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Expression, engineering and characterization of the tumor-targeting heterodimeric immunocytokine F8-IL12

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Proinflammatory cytokines have been used for several years in patients with advanced cancer but their administration is typically associated with severe toxicity hampering their application to therapeutically active regimens. This problem can be overcome by using immunocytokines (cytokines fused to antibody or antibody fragments) which selectively deliver the active cytokine to the tumor environment. Preclinical and recent clinical results confirmed that this approach is a very promising avenue to go. We designed an immunocytokine consisting of the scFv(F8) specific to extra-domain A of fibronectin and the very potent human cytokine interleukin-12 (IL12). The heterodimeric nature of IL12 allows the engineering of various immunocytokine formats, based on different combinations of the two subunits (p35 and p40) together with the scFv. In comparison to monomeric or homodimeric cytokines, the construction of a heterodimeric immunocytokine poses many challenges, e.g. gene dosing, stable high-yield expression as well as good manufacture practice (GMP) purification and characterization. In this paper, we describe the successful construction, characterization and production of the heterodimeric immunocytokine F8-IL12. The positive outcome of this feasibility study leads now to GMP production of F8-IL12, which will soon enter clinical trials.

Keywords: heterodimer/immunocytokine/interleukin 12/scFv antibody fragment

Introduction

Cytokines are key mediators of innate and adaptive immunity. Many cytokines have been used for therapeutic purposes in patients with advanced cancer, but their administration is typically associated with severe toxicity, hampering dose escalation to therapeutically active regimens and their development as anticancer drugs (Janeway's Immunobiology; Fyfe *et al*., 1995; Eggermont *et al*., 1996; Leonard *et al*., 1997). To overcome these problems, the use of 'immunocytokines' (i.e. cytokines fused to antibodies or antibody fragments) has been proposed, with the aim to concentrate the immune-system stimulating activity at the site of disease while sparing normal tissues (Savage *et al*., 1993; Reisfeld *et al*., 1997; Dela Cruz *et al*., 2004; Neri and Bicknell, 2005; Schrama *et al*., 2006).

The group of Reisfeld has pioneered the fusion of interleukin-2 (IL2) at the C-terminal end of the heavy chain of intact immunoglobulins and has brought these products to clinical trials in patients with cancer (Osenga *et al*., 2006). Our group has focused on the engineering of polypeptides consisting of individual cytokines fused to antibody fragments in scFv format, in order to reduce the circulatory half-life of these biopharmaceuticals to a minimum, while retaining their tumor-homing properties (Neri and Bicknell, 2005; Schliemann and Neri, 2007). For pharmacodelivery applications, we have mainly focused on antibodies specific to splice isoforms of fibronectin and of tenascin-C, as these tumor targets are strongly expressed in the stroma and neovasculature of virtually all cancer types, while being undetectable in most normal adult tissues (Neri and Bicknell, 2005; Brack *et al*., 2006; Pedretti *et al*., 2009; Schliemann *et al*., 2009; Schwager *et al*., 2009). Indeed, we have genetically fused scFv(L19) (specific to the alternatively spliced EDB domain of fibronectin; Pini *et al*., 1998), scFv(F8) (specific to the EDA domain of fibronectin; Villa *et al*., 2008) and scFv(F16) (specific to the A1 domain of tenascin-C; Brack *et al*., 2006) to several cytokines and have brought L19-IL2 (Carnemolla *et al*., 2002), L19-TNF (Borsi *et al*., 2003; Halin *et al*., 2003), F16-IL2 (Marlind *et al*., 2008) and F8-IL10 (Schwager *et al*., 2009) to Phase I and II clinical trials. In addition, a number of other immunocytokines have been tested in preclinical models of cancer (Halin *et al*., 2002a,b, 2003; Ebbinghaus *et al*., 2005; Gafner *et al*., 2006; Kaspar *et al*., 2007; Trachsel *et al*., 2007a).

IL12 is a heterodimeric cytokine, consisting of a p35 and a p40 subunit covalently linked by a disulfide bridge (Yoon *et al*., 2000). IL12 is a critical mediator of cell-mediated immune responses and plays an essential role in the interactions between the innate and adaptive arms of immunity acting on natural killer (NK) cells and T cells and enhancing the generation and activity of cytotoxic lymphocytes. IL12 is responsible for the priming of Th1 cell responses and the secretion of large amounts of IFN-γ from T cells and NK cells (Colombo and Trinchieri, 2002). IL12 is required for resistance to bacterial and intracellular parasites, as well as for the establishment of organ-specific autoimmunity.

IL12 that has exhibited a potent antitumor and antimetastatic activity in preclinical studies (Brunda *et al*., 1993; Trinchieri, 1997; Tsung *et al*., 1997; Rodolfo and Colombo,

