Original Research

Evaluation of antibody–chemokine fusion proteins for tumor-targeting applications

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Abstract

There is an increasing biotechnological interest in the 'arming' of therapeutic antibodies with bioactive payloads. While many antibody–cytokine fusion proteins have been extensively investigated in preclinical and clinical studies, there are only few reports related to antibody–chemokine fusion proteins ('immunochemokines'). Here, we describe the cloning, expression, and characterization of 10 immunochemokines based on the monoclonal antibody F8, specific to the alternatively spliced extra domain A (EDA) of fibronectin, a marker of angiogenesis. Among the 10 murine chemokines tested in our study, only CCL19, CCL20, CCL21, and CXCL10 could be expressed and isolated at acceptable purity levels as F8-based fusion proteins. The immunochemokines retained the binding characteristics of the parental antibody, but could not be characterized by gel-filtration analysis, an analytical limitation which had previously been observed in our laboratory for the unconjugated chemokines. When radioiodinated preparations of CCL19-F8, CCL20-F8, CCL21-F8, and CXCL10-F8 were tested in quantitative biodistribution studies in tumor-bearing mice, the four fusion proteins failed to preferentially accumulate at the tumor site, while the unconjugated parental antibody displayed a tumor:blood ratio $>$20:1, 24 h after intravenous (i.v.) administration. The tumor-targeting ability of CCL19-F8 could be rescued only in part by preadministration of unlabeled CCL19-F8, indicating that a chemokine trapping mechanism may hinder pharmacodelivery strategies. While this article highlights expression, analytical, and biodistribution challenges associated with the antibody-based *in vivo* delivery of chemokines at sites of disease, it provides the first comprehensive report in this field and may facilitate future studies with immunochemokines.

Keywords: Immunochemokine, antibody–chemokine fusion protein, chemokine, vascular targeting, oncofetal fibronectin, armed antibody

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Introduction

Monoclonal antibodies represent the largest and fastest growing class of biopharmaceutical products (for cancer therapy).[1] In addition to the use of intact immunoglobulins in IgG format, there is a growing interest in 'arming' antibodies with payloads for therapeutic applications. Several strategies have been proposed, but the ones which are most widely used in industrial and clinical applications feature the use of cytotoxic drugs,[2,3] cytokines,[4,5] and radionuclides[6,7] as payloads for their antibody-based pharmacodelivery to sites of cancer *in vivo*. To the best of our knowledge, only few reports on antibody–chemokine fusion proteins ('immunochemokines') have been published so far.[8–10]

Chemokines are a family of structurally related chemotactic cytokines of small molecular weight that are acting in gradients to promote directional migration of leukocytes, epithelial, and endothelial cells.[11] Some chemokines can have additional activities including HIV inhibitory activity, tumor-promoting or -inhibitory activity, angiogenic or angiostatic activity or the ability to modulate gene expression, phagocyte activation, and cell differentiation.

Based on the relative position of the conserved N-terminal cysteine residues they are classified into CXC, CC, CX3C, or C chemokines. They mediate their signals through G-protein coupled receptors (GPCRs) and are both pleiotropic and redundant in their effects. Internalization and signal transduction are mediated by a number of adapter proteins which dynamically interact with the GPCRs and form a 'chemosynapse' that is vital for directional sensing and polarization of the chemotactic cell.[12–14]

Chemokines contain disulfide bonds, which stabilize the overall protein conformation. Many chemokines form

dimers and higher-order oligomers in solution or upon binding to glycosaminoglycans (GAGs). Despite highly variable sequence homologies, all chemokines share remarkably conserved tertiary structures, consisting of a disordered N-terminus of 6–10 amino acids, which functions as key signaling domain.[15] They undergo post-translational proteolysis by various proteases,[16–24] leading to truncated products with different affinities and specificities for their receptors and therefore different biological properties.

Several reports have indicated that the induction of certain chemokines at the tumor site may lead to a therapeutic benefit. Promising preclinical antitumor effects have been reported for CCL19 and CCL21.[25–29] These promising preclinical results have led to the execution of a Phase I clinical study in melanoma using CCL21-transduced dendritic cells (DCs).[30]

Complete tumor rejection associated with CD8+ and T-cell infiltration could be shown with CXCL11 overexpressing tumor cells.[31] Similarly, mice treated with CCL17 or CCL5 in combination with granulocyte macrophage colony-stimulating factor in a WEHI3B tumor model showed significantly higher survival rates than untreated mice and mice injected with GM-CSF-transduced cells alone.[32] Furthermore, CCL20 overexpression in established murine tumors suppressed tumor growth by attraction of DCs and activation of tumor-specific cytotoxic T cells.[33]

Tumors depend on neoangiogenesis for growth and survival.[34] Many cancer cells secrete proangiogenic factors acting directly on endothelial cells, but there is evidence that chemokines also play an important role by attracting monocyte-derived cells to the site of disease.[35] As a general rule, CXC chemokines regulate angiogenesis either positively or negatively depending on the presence or absence of the ELR (Glu-Leu-Arg) motif in their N-terminus.[36] Members of the CXC family with the ELR motif are angiogenic, while ELR-negative CXC chemokines, are angiostatic.[37] CXCL9 overexpression resulted in the inhibition of non-small-cell lung carcinoma growth and metastasis.[38] CXCL10 inhibits CXCL8 and bFGF-induced angiogenesis.[39] Furthermore, CXCL11 inhibits angiogenesis in a murine model of pulmonary fibrosis.[40]

Collectively, the preclinical findings summarized in the previous paragraphs provide a strong rationale for the implementation of therapeutic strategies, aiming at the pharmacodelivery of certain chemokines to neoplastic sites *in vivo*. However, while cytokines have extensively been studied as partners for the preparation of antibody-based fusion proteins with an impressive therapeutic potential for cancer[4,5] and other diseases,[41,42] there have been only few reports related to the implementation of tumor-targeting strategies with immunochemokines. Human CCL5 (RANTES) was fused to the N-terminus of the heavy chain of an IgG3 antibody specific to the tumor associated antigen HER2/*neu*. A selective *in vivo* localization of the fusion protein to HER2/*neu*-expressing xenografted tumors in SCID mice has been reported, although no biodistribution data have been shown.[43] Specific binding of RANTES.her2.IgG3 fusion protein to HER2/*neu* Ag expressed on EL4 cells and on SKBR3 breast cancer cells could be shown.[44]

Epstein and coworkers[45] described the production of a recombinant immunochemokine, consisting of murine liver-expression chemokine (LEC)/CCL16 fused to the murine antibody chTNT-3 in IgG format, which targets DNA in necrotic regions of tumors. In biodistribution studies with this antibody–chemokine fusion protein, a biologic half-life of 3 h and a tumor uptake of 2.4% injected dose/g, have been reported.[10] The product, administered at a daily dose (five injections) of 20 μg/mouse, mediated a 37–55% reduction in tumor growth rate in immunocompetent mice bearing MAD109, COLON26, and RENCA tumors.

