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ORIGINAL ARTICLE

The antibody-mediated targeted delivery of interleukin-13 to syngeneic murine tumors mediates a potent anticancer activity

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Abstract We describe the expression and in vivo characterization of an antibody–cytokine fusion protein, based on murine Interleukin-13 (IL13) and the monoclonal antibody F8, specific to the alternatively spliced extra domain A of fibronectin, a marker of neo-angiogenesis. The IL13 moiety was fused at the C-terminal extremity of the F8 antibody in diabody format. The resulting F8-IL13 immunocytokine retained the full binding properties of the parental antibody and cytokine bioactivity. The fusion protein could be expressed in mammalian cells, purified to homogeneity and showed a preferential accumulation at the tumor site. When used as single agent at doses of 200 μg, F8-IL13 exhibited a strong inhibition of tumor growth rate in two models of cancer (F9 teratocarcinoma and Wehi-164), promoting an infiltration of various types of leukocytes into the neoplastic mass. This anticancer activity could be potentiated by combination with an immunocytokine based on the F8 antibody and murine IL12, leading to complete and long-lasting tumor eradications. Mice cured from Wehi-164 sarcomas acquired a durable protective antitumor immunity, and selective depletion of immune cells revealed that the antitumor activity was mainly mediated by cluster of differentiation 4-positive T cells. This study indicates that IL13 can be efficiently delivered to the tumor neo-vasculature and that it mediates a potent anticancer activity in the two models of cancer investigated in this study. The observed mechanism of action for F8-IL13 was surprising, since immunocytokines based on other payloads (e.g., IL2, IL4, IL12 and TNF) eradicate cancer by the combined contribution of natural killer cells and cluster of differentiation 8-positive T cells.

Keywords Interleukin 13 · Armed antibody · Cytokine · Targeting · Cancer therapy

Abbreviations

CD4+	Cluster of differentiation 4-positive
CD8+	Cluster of differentiation 8-positive
CHO	Chinese hamster ovary
EDA	Extra domain A
ELISA	Enzyme-linked immunosorbent assay
i.v.	Intravenous
IFN	Interferon
ILx	Interleukin-x
NK	Natural killer
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
s.c.	Subcutaneous
SDS	Sodium dodecyl sulfate
SIP	Signal peptide
Th	T helper
TNF	Tumor necrosis factor

Introduction

Cytokines are a class of proteins capable of modulating the activity of the immune system. Some recombinant cytokines, such as interferon (IFN) α, IFNβ, IFNγ, IL2, tumor necrosis factor (TNF) or granulocyte–macrophage colony-stimulating factor, have received marketing

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authorization for the treatment of patients with various types of diseases. However, cytokines can be toxic at single-digit milligram doses in humans, leading to substantial toxicities, which may prevent the escalation to therapeutically active regimens [1, 2]. Antibody vehicles have been considered as fusion partners for cytokines, with the aim to enhance the therapeutic index [3, 4] of these potent payloads. Many cytokines have successfully been fused to antibodies specific to the alternatively spliced extra domain A and B of fibronectin [5, 6], which represent ideal targets for antibody-based pharmacodelivery strategies, as they are highly conserved from mouse to man, are virtually undetectable in normal adult tissues (except for placenta and for the endometrium in the proliferative stage) and are strongly expressed in the majority of aggressive malignancies [7, 8]. The F8 antibody recognizes with identical affinity the murine and human EDA of fibronectin, which is expressed not only in the sub-endothelial extracellular matrix of most solid tumors [6, 9, 10], but also in lymphomas [11] and in certain acute leukemias [12] and efficiently localizes to the tumor neo-vasculature, following intravenous (i.v.) administration, as revealed both by quantitative biodistribution studies and by microautoradiographic analysis [13].

Many antibody–cytokine fusion proteins (referred to as 'immunocytokines') have been studied in mouse models of cancer and of chronic inflammation, and more than ten immunocytokine products have entered clinical development programs [14–16]. Whenever a curative anticancer activity in mice was observed (e.g., as in the case of combination treatments with F8 fusions with IL2, IL4, IL12 or TNF), lymphocyte depletion studies revealed that the tumor rejection process was dependent *both* on NK cells and on CD8+ T cells [6, 17, 18].

Interleukin-13 is a pleiotropic cytokine, mainly produced by activated T helper (Th) 2 cells and closely related to IL4. IL13 contributes to the regulation of IgE class switching, eosinophilic inflammation, mucus secretion, airway hyperresponsiveness, gastrointestinal parasite expulsion, tissue remodeling and tissue fibrosis. It mediates a variety of different effects on many cell types including B cells, natural killer cells, endothelial cells and fibroblasts, while several cell types beside activated Th2 cells, such as natural killer T cells, dendritic cells, mast cells and macrophages, can produce IL13 [19, 20]. Further, this cytokine has anti-inflammatory effects on monocytes, inhibiting the release of pro-inflammatory cytokines. Together with IL4 and IL10, IL13 contributes to the development of a Th2-dependent humoral response. However, unlike IL4, IL13 does not affect T cells due to the lack of expression of functional IL13 receptors by this cell population [21, 22]. Additionally, some unique effector functions distinguish this cytokine from IL4. For example, IL4 inhibits IFNγ production in large granular lymphocytes and promotes a Th2 response, while IL13 does not inhibit IFNy production but can synergize with IL2 and can have, similar to IL12, a direct effect on IFNγ synthesis and may therefore promote also a cellular Th1 response [23]. IL13 can have profound effects on tumor cell growth and can interact directly with cancer cells, inhibiting their proliferation in a concentration-dependent manner [24–26]. Mice injected with transfected IL13-secreting P815 mastocytoma cells rejected the tumors and developed a systemic long-lasting antitumor immunity mainly by recruiting infiltrating neutrophils and macrophages. Antibody-based depletion of T lymphocytes had shown that IL13-mediated tumor rejection was not mediated solely by T cells, but also requires an indirect nonspecific tumor defense mechanism, such as the action of NK cells or the infiltration of neutrophils and macrophages in HeLa tumors [27]. However, in weakly immunogenic 3LL lung carcinoma, IL13 expression did not induce long-term antitumor protection, while IFNγ did [28]. Recently, also direct IL13-mediated cell killing via induction of an apoptotic pathway in cancer cells has been proposed [29], making this cytokine an interesting payload for a tumor-targeted therapy approach.