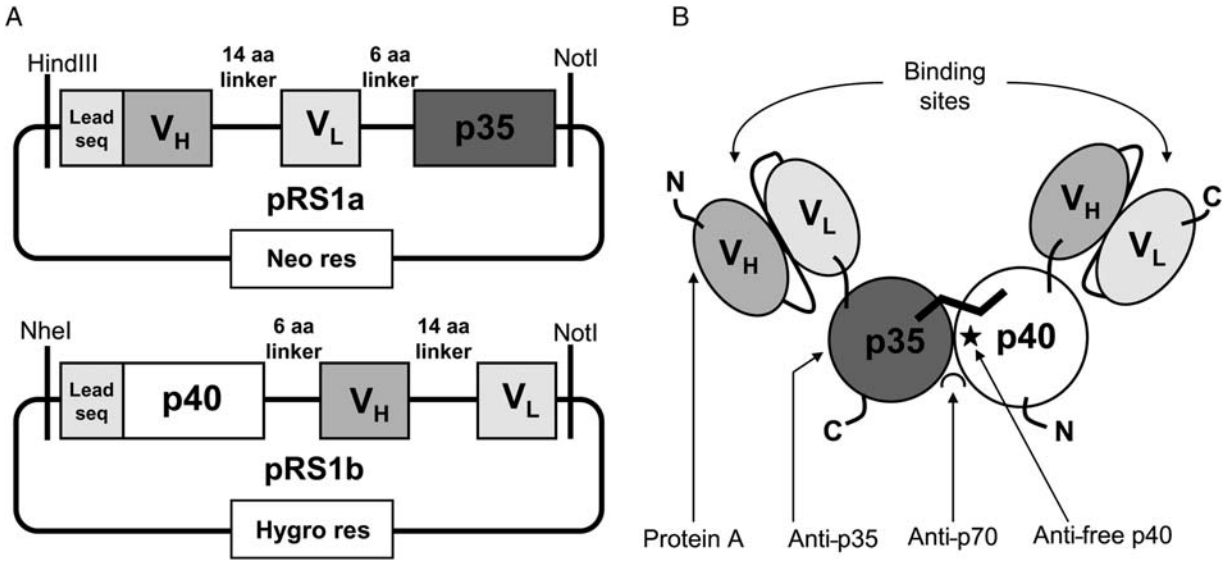


Fig. 1. Structure of F8-IL12. (**A**) Schematic representation of the cloning strategy of the F8-IL12 fusion protein. (**B**) Protein structure and binding sites of antigen, protein A, anti-p35 antibody, anti-p70 antibody and anti-p40 antibody.

1999; Colombo and Trinchieri, 2002). Clinical trials in patients with cancer have revealed promising therapeutic activities, but have also shown that recombinant human IL12 is extremely toxic to humans, with a maximal tolerated dose of 0.5 μg/kg of body weight (Atkins *et al*., 1997; Gollob *et al*., 2000).

We have previously reported that the tumor-homing performance and the antitumor activity of recombinant IL12 can be dramatically improved by fusion to suitable tumor-targeting antibody fragments, such as scFv(L19) (Halin *et al*., 2002b, 2003). The targeted delivery of the murine IL12 to the tumor site by the fusion to an scFv(L19) moiety markedly enhanced the antitumor activity of this cytokine in several models of mice bearing different aggressive murine tumors (Halin *et al*., 2002b). The tumor masses in the treated mice were infiltrated with lymphocytes, macrophages and NK cells and had elevated IFN-γ. The increased therapeutic activity of IL12 when fused to a tumor-homing antibody, compared with the minor effect observed when fused to an irrelevant antibody, showed that therapy correlates with targeting efficiency. Since the fusion of IL12 to scFv(L19) in a single-chain polypeptide format revealed modest tumor-targeting performance (Halin *et al*., 2002b), an engineered fusion protein with enhanced tumor uptake would display even more potent anticancer therapeutic properties.

Indeed, the heterodimeric nature of IL12 allows the engineering of various immunocytokine formats, based on different combinations of p35, p40 and scFv fusions. In a comparative biodistribution analysis, we have previously shown that the heterodimeric scFv-p35/p40-scFv format (Fig. 1), where both p35 and p40 subunits are genetically fused to an scFv fragment, displays optimal tumor-targeting properties and is thus ideally suited for therapeutic applications (Gafner *et al*., 2006). However, the cloning, expression and characterization of this heterodimeric immunocytokine for clinical use poses significant challenges, since clinical-grade material should not contain the peptidic tags that were previously used to facilitate purification of the heterodimer (Gafner *et al*., 2006) and since the p35 subunit is prone to homodimerization. Gene dosage effects on the expression of heavy and light chain have been found to play an important role for the high-titer production of IgG (Schlatter *et al*., 2005) and this issue becomes even more important for the expression and purification of heterodimeric immunocytokines, where one of the cytokine subunits can form both homodimers and heterodimers (see also the 'Results' section).

In this paper, we describe how the heterodimeric immunocytokine F8-IL12, consisting of scFv(F8) fused to both p35 and p40 subunits of human IL12, could be stably expressed in mammalian cells and purified to homogeneity with full retention of cytokine activity. The resulting product exhibited an impressive tumor-targeting activity in a mouse model of cancer. ScFv(F8) is particularly suited for tumor-targeting applications, since the cognate antigen is undetectable in normal adult tissues (exception made for placenta, endometrium and ovaries where physiological angiogenesis takes place; Schwager *et al*., 2009) but is strongly expressed not only in most solid tumors and lymphomas (Villa *et al*., 2008; Schliemann *et al*., 2009), but also on the neovasculature of tumor metastases (Rybak *et al*., 2007). F8-IL12 has now been produced according to good manufacture practice (GMP) and is now ready for pharmaceutical product development.

