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Therapeutic Cancer Vaccines Targeting Molecules Associated with Tumor Angiogenesis

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Abstract
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Induction of an endogenous antibody response by therapeutic vaccination could provide an alternative to cost-intensive monoclonal antibody-based treatments for cancer. Since the target of a cancer vaccine will most likely be a self-antigen, self-tolerance of the immune system must be circumvented. Using fusion proteins consisting of the self-antigen to be targeted and a part derived from a foreign antigen, it is possible to break tolerance against the self-antigen. Furthermore, a potent adjuvant is required to support an immune response against a self-molecule. Currently no adjuvant suitable for this purpose is approved for use in humans.

This thesis describes the development of a therapeutic vaccine targeting the vasculature of tumors. As tumor cells have developed strategies to escape immune surveillance, targeting of molecules associated with the tumor stroma is an interesting alternative. The alternatively spliced extra domain-A and B (ED-A and ED-B) of fibronectin and the glycan-binding protein galectin-1 are selectively expressed during events of tumor angiogenesis. We have designed recombinant proteins to target ED-B, ED-A and galectin-1, containing bacterial thioredoxin (TRX) as a non-self part, resulting in TRX-EDB, TRX-EDA and TRX-Gal-1. Vaccination against ED-B induced anti-ED-B antibodies and inhibited growth of subcutaneous fibrosarcoma. Immunization against ED-A decreased tumor burden and reduced the number of lung metastases in the MMTV-PyMT model for metastatic mammary carcinoma in a therapeutic setting. Analysis of the tumor tissue from ED-B and ED-A-immunized mice indicated an attack of the tumor vasculature by the immune system. Finally, we show that galectin-1 immunization reduced tumor burden and increased leukocyte numbers in the tumor tissue. Galectin-1 is pro-angiogenic and immunosuppressive, and therefore allows simultaneous targeting of fundamental characteristics of tumorigenesis. We furthermore show that the biodegradable squalene-based Montanide ISA 720 combined with CpG oligo 1826 (M720/CpG) is at least as potent as Freund's adjuvant with respect to breaking self-tolerance, when comparing several immunological parameters. Freund's is a potent but toxic adjuvant used in the majority of preclinical studies.

The work presented in this thesis shows that therapeutic cancer vaccines targeting the tumor vasculature are a feasible and promising approach for cancer therapy.

Keywords: tumor, therapeutic, cancer vaccine, angiogenesis, vasculature, fibronectin, galectin-1

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To my parents

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I Huijbers, E.J., Ringvall, M., **Femel, J.**, Kalamajski, S., Lukinius, A., Åbrink, M., Hellman, L. and Olsson, AK. (2010) Vaccination against the extra domain-B of fibronectin as a novel tumor therapy. *FASEB J* 24(11):4535-4544

II Huijbers, E.J., **Femel, J.**, Andersson, K., Björkelund, H., Hellman, L. and Olsson, AK. (2012) The non-toxic and biodegradable adjuvant Montanide ISA 720/CpG can replace Freund's in a cancer vaccine targeting ED-B - a prerequisite for clinical development. *Vaccine* 30(2):225-230

III **Femel, J.**, Huijbers, E.J., Saupe, F., Cedervall, J., Zhang, L., Roswall, P., Larsson, E., Olofsson, H., Pietras, K., Dimberg, A., Hellman, L. and Olsson, AK. (2014) Therapeutic vaccination against fibronectin ED-A attenuates progression of metastatic breast cancer. *Submitted manuscript*

IV **Femel, J.**, Saupe, F., Huijbers, E.J., Verboogen, D.R., Cedervall, J., Thijssen, V.L., Hellman, L., Griffioen, A.W. and Olsson, AK. Targeting galectin-1 by vaccination suppresses tumor growth and promotes leukocyte recruitment to the tumor tissue. *Manuscript*

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The cover picture shows the expression of the extra domain-A of fibronectin (red) in tumor tissue from the MMTV-PyMT model of mammary carcinoma. Blood vessels are depicted in green and nuclei in blue.

Contents

Abbreviations

ANG	Angiopoietin
APC	Antigen-presenting cell
BCR	B cell receptor
CSF	Colony stimulating factor
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DLL	Delta-like ligand
ECM	Extracellular matrix
ED-A	Extra domain-A
ED-B	Extra domain-B
EMT	Epithelial-mesenchymal transition
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
HIF	Hypoxia-inducible factor
IFN	Interferon
IL	Interleukin
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
ODN	Oligodeoxynucleotides
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PlGF	Placental growth factor
PRR	Pattern recognition receptor
RTK	Receptor tyrosine kinase
TAM	Tumor-associated macrophage
TCR	T cell receptor
TGF-β	Transforming growth factor-β
TKI	Tyrosine kinase inhibitor
TLR	Toll-like receptor
Treg	Regulatory T cell
TRX	Thioredoxin
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

Introduction

The tumor microenvironment

Solid tumors arise from transformed cells that have acquired the ability to proliferate in a deregulated manner. Tumor cells have accumulated mutations, which enable them to grow independently from external growth promoting and suppressing signals, replicate unlimitedly and avoid cell death (1). However, in addition to genetic alterations sustaining proliferation, tumors require access to oxygen and nutrients in order to grow. Angiogenesis, the formation of new blood vessels from pre-existing ones, was early associated with tumors (2). It is now known that tumors are not only comprised of malignant cells, but also of other cell types as well as extracellular matrix (ECM), together composing the tumor stroma. It has become increasingly evident that the tumor cells co-opt the components of the stroma and establish a microenvironment that protects from immune recognition and promotes tumor progression. Eventually, tumors invade the surrounding tissue and disseminate to distant sites, where similar mechanisms support growth of metastases (1). The following pages present an overview over the components of the tumor stroma and processes within the tumor microenvironment.

The tumor stroma

Stromal cells include endothelial cells comprising the blood and lymphatic vessels, perivascular cells (smooth muscle cells or pericytes), fibroblasts, adipocytes and different types of immune cells of the innate and adaptive immune system (Figure 1). Together with the stroma the tumor cells form an organ-like structure, although it displays abnormalities with respect to function and structure (3, 4).

The conditions within the tumor stroma, with respect to cellular and matrix composition and activated pathways, resemble the process of wound healing. By exploiting this physiological program, including angiogenesis, inflammation and tissue remodeling, tumor cells manage to create an environment optimal for their proliferation and dissemination to distant sites in the body. However, while the normal purpose of wound healing is a re-establishment of a functional tissue, the structural organization of the stroma becomes more abnormal the further tumor growth progresses, causing tumors to be "wounds that never heal" (5, 6). The impact of the stromal com-

partment on tumor progression is highlighted by the prognostic potential of tumor composition or gene expression profiles of stromal cells (7-10).
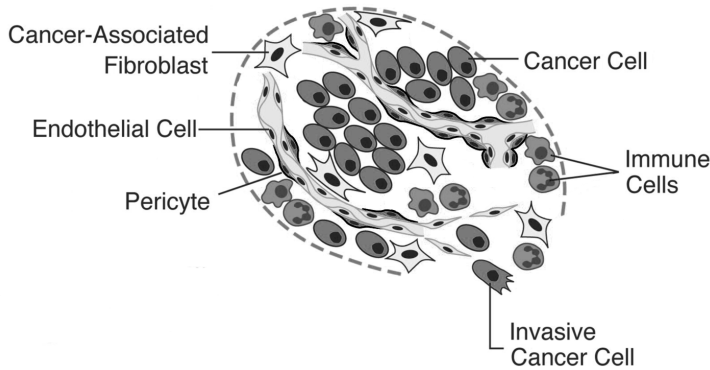

Figure 1. The tumor stroma. In addition to malignant cells, tumors consist of a variety of other cell types, which together with the extracellular matrix form the tumor stroma. Adapted from (2).

Tumor angiogenesis

The normal vasculature is organized in a hierarchic system. Capillaries, the smallest vessels of the vascular system, consist of endothelial cells forming a lumen. The endothelial cells are surrounded by the basement membrane, a specialized type of the ECM, into which pericytes are embedded (11). Pericytes, cells of the smooth muscle-lineage, have direct cell contacts with the endothelial cells. By ECM deposition and surface receptor/ligand interactions pericytes provide structural stability and maintain a quiescent state of the vasculature. (12, 13). Larger vessels, which divide into arteries, arterioles, veins, and venules, show a different architecture. To resist shear stress from pulsatile blood flow, the arteries are located in a collagen and fibroblast-rich ECM and are densely covered by contractile smooth muscle cells. The veins are irregularly enclosed by vascular smooth muscle cells, but contain valves to prevent backflow of the blood due to the low pressure (11, 14).

The angiogenic switch

As long as a tumor is dormant and not bigger than 2-4 mm^3, tumor cells acquire oxygen by diffusion. To grow beyond that size, the tumor requires access to the vascular system to obtain oxygen and nutrients (15). Tumors accomplish this by stimulating growth of new blood vessels from preexisting ones, a process termed angiogenesis. In a healthy tissue blood vessels are kept quiescent through a tightly regulated balance between proangiogenic factors and angiogenesis inhibitors (1, 16). Angiogenesis occurs only under very few conditions in a healthy, adult body, such as wound heal-

ing or the female menstrual cycle. However, in response to hypoxia within a growing tumor, cells increase the expression of molecules able to induce blood vessel growth. When the balance of regulators tips in favor of the pro-angiogenic factors, the angiogenic program will be activated. This event is referred to as the "angiogenic switch" (16). Independently from hypoxia, pro-angiogenic factors can be expressed and anti-angiogenic factors suppressed by tumor cells in response to oncogene activation or tumor suppressor inactivation (16, 17). The key stimulator of angiogenesis is vascular endothelial growth factor A (VEGF-A).

Molecular regulation of angiogenesis

Vascular endothelial growth factor A

The key stimulator of angiogenesis is vascular endothelial growth factor A (VEGF-A). VEGF-A is a member of the VEGF growth factor family, which regulate angiogenesis and lymphangiogenesis. The VEGF family members include VEGF-A, -B, -C, -D and placental growth factor (PlGF). Homodimers of the growth factors are ligands to three VEGF receptor (VEGFR) tyrosine kinases, VEGFR-1, -2 and -3, to which they bind in an overlapping pattern. The VEGF receptors are found as homo- or heterodimers on the surface of endothelial cells. Binding of VEGF-A to VEGFR-2 leads to the activation of different pathways regulating endothelial cell proliferation, migration and survival (17, 18). The VEGF-A/VEGFR-2 pathway is essential for angiogenesis and de-novo formation of blood vessels (vasculogenesis). Knockout of either growth factor or receptor causes severe vascular defects and is embryonically lethal (19-21). VEGFR-1 has been suggested as a negative regulator of angiogenesis during development, as lack of VEGFR-1 in mice is lethal due to excessive endothelial cell proliferation and obstruction of the vessel lumen (22). Different isoforms of VEGF-A, which have different functional implications, are produced through alternative splicing. The larger isoforms VEGF-A$_{165}$ and VEGF-A$_{189}$ are both ligands for neuropilin 1, a co-receptor for VEGFR-2 (17). Furthermore they bind to heparan sulfate proteoglycans, which are components of the ECM. Upon ECM degradation the VEGF molecules are released and form concentration gradients, which guide migrating tip cells of angiogenic sprouts (23, 24).

