Chapter 3

Targeted vaccination against the bevacizumab binding site on VEGF using 3D-structured peptides elicits efficient anti-tumor activity

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ABSTRACT

Therapeutic targeting of the VEGF signaling axis by the VEGF neutralizing monoclonal antibody bevacizumab, has clearly demonstrated clinical benefit in cancer patients. To improve this strategy using a polyclonal approach, we developed a vaccine targeting VEGF using 3D-structured peptides that mimic the bevacizumab binding site. An in-depth study on peptide optimization showed that the antigens 3D-structure is essential to achieve neutralizing antibody responses. Peptide 1 adopts a clear secondary, native-like, structure including the typical cysteine-knot fold, as evidenced by CD spectroscopy. Binding and competition studies with bevacizumab in ELISA and SPR analysis revealed that peptide 1 represents the complete bevacizumab binding site, including the hairpin-loop (β5-turn-β6) and the structure-supporting β2-α2-β3 loop. Vaccination with peptide 1 elicited high titers of cross-reactive antibodies to VEGF, with potent neutralizing activity. Moreover, vaccination-induced antisera displayed strong angiostatic and tumor-growth inhibiting properties in a preclinical mouse model for colorectal carcinoma, whereas antibodies raised with peptides exclusively encompassing the β5-turn-β6 loop (peptides 15 and 20) did not. Immunization with peptide 1 or 7 (murine-analogue of 1) in combination with the potent adjuvant RFASE showed significant inhibition of tumor growth in the B16F10 murine melanoma model. Based on these data, we conclude that this novel vaccination technology, which is currently investigated in a phase I clinical trial (NCT02237638), can potentially outperform currently applied anti-VEGF therapeutics.
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INTRODUCTION

Vascular endothelial growth factor (VEGF) is frequently investigated as a target in anti-cancer therapy (1-3). VEGF contains a ‘cysteine-knot’ motif, which is crucial for proper folding and biological activity (4), and VEGFR2 is the predominant mediator of its pro-angiogenic effects (3). In combination with chemotherapy, treatment with the monoclonal anti-VEGF antibody bevacizumab has shown clinical benefit in a number of different tumor types (5-7). There is growing evidence that long-term treatment with bevacizumab can be beneficial (8,9), even beyond disease progression while on bevacizumab containing therapy. Generating anti-VEGF antibodies through active immunization could offer important advantages. Application would i) only need few intramuscular injections, ii) induce antibodies with superior VEGF neutralizing ability compared to bevacizumab, iii) provide durable VEGF suppression and reduce the number of hospital visits, and iv) be more cost-effective, overcoming the socio-economic problems of prohibitively high treatment costs (10). Over the past decades, the interplay of the immune system and angiogenesis was further clarified (11). Angiogenic growth factors contribute to immune suppression by downregulation of endothelial adhesion molecules (12-14), inhibition of dendritic cell maturation (15) and attraction and proliferation of immunosuppressive cells (16,17). Conversely, anti-angiogenic drugs can help reverse the immunosuppressive state in cancer patients (18-20). Therefore, an angiostatic vaccination approach against VEGF can potentially inhibit tumor growth via multiple mechanisms (21).

Preclinical evidence for the antitumor activity of VEGF-vaccination strategies was provided previously (22,23), showing the safety of this approach. Recently, phase I clinical data with a VEGF-vaccine (24) in patients with advanced cancer was reported. This treatment appeared to be safe and common anti-VEGF related adverse events were absent. However, vaccination with intact VEGF has major drawbacks such as unwanted biological activity and weak immunogenicity, requiring chemical inactivation and/or protein-conjugation (22). Using modified antigens, however, may give rise to antibodies lacking neutralizing properties. Alternative strategies using (h)VEGF-derived peptides (25) appeared moderately successful (26,27). We show that induction of neutralizing antibodies with tumor-growth-inhibiting power was only successful for a 3D-structured 79-mer peptide 1 with a fully intact cysteine-knot fold that covers the complete discontinuous binding site of bevacizumab. Crucial to its activity is that peptide 1 adopts a native-like VEGF structure in solution and that it binds to bevacizumab with near equal affinity as hVEGF165. Eradication of tumor growth using peptide 1 was demonstrated in two different tumor models.
MATERIALS AND METHODS

