



Review

Vaccination approach to anti-angiogenic treatment of cancer



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ABSTRACT

Improvement of patient survival by anti-angiogenic therapy has proven limited. A vaccination approach inducing an immune response against the tumor vasculature combines the benefits of immunotherapy and anti-angiogenesis, and may overcome the limitations of current anti-angiogenic drugs. Strategies to use whole endothelial cell vaccines and DNA- or protein vaccines against key players in the VEGF signaling axis, as well as specific markers of tumor endothelial cells, have been tested in preclinical studies. Current clinical trials are now testing the promise of this specific anti-cancer vaccination approach. This review will highlight the state-of-the-art in this exciting field of cancer research.

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1. Introduction

Angiogenesis is intricately regulated by a balance of many different endogenous activators and inhibitors. While a plethora of pro-angiogenic growth factors has been described, among which vascular endothelial cell growth factors (VEGFs) and fibroblast growth factors (FGFs) are the most prominent, a growing family of anti-angiogenic molecules is also emerging [9,39]. Among the latter are molecules such as certain alpha-chemokines [124], interferon-alpha [127], tissue inhibitors of metalloproteinases [113], bactericidal permeability increasing protein [123], and breakdown products of angiogenic factors such as endostatin [88], tumstatin [43], and the 16k-fragment of prolactin [114]. Since new blood vessel formation is a critical step in the progression of cancer as well as in metastasis formation and outgrowth, it has been realized that inhibition of angiogenesis may be a tool to control cancer. The field of angiogenesis research has expanded explosively since its nativity, mainly after (pre)clinical validation of the concept in the early 1990s. The impact of the field increased with the understanding that also other angiogenic diseases may benefit, such as rheumatoid arthritis [119], inflammatory bowel disease [107] and age-related macular degeneration [104].

Inhibition of angiogenesis as a therapeutic approach was mostly developed in the oncological arena. Over the last decade, this research has led to the FDA approval of several angiogenesis inhibitors, the first one being bevacizumab (Avastin) in 2004. In combination with chemotherapy this drug has shown a clinical benefit for several indications, including metastatic colorectal cancer (mCRC) [52], metastatic renal cell cancer (mRCC) [21] and metastatic non-small cell lung cancer (mNSCLC) [106]. Several other targeted compounds with anti-angiogenic effects received FDA approval in the anti-cancer field in the years thereafter. Among these are aflibercept; a fusion protein binding VEGF; and the small-molecule tyrosine kinase inhibitors (TKI) sunitinib, sorafenib and pazopanib; which among other kinases bind VEGF receptors. Other anti-angiogenic compounds target mTOR. These compounds, e.g., everolimus, have a rather indirect activity and due to their blockade of key signal transduction molecules, may have a broader effect. The mTOR inhibitor everolimus exerts its anti-angiogenic effects by decreasing the levels of hypoxia inducible factor (HIF) and thereby the production of angiogenic growth factors by tumor cells. Moreover mTOR inhibition blocks the growth and proliferation of vascular endothelial cells [41]. However, although proof for the concept of tumoristatic activity of angiogenesis inhibition has been provided, the benefit of these anti-angiogenic agents on the progression-free survival and overall survival of cancer patients are still rather modest. The reason for this limited activity has been suggested to be due to variation among patients as well as in tumors, resistance mechanisms [33], the induction of a more aggressive tumor phenotype [98] and dose-limiting toxicities necessitating discontinuous treatments. In addition, an apparent discrepancy between clinical and preclinical *in vivo* dependence on angiogenesis for tumor growth and metastasis formation, makes translation of promising strategies to the clinic challenging [19].

It has been hypothesized that the limited success of anti-angiogenic treatment may also be due to the generally followed strategy of targeting tumor-derived growth factors and their receptors [35]. This strategy is likely to give growth advantage to mutated tumor cells that can rely on alternative growth factor pathways to attract blood vessels. Therefore, it has been suggested that a direct tumor endothelial cell targeting approach, in view of their genetic stability, should outperform most FDA approved drugs, and drugs currently in clinical testing. Consequently, genomic screening approaches to identify tumor endothelial cell markers are of key importance [121,4,110].

Over the last years it has become clear that tumor-infiltrating immune cells have important prognostic significance in cancer patients. This is in part regulated by angiogenic factors. Tumor infiltration with M2 macrophages [56], myeloid derived suppressor cells (MDSCs) [28], CD4+ T-helper2 (Th2) lymphocytes [12] and regulatory T cells (Treg)

[11] is generally associated with a poor prognosis, whereas patients with tumors that are infiltrated by CD8+ T lymphocytes [20] as well as CD45RO+ memory T cells and Th1 lymphocytes [96,120], usually have a superior clinical outcome. Angiogenic growth factors that also contribute to the immunosuppressive tumor environment include VEGF, placental growth factor (PlGF) and transforming growth factor beta (TGF- β) [83,84]. This was reported in a series of papers in the mid-1990s showing that VEGF, secreted by tumor cells is able to inhibit the functional maturation of dendritic cells (DCs) [30]. These studies show that VEGF overexpression (i) impairs the antigen presenting cell (APC) function of DCs [13], (ii) can induce DCs to undergo apoptosis [116], (iii) inhibits effector T-cell development [89], (iv) increases the number of regulatory T cells in the tumor microenvironment [66], and (v) promotes the formation of tumor promoting MDSCs [29]. Also an indirect, endothelial cell mediated, immunosuppressive activity of pro-angiogenic factors was described, based on their suppression of endothelial adhesion molecules and subsequent suppression of leukocyte infiltration [37,38,78].

This immune suppressive activity of angiogenic growth factors urged researchers to investigate a presumed inflammatory activity of anti-angiogenic compounds. Indeed, there are indications that anti-angiogenic drugs are able to help reverse the immunosuppressive tumor microenvironment. Although the described effects of bevacizumab treatment on DC maturation are inconsistent [95,26,1,31], it seems that bevacizumab as well as sunitinib treatment reduces the number of immature myeloid cells and consequently MDSCs that can arise from them [95,26,61]. Furthermore, there is proof for the fact that treatment with low dose anti-angiogenic drugs polarizes tumor associated macrophages (TAMs) with an M2-skewed phenotype into an immunosupportive M1-like phenotype [50]. Other proposed mechanisms by which anti-angiogenic drugs can promote an immunosupportive tumor microenvironment include the increase of cell adhesion molecules (CAM), which promote leukocyte-endothelium interactions [15,16,14] and the reduction of regulatory T cell numbers [66].

Recent work demonstrated that a high baseline VEGF concentration correlates with poor outcome in metastatic melanoma patients treated with the immune checkpoint inhibitor ipilimumab [137]. Moreover treatment with ipilimumab or the GVAX vaccine can induce antibody responses against different pro-angiogenic players, including VEGF and angiopoietin 1/2 [108]. This suggests that combining anti-angiogenic drugs with immunotherapy is worthwhile to investigate. Pre-clinical studies have shown enhanced effects of immunotherapy when combined with anti-angiogenic drugs [50,112]. Based on these results clinical trials have been initiated to investigate the potential synergy of this combination treatment. Recently the first clinical trial investigating the combination of ipilimumab and bevacizumab in patients with metastatic melanoma was published [46]. In one patient a complete response was achieved, eight patients had a partial response and 22 of the 46 treated patients showed stable disease. Immunohistochemical stainings showed enhanced immune cell infiltration after this combination therapy, as compared to ipilimumab monotherapy [46]. Whether the combination treatment is superior over ipilimumab monotherapy in terms of clinical outcome remains to be assessed in further studies.

The intricate relationship between the immune system and angiogenesis suggests a benefit of developing an immunotherapeutic strategy against angiogenesis. A vaccination approach against the tumor vasculature combines this benefit with enhanced selectivity against specific tumor endothelial markers. An additional advantage may be the possibility to circumvent the disadvantages of current anti-angiogenic compounds. To date, several studies have reported on the efficacy of this promising approach. This review will discuss these studies and will also highlight the results of recent clinical trials investigating this novel treatment strategy. We will end with an outlook on future directions to further this field.

2. Cancer vaccines

A major challenge in the development of cancer vaccines is the fact that one needs to overcome immune self-tolerance to induce a proper immune response towards a self-antigen. In addition, patients included in phase I clinical trials mostly have advanced cancer and have gone through multiple lines of treatment. These patients are likely to have a compromised immune system, resulting in failure of the vaccination. Next to these issues, mechanisms of immune escape and selection of efficient vaccine adjuvants suitable for clinical use remain major challenges. Recent research on the checkpoint inhibitors confirmed that immunomodulation can be used as an efficient anti-cancer strategy. This suggests that the success of an effective anti-cancer vaccine may depend on simultaneous immune checkpoint inhibition.

Over the last decade promising results on cancer vaccines and other immunotherapeutic modalities such as monoclonal antibodies have been achieved in clinical trials and the field is expanding rapidly. To date, there are two prophylactic cancer vaccines that have been clinically approved. Gardasil and Cervarix are vaccines targeting the human papilloma virus, which drives cervical carcinogenesis. In 2010 the first therapeutic cancer vaccine sipuleucel-T (Provenge) which targets prostatic acid phosphatase was approved for the treatment of asymptomatic or minimally metastatic hormone-refractory prostate cancer. The approval was mainly based on the results of the pivotal IMPACT trial, which showed an increase in median overall survival (OS) of 4.1 months in sipuleucel-T treated patients versus placebo treated patients [59].

Promising cancer vaccines that are currently in clinical development include G-VAX, a GM-CSF-transduced autologous or allogeneic tumor cell vaccine [122], DC vaccines and vaccines targeting tumor associated antigens, like an EGF cancer vaccine (NCT02187367) or the IMA901 vaccine (NCT01265901) which targets multiple antigens. However, disappointing results have plagued the field of therapeutic cancer vaccines as several vaccine trials failed to meet their endpoints in late stage development, like MAGE-A3 in NSCLC [125] and Tecemotide (MUC1 vaccine) [8].

