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Tumor-targeting properties of novel immunocytokines based on murine IL1β and IL6

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There is an increasing biotechnological interest in 'arming' therapeutic antibodies with bioactive payloads. Many antibody–cytokine fusion proteins (immunocytokines) have been described and some of these biopharmaceuticals have progressed to clinical studies. Here, we describe for the first time the expression and *in vivo* characterization of immunocytokines based on murine IL1β and IL6. These potent pro-inflammatory cytokines were fused at the N-terminus or at the C-terminus of the monoclonal antibodies F8 (specific to the alternatively-spliced extra-domain A domain of fibronectin, a marker of tumor angiogenesis). All immunocytokines retained the binding properties of the parental antibody and were homogenous, when analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and size-exclusion chromatography, except for the N-terminal fusion of IL1β which revealed the presence of glycosylated species. When analyzed by quantitative biodistribution analysis using radioiodinated protein preparations, F8 fusions with IL6 revealed a preferential accumulation at the tumor site for both cytokine orientations, whereas IL1β fusions exhibited lower tumor to organ ratios and a slower blood clearance profile. The fusion proteins with the cytokine payload at the C-terminus were studied in therapy experiments in immunocompetent mice bearing F9 tumors. Immunocytokines based on IL1β resulted in 10% body weight loss at a 5-μg dose, whereas IL6-based products caused a 5% body weight loss at a 225-μg dose. Both F8-IL1β and F8-IL6 exhibited a <50% inhibition of tumor growth rate, which was substantially lower than the one previously reported for F8-TNF, a closely related pro-inflammatory immunocytokine. This study indicates that IL6 can be efficiently delivered to the tumor neo-vasculature by fusion with the F8 antibody. While F8-IL6 was not as potent as other F8-based immunocytokines that exhibit similar biodistribution profiles, the fusion protein sheds light on the different roles of pro-inflammatory cytokines in boosting immunity against the tumor.

Keywords: cancer/F8 antibody/immunocytokine/interleukin 1β/interleukin 6/targeting

Introduction

Cytokines are a diverse class of soluble proteins that are able to modulate immune responses against tumor cells. Some recombinant cytokines (e.g. IL2, TNF, IFNα, IFNβ, IFNγ, GM-CSF) have received marketing authorization for applications in oncology, infectious disease and autoimmunity. The systemic administration of certain pro-inflammatory cytokines (e.g. IL2) may confer a potent anti-tumoral activity, which can be curative in a small portion of patients with metastatic melanoma or renal cell carcinoma (McDermott *et al.*, 2006; Smith *et al.*, 2008) in young patients, who are fit enough to tolerate this toxic therapeutic procedure. For most other indications, however, recombinant cytokines rarely mediate objective responses (Welander, 1987; Jackaman *et al.*, 2003) and may cause serious adverse events already at low doses, thereby preventing the escalation to therapeutically active dose regimens. For these reasons, tumor-targeting antibodies have been considered as vehicles for pharmacodelivery applications, with the aim of improving the therapeutic index of the corresponding immunomodulatory payload (Halin *et al.*, 2002; Hess *et al.*, 2013; Neri, 2013). Antibody–cytokine fusion proteins are referred to as 'immunocytokines' and are being developed, both clinically and preclinically, for the treatment of cancer and certain inflammatory diseases (Schrama *et al.*, 2006; Pasche and Neri, 2012).

The F8 antibody, directed against the extra-domain A (EDA) expressed in the sub-endothelial extracellular matrix (ECM) of a wide range of malignancies, is one of the most extensively studied antibodies for pharmacodelivery applications. F8 recognizes the cognate antigen of murine and human origin with identical affinity, does not stain normal organs (with the exception of placenta, uterus and some vessels in the ovaries) (Villa *et al.*, 2008), but strongly reacts with many solid malignancies (Frey *et al.*, 2011*a*; Moschetta *et al.*, 2012; Hemmerle and Neri, 2013), lymphomas (Schliemann *et al.*, 2009) and also with certain types of acute leukemia (Gutbrodt *et al.*, 2013). The antibody-based targeting of the sub-endothelial ECM in tumors is particularly attractive, because immunocytokines typically remain bound to their abundant and stable antigen for several days, thus having the opportunity to interact with in-transit receptor-positive leukocytes and to modulate immunity at the site of disease.

Many cytokines (including GM-CSF, IFNα, IFNγ, IL2, IL4, IL7, IL10, IL12, IL15, IL17, IL18, TNF, CD40L, FasL, TRAIL, LiGHT, VEGI, lymphotoxin α, lymphotoxin β and lymphotoxin α1/β2) have already been fused to tumor-targeting antibodies specific to splice variants of fibronectin (Pasche and Neri, 2012; Hemmerle and Neri, 2013). Fusions with IL2, IL4, IL12 and tumor necrosis factor (TNF) were potently active against cancer as single agents and the activity could be further improved in combination therapy modalities (Hemmerle and Neri, 2013; Hemmerle *et al.*, 2013*a*; Schwager *et al.*, 2013), while the other cytokines showed either a modest therapeutic benefit or no benefit at all. Similarly, TNF-based immunocytokines display a potent anti-cancer activity against certain tumor types (especially sarcomas) (Borsi *et al.*, 2003; Balza *et al.*, 2006; Hemmerle *et al.*, 2013*b*).