In a third report, featuring the use of a recombinant murine antibody fragment specific to human acidic isoferritin in single-chain Fv (scFv) format, the fusion protein mCXCL10–scFv could be expressed[8,46] and the immunochemokine retained its antibody-binding specificity and chemokine function, but was not tested *in vivo* for biological activity and for therapeutic applications.

Here, we present the first comprehensive attempt to generate and characterize several antibody–chemokine fusions for tumor-targeting applications. As delivery vehicle, we chose the human monoclonal antibody F8 in diabody or scFv format.[47] The F8 antibody recognizes the alternatively spliced EDA of fibronectin[48] with identical affinity in mouse and man. The tumor-targeting performance of the F8 antibody alone or fused to several different cytokines has previously been studied using radioiodinated protein preparations by quantitative biodistribution analysis in tumor-bearing mice, revealing a selective accumulation on neoplastic masses, with excellent tumor to organ ratios.[49–51] We have fused the F8 antibody to 10 different murine chemokines and attempted the expression of the corresponding fusion proteins by transient gene expression in mammalian cells to ultimately test the *in vivo* applicability of immunochemokines.

Materials and methods

Cloning of chemokine-F8 fusion proteins

Cloning of the CCL5-F8 immunochemokine is described here. Cloning of all other chemokine-F8 constructs was performed in a similar fashion and all the corresponding primer sequences can be found in Supplementary Figure 2(a). A murine CCL5 cDNA clone (Sinobiologicals Inc., Beijing, China) was PCR amplified by Polymerase Chain Reaction (PCR) using primers CCL5-SIP_fw (5′-CCTGTTCCTCGTCGCTGTGGCTACAGGTGTGCACTCG TCACCATATGGCTCGGACACCACTC-3′), that appends a NheI restriction site and part of the signal peptide (SIP) secretion signal sequence, and CCL5-linker_rev (5′-CCGCCAGAGCCACCTCCGCCTGAACCGCCTCCACCG CTCATCTCCAAATAGTTGATGT-3′), that appends part of the 14-amino acid GGGGS linker peptide between the chemokine and the antibody moiety. The F8 diabody gene was PCR amplified using the primers Linker-F8_fw (5′-TCAGGCGGAGGTGGCTCTGGCGGTGGCGGAGAGGT-GCAGC TGTTGGAGTCTGGG-3′) that appends a part of the GGGGS-linker peptide (including a 23 base pair overlap) and F8-NOT_I_rev (5′-TTTTCCTTTTGCGGCCGC TCATTATTTGATTTCCACCTTGGTCCCTTGGCCGAA-3′)

which appends two stop codons as well as a NotI restriction site. The murine CCL5-linker and linker-diabody(F8) DNA fragments were PCR-assembled using primers NheI_Sip_fw (5′-CCCGCTAGCGTCGACCATGGGCTGGA GC CTGATCCTCCTGTTCCTCGTCGCTGTGGC-3′), containing a NheI restriction site followed by the N-terminal part of the SIP sequence (21 base pair overlap with CCL5-SIP_fw), and F8-NOT_I_rev. The PCR-assembled full length CCL5-F8 gene was double digested with NheI/NotI restriction endonucleases and cloned into the mammalian cell expression vector pcDNA3.1(+) (Invitrogen). CXCL10-F8 was cloned also in scFv(F8) format according to the same procedure but with a scFv(F8) template instead of the diabody(F8) template.[52]

The chimeric chemokine termed ITIP, was assembled using the C-terminal part of the IFN-inducible protein 10 (IP10)/CXCL10 and N-terminal part of the IFN-inducible T cell alpha chemoattractant (ITAC)/CXCL11 sequence as previously described.[53] Further cloning steps were performed as described above.

Cell culture/cell lines and animals

CHO-S cells (ATCC) in suspension were cultured in shaker incubators using PowerCHO-2CD medium (Lonza, Switzerland) supplemented with 8 mM Ultraglutamine, HT supplement (Lonza, Switzerland), and antibiotics. The murine teratocarcinoma F9 cell line was used for biodistribution and therapy studies (CRL-1720, ATCC). Cells were grown in 0.1% gelatin-coated tissue flasks in DMEM (Gibco) supplemented with 10% fetal calf serum. Cells were incubated at 37°C and 5% CO_2. Tumor cells were implanted subcutaneously in the flank of 12-week-old female 129/SvEv mice (Charles River, Germany) using 25×10^6 cells. Experiments were performed under a project license granted by the Verterinaeramt des Kantons Zürich, Switzerland (Bew. Nr. 42/2012).

Transient gene expression and characterization

All fusion proteins used in this study were expressed using transient gene expression as previously described[54] with the following modifications: Expression time was reduced to three days and in case of CXCL10-F8 (scFv) expression, heparin was added to the medium (0.1 mg/mL). Proteins were purified by protein A affinity chromatography followed by ion exchange chromatography (IEC) and analyzed by SDS–PAGE, ELISA, or the surface plasmon resonance (BIAcore). For CCL19-F8, CCL20-F8, and CCL21-F8 ELISA experiments, biotinylated EDA antigen or the biotinylated BCD-domain of Tenascin-C was coated (10^{-7} M) on streptavidin stripes (Roche, Switzerland) and different concentrations of the fusion proteins were applied. Detection was done with protein A horseradish peroxidase (HRP) conjugate (Ge Healthcare) and the chromogenic substrate POD (Roche) at 450 nm. The F8 antibody in small immunoprotein format (SIP(F8)) at a concentration of 250 nM served as positive control. In case of CXCL10-F8(scFv), unbiotinylated EDA antigen (10^{-6} M) was coated on a Nunc MaxiSorp flat bottom 96-well plate (Thermo Scientific) and CXCL10-F8(scFv) was added at different concentrations. Detection was done with a biotinylated rabbit anti-mCXCL10 antibody (0.01 mg/mL, Peprotech) followed by streptavidin-HRP (GE Healthcare). BIAcore measurements were performed on an EDA antigen-coated sensor chip.