3. Vaccination as novel anti-angiogenic treatment modality

The interplay between the process of angiogenesis and the immune system suggests that it might be beneficial to use immunity as an anti-angiogenic effector mechanism. An attractive approach in this regard would be the development of a vaccine against the major molecular angiogenic players or directly against molecular markers expressed in the tumor vasculature. As shown before, many anti-angiogenic drugs induce immune infiltration, help restore cancer immunosurveillance and are therefore pro-inflammatory compounds [46]. Inducing an immune response against pro-angiogenic factors has therefore the potential to cause a synergistic anti-tumor effect, by causing simultaneous angiostasis and immune potentiation. The results of several initiatives indeed seem to support this hypothesis. Earlier published reviews on this subject have already noted the potential of this treatment strategy [24,77,91,109]. Next to a high anti-tumor efficacy of such vaccines, there are many more advantages of a vaccination strategy over other anti-angiogenic therapies with monoclonal antibodies. First of all, it is expected that a polyclonal antibody response, as induced by vaccination, has a better antigen neutralizing capacity. This is due to targeting of multiple epitopes of the target molecule, as compared to monoclonal antibodies directed against a single epitope. Second, vaccines do not require to be frequently administered, as is the case with monoclonal antibodies and small molecule drugs. Third, vaccination provides an extremely cost effective strategy, which can be up to a thousand times less expensive than a monoclonal antibody therapy, due to the low amount of protein required for injection/therapy.

The anti-angiogenic and anti-tumor effect of anti-angiogenic vaccines can be achieved by a humoral immune response and/or

cellular immune response. Factors affecting the induced immune response include the format of the vaccine (i.e., DNA vaccine or protein vaccine), mode of administration (i.e., subcutaneous or intramuscular), the choice of adjuvant (i.e., Complete Freund's Adjuvant (CFA) or CpG) and the target antigen localization (i.e., soluble or membrane bound).

Antigen specific antibodies induced by vaccination can have different effector functions, which include ligand or receptor blockade, antibody dependent cellular cytotoxicity (ADCC) and depletion or signaling induction. ADCC as well as complement dependent cytotoxicity (CDC) can only be induced when a non-soluble (e.g., membrane bound) antigen is targeted. This would yield extra benefit when a strictly tumor endothelial cell specific marker is targeted, but could cause toxicity in the case that the antigen is also expressed during physiological angiogenesis.

An important issue in the vaccination approach, similar to other cancer vaccines, is that the targets of anti-angiogenic vaccines are self-antigens. This implies that self-tolerance needs to be overcome in order to induce an effective immune response against these antigens. This can be accomplished in various ways e.g., by inserting small changes in the amino acid sequence of the target antigen, by using a xenologous protein sequence, or by fusing the target self-antigen to a foreign antigen. The former approach would generate immune recognition of the species differences and similarities, leading to immunity to self-antigens by epitope spreading and molecular mimicry. The latter would generate mainly antibody responses due to activation of auto reactive T and B cells by helper T cells that are specific to the foreign antigen. The following chapter gives an overview of the many initiatives worldwide that are undertaken to challenge these issues. In Table 1 the findings of all described studies are summarized, and in Fig. 1 the different targets used for vaccination are visualized.

4. Anti-angiogenic vaccination strategies

4.1. Vaccination against soluble pro-angiogenic factors

Most studies have focused on VEGF as target for active immunization. VEGF-A is the prototype member of the VEGF family and is hereafter referred to as VEGF. The role of VEGF-B in vessel formation is less pronounced, whereas VEGF-C and VEGF-D are crucial in the development of lymph vessels. Through alternative exon splicing multiple VEGF-A isoforms can be generated, determining the heparin binding capacity and biological activity. VEGF₁₂₁ is a soluble isoform whereas VEGF₁₈₉ and VEGF₂₀₆ are fully heparin bound isoforms. VEGF₁₆₅ is an intermediate isoform which can be secreted but is also partly bound to the cell surface or ECM through binding to heparin [25].

Wei et al. [131] were the first to execute a strategy against VEGF by using it as a vaccination target. To overcome immune tolerance, they developed a vaccine to be employed in murine tumor models based on plasmid DNA encoding *Xenopus* VEGF (XVEGF-p). As controls, the corresponding mouse VEGF (MVEGF-p) vaccine and an empty vector (e-p) were used. In prophylactic and therapeutic active immunization experiments it was demonstrated that intramuscular injections with XVEGF-p had tumor growth inhibiting effects in 3 different tumor models. Intravenous (i.v.) passive immunization with pooled antisera of actively immunized mice also induced inhibition of tumor growth, indicating that an induced humoral immune response was important for the observed anti-tumor response. Indeed, anti-*Xenopus* VEGF as well as antibodies binding and neutralizing mouse and human VEGF were found in sera derived from XVEGF-p immunized mice. Both in the active as well as the passive immunization experiments, tumor growth of mice immunized with MVEGF-p was not inhibited, pointing out the necessity of the xenologous *Xenopus* sequence to induce an immune response. This was consistent with the observation that no anti-VEGF autoantibodies were found in sera derived from MVEGF-p immunized mice.

Unlike Wei et al., Gavilondo et al. developed a protein vaccine targeting VEGF. This vaccine was called CIGB-247 (also referred to as P64K-hVEGF_{KDR}) [6,32,80–82] and consisted of a recombinant fusion

Table 1
Studies investigating anti-angiogenic vaccines.

Target	Type of vaccine	Tumor type	Species	Route of administration	Mechanism of action	Adjuvant/carrier system	Antitumor effects	Adverse effects	Reference
<i>Soluble pro-angiogenic factors</i>									
VEGF	Xenogeneic (<i>Xenopus</i>) DNA vaccine	Different tumor models	Murine	Intramuscular	Antibody response	None	Inhibition of primary tumor growth	No effects on gross measures, pathology or biochemistry	[131]
VEGF	Peptide vaccine	N.A.	Murine	Not specified	Antibody response	Primer: CFA booster: IFA	N.A.	Not assessed	[57]
VEGF	Bacterial recombinant antigen	Different tumor models	Murine/rat/rabbit/monkey	Subcutaneously	Antibody and CTL response	VSSP	Inhibition of primary tumor growth and metastasis formation	None	[6,80–82]
VEGF	Bacterial recombinant antigen	Advanced solid tumors	Human	Subcutaneously	Antibody and CTL response	VSSP	12/30 objective clinical benefit	Local injection site reactions, fever	[32]
VEGF	Peptide vaccine	Different tumor models	Murine	Intraperitoneally or intradermally	Antibody response	T-cell epitope of MVF protein	Inhibition of primary tumor growth	Mild serositis of the small bowel mesentery	[129]
VEGF	Protein vaccine	Spontaneous cutaneous soft tissue sarcoma	Canine		Antibody response	Liposome–DNA	3/9 partial response	No injection site reactions, no evidence of coagulopathy	[58]
VEGF	Complex of murine or human VEGF to KLH	Different tumor models	Murine	Intramuscular	Antibody response	Primer: CFA, booster: IFA	Inhibition of metastasis formation (vaccine) and primary tumor growth (transfer of IgGs)	No effects on wound healing	[101]
VEGF	DNA peptide vaccine	CT 26 colon carcinoma	Murine	Intramuscular + electroporation	Antibody response	Hepatitis B virus core (HBc) system	Inhibition of primary tumor growth	Not assessed	[62]
bFGF	DNA vaccine	CT 26 colon carcinoma	Murine	Subcutaneously	Antibody and CTL response	LPD particles	Inhibition of primary tumor growth and lung metastases	Slight delay in wound healing	[138]
bFGF	Peptide vaccine	Different tumor models	Murine	Intramuscular	Unknown	Lipid vesicles with lipid A	95–96% inhibition on metastasis formation.	No effects on wound healing and reproduction	[99,100]
<i>(Whole) endothelial cells</i>									
HUVEC/GEN-T	Paraformaldehyde fixed xenogeneic endothelial cells	Different tumor models	Murine	Intraperitoneal	Antibody response	None	Inhibition of primary tumor growth	No effects on gross measures, pathology	[132]
HUVEC	Viable xenogeneic endothelial cells	Different tumor models	Murine	Intraperitoneal	Antibody and CTL response	None	Inhibition of primary tumor growth and metastasis formation	No effects on gross measures, pathology	[10]
HUVEC	Glutaraldehyde-fixed endothelial cells	Recurrent malignant brain tumor, mCRC	Human	Intradermally	Antibody and CTL response	None	2/9 partial tumor response and 1/9 complete tumor response	DTH-like skin reaction at the injection site	[92]
LSEC	Glutaraldehyde-fixed endothelial cells	CT26 colon carcinoma	Murine	Subcutaneous	Antibody and CTL response	None	Inhibition of metastasis formation	No effects on gross measures	[90]
<i>Endothelial matrix markers</i>									
ED-B of fibronectin	Fusion protein vaccine	T241 fibrosarcoma	Murine	Subcutaneously	Antibody response	Primer: CFA, Boosters: IFA	70% reduction in primary tumor size	No effects on wound healing and cartilage.	[51]
MMP-2	Xenogeneic (chicken) DNA vaccine	Meth A fibrosarcoma, H22 hepatoma	Murine	Intramuscular	Antibody response	None	Inhibition of primary tumor growth and 33–50% reduction of lung metastases	No effects on gross measures, pathology	[115]
<i>Endothelial cell membrane</i>									
Angiotonin	DNA vaccine	TUBO cells/BALB-neuT transgenic breast cancer	Murine	Intramuscular	Antibody response	Electroporation	Inhibition of TUBO tumor growth, no inhibition in transgenic model	No effects on retina vasculature	[3,47]