High levels of TNF in plasma have been associated with poor prognosis for various tumor types, and preclinical studies

have shown that TNF can promote cancer development and dissemination (Balkwill, 2002). TNF is one of the main mediators of acute and chronic inflammation (Balkwill, 2002) and is able to induce the expression of IL1β and IL6 (Mule *et al.*, 1990; Turner *et al.*, 2007). IL1, IL6 and TNF are three related examples of multifunctional cytokines involved in the regulation of the immune response, hematopoiesis and inflammation, with partially overlapping functions. Like TNF, the other two cytokines can have pro- and anti-inflammatory functions, making them a double-edged sword for cancer therapy. Unlike TNF, however, IL1β and IL6 have not been previously fused to antibodies and studied for pharmacodelivery applications.

The IL1 family comprises 11 proteins, whereof the two agonists IL1α and IL1β were discovered first and are therefore the most studied members. While IL1α is membrane-bound, IL1β is secreted and therefore can act systemically. IL1 family cytokines bind to the IL1-receptor, which exists in two different forms. Type I receptor is primarily responsible for transmitting the inflammatory effects of IL1, while Type II receptor may, together with IL1-receptor antagonist, suppress the IL1 activity. IL1β is a pluripotent cytokine involved in normal physiological processes but is also secreted under pathological conditions (e.g. in autoimmune diseases). Recombinant human IL1β (rhIL1β) was evaluated in a Phase I clinical trial in patients with metastatic or unresectable solid tumors (Rinehart *et al.*, 1997). Four dose levels up to 200 ng/kg of rhIL1β were evaluated. The major toxicities were chills, rigors, headache, fatigue and hypotension. IL1β exhibited extensive hematological effects but its usefulness in clinical practice is limited by the extensive toxic side-effects at all tested dose levels, a problem that could be overcome with a targeted delivery to the site of disease.

As for TNF, there are contradictory reports about the effect of IL1β on the growth of tumor cells. Some (more recent) studies have shown stimulatory effects, whereas other (older) investigations have reported an inhibitory activity. IL1β is significantly linked to poor prognosis for patients with esophageal cancer and may be a promising molecular target for therapeutic intervention for esophageal squamous cell carcinoma (Chen *et al.*, 2012). The IL1 gene is frequently expressed in metastases from patients with several types of human cancers (Elaraj *et al.*, 2006). However, there are reports showing anti-cancer activity of IL1β (Kimura *et al.*, 1992; Yip *et al.*, 1995; Ohta *et al.*, 1996). Intraperitoneal administration of rhIL1β led to dose-dependent effects on tumor growth in nude mice bearing human ovarian tumor xenografts. At low dose levels (10 ng/day), a promoting effect on micrometastatic peritoneal implants could be observed, while at 1 μg/day an anti-tumor effect was reported (Malik *et al.*, 1992), in keeping with more recent observations (Roy *et al.*, 2006). The targeted delivery of TNF to the tumor neo-vasculature has been reported to have an even more complex effect, with tumor growth inhibition at ultra-low doses and at the maximal-tolerated dose, but a lower effect in the intermediate dose range (Curnis *et al.*, 2002).

Interleukin 6 is a pleiotropic cytokine involved in the host immune defense mechanism and the modulation of growth and differentiation of cells as well as the regulation of metabolic, regenerative and neural processes. IL6 can be produced by various cell types, including tumor cells. IL6 (and IL6 type cytokines except IL31) share a common receptor and signal transducer, termed gp130. IL6 initially binds to the membrane-bound IL6-receptor (α-receptor) and this complex then subsequently dimerizes with gp130 leading to intracellular signaling events.

In mice injected with IL6, no treatment-related deaths were observed at doses up to 50 μg per injection (maximum tested dose) and no signs of toxicity were reported. Unlike IL2, which was also investigated in the same study, IL6 did not induce vascular leak syndrome (Mule *et al.*, 1990). IL6 possesses multiple biological functions affecting a broad range of cells, including those directly involved in immune responses. The systemic use of this cytokine led to a reduction in the number of micro-metastases in four syngeneic mouse models of cancer (Mule *et al.*, 1990). In addition, tumor regressions and even cure rates were observed, when IL6 was administered in combination with sub-therapeutic doses of TNF (Mule *et al.*, 1990). However, reports on IL6 as being a tumor-promoting agent are predominant in the scientific literature. IL6 has been reported to promote the growth of tumor cells in malignant melanoma, renal cell carcinoma multiple myeloma and cervical cancer. Increased serum IL6 concentrations in patients are associated with advanced tumor stages (e.g. prostate cancer, breast cancer, ovarian cancer, multiple myeloma, non-small-cell lung carcinoma, colorectal cancer, renal cell carcinoma) and short survival (Guo *et al.*, 2012). Therefore, preclinical and clinical studies are currently investigating anti-IL6 therapy as a potential anti-cancer strategy (Guo *et al.*, 2012).

In view of the contrasting tumor-promoting and tumor-inhibitory functions attributed to the two cytokines, it would be important to learn whether IL1β and IL6 can be fused to antibodies and delivered to neoplastic sites, thus observing the effect of the corresponding biopharmaceuticals on the tumor mass. Here we report the cloning, expression and *in vivo* evaluation of IL1β- and IL6-based immunocytokines. The cytokines were fused both to the N- and to the C-terminus of the F8 antibody. Targeting performance of all four immunocytokines was evaluated by quantitative biodistribution experiments in 129/SvEv mice bearing F9 teratocarcinoma tumors. The best-performing immunocytokine formats were then used for therapy studies in the same immunogenic mouse model of cancer.