Cell lines and reagents.
The Ba/F3-VEGFR2 cells were a kind gift from K. Alitalo (Helsinki, Finland) and were licensed by the Ludwig Institute for Cancer Research, New York, NY. Ba/F3 cells are transfected with a receptor consisting of the extracellular domain of VEGFR2 and the transmembrane and cytoplasmic domain of mouse erythropoietin receptor (mEpoR)(45,46), making them dependent on VEGF or mIL3 for proliferation and survival. Ba/F3-VEGFR2 cells were maintained as suspension cultures in complete medium (DMEM (Lonza, Verviers, Belgium) supplemented with 10% heat-inactivated (HI) fetal bovine serum (FBS)(BioWest SAS, Nuaillé, France), Pen-Strep (Lonza, Verviers, Belgium), L-glutamin (2 mM; Scharlab S.L., Barcelona, Spain), mIL3 (4 ng/mL; R&D systems, Minneapolis, MN), zeocin (500 µg/mL; Invitrogen, Carlsbad, CA)) in an atmosphere of 5 % CO₂ at 37 °C. The murine melanoma cell line B16F10 and the human colon cancer cell line LS174T were purchased from the American Type Culture Collection (ATCC; Rockville, MD) and were cultured in DMEM supplemented with 10% HI FBS, Pen-Strep and L-glutamin.

Ba/F3-VEGFR2 bioassay.
Complete medium (see above) containing hVEGF (1.25 ng/mL; R&D systems, Minneapolis, MN) was pre-incubated with serum containing anti-VEGF antibodies and plated in a 96-wells plate for one hour at 37°C. Ba/F3-VEGFR2 cells were added at a concentration of 2*10⁵ cells/mL and incubated for 72 hours. Cell proliferation was quantified with WST-1 (Roche Applied Science, Mannheim, Germany). Optical Density (OD) was measured with a TecanSpectrafluor plate reader at a wavelength of 450 nm and a reference wavelength of 600 nm. The proliferation of cells on serum from vaccinated animals was calculated as percentage of cell proliferation when cultured with hVEGF alone.

Vaccine preparation.
The synthesis of all VEGF-derived peptides, BIACORE affinity measurements as well as detailed description on vaccine preparation are described in the SI Appendix, section 3.2. Briefly, the peptides were mixed with the adjuvants Raffinose Fatty Acid Ester (RFASE), Sucrose Fatty Acid Ester (SFASE)(47,48) or CFA/IFA.

Immunization studies.
Animal experiments were approved by the local ethical review committee. Detailed procedures for these studies are described in the SI Appendix, section 3.5. In summary, studies 1 and 2 were passive immunization studies in which LS174T colon cancer bearing immunodeficient Swiss nu/nu mice were treated with antisera recognizing hVEGF₁₆₅ that
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were generated in Wistar rats. For study 1, rats were immunized with the 3D-structured peptide 1, while for study 2 rats were immunized with either the linear or cyclic peptides 9-13 derived of the β5-turn-β6 region of hVEGF165 (aa’s 69-103). Please refer to Fig. 4A for the study design of both experiments. In study 3, C57BL/6 mice were prophylactically immunized with 175 μL peptide 8/RFASE (group-3), peptide 7/RFASE (group-4) or peptide 1/RFASE (group-5). Control mice received either RFASE (group-2) or PBS (group-1) alone. Ten days after the last immunization the mice were challenged with 5*10^4 B16F10 murine melanoma cells. The tumors were allowed to grow for 21 days (Fig. 5A).

**Histological analysis in xenograft tumors.**

CD31 was detected by immunohistochemical (IHC) staining on solid LS174 T tumors. Five μm sections were stained for CD31 with hematoxylin as counterstain. Tissues were fixed in acetone for 15 minutes at -20°C. Endogenous peroxidase was blocked with 3% H_2O_2 and aspecific binding was prohibited by treatment with 5% BSA. Primary and secondary antibodies used were rat-anti-mouse CD31 (BD Biosciences Pharmingen, Franklin Lakes, NJ; 1:100 dilution) and donkey-anti-rat IgG (Jackson, Westgrove, PA; 1:300 dilution). Hereafter, the tissues were incubated with streptavidin-HRP (Dako, Carpinteria, CA; 1:300 dilution). HRP was detected by the addition of DAB (Envision kit; Dako, Carpinteria, CA; 1:50 dilution). Intratumoral microvessel density (MVD) was calculated by taking the mean of vessel counts in 10 random fields at 400x magnification.