TEM8	DC vaccine	H22 hepatocellular carcinoma	Murine	Subcutaneously	CTL response	None (adenovirus?)	30% inhibition of primary tumor growth	No effects on wound healing	[135]
TEM8	Xenogeneic DNA vaccine	B16F10 melanoma	Murine	Oral	CTL response	<i>S. typhimurium</i>	75% inhibition of primary tumor growth and 60% inhibition of metastasis formation	No effects on wound healing, gross measures, biochemistry	[105]
TEM1	Fusion DNA vaccine	Different tumor models	Murine	Intramuscular and electrogene transfer	CTL response and cross priming	Tetanus toxoid	Significant inhibition of primary tumor growth	No effects on wound healing and reproduction	[22]
FGFR-1	Xenogeneic (<i>Xenopus</i>) DNA vaccine	Different tumor models	Murine	Intramuscular	Antibody response	None	Inhibition of primary tumor growth	Delayed wound healing	[45]
VEGFR1	HLA restricted peptide vaccine	Different tumor models	Murine	Intradermally	CTL response	IFA	Inhibition of primary tumor growth and metastasis	No effects on wound healing, reproduction and hematopoiesis	[53]
VEGFR1	HLA restricted peptide vaccine	Advanced solid tumors	Human	Subcutaneously	CTL response	IFA	5/9 stable disease	No DLT	[44]
VEGFR1	HLA restricted peptide vaccine	Renal cell carcinoma	Human	Subcutaneously	CTL response	IFA	2/18 partial response, 8/18 stable disease	No DLT, but 10/18 local skin reaction	[136]
VEGFR2	DNA vaccine	Different tumor types	Murine	Oral	CTL response	<i>S. typhimurium</i>	Inhibiting of primary tumor and metastases	Delayed wound healing	[70,87]
VEGFR2	Xenogeneic (quail) protein vaccine	Different tumor models	Mice	Subcutaneously	Antibody and CTL response	Aluminum hydroxide	Inhibition of metastases. Inhibition of primary tumor growth with adoptive transfer of IgGs.	No effects on gross measures, pathology, reproduction, hematopoiesis	[70]
VEGFR2	Fusion DNA vaccine	Lewis lung carcinoma, CT26 colon carcinoma	Murine	Intramuscular	Antibody and CTL response	Cationic liposome	Inhibition of primary tumor growth, metastases and angiogenesis	Not assessed	[130]
VEGFR2	Fusion DC vaccine	Different tumor models	Murine	Intravenously	Antibody and CTL response	Alkaline phosphatase	Inhibition of metastasis formation	Inhibition on pregnancy	[68]
VEGFR2	DNA vaccine	B16F10 melanoma	Murine	Oral	CTL response	<i>Salmonella</i> type III secretion system	50% inhibition of primary tumor growth, inhibition of lung metastases	No effects on gross measures	[55]
VEGFR2	Fusion DNA vaccine	Different tumor models	Murine	Subcutaneously	CTL response	Lysteriolysin-O	Inhibition of primary tumor growth	No effects on wound healing and reproduction	[111]
VEGFR2	Peptide vaccine combined with gemcitabine	Metastatic/unresectable pancreatic cancer	Human	Subcutaneously	CTL response	IFA	1/24 partial response and 11/23 stable disease	Injection site reactions	[79]
VEGFR2	Peptide vaccine	Advanced solid tumors	Human	Subcutaneously	CTL response	Not reported	2/10 stable disease	Injection site reactions and proteinuria	[93]
VEGFR2	T4-mVEGFR2 phage vaccine	Lewis lung carcinoma	Murine	Subcutaneously	Antibody response	T4 phage	75% inhibition of growth primary tumors	No effects on gross measures	[103]
VEGFR2	DNA minigene vaccine	Different tumor models	Murine	Oral	CTL response	<i>S. typhimurium</i>	Inhibiting of primary tumor growth	Not assessed	[139]
VEGFR2	Peptide vaccine	MC38 colon cancer	Murine	Subcutaneously	CTL response	IFA	Inhibition of primary tumor growth	No effects on gross measures	[17]
VEGFR2	HLA restricted peptide vaccine	Different tumor models	Murine	Intradermally	CTL response	IFA	Inhibition of primary tumor growth and angiogenesis	Delayed wound healing and reduced fertility	[128]
VEGFR2 DNA fusion vaccine	Fusion DNA vaccine	BTT739 bladder cell carcinoma	Murine	Intramuscular	Antibody response	C3D3	Inhibition of primary tumor growth.	Not assessed	[69]
VEGFR2	DNA vaccine combined with IP-10	B16F10 melanoma	Murine	Oral	CTL response	<i>S. typhimurium</i>	Inhibition of primary tumor growth	No effects on gross measures	[73]
VEGFR2	Minigene DNA vaccine	Different tumor models	Murine	Oral	CTL response	<i>S. typhimurium</i>	Inhibition of metastasis formation	Not assessed	[74]

(continued on next page)

Table 1 (continued)

Target	Type of vaccine	Tumor type	Species	Route of administration	Mechanism of action	Adjuvant/carrier system	Antitumor effects	Adverse effects	Reference
Tie-2	Xenogeneic protein vaccine	H22 hepatoma and B16F10 melanoma	Murine	Subcutaneous	Antibody response	Aluminum hydroxide	Inhibition of primary tumor growth and metastasis formation	No effects on gross measures, pathology	[75]
Tie-2	DNA vaccine	N.A.	Murine	Helios gene gun	CTL response	None	N.A.	Not assessed	[102]
Endoglin	DNA vaccine	D2F2 breast carcinoma	Murine	Oral	CTL response	<i>S. typhimurium</i>	Inhibition of metastasis formation	Not assessed	[63]
Endoglin	DNA vaccine	NT-2 mammary tumor	Murine	Intraperitoneal	CTL response	Carrier: <i>Listeria</i> , adjuvant: Listerolysin O	Inhibition of primary tumor growth and metastasis formation	Not assessed	[133]
Endoglin	DNA vaccine combined with IL-12 or cyclophosphamide	B16F10 murine melanoma	Murine	Oral	CTL response and immunomodulation	<i>S. typhimurium</i>	Inhibition of primary tumor growth	No effects on gross measures, wound healing	[54]
Endoglin	Xenogeneic (porcine) protein vaccine	Different tumor models	Murine	Subcutaneous	Antibody and CTL response	Aluminum hydroxide	Inhibition of primary tumor growth and metastasis formation	Delayed wound healing	[118]
Endoglin	Xenogeneic protein vaccine combined with cisplatin	Lewis lung carcinoma, CT26 colon carcinoma	Murine	Subcutaneous	Antibody and CTL response	Aluminum hydroxide	Inhibition of primary tumor growth	No effects on gross measures, pathology, biochemistry	[117]
PDGFR β	DNA vaccine	Different tumor models	Murine	Oral	CTL response	<i>S. typhimurium</i>	78% reduction in primary tumor growth, 60–73% reduction in number of metastases	Not assessed	[60]
DLL4	Xenogeneic (human) DNA vaccine	D2F2/E2 and TUBO mammary tumor	Murine	Intramuscular/intradermally	Antibody	Electroporation	61–64% inhibition of primary tumor growth	No effects on gross measures, wound healing, liver homeostasis	[42]
<i>Combination vaccines</i>									
Survivin	DNA vaccine	Panc02 pancreas carcinoma, A20 lymphoma	Murine	Intramuscular	Unknown	None	Inhibition of primary tumor growth and increase survival	Not assessed	[140]
Survivin	Combination DNA vaccine	D121 Lewis lung carcinoma	Murine	Oral	CTL response	CCL21 and <i>S. typhimurium</i>		No effects on wound healing and reproduction	[134]
Survivin	DNA vaccine	None	Murine	Intramuscular	Antibody response and CTL response	GM-CSF	No tumor experiments performed	Not assessed	[72]
Survivin	DNA vaccine	B16F10 melanoma	Murine	Intradermal	CTL response	Electroporation	40% of mice protected from tumor	No effects on gross measures	[71]
Legumain	DNA vaccine	Different tumor models	Murine	?	CTL response	<i>S. typhimurium</i>	Inhibition of primary tumor growth and metastasis formation	Not assessed	[76]
Legumain	Minigene DNA vaccine	D2F2 carcinoma	Murine	Oral	CTL response	<i>S. typhimurium</i>	Inhibition of primary tumor growth and metastasis formation	Not assessed	[65]
Different antigens	DC vaccine	B16F10.9 melanoma, MBT-2 bladder carcinoma	Murine	Intravenously	CTL response	None	Inhibition of primary tumor growth and metastasis formation	Reduction of short term fertility	[85]
HP59	Peptide vaccine conjugated to KLH	Lewis lung carcinoma	Murine	?	Antibody response	Primer: CFA, boosters: IFA	61.7% inhibition of primary tumor growth	Infiltration of lymphocytes in the liver and kidney	[27]

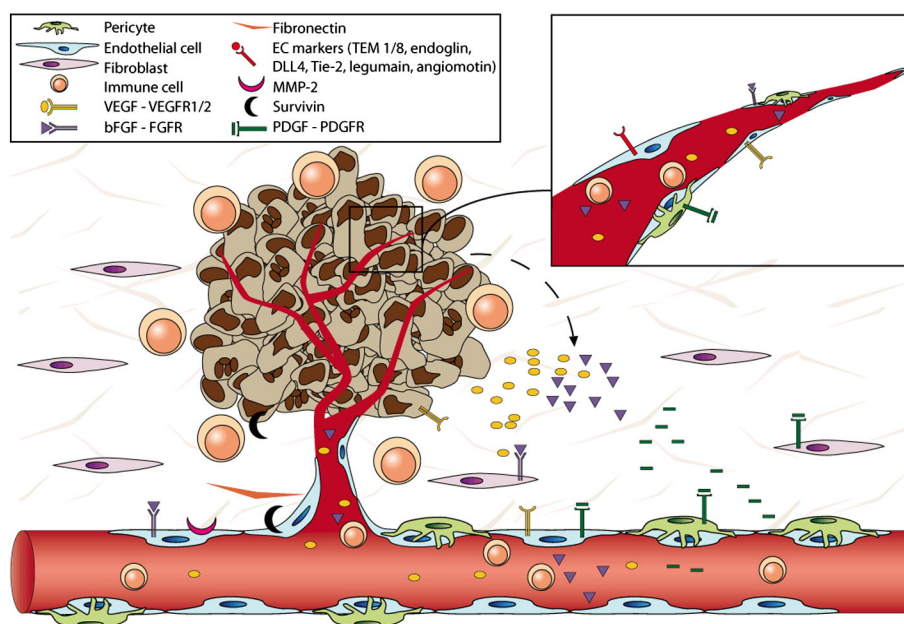


Fig. 1. Targets investigated for anti-angiogenic vaccination.

