Development of a Cancer Vaccine Targeting Tumor Blood Vessels

ELISABETH JM HUIJBERS
A treatment strategy for cancer is the suppression of tumor growth by directing an immune response to the tumor vessels, which will destroy the tissue.

In this thesis we describe the development of a vaccine that targets antigens expressed around angiogenic vasculature in most solid tumors. These antigens are alternative spliced extra domains of glycoproteins present in the extracellular matrix; e.g. the extra domain-B (ED-B) and extra domain-A (ED-A) of fibronectin and the C-domain of tenascin-C (TNCC).

We show that it is possible to break self-tolerance and induce a strong antibody response against ED-B by vaccination. Furthermore, tumor growth was inhibited and the changes observed in the tumor tissue were consistent with an attack of the tumor vasculature by the immune system.

For clinical development of therapeutic vaccines, targeting self-molecules like ED-B, a potent but non-toxic biodegradable adjuvant is required. The squalene-based Montanide ISA 720 (M720) in combination with CpG DNA fulfilled these requirements and induced an equally strong anti-self immune response as the preclinical golden standard Freund’s adjuvant. We have further characterized the immune response against ED-B generated with the adjuvant M720/GpG.

The ED-B vaccine also inhibited tumor growth in a therapeutic setting in a transgenic mouse model of pancreatic insulinoma in which tumorigenesis was already initiated. Furthermore, antibodies against ED-A and TNCC could be induced in mice and rabbits. We analyzed the expression of ED-A in breast tumors of transgenic MMTV-PyMT mice, a metastatic breast cancer model, with the aim to use this model to study the effect of an ED-A vaccine on metastasis. We also detected ED-B in canine mammary tumor tissue. Therefore vascular antigens might also represent potential therapeutic targets in dogs.

All together our preclinical data demonstrate that a vaccine targeting tumor blood vessels is a promising new approach for cancer treatment.

Keywords: Vaccine, Therapeutic, Cancer, Tumor, Angiogenesis, Immunization, Vascular, Extracellular matrix

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Life is what you make of it!
List of Papers

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


§ equal contribution

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Contents

Introduction .................................................................................................................. 13
CANCER .................................................................................................................... 13
THE VASCULAR SYSTEM ........................................................................................ 15
TREATMENT OF CANCER ....................................................................................... 19
VASCULAR TARGETS .............................................................................................. 28
SELF-TOLERANCE .................................................................................................. 39
ADJUVANTS ............................................................................................................. 40
TUMOR MODELS ..................................................................................................... 41

Present investigations ................................................................................................. 44
VACCINATION MECHANISM .................................................................................. 44
DEVELOPMENT OF A POTENT ADJUVANT SAFE FOR CLINICAL USE .......... 46
Paper I ...................................................................................................................... 47
Paper II .................................................................................................................... 49
Paper III ................................................................................................................... 50
Paper IV ................................................................................................................... 51
Discussion ................................................................................................................ 52
Future perspectives and concluding remarks ......................................................... 54

Populär vetenskaplig sammanfattning ................................................................. 57
Nederlandse samenvatting ....................................................................................... 59
Deutsche Zusammenfassung .................................................................................... 61
Acknowledgments .................................................................................................... 63
References ................................................................................................................. 66
Abbreviations

Ab
Antibody

ADCC
Antibody dependent-cell-mediated cytotoxicity

ALL
Acute lymphoblastic leukemia

APC
Antigen-presenting cell

AS
Adjuvant system

B-cell
Bone marrow-lymphocyte; B-lymphocyte

BAFF
B-cell activating factor

BCG
Bacillus Calmette-Guérin

BCR
B-cell receptor

C
Constant Ig domain

CD
Cluster of differentiation

CDC
Complement-dependent cytotoxicity

CEP
Carboxyethylpyrrole

CH
Constant Ig domain of the heavy chain

CIS
Carcinoma in situ

CLL
Chronic lymphocytic leukemia

CML
Chronic myelogenous leukemia

CSF-1
Colony stimulating factor 1

CTL
Cytotoxic T-lymphocyte

CTLA4
Cytotoxic T-lymphocyte antigen 4

DC
Dendritic cell

DLL4
Delta-like ligand 4

E. coli
Escherichia coli

EBRT
External beam radiotherapy

EC
Endothelial cell

ECM
Extracellular matrix

ED-A
Extra domain-A; EIIIA

ED-B
Extra domain-B; EIIIB

EDA+
ED-A containing

EDB+
ED-B containing

EGF
Epidermal growth factor

EGFR
Epidermal growth factor receptor

ELISA
Enzyme linked immunosorbent assay

EMT
Epithelial mesenchymal transition

FDA
American Food and Drug Administration

FGF
Fibroblast growth factor
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>FLK1</td>
<td>murine VEGFR-2</td>
</tr>
<tr>
<td>FLT-3</td>
<td>Fms-related tyrosine kinase 3</td>
</tr>
<tr>
<td>FN</td>
<td>Fibronectin</td>
</tr>
<tr>
<td>GEMM</td>
<td>Genetically engineered mouse model</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal tract</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage-colony stimulating factor</td>
</tr>
<tr>
<td>GMDP</td>
<td>N-acetylglucoseamine-1-4-N-acetylmuramyl-alanyl-D-isoglutamine</td>
</tr>
<tr>
<td>GMDP-A</td>
<td>N-acetylglucoseamine-1-4-N-acetylmuramyl-alanyl-D-glutamic acid</td>
</tr>
<tr>
<td>GMP</td>
<td>Good manufacturing practice</td>
</tr>
<tr>
<td>GST</td>
<td>Glutathione-S-transferase</td>
</tr>
<tr>
<td>GU</td>
<td>Genitourinary tract</td>
</tr>
<tr>
<td>HAV</td>
<td>Hepatitis A virus</td>
</tr>
<tr>
<td>HBV</td>
<td>Hepatitis B virus</td>
</tr>
<tr>
<td>HER</td>
<td>Human epidermal growth factor receptor</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>HMGB1</td>
<td>High-mobility group B1</td>
</tr>
<tr>
<td>HPV</td>
<td>Human papillomavirus</td>
</tr>
<tr>
<td>I&lt;sup&gt;131&lt;/sup&gt;</td>
<td>Radioactive iodine</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule 1</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>ISA</td>
<td>Incomplete Seppic adjuvant</td>
</tr>
<tr>
<td>KHL</td>
<td>Keyhole limpet hemocyanin</td>
</tr>
<tr>
<td>KIT</td>
<td>Stem cell factor receptor</td>
</tr>
<tr>
<td>M720</td>
<td>Montanide ISA 720</td>
</tr>
<tr>
<td>MAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MAC</td>
<td>Membrane attack complex</td>
</tr>
<tr>
<td>MART</td>
<td>Melanoma antigen recognized by T-cells</td>
</tr>
<tr>
<td>MDP</td>
<td>Muramyl dipeptide</td>
</tr>
<tr>
<td>MDSC</td>
<td>Myeloid-derived suppressor cell</td>
</tr>
<tr>
<td>MHC-I</td>
<td>Major histocompatibility complex class I</td>
</tr>
<tr>
<td>MHC-II</td>
<td>Major histocompatibility complex class II</td>
</tr>
<tr>
<td>MLP</td>
<td>Monophosphoryl lipid A</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MMTV</td>
<td>Mouse mammary tumor virus</td>
</tr>
<tr>
<td>M720</td>
<td>Montanide ISA 720</td>
</tr>
<tr>
<td>MTD</td>
<td>Maximum tolerated dose</td>
</tr>
<tr>
<td>NCI</td>
<td>National Cancer Institute, USA</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor κB</td>
</tr>
<tr>
<td>NK cell</td>
<td>Natural killer cell</td>
</tr>
</tbody>
</table>
NOD: Nucleotide-binding oligomerization domain
NSCLC: Non-small cell lung carcinoma
O/W: oil-in-water
ORO: Opossum-rat-opossum
PAP: Prostatic acid phosphatase
PBMC: Peripheral-blood mononuclear cell
PCR: Polymerase chain reaction
PDGF: Platelet derived growth factor
PDGFR: Platelet derived growth factor receptor
pIII: Minor bacteriophage coat protein
PKB/Akt: Protein kinase B
PIGF: Placental growth factor
PRR: Pattern recognition receptor
PSA: Prostate specific antigen
pVIII: Major bacteriophage coat protein
PyMT: Polyoma virus middle T antigen
RA: Rheumatoid arthritis
Rac1: Ras-related C3 botulinum toxin substrate 1
RAGE: Receptor for advanced glycation end products
RCC: Renal cell carcinoma
RGD: Arginine-Glycine-Aspartic acid, Arg–Gly–Asp
RIP: Rat insulin promoter
RIT: Radio-immunotherapy
scFV: Single-chain FV fragment
SCID: Severe combined immunodeficiency
SEM: Scanning electron microscope
SIP: Small immunoprotein
SMC: Smooth muscle cells
SR protein: Serine- and arginine-rich protein
SV: Simian virus
T-cell: Thymus-lymphocyte; T-lymphocyte; CD8 T-cell/lymphocyte
T241: T241 fibrosarcoma
Tag: T-antigen
TAM: Tumor-associated macrophage
TCR: T-cell receptor
TGF-β: Transforming growth factor-beta
Th-cell: T-helper cell/lymphocyte; CD4 T-cells/lymphocyte
TIMP: Tissue inhibitor of matrix metalloproteinase
TKI: Tyrosine kinase inhibitor
TLR: Toll-like receptor
TNC: Tenascin-C
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>TNCC</td>
<td>C-domain of tenascin-C</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor α</td>
</tr>
<tr>
<td>T&lt;sub&gt;reg&lt;/sub&gt;</td>
<td>Regulatory T-lymphocyte</td>
</tr>
<tr>
<td>TSP</td>
<td>Thrombospondin</td>
</tr>
<tr>
<td>tTF</td>
<td>Extracellular soluble domain of tissue factor</td>
</tr>
<tr>
<td>TUR</td>
<td>Transurethral resection</td>
</tr>
<tr>
<td>V</td>
<td>Antibody variable region</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR</td>
<td>Vascular endothelial growth factor receptor</td>
</tr>
<tr>
<td>VLA-4</td>
<td>Very late antigen 4</td>
</tr>
<tr>
<td>VPF</td>
<td>Vascular permeability factor</td>
</tr>
<tr>
<td>W/O</td>
<td>water-in-oil</td>
</tr>
<tr>
<td>WBRT</td>
<td>Whole brain radiation treatment</td>
</tr>
<tr>
<td>TRX</td>
<td>Thioredoxin</td>
</tr>
</tbody>
</table>
Introduction

CANCER

Cancer is after ischemic heart disease the most common cause of death in the Western world and it is the third leading cause of death in the developing countries\cite{1-3}. The most common types of cancer diagnosed in 2009 in Sweden were prostate cancer and breast cancer. Over the last two decades the recorded cancer incidence has slightly increased. Explanations for this are better screening programs and diagnostics that detect more cases as well as increasing age of the population. However, due to better treatment alternatives it is now possible to cure the disease or prolong survival drastically depicted for example by the fact that the relative 5-year survival has increased from 30% to 70% over the last 40 years in Sweden\cite{1}. In North America, Sweden and Japan 80% of women diagnosed with breast cancer survive the disease due to optimized treatment compared to 60% in middle-income and below 40% in low-income countries\cite{4,5}. However, once the disease has disseminated (metastasized) treatment is difficult and often can only prolong survival but not cure the disease.

The formation of a tumor is characterized by uncontrolled cell growth. A tumor can be noninvasive (benign) or invasive (malign). Noninvasive tumors do not grow into the surrounding tissue and usually do not interfere with normal tissue and organ function, whereas malignant tumors invade the surrounding tissue and disturb it. Tumor cells become more malignant by successively gaining new capabilities in the multistep tumor growth pathogenesis. These capabilities defined as the hallmarks of cancer are suggested to include: sustained proliferative signaling, evasion of growth suppression, resistance to cell death, enabling of replicative immortality, induction of angiogenesis, reprogramming of energy metabolism, escape of immune destruction and activation of tumor invasion and metastasis. Genome instability generates genetic diversity, which is the cause of malignancy and inflammation\cite{6,7}. Malignant tumors can eventually develop metastases, when the cancer cells are spread to distant organs enabling development of numerous new tumors within the body. Metastatic disease is more difficult to treat than a localized primary tumor and therefore accounts for 90% of all cancer-associated mortality\cite{8}.

Metastatic cancer cells need to acquire several new traits in order to be able to invade and settle into a new tissue environment. Obstacles that need
to be tackled by metastatic cancer cells are invasion of local tissue environment, intravasation (leaving the tissue and entering into the circulation), to survive shear stress and immune attacks, transport through the body, extravasation (entering new tissue via penetration of the blood vessel wall), micrometastases formation and colonization of the new milieu\textsuperscript{9,10}.

Causes of cancer/risk factors

Factors contributing to the development of cancer are heredity, increasing age and living conditions (physical environment and lifestyle)\textsuperscript{11}. Increasing age is a risk factor for cancer since with the accumulating number of cell divisions during a human lifetime there is a higher chance for mistakes (mutations) to occur. Environmental factors have been found to act tumor promoting by induction of mutagenesis or proliferation. Some environmental factors that have been connected to cancer diseases are virus infections (hepatitis B and C or human papilloma virus (HPV)), exposure to carcinogens or agents that cause chronic inflammation or infections. Chronic inflammation is believed to be the cause of 15-20\% of all malignancies worldwide. Lifestyle factors like use of tobacco, unhealthy diet and physical inactivity were also found to be risk factors for cancer development\textsuperscript{11-14}.

Tumor composition

The majority (80\%) of cancers and the most well studied tumor type are carcinomas, which are derived from epithelial cells\textsuperscript{11,15}. In general a tumor can be seen as a separate ‘organ’ that in addition to tumor cells also consists of stroma. The tumor stroma contains immune cells such as macrophages, neutrophils and T-cells; mesenchymal cells \textit{e.g.} matrix synthesizing fibroblasts, extracellular matrix (ECM) and blood vessels\textsuperscript{16}. Tumor growth can also be described as a process of continues inflammation as compared to sterile self-limiting inflammation observed in wound healing. Therefore a tumor is said to resemble a ‘wound that does not heal’\textsuperscript{17}. Keratinocytes (epithelial cells) in a wound undergo partial epithelial mesenchymal transition (EMT) and gain a mitogenic and motile phenotype to mediate wound closure. Tumor cells however undergo full EMT and adapt an invasive phenotype by losing their cell-cell contacts, obtaining fibroblast-like morphology and expressing mesenchymal proteins\textsuperscript{9,18-21}. Tumors also contain a matrix of fibrin and fibronectin similar to a clot in wounds\textsuperscript{17}. However, in tumors the clot persists due to constant vessel leakiness, whereas vascular hyperpermeability in wounds is transient. The presence of inflammatory cells in a tumor promotes further tumor growth and progression. Since these cells release mediators that maintain inflammation\textsuperscript{18,22,23}. Similar growth factors are found in tumors as are
involved in the process of wound healing such as vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF) and transforming growth factor-beta (TGF-β)\textsuperscript{18,24-26}. A tumor requires blood vessels to sustain growth and therefore turns on angiogenesis, the formation of new blood vessels from preexisting ones\textsuperscript{27}. In a healing wound, angiogenesis is tightly regulated and transient, instead of continuous as in a tumor. Carcinomas contain fibroblasts and myofibroblasts due to release of PDGF, a fibroblast mitogen, by activated platelets and tumor cells\textsuperscript{26}. In a wound these mesenchymal cells produce ECM, growth factors and proteinases for fibrin matrix degradation\textsuperscript{18,28}. Similar functions have been found for these cells in cancer\textsuperscript{18}.