**Statistical analysis.**

The repeated-measured two-way ANOVA with Bonferroni post test was used for repeated measurements at different time points (Fig. 4B, Fig. 5D). For tumor weight comparisons (Fig. 4C) and MVD in tumor sections (Fig. 4D) the one-way ANOVA with Dunnett’s post test was used. P values < 0.05 were considered significant. All analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, California, USA).

**RESULTS**

**Design and synthesis of VEGF peptide mimics.**

As virtually all antibody binding sites are discontinuous in nature (28), a total of 33 peptide mimics of VEGF with varying levels of structural complexity (linear, conformational, and discontinuous) were designed, synthesized and tested for bevacizumab binding and the ability to generate potent antisera with cross-reactivity to and neutralizing ability for hVEGF165 (Fig. 1). The 79-mer peptide 1 (ox-hVEGF26-104, MW=9.1 kDa, ~25% of hVEGF165), starting at C_1 and ending at C_v1 of the cysteine-knot fold (SI Appendix, Table S1), mimics
the bevacizumab binding site in its most native form (Fig. 1A). It contains both the β5-turn-β6 hairpin-loop as well as the β2-α2-β3 loop, which stabilizes and positions the β5-turn-β6 loop for bevacizumab binding (29,30)(Fig. 1B). Cysteines C51 and C60, which covalently link the subunits in hVEGF_{165} via two disulfide bonds (31), were replaced by alanines to block homodimerization and thus prevent agonistic VEGF-like activity (Fig. 1B-C). In addition, the rat- (6) and mouse-variant (7) were synthesized (SI Appendix, Table S1).

FIGURE 1. Graphical representation of all constructed VEGF-mimics. (A) Ball-and-stick and schematic representation of hVEGF_{165}. (B) Ball-and-stick representation of the binding of bevacizumab to VEGF. The picture illustrates that the antibody-binding β5-turn-β6 loop (in light blue) is structurally supported by the β2-α2-β3-loop (in dark blue). (C) Ball-and-stick and schematic representation of folded peptide 1 (including the intact cysteine-knot fold in blue). Cysteines (and mutated Alanines), that form the two disulfide bonds covalently linking the individual subunits in the homodimer, are indicated in green. In its folded state, this peptide provides the smallest possible mimic of the bevacizumab binding site on hVEGF_{165}. (D) Schematic representation of linear and xylene-bridged peptides 9-33.
Folding of peptides 1, 6 and 7 produced the intact cysteine-knot fold in less than two hours (SI Appendix, Fig. S1A), which is considerably shorter than folding of native hVEGF165, which takes ~5 days (32). Both MALDI-TOF MS (SI Appendix, Fig. S1B) and ESI-MS (SI Appendix, Fig. S2 and Table S2) confirmed formation of the cysteine-knot fold as judged from the 6 Da reduction in MW for folded peptide 1 (MW$_{exp}$ = 9060.6) as compared to unfolded peptide 1 (MW$_{exp}$ = 9066.6, SI Appendix, Fig. S1B).

To evaluate the relevance of structural folding for bevacizumab binding and the ability to generate neutralizing anti-VEGF antibodies, we synthesized three SS-deletion variants of peptide 1, each lacking one of three SS-bonds in the native cysteine-knot fold (SI Appendix, Table S1). In addition, a variant lacking all three cysteine-knot SS-bonds (peptide 5) was prepared via reaction of reduced peptide 1 with excess of iodoacetamide (SI Appendix, Table S1).

The bevacizumab epitope is reported to be linear or conformational and located at the top of the β5-turn-β6 loop (a.a. 85-92)(30). Accordingly, we included five linear (9-13) peptides and twenty different side-chain cyclized peptides (14-33) covering this region (Fig. 1D and SI Appendix, Table S3). For each cyclic peptide an adjacent pair of native amino acids was replaced by a pair of cysteines that were subsequently connected using a meta-xylene bridge (33, 34) (SI Appendix, Table S3).