protein comprising mutated hVEGF₁₂₁ incapable of binding to Kinase insert Domain Receptor (KDR) fused to P64K, a recombinant protein derived from *Neisseria meningitidis*. Different adjuvants (aluminum hydroxide, VSSP and CAF01) were used in the pre-clinical experiments with this vaccine [80]. VSSP is an adjuvant consisting of very small proteoliposomes obtained by incorporation of gangliosides into the outer membrane complex of *Neisseria meningitidis* whereas CAF01 is a cationic liposome consisting of dimethyldioctadecylammonium and trehalose dibehenate. The occurrence and growth of B16F10 melanoma were reduced in P64K-hVEGF_{KDR}-immunized mice, with VSSP being the most potent adjuvant, even though antibody titers were found to be relatively low in mice immunized with the VSSP-based vaccine as compared to mice immunized with the aluminum hydroxide-based vaccine. A HUVEC proliferation assay showed that sera from P64K-hVEGF_{KDR}/VSSP immunized mice were more effective in blocking the proliferative effect of VEGF on HUVECs. Mice depleted for CD8⁺ T cells were only partially protected from tumor challenge compared to the control group, indicating that CD8⁺ T cells were (at least in part) responsible for the observed anti-tumor effect. This CIGB-247/VSSP vaccine was tested in other mouse studies and demonstrated to be effective at inhibiting metastasis formation in experimental lung metastasis models [6]. Using the 3LL-D122 spontaneous pulmonary metastasis model it was shown that lungs of mice vaccinated with CIGB-247 exhibited significantly less metastases than lungs of control groups [6]. Experiments in other species (i.e., rats, rabbits and monkeys) showed a good safety profile in terms of behavioral and laboratory parameters, as well as skin wound healing [81,82]. Furthermore, a high anti-human VEGF antibody response in monkeys immunized with CIGB-247 was induced, indicating that the vaccine is well able to break tolerance. Although the antigen dose had no effect on the maximum anti-VEGF antibody titer, titer values dropped at a faster rate in the low antigen dose group. Results from a direct cell cytotoxicity assay in which target cells were labeled with CFSE indicated that an anti-VEGF cellular immune response was induced. CIGB-247 has recently been investigated in a dose escalation phase I clinical trial [32]. In total 30 patients with advanced solid tumors were subcutaneously immunized with escalating doses (50, 100 or 400 µg) of the VEGFA₁₂₁ fusion antigen together with a fixed dose of 200 µg VSSP adjuvant. The vaccine was given weekly for the first 8 weeks and every 4 weeks hereafter. During the dose-limiting toxicity (DLT) period of 16 weeks no vaccine related grade 3 or greater adverse events were

observed. However, 12 vaccination-unrelated severe adverse events were observed during the DLT period, predominantly increases of liver enzymes. Observed side effects were presumably related to the vaccine and included local injection site reactions and fever. After the DLT period of 16 weeks, anti-VEGF seroconversions were observed in 19/30 patients. By using the ELISPOT technique it was found that in 16/30 patient an increased number of IFN-γ spots in comparison to pre-vaccination samples was observed when T cells were exposed to VEGF, which demonstrated the induction of a VEGF specific T cell response. Lastly, a significant decrease in the platelet VEGF value was found in the 400 µg cohort, indicating that the induced anti-VEGF antibodies were able to neutralize VEGF.

A study by Kamstock et al. [58] also yielded results on a protein vaccine against VEGF. This vaccine was studied in dogs with spontaneous soft tissue sarcoma. The vaccine consisted of recombinant human VEGF₁₆₅ together with a liposome–DNA complex as adjuvant. In the majority of dogs that received 5 or more vaccinations, a significant increase in anti-human VEGF antibodies was observed together with a partial tumor response in 3 out of 9 animals. No local reactions or cases of delayed wound healing were observed, indicating an acceptable safety profile of the vaccine.

Another immunization strategy that can be used is the kinoid technology developed by NeoVacs S.A. Kinoids are cytokines chemically linked to keyhole limpet hemocyanin (KLH). Rad et al. [101] used the kinoid technology against VEGF. Immunization with both mVEGF-kinoid and with hVEGF-kinoid induced high anti-mouse VEGF antibody levels and anti-human VEGF antibody levels, respectively. This study showed that it is possible to raise autoantibodies with a homologous vaccine. Vaccination also induced a pronounced reduction in the number of lung metastases in an experimental metastasis model. Interestingly, in-vivo adoptive transfer of purified immunoglobulins isolated from KLH-mVEGF immunized mice, with or without paclitaxel, resulted in marked inhibition of tumor growth in several tumor models. Importantly, no adverse effects on wound healing were observed [101].

The research group of Kaumaya published two papers on VEGF peptides, which can act both as a vaccine [129] as well as a VEGF peptide mimic [126]. Through binding to VEGFR2 these peptides prevent VEGFR2 dimerization and consequently phosphorylation. Wang et al. [129] focused on the use of these peptides as immunogen in ovarian cancer. Two peptides were constructed (VEGF127–144 and VEGF102–122) and

coupled to the promiscuous T-helper cell epitope MVF (measles virus fusion protein). High antibody titers were achieved after immunization with both peptide vaccines and shown to be effective at inhibiting tumor growth and angiogenesis in Rip1-Tag2 transgenic mice as well as in a VEGF-overexpressing ovarian cancer model.

In another paper by Vicari et al. [126] the focus was put on the application of the VEGF102–122 peptide as VEGF mimic. In a VEGF-overexpressing tumor mouse model (double transgenic VEGF^{+/-} Neu2-5^{+/-}) intravenous treatment with the peptide (VEGF-P3 (CYC)) significantly delayed tumor burden.

Kyutoku et al. [62] set out to selectively induce antibody responses by using a VEGF peptide which contained the binding epitopes of bevacizumab, VEGFR2 and VEGFR1. In BALB/c mice, which were intramuscularly immunized with the VEGF peptide vaccine, anti-mVEGF antibodies could be detected, primarily of the IgG2a isotype. This indicated that a Th1 cell skewed immune response was elicited, but neither in an IFN γ ELISPOT nor in a T cell proliferation assay, any proof was obtained for a cellular immune response against VEGF. In a prophylactic immunization experiment it was shown that the growth of CT26 colon carcinoma was inhibited in vaccinated mice.

Jiang et al. [57] synthesized five (a–e) non-native isomers of mouse VEGF₁₁₀ (X-VEGF), which could act as immunogens. All X-VEGF₁₁₀ isomers were able to induce a high anti-mouse VEGF antibody response, with X-VEGF₁₁₀-e being the most potent. As a marker of treatment efficacy, PlGF levels were measured. In all X-VEGF₁₁₀ immunized mice these PlGF levels were increased. The authors claimed that the increased PlGF levels might be the cause of lowered VEGF levels, because the treatment-induced hypoxia promotes the release of other pro-angiogenic growth factors. Unfortunately, the authors did not report data on the VEGF levels in these mice, nor were tumor experiments performed. It therefore remains to be seen how effective this strategy really is.

Only a few papers were published on vaccines that targeted other pro-angiogenic growth factors [99,138]. Two studies have focused on basic fibroblast growth factor (bFGF, also referred to as FGF-2) as target for vaccination. Plum et al. [99] developed a peptide vaccine (L(HBD)) consisting of a 42-mer peptide corresponding to the heparin binding domain (HBD) of murine FGF-2 conjugated to, and encapsulated in, liposome vesicles containing the adjuvant Lipid A. With ELISA performed on sera from treated animals anti-FGF-2 antibody titers of 1:5000 were found. Furthermore, L(HBD) was shown to block the induction of neovascularization by FGF-2 in a B16BL6 melanoma sponge implant model of angiogenesis. Using this model it was also observed that tumor growth was inhibited in L(HBD) vaccinated mice. In two different models, treatment with L(HBD) resulted in 95–96% inhibition of macroscopic metastases in the lungs when compared with the lungs of control mice. Since adoptive transfer of antisera, generated in mice vaccinated with L(HBD), was unable to inhibit tumor development, cellular immunity was likely responsible for the observed tumor inhibition in the active immunization experiments. In a later publication it was shown that this vaccine had no adverse effects on wound healing or reproduction [100].

Zhang et al. [138] developed a vaccine consisting of a human N-, C-terminally truncated bFGF (tbFGF) combined with liposome-polycation–DNA (LPD) particles as adjuvant. In a prophylactic model BALB/c mice were immunized with LPD/tbFGF by subcutaneous injection once a week for 6 consecutive weeks and consequently challenged with CT26 colon carcinoma cells. In LPD/tbFGF immunized mice, high anti-bFGF antibody titers of 1:256,000 were induced. A cytotoxicity assay with LPD/tbFGF immunized mouse splenocytes showed a strong CTL response against mouse MS1 microvascular endothelial cells. Furthermore, tumor growth and lung metastasis formation in LPD/tbFGF immunized mice were significantly inhibited compared to the control groups. IHC staining for CD31 on tumor sections showed a decreased microvessel density in LPD/tbFGF immunized mice.

4.2. Vaccination using (whole) endothelial cells

A year prior to their seminal report on VEGF vaccination, Wei et al. [132] were the first to publish on the use of whole endothelial cell preparations as vaccines to target the tumor vasculature. They showed that immunization with paraformaldehyde fixed xenogeneic endothelial cells inhibited tumor growth both in a prophylactic, as well as in a therapeutic setting. However, immunization with SVEC4–10 cells (derived from mouse endothelial cells) did not inhibit tumor growth, stressing the need for a xenogeneic antigen to break immune self-tolerance. Interestingly, no additional adjuvant was required to induce cross-reactive antibodies. Immune cell depletion experiments indicated that CD4⁺ T lymphocytes were essential for tumor inhibition. By Western Blot analysis peptides within VEGFR2 and αv integrin were identified as the main endothelial cell membrane targets of the cross-reactive antibody response. Passive immunization with immunoglobulins, isolated from mice, actively immunized with a xenogeneic peptide derived from VEGFR2 and αv integrin, also resulted in tumor growth inhibition as well as angiogenesis inhibition whereas immunoglobulins from mice immunized with mouse peptides did not interfere with tumor growth and angiogenesis compared to control mice.

Okaji et al. [90,92] were able to induce an immune response against endothelium upon vaccination with glutaraldehyde fixed autologous endothelial cells. Similar to the vaccine developed by Wei et al., no adjuvant was required to promote the immune response. In BALB/c mice, prophylactically or therapeutically treated with this autologous vaccine, lung metastasis formation was inhibited. Immunogenicity assays indicated that both cross-reactive antibodies and CTL were induced upon immunization. In comparison with a similarly constructed glutaraldehyde fixed HUVEC (xenologous) vaccine, the autologous vaccine was shown to be superior in terms of metastasis inhibition and immunogenicity. No direct anti-tumor immune response was observed in different immunogenicity assays. In 2008 the first clinical results with this vaccine were published [92]. Glutaraldehyde-fixed HUVECs were investigated as immunogen in 6 patients with recurrent malignant brain tumors and 3 patients with metastatic colorectal cancer mCRC. In these patients 5×10^7 HUVECs were administered intradermally every week for the first month and every 2 weeks thereafter. Except for local injection site reactions, no adverse events were observed. A specific anti-HUVEC membrane antigen antibody response was induced in 8 out of 9 patients, whereas in only 6 patients a specific cellular response was induced. In three patients with malignant brain tumors a partial or complete response was observed, which was maintained for at least 9 months after the first immunization. However, no clear correlation could be found between the immunological and anti-tumor response.

In contrast to Wei and Okaji, Chen et al. [10] developed a HUVEC based vaccine in which the HUVECs received no chemical treatment (i.e., fixation with paraformaldehyde or glutaraldehyde). Immunocompetent mice were prophylactically immunized with 1×10^6 of these viable HUVECs once a week for four consecutive weeks and thereafter challenged with either LLC cells or FO myeloma cells. Tumor growth was significantly inhibited in both tumor models. In a spontaneous lung metastasis model it was shown that survival was increased in HUVEC immunized mice compared to control mice. High anti-HUVEC antibody titers were found through ELISA and a CTL assay showed that T lymphocytes isolated from immunized mice were cytotoxic to HUVECs. Interestingly, no antibody response or cytotoxicity was found against tumor cells. The results of passive immunization experiments in immunocompromised tumor bearing mice showed that tumor growth could be inhibited with antibodies or CTLs induced by vaccination. However, the authors claim that CTLs were more potent as opposed to antibodies in terms of tumor growth inhibition. Although this vaccine was able to induce an immune response in mice, it remains to be proven that chemically untreated viable HUVECs will be able to induce a similar immune response as allogeneic vaccine in human patients.