None of the fusion proteins could be recovered after size exclusion chromatography in phosphate buffered saline (PBS) on a Superdex 200 10/300 GL column (GE Healthcare). All fusion proteins could be purified to homogeneity by IEC on a 1 mL ATOLL MiniChrom column in a 20 mM NaP buffer at pH 6.8. Elution was done with a salt gradient (one step for CCL19-F8, CCL21-F8, and CXCL10-F8; two-step gradient for CCL20-F8 purification) of 20 mM NaP buffer with 1 M NaCl. Samples were then dialyzed in PBS over night at 4°C.

Quantitative biodistribution studies

The *in vivo* targeting performance of chemokine-F8 fusion proteins was evaluated by biodistribution analysis with radioiodinated protein preparations as described before.[55] Six to seven days after tumor implantation, mice were grouped and injected into the lateral tail vein with 10 μg of the radiolabeled chemokine-F8 fusion proteins. Mice were sacrificed 24 h after injection, organs were excised, weighed, and radioactivity was measured (Packard Cobra γ-counter). Values are given in percentage of injected dose per gram of tissue (%ID/g ± standard error). With CCL19-F8 another two biodistribution studies were performed. In one experiment, mice were sacrificed 20 min after injection of the radioiodinated protein and in the second experiment 30 μg of unlabeled (cold) fusion protein was injected prior to 10 μg of the radiolabeled protein and the mice were sacrificed after 24 h.

Intratumoral therapy studies

When tumors were clearly palpable, mice were randomly grouped and received on five consecutive days intratumoral (i.t.) injections of 10 μg CXCL10-F8(scFv) in PBS buffer, which was also used for injecting the negative control group. Weight and tumor volume (volume = length × width^2 × 0.5) of all mice was monitored daily.

CCL19-F8 and CCL21-F8 transwell migration assay

To test the chemotactic activity of CCL19 and CCL21, a DC chemotaxis assay was performed with lipopolysaccharide-matured murine dendritic cells in transwell plates (3–5 μm, Corning®). CCL19-F8 and CCL21-F8 were added at 100 nM concentration to the lower transwell chamber, while cells were added to the upper transwell chamber. The number of migrated cells is expressed as % migrated cells in comparison to DCs present in the 100% control well. Cell numbers could easily be monitored by fluorescence-activated cell sorting (Canto) due to their YFP expression. Recombinant murine CCL21 served as positive control. All the materials for this assay were kindly provided by Dr. Alvaro Teijeira Sanchez (ETH Zurich).

Immunofluorescence studies of CXCL10-F8(scFv) treated tumors

Ex vivo detection of tumor-infiltrating cells after intratumoral application of CXCL10-F8(scFv) was done with tumors taken three days after the last injection. Tumors were excised, embedded in cryoembedding medium (Thermo Scientific) and cryostat sections (10 μm) were stained using the following antibodies: CD4 (BioXCell), CD8 (BioXcell), F4/80 (Abcam), CD3e (eBioscience), CD45 (BD Biosciences), CD45R (eBioscience), Asialo GM1 (Wako Pure Chemical Industries), Foxp3 (eBioscience), and CD31 (Santa Cruz Biotechnology). Detection was done with AlexaFluor488 and AlexaFluor594 conjugated secondary antibodies (Invitrogen). Slides were analyzed with an Axioskop2 mot plus microscope (Zeiss).

Results

Since chemokines typically require an intact N-terminus for biological activity, we fused 10 murine chemokines at the N-terminal extremity of the F8 antibody in diabody format[51] and expressed the corresponding immunochemokines in CHO-S cells using a transient gene expression methodology[56] (Figure 1(a)). The chemokines CCL5, CCL17, CCL19, CCL20, CCL21, CXCL4, CXCL9, CXCL10, CXCL11, and the chimeric chemokine ITIP were chosen as possible payloads, on the basis of previous reports on their potential anticancer activity. As positive control for tumor-targeting applications, the F8 antibody was used in SIP format (Figure 1(b)). This recombinant antibody format has previously been shown to display biodistribution properties in tumor-bearing mice similar to the ones of F8 in diabody format.[51] Furthermore, the homobivalent SIP(F8) antibody has a molecular weight of about 80 kDa, which is comparable to the one of non-covalent homobivalent immunochemokines in diabody format (Figure 1(b)). Unfortunately, the yield and purity of all 10 immunochemokines after protein A purification was unsatisfactory, in sharp contrast to our previous experience with F8-based immunocytokines[4] and with SIP(F8) (Figure 1(c)).

In transient gene expression experiments, CXCL10-F8 exhibited the presence of a fragment with the size of the F8 diabody, suggesting that proteolytic degradation had taken place (Figure 1(c)). Since CXCL10 is known to bind to heparin, we repeated the expression of CXCL10-F8 adding heparin to the expression medium in order to protect the fusion protein from the action of proteases. In this case, after protein A purification, 2 mg of protein could be recovered per liter of medium, but only ~30% showed the correct molecular weight of the fusion protein, as revealed by SDS–PAGE and by MS analysis (data not shown). An attempt to recover the intact CXCL10-F8 (diabody) fusion protein by IEC did lead to unsatisfactory purity and yields of the desired fusion product which were not compatible with the execution of *in vivo* experiments. Therefore, another fusion protein with F8 antibody in scFv format was cloned and expressed in CHO-S cells with heparin added to the medium (Figure 4(a)). In this format ~0.4 mg/L of the desired product could be recovered after ion exchange purification and dialysis against PBS. A Western blot analysis of the CXCL10-F8 expression showed that the fusion protein is stable in the culture supernatant and that the addition of heparin can prevent the formation of higher molecular weight disulfide-bonded oligomers (Supplementary Figure 3(b)). In a small scale test-expression the fusion protein could not be observed in the flow-through and wash fractions and did not elute from a protein A column, suggesting that the immunochemokine may aggregate and/or precipitate when reaching high local concentrations in the affinity chromatography procedure or may be degraded during purification procedure (Supplementary Figure 3(c)).

