Phage-Derived Fully Human Monoclonal Antibody Fragments to Human Vascular Endothelial Growth Factor-C Block Its Interaction with VEGF Receptor-2 and 3

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Abstract

Vascular endothelial growth factor C (VEGF-C) is a key mediator of lymphangiogenesis, acting via its receptors VEGF-R2 and VEGF-R3. High expression of VEGF-C in tumors correlates with increased lymphatic vessel density, lymphatic vessel invasion, sentinel lymph node metastasis and poor prognosis. Recently, we found that in a chemically induced skin carcinoma model, increased VEGF-C drainage from the tumor enhanced lymphangiogenesis in the sentinel lymph node and facilitated metastatic spread of cancer cells via the lymphatics. Hence, interference with the VEGF-C/VEGF-R3 axis holds promise to block metastatic spread, as recently shown by use of a neutralizing anti-VEGF-R3 antibody and a soluble VEGF-R3 (VEGF-C/D trap). By antibody phage-display, we have developed a human monoclonal antibody fragment (single-chain Fragment variable, scFv) that binds with high specificity and affinity to the fully processed mature form of human VEGF-C. The scFv binds to an epitope on VEGF-C that is important for receptor binding, since binding of the scFv to VEGF-C dose-dependently inhibits the binding of VEGF-C to VEGF-R2 and VEGF-R3 as shown by BIAcore and ELISA analyses. Interestingly, the variable heavy domain (VH) of the anti-VEGF-C scFv, which contains a mutation typical for camelid heavy chain-only antibodies, is sufficient for binding VEGF-C. This reduced the size of the potentially VEGF-C-blocking antibody fragment to only 14.6 kDa. Anti-VEGF-C VH-based immunoproteins hold promise to block the lymphangiogenic activity of VEGF-C, which would present a significant advance in inhibiting lymphatic-based metastatic spread of certain cancer types.

Introduction

Lymphangiogenesis is the growth of lymphatic vessels from preexisting ones and the extent of lymphangiogenesis in cancers such as malignant melanoma has been shown to be a predictor of disease progression and survival [1]. The growth of peri- and intratumoral lymphatic vessels, which, in contrast to blood vessels, lack a basement membrane as well as coverage by smooth muscle cells and pericytes and are therefore especially easy to be infiltrated by cancer cells, opens up new ways for metastatic dissemination of the primary tumor. Tumors control the growth of blood and lymphatic vessels in their periphery by the secretion of growth factors. Vascular endothelial growth factor-C (VEGF-C) has been shown to be the main lymphangiogenic growth factor [2], together with VEGF-D [3]. In many tumors, the expression of high levels of VEGF-C has been correlated with lymphatic vessel invasion, the emergence of sentinel and distant lymph node metastasis and overall poor prognosis [4]. Today, tumor metastasis still represents the hallmark of malignancy in cancer.

VEGF-C and VEGF-D exert their action via binding to VEGF-receptors 2 and 3 [2,3]. While VEGF-R2 is expressed on blood and lymphatic vascular endothelial cells, VEGF-R3 is in the adult expressed normally only lymphatic endothelial cells. Next to their role in metastasis, VEGF-C and -D might also directly activate VEGF-R3 expressed on tumor cells [5,6], leading to autocrine activation of primary cancer growth and a more aggressive cancer phenotype. VEGF-C and -D are therefore attractive targets for cancer therapy and agents that are capable of blocking VEGF-C/D and reducing cancer aggressiveness and metastatic dissemination are highly needed to prevent disease progression. Interference with the VEGF-C/D – VEGF-R2/3 system has shown promising results in reducing tumor metastasis and/or primary tumor growth in a number of models. Notably, blocking of VEGF-D by a mouse monoclonal anti-human-VEGF-D antibody [7,8] was effective in halting primary tumor growth and suppressing local tumor metastasis in a mouse xenograft tumor model. Similarly, neutralizing antibodies against VEGF-R3 inhibited lymph node metastasis [9–11] and soluble VEGF-R3, that traps both VEGF-C and VEGF-D, blocked lymphangiogenesis and lymph node metastasis in several models [12,13].

However, these strategies have potential drawbacks since VEGF-D and VEGF-R3 function in other cells and tissues may

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also be blocked. VEGF-D is e.g. also expressed in osteoblasts, where it controls bone growth via VEGF-R3 [14]. Blocking of either of these molecules could potentially lead to undesired side effects on bone regeneration.

Blocking of VEGF-C by antibodies has been reported in only a few studies [15–18], none of which involved tumor studies. Furthermore, none of these antibodies are of human origin, which hampers their use in human therapy due to immunogenicity. To directly obtain human antibodies, antibody phage-display libraries based on human germline antibody genes offer an alternative route. The fully human ETH-2 Gold antibody phage-display library has been used to isolate binders against a wide spectrum of antigens [19], and antibodies based on binders isolated from the library (e.g. L19, a fully human IgG against the extra domain B of fibronectin, a vascular tumor neo-angiogenesis marker) are currently under clinical development [20].

VEGF-C undergoes excessive processing by proprotein convertases before and after secretion; this processing trims the full length VEGF-C by a N-terminal and C-terminal propeptide and also affects the pI), we decided to use 100 mM glycosylation also affects the pI), we decided to use 100 mM

![Figure 1. Amino acid sequences of ΔΝΔC-VEGF-C and ΔΝΔC-VEGF-D variants used in the study.](https://www.plosone.org/figure/1)

### Table 1. Enrichment factors during panning.

| Antigen | Round | Input (tu) | Output (tu) | Ratio (out/in) | Enrichment (ratio n/ratio n-1) |
|---------|-------|------------|-------------|----------------|-------------------------------|
| VEGF-C  | 1     | 5.0×10^{12} | 1.2×10^{8}  | 2.4×10^{-7}    | n/a                           |
| VEGF-C  | 2     | 5.4×10^{13} | 1.2×10^{4}  | 2.2×10^{-10}   | 0.0093                        |
| VEGF-C  | 3     | 6.7×10^{13} | 1.5×10^{7}  | 2.2×10^{-7}    | 974                           |
| mock    | 1     | 5.0×10^{12} | 5.0×10^{3}  | 4.0×10^{-8}    | n/a                           |
| mock    | 2     | 6.3×10^{13} | 4.0×10^{4}  | 6.3×10^{-10}   | 0.016                         |
| mock    | 3     | 4.0×10^{13} | 8.0×10^{3}  | 2.0×10^{-8}    | 32                            |

Transducing units (tu) before and after panning rounds were determined using titration of transduced colonies. Ratio of output vs. input in the same round and enrichment, i.e. the factor by which the ratio of rescued phages differed from round n-1 to round n was calculated. “Mock” refers to selections with uncoated immunotubes.

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Figure 2. Binding specificities of anti-VEGF-C scFv. (A) ELISA screening of random clones obtained after 2 or 3 rounds of panning against ∆NAC-VEGF-C. (B) ELISA analysis of representative anti-VEGF-C scFv clones for the 4 different amino acid sequences obtained. Maxisorp or streptavidin-precoated (SA) plates were coated with his-tagged human ∆NAC-VEGF-C derived from P. pastoris or biotinylated his-tagged human ∆NAC-VEGF-C from mammalian cells or P. pastoris, respectively. Control surfaces were left untreated. Antibody fragments and control antibodies were subsequently added and the ELISA was developed as described in Materials and Methods. (C) Cross-reactivity tested by ELISA. Human VEGF-C or mammalian cell-derived VEGF-C was added and the ELISA was developed as described in Materials and Methods. (D) BIAcore profiles from the 4 different anti-VEGF-C scFv clones. Different concentrations of protein-A purified scFv were injected on a streptavidin-precoated sensorchip coated with ca. 2000 RU biotinylated VEGF-C or streptavidin-precoated (SA) plates were coated with his-tagged human ∆NAC-VEGF-C derived from P. pastoris or biotinylated his-tagged human ∆NAC-VEGF-C from mammalian cells or P. pastoris, respectively. Control surfaces were left untreated. Antibody fragments and control antibodies were subsequently added and the ELISA was developed as described in Materials and Methods. (C) Cross-reactivity tested by ELISA. Human VEGF-C or mammalian cell-derived VEGF-C was added and the ELISA was developed as described in Materials and Methods. (D) BIAcore profiles from the 4 different anti-VEGF-C scFv clones. Different concentrations of protein-A purified scFv were injected on a streptavidin-precoated sensorchip coated with ca. 2000 RU biotinylated mammalian cell-derived ∆NAC-VEGF-C.