VEGF-expression, as well as expression of various other pro-angiogenic factors, is regulated by the transcription factor hypoxia-inducible factor 1 (HIF-1) (25). HIF-1 is a protein heterodimer consisting of two subunits, HIF-1α and HIF-1β. While the β-subunit is constitutively expressed, the α-subunit is regulated at the protein level in an oxygen-dependent manner. Under normoxia the oxygen-dependent enzymatic hydroxylation of two prolyl residues in the HIF-1α molecule enables the interaction with the Von Hippel-Lindau E3 ubiquitin ligase complex, which degrades the protein subunit. Hypoxia prevents hydroxylation of the prolyl residues and therefore

degradation of HIF-1α, and both subunits dimerize. The HIF-1 molecule induces gene expression by binding to a sequence-motif termed hypoxia-responsive element, which is contained in the promoter region of various pro-angiogenic factors (26).

Besides VEGF-A other growth factor pathways have the ability to induce and regulate angiogenesis. Among these are angiopoietins (ANG) and the TIE receptors, and the fibroblast growth factors (FGFs) with corresponding receptors (15). ANG-1/TIE-2 signaling has been demonstrated to contribute to vascular quiescence (27), while FGFs represent alternative growth factors that contribute to resistance against anti-VEGF therapy (28).

Sprouting and maturation of blood vessels

A vascular sprout usually consists of a leading cell, the "tip cell", which is followed by a number of "stalk cells". The tip cell follows the gradient of pro-angiogenic molecules, such as VEGF-A, released by cells experiencing hypoxia, and through matrix metalloproteinase (MMP) cleavage of the ECM (14, 15). The stalk cells proliferate to extend the growing sprout. The signaling of Delta-like ligand 4 (DLL-4) and the Notch receptors is one of the crucial pathways determining the stalk cell phenotype. Expression of DLL-4 is elevated in the tip cells upon VEGF-A signaling. DLL-4 then binds to Notch 1 and 4 expressed on the adjacent stalk cells, which suppresses VEGFR-2 expression. Disruption of DLL-4/Notch signaling leads compromised vessel formation (29-31). The selection of a tip cell is not a one-time, but a dynamic process and a previous stalk cell might take over the position as leading cell (32, 33). The formation of a vascular lumen occurs in the stalk of the angiogenic sprout and enables blood flow, which improves oxygenation and results in a decrease in VEGF-A expression (34).

The endothelial cells forming a new vascular lumen secrete platelet-derived growth factor BB (PDGF-BB). PDGF-BB attracts pericytes, which express the PDGF receptor β (PDGFR-β) on their cell surface (12, 13). Pericytes form direct contacts with the endothelial cells, which, together with paracrine signaling, permit cell communication between these cell types. Pericytes are able to cover several endothelial cells through extending long cell processes, which support vascular integrity. While PDGF-BB appears to be the most important growth factor facilitating pericyte recruitment, other pathways, such as the ANG-1/TIE-2 receptor pathway and transforming growth factor-β (TGF-β) signaling have been implied in vessel stabilization by pericytes (11-13).

Besides angiogenesis other mechanisms of blood vessel growth have been observed in tumors. Vessels can be assembled *de novo* from vascular endo-thelial precursor cells, a process termed vasculogenesis, which occurs most commonly during embryogenesis. Furthermore, intussusception can generate new vessels by the longitudinal separation of initially one blood vessel (14, 15, 27). Also incorporation of endothelial progenitor cells recruited from the

bone marrow or "vascular mimicry" by tumor cells has been described (1, 15, 27).

Characteristics of the tumor vasculature

The vasculature that results from tumor angiogenesis is very different from the blood vessels that are product of physiological angiogenesis during embryogenesis or wound healing. Hierarchic organization of venules, arterioles and capillaries is lost in the tumor vasculature, and the vessels appear disorganized and tortuous (Figure 2) (35).
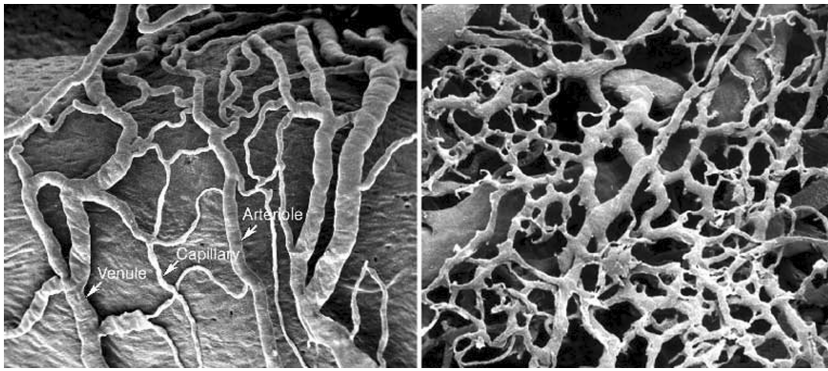

Figure 2. Normal and angiogenic vasculature. Scanning electron microscopy (SEM) image of polymer cast of normal (left) and tumor microvasculature (right). Normal vessels show a hierarchic organization of arterioles, venules and capillaries. Tumor microvasculature is chaotic and vessel hierarchy is lost. Reprinted with permission from (35).

Due to the rapid proliferation of the tumor cells, the growth of the tumor is constantly ahead of the growing vasculature and hypoxia is a permanent state. The persistent high levels of angiogenesis-promoting growth factors prevent appropriate maturation of the vasculature and impair pericyte recruitment, resulting in reduced pericyte coverage or compromised cell contacts to the endothelium (11, 36, 37). This causes tumor vessels to be leaky and poorly perfused, which furthermore sustains hypoxia and an acidic pH in the tumor microenvironment (38, 39).

Another effect of VEGF-A signaling is stimulation of increased permeability. As this was the initial function identified for VEGF-A (40), it is also known as vascular permeability factor (VPF). In response to VEGF-A signaling separated vacuoles in endothelial cells fuse and intercellular junctions between endothelial cells open, which causes plasma and proteins to leak from the blood vessels into the surrounding tissue (41). Additionally, the lymphatic vasculature, which is normally responsible for returning protein-rich fluid back into the blood circulation (42), is often poorly functioning in tumors. The rapid proliferation of the tumor cells results in a high intra-

tumoral pressure, which leads to the collapse of the lymphatic vasculature, disabling them from draining fluid from the tissue. The high permeability of the endothelium, together with poor assembly of the tumor blood vessels and the deficiency of fluid drainage by lymphatic vessels results in tissue swelling (edema) and an elevated interstitial fluid pressure (IFP). High IFP within tumors impairs delivery of chemotherapeutic agents to the tumor cells and thereby reduces therapy efficacy (38).

Continuous presence of VEGF-A furthermore affects expression of other endothelial proteins, such as surface receptors. Endothelial adhesion molecules on the luminal side of endothelial cells are downregulated upon exposure to pro-angiogenic factors such as VEGF-A. This prevents interactions of leukocytes with the endothelium and leads to decreased lymphocyte infiltration into the tumor tissue. For the tumor this is of advantage, as it supports evasion from an anti-tumor immune response (43-47).

Anti-angiogenic therapy

Based on the knowledge that neovascularization was indispensible for tumor growth, in 1971 Judah Folkman proposed the strategy of "anti-angiogenesis" (2). He suggested that by inhibiting angiogenesis, growth of tumors could be stopped, which ultimately would help eradicate them. In fact, he suggested the development of an antibody targeting the so-called "tumor angiogenesis factor", the growth factor inducing vessel growth in tumors (2). Intense research during the following decades led to the discovery of a number of pro-angiogenic factors, the first of which were FGF-2 (48) and VEGF-A (40, 49, 50). Later receptor tyrosine kinases (RTKs) involved in angiogenic signaling and endogenous angiogenesis inhibitors were discovered (51). This enabled the development of drugs targeting the pro-angiogenic molecules and their receptors, such as antibodies and tyrosine kinase inhibitors (TKIs). Due to the significance of the VEGF-pathway for tumor vascularization, drugs targeting this pathway remain the major group of angiogenesis inhibitors approved for clinical use.

Anti-angiogenic drugs

In 2004 Bevacizumab (Avastin), a humanized monoclonal antibody that neutralizes VEGF-A (52), was the first anti-angiogenic drug approved by the American Food and Drug Administration (FDA) for treatment of cancer (metastatic colorectal cancer). Today, bevacizumab is used in combination with chemotherapy as first-line treatment for non-small-cell lung cancer, and as first- and second-line treatment for metastatic colorectal cancer. Furthermore, it is used in combination with cytokine-therapy for metastatic renal cell carcinoma. Monotherapy with bevacizumab has failed in a number of trials, and is currently only used as second-line treatment for glioblastoma multiforme (53, 54). While a number of phase III trials with bevacizumab in combination with chemotherapy demonstrated a significant increase in pro-

gression-free survival, an increase in overall survival was not achieved. In general, the survival benefits, progression-free or overall, were modest and within the range of a few months (53). End of 2011 the FDA revoked the approval for use of bevacizumab in metastatic breast cancer, as no benefit in progression-free or overall survival could be demonstrated and quality of live of breast cancer patients was not improved (54).

Another group of angiogenesis inhibitors are the small-molecule TKIs. These molecules compete with ATP for the ATP-binding site of RTKs, which is highly conserved among all protein kinases. This prevents the kinase from transferring the phosphate group from ATP to their target protein and activating it (55). Unlike an antibody, these small molecule inhibitors can act intracellularly, as they are hydrophobic and can pass the cell membrane, and show a broadened specificity (55). Examples for TKIs are sunitinib (Sutent), sorafenib (Nexavar) and pazopanib (Votrient). Sunitinib and sorafenib were initially designed for VEGFR-2 inhibition, but are know to inhibit PDGFR-β, FGF receptor 1 (FGFR-1), Raf, fms-related tyrosine kinase-3 (FLT-3) and c-Kit. Sunitinib furthermore binds to and inhibits PDGFR-α, and colony stimulating factor 1 receptor (CSF-1R) (53, 55, 56). Pazopanib has been developed as a multi-targeted TKI and inhibits VEGFR-1, 2 and 3, PDGFR-α and β, FGFR-1 and 3, and furthermore c-Kit (57). All three TKIs are approved as monotherapies for renal cell carcinoma. Furthermore, sorafenib is approved for treatment of hepatocellular carcinoma, and sunitinib for gastrointestinal stromal tumors (53, 58). The less restricted specificity of the inhibitors might be an explanation why these drugs have showed clinical benefit in the monotherapy-setting, unlike bevacizumab.