Material and methods

Cloning of F8-IL1β and F8-IL6 fusion proteins

The cloning of IL1β-F8 and IL6-F8 immunocytokines is described here. Murine interleukin 1β and interleukin 6 cDNA clones (Sinobiologicals, Inc.; Beijing, China) were PCR amplified using SIP-IL1β-fw (5′-CCTGTTCCTCGTCGCTGTGG CTACAGGTGTGCACTCGGTTCCCATTA GACAGCTGCA C-3′) and SIP-IL6_fw (5′-CCTGTTCCTCGTCGCTGTGGC TACAGGTG TGCACTCGTTCCCTACTTCACAAGTCCGG-3′) forward primers, which append a NheI restriction site and part of the SIP secretion signal sequence, and IL1β-linker_rev (5′-CCG CCAGAGCCACCTCCGCCTGAACCGCCTCCACCGGAAG ACACGGATTCCATGGTGAAG-3′) IL6-linker_rev (5′-CCG CCAGAGCCACCTCCGCCTGAACCGCCTCCACCGGTTT GCCGAGTAGATCTCAAAGTG-3′) reverse primers, which append part of the 14-amino acid GGGS linker peptide between the cytokine and the antibody moiety. The F8 diabody gene was PCR amplified using the primers Linker-F8_fw (5′-TCAGGCGGAGGTGGCTCT GGCGGTGGCGGAGAGG TGCAGCTGTTGGAGTCTGGG-3′) that N-terminally appends

part of the GGGS-linker peptide (including a 23 amino acid overlap) and F8-NOT_I_rev (5′-TTTTCCTTTTGCGGCCG CTCATTATTTGATTTCCACCTTGGTCCCTTGGCCGAA-3′) which appends two stop codons as well as a NotI restriction site. The murine IL1β-linker/IL6-linker and linker-diabody(F8) DNA fragments were PCR-assembled using primers NheI_Sip_fw (5′-CCCGCTAGCGTCGACCATGGGCTGGAGCCTGATCCT CCTGTTCCTCGTCGCTGTG GC-3′), containing a NheI restriction site followed by the N-terminal part of the SIP secretion sequence (21 bp overlap IL1β/IL6-SIP_fw), and F8-NOT _I_rev containing a stop codon. The PCR-assembled full-length cytokine-F8 gene was double digested with NheI/NotI and cloned into the mammalian cell expression vector pcDNA3.1(+)(Invitrogen). The C-terminal fusion proteins were cloned analogously with a reverse domain assembly.

Cell culture/cell lines

CHO-S (Invitrogen, Zug, Switzerland) cells in suspension were cultured in shaker incubators using PowerCHO-2CD medium (Lonza, Switzerland) supplemented with 8 mM Ultraglutamine, HT supplement (Lonza) and antibiotics. Cells were incubated at 37°C. For biodistribution and syngeneic tumor mouse therapy studies, the murine teratocarcinoma F9 cell line was used (CRL-1720, ATCC, Molsheim-Cedex, France). Cells were grown in tissue flasks coated with 0.1% gelatin in Dulbecco's Modified Eagle's medium (GIBCO®) supplemented with 10% fetal calf serum. Murine B9 cells (ACC-211, DSMZ, Germany), TIB-224 cells (ATCC, Molsheim-Cedex, France) were cultured according to supplier's protocol.

Transient gene expression and characterization

All fusion proteins used in this study were initially expressed in CHO-S cells using transient gene expression as previously described (Pasche *et al.*, 2011). From the transient culture, stable cell lines were obtained upon selection of G418-resistant clones. Positive clones were screened for high fusion protein expression levels by enzyme-linked immunosorbent assay (ELISA). For ELISA experiments biotinylated EDA antigen was coated (10^{-7} M) on streptavidin stripes (Roche, Switzerland) and protein A-HRP (GE healthcare) was used for