Material and methods

Cloning of F8-p35

For the cloning of F8-p35, the scFv(F8), containing a NheI restriction site and a leader sequence (Li *et al*., 1997) at the 5′, was amplified by PCR with primer RS9fo (5′-ctagctag cgtcgaccatgggctggagcctgat-3′) and NP5ba (5′-atcagcgcttccttt gatttccaccttggtcccttg-3′), which appends a part of the human p35 sequence. Human p35 gene was isolated from a human cDNA library (Human immune system MTC panel; Clontech, USA) and was amplified by PCR with primer NP6fo (5′-gtggaaatcaaaggaagcgctgatggaggtagaaac-3′) that

appends an overlapping sequence to the VL of scFv(F8) and RS12ba (5′-atagtttagcggccgctcattaggaagcattcagatagctcatcac-3′) which contains two stop codons and a NotI restriction site at the 3′. The scFv(F8) antibody sequence and the human p35 sequence were PCR assembled. The double-digested NheI–NotI assembly product was cloned into the mammalian cell expression vector pcDNA3.1(+) (Invitrogen, Switzerland) containing the resistance for neomycin.

Cloning of p40-F8

For the cloning of p40-F8, the human p40 gene and its leader sequence were isolated from a human cDNA library (Human immune system MTC panel; Clontech) and was PCR amplified with primer KS5fo (5′-cccaagcttatgtgtcacca gcagttggtcatc-3′) that anneals on the leader sequence and introduces a HindIII restriction site at the 5′ and RS8ba (5′-cccagactccaacagctgcacctcacctccatcagcgcttccactgca-3′) that contains an overlapping sequence with the scFv(F8). The scFv(F8) was amplified with primers RS3 (5′-gaggtgcagc tgttggagtctggg-3′) which partially overlaps with the sequence of human p40 and NP7 (5′cctttgcggccgctcactatttgatttccacc ttggtcccttg-3′) that introduces two stop codons and a NotI restriction site. Human p40 and the scFv(F8) sequences were PCR assembled and double digested with NheI and NotI restriction enzymes. The digestion product was cloned into the mammalian cell expression vector pcDNA3.1(+) (Invitrogen) containing the resistance for hygromycin. All PCRs were performed using the High Fidelity Taq Polymerase (Roche Diagnostics, Germany).

Cell culture

Adherent Chinese hamster ovary (CHO)-S cells were cultured in RPMI 1640 (Gibco, Switzerland) containing 10% FCS (Invitrogen) and incubated at 37°C and 5% CO_2. Suspension CHO-S cells were cultivated in PowerCHO-2 CD (Lonza, Switzerland) containing 8 mM ultraglutamine (Lonza, Belgium) incubated at 37°C and 5% CO_2. When necessary, G418 (Calbiochem, Germany) and Hygromicin (Invivogen, Switzerland) were added to the medium at a concentration of 0.5 mg/ml.

Generation of a cell line expressing F8-IL12

Adherent CHO-S cells were electroporated using an Amaxa nucleofector according to the manufacturer's protocol. Different ratios of plasmids F8-p35/p40-F8 were used to find the best conditions to maximize the expression of the heretodimer. Three days after electroporation, stably transfected cells were selected in the presence of G418 0.5 mg/ml (Calbiochem) and 0.5 mg/ml hygromycin (Invivogen). Monoclonal cell lines were obtained by serial dilutions and screened for expression of the fusion protein by ELISA.

Large-scale culture of F8-IL12 in bioreactor

Stably transfected suspension CHO-S cells driving F8-IL12 expression were grown in PowerCHO-2 CD containing 8 mM ultraglutamine, 0.5 mg/ml G418 and 0.5 mg/ml hygromicin. The cell culture inoculum for the 3 l Labfors bioreactor (Infors AG, Switzerland) was obtained starting from a static culture of the cells of the master cell bank. The cell culture was expanded in roller bottles until cell density reached $2–3 \times 10^6$ cells/ml. The fed batch fermentation process was used for the production of F8-IL12. Cells were seeded at 5×10^5 cells/ml in the bioreactor. Fixed cell culture parameters of F8-IL12 in the bioreactor were defined as 37°C temperature, 50% pO_2, 130 rpm helical stirring, pH 7.25. Glucose concentration was monitored, and manual feeds were applied to bring its concentration up to 2 g/l. In the exponential and plateau growth phase (72–144 h), feeds of fresh medium supplemented with ultraglutamine were introduced to maintain cell vitality. In order to monitor the total protein productivity, 100 ml supernatant samples were daily collected and affinity purified on HiTrap Protein A resin (GE Healthcare, Sweden).

Purification of F8-IL12

The fusion protein could be purified from the cell culture medium by protein A affinity chromatography, as there is a staphylococcal protein A binding site present on most VH3 domains (Sasso *et al*., 1991; Hoogenboom and Winter, 1992; Silacci *et al*., 2005). Ion exchange chromatography (Ceramic hyper D; PALL, USA) and preparative size exclusion chromatography on a HiLoad 26/60 Superdex 200 size exclusion column (GE Healthcare) of the eluted protein A preparation allowed the isolation of the F8-IL12 heterodimer from a mix containing also aggregates and polymers.