Mechanisms of action
Several mechanisms of action have been described for angiogenesis inhibitors, of which a selection will be described below. Jain proposed the concept of vascular "normalization", suggesting that anti-angiogenic therapy might revert the aberrant phenotype of tumor vessels, and by this increase oxygenation and improve the delivery of chemotherapy to the tumor (59). This hypothesis might explain the clinical benefits of combining bevacizumab and chemotherapy. Furthermore, anti-angiogenic treatment has been demonstrated to restore the expression of adhesion molecules on the endothelium, which are required for interaction of leukocytes with the vessels walls. This might improve tumor immunity by increasing the influx of leukocytes including tumor-targeting cytotoxic T lymphocytes (CTLs) (46, 60, 61). In contrast to the expected improvement of drug delivery to the tumor after anti-VEGF therapy, Van der Veldt et al. found that delivery of chemotherapy was rapidly and persistently decreased in cancer patients treated with bevacizumab (62). Therefore, as Jain discussed, optimal scheduling and dosage of anti-angiogenic therapy might be crucial for treatment efficacy, as

prolonged treatment might increase hypoxia and possibly promote invasiveness and metastasis (53, 59).

Mechanisms of resistance

It was expected that by targeting the genetically stable cells of the tumor stroma instead of the mutation-prone tumor cells, resistance to anti-angiogenic therapy could be avoided (63, 64). However, after only modest gains in patient survival in clinical trials, evidence, both preclinical and clinical, for resistance to VEGF-pathway inhibition emerged. It was first shown in mice that inhibition of angiogenesis caused a transient response, but was followed by revascularization and regrowth of the tumors (65). Reports from clinical trials indicated that interrupting or ending anti-angiogenic treatment could cause tumor growth rates to increase (53). Since then a number of mechanisms of resistance to anti-angiogenic therapy have been suggested. In response to hypoxia resulting from VEGF/VEGFR-inhibition, increased expression of other pro-angiogenic factors, such as FGF-2, PlGF and ANG-1, substitutes for the inhibited signaling pathway (28, 66). Recently, the carbohydrate-binding protein galectin-1 has been identified to contribute to resistance to VEGF-blockade. Changes in the glycosylation-pattern on the endothelial cell surface in anti-VEGF resistant tumors facilitated galectin-1 binding and activation of VEGFR-2, thereby circumventing the lack of VEGF (67). Furthermore, it has been demonstrated that in response to HIF-1α bone marrow-derived monocytes were recruited to the tumor tissue, where they differentiate to macrophages and secrete further pro-angiogenic factors and MMPs (28, 68).

Adverse affects

While VEGF is clearly overexpressed during tumorigenesis, it is known that endothelial cell survival is depending on VEGF in an autocrine fashion (69). This might explain the side effects observed from treatment of bevacizumab, such as hypertension, gastro-intestinal perforation, proteinuria, hemorrhage and impaired wound healing (70-72). Increased tumor invasiveness was observed in response to hypoxia caused by anti-angiogenic therapy, causing tumor cells to migrate into the surrounding tissue in order to get access to blood vessels (28, 53, 73, 74). The most concerning adverse affect of angiogenesis inhibition is probably an increased incidence of metastasis, which has been observed in experimental tumor models (74-76), and occasionally in the clinic (53, 77). Singh et al. suggested that the risk for a therapy-induced increase in metastasis might be higher for TKIs in comparison to VEGF-inhibition, as TKIs target more than one pathway (76).

While anti-angiogenic treatment has made an important contribution to improved treatment outcomes for some tumor patients, it has not become the universally effective cancer treatment as it was initially hoped to be. Intense research focuses on finding predictive biomarkers for treatment efficacy, as

patient responses are highly variable (78, 79). Further investigations are required to optimize therapy design for currently available drugs and identify other suitable targets in order to avoid resistance and relapse. The development of an alternative treatment approach targeting the tumor vasculature has been subject of this thesis.

The extracellular matrix of tumors

The ECM is a network of cross-linked proteins, which surrounds the cells of the tissues. It consists of fiber forming proteins, such as collagens, laminin and fibronectin, as well as of hyaluronic acid and proteoglycans. The ECM functions as a supportive network sustaining tissue shape and homeostasis. Furthermore it provides guidance for cell migration and proliferation during physiological tissue remodeling, such as wound healing. Cells are in contact with the ECM via transmembrane receptors, such as integrins. Through proteoglycans the ECM binds a number of growth factors and cytokines, which can be released upon degradation (24, 80, 81).

Remodeling of the extracellular matrix

In tumors the ECM undergoes constant remodeling and displays alterations, which usually favor tumor cell proliferation and migration, and eventually assist metastasis (3, 4). To enable growth of the tumor as well as new blood vessels, the ECM has to be degraded and resynthesized to provide space for the growing tumor and angiogenic blood vessels, as well as signals and guidance for migration of tumor and endothelial cells. The degradation of the existing matrix is carried out by proteinases, such as MMPs. While tumor cells are frequently able to secrete MMPs, the major contributors to MMP expression are stromal cells, such as fibroblasts and inflammatory cells (82). During the degradation process ECM-sequestered growth factors and cytokines become soluble and activated. Among these are VEGF-A, FGF-2, insulin growth factor (IGF), interferon-γ (IFN-γ) and TGF-β, which act as survival factors or chemoattractants for stromal as well as tumor cells (4, 81, 83). Another consequence of ECM remodeling is the alternative splicing of the matrix proteins fibronectin and tenascin-C. These ECM molecules have been shown to contain additional protein domains under conditions of angiogenesis (84-87).

The extra domain-A and B of fibronectin

Fibronectin is a glycoprotein of the ECM, which in its functional form exists as a dimer. Each fibronectin monomer consists of three types of homologous repeating domains. Two of its domains, the extra domain-A and B (ED-A and ED-B), and its type III connecting segment (IIICS element or variable region) undergo alternative splicing (Figure 3) (86, 87).

Figure 3. Schematic illustration of a fibronectin monomer. Each fibronectin monomer consists of three types of homologous repeating domains: type I (hexagons), type II (rectangles) and type III (ovals). The alternatively spliced extra domain-A and B (ED-A and ED-B) and the type III connecting segment (IIICS) are indicated.

This gives rise to twenty fibronectin isoforms in humans, and twelve in mice and rats. Interactions of cells and fibronectin are mediated via integrins, with integrin α5β1 being the key cell surface receptor (86, 87). Fibronectin is essential for vascular development and its absence is embryonically lethal (88). A soluble form of fibronectin, which lacks ED-A and ED-B, is secreted by hepatocytes into the circulation. Fibronectin of the ECM is mainly expressed by fibroblasts. In tumors fibronectin expression has been attributed to fibroblasts of the stroma and tumor cells (87). It has been demonstrated that especially tumor cells, which underwent epithelial-mesenchymal transition (EMT) and acquired a more invasive phenotype, express fibronectin, underlining the importance of this molecule for cell adhesion and migration (83).

Both ED-A and ED-B are incorporated into the fibronectin molecule by alternative splicing (89-91), which is regulated by TGF-β (92, 93). While ED-A (90 aa) shows a sequence identity of 98% between human and mouse, ED-B (91 aa) is completely conserved in various species, such as human, mouse, rat, rabbit and dog (86, 94). This high degree of conservation suggests a significant function for either of the two domains. A combined knockout of both ED-A and ED-B resulted in embryonic lethality, depending on the genetic background of the mice, but at least in severe cardiovascular defects (95). Physiological expression of these domains occurs during embryogenesis, where ED-A and ED-B are detectable around embryonic blood vessels (96, 97). Otherwise their expression in normal tissues is rare: expression of ED-B was confirmed in the female reproductive tract (98) and in cartilage (98, 99). Both domains are transiently expressed during wound healing (100) (96), but differences in proportion (96) and time course of expression (101) suggest distinct roles for each domain. A number of pathologic conditions are associated with re-expression of ED-A and ED-B. Elevated ED-A expression in joints has been shown during rheumatoid arthritis (102). Furthermore, expression of ED-A and ED-B is abundant in many types of solid tumors, such as glioblastoma multiforme, breast carcinoma and colorectal cancer (103-110).

ED-A contains binding sites for the integrins α4β1 and α9β1, and *in vitro* experiments demonstrated a role of ED-A for cell adhesion and matrix assembly (111, 112). ED-A is a ligand for Toll-like receptor-4 (TLR-4), which

suggests an inflammatory role for this domain. In fact, TLR-4 stimulation by ED-A induces nuclear factor κB (NF-κB) activation (113). Furthermore, ED-A activation of TLR-4 has been demonstrated to stimulate cytokine production in mast cells (114), neutrophil migration (115) and DC maturation (116). Exposure of chondrocytes and synovial cells to ED-A activates MMP expression through an IL-1 dependent, autocrine mechanism (Saito, 1999). A xenograft study with colorectal cancer cells suggested a role for ED-A during tumor lymphangiogenesis through an ED-A-mediated increase in VEGF-C expression (117). ED-A was found to sustain the stem cell capacity of CD133$^+$/CD44$^+$ cells in a xenograft model of colorectal cancer (118). Ou et al. and Sun et al. recently demonstrated that ED-A has the capacity to induce EMT of colorectal carcinoma cells and lung cancer cells through α9β1 integrin interaction (119, 120). Muro et al. found that absence of ED-A expression impaired healing of skin wounds, while Tan et al. found no defects in wound healing in mice lacking ED-A (121, 122). Knockout of ED-A in mice revealed alterations in motor-coordination and a reduction in lifespan (121, 123).

The role of ED-B has been less clear. Knockout studies have shown that mice lacking the ED-B exon develop normally, are fertile (124) and display no alterations of phenotype in different pathological settings (125). However, embryonic fibroblasts lacking ED-B grew more slowly *in vitro* and formed shorter and thinner fibrils, although these effects were relatively mild (124). A role for ED-B in stabilization of fibronectin-dimer formation has been demonstrated (126). ED-B-fibronectin expressed and cell surface-bound by T cells was shown to provide co-stimulatory signals for T cell proliferation (127). No binding sites for other molecules of the ECM or receptors have been identified in the ED-B sequence or on sites in fibronectin unmasked by insertion of the domain. ED-B expression has been assigned to wound macrophages, cartilage, endothelial cells and tumor cells (98, 128, 129). Expression of ED-B by tumor cells has been shown to be downregulated upon inhibition of VEGF or VEGF-R-2 (130).