Immunochemokines based on CC-Chemokines CCL19, CCL20, and CCL21 displayed the presence of the correct molecular species in transient gene expression and protein A purification, although with extensive contaminations and/or proteolytic degradation (Figure 1(c)). We repeated the experiment in larger scale (up to 1 L) and shorter (three days) expression times. After protein A and IEC purification (Figure 2(d)), all three immunochemokines revealed not only a band of the expected size in SDS–PAGE analysis (Figure 2(a)) but also the presence of glycosylation for CCL19-F8, which was confirmed by mass spectrometric analysis (Supplementary Figure 1(d)). In addition, CCL19-F8 and CCL21-F8 were characterized by native PAGE analysis, revealing the presence of non-covalent higher order oligomers (Figure 2(b)).

Attempts to characterize the proteins on Superdex S200 gel-filtration columns failed, in analogy to our previous experience with recombinant chemokines (e.g. CCL5; data not shown). However, the fusion proteins could be recovered from shorter columns, such as PD10 desalting columns (GE Healthcare) (e.g. after radiolabeling). Radioiodinated preparations of the four immunochemokines revealed a full retention of immunoreactivity, as assessed by affinity chromatography, which was comparable to the one of the parental antibody in SIP format (Figure 2(c)). BIAcore analysis of all CC-immunochemokines (Figure 2(e)) and the CXC-immunochemokine (Figure 4(e)) on EDA-coated chips confirmed the ability of the fusion proteins to recognize their antigen with kinetic profiles which were comparable to the ones of the parental antibody in diabody format.[51] The chemotactic activity of CCL19− and CCL21−based immunochemokines was confirmed in a DC transwell migration assay, in which the fusion proteins exhibited an activity which was comparable to the one of recombinant murine CCL21 from a commercial source (Figure 2(f)). All four immunochemokines were able to specifically bind to the cognate antigen EDA and did not react with structurally related proteins (e.g. the BCD recombinant fragment of tenascin-C, containing three Type-III fibronectin homology repeats) as evidenced by ELISA experiments performed at various concentrations (Figures 2(g) and 4(d)).

Radioiodinated preparations of SIP(F8), CCL19-F8, CCL20-F8, CCL21-F8, and CXCL10-F8(scFv) (and CCL5-F8; shown in Supplementary Figure 1) were injected (10 μg and 6–12 μCi per mouse) into the tail vein of immunocompetent mice bearing subcutaneously grafted

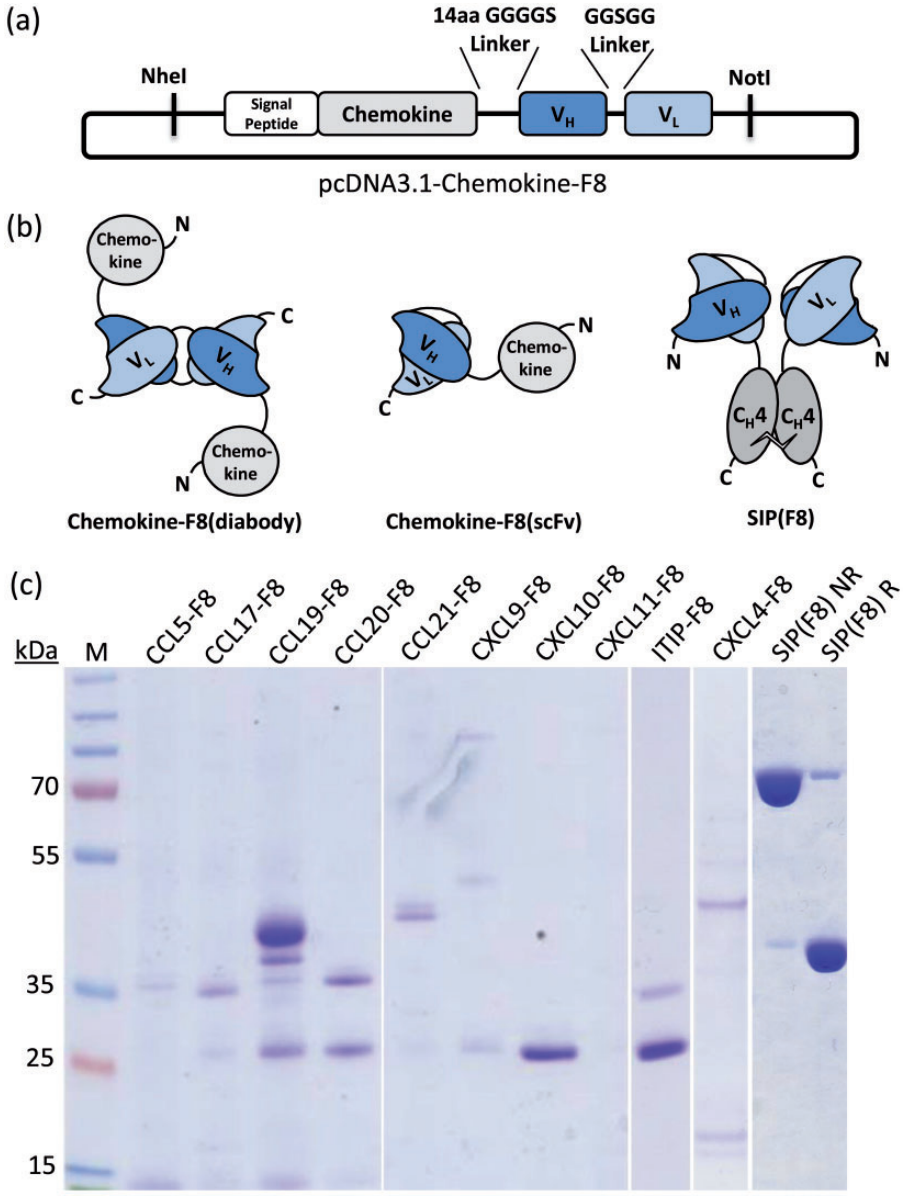

Figure 1 Cloning and expression of chemokine-F8 fusion proteins. The F8 antibody is specific to EDA of human and murine fibronectin. (a) Schematic representation of expression vectors. (b) Schematic representation of chemokine-F8(diabody), chemokine-F8(scFv) and SIP (F8) proteins. (c) SDS–PAGE analysis of protein A purified chemokine-F8(diabody) test expressions and the SIP(F8) antibody used as control in biodistribution studies. M = molecular marker (kDa); NR = non reducing; R = reducing. (A color version of this figure is available in the online journal)

