

The targeted delivery of IL17 to the mouse tumor neo-vasculature enhances angiogenesis but does not reduce tumor growth rate

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Abstract There has been a long controversy as to whether interleukin-17 (IL17) has an impact on tumor growth. In order to assess whether IL17 may affect tumor growth, it would be convenient to achieve high levels of this pro-inflammatory cytokine at the tumor neo-vasculature, since IL17 is known to promote angiogenesis. Here, we have generated and tested *in vivo* a fusion protein, consisting of the F8 antibody (specific to the alternatively spliced EDA domain of fibronectin, a marker of angiogenesis) and of murine IL17 (mIL17). The resulting immunocytokine (termed F8-mIL17) was shown to selectively localize at the tumor neo-vasculature and to vigorously promote tumor angiogenesis, without however reducing or enhancing tumor growth rate both in immunocompetent and in immunodeficient mice.

Keywords Immunocytokines · IL17 · Vascular tumor targeting · EDA domain of fibronectin

Introduction

Interleukin 17 (IL17) is a homodimeric cytokine mostly expressed by Th17 cells [1]. It acts mainly as proinflam-

matory cytokine and was found to play a major role in inflammation and autoimmune diseases [2–5]. In addition, IL17 stimulates angiogenesis [6].

It is controversial whether IL17 favors or inhibits tumor growth [1]. Tumor cells transfected with the IL17 gene grew faster than untransfected cells when implanted in immunodeficient mice [6, 7], but similar experiments performed with IL17 in immunocompetent mice gave ambiguous results [8, 9]. Moreover, MC38 tumor implantation in IL17 knockout mice led to faster tumor growth in one laboratory [10] but not in a second laboratory, where the experiments were repeated [11].

Our group has demonstrated the possibility to deliver cytokines to the tumor environment using fusion proteins based on human monoclonal antibodies specific to splice isoforms of fibronectin [12–18] and of tenascin-C [19, 20]. In particular, the F8 antibody, specific to the alternatively spliced domain of fibronectin EDA (a marker of angiogenesis [21]) was shown to selectively localize at the tumor neo-vasculature following intravenous administration [22, 23].

As knock-out mouse models and tumor cells over-expressing IL17 offer little control over the specific location of IL17 expression within the tumor mass, we reasoned that fusion of IL17 to an antibody capable of selective localization on the tumor neo-vasculature could allow us to more precisely investigate the function of this cytokine when deposited at high local concentration on tumor blood vessels.

Here, a fusion protein consisting of the F8 antibody and of murine IL17 (F8-mIL17) was shown to selectively localize at the tumor neo-vasculature and to vigorously promote tumor angiogenesis, without however impacting on tumor growth rate.

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Materials and methods

Cell lines

CHO-S cells and the F9 murine teratocarcinoma cell line (CRL-1720, ATCC) were cultivated as described before [24]. NIH 3T3 fibroblasts were cultured in RPMI 1640 (Gibco) containing 10% FCS (Invitrogen), 2 mM ultra-glutamine (Lonza), 1 mM sodium pyruvate (Gibco) and 50 μ M β -mercaptoethanol (Gibco) and incubated at 37°C and 5% CO₂.

Cloning, expression, purification and characterization of F8-mIL17

For the cloning of F8-mIL17, containing a *NheI* restriction site upstream of the leader sequence, the (scFv)F8 gene was PCR amplified from the previously described clone F8-SIP [23] using primers RS9 (5'-ctagctagcgtcgaccatggctggagcctgat-3') and NP28 (5'-acctccaccgccagaaccacttccgcctgattgattccaccttggctccttg-3') appending a 10 amino acid linker at the C-terminus of the antibody moiety. The mIL17 gene (Source BioScience) was amplified by PCR with primers NP30 (5'-tcaggcggaagtggcttctggcggtggaggtgcagcgatcatcctcaagctc-3') and NP24 (5'-tcgataagcttcattaggctgctggcgacaatcgag-3') introducing two stop codons and a *HindIII* restriction site. The scFv(F8) and the mIL17 genes were PCR-assembled using primers RS9 and NP24. The double-digested *NheI/HindIII* assembly product was cloned into pcDNA3.1(+) (Invitrogen) vector. A stable CHO-S cell line growing in suspension and expressing F8-mIL17 was generated as previously reported [24]. F8-mIL17 was purified from the cell culture medium by protein A affinity chromatography. F8-mIL17 was analyzed by SDS PAGE, size exclusion chromatography (Superdex200 10/300 GL, GE Healthcare) and BIAcore on an EDA coated sensor chip.

