Endothelial activation and inflammation in the tumor microenvironment

HUA HUANG
Tumors are composed not only of malignant cells, but also of various types of normal cells, including vascular cells and infiltrating immune cells, which drive tumor development and progression. The tumor vasculature is abnormal and dysfunctional due to sustained tumor angiogenesis driven by high levels of pro-angiogenic factors. Proteins differentially expressed in tumor vessels affect vascular function and the tumor microenvironment and may serve as targets for therapy. The tumor is also a site of sustained chronic inflammation. The recruitment and activation of inflammatory cells significantly influence tumor progression and regression. Targeting molecules regulating tumor angiogenesis and inflammation in the tumor microenvironment is therefore a promising strategy for the treatment of cancer. This thesis is aiming to understand and investigate the molecular regulation of these two processes in tumors.

αB-crystallin is a heat shock protein previously proposed as a target for cancer therapy due to its role in increasing survival of tumor cells and enhancing tumor angiogenesis. In this thesis, we demonstrate a novel role of αB-crystallin in limiting expansion of CD11b+Gr1+ immature myeloid cells in pathological conditions, including tumor development. In addition, we show that αB-crystallin regulates leukocyte recruitment by promoting expression of adhesion molecules ICAM-1, VCAM-1 and E-selectin during TNF-α-induced endothelial activation. Therefore, targeting of αB-crystallin may influence tumor inflammation by regulating immature myeloid cell expansion and leukocyte recruitment.

Abnormal, dysfunctional vessels are characteristic of glioblastomas, which are aggressive malignant brain tumors. We have identified the orphan G-protein coupled receptor ELTD1 as highly expressed in glioblastoma vessel and investigated its role in tumor angiogenesis. Interestingly, deficiency of ELTD1 was associated with increased growth of orthotopic GL261 glioma and T241 fibrosarcoma, but did not affect vessel density in any model. Further investigation is warranted to evaluate whether ELTD1 serves a suitable vascular target for glioblastoma treatment.

Anti-angiogenic drugs targeting VEGF signaling is widely used in the clinic for various types of cancer. However, the influences of anti-angiogenic treatment on tumor inflammation have not been thoroughly investigated. We demonstrate that VEGF inhibits TNF-α-induced endothelial activation by repressing NF-κB activation and expression of chemokines involved in T-cell recruitment. Sunitinib, a small molecule kinase inhibitor targeting VEGF/VEGFR2 signaling increased expression of chemokines CXCL10, CXCL11, and enhanced T-lymphocyte infiltration into tumors. Our study suggests that anti-angiogenic therapy may improve immunotherapy by enhancing endothelial activation and facilitating immune cell infiltration into tumors.

Keywords: tumor angiogenesis, endothelial activation, leukocyte recruitment, VEGF-A, αB-crystallin, ELTD1

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ISSN 1651-6206
ISBN 978-91-554-9212-0
urn:nbn:se:uu:diva-247889 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-247889)
I know one thing: that I know nothing. – Socrates, 469-470 B.C.

我知道我一无所知。——苏格拉底

To my family

致我的家人
List of Papers

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


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* Contributed equally to the work
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## Abbreviations

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<tr>
<td>CSF</td>
<td>Colony stimulating factor</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<tr>
<td>CXCL</td>
<td>Chemokine (C-X-C motif) ligand</td>
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<tr>
<td>CXCR</td>
<td>Chemokine (C-X-C motif) receptor</td>
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<tr>
<td>EAE</td>
<td>Autoimmune encephalitis</td>
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<tr>
<td>EC</td>
<td>Endothelial cell</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<td>EMT</td>
<td>Epithelial mesenchymal transition</td>
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<tr>
<td>EndMT</td>
<td>Endothelial mesenchymal transition</td>
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<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
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<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
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<td>HDMEC</td>
<td>Human dermal microvascular endothelial cell</td>
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<tr>
<td>HSP</td>
<td>Heat shock protein</td>
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<tr>
<td>ICAM</td>
<td>Intercellular adhesion molecule</td>
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<tr>
<td>IkB</td>
<td>Inhibitor of κB</td>
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<tr>
<td>IKK</td>
<td>Inhibitor of κB kinase</td>
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<tr>
<td>IMC</td>
<td>Immature myeloid cell</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MDSC</td>
<td>Myeloid-derived suppressor cell</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
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<tr>
<td>MyEnd</td>
<td>Murine myocardial endothelial</td>
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<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
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<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal Transducers and Activators of Transcription</td>
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<tr>
<td>TAM</td>
<td>Tumor-associated macrophage</td>
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<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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<tr>
<td>T-reg</td>
<td>T regulatory</td>
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<tr>
<td>VCAM</td>
<td>Vascular cell adhesion molecule</td>
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<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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Introduction

The tumor microenvironment
Tumors are composed not only of malignant cells, but also of various types of normal cells and extracellular matrix (ECM). The tumor stroma, including vascular cells, infiltrating immune cells, cancer-associated fibroblasts, extracellular matrix, and soluble factors together provide a microenvironment that drives tumor development and progression [Figure 1]. The recently updated “Hallmarks of Cancer”, reviewed by Douglas Hanahan and Robert Weinberg, proposed ten traits that tumors need to acquire during progression [1]. Notably, the tumor stroma cells contribute to several of the hallmarks of cancer. For example, they provide growth factors for sustained cancer cell proliferation, selectivly recruit cells that suppress immune surveillance, produce angiogenic factors such as vascular endothelial growth factor A (VEGF-A) to induce tumor angiogenesis and hence tumor growth, secret proteolytic enzymes to remodel extracellular matrix and thereby evade cell growth suppression and activate tumor cell invasion and metastasis, and reprogram cells such as induce epithelial mesenchymal transition (EMT) and tumor-associated macrophage (TAM) polarization to facilitate tumor invasion and progression [2].

It is becoming evident that although cancer is considered as a genetic disease, the tumor microenvironment plays an important role during tumor progression or regression. Moreover, it also significantly influences the therapeutic response in cancer [3]. The components of tumor microenvironment such as tumor vessels and the properties such as high interstitial fluid pressure constitute barriers for chemotherapeutic drug delivery and protective shells for radiotherapy. On the other hand, therapeutic intervention also changes the tumor microenvironment and leads to complex therapeutic resistances.

In this thesis, we identify molecules in the tumor microenvironment that regulate tumor angiogenesis and explore crosstalk of signaling pathways in the processes of tumor angiogenesis and inflammation during tumor progression, which may serve as potential therapeutic targets and give insights for combinational clinical treatments.
Figure 1. The tumor microenvironment. Tumors are composed not only of malignant cells, but also of various types of normal cells, extracellular matrix and soluble factors. Tumor is also featured by hypoxia, high interstitial fluid pressure and necrosis. (Image kindly provided by Dieterich LC. Institute of Pharmaceutical Science, ETH Zurich, CH-8093 Zurich, Switzerland.)

Tumor vasculature

Tumor blood vessels are structurally and functionally abnormal. Compared to normal vessels, tumor vessels are irregular in size, shape and density, and lack hierarchy of arterioles, capillaries or venules [Fig 2A,B] [4]. Normal blood vessels are composed of endothelial cells and covered by vessel walls of pericytes or smooth muscle cells and basement membrane. While in tumor vessels, the mural cells are loose or missing from the endothelium. Tumor endothelial cells are not connected tightly, and have abundant tiny fenestrations and non-circulating branches or sprouts, which give rise to leaky tumor vessels, and high interstitial fluid pressure in the tumor [Fig 2C,D] [5].
Blood vessel formation

Our body is largely dependent on efficient transport of oxygen, nutrients, molecules and cells. Since oxygen diffusion is limited to 100 to 200μm, all cells need to reside within this distance to a vessel [6]. Vessel formation is achieved by vasculogenesis and angiogenesis. Vasculogenesis is the formation of new vessels from endothelial progenitor cells. It occurs during embryonic development. Angiogenesis is the process of vessel sprouting from pre-existing vessels. It involves remodeling and expansion of the vascular network and does not usually occur in the healthy, full-developed vascular system, except in conjunction with wound healing and the menstrual cycle [7]. However, angiogenesis is frequently induced in pathological conditions such as tumor development, inflammation and ischemia. In fact, tumors rely on a blood supply for maintained tumor cell survival and induction of angiogenesis is one of the hallmarks of cancer [1].

Tumor angiogenesis

Small tumor lesions can remain dormant for a long time. This is due to equal proliferation and apoptosis of tumor cells, and a balance of pro-angiogenic factors and anti-angiogenic factors leading to a quiescent vasculature. However, at a certain stage, this balance can be disturbed. Tumors then progress from a dormant stage to progressive stage with profound vessel outgrowth. The initiation of angiogenesis is termed the ‘angiogenic switch’ [8].