4.3. Vaccination against endothelial matrix markers

The advantage of targeting an extracellular matrix bound antigen as opposed to a soluble antigen is that the antigen cannot be easily engulfed by immune cells, which can lead to an extensive inflammatory response. Huijbers et al. [51] designed an anti-angiogenic vaccine targeting the extracellular matrix bound extra domain B (ED-B) containing isoform of fibronectin. To break self-immune tolerance a fusion protein was constructed, consisting of the autologous ED-B domain of fibronectin fused to the *Escherichia coli*-derived protein thioredoxin (TRX). For primary vaccination complete Freund's adjuvant was used, whereas for booster immunizations the vaccine was adjuvanted with incomplete Freund's adjuvant.

Immunocompetent C57BL/6 mice were prophylactically immunized 3 times with adjuvanted TRX-EDB and subcutaneously challenged with murine T241 fibrosarcoma cells. In two independent experiments, a reduced tumor volume was observed in mice immunized with TRX-EDB as compared to control mice at the end of the experiment. Furthermore, antibodies against ED-B could be measured in sera of TRX-EDB vaccinated mice. Histological analysis of tumor tissue revealed a significant increase in necrotic areas in TRX-EDB immunized mice and electron microscopy analysis showed altered vessel morphology as well as increased leakage of tumor vessels, presumably caused by the frustrated phagocytosis resulting from the inflammatory response. To investigate safety issues a wound-healing assay was performed in which a wound was inserted on the back of immunized and control mice. During a follow-up period of 14 days no differences in wound healing could be observed between the two groups, suggesting that the vaccine does not interfere with physiological angiogenesis. The same group recently showed that a vaccine against the ED-A domain of fibronectin has similar anti-tumor effects in a spontaneous tumor model, through inhibition of angiogenesis and metastasis formation, as well as by inducing an inflammatory response [23,34].

Another way of targeting the ECM is by vaccination against matrix metalloproteinase (MMP). MMPs are responsible for ECM degradation. Su et al. [115] developed a xenogeneic DNA vaccine on the basis of chicken MMP-2 in order to break immune tolerance in mice. Mice intramuscularly immunized with the chicken MMP-2 vaccine were protected from tumor growth in 3 different prophylactic and therapeutic immunization models. In parallel, mice immunized with a mouse MMP-2 vaccine were not protected from tumor growth. As determined by the murine LLC model the number of lung metastases was reduced by 33–50% in immunized mice compared to control mice. In vivo immune cell depletion studies showed that CD4⁺ T cells were most likely responsible for the immune response and the resulting anti-tumor effect. Moreover, treatment with purified immunoglobulins isolated from immunized mice was also effective in inhibiting tumor growth. No apparent adverse events were observed in immunized mice.

4.4. Vaccination against endothelial cell membrane associated targets

The advantage of targeting endothelial cell associated markers is that it constitutes a direct way of targeting and killing of endothelial cells. In addition, these targets are more accessible for antibodies and immune cells. Among these targets are antigens that are more common among all endothelial cells and antigens that seem to be more specifically expressed in the tumor. This has obviously major consequences for the expected activity and side effects.

4.4.1. Angiomin

Angiomin is a membrane-associated protein, which mediates the anti-migratory effects of the endogenous angiogenesis inhibitor angiostatin. Holmgren et al. [47] designed a human DNA vaccine targeting angiomin in which an angiomin plasmid was inserted into the pcDNA3 vector. Immunization of BALB/c mice followed by electroporation to break self-tolerance induced an immune response

against angiomin. In a tumor transplantation model in which BALB/c mice were challenged with a lethal dose of TUBO carcinoma cells, a marked inhibition on tumor growth was observed in the immunized mice. Furthermore, in vivo immune cell depletion studies indicated that the antibody producing B cells were responsible for the anti-tumor response. In a BALB-neuT transgenic breast cancer model monotherapy with the angiomin DNA vaccine did not inhibit tumor growth. However, combination therapy of the angiomin DNA vaccine with a plasmid encoding the extracellular and transmembrane domains of Her2 (EC-TM^{neu}), significantly inhibited tumor growth compared with EC-TM^{neu} plasmid vaccine alone. Moreover, the combination treatment kept 80% of the mice tumor free for over 70 weeks. Inspection of the retina vasculature indicated that this vaccine did not interfere with physiological angiogenesis.

A later publication of the same research group [3] showed that this angiomin DNA vaccine was also able to inhibit the growth of already developed tumors in two transgenic mouse models. Dynamic contrast enhanced-magnetic resonance imaging showed that vessel permeability was increased in vaccinated mice, which potentially could enhance the effect of other anticancer drugs. Combining vaccination with doxorubicin in TUBO tumor bearing mice indeed resulted in a dramatic regression of large tumor masses, which was not observed when vaccination was used as monotherapy.

4.4.2. Tumor endothelium markers (TEM)

As tumor endothelial cells are phenotypically different from their resting counterparts, using these differences is another way of targeting the tumor vasculature. Specific tumor endothelial markers (TEMs) can be used as targets for vaccination. Ruan et al. [105] constructed a xenogeneic DNA vaccine targeting TEM8, which is a transmembrane molecule functioning as the receptor for *Bacillus anthracis* (anthrax) toxin. In immunized mice primary tumor growth was significantly inhibited and survival was prolonged. In an experimental lung metastasis model it was shown that vaccination induced a reduction in the number of metastases. Immunogenicity analyses showed that isolated T cells from immunized mice specifically lysed TEM8 expressing target cells. In vivo immune cell depletion studies indicated that CD8⁺ T cells were primarily responsible for the induced immune response. In particular no negative effects on wound healing were noted in immunized mice, as observed by a cutaneous excision wound model.

Yang et al. [135] developed a DC vaccine in which DCs were transduced with a recombinant adenovirus encoding TEM8. Mature mouse DCs were transduced by a TEM8 encoding adenovirus. Mice were vaccinated with 1,000,000 Ad-TEM8 transduced DCs on days 0, 7 and 14. On day 21, spleens were removed and CD8⁺ T cells were purified. ELISPOT showed that Ad-TEM8 transduced DCs generated strong specific CD8⁺ T-cell responses. Both in prophylactic as well as therapeutic immunization experiments tumor growth was inhibited by approximately 30% in Ad-TEM8 vaccinated mice. Microvessel density was lower in tumors of the Ad-TEM8 group compared to the control groups. Different immune cell subsets were depleted to explore which subsets were the main effectors. These studies revealed that the antitumor effect of Ad-TEM8 transduced DCs was mainly caused by CD8⁺ T cells. A wound healing assay showed that vaccination with Ad-TEM8 transduced DCs had no effect on wound healing, indicating that physiological angiogenesis was not affected.

TEM1 (also referred to as endosialin or CD248) is a family member of TEM8 and was investigated as target for vaccination by Facciponte et al. [22]. TEM1 is overexpressed on tumor endothelium as well as in tumor stroma. This DNA vaccine consisted of TEM1 cDNA fused to the first C domain of fragment C of tetanus toxoid (Tem1-TT). Mice received weekly intramuscular injections after which electrogene transfer was performed. In both prophylactic and therapeutic immunization models Tem1-TT vaccinated mice developed smaller tumors as opposed to control mice. Although the researchers did not measure anti-TEM1 antibodies in serum it is unlikely that the induction of anti-Tem1 antibodies

contributed to the observed antitumor response since adoptive transfer of whole serum did not lead to tumor growth inhibition. On the contrary, adoptive transfer of CD3⁺ T cells derived from Tem1-TT immunized did abrogate tumor growth indicating that the observed tumor growth inhibition in immunized mice was mainly due to the induction of a cellular immune response. Thorough safety studies were conducted, showing no effect on wound healing and reproduction.

4.4.3. FGFR-1

As part of the process of tumor angiogenesis, the endothelium in the tumor upregulates expression of a series of (angiogenic) growth factors receptors. He et al. [45] developed a xenogeneic (*Xenopus*) DNA vaccine targeting FGF receptor 1. The DNA product was subcloned into pcDNA3.1, under control of a cytomegalovirus promoter. FGFR1 is a receptor tyrosine kinase which binds – among other ligands – FGF1 and FGF2. In a prophylactic immunization model vaccinated mice were protected from tumor growth whereas tumor growth was inhibited in a therapeutic immunization model. In sera from mice immunized with the *Xenopus* FGFR1 DNA vaccine-induced auto-antibodies against FGFR-1 could be detected. Depletion of CD4⁺ T cells resulted in abrogation of the anti-tumor response. Furthermore, in-vivo adoptive transfer of purified immunoglobulins effectively protected mice from tumor growth. Although no attempts were made to assess a possible cellular immune response, depletion of CD8⁺ T cells did not abrogate tumor inhibition, suggesting that CD8⁺ T cells are not primarily involved in the immune response. Important to note is that in immunized mice wound healing was delayed, indicating that this vaccine also interfered with physiological angiogenesis.

4.4.4. VEGFR1

VEGF binds both VEGFR1 and VEGFR2. Although the binding affinity of VEGF to VEGFR1 is considerably higher than to VEGFR2, it seems that VEGFR2 is the main driver for the pro-angiogenic effects [25]. VEGFR-1 virtually lacks kinase activity but it appears that signaling can be accomplished through receptor heterodimerization. Moreover it can act as a “trap” for VEGF in order to regulate the accessibility of VEGF to VEGFR2 [94].