Table 2. Amino acid sequences of parental and affinity matured anti-VEGF-C scFv.

| Clone   | CDR-H1 | FR2   | CDR-H2 | CDR-H3 | CDR-L1 | CDR-L3 |
|---------|--------|-------|--------|--------|--------|--------|
| Library | S Y A G L K | X X X X (X) | X X X X X X X | S Y Y | P R F Y P V | 1/64 |
| VC1     | S Y A G P K | E S S M | - - | S Y Y | P I R W A P | 17/64 |
| VC2     | S Y A E L K | E S L P | - - | S Y Y | P R F Y P V | 3/64 |
| VC3     | S Y A G L E | E S L P | - - | S Y Y | P G S E R P | 1/64 |
| VC4     | S Y A E L K | W P A T G | - - | S Y Y | V D A W P G | 2/64 |
| VC2.2   | Q N Y E L K | E S L P | - - | E N W | P R F Y P V | NA  |
| VC2.5   | Q N Y E L K | E S L P | - - | H S Q | P R F Y P V | NA  |
| VC2.15  | K N Y E L K | E S L P | - - | K G W | P R F Y P V | NA  |
| VC2.10  | K N Y E L K | E S L P | - - | K N N | P R F Y P V | NA  |
| VC2.24  | Q N Y E L K | E S L P | - - | S G N | P R F Y P V | NA  |
| VC2.21  | K N A E L K | E S L P | - - | N D Y | P R F Y P V | NA  |
| VC2.27  | G N Y E L K | E S L P | - - | K G Y | P R F Y P V | NA  |
| VC2.23  | N N Y E L K | E S L P | - - | Q N T | P R F Y P V | NA  |
| VC2.16  | S Y A E L K | E S L P | - - | S Y Y | P R F Y P V | NA  |
| VC2.126 | N K Y E L K | E S L P | - - | A H M | P R F Y P V | NA  |
| VC2.13  | Q S L E L K | E S L P | - - | Q W K | P R F Y P V | NA  |

Numbering according to Chothia et al. [43] VC1 to VC4, first generation anti-VEGF-C scFv; VC2.2.2 to VC2.1.6, positive anti-VEGF-C scFv clones from affinity maturation; VC2.1.26, VC2.2.13, negative scFv clones from affinity maturation; X, random amino acid encoded in the CDR-3s of the ETH-2 Gold library.
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Figure 3. Affinity matured anti-VEGF-C scFvs possess a higher affinity. (A, B) ELISA analysis of bacterial supernatant from randomly picked affinity matured clones after 1 to 3 rounds of selection on biotinylated (A) *P. pastoris*-derived or (B) mammalian cell-derived ΔNAC-VEGF-C. (C) BIACore profiles of monomeric affinity matured anti-VEGF-C scFvs. Monomeric fractions of protein-A purified scFv were prepared by FPLC and injected as 2-fold dilution series on a streptavidin-sensorchip coated with 2000 RU biotinylated ΔNAC-VEGF-C derived from mammalian cells.
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Sequencing of 10 positive clones that showed some of the highest ELISA signals and of 2 negative controls was subsequently performed. One clone (VC2.1.6) was identified to be identical to the parental scFv VC2, and clones VC2.2.5 and VC2.1.11 were identical to each other. The positive clones showed a converging selection of CDR-H1 and CDR-L1 sequences (Table 2). In CDR-H1 sequences, X-Asn-Tyr (X-N-Y) was selected in 7 of 8 cases, where X was always a hydrophilic residue (Asp, Glu, Asn, Gln, His, Lys, Arg; D, E, N, Q, H, K, R) or a glycine. In CDR-L1, sequences were more heterogeneous. CDR1s of negative controls exhibited a more random pattern. An amber stop codon (TAG, coding for Stop or Gln) was found in the CDR-H1 of 4 out of 8 positive clones and in the CDR-L1 of 1 out of 8 positive clones and was corrected to CAG by PCR.

Three clones (VC2.2.2, VC2.2.5 and VC2.1.5) were chosen for further characterization, since they showed the strongest ELISA signal. Protein-A purified fractions were further purified by size-exclusion chromatography and monomeric preparations were used for BIAcore analysis. Dissociation constants of 22, 35 and 43 nM, respectively, were measured in this analysis, an improvement by almost 4-fold compared to the parental scFv VC2 (81 nM) (Figure 3C and Table 3). VC2.2.2, which exhibited the lowest Kd, was subsequently used for further analysis of blocking capacity.

VC2.2.2 anti-VEGF-C scFv blocks binding of VEGF-C to VEGF-R2 and VEGF-R3

**BIAcore assay.** Fully processed human VEGF-C (ΔNΔC-VEGF-C) exerts its action via binding to VEGF-R2 and VEGF-R3 and binds the two receptors with affinities of 410 and 135 pM, respectively [22]. Fusions of VEGF-R2 or VEGF-R3 with the crystallizable fragment (Fc) of human IgG (VEGF-R2-Fc or VEGF-R3-Fc) were bound to an anti-Fc coated BIAcore chip to generate a homogenous receptor surface. ΔNΔC-VEGF-C was then passed over the receptor surface and binding occurred. In both cases, the receptor surface was able to bind almost equimolar amounts of VEGF-C (data not shown). To assess the neutralizing capacity of VC2.2.2 scFv, ΔNΔC-VEGF-C was preincubated with different concentrations of VC2.2.2 scFv to allow for the formation of the scFv-antigen complex, and this complex was then injected on the receptor surface.

The binding of ΔNΔC-VEGF-C to immobilized VEGF-R3 was dose-dependently inhibited by anti-VEGF-C scFv VC2.2.2 (Figure 4A), but not by the irrelevant control scFv directed against glutathione-S-transferase (GST) (Figure 4B). With 900-fold molar excess of VC2.2.2 scFv, an 86% reduction of response was achieved.

### Table 3. Comparison of the kinetic constants of the affinity matured anti-VEGF-C scFvs.

| scFv     | k<sub>on</sub> (1/Ms) | k<sub>off</sub> (1/s) | K<sub>d</sub> (nM) | K<sub>d</sub> improvement relative to VC2 |
|----------|-----------------------|-----------------------|-------------------|----------------------------------------|
| VC2      | 4.45 x 10<sup>3</sup> | 3.58 x 10<sup>3</sup> | 81                | -                                      |
| VC2.1.5  | 2.39 x 10<sup>3</sup> | 1.02 x 10<sup>3</sup> | 43                | 1.9                                    |
| VC2.2.2  | 5.65 x 10<sup>3</sup> | 1.22 x 10<sup>3</sup> | 22                | 3.7                                    |
| VC2.2.5  | 2.85 x 10<sup>3</sup> | 1.00 x 10<sup>3</sup> | 35                | 2.3                                    |

The kinetic constants were fitted from dilution series of monomeric scFv preparations with Biacore3.1 software using a 1:1 Langmuir binding model.