Hypoxia, low oxygen tension, is one of the predominant stimuli that induce angiogenesis. When the supply of oxygen in the tissue is too low to satisfy a high proliferation of tumor cells, hypoxia inducible factor 1 (HIF-1)
is up-regulated. HIF-1 needs O₂ as substrate for its hydroxylation that tags the protein for degradation [9]. When the oxygen level is low, HIF-1 accumulates, translocates to the nucleus and induces expression of a specific set of genes, including the pro-angiogenic factor VEGF-A [10, 11]. An increased amount of angiogenic factors in the microenvironment results in the induction of angiogenesis.

Importantly, tumor infiltrating inflammatory cells such as TAMs and myeloid-derived suppressor cells (MDSCs), may also induce the angiogenic switch by secreting multiple pro-angiogenic factors [12, 13].

Molecular regulation of angiogenesis

The VEGF family of pro-angiogenic factors

VEGF is a major regulator of angiogenesis, and one of the most studied pro-angiogenic factors so far. The VEGF family is composed of five members, VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PIGF). VEGF-A is essential for embryonic development, even single allele loss is embryonic lethal due to severe defects in vascular development [14]. Several VEGF-A isoforms exist due to alternative splicing, including VEGF₁₂₁, VEGF₁₆₅, VEGF₁₈₉ and VEGF₂₀₆. VEGF₁₂₁ is an acidic polypeptide, which cannot bind to heparan sulfate and is therefore freely diffusible in the tissue. On the other hand, VEGF₁₈₉ and VEGF₂₀₆ are basic, and bind to heparan sulfate with high affinity. Therefore, they are almost completely sequestered in the ECM, forming concentration gradients to guide endothelial cell migration. VEGF₁₆₅ is an intermediate form, and a large fraction of VEGF₁₆₅ is bound to the ECM. But it is released and activated by plasmin cleavage at the C terminus. When we discuss VEGF in the later chapters of this thesis, we refer to the isoform VEGF₁₆₅ (or VEGF₁₆₄ in mouse) [15].

VEGFs act by binding to receptor tyrosine kinases (RTKs) expressed on endothelial cells (also on other types of cells like bone marrow derived cells) in an overlapping manner. These RTKs, which include VEGFR1, VEGFR2, and VEGFR3, strictly regulate the VEGF signal transduction and cellular functions. VEGFR2 is the major receptor and mediator of VEGF-induced endothelial mitogenesis and angiogenesis. VEGFR2 gene knockout mice are embryonic lethal due to lack of vasculogenesis. VEGFR2 activation regulates multiple biological processes such as endothelial survival, proliferation, sprouting, migration and permeability. VEGFR1 knockout mice are embryonic lethal, and have a phenotype of disorganized vasculature owing to endothelial cell overgrowth. VEGFR1 has been suggested to act as a VEGF decoy, preventing VEGF binding to VEGFR2 and limiting angiogenesis. But VEGFR1 may also positively contribute to angiogenesis by transmitting chemotactic signals for hematopoietic progenitor cells and leukemic cells. VEGFR3 is predominantly expressed in lymphatic vessels. But there is also
evidence of an important role of VEGFR3 in blood vessel formation, as VEGFR3 knockout mice die at an early embryonic stage before the formation of lymphatics due to cardiovascular failure [14].

Several VEGFR co-receptors have been identified, including neuropilins (NRPs) and integrins. NRP1 enhances binding of VEGF to VEGFR2 and increased migration and survival of endothelial cells. NRP2 is a co-receptor for VEGFR3 and regulates VEGF-C-induced lymphatic sprouting [16]. VEGFR signaling is also modified by interaction with certain integrins such as αvβ3 and β1 integrin [17].

VEGFR signaling is tightly regulated through tyrosine phosphorylation. So far, 10 tyrosine phosphorylation sites on VEGFR2 have been described. Y1054 and Y1059 are located in the kinase domain and are critical for the intrinsic kinase activity of the receptor. Phosphorylated Y1175 (Y1173 in mouse) is an important binding site for several adaptor proteins. For example, upon binding to Y1175, phospholipase Cγ activates mitogen-activated protein kinases (MAPK) p42/p44 and p38, as well as extracellular signal regulated kinase 1/2 (ERK1/2) and protein kinase C (PKC), which regulate endothelial survival, proliferation and migration [17]. The substitution Y1173F is embryonic lethal. Y951 (Y949 in mouse) is phosphorylated in activated endothelial cells during vascular development and in tumors. Phosphorylated Y951 recruits the adapter protein T cell-specific adapter molecule (TSAd), and mediates endothelial migration and permeability [18, 19]. Phosphorylated Y1214 (Y1212 in mouse) binds to the non-catalytic region of tyrosine kinase (NCK) adapter protein, and subsequently activates MAPK p38 and mediates endothelial cell migration [20]. In contrast to Y1173, mice with the substitution Y949F or Y1212F are viable and fertile [18, 21].

Aside from VEGF, there are several other pro-angiogenic growth factors that may induce angiogenesis, either in combination with VEGF or alone, including angiopoietins, fibroblast growth factor (FGF), epidermal growth factor (EGF), hepatocyte growth factor (HGF), and insulin like growth factor (IGF) [22-24].

Anti-angiogenic therapies

The idea of targeting angiogenesis was raised by Judah Folkman in the 1970s, with the aim to limit tumor growth by inhibiting tumor angiogenesis, and thereby prolonging patient survival [25]. In the following years, multiple anti-angiogenic regulators were identified and anti-angiogenic drugs were developed, including monoclonal antibodies, tyrosine kinase inhibitors, and aptamers.

Due to the well-established critical role of VEGF in inducing pathological angiogenesis, most of the anti-angiogenic drugs are targeting the VEGF signaling pathway [26]. The first FDA (The Food and Drug Administration)
approved anti-angiogenic drug was Bevacizumab (Avastin®), a monoclonal antibody neutralizing VEGF-A. It is approved for the treatment of metastatic colorectal cancer, advanced non-small cell lung cancer, breast cancer, renal cell carcinoma and glioblastoma, and significantly increases progression-free survival when combined with chemotherapy [27]. However, overall prolonged survival is typically only a few months, and adverse effects such as gastrointestinal perforation, reduced wound healing and serious bleeding have been reported [28-30].

The tyrosine kinase inhibitors represent another important class of anti-angiogenic drugs. Tyrosine kinase inhibitors are small molecules targeting a broad spectrum of receptor kinases such as VEGFR, FGFR, platelet-derived growth factor receptor (PDGFR), c-Kit and so on. By competitive binding of tyrosine kinase inhibitors to the ATP-binding site of receptor kinases, the phosphorylation of the receptor and downstream targets is blocked, and receptor signal transduction is inhibited. Due to the broad specificity of these drugs, they inhibit angiogenesis not only through VEGF signaling but also through repression of other angiogenic signaling pathways. A great effort has been put into the investigation of the clinical benefit of these inhibitors. For instance, sunitinib targeting VEGFR1-3, PDGFR, c-Kit and Flt3, was approved for the treatment of renal cell carcinoma, and gastrointestinal stromal tumor for its significant improvement of progression-free survival [31].

Multiple angiogenic factors have been identified, and several drugs are consequently developed to inhibit VEGF-independent pro-angiogenic factors and pathways. Novel targets include the c-Met (HGF receptor) pathway, the angiopoietin-TIE-2 system, the ALK1/endoglin (TGFβ) pathway, the Notch pathway and ephrins. Several compounds are now in Phase II and III clinical trials [32].

In addition to the selected chemical compound inhibitors, endogenous angiogenesis inhibitors such as endostatin and caplostatin have also been tested with regard to their usefulness in anti-angiogenic treatment. Endostatin is a 20 kD small fragment of collagen XVIII. It is approved in China to treat lung cancer [33]. Several additional endogenous inhibitors are discovered and show promising pre-clinical effects, including histidine-rich glycoprotein which decreases tumor size and normalizes the tumor vasculature in mouse models [34].

Challenges of anti-angiogenic therapies

Side effects

VEGF is not tumor specific. It is also expressed in normal tissues and is important for normal tissue hemostasis. Targeting VEGF disrupts the structure of the vasculature in healthy tissue and leads to hemorrhage or thrombosis [35]. Also it causes problems for wound healing [36] and glomerular filtration barrier damage in kidney [26]. Other side effects including hemato-
logical toxicity (thrombopenia and leukopenia) and skin toxicities (handfoot syndrome) are often seen in tyrosine kinase inhibitors due to the off-target inhibition [26].