THE VASCULAR SYSTEM

As pointed out previously tumors do not only consist of tumor cells but they also contain blood vessels and stroma cells. All cells, normal cells as well as cancer cells, need oxygen and nutrients to survive and proliferate. Cancer cells, however, are extremely fast growing compared to normal cells. This will lead to an increasing consumption of oxygen and nutrients and in order to satisfy this demand tumors recruit blood vessels\textsuperscript{6,7}.

The vascular system is also called the circulatory system and was first described in detail by William Harvey in the year 1628\textsuperscript{29}. It consists of the cardiovascular system that distributes blood and the lymphatic system that transports lymph in the body. Its function is to provide the body with oxygen and other gases, nutrients, hormones, to control homeostasis, pH and body temperature as well as to fight diseases. The cardiovascular system includes the pulmonary circulation and the systemic circulation, which are connected by the heart. In the lungs the blood is oxygenated and then it is pumped through the arteries by the heart to be distributed within the body. The exchange of oxygen, nutrients and waste products between blood and tissue occurs in the capillaries via diffusion through the interstitial fluid. The transport of de-oxygenated (carbon dioxide rich) blood, back to the heart, occurs via venules and veins. In the heart it enters the pulmonary circulation where it will be re-oxygenated\textsuperscript{30}. The lymphatic system consists of a network of lymph vessels carrying the lymph, lymphoid tissues (\textit{e.g.} lymph nodes) and structures involved in the production and circulation of lymphocytes (\textit{e.g.} bone marrow, spleen and thymus). It collects excessive interstitial fluid from the tissues and drains it into the vascular system. Additionally it absorbs and transports fatty acids and fat as chyle to the circulatory system and moves immune cells to and from the lymph nodes\textsuperscript{30,31}.

Arteries, arterioles, veins and venules are composed of endothelial cells and smooth muscle cells (SMCs). The SMCs provide vessel stability and regulate the blood pressure via arteriolar contraction. The endothelial cells
form the vessel lumen and are in contact with the blood. The capillaries are responsible for the exchange with the surrounding tissue and therefore their composition is different from other blood vessels. Capillaries lack SMC coverage and are instead stabilized by pericytes (Figure 1). The pericytes are attached to the endothelial cells and the basement membrane at the abluminal side of the vessel\textsuperscript{32}. All blood vessels are surrounded by ECM, to which the cells in the tissue adhere.

**Figure 1.** Capillary. Endothelial cells (EC) (gray) form the vessel lumen, which is surrounded by basement membrane and pericytes (black)\textsuperscript{32}.

**Angiogenesis**

The vascular system develops *de novo* during embryogenesis and this process is called vasculogenesis. Vasculogenesis is genetically programed, and vessels arise from endothelial cells that originate from progenitors derived from the mesoderm\textsuperscript{33-35}. Blood vessels can also develop from pre-existing vessels, a process called angiogenesis\textsuperscript{27}. The most well studied types of angiogenesis are sprouting and intussusception (the splitting of a mother vessel)\textsuperscript{36,37}. Other proposed mechanisms for angiogenesis are involving circulating endothelial progenitor cells and looping\textsuperscript{38-40}.

Sprouting angiogenesis is initiated by a VEGF-A gradient, which triggers the formation of a tip cell. Local release of proteases such as matrix metalloproteinases (MMPs) by the endothelial cells and other cells causes ECM degradation and creates space for the cells to migrate\textsuperscript{41-46}. The tip cell then develops filopodia to sense its surroundings and to be able to navigate and migrate. Stalk cells, which form a firm cord, will follow the tip cell and proliferate to elongate the sprout. Subsequently, the tip cell will fuse with other tip cells and a vessel will be formed that will be perfused. The vessel will mature and pericytes will be attracted to stabilize it. The process of sprouting is terminated by quiescence of the vessel\textsuperscript{47}.

During intussusception, a process believed to be of importance during vessel remodeling, transluminal pillars (holes) appear that split the vessel. In contrast to sprouting angiogenesis, blood flow is maintained throughout the
whole splitting event. Contractile periendothelial cells, macrophages and blood flow are all believed to be involved in this process\textsuperscript{48}.

Angiogenesis is a rare event in the healthy adult but takes place under physiological conditions like wound healing and the female menstrual cycle, in which it is tightly regulated by a balance between pro- and anti-angiogenic factors\textsuperscript{35,49}. However, under pathological conditions excessive or insufficient angiogenesis can occur. Conditions characterized by excessive angiogenesis are inflammatory diseases (e.g. rheumatoid arthritis), tumor growth and diabetic retinopathy. Insufficient angiogenesis occurs in ischemia and might also be connected to ulcers in the digestive tract as well as infertility\textsuperscript{35}.

A large number of endogenous factors regulating angiogenesis both in a positive and negative manner have been described. Some examples of positive regulators (pro-angiogenic factors) are VEGF, PDGF, fibroblast growth factor (FGF), placental growth factor (PIGF), angiopoietin and toll-like receptors (TLRs)\textsuperscript{49-55}. Negative endogenous regulators (anti-angiogenic factors) are amongst others thrombospondin-1 and -2 (TSP-1, TSP-2), tumstatin, endostatin, angiostatin and histidine-rich glycoprotein\textsuperscript{50,56-60}.

**Vascular endothelial growth factor**

The most extensively studied pro-angiogenic factor is VEGF, which was first discovered under the name vascular permeability factor (VPF)\textsuperscript{61,62}.

The mammalian VEGF family consists of five different isoforms (VEGF-A, -B, -C, -D and PIGF), which exert their effect through binding to different VEGF receptors. The VEGF receptor-family of tyrosine kinase receptors includes three different receptors (VEGFR-1, VEGFR-2 and VEGFR-3)\textsuperscript{51}. Upon binding of VEGF these receptors form hetero- or homodimers, which leads to autophosphorylation of their intracellular domain inducing a signaling cascade. VEGF-A exerts its pro-angiogenic effect through the VEGFR-2 and is involved in endothelial cell survival, mitosis and motility as well as vascular permeability\textsuperscript{51,63-65}. There are five different splice variants of the human VEGF-A monomer, of which VEGF-A\textsubscript{165} is most abundantly expressed\textsuperscript{63}. VEGF-A is essential for development of the vascular system since mice lacking only a single Vegf-a allele die of vascular defects around embryonic day 11\textsuperscript{62,66}. VEGFR-2\textsubscript{\textasciitilde} mice have a phenotype analogous to VEGF-A\textsubscript{\textasciitilde} mice and die around embryonic day 8.5 due to vascular defects\textsuperscript{67}. The VEGFR-1\textsubscript{\textasciitilde} phenotype is characterized by excessive endothelial proliferation and occlusion of the vessel lumen and mice die around embryonic day 8.5-9. These observations indicate that VEGFR-1 is a negative regulator of angiogenesis, at least during development\textsuperscript{63,68}. VEGFR-3 is present on lymphatic vessels and stimulation of the receptor by its ligands VEGF-C and VEGF-D promotes development of lymph vessels from preexisting ones a process defined as lymphangiogenesis\textsuperscript{31}.  


Angiogenesis mediated via endogenous toll-like receptor ligands

It is now clear that angiogenesis also can be induced by mechanisms that are independent of VEGF and some of these pathways are regulated by TLRs, pattern-recognition receptors (PRRs) involved in innate immunity. One example is oxidative stress promoted by myeloid cells in wound healing, inflammation and tumors that causes an accumulation of oxidative products derived from phospholipids in the cell membrane, such as carboxyethylpyrrole (CEP) and other related pyrroles. CEP was found to bind to TLR2 on endothelial cells. CEP-induced signaling through TLR2 in endothelial cells and activated C3 botulinum toxin substrate 1 (Rac1), a protein that mediates cell signaling. Activation of Rac1 promotes endothelial cell migration via integrins and hence angiogenesis.

Another example is the release of the protein high-mobility group B1 (HMGB1) from necrotic cells, which was shown to signal through the receptor for advanced glycation end products (RAGE), TLR2 and TLR4. Activation of these receptors induced upregulation of angiogenic factors such as VEGF in hematopoietic and endothelial cells via activation of nuclear factor κB (NF-κB).

The angiogenic switch

Most tumors remain dormant (1-2 mm³ in size) and do not develop into an aggressive phenotype. In 1971 Judah Folkman postulated that this might be due to the fact that angiogenesis is lacking to provide the tumor with sufficient nutrients and oxygen in order to grow larger. One critical step in the progression of tumor development is therefore called the ‘angiogenic switch’. Hypoxia, hypoglycemia and genetic as well as inflammatory alterations are believed to trigger the angiogenic switch. These triggers might induce the expression of oncogenes in tumor cells that can downregulate anti-angiogenic factors such as thrombospondin-1. But the triggers might also activate the overexpression of pro-angiogenic factors in tumor cells or in the by the tumor recruited inflammatory cells, such as macrophages and mast cells. Eventually there will be a misbalance between pro- and anti-angiogenic factors in favor of the pro-angiogenic factors, which tilts the balance towards the development of tumor blood vessels resulting in a vascular and eventually metastatic tumor type.

Tumor vessels

The blood vessels in a tumor are leaky/highly permeable due to an excess of VEGF-A produced by the tumor cells or inflammatory cells recruited by the tumor. The activated tumor endothelium also overexpresses several proteins and/or receptors such as for example VEGFR-2 or survivin, an anti-apoptotic protein, promoting an angiogenic phenotype and enhanced vessel
growth. Tumor vessels also show reduced pericyte coverage and partial loss of endothelial cells$^{52,86-88}$. All these features lead to formation of a malfunctioning disorganized and chaotic tumor vasculature in which hierarchy is lost and perfusion is impaired. This can be seen in Figure 2 in which tumor vasculature and normal vasculature are depicted.

![Figure 2. Normal vasculature and tumor vasculature. The left panel depicts normal organized microvasculature and the right panel disorganized tumor microvasculature, in which vessel hierarchy was lost. Images of polymer casts were taken in a scanning electron microscope (SEM). Reprinted with permission from the Nature Publishing Group: Nature Medicine$^{89}$, copyright 2003.]

**TREATMENT OF CANCER**

In addition to the actual tumors cells, cancer consists of different types of cells, extracellular matrix and blood vessels, which all represent potential targets for tumor treatment.

Classical cancer treatment is surgery (tumorectomy) to remove the tumor if possible combined with chemotherapy and/or radiotherapy as additive therapy to prevent recurrent tumor growth. In case the cancer cannot be removed chemo- and radiotherapy is used as curative therapy or for palliative care to relieve suffering. Surgery, chemotherapy and radiotherapy are until today the most widely used and most successful methods for cancer treatment. A side effect of chemo- and radiotherapy is the damage to healthy tissue that these agents cause, because they will also affect normal tissue.

During recent years, new treatment options have become available and are also under development. One group of drugs used in the clinic are tyrosine kinase inhibitors (TKIs) that inhibit growth factor receptors on tumor blood vessels and tumor cells$^{90-92}$. Monoclonal antibodies (MAbs) have been developed for the treatment of cancer as well. These monoclonal antibodies target either VEGF$^{93,94}$, which is overexpressed in tumors to
promote tumor angiogenesis, or tumor antigens (HER2/Neu; ErbB2 receptor) or growth factor receptors (EGF-R, HER1)\textsuperscript{95-97}.

Another way of targeting tumor tissue is by immunotherapy. Different strategies are used and there are a number of therapies in preclinical development. Priming of dendritic cells with tumor-associated antigens or proteins/receptors overexpressed on the tumor endothelium, and adoptive T-cell transfer are examples of approaches used. These different strategies will be described in more detail below.

Chemotherapy

The chemical warfare mustard gas was found to suppress hematopoiesis and thus cell growth. In 1942 the related agent ‘nitrogen mustard’ was used as the first chemotherapeutic drug to treat a cancer patient with non-Hodgkin’s lymphoma\textsuperscript{98}. Over 20 years later the first combination of different chemotherapeutic drugs was administered to patients with acute lymphoblastic leukemia (ALL)\textsuperscript{99}. However, until 1975 the genetic and biochemical mechanisms of cancer pathogenesis were still unidentified\textsuperscript{100}, which made target-specific treatment impossible. Since the introduction of the first chemotherapeutic drug many new drugs have been developed. The general mechanism of these agents is the inhibition of cell proliferation. Tumor cells are highly proliferative and are therefore more sensitive to chemotherapeutics than normal cells. In Table 1 the different classes of chemotherapeutic drugs used in the clinic are listed.
Table 1. Chemotherapeutic drugs used as conventional chemotherapy in the clinic\textsuperscript{101}

<table>
<thead>
<tr>
<th>Agent group</th>
<th>Mechanism of action</th>
<th>Drug substance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkylating agents</td>
<td>Crosslinking of DNA strands by alkylation of purine bases, which leads to apoptosis.</td>
<td>CLASSICAL: cyclophosphamide, melphalan</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PLATINATING: cisplatin, carboplatin</td>
</tr>
<tr>
<td>Antimetabolites</td>
<td>Interfere with DNA and/or RNA synthesis.</td>
<td>ANTIFOLATES: methotrexate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PURINE ANALOGS: fludarabine, gemcitabine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>THYMIDYLATE SYNTHASE INHIBITORS: fluorouracil, capecitabine</td>
</tr>
<tr>
<td>Topoisomerase inhibitors</td>
<td>Topoisomerase I inhibitors interfere with DNA unwinding and strand passage.</td>
<td>TOPOISOMERASE I: topotecan, irinotecan</td>
</tr>
<tr>
<td></td>
<td>Topoisomerase II inhibitors interfere with DNA replication, transcription and repair.</td>
<td>TOPOISOMERASE II: etoposide, doxorubicin</td>
</tr>
<tr>
<td>Taxanes</td>
<td>Promote microtubule assembly; antimitotic</td>
<td>Paclitaxel, docetaxel</td>
</tr>
<tr>
<td>Vinca alkaloids</td>
<td>Inhibit microtubule polymerization; antimitotic</td>
<td>Vincristine, vinorelbine</td>
</tr>
<tr>
<td>Mixed bag</td>
<td>DNA intercalating agents; L-asparaginase catalyzes the hydrolysis of asparagine to aspartic acid (ALL leukemic cells are not able to produce asparagine).</td>
<td>ANTIBIOTICS: streptozotocin, Anthracycline antibiotics: doxorubicin, daunomycin ENZYMES: L-Asparaginase</td>
</tr>
</tbody>
</table>

All these therapeutic agents are given as high-dose chemotherapy (at the maximum tolerated dose (MTD)) to avoid drug resistance\textsuperscript{101,102}. This is often accompanied by severe side effects caused by immunosuppression such as anemia, neutropenia or thrombocytopenia, which have to be treated concurrently\textsuperscript{103}. Therefore, low-dose metronomic chemotherapy was developed, which is the continuous oral administration of conventional chemotherapy at relatively low, minimally toxic doses\textsuperscript{104-106}. Metronomic treatment has shown promising results in phase II clinical trials and is now evaluated for use in phase III clinical studies\textsuperscript{107,108}. Low-dose chemotherapy has been shown to activate the immune system rather then to suppress it as well as to inhibit tumor angiogenesis\textsuperscript{13,103,108,109}.