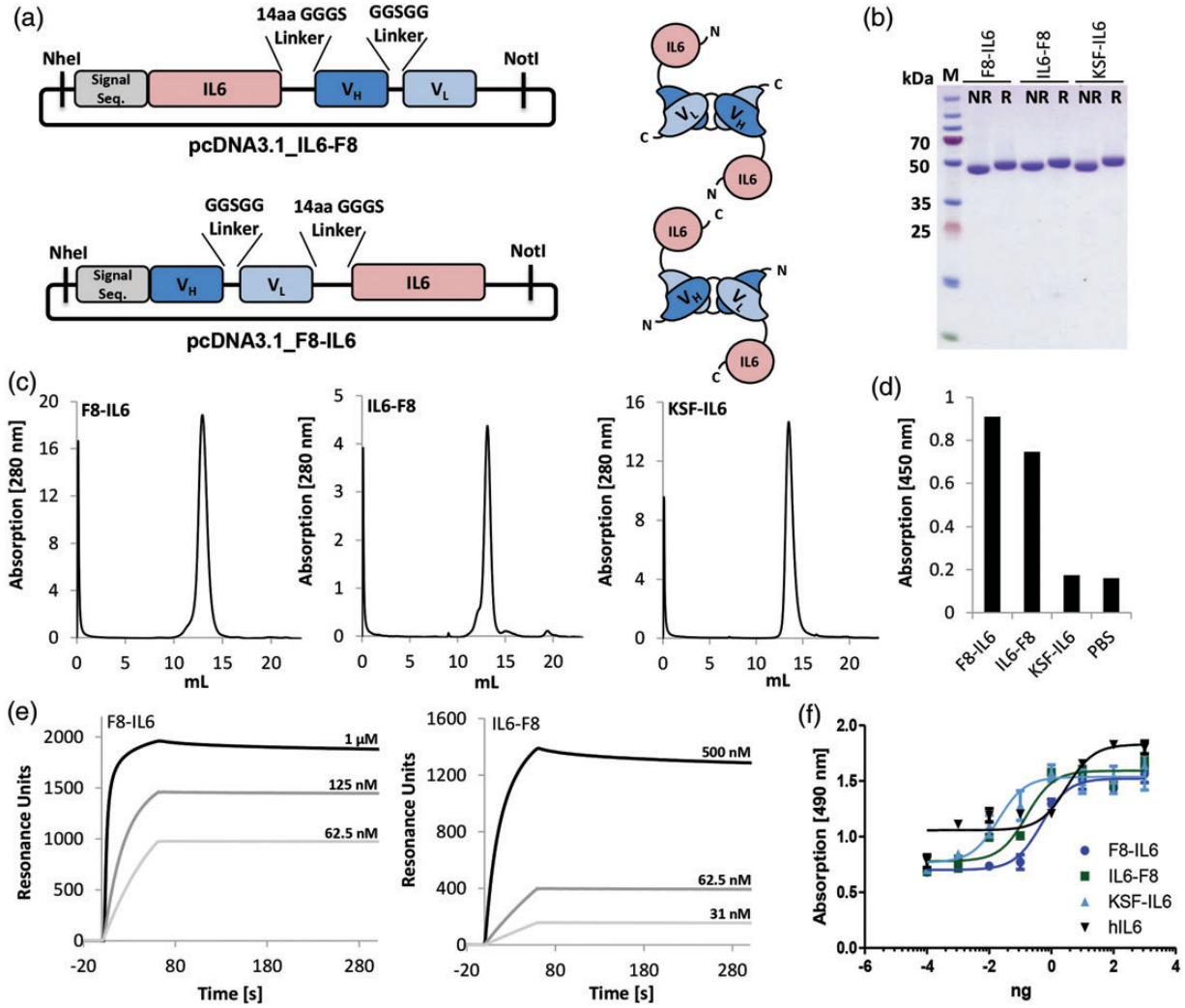

Fig. 1. Cloning, expression and *in vitro* characterization of non-covalent dimers of IL6-F8 and F8-IL6 fusion proteins. (a) Schematic representation of the expression vectors and the corresponding domain assembly schemes. (b) SDS–PAGE analysis of F8-IL6, IL6-F8 and KSF-IL6; M, molecular marker; NR, non-reducing conditions; R, reducing conditions. (c) Size-exclusion analysis of F8-IL6, IL6-F8 and KSF-IL6. (d) ELISA binding assay performed on EDA-antigen-coated wells; proteins were applied at 1 µM concentration. (e) Surface plasmon resonance analysis on an EDA-coated sensor chip. Left: F8-IL6 (1 µM, 125 nM and 62.5 nM); right: IL6-F8 (500, 62.5 and 31.25 nM). (f) IL6 bioactivity assay performed with F8-IL6, IL6-F8, KSF-IL6 and recombinant human IL6 as positive control on IL6-responsive B9 cells (30 000 cells/well). EC_{50}: F8IL6 (0.48 ng), IL6-F8 (0.16 ng), KSF-IL6 (0.02 ng), hIL6 (3.1 ng).

detection. Fusion proteins were purified from the cell culture medium to homogeneity by protein-A chromatography and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE, reducing, non-reducing conditions), size-exclusion chromatography (Superdex 200 10/300GL, GE Healthcare) and surface plasmon resonance (SPR) analysis (Biacore) on an EDA antigen-coated sensor chip.

Bioactivity assay

The biological activity of murine IL1β and IL6 was determined by its ability to stimulate the proliferation of TIB-224 and murine B9 cells, respectively. Cells (30 000 cells/well) were seeded in 96-well plates in culture medium supplemented with varying concentrations of recombinant fusion proteins (protein range IL1β: 2 ng to 1 pg; protein range IL6: 100 ng to 1 fg). After incubation at 37°C for 48 h, cell proliferation was determined with Cell Titer Aqueous One Solution (Promega). Recombinant human IL6 was used as positive control in the IL6 proliferation assay, while medium without IL1β served as negative control in the IL1β assay. EC_{50}: F8IL6 (0.48 ng), IL6-F8 (0.16 ng), KSF-IL6 (0.02 ng), hIL6 (3.1 ng). EC_{50}: F8IL1β (56 pg), IL1β-F8 (317 pg), KSF-IL1β (89 pg).