Resistance
Another major challenge of anti-angiogenic drugs is drug resistance. Following tumor shrinkage after first round of drug administration, tumors re-grow even more aggressively. The mechanisms by which tumors evade anti-angiogenic therapy (mainly anti-VEGF signaling therapy) were reviewed by Hanahan [37] and Folkman [38]. Important mechanisms include: first, alternative pro-angiogenic factors such as EGF, FGF, angiopoietin-1 and ephrins are up-regulated upon anti-VEGF treatment. These anti-angiogenic factors promote angiogenesis independent of VEGF signaling. Second, stromal cells contribute to resistance. Endothelial progenitor cells are recruited and give rise to new vessel formation. Bone marrow derived myeloid cells, TAMs, and cancer-associated fibroblasts promote angiogenesis by releasing cytokines and growth factors. Moreover, pericytes and mural cells protect endothelium from VEGF-targeting therapy. Third, the tumor increases its capacity of invasiveness without angiogenesis. Tumor cells can invade into normal tissues by co-opting normal blood vessels [39]. Interestingly, there are also reports indicating that tumor cells can form tubes that mimic vascular network [40] or transdifferentiate into endothelial cells and pericytes [41-44]. This might be another mechanism of resistance.

Increased invasiveness/metastasis
Disturbingly, in 2009 two pre-clinical studies indicated that anti-angiogenic therapy may be associated with increased tumor malignancy [45-47]. Later, more studies recognized accelerated tumor growth in various tumor types following anti-angiogenic treatment [48]. It is also observed in glioma patients in the clinic that bevacizumab treatment leads to a more infiltrative tumor phenotype [49-52]. The mechanism underlying increased invasiveness and metastasis needs more investigation. However, aggressive anti-angiogenic treatment increased hypoxia conditions may constitute one of the reasons. The low oxygen level in tumors following anti-angiogenic treatment forces tumor escape to a distant location. In glioblastoma, hypoxia leads to a selection of hypoxia resistant cells, particularly cancer stem-like cells [53]. Reports show that hypoxia promotes the expansion of cancer stem-like cells after the treatment of sunitinib or bavacizumab in breast cancer xenograft models [54, 55]. Importantly, hypoxia induces HIF-1α expression, activates c-Met pathway and induces EMT that leads to tumor invasion and metastasis.
Future directions of anti-angiogenic therapy

Since inhibition of the VEGF pathway as a single target has a limited effect, anti-angiogenic cocktails and combination treatment with other anti-tumor therapies may increase the survival benefits. Actually, Bevacizumab as well as tyrosine kinase inhibitors are always combined with chemotherapy and radiotherapy in the clinic. Recently, novel therapies targeting other pathways than the VEGF signaling pathway are under clinical investigation [56]. It is interesting to investigate the clinical benefits of combining anti-VEGF with anti-EGF, anti-FGF2 (targeting alternative pro-angiogenesis), anti-PDGF (targeting pericytes), or anti-c-Met (targeting invasiveness) pathways [56].

Intrinsic properties of the tumor and the tumor microenvironment provide initial resistance to anti-angiogenic therapy. Therefore, it is important to identify biomarkers to select and monitor patients who will respond to anti-angiogenic therapy. Potential biomarkers include circulating growth factors, circulating endothelial cells and tumor cells, or functional imaging dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) and position emission tomography (PET) [57].

Hypoxia is a major contribution to the resistance mechanisms. Hypoxia induces up-regulation of HIF-1α, which is a transcription factor for pro-angiogenic factors FGF2, angiopoietin 1, and cytokine the stromal cell-derived factor 1 (SDF1, also known as CXCL12) which recruits CXCR4+ monocytes. Hypoxia leads to increased invasiveness and metastasis. Therefore, it is likely that hypoxia should be avoided when applying vascular targeting therapy. Instead of aggressive treatment, it is believed that modest treatment to achieve vascular normalization may be a better goal for anti-angiogenic therapy. Anti-angiogenic regimens that normalize the tumor vessels may lead to a more efficient drug delivery into the tumor, and also inhibit continuous angiogenesis by achieving a balance in pro- and anti-angiogenic factors [58, 59].

Another strategy to avoid tumor aggressiveness due to increased hypoxia is by blocking hypoxic signaling. In a mouse model, heterozygous deficiency of prolyl hydroxylase domain-containing protein 2 (PHD2), an oxygen sensing protein that is involved in stabilization of HIF-1α, inhibits tumor invasion and metastasis by inducing endothelial normalization and improves oxygen levels in the tumor [60]. Targeting PHD2 may serve as another approach for normalizing vasculature and improving drug delivery and efficacy.

Tumor endothelial markers

Tumor endothelial cells display morphological and molecular heterogeneity in part due to the exposure to various signals in the tumor microenvironment. The tumor endothelial gene signature varies in different tumor types and in
different stages of tumor growth [61]. By using antibody coated magnetic beads, St. Croix first isolated and characterized tumor endothelial cells from colorectal tumor tissue, in an attempt to identify tumor endothelial specific genes which can serve as tumor vascular targets [62]. In this study, nine novel endothelial genes were identified. Notably, many of these genes are expressed in physiological angiogenesis and in normal vessels. Later, following studies revealed comprehensive genetic profiling of tumor endothelial cells in various tumor types [63]. Recently, we isolated glioma vessels by laser-capture microdissection, and identified a distinct vascular gene expression pattern in high grade glioma compared to normal brain or low grade glioma [64]. Many of the detected genes have not been associated with glioblastoma vessels before, such as ELTD1 (EGF, latrophilin and seven transmembrane domain containing 1), an orphan G-protein coupled receptor (GPCR), which is further studied in manuscript Paper III in this thesis.

αB-crystallin

αB-crystallin is a molecular chaperone that belongs to the α-crystallin type small heat shock protein (α-HSP) family. It was first recognized as one of the main proteins contributing to the transparency of the eye lens together with the closely related αA-crystallin. However, αB-crystallin is also expressed in other tissues, including heart, kidney, brain and skeletal muscle, suggesting a function also in other organs. αB-crystallin is constitutively expressed in several cell types including endothelial cells, and acts as a chaperone participating in many cellular process such as in maintenance of the cytoskeleton architecture, in intracellular redox homeostasis and in protection from apoptosis. Also, αB-crystallin may be induced in response to various types of cellular stress such as heat shock, osmotic stress, and oxidative stress, to prevent misfolding and aggregation of other proteins.

αB-crystallin is a low molecular weight chaperone (~22kD). It has a conserved C terminal α-crystallin domain which is shared by all the α-HSP family. This domain forms a β-sandwich structure and plays an important role in forming dimers which are the building blocks of larger oligomers. It also contains a moderate conserved WDPF domain (an amino acid motif consisting of tryptophane, aspartate, proline and phenylalanine) and a non-conserved flexible C-tail. The flexible region is prone to post-translational modifications. The N-terminal domain contains three phosphorylation sites: Ser19 is phosphorylated by a yet unknown kinase, Ser45 by MAPK p44/p42, and Ser59 by MAPKAPK2 [65]. αB-crystallin may also autophosphorylate on serine in a cAMP-independent manner [66, 67]. Phosphorylation induces a conformational change in αB-crystallin and affects the quaternary structure and thus the chaperone activity [65]. Phosphorylation of Ser45 and Ser59 leads to nuclear translocation [68]. Phosphorylation may also regulate molecular functions, as will be discussed in the following sections.
General cellular functions

αB-crystallin has a well-known function in inhibiting apoptosis and increasing cell survival. αB-crystallin is able to protect the cell from oxidative stress. The anti-oxidant ability comes from up-regulated reducing enzymes such as glucose-6-phosphate dehydrogenase (G6PDH), and decreased intracellular iron levels [69].

Also, αB-crystallin binds to cytoskeletal components and protects the cytoskeletal integrity during cellular stress. When phosphorylated at Ser59 [70], αB-crystallin interacts with actin, binds to vimentin and desmin [71] and protects them from thermal aggregation.

In addition, a direct role for αB-crystallin in regulating the apoptosis machinery has been demonstrated. αB-crystallin binds to pro-apoptotic Bcl2 (B-cell lymphoma 2) family members and p53 protein, and prevents their translocation to the mitochondrial membrane [72, 73, 74]. Moreover, αB-crystallin binds directly to pro-caspase-3 and inhibits its cleavage, thereby reducing the level of caspase-3 activation [75-77].

Additionally, αB-crystallin may facilitate protein ubiquitination and proteasomal degradation. For example, it is reported to promote ubiquitination and degradation of cyclin D1 by interacting with F-box protein 4 (FBX4) [78, 79].

αB-crystallin in cancer

Consistent with a pro-survival function, αB-crystallin is expressed in several types of cancer, including prostate cancer, oral squamous cell carcinomas, renal cell carcinoma [69], glioma [80], and retinoblastoma [81]. Overexpression of αB-crystallin correlates with enhanced tumor growth and increased cell migration in clear cell renal cell carcinoma [82]. αB-crystallin is also detected in highly infiltrative glioma cells where it plays a role in apoptosis resistance [83]. Notably, αB-crystallin is characterized as a novel marker for triple-negative and basal breast cancers [84], and has been shown to act as an oncoprotein, its expression correlating with poor prognosis [85-87].