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Figure 4. VC2.2.2 blocks binding of VEGF-C to VEGF-R2 and VEGF-R3 as measured by SPR. VEGF-R3-Fc was bound to a CM5 sensorchip coated with anti-human IgG antibody. 10 nM ΔNΔC-VEGF-C was then preincubated with a 9 to 900 times molar excess of (A) anti-VEGF-C scFv or (B) control scFv and injected on the VEGF-R3-Fc surface and the amount of binding of ΔNΔC-VEGF-C was measured by SPR. (C) VEGF-R2-Fc was bound to a CM5 sensorchip coated with anti-human IgG antibody. 10 nM ΔNΔC-VEGF-C or VEGF-A with or without preincubation together with anti-VEGF-C scFv were then injected on the VEGF-R2 surface and bound VEGF-A or VEGF-C was measured by SPR.

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On the VEGF-R2 coated surface, binding of ΔNAC-VEGF-C was also dose-dependently inhibited by injection of a mixture of ΔNAC-VEGF-C preincubated for 30 minutes with anti-VEGF-C scFv VC2.2.2 (Figure 4C). With 900-fold molar excess of VC2.2.2 scFv, an 81% reduction of response was achieved. Binding of 10 nM VEGF-A to VEGF-R2 resulted in a response comparable to the binding of 10 nM ΔNAC-VEGF-C but was not blocked by a 900-fold molar excess of anti-VEGF-C scFv VC2.2.2, demonstrating the specificity of the anti-VEGF-C scFv (Figure 4C).

**Competitive ELISA.** The flow-based Biacore VEGF-C neutralization assay measures the neutralizing potency of the VC2.2.2 anti-VEGF-C scFv only during a short time span, since the scFv-bound VEGF-C had only a short lasting possibility (the passage through the flow-cell requires seconds) to dissociate from the scFv and to bind to the immobilized VEGF-R on the chip. The equilibrium between dissociation from the scFv and association to the VEGF-R might not be reached during the passage through the flow cell. Therefore, we next used a competitive ELISA to characterize the neutralization potency of the anti-VEGF-C scFv over a longer time span.

We found that VC2.2.2 anti-VEGF-C scFv dose-dependently blocked the binding of biotinylated ΔNAC-VEGF-C to immobilized VEGF-R, while the anti-GST control scFv 3D6 did not block this binding (Figure 5). Blocking of VEGF-R2 binding was more efficient than blockage of VEGF-R3 binding, in agreement with the findings in the Biacore assay and the fact that affinity of ΔNAC-VEGF-C to VEGF-R3 is higher than to VEGF-R2 [22]. With a 900-fold molar excess of VC2.2.2 anti-VEGF-C scFv, binding of biotinylated ΔNAC-VEGF-C to VEGF-R2 was inhibited by 95%±1.0%, while the binding to VEGF-R3 was blocked by 74%±3.5%. Blocking reached a significance level of p<0.05 (Student’s t-test) vs. 3D6 irrelevant control antibody for VC2.2.2 anti-VEGF-C scFv concentrations ≥200 nM or 100-fold molar excess over biotinylated VEGF-C.

**VC2.2.2 anti-VEGF-C scFv binds to an epitope on ΔNAC-VEGF-C implicated in VEGF receptor binding**

To locate the epitope on ΔNAC-VEGF-C to which VC2.2.2 anti-VEGF-C scFv binds, a peptide microarray consisting of overlapping 15-mer peptides spanning the whole ΔNAC-VEGF-C aa sequence was used. Cy3-labelled VC2.2.2 scFv and Cy5-labelled 3D6 scFv were allowed to competitively bind to the peptide array. Upon scanning, the ratio of median signals from Cy3 vs Cy5 channels were used to generate a list of peptides bound by the respective scFvs.

P. pastoris-derived ΔNAC-VEGF-C, the positive control protein, emerged as the top-hit from this scan, being bound more than 100 times stronger by the anti-VEGF-C scFv than by the irrelevant scFv (Table 4). This validates the usefulness of the peptide-array. Target peptides more strongly bound by the VC2.2.2 anti-VEGF-C scFv vs. the irrelevant scFv 3D6 contained the sequence FFKPCCSVYRC (found more than 22 times stronger by VC2.2.2 than irrelevant control) as well as the C-terminal sequence spanning SCR CMS to RQVHSIRRHHHHH (bound more than 4 times stronger).

Interestingly, the FFKPCCSVYRC sequence maps to the region on VEGF-C that is most important for receptor binding to VEGF-R2 and VEGF-R3 (Figure 6) [24] and contains Cys156, the key-residue responsible for VEGF-R2 binding [25]. Importantly, ΔNAC-VEGF-D, which is not bound by VC2.2.2, features two different residues in the FFKPCCSVYRC region, which might be responsible for the loss of binding (Figure 1). The C-terminal sequence SCR CMS to RQVHSIRRHHHHH lies in proximity to the loop 2 in the site 1 receptor binding interface of VEGF-C (Figure 6) and binding of anti-VEGF-C to this region could therefore also sterically hinder the binding of VEGF-C to its receptors.

The variable heavy domain V<sub>H</sub> of VC2.2.2 is sufficient for binding to VEGF-C

Bacterial supernatant from IPTG induced cultures expressing VC2.2.2 V<sub>H</sub>-myc or a control V<sub>H</sub>-myc were checked by ELISA for reactivity against ΔNAC-VEGF-C. Binding to ΔNAC-VEGF-C could be observed, while no unspecific stickyness to alpha-2-macroglobulin was seen (Figure 7). Detection with protein-A was also successful, pointing to a generally correct folding of the V<sub>H</sub>-since protein-A binds to a conformational epitope on the opposite face of the former dimerization interface between V<sub>H</sub> and the variable light domain V<sub>L</sub> [26].

**Anti-VEGF-C scFvs contain hydrophilic cameld V<sub>H</sub>L-like mutations in the V<sub>H</sub>–V<sub>L</sub> dimerization interface**

Upon reexamination of the anti-VEGF-C scFv, mutations in the framework region 2 (FR2) and adjacent regions of the heavy chain could be identified. In VC2 and its daughter clone VC2.2.2, residue 44 was mutated from glycine (as encoded in the ETH-2 Gold library) to glutamic acid (Table 2), by a purinic single nucleotide mutation from GGG to GAG, making it more hydrophilic. The FR2 region lies within the dimerization interface of the V<sub>H</sub> and V<sub>L</sub> domains and loss of the V<sub>L</sub> domain leads to resurfacing of hydrophobic residues within the former dimerization interface and could lead to decreased solubility and enhanced aggregation. The G44E substitution and other hydrophilic substitutions in the FR2 region are therefore a hallmark of the variable heavy domain V<sub>H</sub>L in cameld heavy chain antibodies, naturally occurring immunoprobs devoid of light chains [27,28]. VC1 contains a mutation in the FR2 region where the hydrophobic leucine at position 45 is replaced by the less hydrophobic proline, caused by a CTG to CCG transition (Table 2). L45 is also one of the typical residues altered in cameld V<sub>H</sub>L, although the canonical residue there is the hydrophilic arginine. However, the CGG arginine codon can only

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**Figure 5. VC2.2.2 blocks binding of VEGF-C to VEGF-R2 and VEGF-R3 as measured by competitive ELISA.** 2 nM biotinylated ΔNAC-VEGF-C was preincubated with varying amounts of anti-VEGF-C scFv or control scFv and added on a VEGF-R2 or VEGF-R3 coated microtiter plate. Plotted datapoints are means from 4 replicates ± SEM. The datapoints were fitted to a sigmoidal dose-response curve model using GraphPad Prism 4. Inhibition of VEGF-C binding by anti-VEGF-C scFv reached significance vs the control scFv (*, p<0.05, Student’s t-test) at molar excess of 100× more anti-VEGF-C scFv vs VEGF-C. #: p=0.067 for anti-VEGF-C/VEGF-R2 vs control at molar excess of 33× more anti-VEGF-C than VEGF-C.