Radiotherapy

Radiotherapy has been used for more than a century to treat cancer. Its mechanism of action is to damage cellular DNA by ionizing radiation. This type of radiation harms the cells directly by photons or electrical charged particles or by formation of free hydroxyl radicals due to ionization of water in the body\textsuperscript{110}. It is either used as curative, additive (together with
chemotherapy) or palliative cancer treatment. Radiotherapy is used for local treatment of tumors to avoid damage of healthy tissues.

Tyrosine kinase inhibitors

Targeted therapy

The mechanism of action of these drugs is the inhibition of the tyrosine kinase activity of receptor tyrosine kinases. In tumors the tyrosine kinase signaling pathways are often overactive and promote tumor growth and angiogenesis. Small molecule tyrosine kinase inhibitors used in the clinic that target multiple kinases are sorafenib (Nexavar®), sunitinib (Sutent®) and imatinib (Gleevec®). Sorafenib blocks preferentially VEGFR-1, VEGFR-2, VEGFR-3, PDGF receptor β (PDGFRβ), and Raf kinases. Sunitinib mainly inhibits VEGFR-2, PDGFRα and PDGFRβ, the stem cell factor receptor (KIT), fms-related tyrosine kinase 3 (FLT-3), colony-stimulating factor 1 (CSF-1) and rearranged during transfection kinase (RET). Imatinib preferentially targets the BCR-ABL kinase involved in the pathogenesis of chronic myelogenous leukemia (CML), KIT and PDGFRβ. Since tyrosine kinase activity is required for tissue homeostasis treatment with TKIs is often accompanied by side effects. Hypertension is for example frequently seen in patients treated with sorafenib or sunitinib. Occurrence of hypertension is used as a measure of treatment effectiveness and related to prolonged disease survival. Recently it was shown that sunitinib related hypertension is caused by concurrent inhibition of the PDGFR on pericytes in the heart vasculature.

Monoclonal antibodies

Over the past decade several MAbs have been developed for treatment of cancer and other diseases. Examples of monoclonal antibodies used as anti-cancer drugs in the clinic are bevacizumab (Avastin®), a VEGF-A binding antibody, trastuzumab (Herceptin®), an anti-HER2 antibody, cetuximab (Erbitux®), an epidermal growth factor receptor (EGFR; HER1) blocking antibody and rituximab (Rituxan®), an anti-CD20 (cluster differentiation 20) antibody. Trastuzumab binds to the HER2/Neu (ErbB2) receptor, a tyrosine kinase receptor frequently overexpressed on solid tumors. HER2 heterodimerizes with EGFR and stimulation of this receptor by its ligand epidermal growth factor promotes cell survival, differentiation, invasion and angiogenesis. HER2 can also act as an oncprotein, which does not need activation by its ligand but instead has constitutive kinase activity. Tumors overexpressing HER2 have a more aggressive phenotype and are more difficult to treat. Cetuximab directly binds to the EGFR and blocks the binding site of the EGF-ligand thereby
preventing receptor signaling. Rituximab for example is used to treat chronic lymphocytic leukemia (CLL). CLL is characterized by accumulation of monoclonal B-lymphocytes (B-cells) in the blood, secondary lymphoid tissues and bone marrow. Binding of rituximab to CD20, a tetraspan phosphoprotein expressed on the surface of B-cells, leads to elimination of all B-cells via antibody dependent-cell-mediated cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC) and direct induction of apoptosis.

Monotherapy with the anti-VEGF-A antibody (Avastin ®) has shown to be ineffective for cancer treatment in humans and only in combination with conventional chemotherapy an overall survival benefit in colorectal and lung cancer patients could be achieved. One explanation for this observation is that anti-VEGF-A therapy does not strangulate the tumor but instead normalizes the leaky tumor vasculature. This leads to a better delivery of chemotherapeutic drugs into the tumor tissue and a greater inhibitory effect on tumor growth.

A monoclonal antibody in clinical trials is the anti-CTLA4 (cytotoxic T-lymphocyte antigen 4) antibody. CTLA4 is a molecule expressed on the surface of activated T-cells by which the extent of the T-cell (T-lymphocyte) response can be regulated. It competes with CD28 for B7 (CD80/CD86) binding (a co-stimulus needed for T-cell activation; see paragraph self-tolerance) and transmits inhibitory signals to shut down T-cell activation. Blocking of CTLA4 shuts down this negative-feedback control loop and was found to enhance antitumor T-cell responses in cancer. The anti-CTLA4 antibody has been shown to give significant treatment responses in patients with cancer although at the expense of severe autoimmune side effects, such as colitis or hepatitis.

Immunotherapies

Three general aspects, which complicates development of a tumor vaccine are firstly the frequently occurring downregulation of major histocompatibility complex I (MHC-I) by tumor cells, which makes them less prone to a cytotoxic T-lymphocyte (CTL) attack. Secondly, tumors commonly cause upregulation of regulatory CD4+ CD25+ T-cells (Tregs) suppressing the host’s immune system. Thirdly cancer patients are often immunocompromised because of chemotherapy, rendering therapeutic vaccination ineffective. One way to circumvent some of the above problems is to induce a humoral immune response, involving CD4+ lymphocytes, in which endogenous antibodies are produced that directly bind to their target. This would create a more specifically directed immune response independent of MHC-I expression on tumor cells.
Preventive tumor vaccines

The only example of a prophylactic tumor vaccine in clinical use is the recombinant peptide vaccine for prevention of cervical cancer caused by HPV. Today there are two HPV vaccines, Gardasil ® and Cervarix ®, available on the market, which both protect against the most common HPV types (HPV-16 and HPV-18) causing cervical cancer. HPV is a foreign antigen, which makes vaccination against it relatively simple, since it will be recognized by the body’s immune system as foreign. However, it is more difficult to vaccinate against tumor-associated antigens because these are self-proteins and the body has developed several mechanisms to avoid an immune response against self-molecules. These mechanisms include that autoreactive T-cells are removed or inactivated during development and thus prevent endogenous tissue in the body from being attacked by the immune system, a phenomenon referred to as self-tolerance (see paragraph self-tolerance).

Therapeutic tumor vaccines

Therapeutic cancer vaccines can be divided into cell-based vaccines and antigen-based vaccines. Cell-based vaccines make use of cell transfer to induce specific tumor immunity. These types of vaccines include whole cells for example allogeneic (genetically similar; from the same species) cancer cells, peripheral-blood mononuclear cells (PBMCs) activated with tumor-associated antigens or adoptive T-cell transfer.

Antigen-based vaccines aim at novel presentation of peptide/protein antigens and methods to enhance the endogenous immune response. These vaccines make use of fusion proteins, whole bacteria or DNA. Proteins or DNA can either be injected directly to evoke an immune response or delivered within a vector to avoid breakdown and give additional immune stimulation. Vectors used are modified viruses (adenovirus or poxvirus of which vaccinia virus is the most widely used), bacteria both alive and attenuated (Salmonella, Mycobacterium, Listeria or Shigella) or yeast (nonpathogenic Saccharomyces cerevisae). The bacteria here listed are all intracellular bacteria, which have the ability to target antigen-presenting cells (APCs) directly and depending on their location in the APC they interfere with distinct antigen-presenting pathways (MHC class I or MHC class II).

The therapeutic vaccines and their mechanism of action are listed in Table 2.
Table 2. Different therapeutic vaccines/ immunotherapies used in the clinic* and in preclinical development. Antibody (Ab), neutral killer (NK) cell, dendritic cell (DC)

<table>
<thead>
<tr>
<th>Vaccine type</th>
<th>Antigen</th>
<th>Mechanism</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ANTIGEN-BASED VACCINES</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>Tumor-associated carbohydrates</td>
<td>Ab formation, T-cell response</td>
<td>FUSION PROTEIN: KHL-carbohydrate\textsuperscript{157, 158} FLK1-AP (pulse DCs by intradermal injection)\textsuperscript{159}</td>
</tr>
<tr>
<td>Whole bacteria</td>
<td>Bacterial antigens</td>
<td>Immunostimulation by infection, CTL and Ab response</td>
<td>TheraCys \textsuperscript{®}\textsuperscript{160}</td>
</tr>
<tr>
<td>DNA</td>
<td>Tumor-associated antigens, antigens on tumor vasculature</td>
<td>CTL response and or Ab response depending on the delivery vector used</td>
<td>pDERMATT\textsuperscript{161} pVAX-DLL4\textsuperscript{162} pcDNA3.1-FLK1\textsuperscript{163}</td>
</tr>
<tr>
<td><strong>CELL-BASED VACCINES</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Allogeneic whole cancer cells</td>
<td>All tumor cell antigens</td>
<td>T-cell response, NK cells, macrophages, eosinophils</td>
<td>\textsuperscript{164, 165}</td>
</tr>
<tr>
<td>APC (DC) vaccine</td>
<td>Tumor-associated antigens</td>
<td>Activation and maturation of DCs to evoke an CTL response</td>
<td>Sipuleucel-T\textsuperscript{®}\textsuperscript{166}</td>
</tr>
<tr>
<td>Adoptive T-cell transfer</td>
<td>Tumor-associated antigens</td>
<td>CTLs response</td>
<td>Selection, generation and activation of anti-tumor T-cells\textsuperscript{167-169}</td>
</tr>
</tbody>
</table>

**Cell-based vaccines**

Recently (in the year 2010) the American Food and Drug Administration (FDA) approved the first therapeutic cancer vaccine Sipuleucel-T (Provenge \textsuperscript{®}) for clinical use\textsuperscript{170,171}. The FDA approval was based on the results of a randomized placebo controlled phase III clinical\textsuperscript{171}. In this study treatment
with Sipuleucel-T gave an antigen-specific cellular and a humoral immune response and showed a 4.1-month median survival benefit and an 8.7% extended 3-year survival in patients with asymptomatic or minimal symptomatic disease compared to placebo. The vaccine is an autologous active cellular immunotherapy used for treatment of metastatic castration resistant prostate cancer. In this method autologous peripheral-blood mononuclear cells, including APCs, which are dendritic cells (DCs) and macrophages, are removed from the patient and stimulated in vitro with a prostatic acid phosphatase-granulocyte macrophage-colony stimulating factor (PAP-GM-CSF) recombinant fusion protein (PA2024). In this fusion protein a prostate antigen (prostatic acid phosphatase) is coupled to GM-CSF, an immunostimulatory agent. The activated APCs are then placed back into the patient and will promote a CTL immune response against the prostate tumor cells. Sipuleucel-T is given as a 60-minute infusion of minimal 50 million activated autologous CD54+ cells (CD54 is a surrogate marker for activated APCs) every two weeks for three doses.

Another strategy under development used for the induction of an antitumor CTL response is adoptive T-cell transfer. By this method large numbers of antitumor T-cells, which specifically recognize tumor antigens, can be selected, generated and activated in vitro and transferred back into the host. Allogeneic whole cell vaccines have also been tested in clinical trials with encouraging results. Here irradiated whole cancer cells from another host with the same tumor type are injected into the patient with the aim to evoke an immune response against the multiple antigens expressed by these cells. Whole tumor cells are poorly immunogenic and therefore additional immunostimulating agents have to be added to the vaccine.

Antigen-based vaccines
TheraCys® consists of Bacillus Calmette-Guérin (BCG), which is a live attenuated strain of Mycobacterium bovis. The whole bacterial vaccine is approved by the FDA for treatment and prophylaxis of carcinoma in situ (CIS) in the urinary bladder and prophylaxis of primary or recurrent stage Ta (noninvasive papillary carcinoma) and/or T1 (invasion of subepithelial connective tissue) papillary tumors following transurethral resection (TUR). The bacteria are administered into the bladder via a catheter (intravesical), which leads to immune stimulation caused by the bacterial infection.

A recombinant DNA vaccine for treatment of melanoma named pDERMATT (plasmid DNA Encoding Recombinant MART-1 and Tetanus toxin fragment-c), consisting of a DNA plasmid encoding melanoma associated antigen ‘melanoma antigen recognized by T-cells’ (MART-1) specific to the melanocyte lineage and an immunostimulatory tetanus toxin fragment-c, was successfully used for induction of a cytotoxic T-cell response against MART-1 in mice. In this study only the immunogenicity
of the DNA vaccine was tested and no data are available on the effect of the vaccine on tumor growth in a mouse model.

Another strategy used is carbohydrate-based vaccines, which consist of a protein carrier (e.g. keyhole limpet hemocyanin (KHL)) coupled to tumor-associated carbohydrate antigens. A foreign protein carrier is used in this strategy to make the tumor-associated carbohydrate antigens, which are self-antigens, visible to the immune system. In a phase I clinical trial the safety of a tumor-associated carbohydrate antigen-KHL vaccine injected with a saponin immunologic adjuvant was tested in patients with relapsed prostate cancer. Antibodies against the tumor antigen could be measured and a decrease in prostate specific antigen (PSA) could be observed. Further vaccine approaches to target carbohydrates on tumor cells are under investigation.