Quantitative biodistribution studies

The *in vivo* targeting performance of N- and C-terminal F8 interleukin fusion proteins was evaluated by biodistribution analysis with radioiodinated protein preparations as described before (Frey *et al.*, 2011*a*). Six to seven days after tumor implantation, mice ($n = 5$ per group) were grouped and injected into the lateral tail vein with 2.5 μg (~3 μCi) of radioiodinated IL1-based fusion proteins or 10 μg (~12 μCi) of the radio-labeled IL6 fusion proteins. Mice were sacrificed 24 h after injection, organs were excised, weighed and radioactivity was

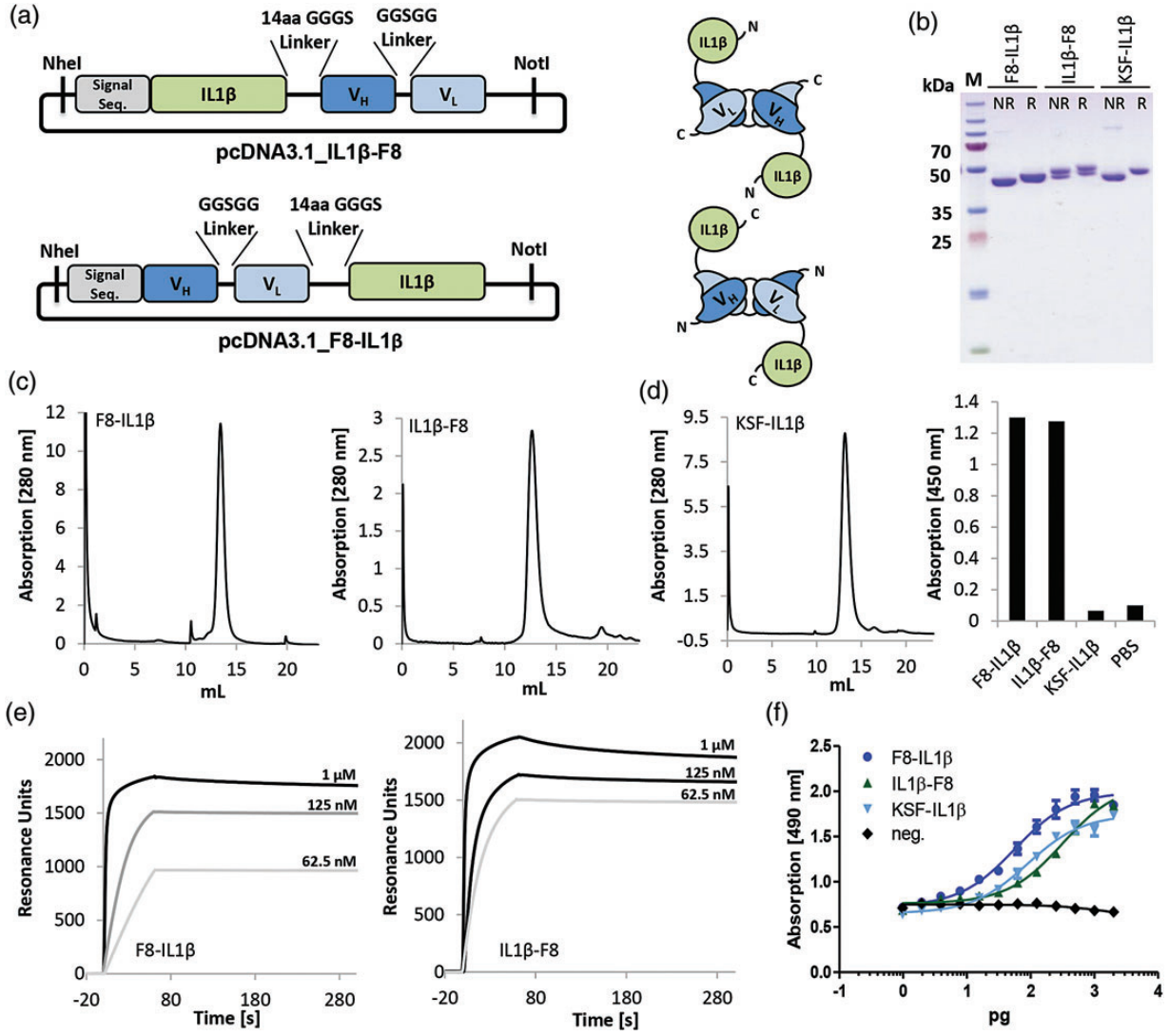

Fig. 2. Cloning, expression and *in vitro* characterization of non-covalent dimers of IL1β-F8 and F8-IL1β fusion proteins. (a) Schematic representation of the expression vectors and the corresponding domain assembly schemes. (b) SDS–PAGE analysis of F8-IL1β, IL1β-F8 and KSF-IL1β; M, molecular marker; NR, non-reducing conditions; R, reducing conditions. (c) Size exclusion analysis of F8-IL1β, IL1β-F8 and KSF-IL1β. (d) ELISA binding assay performed on EDA-antigen coated wells; proteins were applied at 1 μM concentration. (e) Surface plasmon resonance analysis on an EDA-coated sensor chip. F8-IL1β (left) and IL1β-F8 (right) were measured at 3 different concentrations (1 μM, 125 nM and 62.5 nM). (f) IL1β activity assay performed with F8-IL1β, IL1β-F8, KSF-IL1β and TIB-224 cells (30000 cells/well). As negative control culture medium without any addition of IL1β was used. EC_{50}: F8IL1β (55.9 pg), IL1β-F8 (317 pg), KSF-IL1β (89 pg).

measured with a Packard Cobra γ-counter. Values are given in percentage of injected dose per gram of tissue (%ID/g ± SE).