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be reached from the CTG proline codon by a T to G pyrimidine/ purine transition, but transversions are about an order of magnitude less frequent than purinic (A/G) or pyrimidinic (C/T) transitions [29,30]. For a single pyrimidinic transition, proline is less frequent than purine transversion, but transversions are about an order of magnitude less frequent than purinic (A/G) or pyrimidinic (C/T) transitions [29,30]. For a single pyrimidinic transition, proline is less frequent than purine.

Table 4. Epitope mapping.

| Peptide/Protein | log2 ratio | Stdev | aVEGF-C Signal | Stdev | aVEGF-C SNR | Stdev | aGST Signal | Stdev | aGST SNR | Stdev |
|-----------------|------------|-------|----------------|-------|-------------|-------|-------------|-------|---------|-------|
| ANAC-VEGF-C     | 6.26       | 0.19  | 8346           | 5844  | 11.4        | 5.0   | 202         | 79    | 2.9     | 1.1   |
| ANAC-VEGF-C     | 6.18       | 0.12  | 8531           | 5797  | 25.8        | 30.0  | 213         | 78    | 3.5     | 2.1   |
| TNTFFKPCVSYYR    | 5.98       | 0.03  | 51361          | 1256  | 112.7       | 9.5   | 917         | 48    | 16.9    | 5.5   |
| FFKPCVSYYRCGGC   | 4.50       | 0.12  | 45671          | 391   | 120.2       | 89.2  | 2114        | 184   | 46.4    | 17.6  |
| SCRCSKLDVYRQV    | 4.29       | 0.17  | 34911          | 1322  | 332.6       | 215.7 | 1872        | 142   | 49.4    | 12.4  |
| RQVHSIRHHHHHH    | 4.22       | 0.17  | 22414          | 1071  | 166.6       | 43.8  | 1302        | 86    | 34.8    | 6.4   |
| VYRQVHSIRHHHH    | 3.69       | 0.03  | 32602          | 1576  | 63.8        | 29.1  | 2615        | 189   | 26.4    | 3.1   |
| KLDVYRQVHSIRHH   | 2.67       | 0.07  | 25280          | 2821  | 123.0       | 68.9  | 4041        | 269   | 64.3    | 14.5  |
| PPCVSYYRCGGCCNS  | 2.13       | 0.05  | 1555           | 65    | 25.0        | 2.4   | 415         | 37    | 9.0     | 2.1   |
| SFANHTSCRCSKLD   | 1.58       | 0.12  | 1248           | 54    | 16.1        | 4.5   | 459         | 9     | 6.8     | 3.8   |
| NHTRSCRCSKLDVTR  | 1.53       | 0.24  | 7390           | 1362  | 170.9       | 95.4  | 2577        | 126   | 58.3    | 26.9  |
| VTSANHTSCRCSM    | 1.24       | 0.11  | 2945           | 165   | 65.6        | 24.5  | 1272        | 18    | 31.9    | 3.7   |
| human IgG        | 0.07       | 0.01  | 9348           | 531   | 41.3        | 7.5   | 8917        | 583   | 31.3    | 4.8   |
| mouse IgG        | 0.03       | 0.06  | 9071           | 491   | 34.0        | 15.0  | 8833        | 423   | 27.3    | 9.3   |
| human IgG        | −0.05      | 0.04  | 4873           | 474   | 24.4        | 11.2  | 4969        | 363   | 27.1    | 8.6   |
| mouse IgG        | −0.05      | 0.01  | 4885           | 268   | 43.0        | 6.9   | 4994        | 310   | 32.1    | 5.5   |
| CMSKLDVYRQVHSII  | −0.36      | 0.13  | 401            | 43    | 7.0         | 1.1   | 388         | 32    | 4.5     | 3.0   |
| QCMTNTSTYSVLTLF  | −0.96      | 0.16  | 1183           | 81    | 15.8        | 5.8   | 2065        | 121   | 10.4    | 1.2   |

Values are arithmetic means and standard deviations from 3 subarrays. SNR: signal to noise ratio, log2 ratio are log2 (VC2.2.2 anti-VEGF-C/3D6 anti-GST). All peptides that generated data with errors, SNRs <2 in both channels and signal intensities ≤100 in both channels were filtered out. Shown is a representative array from at least 3 replicates. Dye-swap arrays yielded similar results.

Detection

In this study, we describe the development of function-blocking monoclonal antibody fragments against human ΔNAC-VEGF-C. The blocking capabilities were confirmed at the molecular level using BLAcore and competitive ELISA. Antibody phage-display was used to select 4 lead binders from the ETH-2 Gold antibody phage display library. From these 4 binders, one was specific for an epitope only present on P. pastoris-derived ΔNAC-VEGF-C, which was used for selection, while the other 3 clones bound to an epitope in the conserved region of ΔNAC-VEGF-C. Binding to the His-tag could be excluded, since ΔNAC-VEGF-D, which was used to select 4 lead binders from the ETH-2 Gold antibody phage display library. From these 4 binders, one was specific for an epitope only present on P. pastoris-derived ΔNAC-VEGF-C, but not to mammalian cell-derived ΔNAC-VEGF-C. Recently, the three-dimensional crystal structure of ΔNAC-VEGF-C in complex with one of its receptors, VEGF-R2, was solved by X-ray diffraction [32]. The receptor binding domains of VEGF-C were
The VEGF-C residues contacting VEGF-R2 as reported in [32] are represented in yellow (N-terminal helix), red (loop 1), orange (loop 2) and brown (loop 3). The two epitope stretches identified in the peptide scan are colored in blue. From epitope B, only SCRCS5K is shown, the C-terminal end is missing in the reported structure. Overlaps of epitope A (FFKPPCSVYRC) and receptor-contacting residues in loop 1 are colored in purple. Residues in loop 1 found to affect VEGF-R2-binding or VEGF-R3-binding by mutational analysis [24] are shown with the same colors as in (A). The localization of the epitopes within the VEGF-C dimer is shown in (D) and their interference with the boxed interface of VEGF-R2 (cyan) is shown in (E), with magnifications in “side” view (F) and “top view” (G). The pdb file 2X1X was used for the representation (www.pdb.org).

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Figure 6. Possible VC2.2.2 anti-ΔΝΔC-VEGF-C epitope-localization within or near the receptor-binding region on ΔΝΔC-VEGF-C.

The VEGF-C residues contacting VEGF-R2 as reported in [32] are represented in yellow (N-terminal helix), red (loop 1), orange (loop 2) and brown (loop 3). The two epitope stretches identified in the peptide scan are colored in blue. From epitope B, only SCRCS5K is shown, the C-terminal end is missing in the reported structure. Overlaps of epitope A (FFKPPCSVYRC) and receptor-contacting residues in loop 1 are colored in purple. Residues in loop 1 found to affect VEGF-R2-binding or VEGF-R3-binding by mutational analysis [24] are shown with the same colors as in (A). The localization of the epitopes within the VEGF-C dimer is shown in (D) and their interference with the boxed interface of VEGF-R2 (cyan) is shown in (E), with magnifications in “side” view (F) and “top view” (G). The pdb file 2X1X was used for the representation (www.pdb.org).

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Figure 7. The VC2.2.2 V_H is sufficient to bind ΔΝΔC-VEGF-C.

Binding of the single V_H-domain of VC2.2.2 to ΔΝΔC-VEGF-C and an unrelated antigen (alpha-2-macroglobulin, a2MG) was tested by ELISA using either anti-myc and anti-mouse HRP or protein-A-HRP as detection compounds.