αB-crystallin in inflammation

αB-crystallin has been described as an anti-inflammatory factor in neuroinflammatory disease, particularly in experimental autoimmune encephalitis (EAE), a mouse model for multiple sclerosis (MS) [88]. The expression of αB-crystallin is strongly up-regulated during MS and has been demonstrated to trigger innate response [89] and to be an autoantibody target, as well as a major target of the CD4+ T-cells that are attacking the myelin sheath during MS [90]. Interestingly, Th1 and Th17 cell responses are accentuated in αB-crystallin-deficient mice with EAE, associated with aggravated disease. This is connected to increased activation of NF-κB and MAPK p38 in T cells, macrophages, dendritic cells and glial cells [88]. Notably, administration of
recombinant αB-crystallin in different mouse strains with induced EAE proved to be a beneficial therapeutic strategy, leading to reduced infiltration of immune cells and decreased inflammatory cytokine production [88].

We demonstrated that αB-crystallin promotes leukocyte infiltration into tumors and ischemic myocardium. Importantly, we found that αB-crystallin is prominently expressed in CD11b+Gr1+ immature myeloid cells (IMCs), and regulates IMC expansion during F9 tumor development and chronic liver inflammation. IMCs have previously been associated with pro-angiogenic activities and suppression of cytotoxic T cells [91]. Our data suggest that αB-crystallin may regulate inflammatory response in many pathological conditions by limiting expansion of IMCs (Paper I).

**αB-crystallin in regulating tumor angiogenesis**

αB-crystallin is expressed in various types of endothelial cells in culture, such as human dermal microvascular endothelial cells (HDMECs), bovine capillary endothelial (BCE) cells and murine myocardial endothelial (MyEnd) cells. The expression is up-regulated by pro-angiogenic factors FGF and VEGF [92]. Consistent with this, αB-crystallin levels are elevated in endothelial cells in several different types of tumors, including clear cell lung cancer, renal cancer and multiple myeloma [92, 93]. αB-crystallin promotes tumor angiogenesis via both endothelial cell intrinsic and extrinsic pathways. Intrinsically, αB-crystallin reduces caspase-3 dependent apoptosis and increases endothelial cell survival. Additionally, αB-crystallin probably helps with rearrangement of neovascular cell cytoskeletal morphogenesis. Extrinsically, αB-crystallin is reported to be secreted by injured vessels and to be present in plasma as a regulator of platelet function [94]. Platelets are a source of pro-angiogenic and anti-angiogenic molecules during tumor progression. Although the role of αB-crystallin in regulating platelet function is not fully investigated, it is likely that αB-crystallin modulates tumor angiogenesis at least partially through regulating platelet activation. Furthermore, αB-crystallin strongly interacts with nerve growth factor (NGF), FGF, VEGF and insulin in chaperone domains, suggesting a protective role for pro-angiogenic factors [95]. Indeed, Kase et al identified αB-crystallin as a VEGF chaperone. Under hypoxia stress, Ser59 phosphorylated αB-crystallin co-localizes with VEGF in the endoplasmic reticulum, assisting the refolding of misfolded VEGF [96, 97].

**ELTD1**

ELTD1 is an orphan GPCR, and belongs to the adhesion GPCR family. Structurally, ELTD1 is composed of a long extracellular domain with epidermal growth factor-like repeats, a seven-transmembrane domain and a short intracellular domain. It is first identified and described in 2000 by Nechiporuk et al in a cardiac developmental study. ELTD1 is found to be
expressed in myocytes and smooth muscles during cardiac muscle maturation [98].

Through analysis of glioma tissue microarray staining, we found that ELTD1 was highly expressed in glioma vessels, and the expression was associated with tumor grade. Correspondingly, several independent studies reported that ELTD1 is enriched in tumor vessels. Towner et al demonstrated a strong vascular staining pattern of ELTD1 in human glioma [99]. Consistent with a predominant staining in the glioblastoma vessels, and no or minimal expression in tumor cells, Wallgard and co-authors demonstrated that ELTD1 is highly expressed in the brain microvasculature as compared with the rest of the brain tissue [100]. Masiero et al found that ELTD1 is highly expressed in vessels in other type of tumors, such as head and neck squamous cell carcinomas, breast cancers and clear cell renal carcinomas [101].

We identified that ELTD1 mRNA and protein are highly expressed in high grade glioblastoma vessels compared to vessels in low grade glioma or normal brains [64]. Glioblastoma vessels are highly abnormal and characterized by enlarged diameters and thick basement membranes. The multiple layered structure of the pleomorphic microvasculature known as ‘Glomeruloid tufts’ consisting of endothelial cells, pericytes and basement membranes is a unique feature of glioblastoma vasculature and is one of the diagnostic criteria for grade IV glioma [102]. Yet the formation and functional consequences of the abnormal structures of vessels are largely unknown.

**General functions**

The function of ELTD1 has not being investigated. Actually, the function of the adhesion GPCR family is not well studied and most of the members are orphan proteins with ligand not being found yet. Adhesion GPCR is a family of GPCR with long extracellular N-terminus consisting of similar functional domains such as EGF-like domain, lectin-like domain, immunoglobulin and cadherins. These domains are usually found in proteins functioning in regulating cell-cell adhesions. The name ‘adhesion GPCR’ indicates potential functions of this family. Indeed, a lot of the adhesion GPCR members are found to bind cellular matrix and regulate cell migration and leukocyte adhesion [103-105]. Interestingly, several of the members are found regulating angiogenesis. For example, CD97 is found to be expressed on leukocytes and stimulates angiogenesis by binding to integrin on endothelial cells [106]. GPR124 is highly expressed in central nervous system (CNS) endothelium, and is essential for regulating CNS angiogenesis [107-110]. However, little is known about the function of ELTD1.

**ELTD1 in tumor angiogenesis**

Recently, Masiero et al detected that the expression of ELTD1 was increased in tumor vessels in renal cell carcinoma, head and neck cancer and ovarian
cancer. High expression of ELTD1 was associated with better patient survival. Silencing ELTD1 in an ovarian cancer and a colorectal cancer model resulted in decreased tumor growth, accompanied with reduced vessel density, increased tumor hypoxia, apoptosis of endothelial cells and increased pericyte coverage [101]. Contrast to these results, using a different gene silencing method, we found increased tumor growth in GL261 glioblastoma and T24I fibrosarcoma tumors in ELTD1-/- mice compared to wild type. This was not associated with a change in vascular density in both tumor models. More detailed results and discussion are written in manuscript Paper III. Clearly, further studies are needed to demonstrate the mechanism of ELTD1 in regulating tumor growth and whether it plays a role in tumor angiogenesis.

Cancer-related inflammation

Inflammation is a wound healing process. The purpose of inflammation is to remove infection or irritation. A tumor resembles a wound that never heals [111]. Sustained inflammation is a characteristic of the tumor microenvironment [1]. Tumor-associated inflammation has been demonstrated to be important for various processes during tumor progression including induction of oncogene activation, initiation of tumor growth, promotion of angiogenesis and metastatic dissemination [112]. However, it is so far not clear to what extent inflammation correlates to tumor initiation. Possibly, inflammatory mediators and metabolites i.e. reactive oxygen species cause genetic cell instability and induce tumorigenesis. More importantly, tissue damage creates a microenvironment that supports neoplastic cell proliferation and progression. At the inflammation site, it is rich of recruited immune cells and pro-inflammatory cytokines and growth factors such as tumor necrosis factor α (TNF-α), VEGF and matrix metalloproteinases (MMPs), which contribute to tumor growth.

Inflammatory cells in tumor

Cytotoxic T cells

CD8+ cytotoxic T cells are thought to be the major players in tumor immunosurveillance. A high number of infiltrated cytotoxic T cells or a high ratio to CD4+ T cells is associated with good prognosis in many types of cancer [113-116]. However, the immune system fails to eliminate tumor cells. Tumors develop several ways to evade the immune system. For example, tumors down regulate the expression of major histocompatibility complex (MHC) class I [117] or tumor antigens that are recognized by T cells [118]. T-cell activation is inhibited due to the immunosuppressive tumor microen-
vironment mediated by cells such as T regulatory cells, macrophages, and myeloid derived suppressor cells [119, 120]. Current immunotherapy under investigation including vaccine strategies and adoptive cell transfer (dendritic cell transfer and T-cell transfer) are aiming to deliver activated cytotoxic T cells with better tumor-killing capabilities. Furthermore, cytotoxic T-cell recruitment to the tumor site by endothelial cells is inefficient. Inhibited endothelial activation leads to down-regulated expression of chemoattractants and adhesion molecules [121]. We have observed that decreased T-cell infiltration was associated with inhibited endothelial activation and down-regulated chemokine expression CXCL10 and CXCL11. Moreover, sunitinib targeting VEGF/VEGFR2 pathway treatment enhanced T-cell infiltration into the inner mass of the tumors (Paper IV). Combining strategies of endothelial activation with immune cell transfer therapy may enhance immune cell delivery efficacy and give better therapeutic outcome.