As ED-A and ED-B show a high selectivity for the tumor vasculature, they have been described tumor vascular antigens or targets (94). A high affinity antibody against ED- B, L19, has been developed using the phage display technique (131, 132) and used successfully for tumor detection and treatment in mice (133-136). Radiolabelled L19-derivates have shown promising results in an imaging application (109) and phase I and II clinical trials (110, 137, 138). Furthermore, IL-2 and tumor necrosis factor (TNF)-fused derivatives of L19 showed therapeutic efficacy in phase I and II clinical studies (139-141). Similarly to L19, an ED-A targeting antibody, F8, was established. Biodistribution tests and experimental tumor studies have confirmed selectivity and therapeutic efficacy of IL-2 fused F8 (142-145).

Interactions of tumors and the immune system

The role of tumor immunosurveillance, a physiological program of the immune system to prevent expansion of mutated cell clones, has been subject of intense research since it was first proposed by Burnet in 1970 (146). Numerous reports from preclinical studies and data from immunosuppressed transplant patients have confirmed that an impaired immune system leads to a higher incidence of non-virus induced cancers (147, 148). Furthermore, increased numbers of T cells, specifically CD8^{+} CTLs, in tumors have been linked to increased patient survival (9, 147). However, considering the prevalence of cancer, the immune system must frequently fail to prevent tumor development. The reason for this is probably multifaceted. Most of the antigens expressed by the tumor are essentially self-antigens, and are protected from immune attack by mechanisms of self-tolerance. Additionally, tumors are able to employ various strategies to escape recognition by the immune system. This involves various types of immune cells found in the tumor microenvironment, which have been shown to exert immunosuppressive effects.

Self-tolerance

Self-tolerance can be defined as an unresponsiveness to the body's own antigens and is an essential property of the immune system (149). The dramatic results of a loss of tolerance against self-antigens can be seen in patients suffering from autoimmune diseases, who frequently experience destruction of whole tissues by their own immune system. Tolerance of the antigen-specific cells of the immune system, T and B lymphocytes, is maintained by two fundamental mechanisms: central tolerance and peripheral tolerance.

Central tolerance of T cells is controlled in the thymus during embryonic development and post-natal life (149, 150). The specificity of the T cell receptor (TCR) is generated through V(D)J recombination of variable (V), diversity (D) and joining (J) gene segments of the TCR α and β chain. In the thymus self-antigens are presented by thymic epithelial cells to maturing T cells through ubiquitous expression of peripheral antigens. This process is to a large extent controlled by the transcription factor AIRE. How AIRE controls the expression of antigens from virtually all tissues in the thymus has not been completely elucidated. However, mutation of AIRE is connected to severe autoimmune disorder, which demonstrates its absolute necessity for the immune tolerance (149-151). Self-antigens are presented to T cells on major histocompatibility complex (MHC) class I and II molecules. High-avidity recognition of self-antigen/MHC ligands by the TCR leads to apoptosis of the respective T cell, a process termed negative selection. Some of the self-reactive CD4^{+} T cells differentiate into regulatory T cells (Tregs) through a complex process, for which the expression of the transcription factor forkhead box P3 (FoxP3) is essential (149). Peripheral T cell tolerance

prevents activation of T cells recognizing self-antigens and results in functional unresponsiveness, termed anergy. This is usually the result of a lack of co-stimulatory signals, such as interaction of CD28 on the surface of T cells and B7 on antigen-presenting cells (APCs), and a specific cytokine-milieu. Furthermore, Tregs are part of the peripheral T cell tolerance (149, 150).

Central B cell tolerance occurs in the bone marrow, where the B cells develop. New B cells with different specificity are continuously formed due to VDJ recombination of the immunoglobulin (Ig) heavy chain genes, and VJ recombination of the light chain genes. When the IgM molecule of the B cell receptor (BCR) of an immature B cell binds with high avidity to a membrane/cell surface-bound self-antigen, the Ig undergoes a process called receptor editing. This allows the change of the light chain specificity of the Ig through new gene rearrangement (152-154). Failed receptor editing induces apoptosis upon persistent auto-reactivity. However, relatively high numbers of mature B cells with weak avidity for self-antigens not present in the bone marrow are found in the circulation (153, 155). Outside the bone marrow, inside the peripheral lymphoid tissues, self-reactive B cells are controlled by peripheral tolerance mechanisms. B cells recognizing peripheral self-antigens with high avidity will be deleted by apoptosis. B cells showing low avidity for a self-antigen fail to be activated by helper T cells, as no T cell with the same specificity is available due to negative selection of self-reactive T cells in the thymus. These B cells will become anergic. During affinity maturation against T cell-dependent antigens, which occurs in the germinal centers of secondary lymphoid organs, B cells might acquire specificity for a self-antigen through somatic hypermutation. These B cells will not receive further T cell help and become anergic (149, 151, 153).

Tumor antigens

Tumors present antigens on their cell surface that theoretically can be recognized by the immune system. These can be divided into two groups: Tumor-specific antigens, which are derived from proteins that are exclusively expressed by tumor cells, and tumor-associated, which are antigens derived from proteins that can also be found on normal cells, but that are abnormally expressed in tumors (148, 149, 156, 157).

Tumors caused by oncogenic viruses present foreign antigens derived from the virus, which can induce specific CTL responses and antibody responses mediated by B cells. Non-virus induced tumors, as most types of tumors are, can be identified as "abnormal" via presentation of products of mutated genes. A change in the amino acid sequence of a protein, caused e.g. by a point mutation, can induce a T cell response, if the change affects a peptide that is presented on MHC (157). Furthermore, oncogenic fusion proteins formed by chromosomal translocations, such as Bcr-Abl, can generate new antigenic peptides (156, 157), which are foreign to the immune system. Interestingly, tumors commonly display changes in glycosylation of surface

proteins (158, 159). Aberrant activity or expression of glycosyltransferases leads to incomplete glycan synthesis or leaves glycosylation sites unoccupied (158). This creates immunogenic epitopes, despite an unchanged amino acid sequence (158). A number of genes, which are normally restricted to male germ cells, are expressed in a range of tumors, which give rise to the so called cancer/testis antigens. As germ cells do not express MHC class I, these antigens are normally not presented to T cells (156, 157). Antibody and T cell responses against cancer/testis antigens have been detected in patients with certain tumors (160, 161). However, these antigens are not universally expressed in all tumors, or are only weakly immunogenic. Additionally, possible immune responses might be repressed by the tumor.

Tumors can evade immunosurveillance

Tumor cells have developed effective strategies to avoid recognition by the immune system and resist killing mechanisms (147, 148). Therefore immune evasion has been added as one of the "tumor enabling" hallmarks of cancer (1). The most effective endogenously occurring anti-tumor response is believed to be mediated by CD8^{+} CTLs and natural killer (NK) cells. High numbers of these cell types in tumors have been correlated with favorable prognosis (9, 162). CTLs are able to detect altered proteins via MHC class I-presentation and directly kill tumor cells by release of IFN-γ, granzyme and perforin (163, 164). To avoid recognition, tumor cells are able to downregulate the expression of proteins that are not essential for maintaining their transformed state, but might attract CTLs, by promoter regulation. Furthermore, tumor cells can decrease synthesis of MHC class I molecules or expression of other proteins involved in antigen processing, which prevents activation of CTLs (147, 156). Tumor cells, which have downregulated MHC class I molecules are vulnerable to NK cell-toxicity, mediated by granzyme and perforin, as these specifically recognize cells with missing MHC class I expression. Additionally, NK cell express receptors that sense stress-induced self-ligands. A receptor with major importance for tumor immunosurveillance is NKG2D. This receptor recognizes the NKG2D ligand (NKG2DL), which is expressed by cells in response to DNA damage or virus infection. A major mechanism of escape from NK cells is the secretion of soluble NKG2DL and ligands of other NK cell activating receptors by tumor cells (162, 165).

Another mechanism employed by tumors to prevent destruction by CTLs is attraction of CD4^{+} CD25^{+} FoxP3^{+} Tregs by secreting the chemoattractant CCL22, which binds to the CCR4 receptor on Tregs. Tregs are mediators of immunological tolerance and exert their inhibitory function via receptor-ligand-based mechanisms and various effector molecules. Among these IL-10 and TGF-β were found to play an important role in immunosuppression during tumorigenesis (166, 167). In addition to suppressing CTLs, Tregs also inhibit the function of helper T cells (Ths), and APCs (166). Helper T cells

are important activators of antibody production by B cells (adaptive immunity) and macrophages (innate immune system). High numbers of Tregs have been found in various types of tumors and were correlated with poor prognosis (168). Tumor cells also acquire certain traits that enable them to prevent an immune attack without relying on help from the Tregs. One example is the release of TGF-β and IL-10 by tumor cells. Expression of the enzyme indoleamine-2,3-dioxygenase (IDO) by tumor cells leads to inhibition of T cell activation via degradation of tryptophan to catabolites that have immunosuppressive functions on T cells. Normally, interaction of Tregs via their surface molecule CTLA-4 and B7 on APCs leads to expression of IDO by the APCs and is important for maternal tolerance during pregnancy (148, 169, 170). Again, exploitation of physiological mechanisms is a successful tactic for tumors to perpetuate their progression.

Paradoxical immune cells

Growth factors and chemokines released during degradation of the ECM during angiogenesis or secreted by the tumor attract cell types of the innate immune system and adaptive immune system. Abundantly found in the tumor stroma are macrophages, which are an example of the paradoxical interaction of immune cells and tumors. They are recruited as monocytes from the circulation and differentiate into macrophages when they enter the tissue. Chemokines important for macrophage attraction are CCL2, CSF-1 and the cytokine CXCL12, which are released by tumor cells and stromal cells (171-173). While macrophages, as part of the innate immune system, can be effective protagonists in anti-tumor immunity, high numbers of macrophages in tumors have been related to poor prognosis (172-174) and several mechanisms of tumor promotion by tumor-associated macrophages (TAMs) have been identified. TAMs are often polarized towards an M2-phenotype, which is characterized by expression of various growth factors and enzymes that aid tumor progression. Through release of VEGF-A, PlGF and MMPs, especially MMP-9, M2-TAMs are important contributors to tumor angiogenesis (171, 172). Macrophages with the M2-phenotype are also termed "alternatively activated" (through TGF-β and IL-10) and display an immunosuppressive phenotype. The "classically activated" M1-macrophages (through TLR stimulation by LPS and IFN-γ) have a pro-inflammatory phenotype and are able to exert anti-tumor activity (173, 175). A similar polarization towards a tumor-inhibiting and tumor-promoting phenotype has also been reported for neutrophils, thus termed N1 and N2 neutrophils (176, 177).

Galectin-1 and its role in tumors

Galectin-1 is one of fifteen members of the family of β-galactoside-binding proteins. These proteins share an affinity for β-galactosides of glycoproteins or glycolipids and contain at least one conserved carbohydrate recognition

domain (CRD) (178). The CRD, which consists of approximately 130 amino acids, represents the major functional domain of the galectins and enables binding to N- and O-linked glycans. Furthermore, in galectins containing one CRD, this domains allows dimerization (179, 180).