In this article, we describe the production, characterization and antitumor effects mediated by the fusion of murine IL13 to the F8 antibody. The F8-IL13 fusion protein selectively localized to tumors in vivo and potently inhibited tumor growth in two syngeneic immunocompetent models of cancer.

Materials and methods

Cloning of F8-IL13 and KSF-IL13 fusion proteins

Murine Interleukin 13 cDNA clone (Sinobiologicals Inc.; Beijing, China) was polymerase chain reaction (PCR) amplified using linker-IL13_fw (5′-TCAGGCGGAGGTGG CTCTGGCGGTGGCGGACCGGTGCCAAGATCTG TGTCTCTCC-3′) forward primer that appends N-terminally part of the glycine–serine linker sequence, and IL13-NotI_rev (5′-TTTTCCTTTTGCGGCCGCTCATTAGA AGGGGCCGTGGCGAAACAGTTGC-3′) reverse primer containing a NotI restriction site. The F8 diabody gene was PCR amplified using the primers SIP-F8_fw (5′-CCTGTTCCTCGTCGCTGTGGCTACAGGTGTGC ACTCGGAGGTGCAGCTGTTGGAGTCTGGGG-3′) that N-terminally appends part of the signal peptide (SIP) DNA sequence and F8-linker_rev (5′-CCGCCAGAGCC ACCTCCGCCTGAACCGCCTCCACCTTTGATTTCC ACCTTGGTCCCTTGG-3′), which appends part of the linker peptide (including a 23 amino acid overlap) at the C-terminus. The murine IL13 and diabody (F8) DNA fragments were PCR-assembled using primers NheI_Sip_fw (5′-CCCGCTAGCGTCGACCATGGGCTGGAGCCTG

ATCCTCCTGTTCCTCGTCGCTGTGGC-3′), containing a NheI restriction site followed by the N-terminal part of the SIP sequence (21 bp overlap with SIP-F8_fw) and IL13-NotI_rev. The PCR-assembled full-length immunocytokine gene was double digested with NheI/NotI restriction endonucleases and cloned into the mammalian cell expression vector pcDNA3.1(+) (Invitrogen). The KSF-IL13 fusion protein was cloned analogously with the KSF antibody template instead of the F8 antibody template (annealing parts are the same; hence, the same primers could be used). DNA sequences of F8-IL13 and KSF-IL13 can be found in Supplementary Figure 3. The cloning and expression of IL12-F8F8 are described elsewhere [30].

Cell culture/cell lines

Chinese hamster ovary (CHO) cells (CHO-S, Invitrogen, Switzerland) in suspension were cultured in shaker incubators using PowerCHO-2CD medium (Lonza, Switzerland) supplemented with HT supplement (GIBCO®), 8 mM Ultraglutamine (Lonza, Switzerland) and 1 % antibiotics (GIBCO®). Cells were incubated at 37 °C. For biodistribution and syngeneic tumor mouse therapy studies, the murine teratocarcinoma F9 (CRL-1720, ATCC, Molsheim-Cedex, France) and Wehi-164 (CLS cell line service) cell lines were used. F9 cells were grown in tissue flasks coated with 0.1 % gelatin in DMEM (GIBCO®) supplemented with 10 % FCS. Wehi-164 cells were cultured according to supplier's protocol in RPMI (GIBCO®) medium. Murine B9 hybridoma cells (DSMZ, ACC-211, Germany) were cultured according to supplier's protocol.

Transient gene expression and characterization

F8-IL13 and KSF-IL13 fusion proteins were expressed in CHO-S cells using transient gene expression as previously described [31]. Fusion proteins were purified from the supernatant to homogeneity by protein A affinity chromatography and analyzed by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) (reducing and nonreducing conditions, Invitrogen), size-exclusion chromatography (Superdex 200 10/300GL, GE Healthcare), enzyme-linked immunosorbent assay (ELISA) and surface plasmon resonance analysis (i.e., BIAcore) on an EDA antigen-coated sensor chip. ELISA experiments were performed on streptavidin stripes (Roche, Switzerland) coated with biotinylated EDA antigen (10^{-7} M). Detection was done with protein A-horseradish peroxidase (GE healthcare).

Bioactivity assay

The biological activity of murine IL13 was determined by its ability to stimulate the proliferation of murine B9 cells (ACC-211, DSMZ). Ten thousand cells per well were seeded in 96-well plates in culture medium supplemented with varying concentrations of recombinant fusion proteins (protein range 7.5 ng/ml to 1,000 ng/ml). After incubation at 37 °C for 48 h, cell proliferation was determined with Cell Titer Aqueous One Solution (Promega).

