Journal Article

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Publication Date:
2018-04-10

Permanent Link:
https://doi.org/10.3929/ethz-b-000250058 →

Originally published in:
Journal of biotechnology 271, http://doi.org/10.1016/j.jbiotec.2018.02.004 →

Novel antibody-cytokine fusion proteins featuring granulocyte-colony stimulating factor, interleukin-3 and interleukin-4 as payloads

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Submitted as research paper to *Journal of Biotechnology*

Abstract

Neutrophils can strongly influence disease activity in cancer and in chronic inflammation. Here, we report for the first time the construction and characterization of antibody-fusion proteins featuring granulocyte-colony stimulating factor and interleukin-3 as payloads capable of enhancing neutrophil activity and a novel antibody-interleukin-4 fusion protein with neutrophil inhibitory potential. We used the F8 antibody specific to the alternatively-spliced extra domain A (EDA) of fibronectin as a targeting agent, since the cognate antigen is strongly upregulated in diseases characterized by angiogenesis.

The fusion proteins GCSF-F8, F8-IL3 and F8-IL4-F8, were cloned, expressed, and their targeting ability assessed, exhibiting preferential tumor uptake with tumor:blood ratios at 24 hours after injection of 3.3, 18.2 and 27.3, respectively.

In F9 tumor bearing-mice GCSF-F8 and F8-IL3 did not provide a therapeutic benefit, while F8-IL4-F8 showed a potent tumor growth retardation. In the collagen-induced model of arthritis, GCSF-F8 and F8-IL3 induced a worsening of the disease, while F8-IL4-F8 slowed arthritis progression but, surprisingly, exhibited substantial toxicity when used in combination with dexamethasone.

Collectively, the results indicate that the novel fusion proteins could be expressed and efficiently delivered to the site of disease. However, they were not superior to other antibody-cytokine fusions previously described by our laboratory.

Keywords

granulocyte-colony stimulating factor, interleukin-3, interleukin-4, immunocytokines, EDA of fibronectin

1. Introduction

Cytokines are a class of immunomodulatory proteins which play an important role in health and disease by regulating the number, activity and behavior of cells.(Murphy et al., 2012) Besides interleukins, produced by lymphocytes, some hematopoietic growth factors (e.g., Granulocyte-colony stimulating factor (G-CSF) and Granulocyte-macrophage colony-stimulating factor (GM-CSF)) are nowadays also classified as members of the cytokine family.(Murphy et al., 2012) Various recombinant cytokines have gained marketing authorization for the treatment of different types of conditions.(Bootz and Neri, 2016; Kontermann, 2012) In most cases, these agents show activity at sub-milligram doses in patients and the escalation to therapeutically-active regimens is sometimes prevented by the onset of toxicity. For this reason, considerable research efforts have been devoted to the generation of disease-targeting antibody-cytokine fusions ("immunocytokines"), with the aim to increase the therapeutic index of the immunomodulatory payload.(Kontermann, 2012; Neri and Sondel, 2016; Pasche and Neri, 2012) A number of immunocytokine products have been investigated in clinical trials for the therapy of cancer and of other conditions and some agents are currently being investigated in Phase III clinical studies [clinical trial identifier: NCT02938299 (www.clinicaltrial.gov)].(Neri and Sondel, 2016; Pasche and Neri, 2012)

Various types of antibodies can be considered for the generation of cytokine fusions. Our laboratory has previously described the use of the F8 antibody, specific to the alternatively-spliced extra domain A (EDA) of fibronectin, as a vehicle for pharmacodelivery applications.(Schwager et al., 2009; Villa et al., 2008) EDA(+)-fibronectin is virtually undetectable in normal tissues (exception made for placenta, the endometrium and some vessels of the ovaries), while the antigen is strongly expressed at sites of tissue remodeling,

with a distinctive perivascular pattern of staining in various types of cancer and in rheumatoid arthritis.(Rybak et al., 2007; Schwager et al., 2009)