Ishizaki and co-researchers developed a vaccine using peptides covering epitopes derived from VEGFR1 specifically restricted through two HLA types (HLA-A*0201 (A2) and HLA-A*2402 (A24)) [53]. Because the researchers had access to A2/Kb transgenic mice, only peptides restricted to HLA-A*0201 (A2) were used in the mouse experiments. In A2/Kb transgenic mice immunized with three different peptides (VEGFR1-1087, VEGFR1-770 and VEGFR1-417) admixed with IFA, peptide-specific production of IFN γ was induced in T cells. Human PBMCs were obtained from healthy volunteers with the corresponding HLA type and pulsed with the 3 candidate epitope peptides. Only peptides VEGFR1-1087 and VEGFR1-770 were thus shown to lead to priming of specific CTLs in vitro. In three different therapeutic immunization models, significant inhibition of tumor growth was achieved upon immunization with the peptides VEGFR1-1087 and VEGFR1-770. IHC staining for CD31 revealed a significant reduction of tumor vessels. In an experimental B16.F10 metastasis model these peptide vaccines could also inhibit metastasis. In a final study the VEGFR1 peptide vaccine was combined with a VEGFR2 peptide vaccine developed by Wada et al. [128]. The combined immunization resulted in a more potent inhibition of tumor growth as compared to VEGFR1 peptide or VEGFR2 peptide immunization alone. No significant delay in wound healing or diminished fertility was observed in these mice. Furthermore, bone marrow and peripheral blood analysis showed that the bone marrow hematopoiesis was not affected by immunization.

After these promising results a phase I clinical study [44] was initiated to investigate the safety and immunogenicity of one of the HLA-A*24-02 restricted peptides (VEGFR1-1084) identified by Ishizaki et al. [53]. The vaccine, here referred to as OTS11101, was emulsified with IFA and administered subcutaneously once weekly for 4 weeks

(treatment cycle) and was studied in dose escalation. The dose levels used in this study were 1.0, 2.0 and 3.0 mg. If no DLT were observed, patients could be subjected to further cycles of vaccination. In this study 9 patients with advanced solid tumors were enrolled. Although one patient in the 2.0 mg group experienced a grade 3 γ GTP increase, this was not recorded as being a DLT because the adverse event was attributed to disease progression. No other grade 3 adverse events were observed. Five out of 9 patients had stable disease for at least one treatment cycle. No partial or complete responses were observed. To assess a possible cellular immune response, IFN- γ ELISPOT assays were performed using PBMCs collected from the patients. In 2 out of 9 patients, a positive CTL response was observed. Interestingly, in both patients stable disease was achieved. Serum levels of soluble VEGFR1 and VEGFR2 were found to be increased after one treatment cycle. It remains unfortunate that VEGFR1 peptides restricted through HLA-A*24-02 were clinically tested, while the pre-clinical studies only described the effects of vaccination with VEGFR1 peptides restricted through HLA-A*02.

Another clinical trial using the VEGFR1 peptides identified by Ishizaki et al. was performed by Yoshimura et al. [136]. Metastatic renal cell carcinoma patients received either VEGFR1-770 (HLA*0201 restricted) or VEGFR1-1084 (HLA*2401 restricted) emulsified with IFA. The peptide vaccines were investigated in dose escalation (0.5, 1.0 and 3.0 mg) and were administered once weekly for a 4-week treatment cycle. If the safety profile proved to be favorable, immunization was continued once every 2 weeks. In total, 9 patients received the VEGFR1-770 vaccine and 9 patients the VEGFR1-1084 vaccine. No grade >3 adverse events or DLTs were observed in this study. The most commonly observed adverse events were grade 1/2 local skin reaction (10 out of 18 patients). A positive CTL response was observed in 15 out of 18 patients (83%). In two patients partial response (PR) was achieved. Stable disease (SD) for at least five months was achieved in another 9 patients of which 6 patients received the HLA*2402 restricted VEGFR1-1084 peptide vaccine. However, no obvious correlations between CTL response and clinical outcome were found.

4.4.5. VEGFR2

As expected from the important role of VEGF in tumor angiogenesis, VEGFR2 (also referred to as KDR in human or Flk-1 in mice) has been thoroughly investigated as target for vaccination. In 2002, Li et al. [68] were the first to report on a vaccine that targeted VEGFR2. Dendritic cells isolated from bone marrow were pulsed with a fusion protein, consisting of Flk-1 coupled to alkaline phosphatase (AP). When administered prophylactically, the vaccine (DC-flk1-AP) was able to inhibit lung metastasis formation in 2 different experimental metastasis models (i.e., Lewis Lung carcinoma and B16 melanoma). The effect of the vaccine on primary tumor growth was not studied. Immunogenicity assays showed that both a transient antibody response and a CD8⁺ cytotoxic T cell (CTL) response was induced. However, a subsequent in vivo immune cell depletion experiment showed that the CD8⁺ CTL response was primarily responsible for the inhibition of metastasis. An alginate in vivo angiogenesis assay showed significantly decreased tumor vascularization in mice vaccinated with DC-flk1-AP. Although no significant differences in wound healing were observed between vaccinated and control mice, a breeding experiment revealed that the vaccine had striking negative consequences on the number of pregnancies as well as the mean litter number.

In an attempt to overcome immune tolerance, Liu et al. developed a xenogeneic (quail) VEGFR2 vaccine [70]. Immunization with the quail VEGFR2 vaccine inhibited tumor growth in a prophylactic as well as therapeutic setting. Moreover, in an experimental CT26 lung metastasis model the formation of lung metastases was significantly suppressed. A similar homologous mouse VEGFR2 vaccine did not inhibit tumor growth or metastasis. Immunological studies showed that the observed anti-tumor effect was mediated by vaccination-induced anti-VEGFR2 antibodies. Toxicity was thoroughly assessed and no adverse events were observed in quail VEGFR2 vaccinated mice.

Another attractive way of inducing an immune response against VEGFR2 is by DNA vaccination. Niethammer et al. developed a DNA vaccine targeting VEGFR2, carried by attenuated *Salmonella typhimurium* [87]. This orally administered vaccine inhibited primary tumor growth in a prophylactic model as well as experimental and spontaneous metastasis in a prophylactic and therapeutic setting. Surprisingly, when mice were challenged i.v. with CT-26 colon carcinoma 10 months after the last immunization, still a marked decrease in tumor growth could be observed. No antibodies were measured in vivo, but immune cell depletion studies indicated that CD8⁺ T cells were crucial for the anti-tumor response and that CD4⁺ T cells did not play a significant role. Importantly, although vaccination did not interfere with fertility of female mice, wound healing was significantly delayed in vaccinated mice. Currently, a humanized version of this VEGFR2 vaccine is investigated in a phase I clinical trial in patients with advanced pancreatic cancer [86]. Considering the observations made in the pre-clinical phase, it will be crucial to monitor adverse events intensely.

The same research group also reported on another VEGFR2 vaccine [139]. A mini-gene vaccine, based on three minimal H-2D^b-restricted T-cell epitopes from FLK-1 (pHI, pHI-Db, pHI-Kb), was constructed using *S. typhimurium* as a gene delivery vector and investigated in a prophylactic vaccination model. The pHI-Db vaccine was found to be most effective in inhibiting tumor growth, angiogenesis and prolonging survival. Vaccinated mice intravenously challenged with tumor cells 10 months after the last vaccination developed significantly less metastases than control mice. In-depth in vitro immunogenicity studies indicated that CD8⁺ T cells were responsible for the observed anti-tumor effect and that this CD8⁺ T cell immune response was specifically induced against FLK₄₀₀, which is a H-2D^b restricted FLK-1 epitope. In order to provide more proof of the efficacy of this vaccination approach this research group published on a similar VEGFR2 DNA mini-gene vaccine in another mouse strain with a different genetic background (BALB/c) [74]. Five VEGFR2 peptides were included in the studies and all mini-gene vaccines induced tumor protection, showing the general applicability of this approach across different genetic backgrounds with varying MHC restrictions.

Similarly to the group of Niethammer et al., Liang et al. [69] also designed a DNA vaccine aimed at inducing a specific immune response against VEGFR2. In this case, however, the extracellular domain of Flk-1 was fused to C3d3, a complement fragment that enhances the immune response and is applied as adjuvant. The investigators only focused on a humoral immune response and found a relatively high anti-Flk1 specific antibody titer in mice that were immunized with the fusion vaccine. The vaccine was also shown to be effective in inhibiting the growth of BT739 bladder carcinoma and increasing survival in mice.

Wang et al. [130] also created a DNA fusion vaccine against VEGFR2. Here, mFlk-1 was fused to murine β -defensin 2 (MBD2), a peptide involved in both innate and adaptive immunity. In order to induce an immune response in-vivo, the MBD2-mFlk-1 construct was encapsulated with cationic nanoliposomes. Tumor bearing mice prophylactically immunized with the fusion vaccine developed significantly smaller tumors compared to control mice. In an experimental metastasis model therapeutically immunized mice developed significantly fewer lung metastases. Anti-VEGFR2 specific auto-antibodies as well as a VEGFR2 specific CTL response were observed. Although passive immunization experiments with either purified immunoglobulins or isolated T cells tumor growth was significantly inhibited in both cases, immune cell depletion studies demonstrated that tumor growth suppression was mainly dependent on elicited CD4⁺ helper T lymphocytes and to a lesser extent on CD8⁺ T lymphocytes [130].

A vaccine based on an attenuated *Salmonella* strain expressing a fusion gene product consisting of the immunodominant epitope of the murine VEGFR2 fused to the defined N-terminal translocation domain of the type III effector molecule Yersinia outer protein E (YopE) was developed by Jellbauer et al. [55]. In a prophylactic immunization model mice were oro-gastrically immunized once and 30 days later they

were challenged with tumor cells. CD8⁺ KDR2 specific T cells were induced within 6 days after immunization and gradually decreased to pre-treatment numbers on day 31. Further analysis indicated that memory T cells were induced. Tumor growth was inhibited by approximately 50% in vaccinated mice. Furthermore, IHC staining revealed a reduced vessel density. In an experimental metastasis model it was found that this vaccine was also able to inhibit B16 lung metastases. No obvious side effects were detected.

A vaccine consisting of mouse VEGFR2 (mVEGFR2) expressing T4 bacteriophage nanoparticles was developed by Ren et al. [103]. The growth of Lewis lung carcinoma (LLC) was inhibited by 75% in mice that were prophylactically and subcutaneously vaccinated with T4-mVEGFR2 nanoparticles. Through ELISA anti-VEGFR2 antibodies were detected in sera from immunized mice. Moreover, adoptive transfer of these antibodies into tumor bearing mice resulted in marked tumor growth inhibition. Tube formation assays and IHC staining on tumor tissue indicated that this vaccination strategy was able to inhibit angiogenesis. In-vivo depletion of CD8⁺ T cells did not reduce the tumor inhibiting effect whereas depletion of CD4⁺ T cells clearly abrogated the vaccine's anti-tumor activity. This suggests that the induction of a humoral immune response is the main mechanism of action of this vaccine.

Lu et al. [73] combined an orally administered VEGFR2 DNA vaccine with the murine interferon induced protein-10 (IP-10) gene, both carried by a *S. typhimurium* vector. The vaccine was assessed in a therapeutic model in B16.F10 tumor bearing mice. Although monotherapy with the VEGFR2 DNA vaccine or IP-10 already inhibited tumor growth significantly as compared to control mice, the combination treatment clearly enhanced the anti-tumor effects. In a ⁵¹Cr release assay it was shown that the combination treatment induced CTLs with cytotoxic activity against endothelial as well as tumor cells. No noticeable adverse events were observed in immunized mice.