Quantitative biodistribution studies

The in vivo targeting performance of F8-IL13 fusion protein was evaluated by biodistribution analysis with ^{125}I-labeled protein preparations as described before [32]. Six days after tumor cell injection, mice ($n = 5$ per group) were grouped and injected into the lateral tail vein with 10 μg of radioiodinated F8-IL13 fusion protein. Mice were killed 24 h after injection, organs were excised and weighed, and radioactivity was measured with a Packard Cobra γ-counter. Values are given in percentage of injected dose per gram of tissue (%ID/g ± SE).

Syngenic tumor mouse models in immunocompetent 129/SvEv and Balb/c mice

Twelve-week-old female 129/SvEv mice (Charles River, Germany) were subcutaneously (s.c.) injected in the flank with 25×10^6 F9 teratocarcinoma cells. Balb/c mice (Charles River, Germany) were s.c. injected in the flank with 3×10^6 cells Wehi-164 sarcoma cells. Four intravenous injections (every 48 h) of recombinant fusion proteins (200 μg of F8-IL13 and KSF-IL13 as single agents, 150 μg F8-IL13 and 8.75 μg of IL12-F8F8 in combination therapy modality) were given to F9-bearing mice, while three injections were given in the Wehi-164 model ($n = 5$). Animals were killed when weight loss was >15 % or 1 day before tumor volumes were exceeding 2,000 mm^3. Experiments were performed under a project license granted by the Verterinaeramt des Kantons Zürich, Switzerland (Bew. Nr. 42/2012).

In vivo depletion of CD4+, CD8+ and NK cells in Wehi-164-bearing Balb/c mice

In vivo depletion of CD4+, CD8+ and NK cells was performed in Balb/c mice bearing Wehi-164 tumors. Mice ($n = 5$, per group) were repeatedly injected with 250 μg rat anti-CD4 (BioXCell), 250 μg anti-CD8 (BioXCell) and 30 μl rabbit anti-Asialo GM1 (Wako Chemicals) antibodies on day 4, 7, 10 and 13 after s.c. injection of tumor cells. On day 5, 7 and 9 after tumor implantation, mice were injected with 120 μg F8-IL13 in combination with 7 μg IL12-F8F8. Supplementary Figure 1c shows a flow cytometric analysis of depletion efficacy.

Immunofluorescence studies of treated tumors

Ex vivo detection of tumor infiltrating cells after i.v. application of therapeutic proteins was done with tumors taken 1 day after the second injection. Tumors were excised and embedded in cryoembedding medium (Thermo Scientific), and cryostat Sects. (10 μm) were stained using the following antibodies: CD4 (BioXCell), CD8 (BioXcell), F4/80 (Abcam), CD3e (eBioscience), CD45 (BD Biosciences), CD45R (eBioscience), Asialo GM1 (Wako Pure Chemical Industries), forkhead box P3 (eBioscience) and CD31 (Santa Cruz Biotechnology). Detection was done with AlexaFluor488- and AlexaFluor594-coupled secondary antibodies (Invitrogen), and the slides were then analyzed with an Axioskop2 mot plus microscope (Zeiss).

Tumor rechallenge studies

More than 50 days after therapy studies, cured mice were injected again s.c. with 25×10^6 F9 cells/mouse and 3×10^6 Wehi-164 cells/mouse, respectively, to see whether mice acquired a protective immunity against cancer cells upon treatment with immunocytokines.

Statistical analysis (see supplementary material)

Data are expressed as mean ± standard deviation (biodistribution studies) or standard error of the mean (therapy studies). Differences between therapeutic groups were compared using GraphPad Prism's (GraphPad Software Inc., La Jolla, CA, USA) two-way ANOVA multiple-comparison analysis ($P < 0.05$ significance level). Differences