For some biotechnological applications, it is convenient to use antibodies in full IgG format.(Becker et al., 1996; Fallon et al., 2014; Mossner et al., 2010; Xuan et al., 2010) Alternatively, antibody fragments (e.g., scFv fragments or diabodies) may be preferable in order to achieve a long residence time at the site of disease and a fast clearance from circulation.(Holliger et al., 1993; Huston et al., 1988) Our laboratory has previously described the fusion of disease targeting antibody fragments with various cytokine payloads, including several interleukins (e.g., IL1β, IL2, IL4, IL6, IL7, IL9, IL10, IL12, IL13, IL15, IL22), members of the TNF superfamily, interferons and growth factors (e.g., VEGF-A, GM-CSF).(Kiefer and Neri, 2016; Pasche and Neri, 2012)

In this study, we focused on the engineering and characterization of novel antibody-fusion proteins, featuring payloads capable of modulating neutrophil activity. G-CSF is responsible for the induction of neutrophil development, differentiation and mobilization.(Murphy et al., 2012) It is produced by fibroblasts and monocytes and acts on neutrophils by binding to the homodimeric G-CSF-Receptor (G-CSFR).(Bendall and Bradstock, 2014; Murphy et al., 2012) In the clinic, recombinant G-CSF is used for the treatment of congenital and acquired (e.g. due to chemotherapy) neutropenia.(Spiekermann et al., 1997) Similarly, interleukin-3 (IL3) acts on a multitude of cell types in early hematopoiesis and can induce a significant elevation of the neutrophil titer, although neutrophils eventually lose responsiveness at mature stages.(Eder et al., 1997; Murphy et al., 2012; Oster et al., 1991) IL3 was so far both shown to induce a worsening as well as a beneficial effect in models of murine arthritis and osteoarthritis.(Bruhl et al., 2009; Kour et al., 2016; Srivastava et al., 2011)

Interleukin-4 (IL4) is a crucial cytokine for the differentiation of $CD4^+$ T cells into a T_H2 lineage.(Murphy et al., 2012) Furthermore, IL4 has recently been shown to inhibit neutrophil expansion and recruitment by interacting with neutrophil type 2 IL4 receptors to antagonize G-CSF and chemokine mediated signaling. (Woytschak et al., 2016) An F8 diabody IL4 fusion protein was the first immunocytokine to induce complete cures in a collagen-induced arthritis (CIA) model in combination with dexamethasone.(Hemmerle et al., 2014) In this study, a novel format of the fusion protein was generated.

We fused the F8 antibody with G-CSF, IL3 and IL4 (generating GSCF-F8, F8-IL3 and F8-IL4-F8), yielding proteins that could be purified to homogeneity and which retained full biological activity. In quantitative biodistribution studies, the fusion proteins efficiently localized to tumors after intravenous administration. However, in an immunocompetent mouse model of cancer, only the IL4 fusion displayed a potent anti-cancer activity. In the collagen-induced model of arthritis, GCSF-F8 and F8-IL3 worsened arthritis progression, while F8-IL4-F8 provided only a weak anti-arthritic activity.

2. Material and Methods

2.1 Cell culture

For cell cultivation CHO-S cells (Invitrogen, Switzerland) were cultured in suspension in PowerCHO-2 CD (LZ-BE12-771Q; Lonza, Belgium) supplemented with 4 mM Ultraglutamine 1 (Lonza, Belgium), 1X HT Supplement (50X) (Gibco, UK) and 1X Anti-Anti (100X) (Gibco, UK) in shaking incubators. For protein production CHO-S cells were cultured in suspension in ProCHO 4 (LZ-BE12-029Q; Lonza, Belgium) supplemented with 4 mM Ultraglutamine 1 (Lonza, Belgium), 1X HT Supplement (50X) (Gibco, UK) and 1X Anti-Anti (100X) (Gibco, UK) in shaking incubators.

CTLL2 cells were cultivated in RPMI 1640 (Gibco, UK) supplemented with 10% Fetal Bovine Serum (Gibco, UK), 2 mM Ultraglutamine 1 (Lonza, Belgium), 1X Anti-Anti (100X) (Gibco, UK), 25 mM Hepes (Gibco, UK), 0.05 mM β-Mercaptoethanol and 60 Units/mL Interleukin-2, human (Roche Diagnostics GmbH, Germany).