**In-vitro activities of VEGF mimics 1-33.**

Binding of folded peptide 1 to bevacizumab was studied in detail. Bevacizumab binds surface-immobilized 1 even at low concentrations (pEC$_{50}$ = 4.5; Fig. 2A) and with comparable intensity as for hVEGF$_{165}$ (pEC$_{50}$ = 4.8). Competition studies showed that peptide 1 and hVEGF$_{165}$ inhibit the binding of bevacizumab to surface-immobilized hVEGF$_{165}$ with almost similar strength (IC$_{50}$ = 19.4 nM for peptide 1 and 6.2 nM for hVEGF$_{165}$; Fig. 2B). Bevacizumab binding was fully specific for the human VEGF-sequence (i.e. peptide 1), as binding was neither observed with rat- (6) nor the mouse (7) variants (Fig. 2A and SI Appendix, Fig. S3B). The 1:1-affinity constant (K$_D$) for the binding of bevacizumab to peptide 1 was 1.01 ± 1.09 nM using BIACORE affinity measurements (SI Appendix, Fig. S4), which compares well with the reported K$_D$-value for bevacizumab and hVEGF$_{165}$ of 2.2 nM (35).
FIGURE 2. hVEGF₁₆₅ and peptide 1 have similar binding properties whereas the activity of SS-deletion variants is reduced. (A) Peptide 1 strongly binds bevacizumab, peptide 4 has intermediate bevacizumab binding capacities whereas peptides 2, 3, 6 and 7 have virtually no binding capacities with bevacizumab. (B) hVEGF₁₆₅ and peptide 1 are equally well able to inhibit the binding of bevacizumab to plate bound hVEGF₁₆₅ in a competition ELISA. (C) Peptides 1 and 4 are able to inhibit the binding of bevacizumab to hVEGF₁₆₅ whereas peptides 2 and 3 are not able to inhibit the binding of bevacizumab to hVEGF₁₆₅ in a competition ELISA. (D) Peptide 1 does not stimulate cell proliferation of VEGF dependent Ba/F3-VEGFR2 cells nor does it affect the Ba/F3-VEGFR2 cell proliferation promoting effects of hVEGF₁₆₅.
In order to clarify the importance of an intact cysteine-knot fold for bevacizumab binding, and in particular the contribution of each individual SS-bond, we also studied the binding to the three SS-deletion variants (2-4) as well as to the fully unfolded peptide 5 (and unstructured) peptides 9-33. Peptide 4 bound with a ~16-fold lower affinity to bevacizumab (pEC$_{50}$ = 3.3; Fig. 2A and SI Appendix, Fig. S3A), but was virtually as active as peptide 1 at inhibiting the binding of bevacizumab to hVEGF$_{165}$ (IC$_{50}$ = 16 nM for peptide 4)(Fig. 2C), while for variants 2 and 3 binding was almost completely lost (pEC$_{50}$ = 2.0 and <2, and IC$_{50}$ = 432 nM and >10 µM for 2 and 3 respectively, Fig. 2B-C and SI Appendix, Fig. S3A). Neither the linear peptides 9-13, nor the unstructured peptide 5 showed measurable binding up to 10 µg/mL (SI Appendix, Fig. S3A). However, the xylene-bridged peptides, 14-17 (C$_{76}$-C$_{96}$) and 19-22 (C$_{78}$-C$_{94}$) also showed prominent binding to bevacizumab (pEC$_{50}$ up to 3.3), thus confirming the conformational nature of the binding site.

We also studied the ability of peptide 1 to either agonize or antagonize VEGF-dependent cell proliferation of Ba/F3 cells, engineered to express VEGFR2. No detectable increase in cell viability was observed in Ba/F3-VEGFR2 cells when incubated with peptide 1 (Fig. 2D). Similarly, peptide 1 did also not markedly reduce cell proliferation when cultured with 1.25 ng/mL hVEGF$_{165}$ (Fig. 2D). These data show that peptide 1 is fully devoid of agonistic or antagonistic activity towards hVEGF$_{165}$ or VEGFR2.