ELISA of supernatants

ELISA experiments on the conditioned culture media were performed according to Carnemolla *et al*. (1996). To detect different clones expressing F8-IL12, the biotinylated 11-EDA-12 domain of fibronectin, which includes the epitope recognized by scFv(F8), was immobilized on a streptavidin-coated plate (StreptaWell, Roche Applied Bioscience, Germany). Four different detection systems were used and allowed the evaluation of the expression of the different components of the fusion protein. Horseradish peroxidase-conjugated protein A (GE Healthcare), diluted according to the manufacturer's recommendations, was used as agent to detect the V_H domain of the fusion protein. In order to detect the heterodimer p70, the p35 subunit and the free p40 subunit, three different mouse monoclonal antibodies were used ['affinity purified antihuman IL12, p35' (eBioscience, Germany); 'affinity purified antihuman IL12, p40' (eBioscience); 'antihuman IL12 p70 antibody' (R&D Systems, Germany)] and detected with a goat anti-mouse IgG peroxidase conjugate (Sigma-Aldrich, USA). In all cases, the immunoreactivity with the immobilized antigen was detected using the substrate BluePOD (Roche Diagnostics) for peroxidase, and photometric absorbance at 405 nm was measured.

Bioactivity assay

The biological activity of F8-IL12 was determined by a T-cell proliferation assay (Gately *et al*., 2001). Freshly isolated human peripheral blood mononuclear cells were cultured immediately after isolation with 25 μg/ml mitogen phytohemagglutinin-M (Roche Diagnostics) for 3 days. Cells were diluted 1:2 by adding equal volume of supplement medium and further cultivated with 50 IU/ml of human IL2 (Roche Diagnostics). After 24 h, cells were seeded in 96-well plates at a density of 2×10^4 cells/well in 200 μl of medium containing serial dilutions of either F8-IL12 or commercially available, recombinant, human IL12 as a standard

(R&D Systems) or culture medium as a negative control. After 48 h, 20 μl Cell Titer 96 Aqueous One Solution (Promega, USA) was added to each well. The plate was incubated for 4 h and absorbance was read at 490 nm. The experiment was performed in triplicate.

Biodistribution studies

The *in vivo* targeting performance of F8-IL12 was evaluated by biodistribution analysis as described before (Carnemolla *et al*., 2002). Briefly, purified F8-IL12 was radioiodinated and injected into the tail vein of immunocompetent 129SvEv mice bearing s.c. implanted F9 murine teratocarcinoma. Mice were sacrificed 24 or 48 h after injection of the fusion protein (8 μg, 7 μCi/mouse). Organs were weighed and radioactivity was counted with 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).

Microautoradiography

Twenty-four hours after the tail injection of radiolabeled F8-IL12, mice were sacrificed and tumors were embedded in paraffin. Ten-micrometer sections were cut and fixed with paraformaldehyde. Sections were then coated with NBT KODAK autoradiography emulsion (KODAK, USA), dried and stored at 4°C in the dark for ~3 weeks. The autoradiography emulsions were developed (Developer D-19, KODAK, France) and fixed (EASTMAN Fixer, KODAK). Finally, slides were rinsed with deionized water and counterstained with hematoxilin (SIGMA, Switzerland).

Mass spectrometry

Following reduction in F8-IL12 with 10 mM TCEP for 30 min at room temperature, the solution was desalted and concentrated using C_4 microcolumns (OMIX^{TM} tips, Varian Inc., Paolo Alto, CA, USA) according to the manufacturer's guidelines. The reduced and desalted protein was mixed with sinapinic acid (20 mg/ml in 70% ACN, 0.1% TFA) and spotted on a MALDI (matrix-assisted laser desorption/ionization) target plate. The mass spectrometric analysis was carried out using a 4800 MALDI-TOF/TOF Analyzer (Applied Biosystems, Foster City, CA, USA). All spectra were acquired in the linear mode in a mass range of 10 000–100 000 m/z with a solid-state laser (355 nm) at a laser repetition rate of 200 Hz. A total of 1500 laser shots were summed for each spectrum. Spectra were further processed using the Data Explorer software (Applied Biosystems).

Results

Optimization and stable transfection of cells expressing F8-IL12

The heterodimeric protein scFv(F8)-p35/p40-scFv(F8) consists of two polypeptides joined together by a disulfide bond between C74 of p35 and C177 of p40 (Yoon *et al*., 2000; Fig. 1B). The C-terminal fusion of scFv(F8) with the p40 subunit is recommended, since the N-terminus of this subunit should not be chemically modified in order to retain maximal biological activity (Lieschke *et al*., 1997). For immunochemical detection, the scFv moiety can be detected with a recombinant fibronectin fragment containing the EDA domain (Villa *et al*., 2008) and with Protein A (Hoogenboom and Winter, 1992; Silacci *et al*., 2005). The p35 subunit is recognized by monoclonal antibody 'affinity purified antihuman IL12, p35' (irrespective of whether it is free or paired with p40 or p35) and the p40 subunit by monoclonal antibody 'affinity purified antihuman IL12, p40' (only when it is not associated with p35; Fig. 1B). Furthermore, the heterodimeric moiety p70, consisting of the disulfide-linked p35 and p40, can be detected using monoclonal antibody 'antihuman IL12 p70 antibody'.

The genes coding for the two chains, featuring a 14 amino acid linker ($Gly_4SerGly_4SerGly_4$) between V_H and V_L domain and a 6 amino acid linker (GlySerAlaAspGlyGly) between scFv and IL12 subunit, were assembled by PCR and cloned into the pcDNA3.1 expression vectors carrying different antibiotic resistance genes, yielding plasmids pRS1a and pRS1b (Fig. 1A and Supplementary Table S1).

In order to investigate the effect of gene dosage on the correct assembly and production of F8-IL12, CHO cells were stably co-transfected with pRS1a and pRS1b used at different ratios (Fig. 2). The highest protein expression (as assessed by ELISA with protein-A-based detection) and the highest titer of heterodimeric protein (as assessed with the antihuman IL12 p70 antibody) were observed at a pRS1a/pRS1b ratio of 3/7 (Fig. 2). These transfectants were then used for the isolation and characterization of monoclonal cell lines.