2.2 Cloning of novel fusion proteins GCSF-F8, F8-IL3, F8-IL4-F8

The F8 antibody in diabody (F8Db) and scFv (F8scFv) format has been described previously.(Frey et al., 2010; Villa et al., 2008)

GCSF-F8scFv was designed linking a secretion sequence peptide containing an N-terminal NheI restriction site, murine GCSF (AA 31-208), a flexible 15-amino-acid linker $(SSSSG)_3$ and the sequence for the F8 antibody in scFv format with a stop codon and EcoRI restriction site at the C-terminus into the mammalian expression vector pcDNA3.1(+). The assembled construct was ordered from Genscript (United States). GCSF-F8Db (hereon referred to as GCSF-F8) was cloned by amplifying from GCSF-F8scFv the fragment containing the N-terminal secretion sequence, GSCF, the 15-amino-acid linker and the F8 V_H (heavy chain variable domain) using the primer pair 5’

CCCGCTAGCGTCGACCATGGGCTGGAGCCTGATCCTCCTGTTCCTCGTCGCTGTGGC

3’ and 5’ CCCCTCCGCTACCGCCACTCGAGACGGTGACCAGGGTTCCC 3’, generating part of the intra diabody linker C-terminally and by amplifying the F8 V_L (light chain variable domain) with C-terminal stop codon and EcoRI restriction site from GCSF-F8scFv with the primer pair 5’ GTCTCGAGTGGCGGTAGCGGAGGGGAAATTGTGTTGACGCAGTCTCCAGGC 3’ and 5’ . TCCCCGCGAATTCTCATTAAGCTATTTGATTTCCACCTTGGTCCCTTGGCCGAACGTC GG 3’, N-terminally generating part of the intra diabody linker. The fragments were assembled by polymerase chain reaction (PCR), and after digestion via NheI and EcoRI restriction sites were inserted into the mammalian expression vector pcDNA3.1(+) (Invitrogen).

F8Db-IL3 (hereon referred to as F8-IL3) was designed linking a secretion sequence peptide containing an N-terminal NheI restriction site, the sequence for the F8 antibody in diabody format, a flexible 15-amino-acid linker $(SSSSG)_3$ and murine IL3 (AA 27-166) with a stop codon and EcoRI restriction site at the C-terminus into the mammalian expression vector pcDNA3.1(+). The assembled construct was ordered from Genscript (United States).

F8scFv-IL4-F8scFv (hereon referred to as F8-IL4-F8) was cloned by amplifying the first F8scFv fragment containing the secretion signal sequence, using the primer pair 5’ CCTGTTCCTCGTCGCTGTGGCTACAGGTGTGCACTCGGAGGTGCAGCTGTTGGAG TC 3’ and 5’ CTGGACGATGAGCCGGAAGAGCTACTACCCGATGAGGAAGATTTGATTTCCACC TTGGTC 3’ from GCSF-F8scFv, by amplifying the IL4 fragment from F8Db-IL4 (described previously) using the primers 5’ GCTCTTCCGGCTCATCGTCCAGCGGCCATATCCACGGATGCGACAAAAATCAC 3’ and 5’ GCCACTAGAGCTGGATCCCGATGAGGAAGACGAGTAATCCATTTGCATGATGC 3’ and by amplifying the C-terminal F8scFv fragment containing a stop codon and EcoRI restriction site at the C-terminus from GCSF-F8scFv with 5’

CCTCATCGGGATCCAGCTCTAGTGGCTCATCGTCCAGCGGCGAGGTGCAGCTGTT GGAG 3' and 5'CCCCGCGAATTCTCATTAAGCTATTTGATTTCCACCTTGGTCCCTTGGCCGAAC GTCGG 3'.(Hemmerle and Neri, 2014a) The first two overlapping fragments were joined with the primers 5'CCCGCTAGCGTCGACCATGGGCTGGAGCCTGATCCTCCTGTTCCTCGTCGCTGT GGC3' and 5' GCCACTAGAGCTGGATCCCGATGAGGAAGACGAGTAATCCATTTGCATGATGC 3' generating F8scFv-IL4, in which the two parts are joined by a flexible 15-amino-acid linker $(SSSSG)_3$, and the C-terminal F8scFv fragment was added on in a further PCR step, generating F8scFv-IL4-F8scFv, where the C-terminal F8scFv moiety is also joined to IL4 with a flexible 15-amino-acid linker $(SSSSG)_3$. After digestion via NheI and EcoRI restriction sites the construct was inserted into the mammalian expression vector pcDNA3.1(+) (Invitrogen).