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Anti-VEGF-C Antibody Fragments

Figure 6. Possible VC2.2.2 anti-ΔΝΔC-VEGF-C epitope-localization within or near the receptor-binding region on ΔΝΔC-VEGF-C. (A) The VEGF-C residues contacting VEGF-R2 as reported in [32] are represented in yellow (N-terminal helix), red (loop 1), orange (loop 2) and brown (loop 3). The two epitope stretches identified in the peptide scan are colored in blue. From epitope B, only SCRCS5K is shown, the C-terminal end is missing in the reported structure. Overlaps of epitope A (FFKPPCSVYRC) and receptor-contacting residues in loop 1 are colored in purple. Residues in loop 1 found to affect VEGF-R2-binding or VEGF-R3-binding by mutational analysis [24] are shown with the same colors as in (A). The localization of the epitopes within the VEGF-C dimer is shown in (D) and their interference with the boxed interface of VEGF-R2 (cyan) is shown in (E), with magnifications in “side” view (F) and “top view” (G). The pdb file 2X1X was used for the representation (www.pdb.org).

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osteoblasts, where it regulates bone regeneration in an autocrine manner via VEGF-R3 [14]. Interference with the VEGF-D/VEGF-R3 axis might therefore potentially affect bone regeneration. Conversely, VEGF-C is expressed in osteoclasts, where it enhances bone resorption in an autocrine manner via VEGF-R3 [38].

Taken together, we have selected a human antibody fragment that (i) binds to the receptor-binding region of mature, fully processed human VEGF-C (ΔNAC-VEGF-C) and (ii) is capable of blocking the interaction of ΔNAC-VEGF-C with both VEGF-R2 and VEGF-R3 at the molecular level. The V_H of the selected anti-VEGF-C scFvs contain camelid V_H-like mutations and VC2.2.2 V_H is sufficient to bind to ΔNAC-VEGF-C. Upon further engineering of this minimal, 14.6 kDa antibody fragment to enhance solubility and stability, it may serve as the basis for development of bulkier fully human neutralizing anti-VEGF-C immunoproteins with improved half-lives in circulation. Such inhibitors could be useful in treatment of cancers that rely on direct VEGF-C signaling such as Kaposi sarcoma [39], acute myeloid or lymphocytic leukemia [40,41] and cancers that metastasize via the lymphatic vasculature [42] but also against cancers that have become refractory to anti-VEGF-A treatment.

Figure 8. SEC gel-filtration profiles of anti-VEGF-C scFvs. Protein-A purified scFv were injected on a Superdex 75 10/300 GL size-exclusion gel-filtration column. Markers represent the major elution peaks of the molecular mass standards ovalbumin (43 kDa) and ribonuclease A (13.7 kDa). doi:10.1371/journal.pone.0011941.g008
Furthermore, such inhibitors could also be active against VEGF-C-induced bone degeneration and age-related macular degeneration.

Materials and Methods

Bacterial Media

Phage growth medium 2YT was mixed from 16 g tryptone (Fluka, Buchs, Switzerland), 10 g yeast extract (Fluka) and 5 g NaCl (Fluka) per liter. For growth of phage infected *E. coli* TG1 on solid agar plates, 2YT was supplemented with 100 μg/ml ampicillin (Sigma-Aldrich, Buchs Switzerland) and 1% (w/v) glucose (Fluka) to give 2YTAG(1%) and mixed with 17 g of agar (Hanseler, Herisau, Switzerland) per liter.

Plasmids

The pHEN1 plasmid was used for expression of scFv in *E.coli* as described previously [19]. For expression of the *VH* domain, the *VH* sequence was amplified from the VC2.2.2 scFv-encoding pHEN1-based plasmid by PCR using the upstream forward primer LMB3long (attaching 5′ of the scFv coding sequence) and the downstream reverse primer VHrev (annealing at the 3′ end of *VH*) (Table 5). The PCR product was then cut with *NdeI* and *Ncol* and ligated into pHEN1-backbone cut with the same enzymes. The resulting plasmid encoding VC2.2.2 *VH* from Glu1 to Ser113 (Chothia 89–97 numbering scheme [43]) as well as the C-terminal myc-tag and amber stop codon was subsequently checked by sequencing.

Antigens

Human recombinant ΔNΔC-VEGF-C was expressed from a pPICZalpha based expression vector (Invitrogen, Paisly, UK) in the yeast *Pichia pastoris* and contains an N-terminal His-tag (Figure 1). Purification to homogeneity was accomplished by IMAC affinity chromatography and gel filtration as described previously [44]. Human recombinant ΔNΔC-VEGF-C and ΔNΔC-VEGF-D, produced in mouse myeloma cells, were purchased from R&D Systems (Abingdon, UK). Human VEGF-A165 was kindly provided by the National Cancer Institute (Bethesda, MD). Alpha-2-macroglobulin was purchased from Sigma-Aldrich.

Biotinylation

ΔNΔC-VEGF-C from *P. pastoris* or mammalian cells was biotinylated with SS- or LC-NHS-Biotin (Pierce, Rockford, IL). 400 μl of a 500 μg/ml protein solution in PBS (Gibco) was mixed...
with 80 μl of freshly prepared 1 mg/ml solution of biotinylation agent in MilliQ water and incubated for 1 hour at RT. For removal of unreacted biotinylation agent, the mixture was loaded on a PD10 gel filtration column (GE Healthcare, Glattbrugg, Switzerland), topped up with 2.12 ml PBS, eluted with 3.5 ml PBS and fractions of 0.5 ml were collected. The protein containing fractions, as measured per spectrophotometric absorbance at 280 nm, were pooled.

To check the biotinylation of the protein, 100 μl of protein solution in PBS was dotted on a nitrocellulose membrane (Biorad, Hercules, CA) with an Easy-Titer dot-blot system (Pierce). After drying for 15 min, the membrane was blocked for 1 hour with 3% BSA (Fluka) in PBS at RT. Following washing with PBS-0.1% Tween-20 (PBST), the biotinylated protein on the membrane was detected with Streptavidin-HRP (GE Healthcare) and fractions of 0.5 ml were collected. The protein containing fractions, as measured per spectrophotometric absorbance at 280 nm, were pooled.

Table 5. Primers used in this study.

| Primer   | Sequence (5'-3') | Reference          |
|----------|------------------|--------------------|
| LMB3long | CAGGAAACAGCTGATACCAGTATTAC | [19]               |
| fdegqlong | GACTGTTAAGAATTTCCTGTATGAG | [19]               |
| Vhrev    | GAGATGAGTTTTTTTGTCGCCGCCGACctcgagacgggctaggtacgctgaggtcgggggt (NotI site underlined, annealing site to Vh in lowercase) | This study |
| DP47CDR1ba | TGGGTCCGCAGCCCTCCAG | [23]               |
| DP47CDR1for | AGCTGGCCGGACCCGCATATMNMNWNMNGCTAAAGGTGAATCCAGAGGCTGC | [23]               |
| DPL16CDR1ba | TGGTCACCAGAGCCAGGAG | [46]               |
| DPL16CDR1for | TCCTGGCTCTGGTGTTACAGGCTGCMNNMNNMNTCTGTAGGCTGCTCTCTTG | [46]               |
| VC2.2heavybackmutf0 | AGCTGGCCGGACCCGCATATATCATTCTGGCTAAAGGTGAATCCAGAGGCTGC | This study |
| VC2.3lightbackmutf0 | TCCTGGCTCTGGTGTTACAGGCTGCMNNMNNMNTCTGTAGGCTGCTCTCTTG | This study |
| VC2.2.13heavybackmutf0 | AGCTGGCCGGACCCGCATATATCATTCTGGCTAAAGGTGAATCCAGAGGCTGC | This study |

Affinity selections with biotinylated antigen

For selection of affinity-matured binders from the affinity maturation library, panning in solution with biotinylated antigen was used. This was done to prevent selection of binders that bind only to denatured VEGF-C. Up to 3 biopanning rounds with different concentrations of biotinylated P. pastoris-derived ΔNAC-VEGF-C (VC2.1 series; 1st round with 3 nM, 30 nM and 300 nM; 2nd round with 30 pM and 3 nM; 3rd round with 30 pM) and biotinylated mammalian cell-derived ΔNAC-VEGF-C (VC2.2 series; 1st round with 300 pM, 5 nM and 30 nM; 2nd round with 3 nM) were performed. Prior to selection, 50 μl streptavidin coated magnetic beads (M-280 Dynabeads, Dynal Biotech, Oslo, Norway) per selection were blocked with 1 ml 3% BSA in PBS for 1 hour at room temperature and resuspended in 50 μl 2% BSA/PBS. 1 ml of antibody phage library (approximately 10^{12} tu in total) in 2% BSA/PBS was incubated with the biotinylated antigen at the above stated concentrations for 1 hour on a rotator at room temperature. The streptavidin magnetic beads were then added to the phage/antigen mixture and allowed to capture the biotinylated antigen and adhering phages for 15 min on a rotator at room temperature. After rinsing 2 and 3 rounds of panning, washing steps were increased to twenty washes with each buffer. Bound phages were eluted for 15 min by incubating the immunotubes on a rotator at 1 ml of 1 M Tris-HCl pH 8.6. The eluted phages were subsequently used to infect 10 ml of exponentially growing E.coli TG1 at OD_{600} = 0.5 for 35 min at 37°C. Titration of the eluted phage, phage amplification and colony picking were performed as described previously [45].