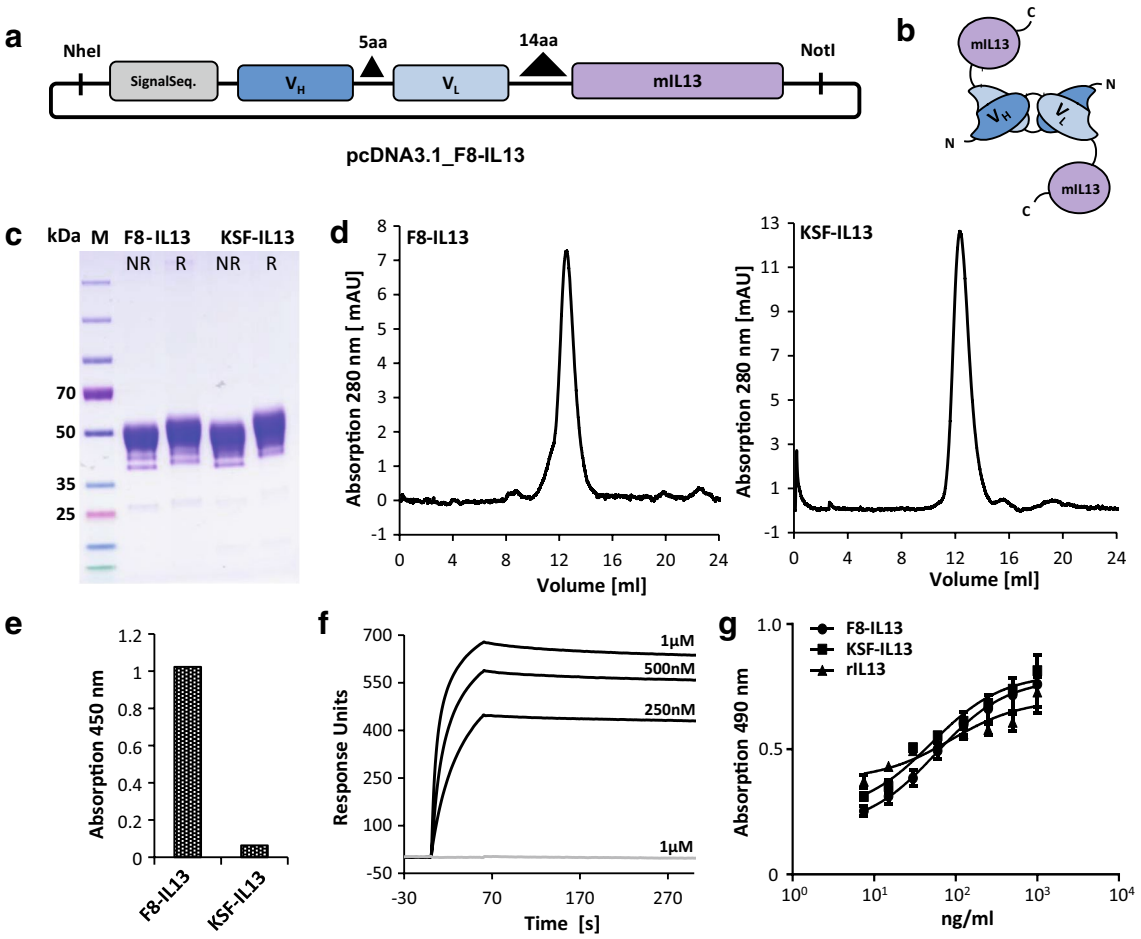

Fig. 1 **a** Schematic representation of the expression vector for F8-IL13 expression in mammalian cells. **b** Schematic representation of F8-IL13 fusion protein. **c** SDS-PAGE analysis of F8-IL13 and KSF-IL13; *M* molecular marker, *NR* nonreducing conditions, *R* reducing conditions. **d** Size-exclusion analysis of F8-IL13 and KSF-IL13. **e** ELISA assay on an EDA-coated surface, 500 nM of the fusion proteins applied. **f** Surface plasmon resonance analysis of three different concentrations (1 μM, 500 and 250 nM) of F8-IL13 (*black*) and 1 μM of KSF-IL13 (*gray*) on an EDA-coated sensor chip. **g** IL13 bioactivity assay performed with F8-IL13, KSF-IL13 and rmIL13 on IL13-responsive murine B9 cells. EC_{50}: F8IL13 (58 ng/ml), KSF-IL13 (50 ng/ml), hIL13 (88 ng/ml)

in cytokine levels were compared using a Mann–Whitney *U* test ($P < 0.05$ significance level).

Results

Interleukin 13 was fused at the C-terminal extremity of recombinant antibodies in diabody format [33], using a flexible 14 amino acid glycine–serine linker (Fig. 1a, b). The recombinant F8 antibody was chosen as tumor-homing vehicle, while the KSF antibody (specific to hen egg lysozyme) served as negative control of irrelevant specificity in the mouse [32]. The genes coding for the fusion proteins were assembled using a PCR-based procedure, cloned into a mammalian expression vector and transiently expressed in CHO-S cells. Both fusion proteins could be expressed at satisfactory yields (up to 14 mg/L in non-optimized conditions) and purified to homogeneity (>95 %, as judged by SDS-PAGE and gel-filtration analysis) after a single protein A affinity chromatography step (Fig. 1c). SDS-PAGE gel analysis revealed the presence of both N-glycosylation and O-glycosylation, since not all glycans could be removed by PNGase treatment (Supplementary Figure 1a). Both fusion proteins were analyzed by size-exclusion chromatography (Fig. 1d) as well as ELISA (Fig. 1e) and surface plasmon resonance studies (Fig. 1f) on EDA-coated surfaces. Cytokine activity was assessed by a proliferation assay with the IL13 cytokine-dependent murine B9 cell line (Fig. 1g). Collectively, these data indicate that both the antibody moiety and the cytokine retained full activity in the F8-IL13 fusion protein.

A radioiodinated preparation of F8-IL13 retained antigen-binding activity, as assessed by affinity chromatography on EDA resin. Ten micrograms of the product were injected into the tail vein of immunocompetent 129/SvEv mice bearing s.c. grafted murine F9 tumors, revealing a preferential accumulation in the neoplastic mass, with over 16 % ID/g in F9 tumors 24 h after injection and with a tumor/blood ratio of 7.35 (Fig. 2a). An ex vivo analysis of tumor sections confirmed a preferential accumulation of F8-IL13 (but not of KSF-IL13) around tumor neo-vascular structures (Fig. 2d).