Figure 3A shows the results of an ELISA characterization of culture cell supernatants derived from different monoclonal cell lines expressing F8-IL12, which retained the high-titer formation of heterodimer observed at the polyclonal level. Clone G11 was used for further subcloning steps and for stability studies. Figure 3B shows that 26/26 daughter clones derived from G11 stably produced F8-IL12, as assessed by anti-p70 ELISA. Subclone 26 was used for cell bank production according to the GMP guidelines (ICH guidelines Q5B, Q5C, Q5D).

Production and characterization of F8-IL12

The stable clone 26, driving F8-IL12 expression, was used for large-scale culture in bioreactor (see also the 'Material and methods' section). A 7-day fed batch fermentation process was found to be compatible with the production of F8-IL12. The cell culture was interrupted after 7 days as the cell viability was drastically decreased (<65%) and no increase in total protein yield could be observed from the previous 24-h measurement (Supplementary Fig. S1).

At the end of the fermentation, the culture supernatant was recovered by cell filtration on vacuum filtration flasks (Stericups, Millipore) and purified by protein-A chromatography. The resulting product was further purified by ion exchange chromatography and size exclusion chromatography, yielding the final product after a sterile filtration step with an overall yield of 7 mg/l. This yield is satisfactory, in consideration of the complex expression of heterodimeric immunocytokines and of the fact that the maximal tolerated dose of IL12 in patients is 0.5 μg/kg of body weight which would correspond to ~70 μg of immunocytokine per patient and administration (yielding 100 doses of fusion protein per liter production).

The resulting purified F8-IL12 was characterized by SDS–PAGE analysis, confirming the presence of a single band of apparent molecular weight equal to 120 kDa in non-reducing

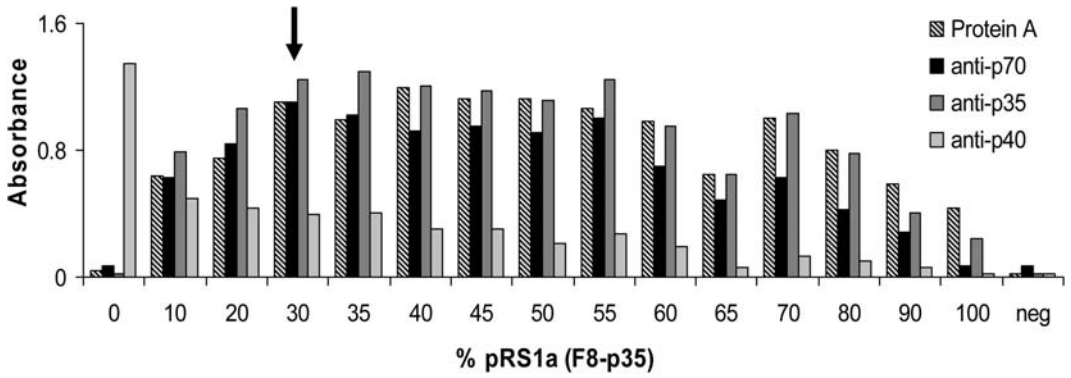


Fig. 2. ELISA on supernatants of co-transfected CHO-S cells with pRS1a and pRS1b at different ratios. The *x*-axis shows the percentage of pRS1a (F8-p35 plasmid) used. The arrow indicates the highest titer of heterodimeric protein (antihuman IL-12 p70 antibody) that was observed at a pRS1a/pRS1b ratio of 3/7.