2.3 Transient gene expression and characterization

All fusion proteins were expressed using polyethyleneimine (PEI)-mediated transient gene expression in CHO-S cells.(Hacker et al., 2013) For 1 mL of production 10^6 CHO-S cells in suspension were centrifuged and resuspended in 0.5 mL ProCHO4. 0.9 μg of desired plasmid DNA followed by 2.5 μg of PEI (Polysciences) was added to the cells and the suspension gently mixed. The cells were incubated for 6 days in a shaking incubator at 31°C. The procedure was scaled up in order to obtain the desired production volume.

The fusion proteins were purified from the culture medium via protein A affinity chromatography and analyzed using SDS-PAGE in non-reducing and reducing conditions, Size exclusion chromatography (Superdex 200 increase, 10/300 GL) (GE Healthcare, UK) and

surface plasmon resonance analysis (BIAcore S200) (GE Healthcare, UK) on an EDA antigen-coated CM5 Biacore sensor chip.

2.4 *In vitro* bioactivity assay

The biological activity of the IL4 moiety was assessed by determination of its capability to stimulate CTLL2 cell proliferation. 20'000 cells were seeded per well in 96-well plates in culture medium supplemented with different concentrations of F8-IL4-F8 or recombinant IL4 (Biolegend, United States). After incubation for 24 h at 37°C in an incubator with 5% CO_2 atmosphere the cell proliferation was assessed with Cell Titer 96 Aqueous One Solution (Promega, Switzerland).

2.5 Immunohistochemistry

8 µm cryostat sections of saline treated F9 teratocarcinoma tumors and saline treated paws from mice with collagen-induced arthritis were fixed in ice-cold acetone and stained with FITC-labelled GCSF-F8, F8-IL3 or F8-IL4-F8 (5 μg/mL) or saline as a negative control. In a second step the sections were stained with rabbit anti-FITC (BioRad, Switzerland) and rat anti-CD31 (ThermoFisher, Switzerland) and detected with anti-rabbit AlexaFluor488 (ThermoFisher, Switzerland) and anti-rat AlexaFluor594 (ThermoFisher, Switzerland). The paw section negative control was additionally incubated with PBS instead of rabbit anti-FITC and rat anti-CD31 in the second step before detection. Slides were then mounted with fluorescent mounting medium (Dako, Agilent, Switzerland) and analyzed with an Axioskop2 mot plus microscope (Zeiss).

2.6 Syngeneic tumor mouse model in immunocompetent 129/Sv mice

For all tumor experiments immunocompetent female 129/Sv mice were purchased from Janvier (France). After at least one week of acclimatization, 9-week old mice were injected subcutaneously in the right flank with F9 teratocarcinoma cells (10 - 20 * 10^6 cells). When average tumor volumes were approximately 100 mm^3, mice were randomly grouped and treatment started. Mice were monitored daily, and tumor volumes calculated with the formula: volume = length × $width^2$ × 0.5 after measurement with a digital caliper. Mice were sacrificed if tumor volumes reached 2000 mm^3 or if weight loss was greater than 15 %. All experiments were performed under a project license granted by the cantonal veterinary office (ZH027/15) in agreement with Swiss regulations.

2.6.1 Quantitative biodistribution studies in F9 tumor-bearing 129/Sv mice

Quantitative biodistribution was used to assess the *in vivo* targeting performance of radiolabeled GCSF-F8 (n=2), F8-IL3 (n=5) and F8-IL4-F8 (n=5) as described previously.(Pfaffen et al., 2010) 0.15 μg of radioiodinated GCSF-F8, 3.1 μg of radioiodinated F8-IL3 and 25 μg of radioiodinated F8-IL4-F8 were injected intravenously into the lateral tail veins of immunocompetent 129/Sv mice bearing subcutaneously grafted F9 tumors ($12*10^7$ cells). After 24 hours, mice were sacrificed, their organs excised, weighed and the radioactivity counted with a Cobra γ counter (Packard, Meriden, CT, USA). The radioactivity of tumors and organs was expressed percentage of injected dose per gram of tissue (%ID/g ± SEM).

