, we have observed that the combination of F8-IL2 and mIL12-F8-F8 led to potent therapeutic activities but only at the expense of severe toxicities. By contrast, promising therapeutic results have been observed combining mIL12-F8-F8 with paclitaxel, a cytotoxic agents which has previously been reported by us and others to potentiate the action of proinflammatory cytokines, particularly when administered simultaneously or before cytokine treatment (39, 43, 44).

The only 2 IL12-based immunocytokines in clinical development featured a fusion of IL12 to an antibody in full IgG format (35). In principle, the Fc portion of this multifunctional molecule can contribute to a long circulatory half-life (22 hours; ref. 35) and may cross-link IL12 to leukocytes carrying Fcγ receptors on their surface. The use of antibody fragments in scFv format may favor shorter half-lives in blood (thus reducing side effects), promote an efficient tumor targeting, and avoid IL12 delivery to non-target cells.

The hIL12-F8-F8 product is potentially applicable for the treatment of a variety of different cancer types, as the F8 antibody recognizes neovascular and stromal structures in the majority of human tumors tested (50), while reacting only with placenta and the endometrium in the proliferative phase in a panel of freshly frozen 36 human normal tissues (51). The first clinical development programs should probably be for the treatment of non-Hodgkin lymphomas, as recombinant hIL12 has exhibited a potent therapeutic effect for this indication (23) and because the alternatively spliced EDA domain of fibronectin is strongly expressed in the majority of different lymphoma types (50).

Disclosure of Potential Conflicts of Interest

D. Neri has ownership interest (including patents) in Philogen, a biotech company that owns the F8 antibody, and is a consultant and advisory board member. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: N. Pasche, S. Wulhfard, D. Neri
Development of methodology: N. Pasche, S. Wulhfard, D. Neri
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N. Pasche, S. Wulhfard, F. Pretto, E. Carugati
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): N. Pasche, F. Pretto, D. Neri
Writing, review, and/or revision of the manuscript: N. Pasche, S. Wulhfard, F. Pretto, D. Neri
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D. Neri
Study supervision: D. Neri

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