The galectin-1 monomer has a molecular weight of 14 kDa and contains a CRD of 135 amino acids. Galectin-1 forms homodimers, which prevents loss of activity by cysteine oxidation in the monomer (180). Galectin-1 can be found intracellularly (181) and in the extracellular space (182), despite the lack of a classical signal peptide (183). The slightly higher molecular weight of secreted galectin-1 (15 kDa) indicates that the protein undergoes post-translational modification, possibly facilitating secretion (184). Furthermore, it has been shown that the presence of β-galactoside containing cell surface receptors is required for secretion of galectin-1 (185). However, the exact mechanism of galectin-1 secretion remains to be elucidated. Galectin-1 shows a variety of physiological functions and is implicated in neural stem cell growth, hematopoietic differentiation and muscle differentiation (180). Furthermore, it plays an important role in endothelial cell function and angiogenesis (179).

Galectin-1 is highly expressed in a number of tumors, such as glioma, breast, ovarian, lung, colon and prostate carcinomas (180, 186-191). Intracellular galectin-1 of tumor cells is involved in oncogenic signaling through enhancing H-Ras binding to the membrane. Interactions of galectin-1 with intracellular proteins are carbohydrate-independent (180). Elevated serum levels of galectin-1 in colorectal or head and neck squamous cell carcinoma patients have been associated with poor prognosis (192, 193), and the degree of invasiveness correlates with levels of galectin-1 expression in several solid tumor types (180).

Galectin-1 expression is markedly increased in tumor vessels compared to normal vasculature (194, 195). High expression of galectin-1 has been detected in the endothelium of experimental tumors and human cancers (191, 196-201). While in endothelial cells of healthy tissues galectin-1 is generally located inside the cell, with only minimal extracellular secretion, galectin-1 is translocated to the endothelial cell surface in the tumor vasculature (195). On the cell surface galectin-1 binds to glycoproteins and glycolipids, and is able to interact with ECM proteins, such as fibronectin, laminin, vitronectin and thrombospondin, and integrins (180), suggesting a role in cell adhesion and migration. Indeed, galectin-1 has been shown to modulate migration of endothelial cells (200, 202), and also tumor cells (203-205). Galectin-1 has been described as an early marker of endothelial activation (195, 198), which describes the phenotype of endothelial cells stimulated by pro-angiogenic factors (15). These findings imply an important role for galectin-1 in tumor progression and tumor angiogenesis.

Evidence for a crucial role of galectin-1 in tumor angiogenesis comes from a number of studies showing that lack or inhibition of galectin-1 im-

pairs tumor growth. Mice lacking galectin-1 showed a reduced tumor volume due to impaired angiogenesis in F9 teratocarcinoma (198) and silencing of galectin-1 impaired tumor growth and decreased the microvascular density in Kaposi's sarcoma xenografts (206). Similar results were observed upon galectin-1 blockade with a monoclonal antibody, reducing microvessel density in a prostate cancer model (191). Croci et al. observed vessel normalization in tumors upon treatment with a galectin-1 specific monoclonal antibody (67). Galectin-1 has been demonstrated to act as a pro-angiogenic growth factor by Thijssen et al., who showed that tumor-secreted galectin-1 was taken up by endothelial cells lacking galectin-1 expression and stimulated endothelial cell proliferation and migration *in vitro* (202). Furthermore, Croci et al. demonstrated that galectin-1 has the capacity to mediate evasion from anti-VEGF therapy by interacting with N-glycans on VEGFR-2, and thereby activating VEGF-like signaling (67). Galectin-1 has also been shown to promote VEGFR-2 signaling via its interaction with neuropilin-1, a co-receptor of VEGFR-2 (200).

In addition to its pro-angiogenic role, galectin-1 is a mediator of tumor immune evasion. Galectin-1 expressed on the tumor cell surface induces apoptosis of T cells in a contact-dependent manner *in vitro* (207). Banh et al. demonstrated that presence of galectin-1 in tumors promotes apoptosis of intratumoral T cells, and furthermore suggest that its immunosuppressive role might surpass a pro-angiogenic role in certain types of tumors (208). Moreover, galectin-1-expressing endothelial cells are able to induce apoptosis of adhering T cells (209) and inhibit T cell transmigration (199). Galectin-1 regulates effector T cell polarization and survival (210). Furthermore, it has been shown that galectin-1 induces IL-10-producing immunosuppressive T cells through stimulation of a tolerogenic phenotype of dendritic cells (DCs) (211) and direct interaction with CD45 on T cells (212). Similarly, neuroblastoma-derived galectin-1 was shown to suppress DC maturation and induce T cell apoptosis (213). Recently, it was demonstrated that glioma overexpression of galectin-1 suppressed NK cell activity (214).

As silencing of galectin-1 in mammary tumor cells reduced the number of Tregs in the tumor, lymph nodes and at the metastatic site, and decreased metastasis, galectin-1 was suggested to promote metastasis by inhibiting immunosurveillance (187). In addition, galectin-1 was demonstrated to accumulate on the tumor cell surface at the site of endothelial cell interaction (215) and augment tumor cell adhesion to the endothelium (194), which might promote tumor cell dissemination. Enhanced invasiveness of galectin-1 expressing oral squamous cell carcinoma has been attributed to an increase in MMP-9 and -2 expression, and invasive capacity of tumor cells was reduced upon galectin-1 silencing (205).

In summary, galectin-1 displays a specific increase in expression and secretion under conditions of tumor angiogenesis and is involved in several

fundamental processes of tumor progression. These characteristics suggest galectin-1 as a highly attractive target for tumor therapy.

Development of a therapeutic cancer vaccine

Immunotherapies and monoclonal antibody-based therapies

The concept of immunosurveillance has encouraged researchers to investigate treatment strategies, which are able to re-establish an immune attack against the tumor by the patient's own immune system, and approaches of passive immunization against molecules associated with tumors. Dendritic cell vaccines are based on isolation of DCs from a patient and subsequent *ex vivo* loading with tumor-associated antigens, to enhance activation of tumor targeting T cells upon reintroduction of the DCs into the body. The cell vaccine Sipuleucel-T (Provenge) is approved for treatment of prostate cancer (216-218). Other approaches of active immunization are DNA or protein vaccines. Examples of passive immunization-approaches against tumor antigens are adoptive T cell transfer (217, 219) or administration of monoclonal antibodies.

Monoclonal antibodies have become an important group of cancer therapeutics for a number of solid tumors over the last decade (217, 220, 221). Evidence accumulates that not only functional inhibition of the target, but also stimulation of an immune response via antibody-depended cell-mediated cytotoxicity contributes to the tumor-targeting activity (ADCC) (217, 221, 222). However, there are some concerns connected to monoclonal antibody-based therapies.

When an antibody of the desired specificity has been raised in a non-human system, such as mouse, a humoral response against this foreign protein will be formed upon administration into humans and the non-human antibody will be removed by the immune system. To minimize an immune reaction, most available monoclonal antibodies are humanized. This is achieved by fusing the antigen-binding complementary determining regions (CDR) of the variable region of the non-human-derived antibody to the constant regions and residues of the variable regions of a human antibody, usually by genetic engineering (223, 224). While this decreases the risk of an immune response to the antibody in human, immunogenic epitopes are left or generated by this method. For example, novel epitopes might be created at the fusion sites (223). Additionally, differences in post-translational modifications, which depend on present amino acids and the expression system, might elicit an immune response (223). The constant regions of endogenous antibodies show a high degree of polymorphisms (allotypy) between individuals. Monoclonal humanized antibodies might therefore cause an anti-allotype response in the treated individual (225). As monoclonal antibodies

have a limited half-life, ranging between days and one month (224), they require frequent administration. For example, the half-life of bevacizumab is 17-21 days and it has to be given every 14 days (52).

Induction of an endogenous immune response by vaccination can circumvent the problems described for monoclonal antibodies. Endogenous antibodies are induced in each vaccinated individual and are not immunogenic. Furthermore, the duration of an immune response exceeds the half-life of monoclonal antibodies by several months (226). As production of monoclonal antibodies is cost-intensive, which is reflected in the high costs of monoclonal antibody-based drugs, therapeutic vaccination can offer a cost-efficient alternative.

Overcoming self-tolerance by vaccination

Vaccination technique

Since the target of a cancer vaccine will most likely be a self-antigen, self-tolerance of the immune system must be circumvented. To successfully break tolerance, a potent vaccination technique and a suitable adjuvant, an immunostimulatory compound, are required. The vaccination technique developed in our lab is based on a fusion protein, comprised of a foreign and a self-part. The foreign part can for example be a bacterial protein. It is of advantage if it facilitates soluble expression of the fusion protein in bacteria. The self-part represents the target antigen and might either contain the complete or parts of the amino acid sequence, depending on the size of the self-antigen. It should be sufficiently long to give several possibilities for presentation on MHC class II.

The fusion protein (non-self + self) will be injected subcutaneously together with the adjuvant and is taken up by APCs, such as macrophages or DCs, in the tissue (Figure 4). These internalize and process the fusion protein and present peptides derived from the non-self and self-part via MHC class II on their surface. The non-self peptides are recognized by the TCR on Type 2 helper T cells (Th2), which become activated. Self-peptides are not recognized by T cells, due to the central T cell tolerance mechanisms. However, self-reactive B cells are present in the circulation and are able to recognize the self-part of the fusion protein with their BCR. These auto-reactive B cells will take up the fusion protein and present peptides derived from both the self- and the non-self part on MHC class II. Because Th2-cells are activated by peptides from the foreign part of the fusion protein, the auto-reactive B cells will receive a stimulatory signal from these helper T cells. This occurs through the interaction of the TCR and peptides of the foreign part of the fusion protein presented on MHC class II on the auto-reactive B cells, which are the same peptides that activated the helper T cells (previously presented by the APCs). The activated B cells will undergo clonal expan-

sion and differentiate into plasma cells, which produce self-antibodies specific for the target antigen (227).
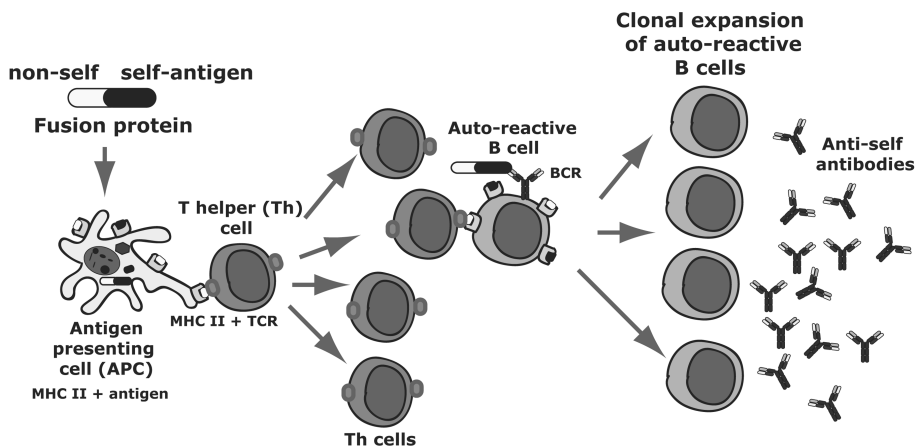

Figure 4. The vaccination technique. Antigen-presenting cell (APC), major histocompatibility complex (MHC), T cell receptor (TCR), B cell receptor (BCR).