2.6.2 Dose escalation study in F9 tumor-bearing 129/Sv mice

Immunocompetent female 129/Sv mice and male DBA/1J mice were purchased from Janvier (France). 129/Sv mice were injected *i.v.* with either 1 μg, 3 μg, 10 μg, 30 μg or 100 μg of GCSF-F8 or F8-IL3 and DBA1/J mice were injected *i.v.* with either 10 μg, 30 μg, 100 μg, 150 μg or 200 μg of GCSF-F8 or F8-IL3 three times, once every 48 hours. Body weights were

monitored daily and after the end of the experiment spleens were excised and measurements compared, to check for splenomegaly as an indication for biological activity of GCSF-F8 and F8-IL3.

2.6.3 Therapy study in F9 tumor-bearing 129/Sv mice

In a first experiment mice were randomly assigned to be administered *i.v.* on days 1, 3 and 5 either 100 µL PBS (n=6, control group), 100 µg GCSF-F8 (n=6) or 100 µg F8-IL3 (n=6). Mice were sacrificed on day 11 (PBS), day 12 (GCSF-F8) or day 12 (F8-IL3) after tumor implantation due to reaching termination criteria, in accordance with local regulations. The administered doses of GCSF-F8 and F8-IL3 were chosen on the basis of the dose escalation study performed.

In a second experiment mice were randomly assigned to be administered *i.v.* on days 1, 3, 5 and 7 either 100 μL PBS (n=5, control group) or 157 μg of F8-IL4-F8 (n=5). Mice were sacrificed on day 13 (PBS) or day 19 (F8-IL4-F8) due to reaching termination criteria, in accordance with local regulations. 4 injections of 157 μg of F8-IL4-F8 correspond to the same molar amount of IL4 given in a study previously described.(Hemmerle et al., 2014) Data is given as mean ± SEM.

2.7 Mouse model of collagen-induced arthritis

Male DBA/1J mice (Janvier, France) were immunized at eight weeks old by subcutaneous (*s.c.*) injection at the base of the tail with 0.05 mL of emulsion of bovine type II collagen and Complete Freud's Adjuvant (Hooke Laboratories, United States). Three weeks thereafter, a 0.04 mL booster injection of the same emulsion was given. After the second immunization mice were inspected daily and monitored for clinical signs of disease. Using a scoring system of 0 = normal; 1 = one toe inflamed and swollen; 2 = more than one toe, but not entire paw,

inflamed and swollen or mild swelling of entire paw; 3 = entire paw inflamed and swollen; 4 = very inflamed and swollen paw, an arthritis score was assigned to each paw. For each animal, a maximum total score of 16 could be reached. Furthermore, the swelling of affected paws was measured daily under isoflurane anesthesia using a caliper. The paw thickness was expressed as the mean of all four paws of each animal.

All experiments were performed under a project license granted by the cantonal veterinary office (ZH253/16) in agreement with Swiss regulations.

2.7.1 Therapy experiments in a mouse model of collagen-induced arthritis

Animals were enrolled in a therapy group on the day of arthritis onset, with arthritic scores ranging between 1 – 6. The arthritic scores, animal weights and paw thicknesses were monitored daily in a blinded fashion and data is given as mean ± SEM.

Mice were randomly assigned to be administered either on days 1, 3 and 5 100 µL PBS intravenously (*i.v.*) (n=8, control group), 100 µg GCSF-F8 (*i.v.*) (n=8), 100 µg F8-IL3 (*i.v.*) (n=8), 175 µg F8-IL4-F8 (*i.v.*) (n=7) or daily from day 1 – 9 100 µg DXM (Dexamethason Helvepharm, Zentiva, Switzerland) intraperitoneally (*i.p.*) (n=8) or a combination of 175 µg F8-IL4-F8 (*i.v.*) on days 1, 3 and 5 with 100 µg DXM intraperitoneally (*i.p.*) daily from day 1 – 9 (n=9).

The dosages and route of administration were chosen on the basis of the dose escalation study (above) or previous reports, in which the same molar amount of IL4 had been tested in the CIA model.(Hemmerle et al., 2014)

Mice were sacrificed on day 5 (PBS), day 4 (GCSF-F8), day 4 (F8-IL3), day 7 (F8-IL4-F8), day 16 (DXM) or day 7 (F8-IL4-F8 + DXM) due to reaching termination criteria based on arthritic score and weight loss in accordance with local regulations.