T regulatory cells
T regulatory (T-reg) cells are a population of T cells that suppress immune response and mediate immune tolerance. T-reg cells are lacking unique recognizing markers. Nevertheless, if not particularly mentioned, T-reg cells are considered a population of CD4+CD25+FOXP3+ cells. While most of the studies reported that a higher T-reg cell number in tumor is associated with worse prognosis [122-124], one report showed that a higher density of T-reg cells correlated with improved prognosis in colorectal cancer [125]. T-reg cells support tumor growth mainly through inhibition of effector T cells, dendritic cells and induction of TAM polarization towards a tumor-promoting phenotype.

Macrophages
Macrophages in the tumor, usually termed tumor-associated macrophages (TAMs), constitute a large population of stromal cells of the tumor microenvironment and play an active role in both tumor initiation and all stages of tumor progression. They are either originally resident in the tissue or differentiated from bone marrow derived mononuclear cells. Macrophage is a very heterogeneous cell type. Due to the high plasticity induced by different stimuli, macrophages in tumors can be both pro-tumoral and anti-tumoral. However, it has been suggested by many studies that the pro-tumoral populations of TAMs are dominant in many types of cancer, as evidenced by several features of the TAMs. First, normal macrophage function, phagocytosis and antigen presenting, is lost in TAMs. Second, TAMs are immunosuppressive by inhibiting T-cell activation. Third, TAMs support tumor progression by releasing cytokines and growth factors to promote tumor angiogenesis and invasion [126, 127]. Giving these pro-tumoral features, therapies targeting TAMs have been investigated. For example, targeting STAT3 signaling in TAMs in pre-clinical models has shown benefits in reducing tumor devel-
opment [128, 129]. However, difficulties are obvious in strategies targeting TAMs as it is hard to distinguish subpopulations due to lack of specific markers.

**Myeloid-derived suppressor cells (MDSCs)**

Myeloid-derived suppressor cells are a mixture of IMCs. In mice, they are identified by two surface markers CD11b and Gr1. However, CD11b is also expressed by neutrophils and commercial Gr1 antibodies recognize Ly6C and Ly6G which are expressed by other myeloid cells. MDSCs are found rich in tumors but not in healthy tissues. The accumulation of MDSCs is probably a result of myeloid proliferation and differentiation that is mediated by cytokines including colony stimulating factor (CSF), Interleukin 6 (IL-6) and Bv8. In Paper I, we found another mediator αB-crystallin also plays a role in regulating myeloid cell expansion. Functionally, MDSCs promote tumor angiogenesis by secreting VEGF and MMPs, and suppress immune responses by production of arginase-1, reactive oxygen species, nitric oxide and other immune suppressive cytokines [130]. Strategies of inhibiting MDSCs which are under pre-clinical and clinical investigations include inhibiting MDSC activation, differentiation of MDSCs into mature cells, blocking development of MDSCs and depletion of MDSCs [120, 131]

Molecular regulation of inflammation

**Endothelial activation**

In non-inflamed tissue, endothelial cells are in quiescent state. Endothelial cells are connected through tight junctions, allowing a stable blood flow. In response to extracellular stimuli, for example pro-inflammatory cytokine TNF-α and toll-like receptor ligand lipopolysaccharide (LPS), endothelial cells are activated and mediate vascular permeability and leukocyte recruitment cascades. Two types of endothelial activation have been described. Type I activation is a rapid response without transduction of new gene expression, and is mediated by GPCR signaling. Upon activation, P-selectin is released to the cell surface via exocytosis of Weibel-Palade bodies (WPBs) [132]. P-selectin is capable of capturing leukocytes and activating signaling to integrins that mediate leukocyte diapedesis. Also, type I activation increases the synthesis of nitric oxide, which relax smooth muscle and increase local blood flow.

Type II activation is a delayed but sustained response accompanied by de novo gene expression in endothelial cells [133]. After cytokines such as TNF-α and IL-1 bind to their receptor TNFR1 and IL1-R1 respectively, the inhibitor of κB kinase (IKK) complex is activated and phosphorylates inhibitor of κB (IκB), which is subsequently tagged for ubiquitination and degradation by the proteasome. IκB degradation releases the NF-κB subunits p50
and p65 (also known as RelA) allowing them to translocate to the nucleus and to induce the transcription of a set of genes including chemokines and adhesion molecules such as E-selectin, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). Both chemokines and adhesion molecules are important mediators of the leukocyte recruitment process. This pathway is called the canonical NF-κB pathway [Figure 3]. However, other cytokines and pro-inflammatory stimuli (i.e. CD40 ligand) also stimulate a similar pathway through activating the homologous subunits of NF-κB, namely p52, RelB and c-Rel (the p52 and RelB complex are also named the NF-κB2 complex). This pathway is called the non-canonical NF-κB pathway.

![Figure 3. Type II activation of the endothelial cells and the canonical NF-κB pathway.](image)

**Leukocyte recruitment**

The leukocyte recruitment cascade has been intensely investigated and is very well understood [134]. However, new mediators and additional mechanisms are continuously being discovered. Leukocytes are recruited through several steps; first capture by the endothelium, then slow rolling, crawling and formation of firm adhesion and in the end transmigration (diapedesis). Each step is mediated by interactions between molecules expressed on endothelial cells and leukocytes [Figure 4].

**Leukocyte capture**

Leukocyte rolling is mediated by selectins, E-selectin, P-selectin and L-selectin. While L-selectin is expressed on leukocytes, E-selectin and P-selectin are expressed on activated endothelial cells. P-selectin is also ex-
pressed on activated platelets. Selectins bind to glycosylated ligands on the leukocytes, most notably P-selectin glycoprotein ligand 1 (PSGL1). Although PSGL1 was first discovered as a ligand for P-selectin, it can interact with all the selectins. E-selectin ligand 1 (ESL1) and CD44 are ligands for E-selectin. The interaction between selectins and their ligands forms and dissolves easily. Therefore selectins capture leukocytes at the site of inflammation but still allow the leukocytes to roll over the surface of the endothelium under shear stress. Interestingly, P-selectin and L-selectin mediated interactions are actually induced by shear stress. Leukocytes detach from the endothelium when the shear stress disappears. Binding of PSGL1 to L-selectin on leukocytes and P-selectins on platelets initiates the secondary leukocyte adhesion. This process acts as a negative regulator inhibiting the expression of E-selectin thereby stopping the leukocyte accumulation. Binding of leukocytes to E-selectin induces integrin activation. Integrins are ligands for adhesion molecules and contribute to the slow rolling of leukocytes on endothelial cells.

**Leukocyte rolling**
Activated endothelial cells express adhesion molecules that can bind to activated integrins on the leukocytes with relatively low affinity. Interaction counterparts that contribute to the slow rolling step include very late antigen 4 (VLA4, also named $\alpha_4\beta_1$-integrin) and VCAM-1, $\alpha_4\beta_7$-integrin and mucosal vascular addressing cell-adhesion molecule 1 (MADCAM1), and lymphocyte function-associated antigen 1 (LFA1) and ICAM-1. There is evidence that shear stress affects the structural conformation of LFA1, thereby influencing the binding affinity to adhesion molecules.

**Leukocyte firm adhesion**
Firm adhesion is dependent on leukocyte GPCR activation triggered by chemokines. Chemokines bind to GPCR with high affinity and induce GPCR signaling within milliseconds. Chemokines induce leukocyte expression of integrins that have a high affinity towards adhesion molecules expressed on endothelial cells. In addition, some loose interactions including LFA1 and ICAM-1 as well as VLA4 and VCAM-1 are strengthened due to a conformational change of the integrins. Notably, integrins expressed in different types of leukocytes are distinct from each other, leading to recruitment of the specific cell type.

**Transendothelial migration**
Once firmly adhered to the endothelial cells, leukocytes start to transmigrate from the luminal side of the vessel to the site of inflammation. Transmigration begins with leukocytes slowly crawling and searching for the endothelial junctions. This process involves ligation of ICAM-1 on endothelial cells and macrophage antigen 1 (MAC1) on leukocytes, which activate pathways
leading to endothelial cell contraction and hence opening junctions between adjacent endothelial cells. Leukocyte migration through junctions is mediated by the adhesion molecules platelet endothelial cell adhesion molecule PECAM1 (also known as CD31), ICAM-2, and junction molecules such as junctional adhesion molecule A (JAM-A), as well as molecules such as CD99, endothelial cell-selective adhesion molecule (ESAM), and vascular endothelial (VE)-cadherin. The majority of leukocytes transmigrate through the paracellular route. However, leukocytes can also transmigrate through the transcellular route, passing through the endothelial cells via vesiculo-vacuolar organelles (VVOs), which are transcellular pores created by fusion of cytoplasmic vesicles and vacuoles.