murine F9 tumors. A biodistribution analysis, performed 24 h after injection, revealed that the parental SIP(F8) antibody was able to selectively localize at the tumor site, with 10% injected dose per gram (%ID/g) in the tumor and a tumor to blood ratio of 29:1 at this time point. By contrast, all four immunochemokines failed to preferentially accumulate in the neoplastic lesion (Figures 3(a) and 4(f)). Analysis of biodistribution of CCL19-F8 20 min after injection revealed that the majority of the fusion protein was in the liver, suggesting the presence of an active hepatobiliary excretion process (Figure 3(b)). Interestingly, preadministration of 30 μg of unlabeled CCL19-F8 prior to the administration of radiolabeled CCL19-F8 (10 μg per mouse) led to a partial rescue of tumor-targeting properties, with 0.5 %ID/g in the tumor at 24 h and a tumor to blood ratio of 5 (Figure 3(c)).

Despite the failure of selective tumor accumulation, CXCL10-F8(scFv) was tested *in vivo* in F9 teratocarcinoma bearing 129/SvEv mice. Four injections of 10 μg of CXCL10-F8(scFv) were administered i.t. and the tumor growth was compared to a control group injected with PBS. A modest but significant ($P = 0.0404$; $n = 4$ mice per group) tumor growth inhibition could be observed with CXCL10-F8(scFv), compared to mice treated with saline (Figure 4(g)). *Ex vivo* detection of tumor-infiltrating cells was done on cryostat sections (10 μm) of tumors taken three days after the last injection, revealing an increased infiltration of Natural Killer and CD45R positive cells in