A suitable adjuvant for application in humans

An adjuvant (from Latin, adiuvare: to aid) is added to a vaccine to stimulate and potentiate the immune response against an antigen. It thereby acts in a non-specific manner, meaning that its contents have an "irritating" effect and cause local inflammation, which attracts APCs (228). Adjuvants often contain an oil phase, which is mixed with the antigen-containing water-phase, forming a water-in-oil emulsion. This results in formation of water-globules in the oil phase, which contain the antigen and allows for a slow release and prolonged antigen-presentation in the tissue (229). At the moment Freund's adjuvant is the "golden standard" that is applied in most preclinical studies. It consists of mineral oil and heat-killed *Mycobacterium tuberculosis*, and is then called "Freund's complete adjuvant". "Freund's incomplete" contains the mineral oil-base only. However, due to its toxicity it is not approved for the use in humans. Nowadays milder adjuvants have been developed, some of them containing squalene, a biodegradable hydrocarbon, in the oil-phase (228, 230).

As demonstrated by Johansson et al. and Ringvall et al. (231, 232), vaccination with a self/non-self fusion protein in presence of a weak adjuvant is not enough to induce a detectable antibody response against the self-antigen. To obtain B cell activation, both the fusion protein and a stimulation of the immune response by a potent adjuvant are required. While adjuvants used for immunization against foreign antigens, such as bacteria or viruses, do not require strong additional stimulators, this seems essential for self-antigens. Additional stimulation of the immune response can for example be achieved by compounds that are recognized by the pattern recognition receptors

(PRRs) of immune cells, such as Toll-like receptors (TLRs) or nucleotide-binding oligomerization domain receptors (NOD receptors).

TLRs are a class of PRRs that recognizes various molecules of microbial origin. TLR-9 is activated by endocytosed DNA that is rich in unmethylated CG-nucleotides, which are a characteristic of bacterial DNA (233). The receptor is expressed intracellularly in the endosomal compartment of mainly plasmacytoid DCs and B cells (234, 235). Activation of TLR-9 stimulates a response of the innate immune system and type I IFN production, which in turn favors a type 1 helper T cell response (236). Simultaneous activation of B cells by helper T cells together with stimulation of TLR-9 and the BCR results in enhanced B cells proliferation and differentiation into antibody-secreting plasma cells (237, 238). It has been shown that auto-reactive B cells become anergic if TLR-9 activation through CpG DNA occurs in absence of helper T cell stimulation (239).

Over the last years synthetic oligodeoxynucleotides (ODNs) containing unmethylated CpG-motifs (CpG ODNs) were shown to be agents able to induce strong immune responses and were used in various applications, among them vaccines (234, 236). CpG ODNs, which are approximately 18-25 bases in length, act through TLR-9 (233) and are divided into three classes: A, B and C. Class-A CpG ODNs strongly stimulate IFN-α production by plasmacytoid DCs, activate NK cells, but have only weak effects on B cell proliferation. IFN-α is a cytokine that stimulates B cells to differentiate into antibody-producing plasma cells, and myeloid DCs to release B cell activating factor (BAFF), which is crucial for survival of B cells (240). Class-B CpG ODNs are strong stimulators of B cell proliferation, but only weakly induce IFN-α secretion (235, 236). Furthermore, class-B CpG ODN possess a phosphorothioate backbone, which increases their half-life due to resistance to nuclease degradation (241). Responses towards C-class CpG ODNs are a combination of the responses towards classes A and B. It has not yet been completely elucidated, how the different CpG ODN classes are able to induce such diverse effects via the same receptor (235, 236). Importantly, the optimal CpG motifs for TLR-9 activation differ between species (235).

The immune response elicited by the vaccine

The polyclonal antibodies induced through vaccination with the fusion protein will recognize and bind to their target antigen. This will lead to activation of the classical pathway of the complement system. The complement system is an effector mechanism of the immune system and exerts its function via three different pathways: the classical, the alternative and the lectin-pathway. The classical pathway is the antibody-dependent pathway and is relevant for the immune response induced by the vaccination. The complement system comprises more than 20 plasma proteins, produced and secreted by the hepatocytes of the liver into the circulation. The cascades of protein cleavage and protein interaction of the three major pathways of complement

activation lead to formation of the membrane attack complex (MAC), which creates pores in the cell wall of microbes and disrupts pathogenic cells. During the proteolytic cleavage of complement proteins, chemoattractants (proteolytic complement fragments 3a and 5a) are released. These attract phagocytosing immune cells, such as neutrophils and macrophages. The complement fragment 3b becomes covalently bound to the surface of cells, and promotes uptake by phagocytes through interaction with the C3b receptor on their surface. Formed immune complexes are taken up by phagocytes through interaction of the Fc regions of the antibodies and Fc receptors on the phagocyte surface. Soluble antigens, such as infected cells, bacteria or soluble proteins, are phagocytosed in this way (242). However, if antigens are not soluble but tissue-bound, phagocytes are not able to engulf these. This leads to induction of a process called "frustrated phagocytosis", which is associated with the release of lysosome contents by the phagocytes into the environment (243). The lysosome contents include different enzymes, such as lysozyme, collagenase and elastase, as well as reactive oxygen species, which will cause tissue degradation.

Experimental tumor models for studying a cancer vaccine

Mouse models of cancer have been essential for studying the role of the tumor microenvironment and different stages of tumor progression, including metastasis to distant organs. Xenograft models in immunocompromised mice are based on transplantation of tissue from one species to another, and are used to grow tumors derived from human tumor tissue. Tumor cells can be transplanted subcutaneously, which simplifies monitoring of tumor growth, or orthotopically, into the tumor-specific tissue of origin. Examples of immunodeficient mouse strains are nude mice, which lack mature T cells and show deficiencies in T cell-mediated responses, and SCID (severe combined immunodeficiency) mice, which display an absence of functional B and T cells. While these models allow studying the response of actual human tumor cells to treatment, it is not possible to study therapeutic approaches requiring a functional host immune system, such as vaccination, in these mice.

Immunocompetent models include syngeneic and genetically engineered mouse models (GEMM). Syngeneic tumor models, where the donor of the tumor cells is genetically identical or immunologically compatible to the host, mimic the interactions of tumor cells and the microenvironment including immune cells to a certain degree. Also in syngeneic models tumor cells are injected either subcutaneously or at the respective orthotopic site. Certain variants of a number of tumor cell lines are known to form spontaneous metastases upon subcutaneous injection, such as Lewis Lung Carcinoma (LLC) and the B16 melanoma, both derived from wild-type C57BL/6 mice (244). The major disadvantage of subcutaneous syngeneic tumor models is their fast growth, with tumors reaching the maximum allowed size according to

animal welfare guidelines within four weeks or less. As induction of a strong antibody response requires up to three weeks or more, mice have to be immunized in advance. This allows studying preventive effects of a cancer vaccine, but it is not possible to address vaccination in a therapeutic setting in these models.

GEMMs resemble the multistage progression of tumors, including metastasis, and are considered to represent the composition of tumors most accurately (245). These models of spontaneous tumorigenesis have been indispensible for understanding the complexity of tumorigenesis and are a useful tool for predicting the effectiveness of therapeutic agents in the clinic (246). The predictive value of mouse tumor models might vary for different treatment approaches (245, 247, 248), and is depending on drug dosing and definition of clinically relevant endpoints for preclinical models (245).

Due to the presence of a functioning immune system and their relatively slow growth, GEMMs are better models for testing the therapeutic effect of a cancer vaccine on established tumors and metastasis. The MMTV-PyMT model is a well-characterized model of multistage mammary tumorigenesis, which shows a high incidence of spontaneous pulmonary metastasis (249). In this model the **p**ol**y**omavirus **m**iddle **T**-antigen is expressed under the control of the **m**ouse **m**ammary **t**umor **v**irus promoter (250). The long terminal repeat (LTR) of the MMTV promoter contains a hormone response element and thereby ensures selective expression of the PyMT oncogene in the mammary epithelium (251, 252). The PyMT oncogene modulates several signaling pathways (e.g. Src family, Ras and PI3 kinase), which are also affected in human breast cancers (253). Therefore expression of the MMTV-PyMT transgene will lead to formation of mammary adenocarcinomas (250). Tumor development in this model can be divided into four stages: hyperplasia, adenoma, and early and late carcinoma (254). Hyperplasia is detectable in mammary tissue from between 4 to 6 weeks of age, which will progress to advanced pre-malignant lesions between 8 and 9 weeks of age. Malignant transition to early carcinomas occurs from week 8 up to week 12 of age, with tumor morphology similar to human ductal carcinoma *in situ*. From 10 weeks of age tumors that have progressed to the advanced carcinoma stage (late carcinoma) can be found. While initially tumors form as a single focus on the main milk-collecting ducts connected to the nipples, secondary tumor foci develop in the distant ducts with increasing age of the mice. The expression of a number of markers in MMTV-PyMT tumors recapitulate the expression statuses observed in human tumors: While expression of steroid hormone receptors and β1-integrin is lost with advancing malignancy, ErbB2 and cyclin D1 are overexpressed (254).

Present investigations

Aims

The aim of this thesis was to study the potential of vaccines directed against molecules associated with tumor angiogenesis to inhibit tumor growth, with the long-term objective to develop a therapeutic vaccine for treatment of cancer. In **paper I** we set out to examine whether a vaccine directed against the tumor vascular antigen ED-B of fibronectin was able to break tolerance and induce anti-ED-B antibodies. Furthermore, we wanted to study the effects of an immune response against ED-B on growth of subcutaneous tumors. The objective of **paper II** was to compare and characterize the immune response against ED-B in the presence of the squalene-based Montanide ISA 720 combined with the CpG ODN 1826 (M720/CpG) or Freund's adjuvant. The aim of **paper III** was to investigate the potential of a vaccine targeting ED-A of fibronectin to inhibit tumor growth and development of metastases in a therapeutic setting. In **paper IV** we aimed to study the effect of a vaccine directed against the pro-angiogenic and immunosuppressive protein galectin-1 on tumor growth.