Figure 4. Leukocyte recruitment cascade and molecules involved in the process.

Interactions between endothelial cells and immune system in tumor

Endothelial cells and immune cells are closely interacting with each other in tumors. For instance, endothelial cells play a pivotal role in recruiting immune cells. Endothelial cells release cytokines and chemokines and express surface adhesion molecules for recruitment of specific subsets of leukocytes. Details of leukocyte recruitment cascade have been discussed above. Besides, endothelial cells have been reported as antigen-presenting cells [135]. Activated endothelial cells express MHC class II, and co-stimulatory proteins such as CD80 and CD86, thereby inducing antigen specific T-cell responses [136]. In turn, T cells release cytokines i.e. TNF-α which modulate endothelial cell antigen presenting as well as other functions such as leukocyte recruiting [137, 138].

Endothelial cell anergy in tumors

Tumor-induced endothelial cell anergy is a phenomenon proposed by Arjan W. Griffioen that angiogenesis factors including VEGF and FGF down regulate adhesion molecules on endothelial cells in tumors, and render endothelial cells less responsive to inflammatory signals [139]. It has been shown that
VEGF and FGF down regulate TNF-α-induced expression of ICAM-1, VCAM-1 and E-selectin on tumor endothelium [121, 140, 141]. The inhibited endothelial activation results in reduced leukocyte infiltration in tumors [142, 143]. Anti-angiogenic treatments overcome the endothelial anergy, and promote leukocyte infiltration into tumors [139, 144]. In Paper IV, we show that VEGF inhibits TNF-α-induced chemokine production, particularly CXCL10 and CXCL11, and sunitinib treatment targeting VEGF signaling enhances T-lymphocyte infiltration into B16 melanomas.

Combination of anti-angiogenic therapy and immunotherapy
Adoptive cell-based immunotherapy has emerged as a promising cancer cell therapy especially for melanoma patients [145-147]. However, one obstacle of adoptive cell therapy is the low efficacy. The dense and tortuous tumor vessels hamper T-cell infusion into tumor. Over-expressed pro-angiogenic factor VEGF within the tumor induces endothelial cell anergy and limits T-cell infiltration. Moreover, VEGF inhibits T-cell and dendritic cell development and restricts cytotoxic T-cell function [148-151]. Interestingly, anti-angiogenic treatment improves the efficacy of immunotherapy in pre-clinical tumor models. Antibodies against VEGF or VEGFR2 enhance T-lymphocyte infiltration into B16 tumors and improve effectiveness of adoptive T-cell therapy [152]. Sunitinib treatment combined with vaccine-based immunotherapy enhances CD8+ T-cell response, and reduces the recruitment of MDSCs and T-reg cells in the tumor microenvironment and reduces tumor growth [153]. Giving the success in pre-clinical models, anti-angiogenic therapy in combination with adoptive cell therapy may offer another promising therapy for the treatment of cancer.
Present investigations

Paper I

αB-crystallin regulates expansion of CD11b+Gr1+ immature myeloid cells during tumor progression.

**Background and aim**

αB-crystallin is a molecular chaperone considered to be potential cancer target due to its expression in tumor cells and its correlation to poor survival in breast cancer and head and neck cancer. Previously, we showed that αB-crystallin is expressed in endothelial cells and promotes tumor angiogenesis by increasing the survival of endothelial cells. A later study demonstrated that αB-crystallin can enhance vessel formation by stabilizing the angiogenic factor vascular endothelial growth factor A (VEGF-A). In this paper, we analyzed the role of αB-crystallin during tumor-associated inflammation.

**Results**

We found a systemic expansion of CD11b+Gr1+ cells in αB-crystallin deficient F9 tumor-bearing mice. Immature myeloid CD11b+Gr1+ cells were found to be increased in the tumor, spleen, and bone marrow, while CD4+, CD8+ T-lymphocytes, B220+ B cells, or CD68+ macrophages were similar in αB-crystallin deficient mice as compared to wild type. We also found increased expansion of CD11b+Gr1+ cells in two other chronic inflammation models, chronic myocardial ischemia and diethylnitrosamine (DEN)-induced liver damage in αB-crystallin deficient mice. CD11b+Gr1+ cells typically represent a mixture of immature cells of the granulocytic and monocytic lineages. Both granulocytic lineage CD11b+Ly6G+ cells and monocytic lineage CD11b+Ly6C+ cells were increased in spleen of αB-crystallin-/- F9 tumor-bearing mice. The expansion of CD11b+Gr1+ cells in αB-crystallin deficient mice was only detected under pathological conditions, no difference was found in naïve mice.

The expansion of CD11b+Gr1+ cells was likely not due to an increased secretion of cytokines that regulate proliferation or recruitment of these cells as there was no difference of intratumor expression of VEGF-A, interleukin 6 (IL-6), granulocyte macrophage colony-stimulating factor (GM-CSF) or CXCL12. Instead, we found that αB-crystallin was robustly expressed specifically in CD11b+Gr1+ cells (but lowly expressed in CD4+, CD8+,
B220+, bone marrow-derived granulocytic cells), and that all trans retinoic acid (ATRA)-induced CD11b+Gr1+ cell differentiation was altered in cells from αB-crystallin/-/ mice. CD11b+Gr1+ differentiated granulocytic Ly6G+ cells were reduced while monocytic Ly6G-Ly6C+ cells were increased. This suggests that αB-crystallin plays a cell-intrinsic role in regulating the expansion of CD11b+Gr1+ cells by altering cell differentiation.

CD11b+Gr1+ cells often referred to as myeloid-derived suppressor cells (MDSCs), have gained considerable interest in the tumor biology field because they have been shown to be immune suppressive by inhibiting cytotoxic T-cell function and to support tumor angiogenesis by secreting tumor angiogenic factors. The CD11b+Gr1+ cells we detected here express arginase-1 and two subunits of NADPH oxidase 2 (NOX2) p91 and p47, which are two molecules that suppress T-cells, and VEGF-A and MMP9 which are two growth factors that promote tumor angiogenesis.

The expansion of CD11b+Gr1+ cells in αB-crystallin/-/ mice under pathological conditions clearly questions the feasibility of using αB-crystallin as a therapeutic target. While targeting of αB-crystallin may inhibit tumor cell survival and potentially reduce tumor angiogenesis, loss of αB-crystallin may also increase the number of CD11b+Gr1+ immature myeloid cells and therefore suppress T-cell immune response and promote expression of pro-angiogenic growth factors.

Paper II

αB-crystallin/HspB5 regulates endothelial-leukocyte interactions by enhancing NF-κB-induced up-regulation of adhesion molecules ICAM-1, VCAM-1 and E-selectin.

**Background and aim**

We have previously shown that αB-crystallin is highly expressed in tumor vessels and regulates tumor angiogenesis by increasing the survival of endothelial cells. However, the role of αB-crystallin in affecting endothelial cell biology and vascular function was not investigated. In this paper, we investigated the role of αB-crystallin in regulating endothelial activation and leukocyte recruitment.

**Results**

αB-crystallin is expressed in several types of endothelial cells, but not in human umbilical vein endothelial cells (HUVECs). Ectopic expression of αB-crystallin in HUVECs was associated with an increase in tumor necrosis factor (TNF-α) -induced surface and mRNA expression of E-selectin. Consistent with this, E-selectin, intercellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule (VCAM-1) mRNA expression was reduced
after TNF-α stimulation in endothelial cells isolated from αB-crystallin deficient mice. NF-κB is a known transcription factor for inducing expression of adhesion molecules E-selectin, ICAM-1 and VCAM-1. The reduced expression of adhesion molecules in αB-crystallin-/- endothelial cells was associated with reduced degradation of IκB, leading to decreased NF-κB activation. In line with the increased expression of adhesion molecules, TNF-α-induced Jurkat cell adhesion to αB-crystallin-expressing HUVECs was enhanced. Moreover, by intravital microscopy, we found that leukocyte rolling velocity and rolling influx were increased in venules in cremaster muscles of αB-crystallin-/- mice after TNF-α injection. This result is compatible with the reduced adhesion molecule expression in the cremaster muscles.

Taken together, our data suggest a novel role of αB-crystallin in endothelial biology in positively regulating endothelial activation and leukocyte recruitment.

Paper III
ELTD1 is dispensable for vascular development and tumor angiogenesis.

Background and aim
ELTD1 (EGF, latrophilin and seven transmembrane domain containing 1) is an orphan G-protein coupled receptor (GPCR) that belongs to the adhesion GPCR family. Previously, we found that ELTD1 is highly expressed in high-grade glioma vessels compared to vessels in low-grade glioma and control brains. Subsequently, several independent studies identified ELTD1 as a new biomarker for glioma due to its high expression levels in brain tumor vasculature. Additionally, ELTD1 was also found highly expressed in other types of tumors, such as head and neck squamous cell carcinomas, breast cancers and clear cell renal cell carcinomas. Taken together, this suggests that ELTD1 may play a role in tumor angiogenesis, and could serve as a new target for glioblastoma treatment. In this paper, we investigated the role of ELTD1 in angiogenesis during tumor progression.