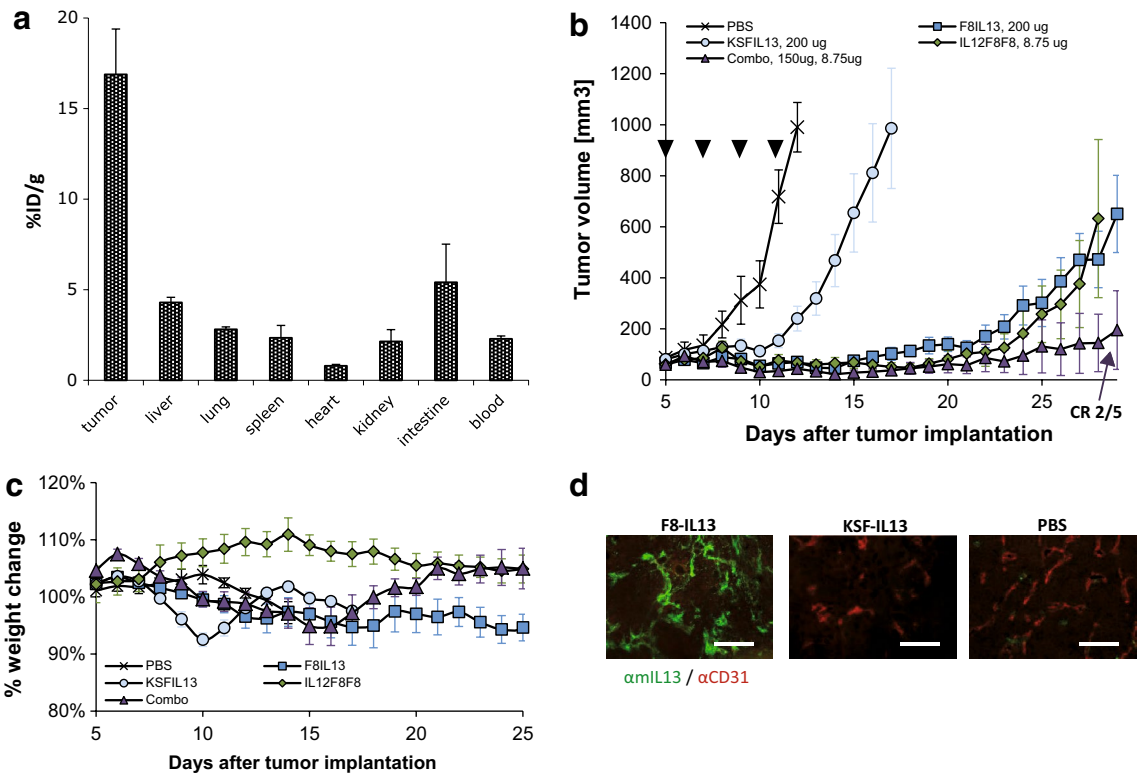

Fig. 2 **a** Quantitative biodistribution study of ^{125}I-labeled preparation of F8-IL13 fusion protein in subcutaneous F9 teratocarcinoma-bearing 129/SvEv mice. Animals were killed 24 h after injection, organs were excised and radioactivity counted, expressing results as percent of injected dose per gram of tissue (%ID/g ± SE). **b** Combination treatment of F8-IL13 with IL12-F8F8 in F9 tumor model. When F9 tumors were clearly palpable, mice were randomly grouped ($n = 5$) and injected four times (every 48 h) with PBS, 200 ug F8-IL13, 8.75 ug IL12-F8F8 or the combination of both agents (150ug F8-IL13 and 8.75 ug IL12-F8F8). **c** Monitoring of toxicity by the measurement of weight of treated mice. **d** In vivo immunofluorescence analysis of F9 tumor sections of mice treated with F8-IL13, KSF-IL13 or PBS; *scale bars* 100 μm

A pilot dose-escalation study, performed in F9 tumor-bearing mice, revealed that doses of F8-IL13 could be administered up to 250 μg by i.v. injection, without any detectable sign of toxicity (e.g., weight loss) and with evidence of potent antitumor activity. As a result, therapy studies in the same model were performed administering four injections of 200 μg F8-IL13 or KSF-IL13, used as single agents, starting treatment when lesions had reached a volume of ~100 mm^3. Additionally, stimulated by recent reports of therapy potentiation by targeted delivery of murine IL12 [6, 17, 34], 150 μg F8-IL13 was administered to a group of mice in combination with 8.75 μg IL12-F8F8. The use of F8-IL13 led to strong tumor growth retardation, with 2/5 cured mice when the product was given in combination with IL12-F8F8 (Fig. 2b). However, upon rechallenge with F9 cells, both animals developed tumors. Some mice receiving F8-IL13 (at a dose of 200 μg) showed mild diarrhea, but body weight loss remained <10 % (Fig. 2c). Therapy experiments were also performed with the combination of F8-IL13 plus IL12-F8F8 in mice with larger tumors (average tumor volume = 990 mm^3, $n = 4$). Some of the treated mice responded well to treatment, in one case with disappearance of the neoplastic mass, but eventually the tumor grew back (Supplementary Figure 1b). A microscopic analysis of tumor sections following treatment revealed a rich infiltrate of a variety of leukocytes in the immunocytokine-treated groups, without obvious changes in vascular density. Moreover, the tumor density of forkhead box P3-positive cells was not increased in any of the treatment groups (Fig. 5a).

Biodistribution studies with F8-IL13 were also performed in immunocompetent Balb/c mice bearing s.c.