**Immunization studies with VEGF-mimics 1-33.**

The immunogenicity of peptide 1 was studied in different species. Adjuvants used included the conventional Complete/Incomplete Freund’s Adjuvant (CFA/IFA) and the novel adjuvant Sucrose Fatty Acid Sulphate Ester (SFASE) (SI Appendix, section 3.2). In Wistar rats immunized with peptide 1 we detected high antibody titers against peptide 1 nine weeks after primer immunization (antibody titer of 5.3, SI Appendix, Table S6), as well as against hVEGF$_{165}$ (antibody titers of 5.2 to >5.4, Fig. 3A and SI Appendix, Tables S5 and S6). Immunization studies in Balb/C mice showed similar results (SI Appendix, Table S5). Moreover, antisera induced in Wistar rats were found to block the binding of bevacizumab to surface-immobilized hVEGF$_{165}$ in a competition ELISA (Fig. 3B) and showed near complete inhibition (>95% compared to hVEGF 1.2 ng/mL) of VEGF-dependent cell proliferation in the Ba/F3-VEGFR2 cell-based bioassay (Fig. 3C, SI Appendix, Fig. S8). Although peptide 1 adjuvated with CFA/IFA or SFASE induced similar antibody titers against hVEGF$_{165}$, SFASE antisera seemed to outperform CFA/IFA antisera in VEGF neutralizing abilities, given the superior results in the competition ELISA as well as the Ba/F3-VEGFR2 cell proliferation assay (Fig. 3B-C).
Immunization with linear and cyclized peptides covering the β5-turn-β6 loop (peptides 9-33) induced high titer antibodies cross-reactive with hVEGF₁₆₅ as well (mean antibody titer ± SD; 4.6 ± 0.7; SI Appendix, Fig. S7). However, antisera induced by immunization with peptides 15 and 20 in a larger experiment were not able to neutralize the biological activity of VEGF in the Ba/F3-VEGFR2 cell proliferation assay (mean cell proliferation compared to hVEGF₁₆₅ 1.25 ng/mL using non-purified sera ± SD; 90.1 % ± 13.9 %; SI Appendix, Table S8). In addition, immunization with SS-deletion variant peptides 2-4 also induced high anti-VEGF antibody titers (mean antibody titer ± SD; 4.6 ± 0.2; SI Appendix, Table S7), while VEGF driven proliferation of Ba/F3-VEGFR2 cells remained unaffected when treated with these sera (mean cell proliferation compared to hVEGF₁₆₅ 1.25 ng/mL ± SD; 104.2 % ± 14.3 %; SI Appendix, Table S7), indicating that antibodies induced with these deletion variants were also devoid of hVEGF₁₆₅ neutralizing properties.
Anti-angiogenic and anti-tumor activity by vaccination against VEGF.

Passive immunization with VEGF based peptides in LS174T human colon cancer xenografts.

The most neutralizing sera in Wistar rats (blue bars in Fig. 3C) were pooled, the IgG fraction purified and used to treat LS174T bearing immunodeficient mice. A very clear and marked inhibition of tumor-growth was observed after treatment with anti-peptide 1 (group-3) or bevacizumab (group-2), as compared to PBS-treated mice (group-1; *p*<0.001, Fig. 4B-C). Both treatments were well tolerated, as evaluated by survival of the animals, overall behavior and body weight (SI Appendix, Fig. S9). As shown in Fig. 4D, the microvessel density in anti-peptide 1 and bevacizumab treated tumors was markedly reduced (*p*<0.001), suggesting that the antitumor effects are due to angiogenesis inhibition.

In parallel, the effects of antisera elicited with β5-turn-β6 loop peptides (15 and 20) immunization were studied in the same tumor model. LS174 T tumor growth again showed to be very sensitive to anti-VEGF treatment, given the clear antitumor effects of bevacizumab treatment (*p*<0.001; SI Appendix, Fig. S10). In contrast, tumor growth of anti-peptides 15 and 20 antisera treated mice was unaffected compared to PBS treated mice (SI Appendix, Fig. S10), showing that these antisera, which were unable to neutralize VEGF *in-vitro* indeed do not exhibit tumor growth inhibition properties.

Active immunization inhibits tumor growth in the B16F10 murine melanoma model.