Results
The role of ELTD in vascular development was investigated in ELTD1-/- mice. The ELTD1-/- mice are viable; have no behavior defects and display normal vascular phenotype. The vascular development in retina, lung, kidney and liver was similar to wild-type mice. Consistent with ELTD1 being dispensable for vascular development, siRNA-mediated knockdown of ELTD1 in endothelial cells did not affect the ability of endothelial cells to form tubes. Endothelial activation and leukocyte adhesion was also not affected by the lack of ELTD1.
ELTD1 is expressed in gliomas of various grades. We analyzed the expression of ELTD1 by immunohistochemistry of human tissue microarrays, and found that ELTD1 was significantly highly expressed in high-grade glioma vessels. However, the level of ELTD1 expression in tumor vessels did not correlate to survival of patients in either high-grade or low-grade gliomas.

To investigate the role of ELTD1 in tumor angiogenesis, we inoculated GL261 cells intracranially in wild-type and ELTD1-/- mice. GL261 is a brain tumor model that displays a similar vascular phenotype as human grade IV gliomas. GL261 tumor growth and tumor incidences were increased in ELTD1-/- mice. This is consistent with decreased survival time after tumor inoculation in ELTD1-/- mice. However, no difference was found regarding the phenotype of tumor vessels. Vascular density, vascular morphology, pericyte coverage and vascular permeability were similar in GL261 tumors grown in ELTD-/- mice and tumors grown in wild-type mice. Also, we did not detect any difference in CD3+ T-cell infiltration. Further studies will focus on elucidating the mechanism of ELTD1 in regulating reduced tumor growth.

To investigate the role of ELTD1 in other types of tumors, we studied if ELTD1 deficiency affected T241 fibrosarcoma, MB49 bladder cancer and B16 melanoma tumor progression. Similar to the GL261 model, we found that T241 tumor growth was increased in ELTD1-/- mice compared to wild type. However, MB49 and B16 tumor growth was similar in ELTD1-/- and wild-type mice. This suggests that stromal expression of ELTD1 may play a different role in regulating tumor growth in different tumor models. In contrast to the apparent lack of effects of ELTD1 deficiency on the tumor vasculature in GL261 tumors, we found a reduction in vascular leakage and decreased pericyte coverage in T241 tumors in ELTD1-/- mice. In addition, we found that CD3+ T-cell infiltration was decreased in T241 tumors which may at least partially explain the enhanced tumor growth in these mice.

Taken together, we show that ELTD1 is dispensable for vascular development and tumor angiogenesis. Although ELTD1 is highly expressed in high-grade glioma vessels, it does not correlate to patient survival. Knockdown of ELTD1 increases GL261 and T241 tumor progression, and has no effect on the growth of B16 or MB49 tumors. Our data is, however, in stark contrast with a recent study where Masiero et al showed that a high level of ELTD1 was correlated to better survival in head and neck, renal and colorectal cancer patients. By injecting siRNA in vivo, they found that knockdown of ELTD1 resulted in reduced tumor growth and better survival in mice bearing ovarian carcinoma. They also showed that silencing of ELTD1 impaired vascular development in zebrafish. The apparently contradictory results that we obtained may be due to the use of different tumor models and a different approach to decrease ELTD1 in tumor vessels. Further studies are required to pinpoint the role of ELTD1 in endothelial biology and to deter-
mine how ELTD1 regulates tumor progression. Nevertheless, we conclude that ELTD1 is dispensable for vascular development. Targeting of ELTD1 may result in faster tumor growth and may therefore not be a suitable strategy for glioblastoma treatment.

**Future plans**

ELTD1 deficiency correlates to increased GL261 and T241 tumor growth but does not affect vascular density or vascular morphology in both models. One question that needs to be addressed is how ELTD1 regulates tumor growth. Endothelial cells are not only key players in tumor angiogenesis, but also actively involved in tumor inflammation. Next step is to investigate whether ELTD1 interfere with tumor associated inflammation. Future studies will focus on infiltrating immune cells such as tumor-associated macrophages (TAMs) and T-regulatory (T-reg) cells, and investigate whether lack of ELTD1 influences their recruitment and phenotypic change in the tumor microenvironment.

To explore the general functions of ELTD1, another important subject that requires extensive study is to identify the ligand and interacting partners. The endothelial intracellular interacting partners may reveal downstream signaling pathways of ELTD1. To determine the interacting proteins, we can use technologies such as co-immunoprecipitation pull-down assays, and analyze the protein fragments by mass spectroscopy. However, the interacting proteins may reside in endothelial cells or other types of cells. Therefore, screening a protein pool which contains proteins not only expressed in endothelial cells would give more insights in biological roles of ELTD1.

**Paper IV**

**VEGF suppresses T-lymphocyte infiltration in the tumor microenvironment through inhibition of NF-κB-induced endothelial activation.**

**Background and aim**

VEGF is a prominent growth factor that promotes tumor angiogenesis. High level of VEGF induces abnormal vessels in tumor and correlates to poor survival. Anti-angiogenic treatment targeting VEGF signaling is in clinical use for various types of cancer. However its effect on endothelial activation and immune cell infiltration is not well studied. In this paper, we investigated the effect of VEGF on TNF-α-induced endothelial activation and the effect of the tyrosine kinase inhibitor sunitinib on the tumor microenvironment.
Results

Using a microfluidic device, we found that VEGF reduced TNF-α-induced Jurkat cell adhesion on human dermal microvascular endothelial cells (HDMECs). By employing affymetrix microarray and principle component analysis, we found global effects of VEGF on TNF-α-induced inflammatory genes in HDMECs. We identified 86 genes which were significantly up-regulated or down-regulated by TNF-α and that were repressed by co-treatment with VEGF. Among these genes, we confirmed CXCL10 and CXCL11, which are two chemokines that are involved in regulating T-cell trafficking. Interestingly, the global effect of VEGF on TNF-α-induced gene expression was restored by treatment with the small molecule kinase inhibitor sunitinib.

We further investigated through which signaling pathway VEGF represses TNF-α-induced gene expression and Jurkat cell adhesion. We found that the TNF-α-induced NF-κB pathway was inhibited by co-treatment with VEGF. VEGF down regulated the phosphorylation of inhibitor of κB kinase (IKK) complex and inhibited IκB degradation. Moreover, VEGF stimulation down regulated TNF-α-induced interferon regulatory factor-1 (IRF1), which is a transcription factor regulating expression of CXCL10 and CXCL11. VEGF treatment also decreased the level of tyrosine 701 phosphorylated STAT1 (p-STAT1) and up regulated the level of tyrosine 705 phosphorylated STAT3 (p-STAT3). P-STAT1 is an inducer for CXCL10 and CXCL11 expression, while p-STAT3 counteracts p-STAT1 and inhibits the induction. Additionally, VEGF enhanced TNF-α-induced phosphorylation of p38. The p38 inhibitor SB203580 abolished VEGF-induced inhibition of IκB degradation.

Consistent with these results, we found that sunitinib treatment enhanced the infiltration of CD3+ T cells into B16 tumors. This was associated with increased CXCL10 and CXCL11 expression in the tumors but not with the changes in adhesion molecule ICAM-1, VCAM-1 or E-selectin expression.

In conclusion, our data show that VEGF suppresses TNF-α-induced endothelial activation and limits T-cell adhesion on endothelial cells. In contrast to previous studies reporting that VEGF suppresses TNF-α-induced endothelial activation by inhibiting ICAM-1 and VCAM-1 expression, we found that VEGF-induced repression was associated with a global down-regulation of inflammatory gene expression, including chemokines CXCL10 and CXCL11. Sunitinib treatment reverses the VEGF-induced repression of endothelial activation in B16 tumors, suggesting that anti-angiogenic therapy may restore endothelial activation and leukocyte recruitment. Further studies are warranted to determine if sunitinib co-treatment would enhance the efficacy of immunotherapy, including adoptive T-cell therapy for cancer patients.
Concluding remarks and future perspectives

It is clear that the tumor microenvironment contributes greatly to tumor initiation and progression. Malignant tumor cells and tumor stromal cells, including vascular cells, infiltrating immune cells and fibroblasts constantly communicate with each other. Understanding the interplay between tumor cells and stromal cells will give insights into tumor targeting therapies. The interplay between endothelial cells, immune cells and tumor cells is indicated below:

In this thesis, we investigate the molecular regulation of the tumor microenvironment, particularly during tumor angiogenesis and inflammation, two important processes that are involved in the interplay between multiple tumor components.