ELISA screening

After 2 and 3 rounds of panning, individual bacterial colonies containing the phagemid were picked and inoculated into 150 μl 2YTAG(0.1%) and grown for 3 hours in a 37°C shaking incubator. Then, the cells were induced by addition of 50 μl of 2YT containing 4 mM isopropyl-thio-galactopyranoside (IPTG; Applichem, Darmstadt, Germany), to give a final concentration of 1 mM IPTG, and grown overnight for 30°C.

A 96-well maxisorp plate (Nunc) was coated overnight at RT with 50 μg/ml of ΔNAC-VEGF-C in PBS as described above. The next day, the ELISA plate was washed three times with PBS...
and blocked with 300 µl 4% MPBS for 2 hours at RT. The plate was then washed again three times with PBS and each well was supplemented with 20 µl of 10% MPBS containing a 1:200 dilution of mouse anti-myc 9E10 antibody (Sigma, Cat No M5546) and 1:200 dilution of anti-mouse horseradish peroxidase labelled sheep antibody (GE Healthcare) as secondary reagents. The bacterial supernatant was centrifuged for 10 min at 1800 g and 80 µl of supernatant from each well was added to the corresponding ELISA well. The plate was then incubated for 1 hour at RT on an orbital shaker. After three washes each with PBST and PBS using a squirt bottle, 100 µl Blue-POD peroxidase substrate (Roche, Mannheim, Germany) was added to each well and the chromogenic reaction was stopped with 50 µl 1 M H2SO4 after 10 min. The plates were then read with a spectrometer at 450 nm and 650 nm. To screen for false positives, the supernatants were also tested on maxisorp plates coated with PBS alone. Since ΔΝΑC-VEGF-C derived from P. pastoris was used for the panning, the supernatants were also tested on streptawell plates (Roche) coated with 100 µl 100 0.22 M biotinylated ΔΝΑC-VEGF-C derived from mammalian cells (R&D Systems) in PBS overnight at 4°C.

**SPR analysis**

All solutions to be injected into the BLACore 3000 (GE Healthcare) were filtered using a 0.22 µm filter (Millipore, Zug, Switzerland). A streptavidin-coated sensorchip flowcell (GE Healthcare) was coated at a flow rate of 5 µl/min with 25 µl of 100 nM biotinylated ΔΝΑC-VEGF-C derived from either P. pastoris or mammalian cells in PBS, 0.01% azide, 0.005% Surfactant P-20 (GE Healthcare). For both antigens, a stable regeneration was done by injection of 5–10 µl 10 mM HCl. Positive supernatants from the ELISA were filtered through a 0.22 µm filter and injected as is.

**Affinity maturation**

Parental antibodies obtained after 3 rounds of panning were randomized in the CDR1 at the following positions (numbering according to the Chothia 89–97 scheme [43]): 31, 32, 33 for VH, 31, 31a, 32 for Vκ-kappa and 31a, 31b and 32 for Vλ-lambda using degenerate primers DP47CDR1for, DP47CDR1ba, and DPL16CDR1for respectively, together with DP47CDR1ba, DPK22CDR1ba, DPL16CDR1ba, LMB3long and fbeqlong respectively (Table 5). The three amplicons were assembled by PCR assembly essentially as described [19]. All primers were purchased from Sigma.

Affinity matured clones that contained a TAG amber stop codon in the mutated CDRs (coding for glutamine in suppressor strains and stop in non-suppressor strains), were backmutated to CAG by using PCR backmutation primers as specified in Table 5 and essentially the same assembly procedure as described above.

**Expression and purification of scFv antibody fragments**

An overnight starter culture of 2YTAG(5%) inoculated with a single colony of phagemid bearing E.coli TG1 was diluted 1:100 in 1 l of 2YTAG(0.1%) and grown at 37°C, 225 rpm until OD600 = 0.5. The cells were then induced by addition of IPTG (final concentration 1 mM) and grown overnight at 30°C, 225 rpm. Bacterial supernatants were clarified by centrifugation at 5000 g, 4°C for 45 min and filtered through a 0.2 µm filter (TPP, Trasadingen, Switzerland). Supernatants were then loaded on a protein-A affinity column (Biorad) using the Profinia automated protein purification system (Biorad), according to the manufacturer's recommendations. The columns were washed with Buffer A (100 mM NaCl, 0.1% Tween-20 (Sigma), 0.5 mM EDTA (Sigma) in PBS) and subsequently Buffer B (500 mM NaCl, 0.5 mM EDTA in PBS). Bound scFv were then eluted with 100 mM triethylamine (Sigma) and immediately neutralized in 1 M Tris-HCl, pH 7.0. The eluate was dialyzed overnight against PBS using Spectra/Por dialysis tubing with 12–14 kDa cutoff (Spectrum Labs, Breda, The Netherlands) and concentrated to about 1 mg/ml with Amicon Ultra 15 ultrafiltration devices with 10 kDa cutoff (Millipore). For sterilization, the scFv preparation was finally filtered through a 0.22 µm filter (Pall, Basel, Switzerland).

**SDS-PAGE and immunoblotting**

Proteins were resolved on 4–12% gradient bis-tris Novex precast gels in MOPS running buffer, using LDS loading buffer with or without reducing agent as indicated by the manufacturer (all components from Invitrogen). Precision Plus molecular weight standards were from Biorad. Staining of SDS-PAGE gels was accomplished using Bio-Safe Coomassie (Biorad). For immunoblotting, the proteins resolved with SDS-PAGE were transferred to nitrocellulose membranes (Biorad). Membranes were subsequently blocked for 2 h with 5% skimmed milk powder (Coop, Basel, Switzerland) in phosphate buffered saline (PBS, Gibco) containing 0.1% (v/v) Tween-20 (Sigma), referred to as MPBST. Immunoblotting was performed using mouse anti-myc 9E10 antibody (1:1,000, Sigma) and secondary anti-mouse HRP-labeled sheep antibody (1:20,000, GE Healthcare), diluted in MPBST. Bands were revealed using ECL Plus detection reagent (GE Healthcare).

**Size-exclusion chromatography**

Size-exclusion chromatography (SEC) and isolation of monoclonal scFv was performed on an Akta FPLC system using the Superdex 75 10/300 GL column (GE Healthcare) at a flow of 0.5 ml/min. The column was calibrated with molecular mass standards ovalbumin (43 kDa) and ribonuclease A (13.7 kDa) (GE Healthcare).

**Epitope mapping using peptide array**

A peptide array (PepStar, JPT, Berlin, Germany) was used to map the epitope of the anti-VEGF-C antibody. The amino acid sequence spanning human ΔΝΑC-VEGF-C from T103 to R227 and an appended C-terminal 6xHis-tag was used to define 40 overlapping, consecutive 15-mer peptides with 3 residues shift each. Synthesis of the peptides, using SPOT peptide synthesis, and peptide printing on glass slides were conducted at JPT. The myc-tag-epitope AEQIISSEDL human and murine IgGs as well as P. pastoris-derived ΔΝΑC-VEGF-C were printed as additional controls. Each slide features 3 identical subarrays, corresponding to 3 technical replicates per condition.