In a final experiment, we investigated if vaccination in a syngeneic tumor model with peptides 1 (group 5), 7 (mouse-variant of 1; group 4) and 8 (unstructured variant of 7; group 3) in mice could inhibit tumor growth. For this study, RFASE adjuvant was used instead of SFASE adjuvant because of its more optimal properties for clinical development (SI Appendix, section 3.2). Mice treated with PBS (group 1) or RFASE alone (group 2) served as controls. C57BL/6 mice received four intramuscular injections with these peptides adjuvated with Raffinose Fatty Acid Sulphate Ester (RFASE) with 2 week intervals (Fig. 5A and SI Appendix, section 3.5). One mouse in group 5 died shortly after the 3rd immunization. Moreover, 50% of the mice of groups-4 and -5 displayed hunched posture, lethargy and pilo-erection immediately after the 3rd immunization. Moreover, these symptoms were observed in all animals of groups 3-5 right after the 4th immunization and also increased in severity. In total, 11 mice (six in group 5, four in group 4 and one in group 3) died after the final booster. All remaining mice recovered within one hour after immunization. Body weights were unaffected in all mice throughout the study (SI Appendix, Fig. S11). Furthermore, other toxicities were not observed in any of the immunized mice.
FIGURE 4. Pooled rat peptide 1 antisera have potent tumor inhibiting and anti-angiogenic properties. (A) Design of the study. (B and C) Treatment with pooled antiserum as well as bevacizumab inhibited the growth of LS174T human colon carcinoma compared to PBS treated tumors, as quantified by tumor volume and tumor weight. Data are expressed as mean ± SEM (n = 9-10 per group). (D) Intratumoral MVD in pooled antiserum and bevacizumab treated tumors was reduced compared to PBS treated tumors. Data are expressed as mean (± SEM for B) (n = 5-10 per group). # is animals out of study.
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FIGURE 5. Active immunizations with peptides 1 and 7 inhibit the growth of B16F10 melanoma. (A) Design of the study. (B) Immunization with peptide 8/RFASE, peptide 7/RFASE and peptide 1/RFASE induces cross-reactive antibodies against hVEGF₁₆₅ (day 74). (C) Immunization with peptide 7/RFASE and peptide 1/RFASE induces an increase in serum mouse VEGF concentration. (D) Immunization with peptide 7/RFASE and peptide 1/RFASE significantly inhibits the growth of B16F10 melanoma whereas peptide 8/RFASE immunized mice developed tumors not statistically significant smaller than PBS treated mice. Data are shown as mean (± SEM for D) (n = 3-10 per group).
Ten days after the 4<sup>th</sup> immunization mice were inoculated with 10<sup>5</sup> B16F10 murine melanoma cells (Fig. 5A). Cross-reactive antibodies against mVEGF<sub>164</sub> were found in all mice of groups 3-5 (Fig. 5B). Counter-intuitively, serum mouse VEGF concentrations were markedly increased in mice of groups 4 and 5 (Fig. 5C). On day 21, tumor growth of mice immunized with peptides 1 or 7 (groups 4 and 5), but not those immunized with unstructured peptide 8, was significantly inhibited, as compared to PBS treated mice (p<0.001, Fig. 5D).

**DISCUSSION**

Treating advanced cancer using anti-angiogenesis therapy (bevacizumab, VEGF-trap) combined with chemotherapy is known to prolong survival in several tumor types (5-7). However, the clinical benefit is fairly modest, urging researchers to discover improved treatment strategies. (Pre-) clinical studies have shown that continuous and prolonged exposure as opposed to discontinuous VEGF suppression provides maximum benefit (8, 36). The ideal way to achieve this is by targeting vascular epitopes via active immunization (10).

In this paper, we describe data from both immunization and tumor-suppression studies using the 79-mer peptide 1 (ox-hVEGF<sub>26-104</sub>) that reconstitutes the conformational and discontinuous binding site of bevacizumab on hVEGF<sub>165</sub>. We provide evidence that enforcing a native-like, secondary structure in 1 is the key to success for inducing neutralizing anti-hVEGF antibodies with tumor-inhibiting power. According to X-ray data, the β2/α2-loop residues E<sub>42</sub>/E<sub>44</sub>/Y<sub>45</sub>/F<sub>47</sub> in 1 have a structure-stabilizing interaction with the β5-turn-β6 loop residues R<sub>82</sub>/K<sub>84</sub> (Fig. 1C), which correctly positions this loop for bevacizumab binding (30). We anticipated that the presence of this structure-stabilizing loop is critical for generating a potent and neutralizing antibody response.