Despite the numerous different therapeutic vaccines in preclinical development only two are clinically approved for cancer treatment. Rosenberg et al. evaluated the overall objective response rate to different types of antigen-based cancer vaccines, in patients with metastatic cancer, as 3.3%. Data in this study were obtained from trials performed at the Surgery Branch of the National Cancer Institute (NCI) and 35 other reports of clinical vaccine trials. The majority of therapeutic antigen-based cancer vaccines activate CD8+ lymphocytes (CTLs) initiating a cellular immune response against the tumor. A vaccine-induced cellular immune response is often insufficient since the achieved circulating levels of high avidity immune cells are too low. Furthermore, these cells have to reach their tumor target and must be activated correctly to be able to destroy it.

Combination of chemotherapy or radiotherapy with immunotherapy

Chemotherapy and radiotherapy are ways to activate the immune system and it might therefore be an advantage to combine these treatments with immunotherapy. In the beginning of the era of chemotherapy it was believed that treatment with chemotherapeutic agents was immunosuppressive due to its anti-proliferative and cytotoxic properties. However, high-dose chemotherapy has also been found to have immunostimulating properties, which might be of advantage for combination treatment with immunotherapy. Many types of chemotherapeutic drugs cause lymphocytopenia, which can create a positive environment for expansion of tumor-specific CTLs. Therefore chemotherapy in combination with a therapeutic CTL inducing vaccine might promote expansion of effector T-cells and enhance antitumor T-cell responses.

Since cancer suppresses the immune system of the host dendritic cells in the tumor tissue and lymph nodes are often immature. Mature DCs,
however are needed for proper CD4\(^+\) and CD8\(^+\) T-cell activation. This can be a hurdle for therapeutic vaccines that aim at induction of a CTL or antibody response for which proper DC and T-cell activation is essential. Furthermore, DC function is suppressed by myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs), which are recruited by the tumor. Mechanisms of actions suggested for anthracycline and cyclophosphamide are the promotion of cancer cell death and thereby release of uric acid. The release of uric acid (crystalline uric acid) was proposed to activate DCs and to promote tumor rejection\(^{191}\). Proteins secreted by dying tumor cells are also believed to promote immunogenic cross-presentation due to interaction with the pattern-recognition receptor TLR4 on DCs\(^{13,192}\). Toll-like receptors alert the immune system and bind to pathogen-associated molecular patterns, which can be of microbial or endogenous origin. Activation of TLRs initiates both innate and adaptive immune responses\(^{193}\). Cross-presentation is the ability of one cell (the DC) to present antigens from another cell (e.g. a virus-infected or tumor cell) to activate antigen-specific T-cells\(^{194}\). It has also been shown that tumor apoptosis and/or necrosis induced by chemotherapy promotes the release of tumor-associated proteins and cross-presentation of these proteins by DCs\(^{195,196}\). Another effect attributed to anthracyclines is the stimulation of calrecticulin translocation to the cell surface. Calrecticulin is involved in the MHC-I processing pathway and its translocation enhances phagocytosis of cancer cells by dendritic cells and promotes their activation\(^{13,197}\). Cyclophosphamide was also found to promote a systemic release of type I interferon (IFN-\(\alpha\), IFN-\(\beta\)), which results in DC, T-cell and B-cell activation leading to an antitumor response\(^{13,198-200}\). Furthermore, chemotherapy is believed to delete regulatory T-cells, which are upregulated by the tumor, TAMs and MDSCs, alleviating tumor induced immune suppression\(^{13,195,196,201,202}\).

Low-dose radiation as well as certain chemotherapeutic drugs may additionally alter the phenotype of tumor cells by upregulation of the expression of Fas (death receptor), MHC-I, ICAM-1 (intercellular adhesion molecule 1) and tumor-associated antigens. This will render the cancer cells more sensitive to a T-cell mediated attack by the immune system\(^{203-206}\). Finally, there are also studies that support that tumor cell death caused by radiotherapy can promote cross-presentation\(^{13,192,207}\).

**VASCULAR TARGETS**

**Tumor endothelial cells as targets**

The previously described vaccines all aim at destroying tumor cells directly, but tumor endothelial cells can also be used as targets. Tumor vessels are believed to be more genetically stable than tumor cells and are therefore
more prone to immune recognition\textsuperscript{35,150,208-210}. This is in contrast to the transformed tumor cells that have developed mechanisms to avoid recognition by the immune system such as downregulation of MHC-I\textsuperscript{149}. However, one should bear in mind that chromosomal abnormalities have also been discovered in endothelial cells of solid tumors\textsuperscript{211,212} and in glioblastomas endothelial cells have even been found to originate from cancer stem-like cells\textsuperscript{213,214}. An advantage of targeting endothelial cells is that they line the vessel lumen and are therefore easily accessible for the immune system compared to tumor cells, which are located at distance from the vessels\textsuperscript{77}. Furthermore, targeting of a single endothelial cell will have an amplified effect due to the inhibition of as many as 100 tumor cells\textsuperscript{215,216}. The reason for this is that oxygen and nutrient supply is guaranteed by blood vessels and by this means one endothelial cell supports several tissue cells.

\textbf{Anti-angiogenic vaccines targeting tumor endothelial cells}

A bacterial DNA vaccine targeting VEGFR-2 has been evaluated\textsuperscript{163}. The vaccine contains attenuated \textit{Salmonella typhimurium} transformed with murine VEGFR-2 (FLK1) encoding DNA (pcDNA3.1-FLK1). Immunization of mice prior to tumor challenge promoted a CTL response against VEGFR-2 and tumor growth was reduced in a murine melanoma, colon carcinoma and lung carcinoma model. Furthermore, a bacterial vector vaccine has been designed for targeting VEGFR-2 in a HER2/neu\textsuperscript{+} breast tumor mouse model. The vaccine consisted of \textit{Listeria monocytogenes} containing a plasmid encoding for polypeptides from mouse VEGFR-2 fused to the microbial adjuvant listeriolysin-O. A strong antitumor CTL response was observed. However, tumor eradication in this model was not solely due to targeting of the tumor vasculature but was also dependent on epitope spreading to HER2/neu\textsuperscript{217}. Li \textit{et al.} engineered a FLK1- alkaline phosphatase (FLK1-AP) fusion protein to pulse dendritic cells. With this method antitumor activity was observed in a preventive vaccine approach, which was mediated by antibodies and CTLs\textsuperscript{159}. Another anti-angiogenic DNA vaccine in preclinical development targets the endothelial tip cells in the tumor vasculature. The vaccine consists of an expression plasmid pVAX1 into which the human Notch ligand delta-like ligand 4 (DLL4) is inserted (pVAX1-DLL4)\textsuperscript{162}. DLL4 regulates vessel sprouting and is present in the activated endothelium of malignant tissues\textsuperscript{218,219}. The vaccine induced a humoral immune response against DLL4 and inhibited tumor growth in two different mouse mammary carcinoma models.

A phase I clinical trial of combination therapy of gemcitabine and a human VEGFR-2 peptide vaccine in patients with metastatic non-resectable pancreatic cancer showed a CTL response in 61% of treated patients and a disease control rate of 67%. Patients in this trial developed immunological reactions at the injection site but no severe adverse effects could be observed\textsuperscript{220}. A potential problem of targeting VEGFR-2 is that this receptor
is also expressed on normal vasculature and is needed for endothelial cell survival and vascular homeostasis\textsuperscript{221}. Therefore it is surprising that no severe side effects were observed in a phase I clinical study when targeting VEGFR-2. However, no long-term side effects were investigated in this study.

Vascular antigens

One group of molecules with a highly restricted expression pattern is the extra domains of splice variants of extracellular matrix molecules present in the neovasculature of tumors or tumor stroma.

**Extracellular matrix**

All cells in the body are surrounded by extracellular matrix, a three dimensional network/meshwork of proteins, consisting of glycoproteins, proteoglycans and collagens\textsuperscript{222}. Examples of glycoproteins within the ECM are fibronectin and tenascin-C. The ECM provides organs and tissues with stability and it is important for tissue and cell adhesion, cell growth, migration, embryogenesis, hemostasis and wound healing\textsuperscript{223,224}. Cell adhesion to the matrix is mediated by integrin receptors, cell surface proteoglycans (such as syndecans) and glycoproteins (\textit{e.g.} discoidin domain receptors)\textsuperscript{222,225,226}. In the ECM many different growth factors are sequestered, which can be released and activated by protease cleavages under certain physiological or pathophysiological conditions leading to cell proliferation and migration, cell communication, tissue homeostasis or matrix remodeling\textsuperscript{223,227,228}. Endothelial cells forming the lumen of blood vessels and capillaries are surrounded by a basement membrane (see Figure 1), which is a specialized type of ECM and mainly composed of laminin, collagen type IV, perlecan and nidogen proteins\textsuperscript{224,227,228}.

Three molecules with a highly restricted tumor vascular expression are the extra domain-B (ED-B) and extra domain-A (ED-A) of the extracellular matrix protein fibronectin and the C-domain of tenascin-C (TNCC). These antigens are highly expressed during embryonic development\textsuperscript{229-234} and around angiogenic vasculature and in the stroma of most solid tumors\textsuperscript{235-238} but are essentially undetectable in normal adult tissue. Exceptions are situations of physiological angiogenesis such as wound healing and the female menstrual cycle during which these extra domains also reoccur\textsuperscript{235,239,240}. Thus the specific expression of these molecules in tumor tissue renders them excellent for tumor-specific targeting. The expression of vascular antigens is more restricted compared to VEGFR-2, which is present on all endothelial cells in the body and essential for blood vessel homeostasis\textsuperscript{221}. Therefore targeting of these extra domains is likely to give fewer side effects since only the tumor vasculature is targeted and not all blood vessels.
Fibronectin

Fibronectin (FN) is a plasma and ECM glycoprotein consisting of two identical 220-250 kDa subunits connected by two disulphide bridges forming a dimer\textsuperscript{241,242}. Furthermore, fibronectin is located in the basement membrane of blood vessels, incorporated between endothelial cells and perivascular cells in vessel morphogenesis, where it provides vessel stability\textsuperscript{243}.

In humans there are 20 different isoforms and in rats and mice there are 12 fibronectin variants, which are formed by alternative splicing of the fibronectin mRNA\textsuperscript{244,245}. Each monomer consists of three different types of homologous repeating domains, the type I, II and III repeat (see Figure 3A)\textsuperscript{242,244,245}. Plasma fibronectin is essentially devoid of the type III repeats termed extra domain-A (EIIIA or ED-A) and extra domain-B (EIIIB or ED-B) (only $< 1\%$ of plasma fibronectin contains these repeats)\textsuperscript{246-248}. Both ED-A and ED-B containing fibronectin are highly expressed during embryogenesis but undetectable in normal adult tissue\textsuperscript{249-251}. However, the extra domains are re-expressed during physiological and pathophysiological angiogenesis\textsuperscript{235,239,240,249,250}. Fibronectin including one or both of the extra domains is also called ‘oncofetal fibronectin’, which refers to its expression during tumor angiogenesis and embryonic development\textsuperscript{235,249,250,252}. To date the function of these extra repeats is unknown but it is hypothesized that incorporation of ED-A and/or ED-B into the fibronectin molecule leads to a conformational change, which makes the RGD (Arg-Gly-Asp) cell-binding site accessible for integrin binding\textsuperscript{242,253,254}. Except for alternative splicing cryptic sequences in the fibronectin can also be exposed by simple stretching or proteolysis of the fibronectin molecule\textsuperscript{255,256}. Splicing of fibronectin mRNA is regulated by SR proteins (proteins rich in serine- and arginine), whose activity is controlled by SR protein kinase and PKB/Akt (protein kinase B)\textsuperscript{242,257-259}. 
Figure 3. Structure of a fibronectin and tenascin-C monomer. (A) Fibronectin monomer. The different types of homologies (12 Type I, two Type II and 15 Type III) are represented. The ED-A and ED-B fragment are excluded in the numbering of the Type III repeats. The splicing pattern for ED-A and ED-B is similar in all species, whereas the splicing of the IIICS region is species-specific (five variants in humans, three in rodents and two in chickens)\textsuperscript{242,260}. (B) Tenascin-C monomer. The structure depicted contains an assembly domain, 14.5 epidermal growth factor like (EGF-like) repeats, 16 type III homology repeats, and a fibrinogen globe\textsuperscript{232,260-262}.

Extra domain-B

The extra domain-B of fibronectin is a 91 amino acid domain, which is highly conserved between species and identical in mouse, rat, rabbit, dog, monkey, human and several other species\textsuperscript{260,263,264}. Due to its high conservation it was believed that in vivo generation of antibodies targeting ED-B would be difficult or even impossible. ED-B is incorporated into fibronectin by alternative splicing during embryogenesis, tumorigenesis and angiogenesis\textsuperscript{229,235,240,242,250,251,265}.

Despite the high conservation of the extra domain-B, its regulation and function is still unknown. Both ED-A\textsuperscript{−/−} and ED-B\textsuperscript{−/−} mice are viable\textsuperscript{266} but double knock out mice (ED-A\textsuperscript{−/−} and ED-B\textsuperscript{−/−}) are 80% lethal on a C57BL6 background\textsuperscript{243,267}. ED-B\textsuperscript{−/−} mice have a similar phenotype as wild type mice. However, fibroblasts derived from these mice showed diminished proliferation and less fibronectin deposition in the pericellular matrix in vitro\textsuperscript{251,266}. Transforming growth factor beta (TGF-β) has been found to regulate the splicing of the extra domain-B\textsuperscript{240,268-271}. Other regulators suggested are high glucose levels\textsuperscript{268} and reduced intracellular pH\textsuperscript{240}, one possible effect of hypoxia. Fibronectin containing the extra domain-B is expressed around the vasculature of most solid tumors or in the tumor
stroma. Examples of ED-B expressing tumors are lung squamous carcinoma, lung alveolar adenocarcinoma, glioblastoma multiforme and invasive ductal breast carcinoma. The expression of ED-B containing (EDB\(^{+}\)) fibronectin is tumor type dependent and several different cell types such as endothelial cells, tumor cells or tumor-associated myofibroblasts have been found to produce this isoform. The increased permeability of tumor vessels is believed to enhance the accessibility of ED-B from the blood stream. Wagner et al. suggest an immunological role for ED-B and found ED-B containing fibronectin to be synthesized by activated T-cells and to be associated to their surface in vitro. They propose that EDB\(^{+}\) fibronectin binds to very late antigen-4 (VLA-4) on adjacent T-cells and thereby provides a co-stimulus for T-cell proliferation.

In early studies the monoclonal antibody BC-1, which recognizes human ED-B\(^{+}\) fibronectin was used to analyze ED-B expression in tissue. This antibody does not recognize ED-B directly but instead a cryptic epitope of the FN type III\(_{7}\) repeat, which is unmasked by insertion of ED-B\(^{+}\). An additional cryptic sequence on the FN type III\(_{8}\) was discovered recently and a high-affinity murine monoclonal antibody C6, specific for ED-B\(^{+}\) fibronectin is available. Bencharit et al. solved the crystal structure of the interface between the ED-B domain and the adjacent FN type III\(_{8}\) repeat in 2007. The crystal structure revealed an acidic groove between the two repeats rendering it a potential binding site for fibronectin interacting molecules. Thus the insertion of ED-B into fibronectin leads to conformational changes in the fibronectin molecule, which unmask hindered sequences but in turn masks others.