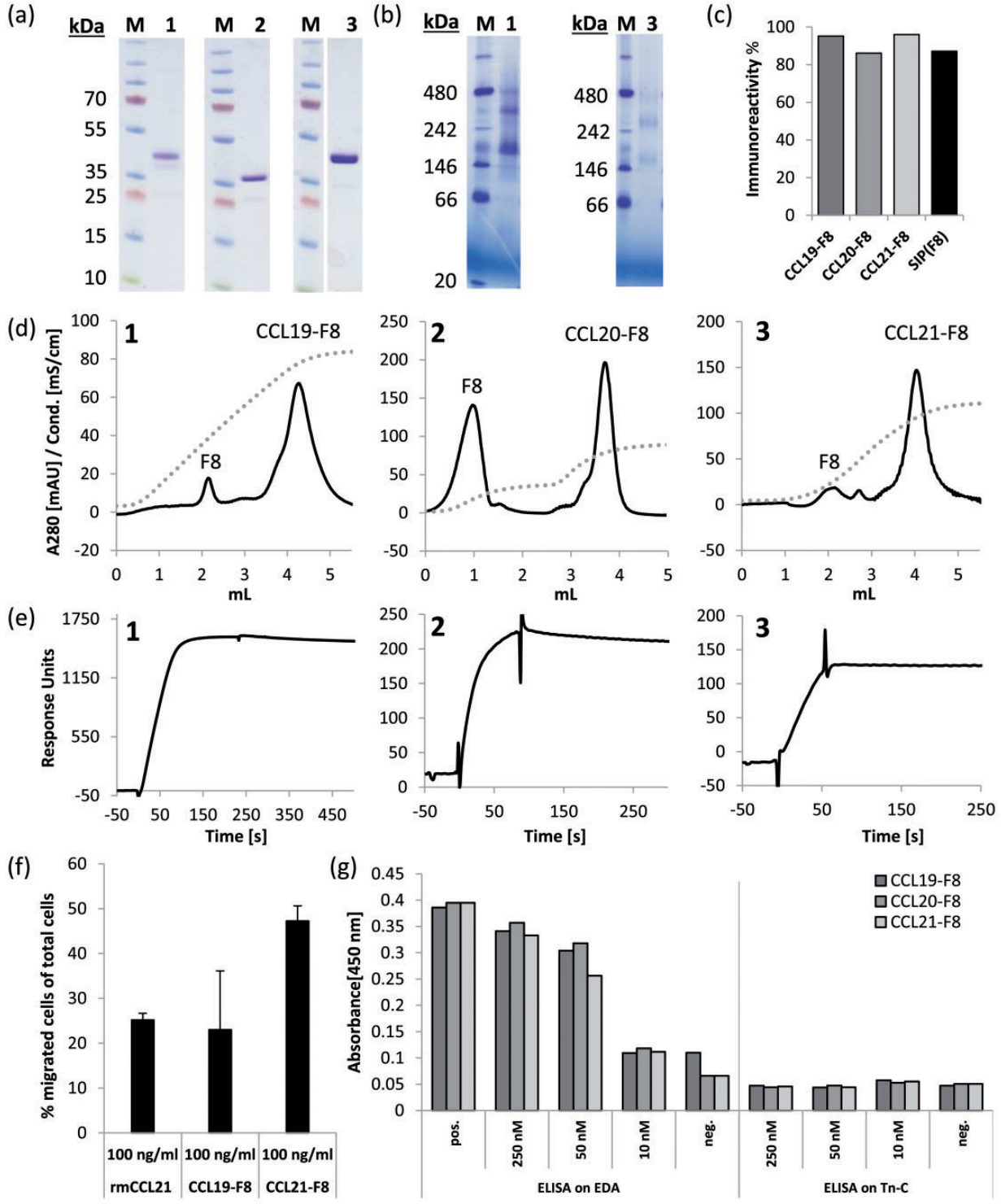

Figure 2 Expression and characterization of CCL20-F8 = (1); CCL21-F8 = (2); CCL21-F8 = (3). (a) SDS–PAGE analysis of protein A and IEC purified preparations of (1), (2), and (3). (b) Native PAGE analysis of (1) and (3). (c) Immunoreactivity (%) of ^{125}I-labeled preparations of CCL19-F8, CCL20-F8, CCL21-F8, and SIP(F8) in EDA affinity chromatography. Values expressed as percentage of eluted radioiodinated protein after protein A affinity chromatography of the total amount applied on the resin. (d) IEC of CCL19-F8, CCL20-F8 and CCL21-F8 in 20 mM NaP buffer, pH 6.8; Adsorption at 280 nm is depicted as black line; conductivity is depicted as grey dashed line. (e) BIAcore analysis of 1 μM (1), 150 nM (2), and 100 nM (3) on an EDA-coated sensor chip. (f) CCL19 and CCL21 chemotactic assay with LPS-matured dendritic cells in transwell plates (3–5 μm, Corning®). CCL19-F8 and CCL21-F8 were added at 100 nM concentration to the lower transwell chamber, while cells were added to the upper transwell chamber. The number of migrated cells could be monitored by fluorescence-activated cell sorting (Canto) due to their YFP expression. Recombinant murine CCL21 served as positive control. Values expressed as percentage migrated cells in comparison to the cells present in the 100% control well. (g) Comparative ELISA of CCL19-F8, CCL20-F8 and CCL21-F8 on EDA and Tenascin-C coated surfaces at concentrations of 250 nM, 50 nM, and 10 nM. Positive = 250 nM SIP(F8) positive control; negative = PBS buffer negative control (A color version of this figure is available in the online journal)

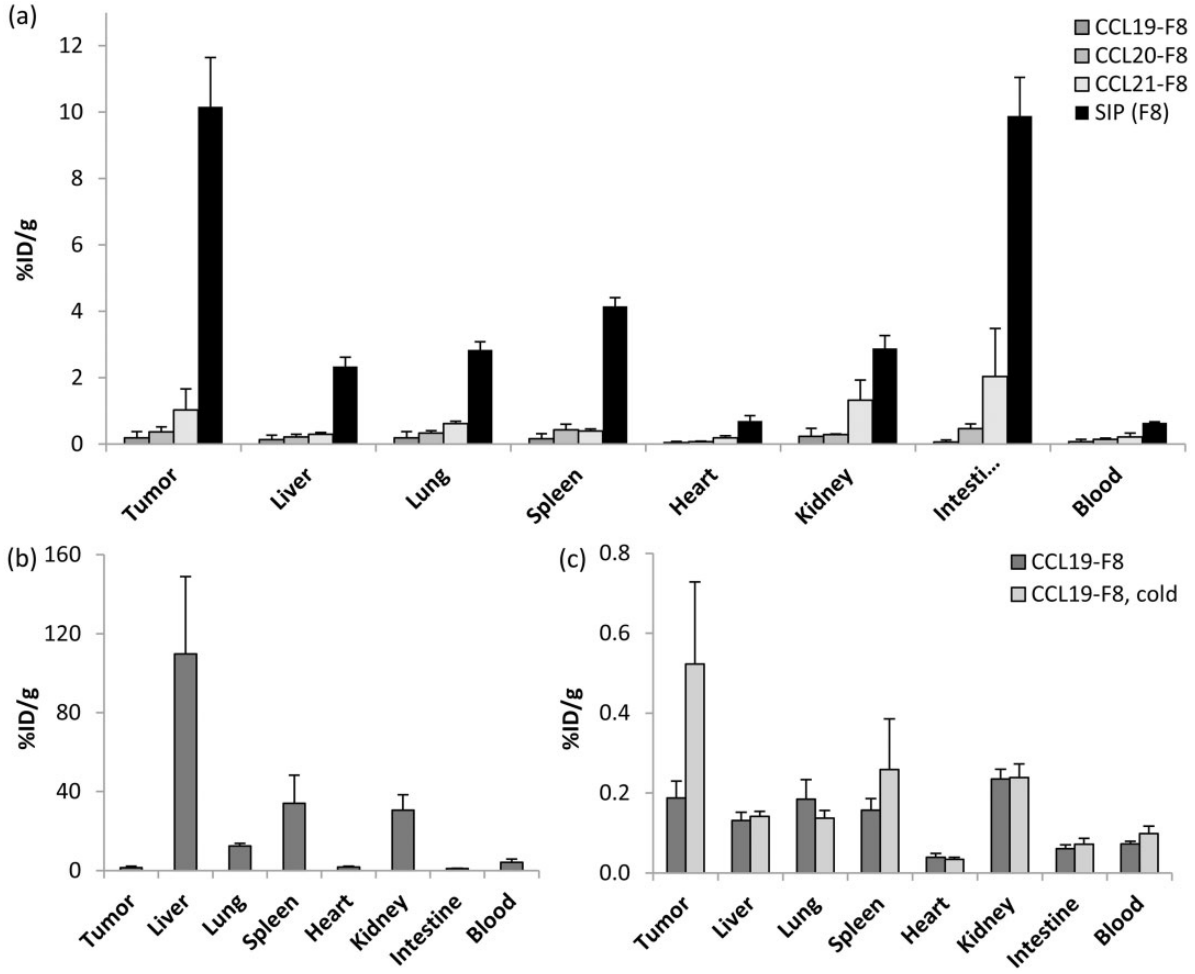

Figure 3 Biodistribution studies of CCL-chemokine-F8 fusion proteins. (a) Biodistribution studies of radioiodinated preparations of CCL19-F8, CCL20-F8, and CCL21-F8 compared to SIP(F8). 129Sv mice bearing subcutaneous F9 teratocarcinoma tumors were injected i.v. with 10 μg ^{125}I-SIP(F8) (black bar, $n = 5$) or with radioiodinated chemokine-F8 preparations (gray bars, $n = 5$). Mice were sacrificed after 24 h. Organs were excised and radioactivity counted, expressing results as percent of injected dose per gram of tissue (%ID/g ± SE). (b) 129/SvEv mice bearing subcutaneous F9 teratocarcinoma were injected i.v. with 10 μg radiolabeled CCL19-F8. Mice were sacrificed after 20 min and organs were excised and radioactivity counted (c) 129/SvEv mice bearing subcutaneous F9 teratocarcinoma were injected i.v. with 30 μg unlabeled CCL19-F8 followed (15–20 min later) by 10 μg of radioiodinated CCL19-F8 (light grey). Biodistribution of 10 μg of radioiodinated CCL19-F8 is depicted as comparison (dark grey). Mice were sacrificed after 24 h, organs were excised and radioactivity counted

CXCL10-F8 treated tumors compared to tumors treated with saline (Supplementary Figure 3(a)).