3. Results

3.1 Production and characterization of novel fusion proteins

Previous work from our laboratory had shown that cytokine payloads could be fused either at the C-terminus or at the N-terminus of antibody fragments with retention of targeting ability.(Bootz et al., 2016; Hemmerle and Neri, 2014a; Pasche et al., 2011; Schwager et al., 2009) We fused G-CSF at the N-terminus and IL3 at the C-terminus of the F8 antibody in homodimeric diabody format (**Figure 1A and 1B**), since a similar topology had previously been described for fusions of these cytokines with other polypeptides.(Cox et al., 2004; Feuring-Buske et al., 2002; Frankel et al., 2000). For interleukin-4, we had previously described diabody fusions and we decided to clone and express the cytokine payload flanked by two scFv fragments (**Figure 1C**). The proteins were produced by transient gene expression in CHO-S cells and purified to homogeneity, as evidenced by SDS-PAGE and size exclusion chromatography analysis. The products retained high binding affinity to the cognate EDA antigen, as detailed in BIAcore real-time interaction analysis (**Figure 1**), and were biologically active (**Supplementary Figure 1**). Both F8-IL3 (**Figure 1B**) and F8-IL4-F8 (**Figure 1C**) showed a smeared band in SDS-PAGE analysis due to protein glycosylation.

The ability of GCSF-F8, F8-IL3 and F8-IL4-F8 to target EDA *in vitro* was assessed with FITC-labelled fusion proteins using immunohistochemistry on both F9 teratocarcinoma and CIA paw sections (**Figure 2B – E**) and *in vivo* in biodistribution studies using radioiodinated fusion proteins (**Figure 2A**). Although compared to the arthritic paw sections the staining of the CD31-positive neo-vascular structures was faint in the tumor sections, the ability of the novel fusion proteins to target EDA *in vitro* was clearly evident in both tumor and paw samples (**Figure 2B – E**). *In vivo*, the radiolabeled proteins showed preferential tumor accumulation 24 h after *i.v.* administration in immunocompetent F9 tumor-bearing 129/Sv mice with tumor:blood ratios of 3.3 for GCSF-F8, 18.2 for F8-IL3 and 27.3 for F8-IL4-F8 (**Figure 2A)**.

Due to the results above, all novel immunocytokines were tested in two different animal models for therapeutic activity.

3.2 Dose finding experiments for GCSF-F8 and F8-IL3

As literature reports on dosing of G-CSF and IL3 were variable, a dose-finding study was performed in both 129/Sv and DBA1/J mice.(Bruhl et al., 2009; Halpern et al., 2002; Powell et al., 2003; Srivastava et al., 2011; Woytschak et al., 2016; Ziltener et al., 1994) Dosages ranging from 1 μg to 200 μg of GCSF-F8 and F8-IL3 were administered *i.v.* three times and the body weights and spleens monitored for signs of toxicity (**Supplementary Figure 1 and 2**). From these experiments doses of 100 μg administered 3 times were deemed appropriate for both GCSF-F8 and F8-IL3 for future therapy experiments.

3.3 Therapy experiments in F9 tumor-bearing mice

The therapeutic activity of GCSF-F8, F8-IL3 and F8-IL4-F8 was assessed in immunocompetent 129/Sv mice bearing subcutaneously grafted F9 tumors. Three injections of either 100 μg of GCSF-F8 or F8-IL3 or 100 μL of PBS were administered *i.v.* (one every 48h). On day 11 after tumor implantation there was a modest but statistically significant treatment effect of F8-IL3, but not GCSF-F8, in tumor growth retardation (**Figure 3A**). Nonetheless, both treatment groups had to be sacrificed just one day after the control group due to reaching termination criteria. In a second experiment four injections of either 157 μg of F8-IL4-F8 or 100 μL of PBS were injected *i.v.* (one every 48h). Treatment with F8-IL4-F8 led to statistically significant retardation of tumor growth (**Figure 3C**). In both experiments, the therapeutic agents were well tolerated and did not lead to excessive weight loss (**Figure 3B, 3D**).