Bioactivity assay

The biological activity of F8-mIL17 was determined by its ability to induce IL6 production in NIH 3T3 fibroblasts. 4×10^4 cells were seeded in 96-well plates in medium containing serial dilutions of F8-mIL17 or recombinant mIL17 (standard, Chemie Brunschwig) or culture medium (negative control). After 48 h, cytokine expression in supernatants was determined with Mouse IL6 ELISA Ready-SET-Go! (BD Biosciences).

Quantitative biodistribution studies

The in vivo targeting performance of F8-mIL17 was evaluated by biodistribution analysis as described before [22]. F8-mIL17 was radiiodinated and injected into the

tail vein of female 129/SvPas (Taconic) (14.5 μ g radiolabeled protein or 7.5 μ g radiolabeled plus 60 μ g unlabeled protein) or Balb/c nude mice (Charles Rivers) (14.5 μ g radiolabeled protein) bearing s.c. implanted F9 tumors [22–25]. Mice were sacrificed 24 h after injection.

Syngeneic tumor mouse models

Tumor bearing mice were obtained by subcutaneous injection of F9 cells (10^7) in 12 week-old female 129/SvPas or Balb/c nude mice [22, 24]. Four days after tumor implantation, mice were grouped and injected into the lateral tail vein three times every 48 h with F8-mIL17 (100 μ g) or saline. Mice were monitored daily and tumor growth was measured with a caliper using the formula: volume = length \times width² \times 0.5. Animals were sacrificed when tumor volumes reached 2000 mm³. The experiment was repeated in immunocompetent mice, which were sacrificed 3 days after last injection. Tumors were excised, embedded in cryoembedding medium (Thermo-Scientific) and stored at -80°C . Experiments were performed under a project license granted by the Veterinäramt des Kantons Zürich, Switzerland (169/2008).

Immunofluorescence analysis on tumor sections

Immunofluorescence staining of cryostat sections (10 μ m) of F9 tumors was performed essentially as described [22] using as primary antibodies rat anti F4/80 (macrophages, Abcam), rat anti CD45 (leukocytes, BD Biosciences), rat anti CD31 (endothelial cells, BD Biosciences), rat anti CD4 (CD4 T cells), rat anti CD8 (CD8 T cells), rabbit anti Asialo/GM1 (NK cells, Wako Pure Chemical Industries) antibodies and biotinylated F8-mIL17. Anti rat IgG-AlexaFluor488, anti rat IgG-AlexaFluor594, anti rabbit IgG-AlexaFluor488, as well as streptavidin-AlexaFluor488, were used as secondary reagents for microscopic detection. Images were obtained with an Axioskop2 mot plus microscope (Zeiss), the staining areas were analyzed using ImageJ software and expressed as percentage of measurement area.

Results and discussion

We cloned and expressed in mammalian cells a fusion protein consisting of scFv(F8) sequentially fused to a murine IL17 monomer (Fig. 1a). The two mIL17 monomers drive the disulfide-linked covalent homodimerization of the fusion protein, forming a stable immunocytokine termed “F8-mIL17” (Fig. 1b). F8-mIL17 was purified by protein A chromatography, exhibited a homogeneous profile in size-exclusion chromatography with a retention time

corresponding to the expected dimeric structure (Fig. 1c), ran as a covalent homodimer in SDS-PAGE in non-reducing conditions and as a monomer in reducing conditions (Fig. 1d). F8-mIL17 was shown to retain the binding affinity of the parental antibody in a BIAcore assay (Fig. 1e) [23] and to be fully active in an in vitro cytokine production assay (Fig. 1f). A radioiodinated preparation of F8-mIL17 was analyzed in a quantitative biodistribution study in immunocompetent mice bearing subcutaneous F9

tumors, a model frequently used to study vascular-targeting antibody-based fusion proteins [22–25], confirming a preferential accumulation in the tumor mass [4.3% injected dose per gram (%ID/g) at 24 h], compared to normal organs [tumor to blood ratio of 14:1 (Fig. 1g)]. Similar results were obtained in athymic mice (Fig. 1g). As expected and in line with previous experiments based on the F8 antibody [23], the F8-mIL17 immunocytokine localized around tumor blood vessels (Fig. 1h).