ScFvs for epitope mapping were fluorescently labelled using Cy3 and Cy5 monoreactive dye (GE Healthcare). Dye aliquots were prepared by dissolving monoreactive Cy3 or Cy5 dye, intended for labeling of 1 mg protein, in 10 µl of DMSO, dividing into 1 µl aliquots and vacuum-drying in a speed-vac. Dye aliquots were stored at 4°C in a desiccator under vacuum in the dark.

20 µl of 1 M sodium bicarbonate buffer, pH 9, was added to 200 µl of a 1 mg/ml scFv solution in PBS to bring the final pH between 8.5 and 9.5. Then, this solution was added to 2 aliquots of Cy3 or Cy5 monoreactive dye and left to incubate for 1 hour at room temperature. Non-reacted dye was subsequently quenched with 100 µl Tris buffered saline (TBS), pH 7.5. To separate free dye from the scFv-bound dye and to exchange the buffer to PBS, the solution was loaded on a microcon concentrator column YM-10 (Millipore) with a membrane-cutoff of 10 kDa. The concent-
trator with the scFv/dye solution was then centrifuged for three times at 14 krpm for 30 min and washed with 500 μl PBS between spins. Finally, the dye-labelled scFv was resuspended in 200 μl PBS and the protein concentration as well as the dye absorption was measured using a spectrophotometer (Nanodrop; Thermo Scientific, Wilmington, DE).

Arrays were competitively incubated essentially according to the manufacturer’s instructions in a sandwich-like fashion with a Cy3-labelled anti-VEGF-C scFv and a Cy5-labelled irrelevant scFv at manufacturer’s instructions in a sandwich-like fashion with a Cy3-of monoclonal anti-Fc antibody at 25°C overnight in a humid atmosphere in the dark. Then, the microrarrays were washed 3 times for 6 min with binding buffer and 3 times for 6 min with MilliQ water in the dark. Slides were dried by spinning for 2 min at 300 rpm and scanned using a Genepix 4200A scanner (Axon Instruments, MDS Analytical Technologies, Concord, ON, Canada) with photo-multiplier tube (PMT) gain values for both channels set to prevent saturated pixels and to yield a pixel-count ratio of 1:1 for the two channels. Scanning was done using 2 line ratio of 1:1 for the two channels. Scanning was done by injection of two times 70 μl EDC/NHS mixture at a flow rate of 5 μl/min, resulting in a ARU of 214. Then, 68 μl of monoclonal anti-Fc antibody at 25 μg/ml diluted in 10 mM sodium acetate pH 5.0 immobilization buffer was injected. After 5 min, 50 μl ethanolamine was injected to deactivate the remaining binding sites. A stable immobilization level of about 16000 RU was achieved. Surface regeneration between cycles was done by injection of 5 μl of 3 M MgCl2. Injections on the anti-Fc coated CM5 chip were done at a flow rate of 5 μl/min and the different proteins were diluted in HBS-P buffer. For VEGF-C/VEGF-R3 binding experiments, 100 μl of 100 nM VEGF-R3-Fc recombinant protein (R&D Systems), resulting in a response of about 2500 RU were immobilized on the anti-Fc coated CM5 chip. For VEGF-C/VEGF-R2 binding experiments, 100 μl of 100 nM VEGF-R2-Fc recombinant protein (R&D Systems), resulting in a response of about 1200 RU were immobilized on the anti-Fc coated CM5 chip.

VEGF-C neutralization assay using BIAcore

A maxisorp plate was coated in quadruplicate with 100 μl of 2 μg/ml monoclonal mouse anti-human IgG (Chemicon, Temecula, CA) in PBS overnight at room temperature. The next morning, the plate was washed 3 times with PBS and blocked for 2 hours at room temperature with 200 μl blocking buffer (3% BSA (Probumin; Millipore) in PBS). The plate was then washed again 3 times with PBS and incubated with 100 μl 1 μg/ml VEGF-R2-Fc or VEGF-R3-Fc in blocking buffer for 2 hours at room temperature. After washing with 3 times PBS, 100 μl of a mixture of 2 nM biotinylated P. pastoris-derived ΔΝΔC-VEGF-C preincubated for 30 min with a variable concentration of VC2:2.2 anti-VEGF scFv or irrelevant scFv in blocking buffer was added to the ELISA plate for 1 hour at room temperature. Subsequently, the ELISA plate was washed for 2 times with PBST and 1 time with PBS and a mixture of streptavidin-horseradish peroxidase conjugate diluted 1:1000 in blocking buffer was added and incubated for 1 hour at room temperature. Finally, the plate was washed again 3 times with PBST and 3 times with PBS and developed as detailed above.

Author Contributions

Conceived and designed the experiments: MR AV KBH DN MD. Performed the experiments: MR AV. Analyzed the data: MR AV. KBH DN MD. Contributed reagents/materials/analysis tools: KBH DN MD. Wrote the paper: MR MD.

References

1. Dadras SS, Paul T, Bertocini J, Bresen LF, Muzikansky A, et al. (2003) Tumor lymphangiogenesis: a novel prognostic indicator for cutaneous melanoma metastasis and survival. Am J Pathol 162: 1951–1960.
2. Joukov V, Pajusola K, Kaipainen A, Chilov D, Lahinen I, et al. (1996) A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. Embo J 15: 171–181.
3. Achen MG, Jeltsch M, Kukk E, Makinen T, Vitali A, et al. (1998) Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flk4). Proc Natl Acad Sci U S A 95: 548–553.
4. Rinderknecht M, Detmar M (2008) Tumor lymphangiogenesis and melanoma metastasis. J Cell Physiol 216: 347–354.
5. Matsuura M, Osamaru M, Yonemitsu Y, Suzuki H, Nakano T, et al. (2009) Autocrine loop between vascular endothelial growth factor (VEGF)-C and VEGF receptor-3 positively regulates tumor-associated lymphangiogenesis in oral squamous cell carcinomas. Am J Pathol 175: 1709–1721.
6. Kodama M, Kitada Y, Tanaka M, Kawai T, Tanaka S, et al. (2008) Vascular endothelial growth factor C stimulates progression of human gastric cancer via both autocrine and paracrine mechanisms. Clin Cancer Res 14: 7205–7214.
7. Stacker SA, Caesar C, Baldwin ME, Thornton GE, Williams RA, et al. (2001) VEGF-D promotes the metastatic spread of tumor cells via the lymphatics. Nat Med 7: 186–191.
8. Achen MG, Roufail S, Domagala T, Cajeml B, Nice EC, et al. (2000) Monoclonal antibodies to vascular endothelial growth factor-D block its interactions with both VEGF receptor-2 and VEGF receptor-3. Eur J Biochem 267: 2505–2515.
9. Roberts N, Kloos B, Cassella M, Podgrabinska S, Persaud K, et al. (2006) Inhibition of VEGF-3 activation with the antagonistic antibody more potently suppresses lymph node and distant metastases than inactivation of VEGFR-2. Cancer Res 66: 2560–2567.
10. Burton JR, Priceman SJ, Sung JH, Braekenhien E, An DS, et al. (2008) Suppression of prostate cancer nodal and systemic metastasis by blockade of the lymphangiogenic axis. Cancer Res 68: 7628–7637.
11. Shimizu K, Kabo H, Yamaguchi K, Kawaiashi K, Ueda Y, et al. (2004) Suppression of VEGFR-3 signaling inhibits lymph node metastasis in gastric cancer. Cancer Sci 95: 328–333.
12. Lin J, Laiani AS, Harding TC, Gonzalez M, Wu WW, et al. (2005) Inhibition of lymphangiogenesis using antibodies against the Flt4 (VEGFR-3) receptor. Cancer Res 65: 6901–6909.
13. He Y, Kozaki K, Karpanen T, Koshikawa K, Yla-Herttuala S, et al. (2002) Suppression of tumor lymphangiogenesis and lymph node metastasis by blocking vascular endothelial growth factor receptor 3 signaling. J Natl Cancer Inst 94: 819–825.
14. Orlandini M, Spreafico A, Bardelli M, Rocchigiani M, Salanek A, et al. (2006) Vascular endothelial growth factor-D activator VEGF-3 expressed in osteoblasts inducing their differentiation. J Biol Chem 281: 17961–17967.
15. Albaquerque RJ, Hayashi T, Cho WG, Kleinman ME, Druth S, et al. (2009) Alternately spliced vascular endothelial growth factor receptor-2 is an essential endogenous inhibitor of lymphatic vessel growth. Nat Med 15: 1023–1030.
16. Shin HY, Smith ML, Toy KJ, Williams PM, Buiss R, et al. (2002) VEGF-C mediates cyclic pressure-induced endothelial cell proliferation. Physiol Genomics 11: 245–251.
17. Timoshenko AV, Rastogi S, Lala PK (2007) Migration-promoting role of VEGF-C and VEGF-C binding receptors in human breast cancer cells. Br J Cancer 97: 1090–1096.
18. Tomasek RJ, Holfeld JS, Reiter RS, Sandra A, Lin JJ (2002) Role of VEGF family members and receptors in coronary vessel formation. Dev Dyn 223: 233–240.
19. Silacci M, Brack S, Scharr G, Marling J, Ettorre A, et al. (2005) Design, construction, and characterization of a large synthetic human antibody phage display library. Proteomics 5: 2340–2350.
20. Schliemann C, Palmbo A, Zuberbuhler K, Villa A, Kaspar M, et al. (2009) Complete eradication of human B-cell lymphoma xenographs using rituximab in combination with the immunocytokine L19-IL2. Blood 113: 2275–2283.
21. Siegfreid G, Basak A, Cronnish JA, Benamet S, Marckinewicz J, et al. (2003) The secretory propionate convertases furin, PC5, and PC7 activate VEGF-C to induce tumorigenesis. J Clin Invest 111: 1723–1732.
22. Joukov V, Sorsa T, Kumar V, Jeltsch M, Claesson-Welsh L, et al. (1997) Proteolytic processing regulates receptor specificity and activity of VEGF-C. Embo J 16: 3898–3911.