Discussion

This article presents the first comprehensive characterization of several antibody–chemokine fusions for tumor-targeting applications. Ten immunocytokines were cloned and expression was attempted in mammalian cells, such as CHO-S, HEK293, and Sf9 cells. Four immunochemokines could be expressed in CHO-S cells with yields >1 mg/L and purified to an acceptable degree of homogeneity. Expression of the fusion proteins in HEK293 and Sf21 cells (data not shown) was inferior to expression in CHO-S cells (data not shown). Yields and downstream processing of immunochemokines were substantially worse, compared to the use of cytokine payloads (which can easily be appended at the N− or C−terminus of antibody fragments in scFv or diabody format).

Importantly, fusion to chemokines abrogated the tumor homing properties of the F8 antibody and no preferential accumulation in the neoplastic mass could be observed following i.v. administration. In the case of CXCL10-F8, the addition of heparin to the culture medium facilitated the isolation of an intact fusion protein. Immunochemokines appear to be proteolytically unstable during production in mammalian cells. Post-translational processing of chemokines by various proteases has been reported in *in vivo* studies.[57,58] However, Western blot analysis of CXCL10-F8 culture supernatants not only confirmed that the fusion protein is stable in the culture medium and that addition of heparin improves the biochemical purity of the immunochemokine but also that the fusion protein could not be observed in the flow-through and wash fractions and did not elute from a protein A column (in a low volume test-expression), suggesting that it may aggregate and/or precipitate when reaching high local concentrations in the