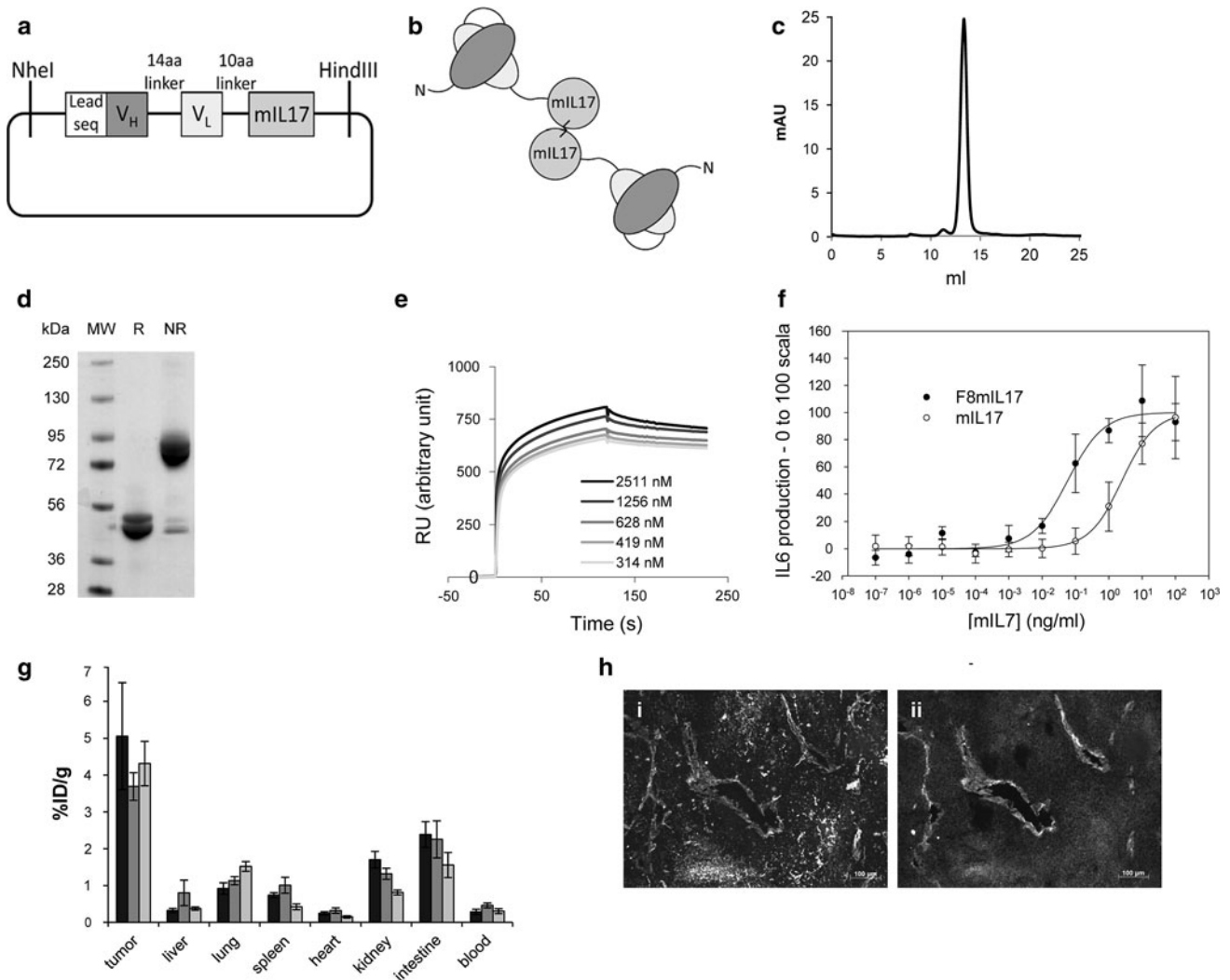


Fig. 1 Cloning, expression and characterization of F8-mIL17. **a** Schematic representation of the cloning strategy of F8-mIL17 with a 14-amino acid linker between variable heavy (VH) and light (VL) chain. **b** Schematic representation of the domain assembly of F8-mIL17. **c** Gel filtration analysis of affinity-purified F8-mIL17. The peak corresponds to the homodimeric form of the fusion protein. **d** SDS-PAGE analysis of purified F8-mIL17: molecular weights (MW), reducing (R) and non reducing (NR) conditions. The fusion protein is present in different glycosylation forms. **e** BIAcore analysis of F8-mIL17 on EDA-coated chip. **f** IL6 production bioactivity assay on NIH 3T3 fibroblasts. F8-mIL17 displayed biological activity comparable with the one of recombinant murine IL17 (mean of 3 replicates \pm SD). **g** Tumor targeting properties of F8-mIL17 in