23. Villa A, Trachsel E, Kaspar M, Schliemann C, Sommariva R, et al. (2008) A high-affinity human monoclonal antibody specific to the alternatively spliced EDA domain of fibroectin efficiently targets tumor neo-vasculature in vivo. Int J Cancer 122: 2405–2413.

24. Jeltsch M, Karpanen T, Strandli T, Also K, Lankinen H, et al. (2006) Vascular endothelial growth factor (VEGF)/VEGF-C mosaic molecules reveal specificity determinants and feature novel receptor binding patterns. J Biol Chem 281: 12187–12195.

25. Joukov V, Kumar V, Sorsa T, Arighi E, Weich H, et al. (1998) A recombinant mutant vascular endothelial growth factor-2 binding, activation, and vascular permeability activities. J Biol Chem 273: 6399–6402.

26. Starovasnik MA, O’Connell MP, Fairbrother WJ, Kelley RF (1999) Antibody variable region binding by Staphylococcal protein A: thermodynamic analysis and location of the Fv binding site on E-domain. Protein Sci 8: 1423–1431.

27. Hamers-Casterman C, Atarhouch T, Muyldermans S, Robinson G, Hamers C, et al. (1993) Naturally occurring antibodies devoid of light chains. Nature 363: 446–448.

28. Muyldermans S, Lauwereys M (1999) Unique single-domain antigen binding fragments derived from naturally occurring camel heavy-chain antibodies. J Mol Recognit 12: 131–140.

29. Konid R, Horai S, Satta Y, Takahata N (1993) Evolution of hominoid mitochondrial DNA with special reference to the silent substitution rate over the genome. J Mol Evol 36: 517–531.

30. Vogel F, Kopun M (1977) Higher frequencies of transitions among point mutations. J Mol Evol 9: 159–180.

31. Barthelemy PA, Raab H, Appleton BA, Bond CJ, Wu P, et al. (2008) Comprehensive analysis of the factors contributing to the stability and solubility of autonomous human VH domains. J Biol Chem 283: 3639–3654.

32. Leppanen VM, Prota AE, Jeltsch M, Anisimov A, Kalkkinen N, et al. (2010) Structural determinants of growth factor binding and specificity by VEGF receptor 2. Proc Natl Acad Sci U S A 107: 2425–2430.

33. Courath KE, Lauwereys M, Galleni M, Matagne A, Frec JM, et al. (2001) Beta-lactamase inhibitors derived from single-domain antibody fragments elicited in the camelidae. Antimicrob Agents Chemother 45: 2807–2812.

34. Lauwereys M, Arbab Ghahroudi M, Desmyter A, Knie J, Holzer W, et al. (1998) Potent enzyme inhibitors derived from dromedary heavy-chain antibodies. Embo J 17: 3512–3520.

35. Muyldermans S, Cambillau C, Wyna L. (2001) Recognition of antigens by single-domain antibody fragments: the superfluous luxury of paired domains. Trends Biochem Sci 26: 230–235.

36. Griebner JG, Deverreux S, Thomas NS, Keim M, Jones HM, et al. (1990) Development of antibodies to unprotected glycosylation sites on recombinant human GM-CSF. Lancet 335: 434–437.

37. Karpusas M, Whitey A, Runkel L, Hochman P (1998) The structure of human interferon-beta: implications for activity. Cell Mol Life Sci 54: 1203–1216.

38. Zhang Q, Guo R, Lu Y, Zhao L, Zhou Q, et al. (2008) VEGF-C, a lymphatic growth factor, is a RANKL target gene in osteoclasts that enhances osteoclastic bone resorption through an autocrine mechanism. J Biol Chem 283: 13491–13499.

39. Stacker SA, Baldwin ME, Achen MG (2002) The role of tumor lymphangiogenesis in metastatic spread. Faseb J 16: 922–934.

40. Dias S, Choy M, Altaldo K, Rafii S (2002) Vascular endothelial growth factor (VEGF)-C signaling through FLT-4 (VEGFR-3) mediates leukemic cell proliferation, survival, and resistance to chemotherapy. Blood 99: 2179–2184.

41. Moehler TM, Ho AD, Goldschmidt H, Barlogie B (2003) Angiogenesis in hematologic malignancies. Crit Rev Oncol Hematol 45: 227–244.

42. Rinderknecht M, Demar M (2009) Molecular mechanisms of lymph-node metastasis. In: Stacker SAA, M G, eds. Lymphangiogenesis in Cancer Metastasis, 1 ed : Springer Nielsen. pp 55–82.

43. Chothia C, Lesk AM, Tramontano A, Levitt M, Smith-Gill SJ, et al. (1989) Conformations of immunoglobulin hypervariable regions. Nature 342: 877–883.

44. Scheidegger P, Weighofer W, Suarez S, Kaser-Hotz B, Steiner R, et al. (1999) Vascular endothelial growth factor (VEGF) and its receptors in tumor-bearing dogs. Biol Chem 380: 1449–1454.

45. Viti F, Nilsson F, Demartis S, Huber A, Neri D (2000) Design and use of phage display libraries for the selection of antibodies and enzymes. Methods Enzymol 326: 480–505.

46. Brack SS, Shalchi M, Birchler M, Neri D (2006) Tumor-targeting properties of novel antibodies specific to the large isoform of tenascin-C. Clin Cancer Res 12: 3200–3208.