Peters et al. determined in 1996 the expression pattern of total fibronectin, ED-B\(^{+}\) fibronectin and ED-A containing (ED-A\(^{+}\)) fibronectin in the adult mouse. In this study they found ED-B\(^{+}\) fibronectin expression in the walls of smaller blood vessels, in the smooth muscle of the gastrointestinal tract (GI), genitourinary (GU), and respiratory tracts, as well as in cartilage and the eye. Tissue staining was performed with anti-ED-B antibodies raised against an EDB-GST (glutathione-S-transferase) fusion protein and the tissue was treated with N-glycanase (Peptide: N-glycosidase F, PNGase F) to remove N-linked oligosaccharides on Asn\(^{1359}\) from ED-B\(^{+}\). Expression of ED-A\(^{+}\) fibronectin was detected in vessel walls, the lung interstitium, and smooth muscle associated with the GI, GU and respiratory tracts of mouse when staining with a human anti-ED-A antibody reactive to mouse tissue. However, several others could not confirm the expression of these segments in normal tissue.

To date one monoclonal anti-ED-B antibody, termed L19, has been developed for clinical use by the phage display technique in which binding to the ED-B fragment was screened.
Antibody phage display technique

The antibody phage display technique, which was first described by McCafferty in 1990, is based on the expression of antibody variable (V) region fragments on the surface of bacteriophages\textsuperscript{283}. For antibody development the immunoglobulin variable genes are amplified from hybridoma- or B-cell lines with the help of the polymerase chain reaction (PCR) and cloned into a phage expression vector containing bacteriophage virus genes. Bacterial cells are then infected with the bacteriophages, which will replicate in the host but are unable to assemble their coat since they are devoid of the genes necessary for assembly. However, insertion of the phage expression vector into the bacterial host will enable packaging of the phage DNA and the assembly of their coat containing the antibody fragment of interest as part of either the minor (pIII) or major coat protein (pVIII). By this means a phage display library can be generated. The specific clones are then selected upon the ability of the expressed surface antibody fragment to bind to antigen-coated wells in an enzyme-linked immunosorbent assay (ELISA) or to an antigen-coated column. The desired bacteriophage clone can then be amplified in \textit{E.coli}. This process of selection is called ‘panning’ and usually 2-4 rounds are needed to select for a high affinity antibody. The antibody fragments derived from the phage display will be secreted into the bacterial periplasm and culture medium from which they can be purified further\textsuperscript{264,284}. The diversity of antibodies generated in a phage display is only around \(10^{12}\), whereas the human body can generate a much higher variety of \(10^{15}-10^{16}\) by rearrangement and somatic mutation\textsuperscript{285}. Generation of a specific high affinity antibody by the phage display technique can therefore be hard to achieve.

For production of monoclonal antibodies the genes encoding the specific antigen-binding site can be isolated from the phage DNA and used for the construction of a complete immunoglobulin (Ig) gene by fusing them with the antibody’s invariant parts. The constructed antibody gene can then be inserted into hybridoma cells, which then will secrete monoclonal antibodies\textsuperscript{286-288}.

Monoclonal anti-ED-B antibodies

The monoclonal phage antibody L19 is a 150 kDa IgG1 antibody, which recognizes ED-B. In addition derivatives of this antibody have been produced, which are a dimeric single-chain FV(scFV) 50 kDa fragment (two antibody variable (V) regions of an IgG fused) and a small immunoprotein (L19-SIP) (80 kDa). The L19 small immunoprotein consists of two scFV fragments each fused to a CH4 domain derived from human IgE that mediates their homodimerization (\textit{Figure 4})\textsuperscript{264,282,289}.
Figure 4. L19 antibody derivatives (adapted from\textsuperscript{289}). (A) The L19-IL2 construct consists of an scFV fragment fused to the cytokine IL-2. (B) Single-chain FV dimer. (C) L19-SIP is composed of two scFV fragments fused each fused to a CH4 domain derived from human IgE that mediates their homodimerization\textsuperscript{264,282,289}.

To date there are many publications available, in which targeting of ED-B with the help of the L19 antibody coupled to different effector molecules has been proven to be a successful anti-cancer strategy in preclinical models and in clinical studies. In a study performed by Borsi \textit{et al}. L19 coupled to tumor necrosis factor (TNF) $\alpha$ induced tumor necrosis\textsuperscript{290}. The L19-TNF$\alpha$ (monoclonal antibody cytokine fusion protein) is currently evaluated in a phase Ib/II study in combination with doxorubicin for treatment of advanced solid tumors. In a phase I study for dose finding L19-TNF$\alpha$ in combination with melphalan is evaluated for treatment of III/IV melanoma\textsuperscript{291}. Another approach in which the L19 antibody was coupled to the soluble extracellular domain of tissue factor (tTF), to induce specific thrombosis of tumor blood vessels, showed infarction of solid tumors and complete tumor eradication in 30\% of mice treated with the highest dose\textsuperscript{292}. The L19 antibody coupled to interleukin-2 (IL-2); a cytokine involved in neutral killer cell and macrophage recruitment, has been tested in combination with rituximab, in a mouse model of human B-cell lymphoma xenografts. Combination treatment caused complete remission of localized lymphomas and showed increased infiltration of immune cells into the tumor tissue\textsuperscript{293}. In several different subcutaneous and orthotopic mouse models treatment with L19-IL2 had a significant effect on tumor growth\textsuperscript{281,294}. In the preclinical models tested no side effects were reported. In a phase I/II clinical trial to evaluate the safety, tolerability and recommended dose, for further use in a phase II study with the L19-IL2, manageable and reversible toxicity could be found. Furthermore, the L19-IL2 antibody showed activity in humans and a stabilized disease level could be observed in 51\% of the patients with solid tumors and in 83\% of the patients with advanced renal cell carcinoma (RCC) after two treatment cycles. With the recommend dose a progression free survival of eight month could be seen in patients with RCC. Upregulation of
natural killer (NK) cells and CD8\(^+\) cells in the blood could also be detected\(^{295}\). Currently the L19-IL2 antibody is tested in a phase II clinical trial in patients with grade III/IV melanoma and in combination with dacrabazine for metastatic melanoma. A combined treatment of the L19-IL2 antibody with gemcitabine is tested in a phase I/II clinical trial for treatment of advanced pancreatic cancer\(^{291}\). A clinical study with L19-SIP coupled to radioactive iodine (I\(^{131}\)) induced a sustained partial response in two out of three relapsed Hodgkin lymphoma patients tested\(^{273}\). According to the Philogen website recently the efficacy and dose of L19-SIP-I\(^{131}\) were evaluated in patients with cancer (phase I/II clinical trial)\(^{291}\). No data on the type of cancer patients treated in this study are available on the website. In a prospective non-randomized study in patients with multiple brain-metastasis from solid tumors L19-SIP-I\(^{131}\) radio-immunotherapy (RIT) is combined with Whole Brain Radiation Treatment (WBRT). Another study performed is the combination of L19-SIP-I\(^{131}\) radio-immunotherapy, External Beam Radiotherapy (EBRT) and chemotherapy for treatment of inoperable, locally advanced (stage III) non-small cell lung carcinoma (NSCLC)\(^{291}\).

**Extra domain-A**
The extra domain-A of fibronectin is a 90 amino acid domain, which is highly conserved between species. However, unlike ED-B, which is 100% identical in mouse and human, mouse and human ED-A are only 98% identical, which includes a two amino acid difference between the species\(^{242,296}\). The expression of the ED-A domain is also regulated by alternative splicing of the fibronectin molecule. The inclusion of ED-A into fibronectin of fibroblasts is regulated by TGF-β\(^1\)\(^{269,270,297}\). Furthermore, TGF-β1 was found to promote inclusion of ED-A in bovine granulose cells\(^3\) and tubular epithelial cells\(^{298,299}\). The ED-A segment binds to α\(_4\)β\(_1\) and α\(_9\)β\(_1\) integrins (cell-surface adhesion receptors that bind to ECM) promoting cell adhesion and migration\(^{239,242,296}\).

ED-A\(^+\) fibronectin is re-expressed during wound healing and under pathological conditions, such as occurrence of solid tumors and rheumatoid arthritis (RA)\(^{216,300}\). ED-A\(^-/-\) mice have defective skin wound healing\(^{301}\). ED-A was found to stimulate keratinocytes to progress into the cell cycle\(^{302}\). In individuals with psoriasis hyperproliferating keratinocytes are suggested to be the source of ED-A\(^+\) fibronectin\(^{302,303}\). Muro and colleagues showed that activation of TGF-β is impaired in ED-A\(^-/-\) mice\(^{304}\). Furthermore, ED-A\(^+\) fibronectin in combination with TGF-β and mechanical tension was proposed to promote *in vitro* differentiation of fibroblasts into myofibroblasts\(^{305-307}\). ED-A containing fibronectin promotes cell adhesion and spreading better than fibronectin lacking this domain\(^{254}\). Another function, which has been suggested for ED-A is the signaling through TLR4\(^{243,308}\). It has also been proposed that ED-A can activate dendritic cells via direct binding to TLR4 and promote a T-helper 1 (Th1) cell directed
immune response\textsuperscript{309}. In vitro activated Th1 cells express mainly fibronectin containing the ED-A domain, which promotes expression of the pro-inflammatory cytokine IL-6 in monocytes\textsuperscript{310}. ED-A\textsuperscript{+} fibronectin is also elevated in plasma of RA, rheumatoid vasculitis, psoriasis, diabetes and cancer patients as well as in acute vascular injury\textsuperscript{236,242,300,303,311-313}. In the pathological condition of rheumatoid arthritis ED-A\textsuperscript{+} fibronectin can additionally be detected in synovial membrane, synovial fluid and cartilage extracts. Therefore Przybysz et al. suggest it as a potential diagnostic marker in the synovial fluid of RA patients\textsuperscript{300}. Kriegsman et al. found both ED-A and ED-B to be expressed in the synovium of rheumatoid arthritis and osteoarthritis patients, which was determined with the murine monoclonal antibody IST-9 (ED-A) and BC-1 (ED-B) antibody\textsuperscript{314}. Pro-inflammatory roles suggested for ED-A in rheumatoid arthritis, mediated via TLR4 interaction, are the stimulation of mast cells\textsuperscript{315} and priming of leukotriene synthesis in neutrophils and monocytes\textsuperscript{316}. In idiopathic pulmonary fibrosis ED-A\textsuperscript{+} fibronectin was found to deposit prior to collagens in regions of active fibrosis and to correlate with increased \(\alpha\)-smooth muscle actin expression in activated fibroblasts\textsuperscript{242,317}. ED-A\textsuperscript{+} fibronectin could also be found in intimal thickening and atherosclerotic plaques of human arteries, in an experimental model of intimal thickening of the rat aorta and in cultured vascular smooth muscle cells in vitro\textsuperscript{230,318}.

**Monoclonal anti-ED-A antibodies**

Recombinant antibody fragments directed against human ED-A have been developed by the phage display technique\textsuperscript{249}. Based on the anti-ED-A antibody fragment (F8) the F8 small immunoprotein was designed\textsuperscript{250} (see Figure 4C). The F8-SIP antibody stained both murine F9 teratocarcinoma and Ramos lymphoma xenografts but not normal human tissue\textsuperscript{250}. With the same antibody fragment ED-A\textsuperscript{+} fibronectin was found in different primary tumors and metastases\textsuperscript{236}. The F8 monoclonal antibody coupled to IL-10 (Dekavil) in combination with methotrexate is tested in a phase I clinical trial for treatment of rheumatoid arthritis\textsuperscript{291}.

**Expression of ED-B and ED-A in wound healing and cartilage**

Clark et al. detected an increase of total fibronectin levels in the basement membrane of blood vessels adjacent to cutaneous wounds between day 3 and 7 post-injury\textsuperscript{319,320}. Singh P et al. determined the expression pattern of the ED-A and ED-B containing isoforms in cutaneous wound healing\textsuperscript{239} and revealed an increase in expression of both isoforms in granulation tissue from day 4 to day 7 after wounding. Expression of ED-B remained elevated through day 14 whereas ED-A expression was reduced. ED-A\textsuperscript{+} fibronectin was more abundant in the wound area than ED-B\textsuperscript{+} fibronectin\textsuperscript{229}. Low amounts of ED-A\textsuperscript{+} and ED-B\textsuperscript{+} fibronectin might even be present immediately after injury in the wounded area due to release of these
isoforms by activated platelets, which contain a mixture of plasma and cellular fibronectin in their α-granules.

Both ED-A and ED-B containing fibronectin are expressed in human articular cartilage where it is probably produced by chondrocytes.

**Tenascin-C**

Tenascin-C (TNC) is an extracellular matrix glycoprotein, which consists of six similar subunits joined together by disulphide bonds at their NH₂ terminus. In the literature different suggestions for the structural composition of a TN-C subunit can be found. One monomer is suggested to contain either 14.5 or 15 epidermal growth factor-like repeats, 15-17 type III homology repeats, one assembly domain and one fibrinogen globe (see Figure 3B). A single gene encodes the protein and its expression is regulated by a single promoter. Tenascin-C exists as several different isoforms, depending on the alternative splicing pattern of the nine fibronectin-like type III repeats (A1, A2, A3, A4, B, AD2, AD1, C, D). In humans 32 different splice variants have been detected to date. In mouse brain however, only six alternatively spliced fibronectin-like type III repeats could be detected. Splicing is cell cycle-dependent and regulated by environmental conditions such as extracellular pH. Pearson et al. showed that expression of TN-C in cultured fibroblasts is induced by TGF-β. Further, it was found that tenascin-C expression can be induced by, FGF-2, EGF, PDGF-BB, mechanical stress exerted by fibroblasts, pro- and anti-inflammatory cytokines, hypoxia or reactive oxygen species.