Peptide 1 is strongly immunogenic and does not require conjugation to a carrier protein, despite the fact that it is derived from an endogenous protein. Truncation of native hVEGF<sub>165</sub> to 79 amino acids (hVEGF<sub>26-104</sub>) as well as substitution of C<sub>51</sub> and C<sub>60</sub> for alanines likely gives rise to neo-epitopes that increase its immunogenicity (37). Immunizations with peptide 1 raised potent anti-VEGF antisera with strong neutralizing activity in VEGF-dependent Ba/F3-VEGFR2 bioassay (Fig. 3C and SI Appendix, Fig. S8) and the ability to inhibit bevacizumab-binding to hVEGF<sub>165</sub> in ELISA (Fig. 3B). Peptide 1 is monomeric in nature, unlike the homodimeric VEGF<sub>165</sub> protein, and therefore neither agonizes nor antagonizes VEGF-dependent proliferation in Ba/F3-VEGFR2 cells (Fig. 2D). This makes peptide 1 the perfect immunogen for development of an anti-VEGF vaccine. Peptide 1 can be administered safely in vivo without the risk of interfering with endogenous VEGF-signaling. Interestingly, antisera
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raised against peptide 1 showed significantly weaker binding to unstructured peptides 2, 3, and 5 in ELISA (SI Appendix, Table S7), indicating that anti-peptide 1 antibodies mainly recognize conformational and/or discontinuous epitopes.

The type of adjuvant used also appears to influence the neutralizing capacity of the resulting antisera to an appreciable extent. Immunization with the ‘oil-in-water emulsion’ SFASE adjuvant induced neutralizing antisera in 9/10 animals (animals 29-38, Fig. 3C), while appreciable VEGF neutralizing ability was only observed in 4/10 antisera using the ‘water-in-oil emulsion’ adjuvant CFA/IFA (animals 19-28, Fig. 3C). This marked difference might be due to the oily component of CFA/IFA causing partial or complete unfolding of peptide 1, thus generating antibodies that no longer recognize nor neutralize endogenous VEGF (see below).

Passive immunization of Swiss nu/nu mice with a cocktail of the 13 most potent antisera raised against peptide 1 completely inhibited the growth of LS174T colon carcinoma, to the same extent as bevacizumab treatment (Fig. 4B-D). Additionally, active immunization with the 3D-folded peptides 1 (human variant) and 7 (mouse-variant) significantly inhibited the growth of B16F10 melanoma in C57BL/6 mice (Fig. 5D), while the analogous unstructured peptide 8 was totally ineffective. These data emphasize that only correctly folded peptides are able to induce antibodies that neutralize VEGF and thus able to inhibit tumor angiogenesis. Further, this experiment confirms that truncation is sufficient to break immune self-tolerance, since immunization with the mouse homologue peptide 7 induced antibodies with similar anti-tumor properties as peptide 1. Much to our surprise, an increase instead of a significant decrease in serum mVEGF-levels was observed for peptide 1 and 7 immunized mice. This is most likely due to reduced clearance of the antibody mVEGF immune complexes formed, which are known to clear slower than free mVEGF (38). Again, the fact that serum mVEGF levels were unaffected in peptide 8 treated mice suggests that the antibodies induced in this group are unable to recognize endogenous mVEGF. Since we did observe a significant reduction in tumor growth for peptide 1 and 7 immunized mice, it appears that these immune complexes do not interfere with tumor growth inhibition and apparently have no biological function. A positive correlation between mVEGF-levels and anti-mVEGF antibody titers was only observed for peptide 7 immunized mice (SI Appendix, Fig. S12B), while this was not the case in peptides 1 and 8 (SI Appendix, Fig. S12A and S12C). Even though our conclusions for peptide 1 might be somewhat premature as a result of the low number of animals in group 5, the lack of correlation for peptide 8 immunized mice clearly stresses the inability of peptide-8 induced antibodies to bind mVEGF.