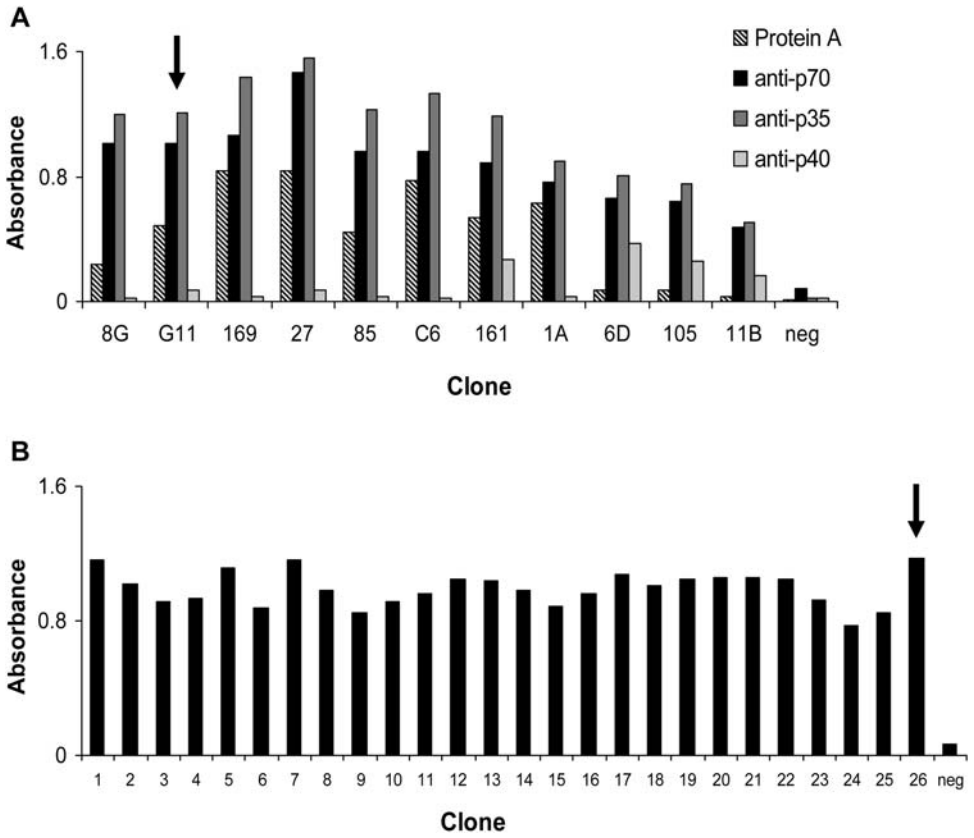


Fig. 3. Monoclonal cell lines. (**A**) ELISA on 11 monoclonal cell line supernatants using four different detection systems. The arrow indicates clone G11 chosen for subcloning. (**B**) ELISA on 26 monoclonal cell line supernatants with an antihuman IL-12 p70 antibody that recognizes the heterodimeric protein. The arrow indicates clone 26 that was used for large-scale production of F8-IL12.

conditions and of two bands of ~55 and 65 kDa in reducing conditions, as expected (Fig. 4A). The product eluted as a single peak in size-exclusion chromatography on a Superdex-200 column, with no evidence of protein aggregation (Fig. 4B). The heterodimeric F8-IL12 exhibited a high functional affinity toward the cognate antigen, with flat dissociation profile (apparent $k_{off} = 3.3 \times 10^{-4}\ s^{-1}$) as assessed by a real-time interaction analysis on a Biacore 3000 instrument (Fig. 4C).

A mass spectrometric characterization on a 4800 MALDI-TOF/TOF analyzer revealed that the two subunits F8-p35 and p40-F8 of the heterodimer F8-IL12 have molecular weights of 53 969 ± 88 and 64 246 ± 46 Da, respectively (Fig. 5). We experimentally detected an increased molecular weight for both subunits (Fig. 5) compared with the calculated molecular mass of the amino acid sequence (Fig. 5 and Supplementary Table S1). The difference in molecular weight for both subunits may be the consequence of a post-translation glycosylation, in agreement with the previously reported glycosylation patterns of unmodified IL12 (Carra *et al*., 2000). Carra *et al*. showed that both p40 and p35 subunits of IL12 undergo a complex glycosylation modification which is an essential requirement for the correct folding, assembly and secretion of the mature heterodimer. Indeed, the incomplete glycosylation of the p35 and p40 subunits deeply alter the formation and secretion of the heterodimer

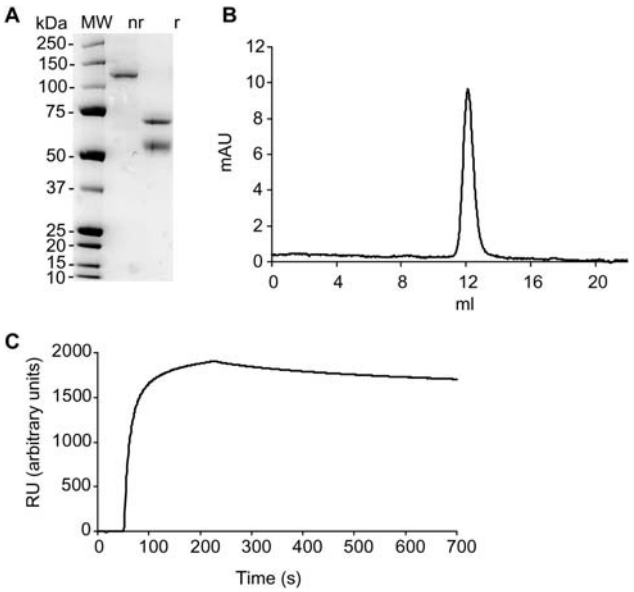
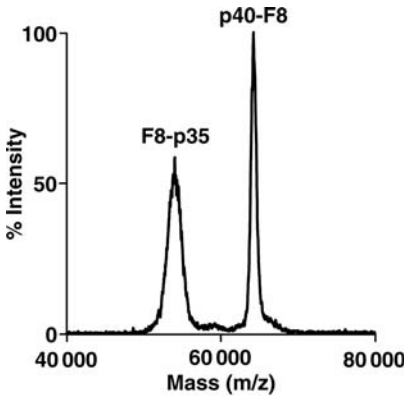


Fig. 4. Characterization of F8-IL12. (**A**) SDS–PAGE analysis of the protein under non-reducing and reducing conditions. (**B**) Gel filtration analysis reveals one single peak when analyzed under native conditions for the heterodimer F8-IL12. (**C**) Surface Plasmon resonance analysis shows flat dissociation phase of F8-IL12 (Biacore 3000, flow 20 µl/min, F8-IL12 5×10^{-7} M).



	Mass AA	Mass MS
F8-p35	48 299Da	53 969Da ±88Da
p40-F8	60 454Da	64 246Da ±46Da

Fig. 5. Mass spectrometric analysis of F8-IL12. Following reduction in the heterodimer F8-IL12, the two subunits F8-p35 and p40-F8 were identified by MALDI-TOF mass spectrometry. The determined mass of both subunits (Mass MS) was considerably higher than the mass calculated according to the amino acid composition (Mass AA). This difference is due to glycosylation of the two subunits.

and determines the formation of unsecreted p35 aggregates, confirming the critic role of the glycosylation for production and secretion of the mature heterodimer (Carra *et al.*, 2000).