Syngeneic tumor mouse models in immunocompetent 129/SvEv mice

Eleven to twelve weeks old female 129/SvEv mice (Charles River, Germany) were subcutaneously injected in the flank with 25×10^6 cells F9 teratocarcinoma cells. Therapeutic doses of recombinant fusion proteins (5 μg of IL1β-based fusion proteins or 100–225 μg of IL6-based fusion proteins were given every 72 or 48 h, three to four injections, $n = 5$ per group). When weight loss was >15% or tumor volumes exceeded 2000 mm^3 animals were sacrificed. Experiments were performed under a project license granted by the Verterinaeramt des Kantons Zürich, Switzerland (Bew. Nr. 42/2012).

Results

The published structures of the receptor–cytokine complexes revealed that both termini of IL1β and IL6 were available for coupling to the tumor targeting F8 antibody. Therefore, each cytokine was fused with a flexible 14 amino acid linker to either the N-terminal or the C-terminal extremity of the recombinant F8 antibody in noncovalent homodimeric diabody format. The four F8-based immunocytokines were denominated F8-IL1β, IL1β-F8, F8-IL6 and IL6-F8, in order to represent the N- or C-terminal position of the cytokine. In addition, the KSF antibody in diabody format, specific to hen egg lysozyme, was fused to IL1β and IL6, serving as negative control of irrelevant specificity in the mouse (Frey *et al.*, 2011*b*). The immunocytokine genes were PCR-assembled, cloned into a mammalian expression vector, expressed in stably transfected CHO-S cells and purified to homogeneity (Figs. 1 and 2). All fusion proteins could be expressed with good yields (up to 10 mg/l) and were highly pure (>95%) after a single protein-A affinity chromatography step. SDS–PAGE gel analysis revealed the existence of a small covalently linked dimer fraction for IL1β-based immunocytokines (in Fig. 1 only barely visible and only for KSF-IL1β) due to an unpaired, poorly accessible cysteine in the murine IL1β protein (van Oostrum *et al.*, 1991). Further, IL1β-F8 shows a slightly different appearance (two bands) on the SDS–PAGE gel, compared with F8-IL1β and control proteins which migrated as a single band. All fusion proteins exhibited excellent biochemical properties in size-exclusion chromatography, ELISA assays and SPR studies (Figs. 1 and 2). Cytokine activities were assessed using cell proliferation assays with cytokine-dependent cell lines (Figs. 1f and 2f). Furthermore, radioiodinated preparations of the four immunocytokines revealed retention of immunoreactivity, as assessed by affinity chromatography procedures (Supplementary Data 2a).

Radioiodinated preparations of F8-IL1β, IL1β-F8, F8-IL6 and IL6-F8 were injected into the tail vein of immunocompetent 129/SvEv mice bearing subcutaneously grafted murine F9 tumors (IL6-based products: 10 μg per mouse; IL1β-based immunocytokines: 2.5 μg per mouse). A biodistribution analysis performed 24 h after injection revealed differences in the tumor targeting performance not only between the two different payloads, but also between the orientations of the two cytokines (Fig. 3a and b). IL1β-based immunocytokines exhibited a slow clearance and a relatively high accumulation in most of the measured organs. Tumor uptakes of 3.39% (N-terminal fusion protein) and 5.8% (C-terminal fusion protein) injected dose per gram (%ID/g) and tumor-to-blood ratios of 1 and 7.1, respectively, were measured. IL6-based immunocytokines showed generally better profiles with low organ values, tumor uptakes of 2.28% (N-terminal fusion) and 4.13%ID/g (C-terminal fusion) and tumor-to-blood ratios of 13.4 and 21.7. Due to better biodistribution performance of the C-terminal fusion proteins, subsequent therapy studies in the same model of cancer were performed with F8-IL1β and F8-IL6.

As literature reports that IL1β can lead to severe toxicity already when used at sub-microgram doses, a dose-finding study was performed in Balb/c mice (Supplementary Data 1b and d). Different concentrations of IL1β and IL6 were administered and the body weight was monitored as a measure for toxicity, leading to the determination of a maximum-tolerated dose of 5 μg of F8-IL1β per intravenous injection. F8-IL6 could be given repeatedly up to 100 μg (maximum dose administered) per injection, without any detectable sign of toxicity.

Therapy studies in F9 tumor-bearing 129/SvEV mice were performed with three injections of 5 μg F8-IL1β or 100 μg F8-IL6 (every 72 h), starting at an average tumor size of ~100 mm^3. Both, F8-IL1β and F8-IL6, exhibited only a modest (<50%) reduction of tumor growth rate (Fig. 4a) and only a modest increase in survival (Supplementary Data 2b). In the case of IL1β-based immunocytokines there is a significant (**, $P = 0.0047$) shift between the specifically targeted (F8-IL1β) and the control (KSF-IL1β) fusion protein from Day 13 onwards. However, the weight loss accompanying therapy with