Even though repeated immunization with peptide 1/RFASE did not lead to overt toxicity in Wistar rats, several C57Bl/6 mice died shortly after the 3rd or 4th booster immunizations. We
hypothesize that this was caused by an antigen-specific IgE or IgG mediated anaphylactic response, which only occurred in mice due to the relatively high doses of antigen given (~10 µg/g for mice versus <1 µg/g body weight for all other animals). Interestingly, mice immunized with RFASE adjuvant alone did not develop similar symptoms, excluding the possibility that the adjuvant was responsible for the observed adverse effects.

We did not specifically address the duration of the immune response, but various reports evidently show that immune responses raised against endogenous antigens are fully reversible (39-43). Long-term data with a peptide-based GnRH-targeting cancer vaccine for testosterone deprivation, also developed by us (44), showed that testosterone levels reappeared approximately 30 weeks later (44), showing that anti-GnRH immunity is indeed reversible. We therefore anticipate that duration of the peptide 1/RFASE immune response will be comparable, which require regular re-vaccination in order to maintain high antibody titers. Importantly, it is highly unlikely that endogenous VEGF will serve as booster after immunization with peptide 1/RFASE, because i) exposure to endogenous VEGF occurs in the absence of RFASE adjuvant and ii) it is improbable that CD4 T cell help for endogenous VEGF is generated as activation of CD4 T cells was only possible through truncation and modification of endogenous VEGF into the immunogen peptide 1.

It is not likely that our vaccination strategy results in cellular immunity. First, the vaccine is administered through intramuscular injections, a route more likely to skew towards humoral immunity. Second, long peptides as immunogens are less prone to induce cellular immunity as compared to DNA vaccines; exogenous peptides require cross-presentation to be able to be presented by MHC I to induce cellular immunity, whereas they can be directly presented in the context of MHC II to CD4 helper T cells.

Several studies have claimed success with vaccines based on peptides derived from either hVEGF165 or VEGFR2 (22,25,27). Most of these involve the use of linear or unstructured peptides. However, all our efforts to obtain antisera with notable neutralizing capacity in the Ba/F3-VEGFR2 cell assay (SI Appendix, Table S8) and/or tumor-inhibiting power (SI Appendix, Fig. S10) using constrained β5-turn-β6 loop peptides 15 and 20 failed. This clearly disqualifies the hypothesis that the binding site of bevacizumab is linear or conformational (30) and supports our view that a structurally complex peptide like 1 is indeed required to reconstitute the bevacizumab binding site entirely. Immunization with peptides 2-5, lacking either one (2-4) or all (5) SS-bonds of the structure-supporting cysteine-knot fold in peptide 1, only raised antisera completely devoid of neutralizing activity for hVEGF (SI Appendix, Table S7).
There is a lack of effective methods classifying good or poor peptide immunogens for hVEGF\textsubscript{165}. We learnt that measuring hVEGF\textsubscript{165} cross-reactivity of peptide antisera in ELISA is clearly not a good indicator, as antisera elicited by mis- or unfolded peptides, e.g. 5 and 14-18, also harbored antibodies binding extremely strong to hVEGF\textsubscript{165} in ELISA (titers 5.4 or higher; SI Appendix, Table S7 and Fig. S7). This is likely due to (partial) denaturation of hVEGF\textsubscript{165} upon surface-immobilization to the ELISA-plate. Instead, we discovered that measuring i) the binding of bevacizumab to the peptides, or ii) the ability of the peptides to inhibit binding of bevacizumab to hVEGF\textsubscript{165}, provides a much better predictor for success. As expected, peptide 1 was the minimal peptide to show high-affinity binding to bevacizumab with SPR and in ELISA and showed strong competition of bevacizumab-binding to surface-immobilized hVEGF\textsubscript{165}, whereas unstructured peptides like 2, 3, 5 and 14-33 showed much weaker or no binding to bevacizumab in ELISA (SI Appendix, Fig. S3A and S3C), nor did they inhibit binding of bevacizumab to hVEGF\textsubscript{165}.

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*Supplementary material is available at https://doi.org/10.1073/pnas.1610258113*
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