In order to confirm that the engineering of the p70 moiety into scFv-p35/p40-scFv format (Fig. 1) did not impair the biological activity of IL12, we compared the immunostimulatory properties of recombinant human IL12 and of F8-IL12 in a lymphocyte proliferation assay. The two proteins

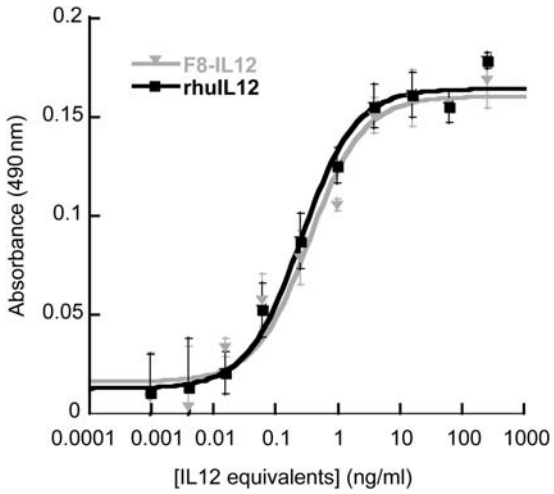


Fig. 6. Bioactivity assay. Comparison of the lymphoproliferative activity of rhuIL12 (in black) and F8-IL12 (in grey).

exhibited superimposable mitogenic profiles, when plotted on an equimolar basis, with a half-maximal activity at 600 pM IL12 equivalents (Fig. 6).

Finally, we investigated the tumor-targeting properties of F8-IL12 by performing a quantitative biodistribution analysis in mice bearing F9 tumors (Villa *et al.*, 2008) with a radioiodinated protein preparation. Cohorts of four mice were sacrificed 24 and 48 h after intravenous injection, and the resulting organs and body fluids were weighed and counted. F8-IL12 selectively accumulated in the tumor, with percent injected dose per gram (%ID/g) values of 22 and 15 at the two time points, respectively (Fig. 7A). When correcting for tumor growth during the experiment (F9 tumors have a doubling time of 40 h; Tarli *et al.*, 1999), one observes a stable accumulation of immunocytokine in the tumor at high levels (approx∼35%ID/g), which is consistent with the inert nature of EDA-containing fibronectin fibers (Fig. 7B). Furthermore, an *ex vivo* microautoradiography of tumors from biodistribution revealed selective accumulation of the radiolabeled F8-IL12 protein around tumor vessels (Fig. 7C).

Discussion

In this article, we have described the cloning, expression and characterization of a novel fully human immunocytokine, suitable for clinical development programs in patients with cancer. Compared with monomeric immunocytokines, the fusion of heterodimeric cytokines to a full antibody or antibody fragment presents several biotechnological challenges. First, multiple formats can be considered for the construction of fusion proteins, which do not exhibit identical *in vivo* biodistribution properties (Gafner *et al.*, 2006). Moreover, vector design and gene dosage effects influence the stability of the resulting stably transfected cell lines and product quality.

We used the strategy of Gafner *et al.* (2006) for the cloning of the heterodimeric cytokine IL12 to the tumor-targeting antibody fragment scFv(F8). The resulting immunocytokine consists of the disulfide-linked IL12 subunits p35 and p40, each joined to an scFv fragment. In contrast to the cloning strategy of Gafner *et al.*, we omitted all C-terminal