biodistribution studies in Balb/c nude and 129/SvPas mice ($n = 5$) bearing subcutaneous F9 tumors. 14.5 μ g radiolabeled 125 I-F8-mIL17 were injected i.v in Balb/c nude (black square) and 129/SvPas mice (grey square), 7.5 μ g radiolabeled 125 I-F8-mIL17 and 60 μ g unlabeled F8-mIL17 were mixed and injected i.v. in 129/SvPas mice (light grey square), which were sacrificed after 24 h. Organs were excised and radioactivity counted, expressing results as percent injected dose per gram of tissue (%ID/g) \pm SE. A selective accumulation of F8-mIL17 in F9 tumors could be observed over time. **h** Immunofluorescent staining of F8-mIL17 on F9 tumors. F8-mIL17 was biotinylated and used as staining reagent in an immunofluorescence procedure. F8-mIL17 shows a vascular staining pattern. Scale bars 100 μ m

When administered intravenously at doses of 100 μg (day 5, 7, 9), F8-mIL17 displayed no anti-cancer activity in immunocompetent (Fig. 2a) and athymic mice (Fig. 2b) bearing subcutaneous F9 tumors, compared to the saline control group. There was no weight loss in immunocompetent mice, but up to 15% weight loss in nude mice [Supplementary Figure 1]. Figure 2c shows a representative immunofluorescence analysis of the tumor masses at the end of the therapy. Staining of the tumors with an anti-CD45 antibody (Fig. 2ci, vi), F4/80 antibody (Fig. 2cii, vii), Asialo-GM1 antibody (Fig. 2ciii, viii) and CD4 antibody (Fig. 2civ, ix) revealed a higher leukocytes, macrophages, NK cells and CD4 T cells (Fig. 2civ, ix) infiltration in the tumors treated with F8-mIL17 (Fig. 2ci–iv), compared to the saline control group (Fig. 2cvi–ix).

By contrast, CD8 T cells were found to be low in both treatment groups (Fig. 2cv, x) (more pictures are available in Supplementary Figures 2–6). Figure 2d shows representative images (the experiment was repeated twice) of tumor blood vessels 3 days after the last injection, from mice treated with F8-mIL17 (Fig. 2di–vi) or with saline (Fig. 2dvi–xii). F8-mIL17 treatment resulted in a significant ($P < 0.01$) increase in blood vessel density (Fig. 2e).

We can conclude that the antibody-based targeted delivery of mIL17 to the tumor blood vessels enhances tumor angiogenesis and leukocyte infiltration into the tumor mass, but this (in contrast to fusion proteins based on other cytokines such as IL2, IL7, IL12, IL15, GM-CSF, TNF [12, 13, 15–20, 24]) does not lead to a measurable anti-cancer activity in immunocompetent or in nude mice.