TNC⁻/⁻ mice are viable but have several defects such as abnormal behavior, abnormalities in brain chemistry and low fibronectin expression in wounds. The tenascin-C isoforms containing fibronectin-like type III repeats are also referred to as large tenascin-C, whereas the isoform devoid of these repeats is called small or little tenascin-C. The large isoform is mainly expressed in processes as cell migration, proliferation or tissue remodeling, which occur in embryonic development, wound healing or neoplasia. Throughout the course of wound healing the expression of tenascin-C is highest 48h after injury. Tenascin-C is synthesized by osteoblasts in bone formation but not present in mature bone and cartilage. The molecule is often co-localized with fibronectin in the ECM and it has been shown in vitro that small tenascin-C binds to fibronectin, whereas large tenascin-C does not. In rheumatoid arthritis small tenascin-C is highly expressed in the synovium and maintains inflammation by activation of TLR4. Tenascin-C has been shown to upregulate the expression of MMP-13 and tissue inhibitor of matrix metalloproteinase-3 (TIMP-3) in breast cancer cells and fibroblasts in vitro, which could play a role in tumor growth and invasion.
**C-domain of tenascin-C**

Similar to the above-mentioned domains of fibronectin the C-domain (type III repeat C)\(^{232,330,331,355}\) of tenascin-C is a 91 amino acid domain inserted into tenascin-C by alternative splicing. The identity between the mouse and human variant is 95%\(^{232}\). The oncofetal tenascin-C including the C-domain is expressed during embryogenesis but essentially undetectable in normal adult tissue\(^{326,356,357}\). However, it is upregulated in solid tumors such as high-grade astrocytoma (grade III), glioblastoma and the majority of lung cancers\(^{238,326,355}\). Its expression pattern is mainly around angiogenic vasculature, proliferating cells and in the tumor stroma\(^{238,326,355}\). Accumulation of the large tenascin-C isoform in malignant cells is due to pH-insensitivity, which prevents that splicing of the mRNA occurs\(^{336}\). No data are at present available in the literature about the expression of the C-domain in wound healing.

**SELF-TOLERANCE**

The immune system is only supposed to be activated by foreign molecules and not by self-molecules. This is to prevent harmful attacks on the body’s own tissues. A number of mechanisms has therefore been developed to avoid recognition of self-molecules and this is termed immunological self-tolerance\(^{358}\). These mechanisms include central (in the thymus and bone marrow) and peripheral (in the lymph nodes and spleen) T-lymphocyte and B- lymphocyte tolerance.

Central T-cell tolerance is the negative selection of autoreactive T-cells in the thymus during embryonic development. After birth there is the peripheral tolerance characterized by that T-cell activation requires several different simultaneous stimuli. The sole presentation of the antigen on MHC by APCs, e.g. dendritic cells and macrophages, and recognition by the T-cell receptor (TCR) is insufficient for proper T-cell activation. In order to get correctly activated T-cells additional co-stimuli such as the surface receptors B7-1 (CD80) and B7-2 (CD86) on the APC and CD28 on the T-cell, as well as cytokine (IL-6, IL-12, IL-7 and TGF-β) secretion by the APC are needed\(^{142,358,360}\). Without presence of these co-stimulatory molecules, the T-cell will be inactivated, a process supported by regulatory T-cells\(^{358,361,362}\). Downregulation of these co-stimulatory molecules is an important feature of peripheral T-cell tolerance.

Central B-cell tolerance is mediated when autoreactive B-cells undergo apoptosis in the bone marrow. This negative selection is triggered when the immature B-cell recognizes and binds to a self-antigen in the bone marrow; through an interaction with its receptor (BCR) that is a surface anchored IgM molecule\(^{358,363}\). Peripheral B-cell tolerance is the consequence when a B-cell
encounters its antigen in the body but does not receive any stimuli from T-helper cells. The lack of the co-stimulatory signal leads to downregulation of the BCR and to B-cell anergy. New B-cells are produced throughout the whole time course of life, which continuously generates new B-cell specificities.

**ADJUVANTS**

An adjuvant is a component added to a vaccine to accelerate, prolong or enhance the immune response but that has no immunogenic effect by itself. The word adjuvant is derived from the Latin *adjuvare*, which means to help or aid. The classification of adjuvants is difficult since their application and mechanism of action is so diverse. For example cytokines such as granulocyte macrophage colony stimulating factor, interleukins and interferons are sometimes also named adjuvants. The attempt made by O’Hagan and De Gregorio to divide the adjuvants into different generations however is in my opinion the clearest one. The first generation adjuvants are alum (aluminum salts) and Freund’s adjuvant. Both adjuvants are composed of particulate dispersions (alum forms aggregates and Freund’s emulsion droplets) to which the antigen adheres. In case of Freund’s adjuvant the oil base forms a water-in-oil (W/O) emulsion when mixed with the water phase (the antigen). In an emulsion the water phase is dispersed as globules in the continuous phase, which functions as a depot to promote a slow release of the antigen. This leads to a gradual exposure of the antigen to the immune system, which is believed to give a more profound immune response. Another aspect is that first-generation adjuvants induce local inflammation, which enhances APC recruitment to the injection site. Polymeric particles and liposomes can also be classified as first generation adjuvants since their particle size is equal to alum and appropriate for uptake into immune cells. These newer adjuvants also adsorb or encapsulate the antigen to enhance slow release and delivery. Furthermore, the first-generation adjuvants have been optimized and to increase tolerability in humans oil-in-water emulsions (O/W) have been developed, of which the squalene-based MF59 is an example. The second-generation adjuvants consist of the first generation adjuvants to which additional components have been added. Examples of agents added to the first-generation base are muramyl dipeptide (MDP), derived from the bacterial cell wall, lipopolysaccharides (monophosphoryl lipid A (MPL)) and oligonucleotides. Adjuvants approved for clinical use today in preventive vaccines are alum, MF59, AS03, AS04 and liposomes (Table 3). Squalene, a natural cholesterol derivative, composes the oil phase of MF59 and of the by GlaxoSmithKline developed Adjuvant System (AS) AS03 adjuvant.
Table 3. Adjuvants approved for clinical use (adapted from\textsuperscript{365,373}). Hepatitis B virus (HBV), Hepatitis A virus (HAV)

<table>
<thead>
<tr>
<th>Name</th>
<th>Class</th>
<th>Indications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alum</td>
<td>Mineral salts</td>
<td>Various (\textit{e.g.} Gardasil\textsuperscript{®})</td>
</tr>
<tr>
<td>MF59</td>
<td>O/W emulsion</td>
<td>Influenza (Fluad \textsuperscript{®}/pandemic flu</td>
</tr>
<tr>
<td>AS03</td>
<td>O/W emulsion + $\alpha$-tocopherol</td>
<td>Pandemic flu (Pandemrix \textsuperscript{®})\textsuperscript{374}</td>
</tr>
<tr>
<td>AS04</td>
<td>MLP + alum</td>
<td>HBV (Fendrix \textsuperscript{®})\textsuperscript{375}</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HPV (Cervarix \textsuperscript{®})\textsuperscript{376}</td>
</tr>
<tr>
<td>Liposomes</td>
<td>O/W emulsion</td>
<td>HAV, Flu</td>
</tr>
</tbody>
</table>

All clinically approved adjuvants are weak immunostimulators and therefore not potent enough to break immune tolerance against self-antigens, which is required for development of a therapeutic cancer vaccine. An adjuvant potent enough to evoke an immune response against a self-antigen is Freund’s adjuvant, which is widely used in animal studies. However, due to its mineral oil base and addition of heat-killed \textit{Mycobacterium tuberculosis} (Freund’s complete adjuvant only), it is toxic for humans and cannot be used in clinical studies\textsuperscript{377-380}. Therefore the identification of new adjuvants, which can be used in therapeutic vaccines to enhance the immunogenicity against self-antigens, is an important aspect to address.

**TUMOR MODELS**

\textit{In vivo} tumor models in mice have been used extensively to study the effect of drugs for cancer treatment. A model that can be used is the subcutaneous injection of tumor cells into mice. The tumor cells have to be \textit{syngeneic}, which means that they are genetically similar to the host and therefore accepted, when injected into immunocompetent mice\textsuperscript{381,382}. A disadvantage of these types of models is that the injected tumor cells are fast growing and hardly resemble slow growing multi-step tumor progression in humans, which often proceeds over decades. For studies of therapeutic effects of tumor vaccines, fast growing tumor models are difficult to use since vaccines need at least two weeks to induce an antibody response and therefore it is difficult to study the effect of a vaccine in a therapeutic setting in subcutaneous disease models.

Tumor cells can also be injected into athymic nude mice or severe combined immunodeficiency (SICD) mice, which accept cells from another species (\textit{xenografts}\textsuperscript{381,382}). Xenograft models are commonly used in cancer research. However, these models cannot be used for vaccine studies since
they lack B- and/or T-cells and therefore an immune response will not be activated.

Tumor cells can be injected into their site of natural origin (orthotopic) or grown at a different site (heterotopic). Heterotopic subcutaneous tumor models have made an important contribution to the development of cancer treatments but to answer more complex biological questions new physiological models may be required. Studies performed in orthotopic tumor models may be preferred for investigating new treatment strategies since the microenvironment into which the cells are placed determines the tumor phenotype and therefore tumors might behave differently in different sites in the body. Models that probably better mimic the stepwise tumor progression in humans are believed to be spontaneously arising tumors\textsuperscript{381,382}. However, the genetically engineered mouse models (GEMMs) of spontaneous cancer have not yet been shown to be superior to human xenograft models in predicting treatment effects in humans, which have been used for development of the most important drugs in the clinic today (chemotherapy)\textsuperscript{382-385}.

As stated before patients often die from metastatic disease, and therefore models that mimic the spread of a tumor to distant sites in the body can be useful tools for treating more progressed cancers.

RIP1-Tag2 transgenic mouse model of insulinoma

A mouse model that is believed to reflect multi-step human tumorigenesis is the transgenic RIP1-Tag2 model of pancreatic insulinoma\textsuperscript{386}. These mice express the Simian Virus-40 (SV40) large Tumor antigen (Tag) oncoprotein under the control of the Rat Insulin Promoter (RIP) in the insulin producing \(\beta\)-cells of the pancreatic Langerhans islets. About 50\% of the pancreatic islets will become hyperplastic with the histological characteristics of carcinoma in situ. However, in order to proceed from hyperplasia to neoplasia the islets need to turn on angiogenesis and only 10\% of the hyperplastic islets succeed to do so at 7-8 weeks of age. Eventually only approximately 3\% of the in total 400 islets develop into solid tumors, adenomas and/or invasive carcinomas, by 12-15 weeks. The mice die around 15 weeks of age from hypoglycemia due to overproduction of insulin\textsuperscript{386-389}.

MMTV-PyMT transgenic mouse model of metastatic breast cancer

Another mouse model, which is believed to reflect multi-step human tumorigenesis, is the mouse mammary tumor virus-polyoma virus middle T antigen (MMTV-PyMT) transgenic mouse\textsuperscript{390}. These mice carry the mouse polyoma middle T antigen (PyMT) under the control of the mouse mammary
tumor virus (MMTV), which only is expressed in the breast tissue. By 8-10 weeks of age the animals develop adenocarcinomas in the mammary epithelium and around week 12-13 a high incidence of pulmonary metastasis can be detected. Tumor development is characterized by four different stages: hyperplasia, adenoma/mammary intra-epithelial neoplasia, and early and late carcinoma. The model resembles human breast cancer in that the mice gradually lose steroid hormone receptors and β1-intergin, which is associated with overexpression of ErbB2 and cyclin D1. Cyclin D1 is important for the stimulation of the cell cycle (mitosis) and the ErbB2 receptor is required for EGF growth factor signaling.
Present investigations

The present investigation has focused on development of a therapeutic cancer vaccine targeting tumor angiogenesis, with the aim to find a novel and efficient treatment for cancer and prevent spreading.

The technique that has been used for immunization described in all four papers of this thesis is based on the injection of a fusion protein together with a potent adjuvant. Both the fusion protein and the adjuvant are required to be able to circumvent the body’s self-tolerance\(^{396,397}\).

VACCINATION MECHANISM

How to break self-tolerance

A flow chart of the vaccination mechanism we used is depicted in *Figure 5*. The non-self part of the fusion protein can be of bacterial origin or any other origin as long as it is recognized by the immune system as foreign. The self-part is the host protein, which is aimed to be targeted. After injection of the fusion protein, so called antigen-presenting cells (APCs), macrophages and dendritic cells, will take up the protein and digest it into peptides. These peptides will be presented via MHC-II to T-helper 2 lymphocytes (Th2-cells).
As previously described, autoreactive T-cells are removed in the thymus during embryonic development or inactivated (anergized) in the periphery. The T-cell receptors (TCRs) on the surface of the T-cells present in the circulation will thus only recognize non-self peptides presented by APCs on the MHC-II molecules. Recognition of the antigen (Ag) by the TCR then leads to activation and clonal expansion (proliferation) of the specific T-cell. Simultaneously, the self-part of the fusion-protein is recognized by the B-cell receptor (BCR) on autoreactive B-cells, which are present in the circulation. Normally these autoreactive B-cells do not become activated since they do not receive any help from autoreactive T-cells. The autoreactive B-cells will also present the foreign peptides of the fusion protein on their surface via MHC-II. In this case the previously activated T-cells will recognize the presented non-self peptides in conjunction with the MHC-II. These T-cells will stimulate the autoreactive B-cells to clonally expand and mature into antibody-producing plasma cells. By this means the T-cells get ‘tricked’, into providing help to autoreactive B-cells, and antibodies against the self-protein will be produced.

The antibodies produced in the spleen will enter the circulation and bind to the self-antigen and make it visible for the immune system. The formed antigen-antibody complexes, are mainly composed of immunoglobulin M (IgM) or immunoglobulin G (IgG) bound to the self-antigen. The immunoglobulins will activate the classical complement pathway, which is the part of the complement system initiated by antigen-antibody complexes (immune complexes). The complement system is part of the innate immune system.
response and consists of three different pathways: the classical, lectin and alternative pathway. In total more than 20 serum glycoproteins, synthesized by hepatocytes in the liver are involved this system, which is important for the recruitment of inflammatory cells and the killing or opsonization of pathogens. The antigen-bound Igs will initiate the classical pathway via their CH2 (IgG) or CH3 (IgM) domains in their Fc portion. C1, a complex consisting of one C1q and two C1s and C1r molecules, will be recruited and bind to the immunoglobulin via the C1q component. This will trigger the classical complement cascade leading to formation of C3 convertase (the C4b/C2a complex). The C3 convertase acts as a major amplification step in the complement pathway because of its ability to produce large quantities of C3b. C3b will bind directly to the antigen and to C3b receptors on phagocytes (macrophages and neutrophils), a process called opsonization. By this means the C3b coated antigen can be phagocytosed. Additionally C3a and C5a, formed by C3 convertase, act as chemoattractants for neutrophils and macrophages to the site of the antigen. In a consecutive cascade the C5b6789 complex, also named the membrane attack complex (MAC), will be formed. The MAC forms ion-permeable pores in the cell membrane of the target cell resulting in osmotic cell lysis.