Paper I

Vaccination against the extra domain-B of fibronectin as a novel tumor therapy

To examine whether vaccination based on the fusion protein technique was able to break self-tolerance against ED-B, we designed and purified the recombinant protein TRX-EDB, consisting of the *E.coli*-derived protein thioredoxin (TRX) fused to the amino acid sequence of ED-B. C57BL/6 mice were immunized with either TRX-EDB or vehicle control (PBS) together with Freund's adjuvant, and boosted twice. Subsequently, T241 fibrosarcoma cells were injected subcutaneously. The study was terminated three weeks after tumor cell inoculation and the tumors were dissected. Analysis of mouse sera with ELISA showed that nineteen out of 20 mice immunized with TRX-EDB developed antibodies against ED-B. We found that the tumor volume was reduced by about 70% in mice carrying anti-EDB-antibodies. To test the ability of the anti-ED-B antibodies to recognize native

ED-B in tissue, we incubated sections of grade III glioma tissue and normal brain with anti-ED-B serum. We obtained a strong staining with a vascular pattern in the glioma tissue, while no staining was visible in the sections of normal brain. T241 tumor sections were immunostained for CD31 and hematoxylin/eosin, and analyzed for necrosis and vascularization. Tumors from the ED-B vaccinated group showed larger areas of necrosis than the control group, but no difference in the extent of tumor vascularization was detected. However, ED-B immunized mice displayed an impaired functionality of the tumor vessels, as judged by increased amounts of extravasated fibrinogen, a measurement for vascular permeability. Electron microscopy revealed morphological changes of the tumor vasculature in mice carrying anti-ED-B antibodies and showed macrophages engulfing the endothelium. Furthermore, an increased number of Gr-1-positive neutrophils were detected in tumors from the ED-B-immunized group. As ED-B is transiently expressed during wound healing, we addressed possible side effects of the ED-B immunization in a wound-healing assay. However, in mice immunized against ED-B no impairment of wound closure was observed. ED-B expression has also been reported in hyaline cartilage, but since cartilage is not vascularized the anti-ED-B antibodies are not able to reach this site. Still, ED-B immunized mice were examined for arthritis on a macroscopic and cellular level and showed no symptoms of arthritis.

In conclusion, we show that it is possible to break self-tolerance against ED-B using vaccination with the fusion protein TRX-EDB. We demonstrated a preventive effect of the ED-B-immunization against tumor growth. Our data suggest that this is caused by an immune response against the tumor vasculature in mice carrying anti-ED-B antibodies.

Paper II

The non-toxic and biodegradable adjuvant Montanide ISA 720/CpG can replace Freund's in a cancer vaccine targeting ED-B – a prerequisite for clinical development

A requirement for a successful transfer of therapeutic vaccines targeting self-antigens to the clinic is a sufficiently potent, but non-toxic adjuvant. Freund's adjuvant is a strong but toxic adjuvant able to stimulate immune responses against self-antigens. The squalene-based Montanide ISA 720 combined with the CpG ODN 1826 (M720/CpG) has previously been identified as potent enough to break tolerance against a self-molecule (232) and is safe for use in humans. In this study, mice were immunized with the fusion protein TRX-EDB and either M720/CpG or Freund's adjuvant to compare the immune responses elicited by the two different adjuvants. Blood samples were drawn regularly. Analysis of mouse sera with ELISA revealed that

M720/CpG induced anti-ED-B antibody titers with less variation between individuals than Freund's. Determination of the IgG subclass using monoclonal antibodies specific for the different IgG heavy chains showed that both adjuvants mainly induced the subclass IgG1, indicating a TH2-response. The immune responses against ED-B elicited by Freund's or M720/CpG are reversible, with antibody levels decreasing over time. However, anti-ED-B antibodies in mice treated with M720/CpG persisted for a longer time than antibodies generated with Freund's, resulting in an approximately 6-fold difference in antibody levels at 7 months after the second boost. Re-immunization of mice whose antibody-levels had decreased to baseline-levels showed that both adjuvants induce a similar memory response. Using a Biacore Biosensor system for affinity measurements, we show that M720/CpG-induced antibodies had an approximately 10-fold higher avidity for ED-B than antibodies stimulated with Freund's. When immunizing naïve mice with TRX-EDB and either of the two adjuvants, we found that anti-ED-B antibodies were detectable earlier when M720/CpG was used. In the M720/CpG group antibodies were present already nine days after immunization, while a booster injection was required for the mice immunized with Freund's to obtain similar antibody levels.

With M720/CpG we have identified a non-toxic alternative to Freund's adjuvant, which is at least as potent with respect to inducing an immune response against a self-antigen.

Paper III

Therapeutic vaccination against fibronectin ED-A attenuates progression of metastatic breast cancer

The MMTV-PyMT model of mammary carcinoma is a well-established model for multi-step mammary tumorigenesis and spontaneous metastasis, and is thought to closely resemble human breast cancer with respect to morphology and molecular signature. We purified the fusion protein TRX-EDA, containing the mouse-specific sequence for ED-A. To evaluate the expression of tumor vascular antigens in this model by immunostainings, we generated species-specific antibodies against ED-A and ED-B, since no antibodies against these domains were commercially available at the time. Rabbits were immunized with the respective fusion proteins, and antibodies specific for ED-A and ED-B were affinity-purified from collected sera. When using the affinity-purified rabbit antibodies to stain MMTV-PyMT tissue, we detected expression of both ED-A and ED-B around blood vessels of primary tumors. Consistent with reports from ED-A expression in human ductal carcinoma, we found that ED-A was more prominently expressed than ED-B. Vascularized lung metastases showed staining for ED-A, but not for ED-B.

We furthermore showed that ED-A expression is detectable in hyperplastic lesions of 5-week-old mice. With an affinity-purified rabbit antibody specific for human ED-A we detected expression of this antigen in human ductal mammary carcinoma. These results confirmed ED-A as a suitable target for therapeutic vaccination in this model.

MMTV-PyMT mice were immunized with TRX-EDA or TRX (control) and M720/CpG at five weeks and boosted at week 7. ELISA revealed that anti-ED-A antibodies are undetectable at week 6.5, but evident at week 8, with levels increasing further until week 11 in all individuals vaccinated with TRX-EDA. At week 13 mice were perfused with a FITC-lectin solution to enable analysis of vascular function. Mice were dissected and the total tumor weight was determined. In ED-A immunized mice tumor burden was significantly decreased. In addition, three mice from the control group had to be euthanized before completion of the study due to high tumor burden. Tumor sections were immunostained for the vascular marker CD31 and different leukocyte markers. No difference was found in the amount of CD31-positive vessels. However, a decreased number of FITC-lectin perfused vessels and increased amounts of extravasated fibrinogen indicated a compromised function of the tumor vasculature in mice with anti-ED-A antibodies. Additionally, increased amounts of infiltrating CD45-positive leukocytes and CD68-positive macrophages were detected in tumors of ED-A immunized mice. Lastly, mice immunized with TRX-EDA displayed fewer lung metastases than control-immunized mice.

Taken together, we show that vaccination targeting a tumor vascular antigen can decrease tumor burden and suppress development of metastases in a therapeutic setting.

Paper IV

Targeting galectin-1 by vaccination suppresses tumor growth and promotes leukocyte recruitment to the tumor tissue

Galectin-1 is an interesting candidate for vaccination, as it is found in increased amounts on the surface of tumor endothelial cells and in the circulation of tumor patients, and promotes several key characteristics of tumors such as angiogenesis and immunosuppression. For immunization against galectin-1 we generated the fusion protein TRX-Gal-1. We could show that vaccination with TRX-Gal-1 and M720/CpG induced anti-galectin-1-antibodies. Analysis of conditioned media from B16 melanoma, T241 fibrosarcoma and Lewis Lung Carcinoma (LLC) culture by Western blot revealed expression of galectin-1 by all three cell-lines. This was confirmed by immunostaining for galectin-1 in the corresponding tumor tissue. A vascular pattern of galectin-1 staining was occasionally visible in B16 tumors. Based

on the expression pattern of galectin-1 and previous reports in the literature, we selected the B16 cell-line to study the effects of the galectin-1 vaccine on tumor growth *in vivo*. Mice were immunized with TRX-Gal-1 and M720/CpG and received two booster injections. Presence of anti-galectin-1 antibodies in sera of immunized mice was confirmed with ELISA and mice were subsequently inoculated subcutaneously with B16 melanoma cells. Tumors were allowed to grow for fourteen days. Volume measurements on day 12 and 14 showed that tumor growth was significantly decreased in galectin-1 immunized mice. We analyzed galectin-1 serum levels in galectin-1 and control-immunized mice with B16 tumors, and in mice without tumors using a sandwich ELISA. While galectin-1 was low in serum from healthy mice, mice with B16 tumors generally displayed elevated galectin-1 serum levels. However, serum levels were significantly reduced in mice carrying anti-galectin-1 antibodies. Tumors from galectin-1 immunized mice showed an increased amount of infiltrating CD45-positive leukocytes and a tendency, although not significant, towards decreased vascularization.

This study shows that it is possible to induce antibodies against the glycan-binding protein galectin-1 using our vaccination technique, and that tumor growth is reduced in mice immunized against galectin-1.

Discussion

Paper I constitutes an important proof-of-concept that vaccination with a fusion protein consisting of a non-self part and a self-part, in combination with a sufficiently potent adjuvant, can break tolerance and induce an antibody response against a self-molecule. We showed that an antibody response against a molecule expressed by and around tumor blood vessels, ED-B of fibronectin, suppresses tumor growth in a preventive approach. We extended these findings in **paper III**, where we showed that an antibody response against another tumor vascular antigen, ED-A of fibronectin, can be induced using the same vaccination technique. Vaccination against ED-A significantly decreased tumor burden and attenuated metastasis in a therapeutic setting. This is an important finding, as the vaccine is intended as a treatment option for patients diagnosed with cancer and possibly metastatic disease. To be able to address the therapeutic effect in the MMTV-PyMT model, mice were immunized at week 5 and received a booster at week 7, to allow for the development of a sufficiently strong immune response and an exposure of tumors to the antibodies before the tumors reached the maximum allowed size. No anti-ED-A antibodies were detectable 1.5 weeks after the first immunization in sera from vaccinated mice. At week 8, when early malignant transition occurs (254) and tumors begin to be palpable, anti-ED-A antibodies were present in the circulation. Antibody levels further increased until week

11, a time point when most of the primary tumors will have progressed to a late carcinoma stage in the majority of mice.