3.4 Therapy experiments in murine CIA

The novel fusion proteins' therapeutic activity was furthermore assessed in a model of collagen-induced arthritis in male DBA/1J mice. GCSF-F8, F8-IL3 and F8-IL4-F8 were all tested for single agent activity and F8-IL4-F8 was additionally assessed in combination with dexamethasone, as F8-IL4 had previously been shown to induce complete cures in mice with CIA when used in combination with the aforementioned corticosteroid.

As can be seen from the scoring of disease severity and measurement of paw swelling, treatment with three *i.v.* injections (one every 48h) of 100 μg GCSF-F8 or F8-IL3 led to a worsening of the disease compared to treatment with PBS, while three administrations of 175 μg F8-IL4-F8 did not substantially improve disease scoring (**Figure 4A and 4C**). F8-IL4-F8 alone did, however, lead to prevention of weight loss and weight stabilization (**Figure 4B**). Intraperitoneal administration of dexamethasone led to a transient disease regression, but conditions worsened and disease progressed, causing the group to reach termination criteria (**Figure 4**). The combination of F8-IL4-F8 with dexamethasone showed a beneficial tendency with strong reduction of arthritic score and paw swelling, however, the group needed to be terminated early due to toxicity in a proportion of the mice (**Figure 4**).

4. Discussion

Our laboratory has a long-term goal to fuse and characterize most known immunomodulatory payloads (e.g., cytokines, chemokines) to antibodies of proven disease-homing activity (e.g., the anti-EDA F8 antibody). In this study, we reported the first antibody fusions with G-CSF and IL3, two cytokines which potently increase the number and activity of neutrophils. In addition, we described a novel format for IL4-based immunocytokines, as interleukin-4 is able to antagonize neutrophil activity.(Woytschak et al., 2016) Interleukin-4 had previously been fused to the F8 antibody in diabody format and had exhibited encouraging results in mouse models of cancer and of arthritis.(Hemmerle et al., 2014; Hemmerle and Neri, 2014a) However, a homologous fusion protein featuring human IL4 as payload had exhibited suboptimal pharmacokinetic results in mice and in *Cynomolgus* monkeys (unpublished data), prompting us to investigate novel molecular formats.

The production of a large number of antibody-cytokine fusions, specific to splice isoforms of fibronectin, has shown that many (but not all) immunomodulatory payloads can be selectively delivered to neoplastic lesions or to sites of arthritis *in vivo*. In this article, we have seen that G-CSF, IL3 and IL4 can be efficiently targeted to EDA(+)-fibronectin, which is abundantly expressed in tumors and in the collagen-induced model of arthritis.(Rybak et al., 2007; Schwager et al., 2009) The observed pharmacokinetic behavior is reminiscent of the one previously reported for F8-TNF, F8-IL2, F8-IL4, F8-IL10 F8-IL12 or IL22-F8.(Bootz et al., 2016; Frey et al., 2010; Hemmerle and Neri, 2014a; Hemmerle et al., 2013; Schwager et al., 2009) Other payloads (e.g., IFNγ and IL15) are more difficult to deliver, as the cognate receptor needs to be saturated in normal organs before a preferential uptake in tumors is observed.(Ebbinghaus et al., 2005; Hemmerle and Neri, 2014b; Kaspar et al., 2007)

The *in vivo* characterization of F8-IL3, GCSF-F8 and of F8-IL4-F8 revealed that the three proteins were well tolerated at doses of 100 μg, 100 μg and 157 μg, respectively. Among these products, only the IL4 fusion exhibited a potent anti-tumor single-agent activity. Since F8-IL3 and GCSF-F8 increase neutrophil counts but did not display any detectable anti-cancer activity, we conclude that polymorphonuclear cells do not contribute to tumor growth inhibition, at least in the F9 murine teratocarcinoma model used in this study. Neutrophils have been shown to have both multifaceted beneficial as well as detrimental roles in tumor pathogenesis. On the one hand, they can secrete cytotoxic mediators that harm malignant cells and can recruit other anti-tumor effector cells to the site of disease. On the other hand, however, neutrophils may assist both the angiogenic growth as well as metastasis of the tumor lesion and studies have increasingly shown that high neutrophil counts at the tumor site correlate with poor prognosis.(Coffelt et al., 2016; Uribe-Querol and Rosales, 2015)