If the antigen is soluble the attracted macrophages and neutrophils will engulf the protein and destroy it, a process called phagocytosis. In the case of a tissue-bound antigen, macrophages and neutrophils will not be able to fully engulf the protein. In this situation a process called frustrated phagocytosis is initiated, where these cells release their granule content instead. The granules contain proteolytic enzymes for lysosomal degradation and oxidases for production of reactive oxygen species promoting tissue damage and necrosis.

DEVELOPMENT OF A POTENT ADJUVANT SAFE FOR CLINICAL USE

As stated before the second requirement for development of a therapeutic vaccine for human use is a potent adjuvant able to break self-tolerance.

The non-toxic and biodegradable squalene-based Montanide incomplete Seppic adjuvant (ISA) 720 (M720) can be used to induce an immune response against a non-self antigen. However, to break self-tolerance additional stimulators of the immune system are needed. To accomplish this, additional substances can be added to the Montanide base. One such component is repetitive unmethylated CpG DNA (CpG oligonucleotides) that is present in genomes of bacteria and certain DNA viruses. CpG oligonucleotides can stimulate the innate immune response that in turn
enhances the adaptive immune response and thereby promotes antibody production\textsuperscript{414,415}. Other immunopotentiating components, which can be added to the Montanide base, are double stranded RNA, a component of viral genomes, or muramyl dipeptides derived from bacterial peptidoglycan that is present in the bacterial cell wall\textsuperscript{416,417}. The effects of these different immunostimulators are believed to be mediated via stimulation of antigen presenting cells, mainly dendritic cells\textsuperscript{414,418}. CpG oligonucleotides exert their effect through TLR9\textsuperscript{415,419-422}; double stranded RNA is known to trigger TLR3 and the muramyl dipeptides activate Nucleotide-binding oligomerization domain 2 (NOD2). TLR9, TLR3 and NOD2 are all receptors present on dendritic cells\textsuperscript{416,423}. In \textit{paper II} we have identified an adjuvant that is potent enough to break self-tolerance and safe to use in humans. In \textit{paper III} we have further characterized the immune response induced with the newly developed adjuvant against the self-antigen ED-B.

\textbf{Paper I}

\textbf{Vaccination against the extra domain-B of fibronectin as a novel tumor therapy}

\textbf{Aim}

In this study we addressed if immunization against ED-B was possible and could inhibit tumor growth.

\textbf{Results}

In our approach we have made a recombinant fusion protein consisting of a bacterial thioredoxin (TRX) part fused with the extra domain-B (ED-B), termed TRX-EDB. This fusion protein was injected together with Freund’s adjuvant into eight-weeks old female wild type C57BL6 mice. Mice were boosterized twice in a period of five weeks before they were inoculated subcutaneously with T241 fibrosarcoma cells, a tumor type known to express ED-B. After a tumor growth period of three weeks animals were sacrificed and blood and tumors were removed. Nineteen out of 20 vaccinated mice responded with production of anti-ED-B antibodies and showed a 70\% reduction in tumor size compared to the animals in the control group vaccinated with Freund’s adjuvant and vehicle and lacking anti-ED-B antibodies.

Staining of murine grade III glioma tissue was done to examine whether the serum from TRX-EDB mice could detect native ED-B. It showed an extensive vascular staining pattern compared to normal brain tissue, which is
devoid of ED-B. This proves that the anti-ED-B antibodies are able to detect native tissue ED-B. To determine the type of immune response induced by the ED-B vaccine we analyzed the anti-ED-B IgG subclasses present in the serum from control and ED-B vaccinated mice. We found that the main immunoglobulin G subclass induced was IgG1, which is characteristic of a Th2 response, thus an antibody-mediated immune response.

Stereological analysis did not reveal any difference in vessel number, vessel density or area between tumors from control and ED-B vaccinated mice. Quantification of tumor necrotic area revealed a greater necrotic area in TRX-EDB vaccinated compared to control animals. Further investigation of the tumors with electron microscopy revealed morphological changes in the tumor vasculature of ED-B vaccinated animals, which was consistent with an immune response towards the tumor vasculature expressing ED-B. Immunohistochemical analysis of tumors from ED-B vaccinated animals showed an increased number of infiltrating neutrophils compared to controls, in agreement with an immune response in the tumor. An increased amount of extravasated fibrinogen, indicative of vascular leakage, was also detected in tumors of ED-B vaccinated animals compared to controls.

Since ED-B is also expressed during physiological angiogenesis we addressed the effect of the presence of anti-ED-B antibodies on wound healing, but could not detect any alterations in the healing process between ED-B vaccinated mice and controls. Fibronectin containing ED-B is also present in cartilage and we scored control and TRX-EDB vaccinated animals for arthritis development but could not detect any differences between the treatment groups.
Paper II
Identification of potent biodegradable adjuvants that efficiently break self-tolerance – a key issue in development of therapeutic vaccines

Aim
The aim of this study was to identify an adjuvant safe for clinical use that is potent enough to break self-tolerance.

Results
In this investigation we screened a number of adjuvants for their ability to induce an immune response against a self-protein. We immunized 40 female Wistar Furth rats, 8-10 weeks old, with a recombinant opossum-rat-opossum (ORO) IgE protein together with Freund’s adjuvant or Montanide ISA 720 (M720) with or without additional immunostimulators. The ORO-protein contains a self-part (C3 domain of rat ε-heavy chain) flanked with two non-self parts (C2 and C4 domain of opossum ε-heavy chain).

We used either M720 alone or with addition of different immunostimulatory compounds: two different CpG oligonucleotides, double stranded RNA, GMDP (N-acetylglucoseamine-1-4-N-acetylmuramyl-alanyl-D-isoglutamine) or GMDP-A (N-acetylglucosamine-1-4-N-acetylmuramyl-alanyl-D-glutamic acid). Freund’s adjuvant was used as reference in this study. M720 is a squalene-based biodegradable mineral oil, which functions as a depot to facilitate a slow release of the protein component of the vaccine, which enhances the immune response. Bacteria and certain DNA viruses contain repetitive unmethylated CpG DNA sequences that stimulate the immune response.

We found that the combination of Montanide ISA 720 and CpG oligonucleotide is as effective as Freund’s adjuvant in inducing an immune response against self-antigens. Even without additions, Montanide ISA 720 was as potent as Freund’s adjuvant with respect to induction of an immune response against a non-self protein.
Paper III

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

Aim

The objective of this paper was to analyze if the adjuvant M720/CpG identified in paper II could be used for induction of an immune response against the self-antigen ED-B. In addition the aim was to compare the immune response induced with M720/CpG with the response generated with Freund’s adjuvant, which was used as adjuvant in paper I.

Results

The TRX-EDB fusion protein was injected together with M720/CpG or Freund’s adjuvant into four- to eight-weeks old female wild type C57BL6 mice. The immunization period was five weeks and booster injections were given at day 14 and 28 of the experiment. Control animals were injected with vehicle or TRX mixed with M720/CpG. Comparable anti-ED-B antibody levels could be measured one week after the second boost in mice vaccinated with Freund’s adjuvant and M720/CpG. However, the variation in individual antibody levels measured was less with M720/CpG compared to Freund’s. Surface plasmon resonance was used to determine the avidity of the antibodies generated with the different adjuvants. We found that the anti-ED-B antibodies induced with M720/CpG were of higher avidity than antibodies generated with Freund’s adjuvant. Anti-ED-B IgG subclass analysis revealed IgG1 as the major subclass induced by both adjuvants. To compare the duration of the immune response induced by the different adjuvants, anti-ED-B antibody levels were measured three months and seven months after the second boost. At the three-month time point antibody levels were comparable in both groups. However, at the seven-month time point anti-ED-B antibody levels had decreased to baseline in the Freund’s group whereas in the M720/CpG group antibodies were still clearly detectable. No difference in induction of a memory response against ED-B between the two adjuvants could be detected. We also investigated the kinetics of induction of anti-ED-B antibodies in naïve mice with M720/CpG and Freund’s as adjuvants. Six-week old female C57BL6 mice were immunized with the TRX-EDB fusion protein mixed with Freund’s adjuvant or M720/CpG. The mice received booster injections at day 14 and 28 of the experiment and blood samples were drawn prior to immunization and at day three, six, nine, 17, 21, 31, and 35 after the first injection. At day nine after the first
immunization anti-ED-B antibodies were detectable in both groups but the levels in the M720/CpG group were significantly higher than those detected in the Freund’s group. In the Freund’s group a booster injection was needed to reach comparable levels. We conclude that the production of anti-ED-B antibodies in naïve mice was faster when M720/CpG, was used as adjuvant compared to using Freund’s.

Paper IV
Development of a therapeutic vaccine targeting blood vessels in primary tumors and metastases

Aim
In the present studies we sought to investigate the effect on the immune response by immunization against the three target molecules ED-B, ED-A, TNCC simultaneously in one vaccine. We also aimed to determine the expression pattern of the tumor vascular antigens in different tumor models. Furthermore, we wanted to address the therapeutic effect of the ED-B vaccine on already established tumors in the orthotopic RIP1-Tag2 insulinoma model.

Results
We show that self-tolerance against ED-A and TNCC can be broken in mouse and rabbits. Anti-ED-B antibody levels generated were significantly reduced by addition of one (TRX-EDA) or two (TRX-EDA and TRX-TNCC) vaccine protein components to the ED-B vaccine. The TRX part alone also significantly suppressed the antibody production against ED-B. When all three vaccine-proteins (TRX-EDB, TRX-EDA and TRX-TNCC) were combined into a single fusion protein (TRX-EDB-EDA-TNCC) the suppressive effect of the foreign fusion part TRX on the antibody production was reduced. Using affinity-purified anti-mouse antibodies from rabbit serum we found that ED-B, ED-A and TNCC was expressed in T241 fibrosarcoma but not in normal mouse tissue.

ED-B was also detected in insulinosomas of RIP-Tag2 mice. Immunization against ED-B induced a significant reduction in the number of tumors in the pancreas compared to control mice. This indicates that the ED-B vaccine can be used in a therapeutic setting when tumorigenesis already has been initiated.

We identified ED-A in tumor tissue of transgenic MMTV-PyMT mice, a model for metastatic breast cancer, but not in normal breast tissue. The aim
is to use this model to study the effect of an ED-A vaccine on metastasis. We also found that ED-B is expressed in canine breast tumors and this domain may represent a potential therapeutic target in dogs. Dog-specific anti-ED-A and anti-TNCC antibodies could also be generated in rabbits and are important tools to establish the expression pattern of these molecules in dog tissue.

**Discussion**

Monoclonal antibodies against ED-A and ED-B alone or in combination with chemotherapy or radiation are tested in phase I/II clinical trials, to deliver coupled agents specifically to the tumor tissue. This supports our choice of target molecules and their disease specificity.

Since the clinically tested antibodies are monoclonal no immune complexes will be formed and thus no effective immune response can be induced with these antibodies. Our strategy however is aiming to induce an endogenous production of antibodies against the vascular antigens. This will give a polyclonal immune response and consequently more binding possibilities to the target antigen. A polyclonal serum will induce immune complex formation leading to a more effective immune response. This explains why monoclonal antibodies alone are insufficient to evoke an immune response against the tumor vasculature and have to be coupled to other agents to obtain an effect.

Mice with anti-ED-B antibodies present in their circulation have normal life span (we kept mice for a time period of almost two years), normal physical appearance and they show no signs of pathological changes in their tissues such as in cartilage. Furthermore, we could not observe any impaired wound healing in full-thickness wounds, a model of physiological angiogenesis, in these mice.

An explanation for the outcome that the cartilage did not seem to be attacked by the immune system in TRX-EDB immunized mice could be the lack of blood vessels in this tissue. This reduces the accessibility of the anti-ED-B antibodies for their target molecule ED-B.

The reoccurrence of ED-B during wound healing could cause a problem in vaccinated animals with anti-ED-B antibodies in their blood and promote an immune reaction directed against the provisional matrix present in wounds. We found that wound healing was not impaired in mice with antibodies against ED-B present in their circulation. An explanation for this could be that ED-B fibronectin expression is transient in a wound compared to the permanent expression observed in tumors. Furthermore, the blood vessel quality might be different in physiological angiogenesis (wound healing) compared to pathophysiological angiogenesis (tumorigenesis). Tumor vessels are constantly leaky whereas the blood vessels in a wound are
only leaky within a transient time-interval\textsuperscript{18}, which might not be long enough to sustain an immune response leading to tissue damage. The antibodies also need to cross the endothelium to reach their target in the ECM and therefore targeting of ED-B in a tumor might be easier. Another possibility could be that ED-B\textsuperscript{+} fibronectin is less abundantly expressed in a healing wound than ED-A\textsuperscript{+} fibronectin\textsuperscript{229,239}. Therefore an immune attack directed towards ED-B might not have any impact on wound healing because ED-A\textsuperscript{+} fibronectin might be the crucial isoform required for this process.

CpG oligonucleotide binds to TLR9. TLR9 is expressed by B-cells and plasmacytoid DCs and stimulation of this pattern recognition receptor leads to production of IFN\alpha\textsuperscript{414,425,426}. IFN\alpha has been shown to induce the secretion of B-cell activating factor (BAFF) by myeloid dendritic cells and the differentiation of B-cells into antibody producing plasma cells\textsuperscript{427}. BAFF was also found to promote survival and antibody secretion by autoreactive B-cells\textsuperscript{427,428}. This mechanism might explain why CpG oligonucleotides can be used to break immune tolerance and induce autoantibodies. TLR9 stimulation by CpG oligonucleotides has been shown to promote B-cell proliferation, inhibit B-cell apoptosis and induce IgM production\textsuperscript{415,429}. Simultaneous triggering of TLR9 and the B-cell receptor was shown to stimulate proliferation of autoreactive B-cells\textsuperscript{422,427}, which might be attributed to the production of IFN\alpha induced by TLR9 stimulation. When systemically administered type I interferons (IFN\alpha and IFN\beta) can induce autoimmune diseases such as Systemic lupus erythematosus\textsuperscript{427}. This observation might therefore provide an explanation for the potent effect of TLR9 stimulation on breaking self-tolerance.

CpG oligonucleotide was also found to directly activate NK cells\textsuperscript{414} and therefore the addition of CpG oligonucleotide to the vaccine maybe also promotes NK cell activation in our vaccination approach.

The anti-ED-B antibodies induced with the help of the adjuvant M720/CpG had a 10-fold higher avidity for ED-B than those induced with Freund’s adjuvant. An explanation for this observation could be the presence of CpG. In a study by Siegrist \textit{et al.} the addition of human specific CpG oligonucleotides (CpG 7909) to a prophylactic hepatitis B vaccine was shown to enhance affinity maturation of the human anti-hepatitis B antibodies induced by the vaccine\textsuperscript{430}. The CpG 7909 significantly increased both antibody levels and antibody avidity. The adjuvant used in this study was alum and human-specific CpG oligonucleotide with a sequence distinct from the mouse-specific CpG 1826 used in our studies. However, the results of Siegrist \textit{et al.} are in agreement with ours.