A highly interesting candidate molecule for targeted cancer therapy is galectin-1, as it has pro-angiogenic, immunosuppressive and pro-metastatic activities, and therefore allows simultaneous targeting of several mechanisms promoting tumor progression. In **paper IV** we demonstrated that vaccination with the fusion protein TRX-Gal-1 induced antibodies against galectin-1. In galectin-1 immunized mice tumor growth was significantly reduced. The high galectin-1 serum levels detected in tumor-bearing mice were significantly decreased in mice with anti-galectin-1 antibodies. The potential of the anti-galectin-1 vaccine to suppress galectin-1 serum levels is highly relevant from a clinical perspective, as high galectin-1 serum levels have been linked to poor prognosis in cancer patients (192, 193). Despite many reports on anti-angiogenic effects of galectin-1 inhibition (179, 180), we did not see a significant reduction in tumor vascularization. However, anti-galectin-1 treatment has also been shown to normalize the tumor vasculature (67), thereby facilitating leukocyte infiltration into the tumor (46, 60, 61), and improve tumor immunity by reducing immunosuppression mediated through galectin-1 (179, 180). We found increased amounts of infiltrating CD45-positive leukocytes in tumors of galectin-1 immunized mice, which might be responsible for the anti-tumor effect of the galectin-1 vaccination.

The ability of anti-galectin-1 antibodies to neutralize soluble galectin-1 in serum indicates that antibodies induced by vaccination with the fusion protein technique are able to bind their target *in vivo* (paper IV). Immunostaining of tumor tissue using serum from anti-ED-B immunized mice (paper I) and affinity-purified rabbit anti-ED-A and ED-B antibodies (paper III) showed specific staining patterns for the respective molecules. These results demonstrate that the polyclonal antibodies induced by vaccination with a fusion protein are functional and able to recognize their native target. This further supports the ability of the antibodies to form immune complexes and mediate an immune response against the target molecule in the tumor tissue. Indeed, we found increased amounts of different types of leukocytes infiltrating the tumors of mice immunized against ED-B, ED-A or galectin-1, indicating an ongoing immune response. An attack on the blood vessels by immune cells is a likely explanation for the impaired vascular functionality observed in tumors from ED-B and ED-A immunized mice. Electron microscopy analysis showing macrophages attempting to phagocytose the endothelium supports this conclusion (paper I). We detected fewer metastases in mice immunized against ED-A (paper III). Anti-ED-A antibodies probably mediate a protective effect against metastasis by attracting an immune response to angiogenic blood vessels expressing ED-A in growing metastases.

To strengthen the clinical feasibility of a therapeutic vaccination strategy targeting tumor blood vessels, we aimed to find a suitable adjuvant. The

adjuvant used in the majority of preclinical studies remains Freund's, which is able to support an immune response against a self-molecule. However, due to its toxicity, it is not approved for use in humans. In a systematic screen, the biodegradable squalene-based Montanide ISA 720 combined with the class B CpG ODN 1826 (M720/CpG) was identified as an adjuvant able to break self-tolerance against IgE in rats when injected together with a chimeric protein consisting of IgE sequences derived from rat and opossum (232). Comparison of immunostimulatory properties of Freund's and M720/CpG in **paper II** demonstrated that M720/CpG is at least as potent as Freund's with respect to inducing an immune response against the self-antigen ED-B. Interestingly, M720/CpG was superior to Freund's in several aspects. Antibodies induced by M720/CpG had a higher avidity for ED-B than antibodies generated in the presence of Freund's. Two studies investigating responses to foreign antigens, a hepatitis B and an anthrax antigen, have shown that the class B CpG 7909, containing a human-specific recognition sequence, is able to increase antibody-avidity (255, 256). Siegrist et al. proposed that CpG ODNs might affect affinity maturation in the germinal centers, resulting in enhanced antibody-avidity (256). Furthermore, presence of M720/CpG induced an anti-ED-B response with a longer duration than the response stimulated by Freund's. The reason behind this difference is not known, but presence of high-avidity anti-ED-B antibodies in M720/CpG-treated mice might contribute to this effect. A longer duration of an immune response might be of advantage in a clinical situation, as fewer immunizations would be required. M720/CpG has been used in the studies investigating anti-ED-A and galectin-1 vaccination (paper III and IV). High levels of anti-ED-A and anti-galectin-1 antibodies were measured in the respective studies, confirming that M720/CpG has the ability to induce an antibody response against other self-antigens if combined with the according self/non-self fusion protein.

Anti-ED-B antibody levels decreased over time, demonstrating reversibility of the immune response (paper II), in agreement with a previous report (226). A reversible immune response minimizes the risk of long-term side effects. Still, possible adverse effects of an antibody response against ED-A and ED-B have to be considered. Transient expression of ED-A and ED-B during wound healing has been shown (96, 98-100). Furthermore, a role for ED-A during wound healing has been suggested, as absence of ED-A expression slightly impaired healing of skin wounds (121). However, Tan et al. found no defects in wound healing in mice lacking ED-A (122). Wound healing in mice carrying anti-ED-B antibodies was not affected (paper I). An explanation for this might be the limited duration of ED-B expression during wound healing and possibly a less leaky vasculature in this situation of physiological angiogenesis, as opposed to deregulated tumor angiogenesis. In comparison to the overly leaky tumor vasculature, vessels in wounds might therefore limit the efficiency of the antibodies to pass the endothelium and reach ED-B.

Expression of ED-B has also been described in hyaline cartilage, and an immune response in this tissue might therefore damage the cartilage tissue. We found no signs of arthritis in ED-B immunized mice. The lack of vascularization in hyaline cartilage prevents contact of anti-ED-B antibodies with this tissue. Mice immunized with TRX-EDB were kept for more than twelve months to follow the duration of the immune response, and anti-ED-B antibodies remained detectable during that time (paper II). No adverse effects on lifespan and appearance were observed in these mice. Furthermore, mice showed no adverse reaction to vaccination with Montanide ISA 720 in combination with CpG 1826 (paper II-IV). In several clinical trials Montanide ISA 720 and CpG 7909 have been tested alone or in combination. No severe toxicity was observed in two phase I studies investigating a CTL-stimulating melanoma vaccine containing Montanide ISA 720 and CpG 7909 (257, 258). Similarly, only mild to moderate adverse effects, ranging from skin toxicity (redness, pain) to fatigue and flu-like symptoms have been seen in phase I and II trials involving single applications of either Montanide ISA 720 or CpG 7909 (256, 259-262).

Reports of increased tumor invasiveness and metastasis after anti-angiogenic treatment targeting the VEGF-pathway with monoclonal antibodies or the TKI sunitinib (53, 74, 75) raise concerns about similar effects when targeting tumor blood vessels by vaccination. However, we observed a reduction in metastatic burden in mice immunized against the tumor vascular antigen ED-A (paper III). In contrast to strategies targeting VEGF or its receptor, which are based on inhibition of a signaling pathway, vaccination against tumor vascular antigens attracts an immune response towards the tumor vasculature. Targeting of galectin-1 will, in addition to attracting immune cells towards the tumor, inhibit galectin1-1 signaling. Several preclinical tumor studies have described a therapeutic benefit of treatment with a monoclonal anti-galectin-1 antibody in absence of apparent-side effects or resistance (67, 191, 206, 263). Whether long-term targeting of galectin-1 might cause resistance remains to be elucidated.

Reports from phase I and II clinical trials investigating safety and therapeutic efficacy of radiolabelled or cytokine-fused monoclonal ED-B antibodies (L19) confirm a high selectivity of ED-B targeting for tumors (110, 137-141). This suggests that targeting ED-B and possibly other tumor vascular antigens by therapeutic vaccination is feasible.

Concluding remarks and future perspectives

The work presented in this thesis shows that eliciting an antibody response against molecules associated with the tumor vasculature is a promising strategy for treatment of primary tumors and metastatic disease.

We demonstrate that using a fusion protein consisting of the antigen to be targeted and a part derived from a foreign antigen, such as a bacterial protein, in combination with a potent adjuvant, an antibody response can be elicited. Using the fusion protein technique we were able to induce antibody responses against three different antigens, suggesting that the method can be used to elicit an immune response also against other self-proteins. We show that vaccination against antigens expressed around the tumor vasculature can inhibit tumor growth in a preventive setting, targeting ED-B and galectin-1 in subcutaneous tumor models, and in a therapeutic setting, when targeting ED-A in a transgenic model of tumorigenesis. Furthermore, our results show that targeting tumor vascular antigens might have a protective effect against metastasis. We have characterized the immunostimulatory properties of an adjuvant, M720/CpG, and confirmed its ability to aid an immune response against self-antigens when using it for vaccination against ED-A and galectin-1. As it is safe for use in humans, it should increase the feasibility of therapeutic vaccination in the clinic.

The expression of ED-A, ED-B and galectin-1 is associated with the tumor vasculature. By targeting the tumor stroma, several mechanisms employed by the tumor cells to avoid immune recognition can be circumvented. Targeting of ED-A and ED-B is mainly based on attraction of an immune response towards the tumor vasculature, which will lead to tissue destruction. The risk for development of resistance mechanisms, as seen when inhibiting the function of a growth factor, is less likely. The targeting effect for galectin-1 is more multifaceted, as it is found on the surface of endothelial cells as well as soluble in the circulation. We have demonstrated that anti-galectin-1 antibodies are able to neutralize soluble galectin-1 in the circulation. Furthermore, it has been demonstrated to act as a pro-angiogenic growth factor. Therefore targeting galectin-1 by vaccination combines functional inhibition and the targeting effect.

We have shown that immunization against galectin-1 increased the amount of infiltrating leukocytes into the tumors. Since galectin-1 has been shown to directly suppress the function of a number of immune cells, we will further study the mechanisms behind the reduction in tumor volume

caused by the immunization. Galectin-1 has been demonstrated to mediate resistance of tumors to anti-VEGF treatment by directly interacting with VEGFR-2 and stimulating signaling via this receptor. It would therefore be interesting to test a combination of anti-VEGF treatment and an immunization against galectin-1 in anti-VEGF resistant tumors.

We have generated species-specific antibodies against ED-A, ED-B and TNCC by immunization of rabbits and subsequent affinity purification. Using these antibodies we plan to analyze expression patterns of these molecules in human tumors, such as breast, prostate, colon and brain tumors. This will establish whether these antigens are expressed simultaneously. To increase the efficacy of the immunization approach it could be an advantage to target several molecules associated with the tumor vasculature at the same time. Besides the molecules presented in this thesis there are further antigens that have been reported to be highly expressed during tumor angiogenesis. One example is the C-domain of tenascin-C (TNCC).

As dogs are affected by tumors in a similar fashion as humans, they represent an interesting patient group for a proof-of-concept study with a therapeutic vaccine. Expression of ED-A, ED-B and TNCC in dogs has not been analyzed previously. We have generated dog-specific antibodies for ED-A and TNCC. In collaboration with veterinarians we are collecting tumor tissue from dogs to map the expression of these vascular antigens in dog tumors. The approach of targeting tumor vascular antigens might be applicable for treatment of dogs with cancer, for which additional treatment alternatives are needed.

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