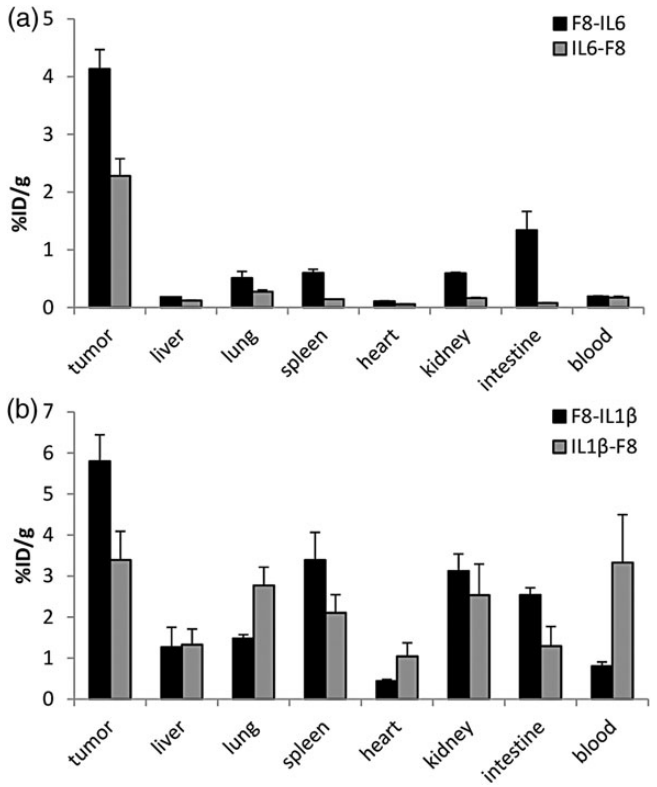

Fig. 3. Quantitative biodistribution study of ^{125}I-labelled preparations of (a) F8-IL6 (black) and IL6-F8 (grey) and (b) F8-IL1β (black) and IL1β-F8 (grey) fusion proteins in subcutaneous F9 teratocarcinoma bearing 129/SvEv mice. Animals were sacrificed after 24 h, organs were excised and radioactivity counted, expressing results as percentage of injected dose per gram of tissue (%ID/g ± SE).

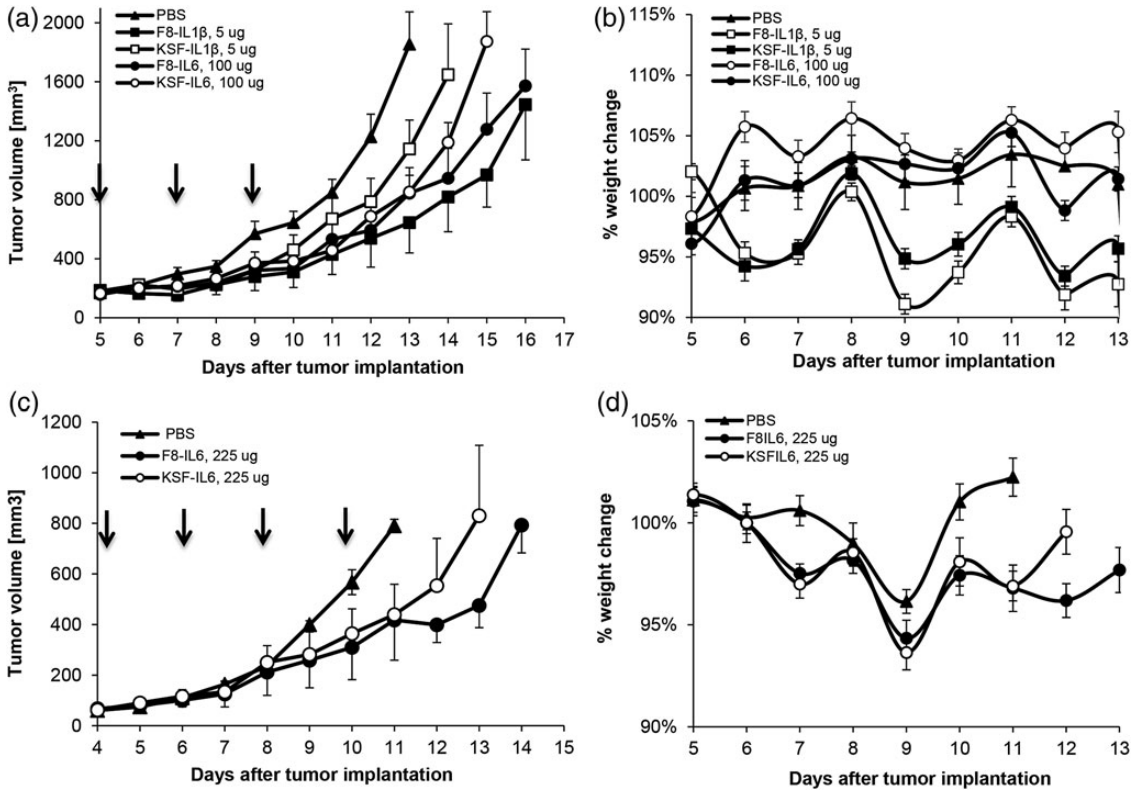

Fig. 4. (a) Evaluation of therapeutic activity of F8-IL1β and F8-IL6 against F9 teratocarcinoma in comparison to the negative control fusion proteins KSF-IL1β and KSF-IL6, specific for egg lysozyme. When F9 tumors were clearly palpable, mice were randomly grouped and injected three times (every 72 h; indicated by arrows) with PBS, F8-IL1β (5 μg) or F8-IL6 (100 μg). Data represent mean tumor volumes (± SEM), $n = 5$. The difference between F8-IL1β and KSF-IL1β is significant (**, $P = 0.0047$) from Day 13 on while there is no significance between F8-IL6 and KSF-IL6 (b) Analysis of toxicity by monitoring changes in the weight of treated mice. (c) Evaluation of therapeutic activity of F8-IL6 (high dose) against F9 teratocarcinoma in comparison to the neg. control fusion protein KSF-IL6, specific for egg lysozyme. When F9 tumors were clearly palpable, mice were randomly grouped and injected four times (every 48 h) with PBS, KSF-IL6 (225 μg) or F8-IL6 (225 μg). Data represent mean tumor volumes (± SEM), $n = 5$. (d) Analysis of toxicity by monitoring changes in weight of treated mice.