As reported for the F8-IL4 fusion protein in the CIA model previously, a beneficial treatment tendency was observed for the combination of F8-IL4-F8 with dexamethasone, alluding to the neutrophil-inhibitory capacity of IL4.(Hemmerle et al., 2014) The targeted delivery of IL3 and G-CSF, however, did not improve (and indeed worsened) inflammation in mice with arthritis. Neutrophil counts are elevated in the joints and synovial fluid of patients with rheumatoid arthritis and are thought to contribute to the pathology of the disease in many ways.(Konttinen et al., 1985; Wright et al., 2010) Through the secretion of inflammatory mediators, antigen presentation, the release of neutrophil extracellular traps and the attraction of immune cells, neutrophils can influence the inflammatory processes, structural damage and disease course of arthritis.(Wright et al., 2014)

While F8-IL3 and GCSF-F8 did not display therapeutic activity in the mouse models of cancer and of arthritis described in this study, their selective accumulation at the site of disease suggests that these biopharmaceuticals may be useful in conditions which require an increase in neutrophil number and activity. For example, patients with atopic dermatitis often suffer from recurrent bacterial skin infections. Although their blood neutrophil counts are normal, skin lesions in patients often show a lack of neutrophils to contain the infections, even though the colonizing bacteria should induce neutrophil recruitment.(Woytschak et al., 2016) Drugs that could elevate neutrophil numbers at the sites of disease may provide a benefit to these patients.

The addition of IL3 and G-CSF to the list of immunomodulatory payloads that can be fused to antibodies and used for disease-homing, expands the type of "armed" antibody products which can be considered for pharmaceutical applications. While the scope of antibody-drug conjugates is limited in practice by high cost-of-goods and by the requirement to use highly-potent (small) agents, which stoichiometrically match the (large) antibody moiety, cytokines appear to be versatile fusion partners for biopharmaceuticals of the future. The EDA domain of fibronectin is virtually undetectable in normal adult tissues, but is strongly expressed in many diseases characterized by intense tissue remodeling.(Schwager et al., 2009; Villa et al., 2008) As a consequence, F8-IL3, GCSF-F8 and of F8-IL4-F8 may represent new tools for the manipulation of immune activity in health and in disease, both in rodents and in patients.

Abbreviations

CD4+	cluster of differentiation 4 positive
CD31	cluster of differentiation 31
CHO cells	Chinese hamster ovary cells
CIA	collagen-induced arthritis
DXM	dexamethasone
EDA	extra domain A
Fc	fragment crystallizable
FITC	fluorescein isothiocyanate
G-CSF	granulocyte colony-stimulating factor
G-CSFR	granulocyte colony-stimulating factor receptor
GM-CSF	granulocyte-macrophage colony-stimulating factor
IgG	Immunoglobulin G
IL2	interleukin-2
IL3	interleukin-3
IL4	interleukin-4
IL6	interleukin-6
IL10	interleukin-10
IL12	interleukin-12
IL22	interleukin-22
i.p.	intraperitoneal
i.v.	intravenous
PBS	phosphate buffered saline
s.c.	subcutaneous
scFv	single-chain variable fragment

SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
T_H2	T helper cell type 2
TNF	tumor necrosis factor

Acknowledgments

We thank Dr. Francesca Pretto for the support of the therapy experiments in the collagen-induced model of arthritis.

We are also grateful to the laboratory staff of the Laboratory for Animal Model Pathology, (Institute of Veterinary Pathology, Vetsuisse Faculty, University of Zurich, Switzerland) for skillful technical support.

Funding

We are grateful to ETH Zürich, the Swiss National Science Foundation [Project Nr. 310030B_163479/1], the Swiss Federal Commission for Technology and Innovation (KTI) [Project Nr. 17072.1] and the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program [grant agreement No 607603] for the financial support.

Conflict of interest statement

DN is a cofounder and shareholder of Philogen SpA (Siena, Italy), the company that owns the F8 antibody and developed Dekavil (F8-huIL10). The experiments of this article have been co-financed by Philochem AG (Otelfingen, Switzerland), a fully-owned company of the Philogen group, in the frame of a collaborative Swiss Federal KTI MedTech Project with ETH (Kommission für Technologie und Innovation). All other authors declare that they have no conflicts of interest.

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