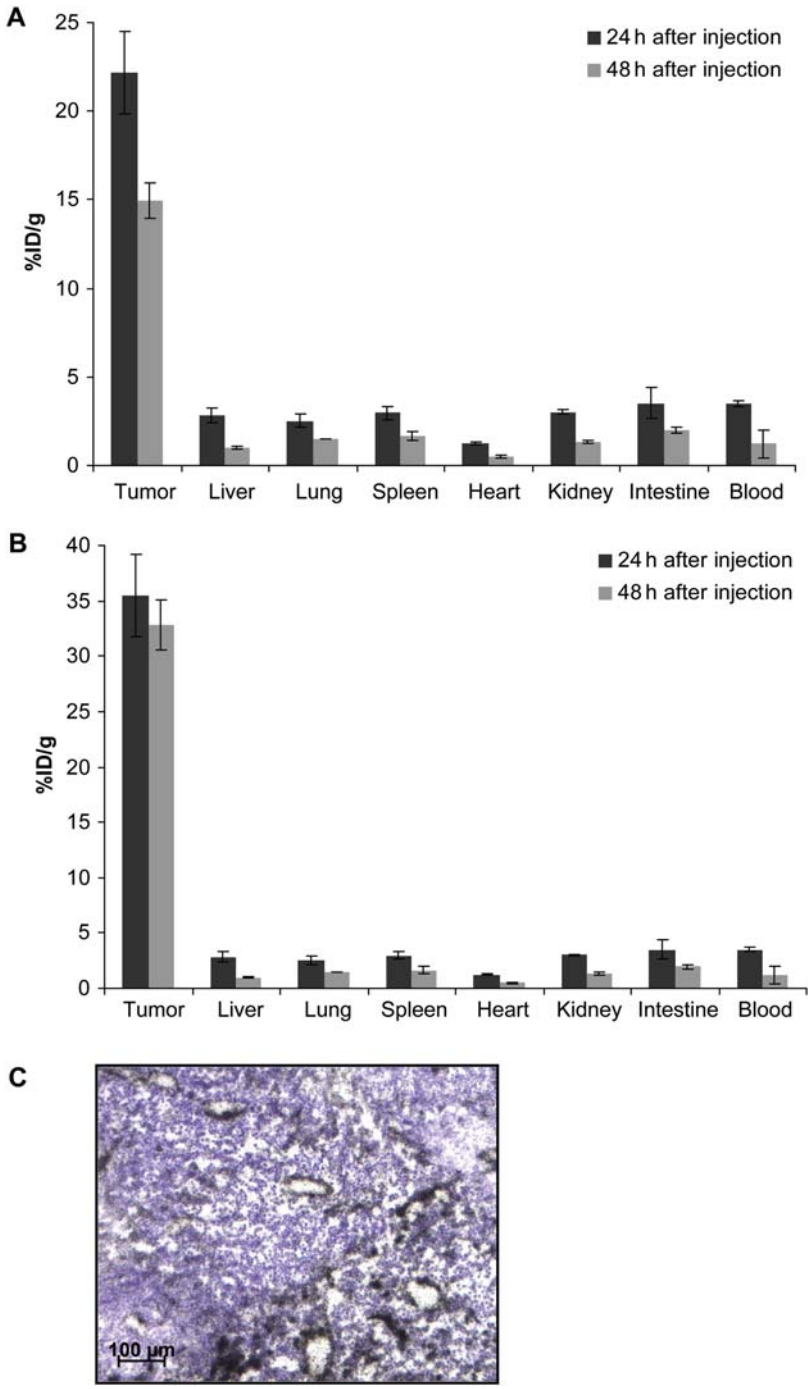


Fig. 7. Biodistribution studies. (**A**) F9 tumor bearing mice were injected intravenously with 125I-labeled F8-IL12 and sacrificed 24 or 48 h after injection. (**B**) Tumor values are corrected for tumor growth during the experiment (F9 tumors have a doubling time of 40 h). (**C**) An *ex vivo* microautoradiography shows a selective accumulation of 125I-labeled F8-IL12 around tumor vessels.

peptide tags, an essential requirement for compatibility with current GMP guidelines and subsequent clinical development of this biopharmaceutical. In spite of the complex cloning strategy with two independent vectors, the absence of peptidic tags for affinity purification and the need for cell line stability over multiple passages, we were able to generate a clone expressing the F8-IL12 heterodimer at 7 mg/l. These yields are compatible with clinical development programs, in consideration of the fact that the Maximal Tolerated Dose of IL12 in cancer patients is as low as 0.5 μg/kg of body weight. The fusion protein retained its full bioactivity as shown by *in vitro* proliferation assay and kept its high binding affinity to the cognate antigen, the alternatively spliced extradomain A of fibronectin. Biodistribution studies revealed an impressive tumor uptake of F8-IL12, with excellent tumor to organ ratio. Microautoradiography confirmed the selective accumulation of F8-IL12 at vascular structures of the tumor.

Our group has generated and investigated in clinical trials the human monoclonal antibodies F8, L19 and F16 (Pini *et al*., 1998; Brack *et al*., 2006; Villa *et al*., 2008), specific to the alternatively spliced EDA and EDB domains of

fibronectin and to the alternatively spliced A1 domain of tenascin-C, respectively. The tumor-targeting properties of these antibodies have extensively been studied in animal models of pathology (Carnemolla *et al*., 2002; Brack *et al*., 2006; Kaspar *et al*., 2007; Marlind *et al*., 2008; Villa *et al*., 2008). In addition, a radioiodinated version of the L19 antibody in the scFv format (Santimaria *et al*., 2003) and in the SIP format (Sauer *et al*., 2009) have been studied in imaging and radioimmunotherapy clinical trials in patients with cancer. Similar studies are currently being conducted also for SIP(F16) and SIP(F8).

The clinical-stage L19, F8 and F16 antibodies have been used as modular building blocks for the construction of immunocytokines based on IL2 (Carnemolla *et al*., 2002; Marlind *et al*., 2008), IL10 (Trachsel *et al*., 2007a; Schwager *et al*., 2009), IL12 (Halin *et al*., 2002b; Gafner *et al*., 2006), TNF (Borsi *et al*., 2003), IFN-gamma (Ebbinghaus *et al*., 2005), IL15, GM-CSF (Kaspar *et al*., 2007) and other cytokines.

IL12-based immunocytokines appear to display potent antitumor effects, without worsening inflammation in animal models of chronic autoimmune diseases (Trachsel *et al*., 2007b).

At present, the immunocytokines L19-IL2, F16-IL2, L19-TNF and F8-IL10 are being investigated in multiple clinical trials at 18 European centers. The development activities for F8-IL12 described in this article represent the basis for the future clinical studies of this product. Investigations in patients with cancer are particularly encouraged by the excellent biodistribution results observed with F8-IL12 (Fig. 7) and by the fact that the EDA domain of fibronectin is virtually undetectable in normal adult organs, while being strongly expressed in the majority of aggressive solid tumors and lymphomas (Pedretti, Rancic *et al*.; Rybak *et al*., 2007; Villa *et al*., 2008; Schliemann *et al*., 2009).

Supplementary data

Supplementary data are available at *PEDS* online.

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