5 μg F8-IL1β per injection did not allow escalation to higher doses of this tumor-targeted cytokine (Fig. 4b). However, since there were no noticeable signs of toxicity at a dose of 100 μg of F8-IL6, a second therapy study with four injections (every 48 h) of 225 μg was performed in the same model of cancer. A slight (∼5%) weight loss was observed at this dose regimen, but the tumor inhibitory effects remained modest (Fig. 4c and d).

Discussion

In this article, we have described, for the first time, the production and characterization (*in vitro* and *in vivo*) of tumor-targeting immunocytokines, based on the potent pro-inflammatory cytokines IL1β and IL6. These payloads are particularly attractive for the development of therapeutic immunocytokines, since they are functionally related to TNF, a pro-inflammatory agent which has been successfully used in clinical-stage products (van Horssen *et al.*, 2006). Like TNF, the clinical use of IL1β as payload should be facilitated by the availability of an approved blocking antibody (Ilaris®), which could serve as an antidote in case of over-dosing or in case of severe unexpected adverse events. In our experiments, however, the anti-cancer activity of the new immunocytokines in the F9 teratocarcinoma model of cancer was not as potent as the one of other products based on the F8 antibody (e.g. F8-IL2 (Moschetta *et al.*, 2012), F8-TNF (Hemmerle *et al.*, 2013*a*) F8-IL4 and IL12-F8F8 (Hemmerle and Neri, 2013)).

Prior to this study, conflicting reports had indicated either a beneficial or a detrimental action of IL1β and IL6 on tumor growth. We expected that high cytokine concentrations at the tumor site, which can be reached using antibody-mediated targeted delivery strategies, could contribute to a strong pro-inflammatory environment at the neoplastic site. We have recently observed that certain cytokines (e.g. IL4) can exert a potent anti-tumor effect, with a mechanism which could not be predicted based on available literature data. We believe that antibody-based pharmacodelivery strategies are ideally suited to investigate whether a payload displays tumor-promoting or tumor-inhibitory activities.

The biodistribution profile of F8-IL6 and IL6-F8 in F9 tumor-bearing mice was comparable with the one previously reported for other F8-based immunocytokines (Pasche and Neri, 2012). The higher tumor uptake (∼4%ID/g vs. ∼2%ID/g) and better tumor-to-blood ratio (21.7 vs. 13.4) observed with the C-terminal fusion protein compared with the N-terminal fusion protein convinced us to use F8-IL6 for therapy experiments, in spite of a somewhat higher organ uptake in the intestinal tract, an organ for which variable uptake has been previously observed with F8 derivatives. In the case of IL1β-based fusion proteins, the difference in biodistribution results for the two domain orientations was pronounced, with a tumor-to-blood ratio of 7.2 for the C-terminal fusion, compared with a tumor-to-blood ratio of 1 for the N-terminal fusion protein, 24 h after intravenous injection.

With high levels of fusion protein observed in the blood and normal organs, the profiles of both IL1β-based immunocytokines were unusual, compared with other F8 derivatives previously studied in our laboratory. This observation could not have been anticipated on the basis of biochemical data and on the results obtained incubating a radiolabeled protein preparation with murine blood. Most of the immunocytokine remained in the supernatant, suggesting that the fusion protein had not been trapped by leukocytes carrying the IL1-receptor (Supplementary Data 1a). For certain payloads (e.g. IFNγ), we have previously observed that immunocytokine trapping by the cognate receptor may prevent an efficient accumulation at the tumor site *in vivo* (Ebbinghaus *et al.*, 2005). Furthermore, protein extravasation can be inhibited for fusion proteins with extreme pI values (Melkko and Neri, 2003). Finally, protein glycosylation can have a serious impact on the clearance, extravasation, tumor targeting and therapy performance (Hemmerle *et al.*, 2012).

The ultimate goal of our laboratory is to fuse and characterize (*in vitro* and *in vivo*) all cytokines to antibodies specific to fibronectin splice isoforms, which have proven to selectively localize on the tumor sub-endothelial ECM. The comparative evaluation of different immunocytokines remains of fundamental importance, in order to derive general principles for antibody-based pharmacodelivery applications and to select the most promising candidates for clinical development activities. The therapeutic results obtained with F8-IL6 (and, to a lesser extent, with F8-IL1β) were unexpected, particularly in view of the excellent biodistribution results obtained in the syngeneic immunocompetent F9 mouse model of cancer. It is possible that this fusion protein, of proven targeting performance *in vivo*, may find an application for therapeutic intervention in a non-oncological indication. Although it might be that F8-IL1β and F8-IL6 immunocytokines are more effective in other mouse models of cancer (e.g. fibrosarcoma for F8-IL6 and ovarian carcinoma for F8-IL1β), at this moment in time, we do not believe that IL1β and IL6 may represent promising payloads for the development of anti-cancer immunocytokines, despite their functional similarity to TNF.

Supplementary data

Supplementary data are available at *PEDS* online.

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Conflict of interest

D.N. is a cofounder and shareholder of Philogen SpA (Siena, Italy), the company that owns the F8 antibody.

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