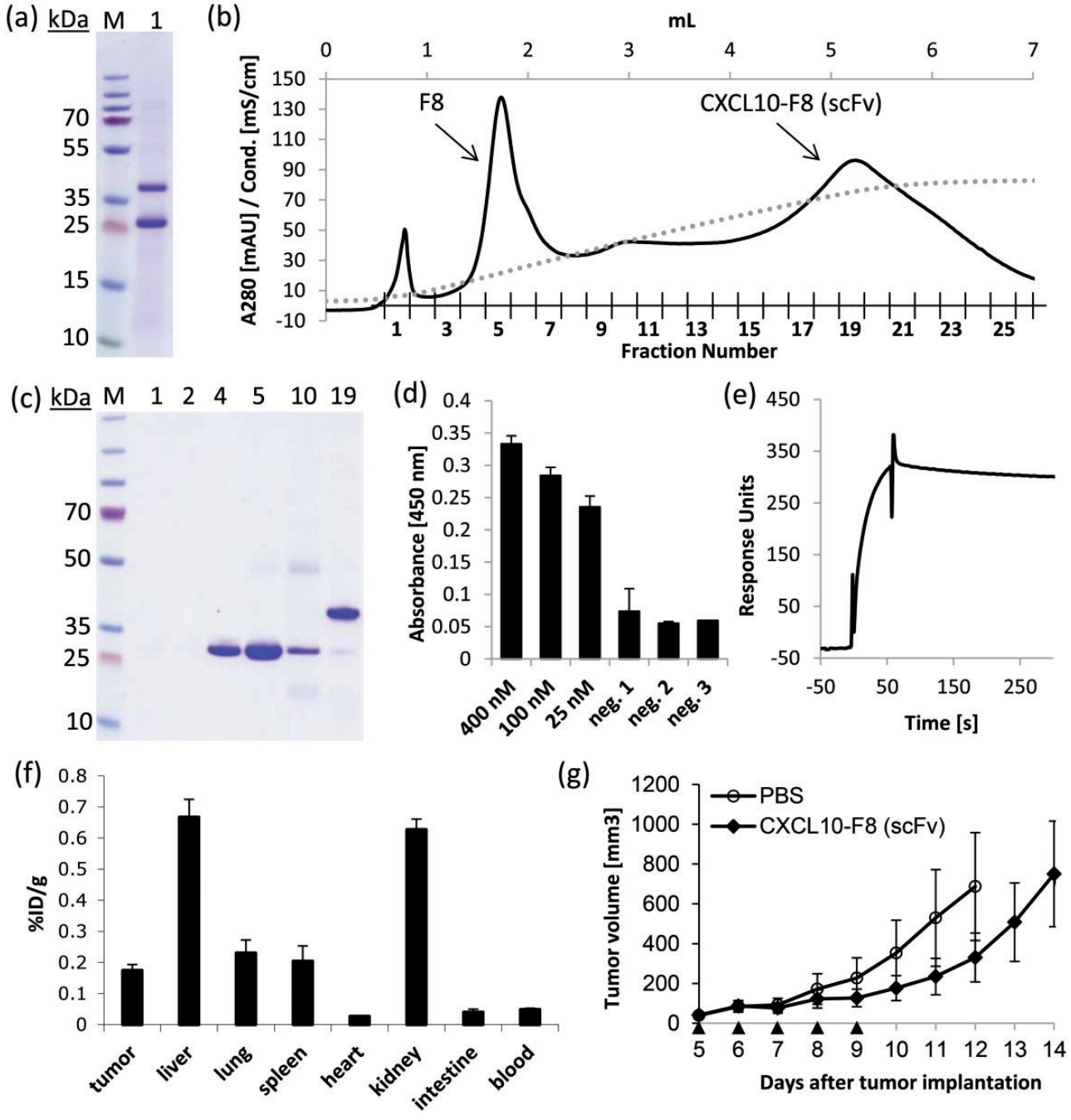

Figure 4 CXCL10-F8(scFv) expression, purification, and *in vivo* characterization. (a) SDS-PAGE analysis of CXCL10-F8(scFv) expressed with addition of heparin. (b) IEC purification of CXCL10-F8(scFv) in 20 mM NaP buffer, pH 6.8, absorbance (280 nm) black line, conductivity (mS/cm) dashed, grey line. Fraction numbers are indicated below. (c) SDS–PAGE analysis of IEC fractions, M = molecular marker (kDa), numbers correspond to the fractions numbers in (b), fraction 19 = CXCL10-F8(scFv). (d) ELISA of CXCL10-F8(scFv) on immobilized EDA antigen at concentrations of 400 nM, 100 nM, and 25 nM, detection of the chemokine moiety was done with a biotinylated anti-mCXCL10 antibody and HRP-conjugated streptavidin, negative 1 = no primary antibody, negative 2 = no secondary antibody, negative 3 = no primary and secondary antibody. (e) BIAcore analysis of 400 nM CXCL10-F8(scFv) on an EDA-coated sensor chip. (f) Biodistribution study of radioiodinated CXCL10-F8(scFv). Immunocompetent 129/SvEv mice bearing subcutaneous F9 teratocarcinoma tumors were injected i.v. with 10 μg radiolabeled protein ($n = 5$). Mice were sacrificed after 24 h. Organs were excised and radioactivity counted, expressing results as percent of injected dose per gram of tissue (%ID/g ± SE). (g) Immunocompetent 129/SvEv mice ($n = 4$) bearing subcutaneous F9 teratocarcinoma tumors were injected daily (i.t.) with 10 μg CXCL10-F8(scFv) (dissolved in PBS) or PBS on five consecutive days (black arrows) and tumor growth over time was monitored. (A color version of this figure is available in the online journal.)

affinity chromatography procedure or degrade during purification procedure.

Immunochemokines could be analyzed by SDS–PAGE, but their size-exclusion chromatographic characterization proved to be difficult, due to the interaction of the chemokine moiety with standard Superdex 200 size exclusion dextran matrix. We had observed similar problems with recombinant chemokines (e.g. CCL5; data not published). Size exclusion chromatography profiles are typically not shown in publications which describe the

production and biological function of chemokines. However, chemokines can be purified by reversed phase, ion exchange, and heparin affinity chromatography methods.[59,60]

For biodistribution studies, radioiodinated immunochemokine preparations were found to be immunoreactive when tested by affinity chromatography on antigen columns, but none was found to display a preferential accumulation at the tumor site following i.v. administration. Unfavorable biophysical properties, such as a high pI value and glycosylation,[61–63] as well as trapping by high expression of the cognate receptor in a tissue (e.g. CXCR3 expression in the liver) or decoy receptors such as D6, DARC, or CCX-CKR lacking classical signaling activities after chemokine binding,[64,65] may prevent an efficient tumor-targeting process. Chemokine receptor expression in the liver or clearance of the immunochemokines via the hepatobiliary route may explain the high liver uptake 20 min after i.v. administration of CCL19-F8. Moreover, tumor to organ ratios improved by prior application of unlabeled CCL19-F8 in biodistribution experiments suggesting that both a receptor-mediated trapping mechanism and a fast clearance of the fusion protein may hinder an efficient tumor-homing process.

The tumor-targeting performance of antibodies specific to splice isoforms of fibronectin (e.g. L19 or F8)[52,66] are dramatically different, when these antibodies are fused to cytokines or chemokines. Most L19 and F8-based immunocytokines exhibit a preferential tumor accumulation in preclinical models of cancer,[67] rendering the findings with immunochemokines even more surprising and unexpected. However, certain payloads have previously been reported to completely abrogate the tumor-targeting potential of the parental antibody in mouse models of cancer.[54,55]

It remains to be investigated whether the choice of alternative antibody formats (e.g. IgG) may improve biodistribution results. In one published report, the IgG-based LEC/chTNT-3 immunochemokine was found to accumulate with 2.4 %ID/g at 12− and 24−h postinjection in MAD109 tumors,[68] while the parental antibody exhibited a tumor uptake of 13.5 %ID/g on day 3 after injection in MAD109 lung adenocarcinoma bearing BALB/c mice.[69] This protein mediated cancer cures, when used in combination with CD25+ depletion.[70] However, CD25+ depletion itself led to a significant reduction of tumor growth and a recently published non-targeted LEC fusion protein consisting of human LEC and soluble Fc (LEC-Fc) showed similar tumor regression as the targeted LEC fusion protein (LEC/chTNT-3).[71]

At this moment in time, it is not known whether immunochemokines may establish a concentration gradient *in vivo*, which is permissive and conducing for the migration of leukocytes. It is also not known whether immunochemokines can alter the vasculature at the site of disease, or regulate the function of immune cells, affecting tumor fibrosis, necrosis, and immune regulation. The fusion proteins tested in this article exhibited a chemotactic activity in *in vitro* experiments, which was comparable to the one of a commercial recombinant chemokine. However, it will be possible to study the full therapeutic potential of immunochemokines only when *in vivo* pharmacodelivery problems can be solved. The target antigen for the F8 antibody is located in the subendothelial extracellular matrix of tumor blood vessels.[52,72–74] Thus, in order to achieve preferential tumor localization, F8-based fusion proteins need to cross the endothelial cell layer and bind to the cognate antigen. The pI values of the 10 chemokines which we fused to the F8 antibody ranged between 8.76 (mCCL5) and 10.62 (mCXCL9). Our group has previously reported that highly positively charged (e.g. murine VEGF-164[75]) or negatively charged (e.g. calmodulin[61]) payloads can abrogate the tumor-targeting properties of antibodies, probably because the many charges inhibit the extravasation process.

Immunochemokines capable of selective *in vivo* targeting of tumor blood vessels may alter the expression of vascular cell adhesion molecules in the lumen to enable the migration of responsive cells into the tumor mass. Furthermore, chemokines with proven antiangiogenic functions (e.g. CXCL10[76]) should be able to exert direct effects on endothelial cells potentially leading to fibrosis and tumor necrosis. The characterization of protein production and the results of quantitative biodistribution studies, as reported in this article, should facilitate future research efforts in the field of immunochemokines, while critically increasing awareness about the impact which payloads may have on the efficiency of antibody-based pharmacodelivery strategies.

AUTHORS CONTRIBUTIONS

Both authors participated in the design, interpretation of the studies, analysis of the data, and review of the manuscript; CH conducted the experiments, DN and CH wrote the manuscript.

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