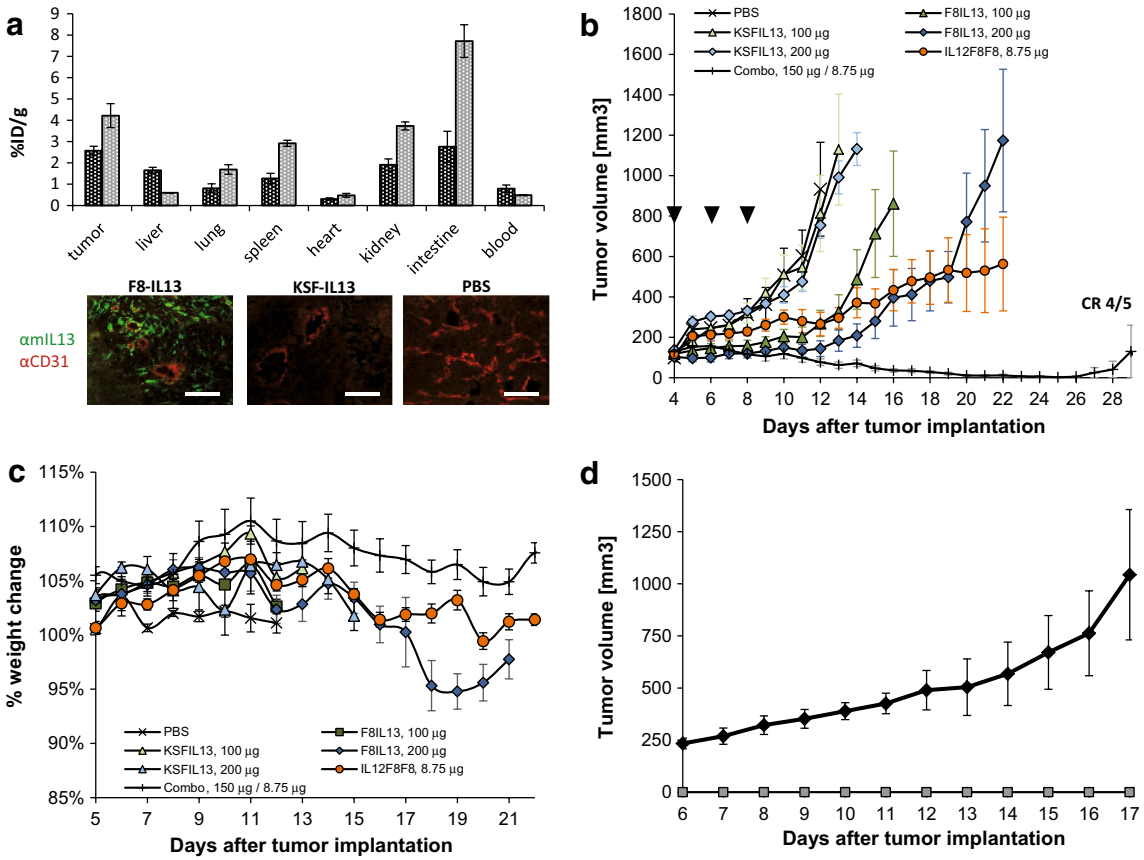

Fig. 3 **a** Quantitative biodistribution study of ^{125}I-labeled preparation of F8-IL13 (*black*, $n = 5$) and SIP(F8) control (*gray*, $n = 5$) in subcutaneous Wehi-164 sarcoma-bearing Balb/c mice. Animals were killed 24 h after injection, organs were excised and radioactivity counted, expressing results as percent of injected dose per gram of tissue (%ID/g ± SE) and below an in vivo immunofluorescence analysis of Wehi-164 tumor sections of mice treated with F8-IL13, KSF-IL13 or PBS is shown; *scale bars* 100 μm. **b** Therapy study in Wehi-164 sarcoma-bearing mice performed with F8-IL13 (two different doses of 100 and 200 μg) IL12-F8F8 (8.75 μg) and the combination of F8-IL13 with IL12-F8F8 (150 and 8.75 μg) in comparison with the negative control fusion protein KSF-IL13 (two different doses of 100 and 200 μg) and the saline group. When F9 tumors were clearly palpable, mice were randomly grouped and injected three times (every 48 h; indicated by *black arrows*). Data represent mean tumor volumes (±SEM; $n = 5$). **c** Analysis of toxicity by monitoring changes in weight of treated mice. **d** Mice cured by the combination treatment of F8-IL13 and IL12-F8F8 (*gray*, $n = 4$) as well as naïve mice (*black*, $n = 4$) were injected with Wehi-164 sarcoma cells, and the tumor growth was measured over time

grafted murine Wehi-164 sarcoma tumors. In this model, a substantial reduction in antibody uptake in the tumor was observed, compared to the F9 teratocarcinoma model. The tumor/organ ratios for F8-IL13, however, were similar to the ones observed for the parental F8 antibody in small-immune protein format (Fig. 3a) [13], but an example of potent antitumor activity has previously been reported for another F8-based immunocytokine in Wehi-164 tumor-bearing mice [35] and an ex vivo analysis of tumor sections confirmed a preferential accumulation of F8-IL13 (but not of KSF-IL13) around tumor neo-vascular structures (Fig. 3a). Three injections were given, and F8-IL13 was tested as single agent at two different doses (100 and 200 μg). All other experimental conditions were identical as for the study in F9-tumor-bearing mice. F8-IL13 exhibited a strong reduction in tumor growth rate (Fig. 3b) and led to cures in 4/5 mice, when administered in combination with IL12-F8F8. As in the F9 model, F8-IL13 was generally well tolerated (Fig. 3c), but few mice developed diarrhea. Unlike what we previously observed in teratocarcinoma-bearing mice, the induction of protective immunity was observed in the Wehi-164 model of sarcoma, as none of the cured mice developed a tumor when rechallenged with tumor cells 75 days after treatment (Fig. 3d). Also in this model, therapy experiments were performed in mice with larger tumors (i.e., above 500 mm^3). A marked reduction in tumor size was observed for F8-IL13 (when used alone or in combination with IL12-F8F8 or F8-IL4), but not for the IL12-F8F8 treatment group (Fig. 4a). The selective in vivo depletion of immune cells with antibodies revealed that the antitumor effect in mice with Wehi-164 sarcomas was predominantly mediated by CD4+ T cells (Fig. 4b). Cytokine levels in Wehi-164 sarcoma tissue samples were measured using a multiplex bead-based assay in tumors 1 day after the second injection, but no obvious changes in cytokine concentrations could be observed in any of the treatment groups (Supplementary Figure 2). A microscopic analysis of tumor sections following treatment revealed the presence of an infiltrate of various types of leukocytes (Fig. 5b).