αB-crystallin is a pro-survival protein previously found to be expressed in tumor cells and tumor endothelial cells, and enhances cell survival and tu-
mor angiogenesis. However, αB-crystallin is expressed in other cells, including inflammatory cells. To be able to employ αB-crystallin as a therapeutic target, it is important to understand its role in regulating endothelial cell activation and inflammatory response. We investigated different roles of αB-crystallin in endothelial cells and inflammatory cells. In paper I, we found that αB-crystallin is expressed in CD11b+Gr1+ immature myeloid cells, and negatively regulates expansion of these cells during tumor development through a myeloid-cell intrinsic mechanism. There is a risk that since CD11b+Gr1+ cells have immunosuppressive and tumor promoting properties, targeting αB-crystallin may result in increased tumor growth. Additionally, we found that αB-crystallin positively regulates endothelial activation and leukocyte rolling in inflamed tissue. Inhibition of αB-crystallin may reduce leukocyte recruitment and therefore affect immune response in the tumor. αB-crystallin is expressed in various kinds of cells including tumor cells, endothelial cells and myeloid cells, and plays multiple roles in processes such as tumor angiogenesis, inflammation, and leukocyte recruitment. It is therefore necessary to evaluate which role of αB-crystallin is predominant under different pathological conditions, and whether targeting αB-crystallin will be beneficial.

Glioblastoma is an aggressive type of brain tumor. It is one of the most fatal cancers with a median survival of only one year despite intensive clinical treatments. Due to the high invasiveness, glioblastoma is very hard to target. Avastin has been approved as anti-angiogenic therapy for recurrent glioma, but it does not improve survival of patients as a first-line therapy. The glioblastoma vasculature is morphologically and functionally abnormal, and the mechanisms underlying the formation as well as its contribution to tumor progression are largely unknown. Through laser-capture microdissection of glioblastoma vessels and transcriptional profiling, we identified the G-protein coupled receptor ELTD1 as differentially expressed in glioblastoma vessels. Expression of ELTD1 positively correlated with tumor grade in glioma. In the GL261 glioma mouse model as well as the T241 fibrosarcoma mouse model, tumors grew faster in ELTD1 deficient mice. This suggests a role of ELTD1 in regulating tumor growth. Nevertheless, loss of ELTD1 did not affect vascular density or permeability in GL261 tumor, nor vascular tube formation in vitro. Furthermore, no change in vascular phenotype was observed in ELTD1-/- mice. Taken together, these data indicate that ELTD1 is dispensable for developmental and tumor angiogenesis. It is still not clear how ELTD1 regulates tumor growth. As a role in immune modulation has previously been shown for other adhesion GPCRs, it would be interesting to known whether ELTD1 also has a role in immune modulation in glioblastoma.

Recently, we and Towner et al have found ELTD1 as a potential marker for glioblastoma. A recent publication in Cancer Cell demonstrated that ELTD1 plays a role in tumor angiogenesis suggesting ELTD1 as a candidate
for anti-angiogenic targeting. However, our study challenges this hypothesis, and warrants more investigation using different tumor models. Also, other aspects regarding the function of ELTD1 need to be determined. Several questions remains to be addressed in the future such as what is the ligand for ELTD1, what is the downstream signaling of ELTD1 and what is the potential function of soluble circulating ELTD1?

VEGF is prominently expressed in most solid tumors, and promote tumor angiogenesis. Anti-angiogenic treatment targeting VEGF signaling is widely used in the clinic for treatment of various types of cancer. However, the effect of anti-angiogenic treatment on other components of the tumor microenvironment must be considered. We demonstrated a global effect of VEGF on repression of TNF-α-induced endothelial activation as indicated by inhibition of TNF-α-induced regulation of 86 inflammatory genes in HDMECs. Notably, VEGF inhibited TNF-α-induced regulation of CXCL10 and CXCL11, two chemokines mediating T-lymphocyte infiltration. Consistent with this, TNF-α-induced Jurkat adhesion on endothelial cells was inhibited by VEGF co-treatment in vitro. Correspondingly, sunitinib treatment restored CXCL10 and CXCL11 expression, as well as T-lymphocyte infiltration into the inner mass of B16 tumors. It is interesting to investigate whether a short period treatment of sunitinib or other anti-angiogenic agents may enhance cytotoxic T-cell infiltration and activation in the tumor. Since VEGF has been reported to inhibit T-cell activation, treatment of sunitinib may also enhance activation of cytotoxic T cells. Immunotherapy including adoptive T-cell transfer is emerging as a promising therapy particularly for melanoma patients, but a hurdle to effective T-cell therapy is that only a small fraction of T cells get to the tumor site, and very few remain active. It would be extremely interesting to investigate if vascular normalization by the treatment of anti-angiogenic agents such as sunitinib would improve the efficacy of adoptive T-cell transfer and enhance immunotherapy by facilitating T-cell infiltration and activation in cancer patients.

Even though the VEGF and TNF-α signaling pathways have been well defined, little is known about the crosstalk between these two pathways. We found that VEGF inhibits TNF-α-induced endothelial activation through repression of the NF-κB pathways, by inhibiting IKK phosphorylation and subsequently IκB degradation. This is at least partly due to enhanced p38 phosphorylation. Moreover, VEGF down regulates TNF-α-induced IRF1 expression and interferes with TNF-α-induced STAT1/3 phosphorylation, which regulates CXCL10 and CXCL11 expression. Further studies are needed to elucidate the crosstalk pathways, and pinpoint the proteins that form the bridge between VEGF and TNF-α signaling.
Acknowledgements

The studies were supported by grants from the Swedish Cancer Society, the Swedish Childhood Cancer Society and the Swedish Research Council. My first three year PhD study was financed by LiSUM program (Linking Sino-European Universities through Mobility). I am grateful for the opportunity to carry out my PhD study at the Department of Immunology, Genetics and Pathology (IGP), Rudbeck Laboratory, Uppsala University.

I would like to express my gratitude to everyone who has helped me or being a lovely friend with me during these years at Rudbeck. Special thanks to:

My supervisor Anna Dimberg, thank you for guiding me into scientific research. Without you, I am still a curious child wandering around the lake of science, but will never be one of the participants in pursuing tumor biology. Thanks for all these years support, and fighting together with me for the deadlines. I am also grateful for your kindness during my pregnancy and life afterwards. You have considered many situations for me more than myself can imagine! Maybe you know already, but, we Love you! 😊

My co-supervisor Lena Claesson-Welsh, you are a strict supervisor, but all for the good of the students. Thank you for the discussion and inspiration on my projects, and great support for my half-time and PhD defense.

My second co-supervisor Elise Langenkamp, thank you for being a good supervisor, colleague and friend. You always have the best organized experiment data templates and never hesitate to share them with us. Thank you for the great help on writings and figures. Your figures and power point slides are beautiful just as your dancing and you yourself.

My former colleague Lothar Dieterich, you introduced me to this group, and were supervising me on the bench. Thank you for being so patient to a biology freshman. I enjoyed a lot talking and discussing with you. You are ‘Wikipedia’ to us. Hope we will always keep in touch.

My colleagues Roberta, you are such a lovely girl, thank you for the great help in genotyping during the special days; Maria, you are my best student, and later best friend. It is happy hours when you are in the lab. Luuk, you are a considerate man, thanks for keeping offering help when I have Estella in me. Lei Zhang, thanks for being a relax person, you make the PhD less stressful. Former colleague Sofie, you are such as kind person who cares a lot about people, environment and all nature creatures.
Sara Mangsbo and Angelica Loskog, for the discussion about T-cell therapy.

Johan Kreuger and Peder Fredlund Fuchs, for helping with setting up the microfluidic system.

All the people in vascular biology group, especially Charlotte, Xiujuan, Oriol, Anja, Jeremy, for the help with confocal microscope settings.

Neuro-oncology group, for great Friday seminars and every year wonderful kickoff.

Anna-Karin Olsson and Maria Ringvall group, for nice discussions at Monday seminars.

Peetra’s group, for being a nice lab neighbor.
The SciLife Lab facility for the help with flow cytometry.
IGP administration specially Christina Magnusson.
The former Rudbeck animal facility and BMC animal facility.
My Chinese friends, Di, Chuan, Yuan, Yiwen, Anqi, Jing, Lei, Liqun, Dan, Shujin, Xiang, thank you for the time around the lunch table, and all the help outside science. 谢谢阳光美少女们的陪伴！

My parents, thank you for all these years’ understanding, and thank you Mom for taking care of Estella when I preparing this thesis. You have a great contribution to this thesis. 谢谢爸妈的支持和理解，谢谢妈妈在我准备答辩期间对小甜橙的照顾，你们对我的爱，无以为报。

My dearest Lazzy, you are always there for me, you are a good husband and father. It is hard to express my love by words, 一切尽在不言中，你懂的。我和小甜橙都爱你！

And last but not least, my little baby daughter Estella, thank you for coming to us and being healthy and lovely. Mommy loves you! 小甜橙健康快乐地成长，妈妈爱你！
References


A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)