Dong et al. [17] aimed at developing an H-2D^b restricted peptide vaccine targeting VEGFR2. Three peptide epitopes (KDR1, KDR2, KDR3) with predicted high affinity binding to H-2Db molecules were synthesized and emulsified in IFA. In mice immunized with KDR2 and KDR3 CD8⁺ T cells were induced that could lyse VEGFR2 expressing endothelial cells without addition of peptide. In a therapeutic immunization model, mice were challenged with MC38 tumor cells and thereafter immunized three times with the KDR2/KDR3 combined peptide vaccine. The mice immunized with the KDR2/KDR3 vaccine developed significantly smaller tumors. Since this tumor cell line does not express VEGFR2 the observed anti-tumor effect is likely due to the inhibition of tumor angiogenesis. No adverse events were observed in the immunized mice.

Another MHC haplotype restricted peptide vaccine was designed by Wada et al. [128]. They used HLA-A*0201 and HLA-A*2402 restricted VEGFR2 peptides that could induce CTL clones with specific cytotoxicity against target cells expressing VEGFR2. Selected peptides adjuvanted with IFA were administered intradermally to HLA-A2/Kb transgenic mice or C57BL/6 mice after challenging them with MC38 colon carcinoma or B16 melanoma. This vaccination strategy was effective in inhibiting tumor growth and tumor angiogenesis in HLA-A2/Kb transgenic mice, whereas no tumor inhibition was observed in wtC57BL/6 mice demonstrating the restriction of the immune response to the HLA-A2 haplotype.

In 2010 the first clinical results of a trial testing the HLA-A*2402 restricted peptide vaccine were published by Miyazawa et al. [79]. The safety and immunogenicity of this VEGFR2-169 peptide adjuvanted with IFA were studied in dose escalation in combination with gemcitabine in HLA-A*2402 positive patients with unresectable pancreatic cancer. In total 18 patients received weekly subcutaneous injections for at least 4 weeks and were eligible for further analysis. 15 out of 18 patients developed immunological reactions, erythema, and/or induration at the injection sites. In total 24 grade 3 adverse events were recorded (primarily neutropenia). However, no vascular adverse events

were observed. Positive CTL responses specific to the vaccinated peptide were observed in 11 out of 18 patients after one cycle of vaccination. In those patients a significant decrease in regulatory T cells was also detected, indicating that vaccination may help restore cancer immunosurveillance. Importantly, one patient achieved a partial response and 11 other patients achieved stable disease. However, there was no correlation found of CTL response and clinical outcome.

The VEGFR-169 peptide vaccine, here referred to as elpamotide, was studied in another phase I dose-escalation clinical trial in patients with advanced solid tumors [93]. The same dose levels and vaccination schedule as in the study of Miyazawa et al. [79] were applied. In total 10 patients enrolled in this study. No grade 3 or higher adverse events were recorded. Five patients experienced a reaction at the injection site. Furthermore, 2 cases of grade I proteinuria were observed. Microarray analysis on PBMCs isolated pre-treatment and on days 8 and 29 after the first immunization, revealed that primarily genes associated with angiogenesis and T cell functional pathways were differentially expressed. A significant decrease in soluble VEGFR2 concentration in serum was found after 4 weeks. Two patients had stable disease for at least 8 weeks.

These 2 trials indicate that the VEGFR2-169 peptide vaccine has an acceptable safety and tolerability profile. Further clinical studies are being carried out to assess safety, immunogenicity and clinical response in larger patient cohorts.

As live vaccines can help break immune self-tolerance. Seavey [111] developed a fusion vaccine consisting of one of three fragments of the mouse VEGFR2 molecule (E1, E2 or I1) coupled to listeriolysin-O (LLO) (adjuvant) and expressed it in *Listeria monocytogenes* (Lm). The growth of NT 2 Her 2/neu expressing tumors was inhibited in mice vaccinated with Flk-E1 and Flk-I1 whereas Flk-E2 was unable to inhibit tumor growth. A significant reduction in vessel density was found in tumors derived from Flk-E1 and Flk-I1 vaccinated mice. IFN γ ELISPOT data indicated that Flk-E1 and Flk-I1 vaccination induced epitope spreading against Her-2/neu epitopes. Immunization of Flk-E1 and Flk-I1 failed to inhibit tumor growth in Her-2/neu transgenic mice, in which epitope spreading to Her-2/neu was inhibited by self-tolerance, indicating that the induced anti-angiogenic effects were not sufficient to inhibit tumor growth and that this vaccine has direct anti-tumor effect in addition to the anti-angiogenic effects. No negative effects on wound healing, pregnancy or fertility were found.

4.4.6. Tie-2

Tie-2 is overexpressed on activated endothelial cells and is important in tumor angiogenesis. Ligands for Tie-2 are the angiopoietins [49]. Luo et al. [75] investigated a chicken Tie-2 protein vaccine as a model to break immune tolerance. This vaccine, adjuvanted with aluminum hydroxide was assessed in an experimental metastasis model. It induced anti-Tie-2 antibody titers and prevented tumor growth, as well as reduced the number of lung metastases. No adverse events were recorded.

Ramage et al. [102] constructed a DNA vaccine containing one of four modified Tie-2 MHC class I epitopes, as predicted by two computer programs. The vaccine containing the modified Z84 epitope was shown to induce CTLs, which could specifically lyse human endothelial cells overexpressing Tie-2. No tumor experiments were conducted with this vaccine, but the authors did not observe any side effects in immunized mice.

4.4.7. Endoglin

Endoglin (also referred to as CD105) is a transmembrane homodimeric glycoprotein, which is primarily expressed on endothelial cells. It acts as a co-receptor for TGF-beta. Different studies have described the importance of endoglin in angiogenesis and tumor growth [67,97]. Although the specificity and the function of endoglin overexpression on tumor endothelial cells can be disputed [36], several studies report overexpression [7]. Tan et al. [118] explored the efficacy of a xenogeneic

(porcine) protein vaccine adjuvanted with aluminum hydroxide targeting endoglin. When administered prophylactically mice were significantly protected from tumor growth and the number of lung metastases was reduced by almost 80%. Moreover, therapeutic immunization of tumor bearing mice also resulted in apparent tumor growth inhibition. As measured by ELISA, the vaccine induced antibodies, which recognized porcine endoglin as well as mouse endoglin. Adoptive transfer of these antibodies in mice resulted in effective protection from tumor growth. Depletion of CD4⁺ T cells resulted in abrogation of tumor growth whereas depletion of CD8⁺ T cells or NK cells did not affect tumor growth. Importantly, this vaccine did induce a delay of wound healing. This research group also published on a combination treatment of the porcine endoglin vaccine (pEDG) together with low-dose cisplatin [117]. This combination strategy synergistically inhibited tumor growth and prolonged survival, while no other adverse events were observed.

All other reports on endoglin vaccines used DNA vaccination technology in order to induce an anti-endoglin immune response. Lee et al. [63] a DNA vaccine encoding murine endoglin with *S. typhimurium* as carrier. The hypothesis was that the vaccine would be delivered to secondary lymphoid organs, such as the Peyer's patches in the small intestines to facilitate subsequent priming of specific T cells. BALB/c mice were immunized once weekly for 3 consecutive weeks by oral gavage. One week after the last immunization all mice were challenged with D2F2 murine breast carcinoma cells. The endoglin vaccine was shown to be effective in suppressing pulmonary metastases and in vivo depletion studies showed that the anti-tumor response was primarily dependent on CD8 + T cells.

Wood et al. [133] also developed two endoglin DNA vaccines (two different regions of endoglin) with *Listeria* as carrier. Truncated listeriolysin O (LLO) was used as adjuvant protein. Both vaccines proved to be effective at inhibiting tumor growth and metastasis in 3 different mammary tumor models. Different in vitro assays suggested that the anti-tumor response was primarily mediated by CD8 + T cells. qPCR showed a reduced expression of CD31, suggestive of an anti-angiogenic effect.

Recently Jarosz et al. [54] published a paper in which an endoglin-based DNA vaccine was investigated in combination with an immunomodulatory agents. As a carrier of this DNA vaccine, an attenuated strain of *S. typhimurium* SL7207 was used. IL-12 and cyclophosphamide were used as immunomodulating agents. Tumor growth was inhibited by this vaccine in 2 different tumor models in prophylactic and therapeutic settings. Combining the vaccine with either IL-12 or cyclophosphamide resulted in an even more significant inhibition of tumor growth and a marked extension of the durability of the anti-tumor effects compared to vaccine, IL-12 or cyclophosphamide monotherapy.

4.4.8. Delta-like ligand 4 (DLL4)

As the endothelial tip cell is an indispensable player in sprouting during angiogenesis, it has been hypothesized to be an attractive target for effective immunization. Haller et al. [42] reported on a xenogeneic cDNA vaccine encoding human DLL4, an essential component of tip cell integrity. Mice received 3 intradermal injections followed by in vivo electroporation. Mice were then challenged with D2F2/E2 or TUBO cells in the mammary fat pad. DLL4 immunization resulted in anti-DLL4 specific antibody responses as well as a significant 61–64% inhibition of tumor growth. In vivo depletion of CD4⁺ or CD8⁺ T cells after immunization did not abrogate tumor growth inhibition, indicating the major role of anti-DLL4 antibodies. Adoptive transfer of induced antibodies resulted in marked tumor growth inhibition, confirming this hypothesis. No prolonged wound healing or other adverse effects were observed.

4.4.9. PDGFR β

Targeting tumor stroma can also induce anti-angiogenic effects. This strategy was investigated by Kaplan et al. [60], who developed a DNA

vaccine targeting platelet derived growth factor receptor-beta (PDGFR β). This receptor tyrosine kinase (RTK) is overexpressed by tumor stromal fibroblasts and pericytes. A ^{51}Cr release assay revealed that the vaccine was able to induce CTLs that specifically lysed mouse PDGFR β (mPDGFR β) expressing target cells whereas no cytotoxicity was observed against target cells that did not express mPDGFR β . In both prophylactic as well as therapeutic immunization experiments primary tumor growth and metastasis formation was greatly inhibited. In an in-vivo matrigel plug assay it was demonstrated that activity was mainly through an anti-angiogenic effect. The authors did not report on potential adverse events.