Discussion

In this article, we describe for the first time the production and characterization (in vitro and in vivo) of an immunocytokine based on IL13. We used the F8 antibody, which is specific to the alternatively spliced EDA domain of fibronectin (a marker of angiogenesis) and which has previously been shown to selectively localize on tumor blood vessels in vivo, as tumor-targeting moiety. Interleukin-13 was considered to be an attractive payload for the study, since it is functionally related to IL4, a cytokine which has successfully been used for the preparation of immunocytokines with potent activity in mouse models of cancer [6] and of rheumatoid arthritis [36].

F8-IL13 was able to selectively home to tumor blood vessels in the two immunocompetent mouse models of cancer tested (F9 and Wehi-164) and displayed a potent single-agent activity, which however did not result in cancer cures. When the product was combined with an immunocytokine based on F8 and murine IL12 (IL12-F8F8), long-lasting tumor eradications could be observed in both models in a high proportion of treated mice. Balb/c mice cured from Wehi-164 tumors were able to reject subsequent challenges with tumor cells, indicating the induction of a protective immunity. By contrast, a similar effect could not

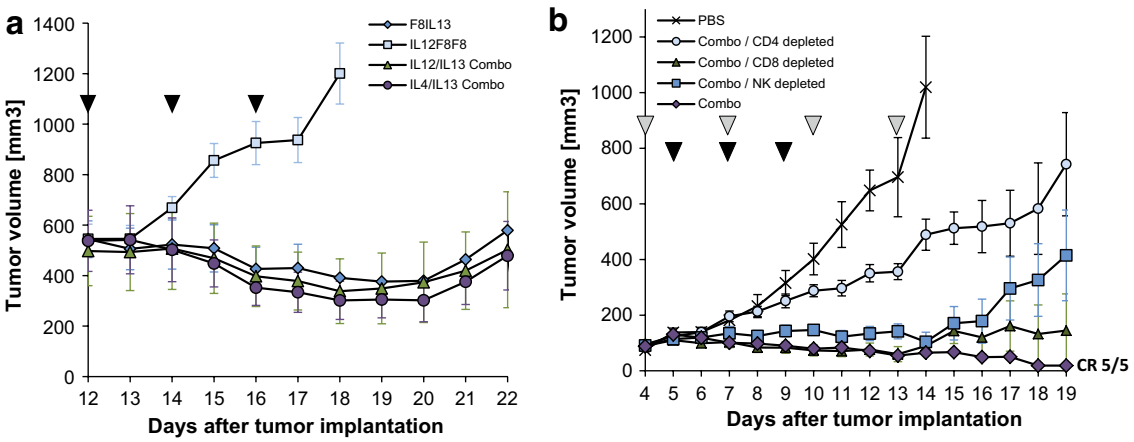

Fig. 4 **a** Wehi-164-bearing mice were injected three times (i.v., every 48 h) with F8-IL13 (150 μg), IL12-F8F8 (8.75 μg), or the combination of F8-IL13 (150 μg) with IL12-F8F8 (8.75 μg) or IL4 (70 μg) starting when the average tumor size exceeded 500 mm^3. **b** In vivo depletion of CD4+, CD8+ and NK cells in Wehi-164-bearing Balb/c mice. Depletion antibodies were injected intraperitoneal on days 4, 7, 10 and 13 (*gray arrows*) while therapeutic antibody combination (120 μg F8-IL13 and 7 μg IL12-F8F8) therapy as well as the PBS control group was injected i.v. on days 5, 7 and 9 (*black arrows*). Data represent mean tumor volumes (±SEM)