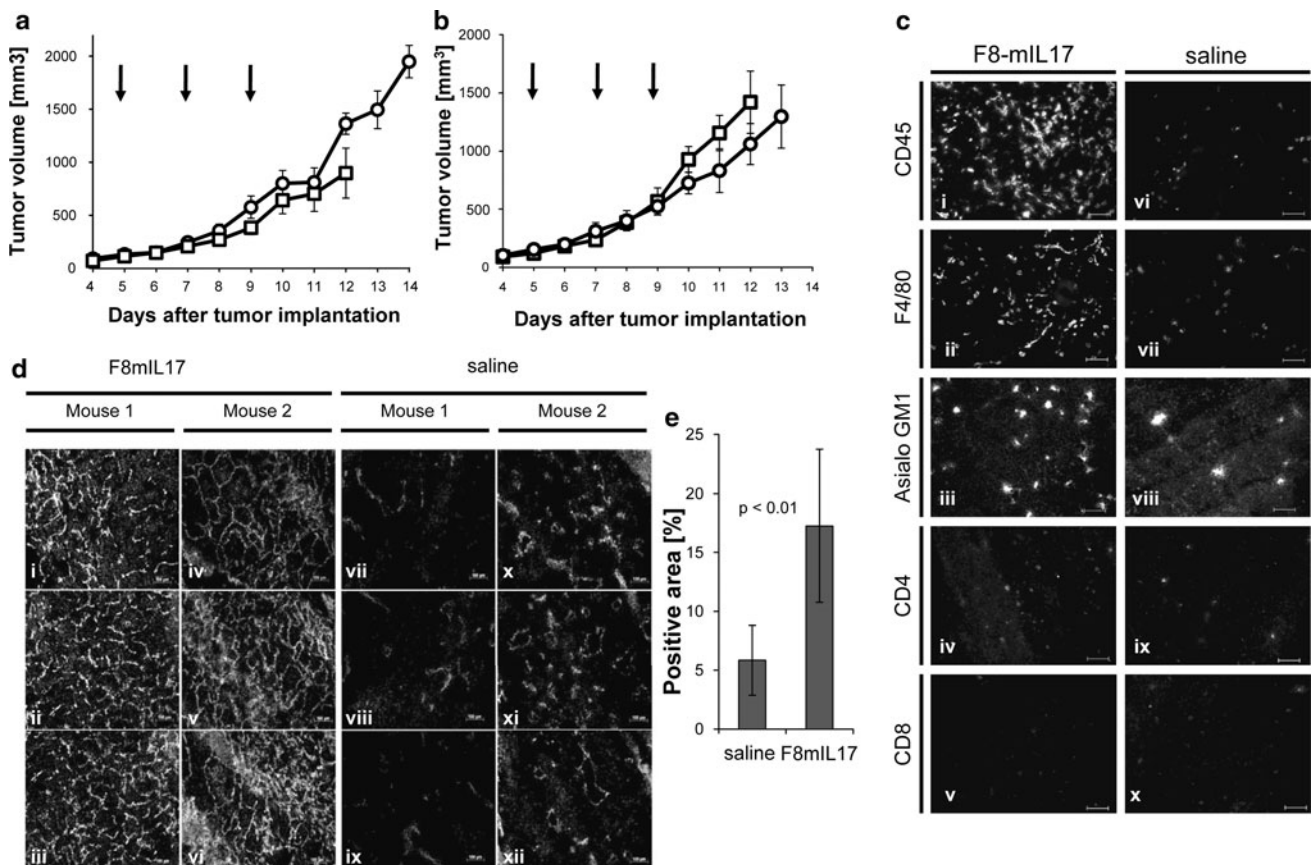


Fig. 2 Effects of F8-mIL17 in mice bearing subcutaneously-grafted F9 tumors. Tumor growth curves of **a** immunocompetent 129/SvPas or **b** athymic Balb/c nude mice bearing F9 tumors after i.v. treatment with three injections of 100 μg F8-mIL17 (square; $n = 5$, $n = 5$) and saline (circle; $n = 3$, $n = 4$). Days of treatment (day 5, 7, 9 after tumor implantation) are indicated by arrows. Data represent mean tumor volumes \pm SE. **c** Immunofluorescence analysis of tumor-infiltrating immune cells. Tumor sections of therapy mice were stained for leukocytes (CD45), macrophages (F4/80), NK cells (Asialo/GM1), CD4 T cells and CD8 T cells. Scale bars = 100 μm . Representative images of leukocytes (i, vi), macrophages (ii, vii), NK

cells (iii, viii), CD4 T cells (iv, ix) and CD8 T cells (v, x) staining in F8-mIL17 (i–v) and saline (vi–x) treated tumors. **d** Immunofluorescence analysis of vasculature (CD31 staining). Immunocompetent F9 tumor-bearing mice were injected i.v. three times every 48 h with 100 μg F8-mIL17 or saline. Mice were sacrificed 3 days after last injection. Tumor sections were stained for endothelial cells. Representative images of 2 mice treated with F8-mIL17 (i–iii, iv–vi) and 2 mice treated with saline (vii–ix, x–xii). **e** F9 tumor sections were evaluated for area percentage positive staining using ImageJ ($P < 0.01$, Student *t* test)

Our findings are consistent with previous publications, in which no difference in tumor growth rate was observed in IL17 $-/-$ mice compared to wild-type mice [11], or in which tumor cells transfected with mIL17 displayed no anti-cancer activity [6, 7, 9]. It remains to be seen whether the activity of IL17 could be different in an orthotopic setting. In spite of these considerations, it would be conceivable to use IL17-based immunocytokines to stimulate therapeutic angiogenesis at sites of disease [26].

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Conflict of interest DN is founder and shareholder of Philogen, a biotech company that owns the F8 antibody.

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