4.5. Dual targeted vaccines: anti-angiogenic and anti-tumor vaccination

Several angiogenesis markers are expressed on tumor cells as well as on endothelial cells, such as survivin and legumain. Immunization against such antigens has the advantage that both endothelial cells and tumor cells are being targeted, which could enhance tumor growth inhibition.

4.5.1. Survivin

Survivin is an apoptosis inhibitor, overexpressed in tumor cells as well as activated endothelial cells. Xiang et al. [134] developed a DNA vaccine encoding survivin and chemokine CCL21 with the aim to induce a T cell response against both tumor cells and tumor vasculature. In an experimental lung metastasis model in which mice were intravenously challenged with tumor cells, the vaccine proved effective in reducing metastasis formation in both prophylactic and therapeutic immunization settings. The effect on primary tumor growth was not studied. Splenocytes obtained from immunized mice and control mice were assessed for cytotoxicity in a standard ^{51}Cr release assay. Splenocytes from immunized mice induced specific lysis of survivin expressing D121 tumor cells and survivin expressing murine endothelial cells, whereas splenocytes isolated from control mice were ineffective at inducing a CTL response. An in-vivo matrigel plug assay demonstrated suppression of tumor neovascularization. No adverse effects on wound healing and fertility were observed, suggesting that physiological angiogenesis was not impaired upon vaccination.

Ladser et al. published two reports on a naked DNA vaccine targeting survivin [72,71]. In the first report mice were given three intramuscular injections with human survivin expressing plasmids together with a plasmid coding for the murine granulocyte-macrophage colony-stimulating factor (pGM-CSF). Two different expression plasmids (pcDNA3.1 and pSecTag2) were engineered in order to yield respectively either intracellular or secreted human survivin protein. In the majority (9/16) of mice immunized with the pSecTag2/Survivin vaccine anti-survivin antibodies were induced, particularly of the IgG2a isotype. In contrast, in only 2 out of 11 mice immunized with the pcDNA3.1/Survivin vaccine anti-survivin antibodies could be detected. Both vaccines induced an increase in IFN γ producing CD8 $^{+}$ T cells. These results indicate that the pSecTag2/Survivin vaccine induces primarily a Th1 CD4 $^{+}$ cell driven immune response. Unfortunately, no tumor protection experiments were performed. In the second report, the pcDNA3.1/human survivin vaccine was administered through intradermal injection followed by electroporation. The amino acid sequence survivin $_{20-28}$ was identified as a good candidate epitope to be presented by MHC class I molecules. Lymphocytes isolated from vaccinated mice in vitro stimulated with human survivin $_{20-28}$ peptide produced significantly more IFN γ as opposed to lymphocytes stimulated with a control peptide. A ^{51}Cr release assay demonstrated cytotoxic activity against survivin $_{20-28}$ -pulsed target cells. In an in-vivo matrigel plug assay anti-angiogenic properties of this vaccine were demonstrated. Unfortunately, no attempts were made to investigate the humoral immune response and its possible role in tumor protection [71].

Zhu et al. [140] developed another naked DNA vaccine targeting survivin. Prophylactic immunization resulted in slower tumor growth

and prolonged survival in the Panc02 pancreatic cancer model as well as the A20 lymphoma model. Tumor tissue derived from immunized mice contained significantly more CD3-positive T lymphocytes than control tumor tissue. In an ELISPOT no IFN γ production could be measured, leaving the exact mechanism of action of this vaccine unclear. However, this study did indicate that xenogeneic DNA vaccines may not always be more immunogenic than autologous DNA vaccines.

Several clinical studies already have been performed investigating survivin vaccines in cancer patients [5,48,64]. These studies showed that it is possible to induce survivin specific CD8 $^{+}$ T cells. However, the question whether these vaccines had an anti-angiogenic effect was not addressed.

4.5.2. Legumain

Legumain is a cysteine endopeptidase, which is overexpressed on tumor cells, tumor associated endothelial cells and tumor associated macrophages (TAMs). Luop et al. [76] have designed a vaccine targeting legumain in order to reduce the amount of TAMs. This DNA vaccine was administered orally and carried by attenuated *S. typhimurium*. Prophylactic vaccination reduced the number of lung metastases in an experimental D121 NSCLC model. In a therapeutic immunization model it was shown that 6 out of 8 immunized mice survived for 3 months. A ^{51}Cr release assay showed that splenocytes isolated from immunized mice had cytotoxic activity against macrophages with high expression of legumain. In vivo immune cell depletion experiments revealed that in particular CD8 $^{+}$ T cells were important for the anti-tumor response. The elimination of TAMs induced a reduction of pro-angiogenic growth factors and immunosuppressive cytokines measured in serum. The inhibition of angiogenesis was confirmed in an in vivo Matrigel plug assay, by a reduction in blood vessels in immunized mice.

The same group published a DNA minigene vaccine, only inducing an immune response against the CTL epitope of interest [65]. Three legumain peptides restricted through H-2 D d and three peptides restricted through H-2 K d were investigated for their ability to induce an anti-TAM immune response. Mice immunized with the legumain H-2 K d DNA minigene vaccine, but not the H-2 D d vaccine, developed an anti-angiogenic activity giving rise to smaller primary tumors and a reduced number of metastases as compared to control mice.

4.5.3. Other targets

Nair et al. [85] designed a dendritic cell (DC) based combination vaccine targeting different angiogenesis-associated antigens and tumor antigens. Mice received intravenous injections with DCs transfected either with VEGF, VEGFR2, Tie-2, tyrosine-related protein 2 (TRP-2), telomerase reverse transcriptase (TERT), the murine bladder tumor antigen MBT-2, or with a combination of 2 of these antigens. A dual DC vaccine targeting both an angiogenesis-associated antigen (i.e., VEGF) and a tumor antigen (i.e., TERT) was shown to be superior in providing tumor growth inhibition and inhibition of metastasis formation. Interestingly, short term fertility (2 weeks after immunization) was unaffected in mice immunized against VEGF, whereas upon vaccination against VEGFR2, fertility and litter size were temporarily significantly reduced.

Fu et al. described a vaccine against HP59, the target of the anti-angiogenic polysaccharide CM101, with anti-angiogenic and antitumor properties [27]. Mice that received 3 immunizations with peptides derived from the amino acid sequence of HP59 conjugated to KLH, resisted a challenge with Lewis Lung Carcinoma cells with over 60% tumor growth inhibition, through inhibition of angiogenesis.

Very recently a promising therapeutic vaccine has been developed against the extracellular domain of Robo4, a member of the magic roundabout family, which is expressed exclusively at sites of active angiogenesis. Fusion of this domain to the Fc-portion of human immunoglobulin and vaccination of Lewis lung carcinoma bearing mice, resulted in impaired fibrovascular invasion and angiogenesis [141].

5. Future perspectives

As evidenced by the impressive body of data described in this review and the sometimes very promising results emerging, it can be concluded that targeting of angiogenesis by vaccination is an emerging new treatment strategy that combines the advantages of targeting endothelial cells over tumor cells and active immunization over monoclonal antibodies or TKIs. As shown in this review a wide range of pre-clinical studies has shown the validity of this concept. Furthermore, the first Phase I/II clinical trials on anti-angiogenic vaccination show that this treatment strategy is in general well tolerated and that it is possible to induce immune responses against self-antigens upon vaccination, even in heavily pretreated advanced cancer patients. Some complete and partial responses were achieved in the different Phase I clinical trials, and the field of research is currently on the verge of moving into later stage clinical trials to confirm this gleaned clinical efficacy. These efforts have to demonstrate whether anti-angiogenic vaccination will ultimately result in clinical benefit and improved survival in larger cohorts of advanced cancer patients.

Currently available anti-angiogenic drugs have proven to result in only limited clinical benefit. An emerging finding of previous clinical studies is that prolongation of progression free survival can be achieved, but overall survival is in most cases unaffected. In addition, when tested in early stage cancer, there seems to be no benefit of angiogenesis inhibitors, possibly due to the low angiogenic potential at this stage [2]. One argument is that in most cases anti-angiogenic drugs are supplied in combination with chemotherapy. Early stage and non-metastasized tumors may be more sensitive to the chemotherapy, masking the limited effect of the anti-angiogenic drug. A very important issue in the field of vaccination is the fact that for breaking self-tolerance a vaccine should be given in the context of a very efficient adjuvant or checkpoint inhibitors. Of note, successful vaccination strategies with certain adjuvants in mice may not be directly translatable to success in patients. This issue was deemed beyond the scope of this review and has therefore been omitted from the discussions contained within this review. In this regard one is referred to the review by Dubensky et al. [18].

An important issue in cancer vaccination strategies is the reversibility of the immune response induced by these vaccines. This issue is mainly relevant in situations where the target of vaccination is not only expressed at the tumor site. In case of vaccination approaches against tumor endothelial cell targets that are expressed elsewhere in the body as well, it will be crucial to find ways to temporarily block the induced immune response when toxicity issues would dictate so. Also, when vaccination related toxicities evolve, this would be an important strategy. This could for example be accomplished by intravenous injection of target antigen derived peptides, which can bind and neutralize vaccination-induced antibodies. Alternatively, immune suppressive compounds such as dexamethasone and cyclosporine may be supplied. Nevertheless, it would be preferential to vaccinate against antigens that are exclusively expressed in the tumor vasculature. This way physiological angiogenesis remains unaffected, which will decrease the chances of adverse events. The search for such antigens is currently an essential focal point for future studies [4,121].

An interesting observation in some of the reviewed studies was that in the majority of the clinical trials no correlation between the induced immune response and the clinical outcome was found. This may be explained by other immunological mechanisms underlying the clinical responses than the ones that are monitored. It is therefore of utmost importance to elucidate the mechanisms of action of the observed anti-tumor effects and to identify robust biomarkers to predict clinical outcome.

Finally, it is generally expected that anti-angiogenic vaccination strategies will be applied in combination with conventional anti-cancer strategies, as is the case for the current anti-angiogenic drugs. If this is the case, it will be essential to investigate what are the most efficient combination treatment strategies. Miyazawa et al. [79] already

showed that VEGFR2 vaccination in combination with gemcitabine is safe but future studies should assess whether combination therapy is superior over vaccination as monotherapy. In terms of combination therapies, it is very feasible to develop vaccines directed at multiple targets. Such strategies could combine different vascular targets, vascular targets and tumor cell targets, or, e.g., a combination of these with a vaccination strategy against the immune checkpoint molecules, such as CTLA-4 and PD/PDL-1. A review on the quest for anti-angiogenic combination therapy has been published recently [40]. A major attraction of anti-angiogenic cancer vaccination is that it may result in the near future in dual- or even triple therapy strategies, combined in a single vaccine.

Transparency document

The [Transparency document](#) associated with this article can be found, in the online version.

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