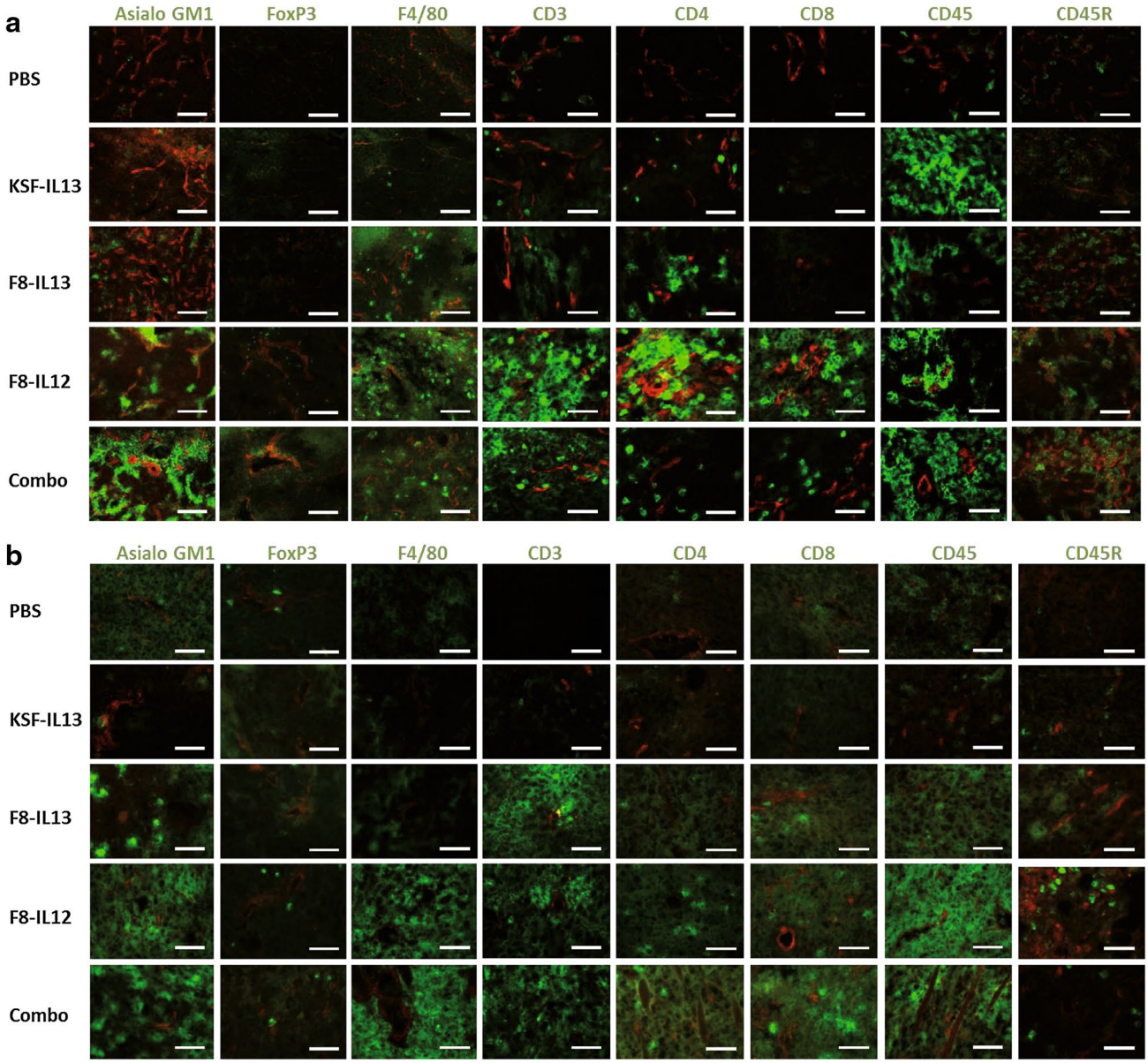

Fig. 5 **a** Immunofluorescence analysis of tumor infiltrating cells on 10 μm F9 teratocarcinoma tumor sections following treatment with PBS, KSF-IL13, F8-IL13, IL12-F8F8 and F8-IL13 in combination with IL12-F8F8 (*red*: anti-CD31); *scale bars* 100 μm. **b** Immunofluorescence analysis of tumor infiltrating cells on 10 μm Wehi-164 sarcoma tumor sections following treatment with PBS, KSF-IL13, F8-IL13, IL12-F8F8 and F8-IL13 in combination with IL12-F8F8 (*red*: anti-CD31); *scale bars* 100 μm

be observed in 129/SvEv mice bearing F9 teratocarcinomas, indicating that the mouse strain or the intrinsic tumor characteristics may contribute to the induction of anticancer immunity.

We have previously reported the induction of cancer cures and of protective immunity for F8-based immunocytokines containing IL2, IL4, IL12 or TNF as payloads, whenever these products were used in combination with other cytokines [6, 37], cytotoxic agents [10, 17, 18, 35, 37, 38] or IgG-based therapeutic antibodies [5]. In all tested cases, the antitumor effect depended on the action of both NK cells and CD8+ T cells. Surprisingly, F8-IL13 conferred a potent antitumor activity mainly through the action of CD4+ T cells, as revealed by in vivo depletion experiments (Fig. 4b). The significance of these findings still remains to be investigated, but examples of potent antitumor activity mediated by CD4+ T cells have previously been described. Transferred naïve tumor-/self-specific CD4+ T cells expanded, differentiated and eradicated established melanoma tumors. Surprisingly, CD4+ T cells developed cytotoxic activity leading to a class II-restricted recognition of tumors cells [39, 40].

Interleukin-13 is the last, in order of time, among many cytokines, which have been fused to tumor-targeting antibodies, including the L19 and F8 antibodies, specific to splice isoforms of fibronectin (for a review, see [4]). The targeted delivery of a potent immunomodulatory payload to the sub-endothelial extracellular matrix results in a high local concentration of cytokine at the site of disease, where this agent can interact with *in transit* leukocytes. The study of many different cytokines as partners for antibody fusion is justified by the potent antitumor activity, which has been observed for some payloads (e.g., IL2, IL4, IL12, IL13, TNF), and by striking differences in tumor-homing properties (e.g., [4, 41, 42]). Similar to other types of armed antibody products (e.g., antibody–drug conjugates), also immunocytokines can mediate cancer eradication in immunocompetent mouse models of cancer. However, cytokine-based products typically do not exhibit a direct toxicity to clearance-related organs and to the bone marrow, thus making them ideally suited for combination with conventional anticancer cytotoxic agents.

The therapeutic activity of F8-IL13 was encouraging, particularly when this product was combined with an IL12-based immunocytokine. Similar to previous reports on F8-IL4, the strong synergy observed with IL12-F8F8 was unexpected, as IL13 and IL12 are thought to control mutually exclusive fates of T cell development and activation. The F8 antibody recognizes the majority of human cancer types [13], and its cognate antigen is conserved in mouse and man, making F8-IL13 an attractive candidate for industrial development activities in oncology. As for other immunocytokine products, it is difficult to predict clinical activity on the basis of preclinical data, as cytokine function can be different in mouse and man.

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Conflict of interest Dario Neri is a cofounder and shareholder of Philogen SpA (Siena, Italy), the company that owns the F8 antibody. Other authors declare no conflict of interest.

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