The difference with the monoclonal antibodies targeting VEGF used in the clinic, which have to be administered frequently and at high doses, is that our vaccination approach triggers the endogenous production of antibodies. By this means the immune system can be directed against the tumor blood vessels, which will lead to vessel destruction and inhibition of tumor growth.
Additionally, the production of monoclonal antibodies is costly and therefore treatment with these agents is expensive. A vaccine in comparison requires lower doses because the body will produce the antibodies against the administered target antigen. Monoclonal antibodies however need frequent administration (every 2-3 weeks) due to their short half-life (approximately 20 days)\textsuperscript{119}. The immune response induced by our vaccine is reversible, which is of advantage. Reversibility is desirable in case the endogenously produced antibodies give side effects. After vaccination against ED-B the induced anti-ED-B antibodies stayed present in the circulation for seven to 12 month, depending on which adjuvant was used. The reason for the difference in duration of the immune response is unknown. One explanation might be that the higher avidity of the antibodies induced with M720/CpG aids to keep the antigen on the surface of follicular dendritic cells (fDCs) promoting a more long-lived response. Long-lived fDCs help to present the injected fusion protein TRX-EDB to immune cells by Ig, which is bound to Fc-receptors. Furthermore, greater antibody avidity might require a lower number of cross-linked Ig molecules on the surface of the B-cell to trigger its activation. Both Montanide ISA 720 and CpG oligonucleotide 7909 have been tested separately in clinical studies and single use of these agents seems to be safe since only mild or moderate side effects were observed\textsuperscript{430-435}. We observed a significant decrease in the number of tumors in RIP-Tag2 mice. The total tumor volume was also reduced although this was not statistically significant in the present study group. Nevertheless the observation indicates that the ED-B vaccine also is effective in a therapeutic setting. The other target molecules ED-A and TNCC have an expression pattern similar to ED-B and are therefore interesting to target with the same approach as used for ED-B. Our data show that it is possible to break self-tolerance against ED-B, ED-A and TNCC in mice and rabbits and therefore our vaccination technology might be an interesting approach for production of antibodies against antigens with low immunogenicity.

**Future perspectives and concluding remarks**

The overall aim of our studies is to develop a new treatment strategy for patients with solid tumors. To achieve this goal the strategy has to be validated in additional preclinical as well as clinical studies. The high degree of conservation of ED-A, ED-B and TNCC should facilitate clinical translation of the approach.

The results described in this thesis show that it is possible to break self-tolerance against vascular antigens, which are present in angiogenic vasculature of most solid tumors. When ED-B was used as a target molecule tumor growth could be inhibited in a preventive (subcutaneous T241 fibrosarcoma) and therapeutic setting (orthotopic RIP-Tag2 tumor model).
Furthermore, we found a non-toxic and biodegradable (M720/CpG) adjuvant potent enough to break the immune tolerance against self-antigens. This adjuvant was also effective in breaking self-tolerance against the vascular antigens ED-A, ED-B and TNCC. This demonstrates that there are less toxic alternatives to Freund’s adjuvant in experimental animal work. In addition, this finding makes M720/CpG an interesting adjuvant for development of therapeutic cancer vaccines. ED-A was found to be expressed in breast tumors of MMTV-PyMT mice. This model will be used to determine the effect of an ED-A vaccine on tumor growth and metastasis. We also found that addition of TRX suppressed the formation of anti-ED-B antibodies in mice immunized with TRX-EDB and that this was also the case when mice were immunized with a vaccine consisting of TRX-EDB, TRX-EDA and TRX-TNCC. The immune response to ED-B however could be partly restored when only one molecule TRX was fused to all three antigens in one fusion protein. Further optimization is needed to obtain equal levels of specific antibodies directed to each of the three antigens when administering them simultaneously as obtained after immunization with ED-B only. We would like to perform a tumor study to address the effect of the vaccine on tumor growth after a combined immunization with all three antigens. The T241 fibrosarcoma model expresses all three vascular antigens. Simultaneous targeting of these antigens might therefore have a more profound effect on tumor growth compared to immunization against a single antigen.

During the past era of cancer treatment it has become clear that combination therapy is essential for tumor eradication and to avoid drug resistance\textsuperscript{101}. One situation when a tumor vaccine could potentially be useful is after resection of the primary tumor to prevent tumor recurrence and metastatic disease. However, this topic requires further investigation. In combination with chemotherapy or radiotherapy the vaccine might be used to treat non-resectable established tumors, since chemotherapy and radiation have been shown to stimulate the immune system\textsuperscript{13,108,189,190}. The vaccine might also be applied to treat metastatic disease in combination with chemotherapy.

We could not observe any changes in wound healing, an example of physiological angiogenesis, between TRX-EDB and control-vaccinated animals.

A clinical situation in which application of a therapeutic vaccine cancer would be highly interesting is for prevention of metastases formation in patients diagnosed with a primary tumor. It has been reported previously that ED-A is expressed in primary human breast tumors and metastases\textsuperscript{226}. We found that ED-A is expressed in a similar pattern in primary tumors and metastases of the transgenic MMTV-PyMT breast cancer model. This makes the MMTV-PyMT mice a suitable model to address the potential of an ED-A vaccine in prevention of breast cancer metastases.
To confirm the therapeutic effect of our vaccine a clinical trial has to be conducted. Dogs with tumors represent a potentially interesting group of patients for a clinical study, since they are to a large extent affected by the same type of tumors as humans. We found ED-B also to be expressed in mammary tumors of dogs and thus ED-B is also a potential target in this common dog tumor type.
Populär vetenskaplig sammanfattning

Utveckling av ett cancervaccin som angriper tumörblodkärl


I denna avhandling beskrivs utvecklingen av ett vaccin som angriper nybildningen av blodkärl i tumörer (tumörangiogenes). Det är en metod som kan hjälpa till att hämma tumörövärkten genom att man kan stoppa blodflödet och därmed svälta ut tumören genom att den inte får tillgång till viktiga näringsämnen och syre. Vaccinet riktar sig specifikt mot molekyler som uttrycks vid blodkärlsyebildning kring blodkärl i de flesta solida tumörer. Dessa molekyler utgör bland annat delar (extra domäner) av proteiner som bygger upp den vävnad som alla celler i kroppen sitter på (extracellulära matrixen). Dessa molekyler är bl.a. extra domän-B (ED-B) och extra domänen-A (ED-A) i fibronectin och C-domänen i tenascin-C (TNCC).

Vi visar att det går att bryta självtolerans genom vaccinering och stimulera produktion av antikroppar mot kroppseget ED-B i det första delarbetet. Immunförsvarvaret är normalt inställt på att inte reagera mot kroppsega molekyler (antigener) utan bara mot det som är främmande. Vaccinet hämmade även tumörövärkten i möss och de observerade förändringarna i tumörvävnaden stämmer väl överens med att immunförsvarvaret angriper blodkärl i tumören.

För att kunna använda vaccineringsmetoden kliniskt behöver man förstärka vaccinets förmåga att sätta igång ett immunsvar. Det gör man genom att tillsätta ett s.k. adjuvans och det måste i det här fallet vara tillräckligt kraftigt för att man skall kunna bryta självtoleransen mot kroppsega antigener som ED-B men det måste även vara ofarligt att
användas på människor. I det andra delarbetet visar vi att det skvalen-baserade adjuvanset Montanide ISA 720 (M720) i kombination med CpG DNA (ometylerade CG rika DNA molekyler isolerade från bakterier eller virus; mänskligt DNA är metylerat) uppfyller dessa krav och skulle kunna användas kliniskt som adjuvans.


Sammanfattningsvis visar våra prekliniska studier att ett vaccin som angriper blodkärlsbildningen via en eller flera vägar är en ny mycket lovande strategi för behandling av cancer i olika former.
Nederlandse samenvatting

De ontwikkeling van een kankervaccin dat aangrijpt op de tumorbloedvaten

Kanker is na ischemische hartziekten de meest voorkomende doodsoorzaak in de Westerse wereld en staat op een derde plaats in de ontwikkelingslanden. De ziekte wordt gekenmerkt door kwaadaardige gezwel, die ontstaan door dat tumorcellen ongecontroleerd groeien en gezond weefsel kunnen binnendoor. Gedurende de vorige eeuw zijn voor een groot deel de oorzaken van kanker in kaart gebracht en werd behandeling mogelijk. Het merendeel van de kanker patiënten sterft echter niet aan het oorspronkelijke gezwel (tumor) maar aan ongeneeslijke uitzaaiingen (metastasen) die met de ziekte gepaard gaan. Met de huidige beschikbare behandelmethode is het moeilijk om kanker die is uitgezaaid te behandelen. Daarom is er behoefte aan nieuwe mogelijkheden ter behandeling van zowel metastasen als primaire tumoren.

In dit proefschrift wordt de ontwikkeling van een vaccin beschreven dat aangrijpt op de bloedvatvorming in tumoren (de tumorangiogenese). Dit is een methode die kan bijdragen aan het remmen van de tumorgroei en de bloedtoevoer naar de tumor kan stoppen, waarmee deze van voedingsstoffen en zuurstof kan worden onttrokken.

Het vaccin richt zich op moleculen die aanwezig zijn tijdens de aanmaak van nieuwe bloedvaten in de meeste solide tumoren. Deze moleculen zijn o.a. extra domeinen van eiwitten die voorkomen in de extracellulaire matrix (het weefsel waarop cellen hechten); voorbeelden zijn het extra domein-B (ED-B) en extra domein-A (ED-A) van fibronectine en het C-domein van tenascine-C (TNCC).

In het eerste artikel laten we zien dat het mogelijk is om door middel van vaccinatie de immuuntolerantie te doorbreken en om de productie van antilichamen tegen het lichaamseigen ED-B te stimuleren. Het immuunsysteem reageert normaalgesproken namelijk niet op lichaamseigen moleculen (antigenen) maar alleen op lichaamsvreemde. Bovendien kon met het vaccin de tumorgroei in muizen worden geremd en de veranderingen, waargenomen in het tumorweefsel, waren overeenkomstig met het aangrijpen van het immuunsysteem op de tumorbloedvaten.

Om de vaccinatietechnologie in de kliniek toe te kunnen passen moet het vermogen van het vaccin om een immuunreactie op gang te brengen worden
versterkt. Dit kan worden bereikt met behulp van een zogenaamd adjuvans, dat krachtig genoeg is om de immuuntolerantie tegen lichaamseigen antigenen zoals ED-B te doorbreken. Tevens moet het adjuvans geschikt zijn voor humaan gebruik. In paper II laten we zien dat het squaleen afgeleide Montanide ISA 720 (M720) in combinatie met CpG DNA (ongemethyleerd DNA rijk aan CG van bacteriën en virussen; humaan DNA is gemethyleerd) voldoet aan deze voorwaarden en mogelijk als adjuvans in de kliniek kan worden toegepast.

Bovendien laten we zien dat M720/CpG even krachtig is als de preklinische ‘gouden standaard’ Freund’s adjuvans in het opwekken van een immuunreactie tegen een lichaamseigen antigeen. In paper III hebben we de met behulp van M720/CpG opgewekte immuunreactie tegen ED-B onderzocht. Het ED-B vaccin bleek tevens effectief in een therapeutische toepassing en remde de tumorgroei in een transgeen muismodel met insulinenomen (insuline producerende tumoren) in de pancreas waarin de tumorgroei al op gang was gekomen. Tevens konden we met onze methode antilichamen tegen ED-A en TNCC opwekken in muizen en konijnen. We hebben ook de expressie van ED-A bepaald in tumorweefsel van muizen met borstkanker. In dit muismodel willen we in de toekomst het effect van een ED-A vaccin op metastasen bestuderen. ED-B kon tevens worden aangetoond in tumorweefsel van honden. Honden met kanker zijn daarom potentieel geschikt voor inclusie in een klinische studie ter analyse van het effect van het kankervaccin.

Samenvattend laten onze preklinische studieresultaten zien dat een vaccin dat aangrijpt op de bloedvatvorming een veelbelovende nieuwe strategie voor de behandeling van kanker is.
Die Entwicklung eines Krebsimpfstoffes der die Blutgefäße im Tumor angreift


In dieser Doktorarbeit wird die Entwicklung eines Impfstoffes, welcher die Bildung von neuen Blutgefäßen durch den Tumor (Tumor-Angiogenese) verhindert, beschrieben. Die Methode kann bei der Hemmung des Tumorwachstums behilflich sein indem sie die Blutzufuhr zum Tumor stoppt und damit dessen Nährstoff- und Sauerstoffzufuhr einschränkt.

Der Impfstoff richtet sich gegen Moleküle, welche nur in unmittelbarer Nähe zu neugebildeten Gefäßen gefunden werden können und die während der Tumorangiogenese von den meisten Krebsarten gebildet werden. Zu diesen Molekülen gehören unter anderem die Extra-Domänen von Eiweißen in der extrazellulären Matrix (das Gewebe worauf Zellen haften); wie zum Beispiel die Extra-Domäne-B (ED-B) und Extra-Domäne-A (ED-A) von Fibronectin und die C-Domäne von Tenascin-C (TNCC).

Im ersten Artikel zeigen wir, dass es möglich ist die Immuntoleranz mit einem Impfstoff zu durchbrechen und die Produktion von Antikörpern gegen das körpereigene ED-B zu stimulieren. Das Immunsystem reagiert normalerweise nur auf körperfremde Moleküle (Antigene) aber nicht auf körpereigene. Außerdem konnte das Tumorwachstum in Mäusen gehemmt werden und die beobachteten Veränderungen im Tumorgewebe erschienen übereinstimmend mit einer Reaktion des Immunsystems auf die Tumorblutgefäße.
Um die Impfung als Therapie im Menschen anwenden zu können muss die Wirkung des Impfstoffes verstärkt werden. Dies erreicht man indem man ein Adjuvans hinzufügt. Das Adjuvans muss in diesem Fall ausreichend strak sein um die Immuntoleranz gegen körpereigene Antigene wie ED-B zu durchbrechen. Außerdem benötigt man ein für den Menschen ungefährliches Adjuvans. Das von Squalen abgeleitete Montanide ISA (M720) in Kombination mit CpG DNA (unmethylierte CG reiche DNA von Bakterien und Viren; menschliche DNA ist methyliert) entspricht diesen Anforderungen und kann möglicherweise in der Klinik als Adjuvans angewendet werden.


Zusammenfassend zeigen, unsere Studien, dass ein Impfstoff der die Tumorblutgefäße angreift eine vielversprechende neuartige Strategie zur Krebsbehandlung darstellt.
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