VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTOR–3
SPECIFIC SINGLE CHAIN ANTIBODIES

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Zürich, 2005
For Stüfi
and my family

Always look on the bright side of life!
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SUMMARY

Vascular endothelial growth factor receptor-3 (VEGFR-3) has been found to play a major role in lymphangiogenesis, which has been implicated in tumor metastasis. The receptor is overexpressed on lymphatics in the vicinity of tumors and even on tumor vasculature itself and is therefore believed to be an effective target for anti-cancer therapy. In order to take a step towards this goal, single chain antibody fragments (scFv) recognizing either human or both human and murine VEGFR-3 were selected by phage display technology. All selected scFvs bind to purified native antigen in enzyme-linked immunosorbent assays and Western blots. Binding of the scFvs to the surface of endothelial cells overexpressing human VEGFR-3 (HEK293T/VEGFR-3) was demonstrated by immunofluorescence. Additionally, the scFv antibodies recognise VEGFR-3 on U937 cells, a human erythroleukemia cell line endogenously expressing the receptor. Pre-incubating the scFvs with purified antigen inhibited their binding to the receptor on lysate samples of cell lines expressing VEGFR-3. Specific binding was shown at scFv concentrations as low as 1 nM. Further characterisation of the scFvs in vitro showed the possible potential of AFC5 to competitively block VEGF-C binding to VEGFR-3, which could inhibit receptor signalling. The scFv AFC5 was able to significantly block both proliferation and migration of human lymphatic endothelial cells in preliminary in vitro experiments. Immunohistochemistry on human neonatal foreskin sections proved the diagnostic potential of the scFv AFC5. The single chain antibody AFC5 is therefore promising both as a diagnostic as well as a therapeutic tool for lymphangiogenesis.

The single chain antibodies were coupled to liposomes to investigate their potential as targeted drug carriers. In vitro results showed specific binding of these immunoliposomes to the receptor on HEK293T/VEGFR-3 cells as seen by immunofluorescence and on U937 cells as seen by flow cytometry. The murine teratocarcinoma cell line F9 was stably transfected with a hVEGFR-3 containing plasmid (F9/VEGFR-3) to create a mouse model to test the specific binding of the scFvs in vivo. The immunoliposomes were loaded with the cytotoxic drugs ETC-NOAC or 5-FdU-NOAC and tested for selective killing of the F9/VEGFR-3 in vitro. Survival rates could only be reduced by 30 % with the specific cytotoxic immunoliposomes in comparison to the control liposomes, because no internalisation of the liposomes could take place. Both, the scFvs alone as well as
immunoliposomes were tested *in vivo* for their biodistribution using the F9/VEGFR-3 mouse model. The specific scFv AFC5 was rapidly eliminated from blood and accumulated in the tumor in a specific manner, resulting in 2.5-3 %ID per organ between 1-4 h after administration. The unspecific scFv A1cys showed similar blood elimination, but tumor accumulation was only 1.7 %ID in the tumor after 30min, with 0.5 %ID remaining after 3 h. The tumor accumulation pattern of A1cys is typical for unspecific organ distribution and elimination. The pattern of scFv AFC5 biodistribution was visualised by SPECT analysis, which convincingly showed the specific accumulation of the antibody in the receptor expressing tumors.

The α-VEGFR-3-AFC5-immunoliposomes were rapidly eliminated from blood and accumulated at high amounts in liver and spleen. This behaviour could be due to a high number of single chain antibody molecules bound to the liposome surface, or more probably, due to the low receptor expression in the mouse model used.

In summary, we have selected and produced highly specific anti-VEGFR-3 single chain antibodies, which recognise both recombinant as well as endogenously expressed VEGFR-3 *in vitro*. The scFv AFC5 can significantly reduce VEGF-C induced migration and proliferation of human lymphatic endothelial cells and specifically recognises the receptor on tissue sections, suggesting that this antibody is a promising diagnostic and therapeutic tool against lymphangiogenesis.
ZUSAMMENFASSUNG


Die anti-VEGFR-3-AFC5-Immunoliposomen wurden sehr schnell aus dem Blut ausgeschieden und akkumulierten in Leber und Milz. Dies könnte darauf zurückzuführen sein, dass eine zu hohe Anzahl von Antikörpern an die Oberfläche der Liposomen gekoppelt wurde, oder weil die Dichte der Rezeptoren auf den Tumorzellen zu gering war.

1 INTRODUCTION

1.1 Cancer: an overview

Cancer is one of the highest causes of death in the industrial world. Every third person is struck by cancer in her or his lifetime and 50-60% die thereof. Cancer has always been thought to be a disease of old age, but it has become increasingly obvious that it is not exclusively so. Because mutations accumulate with age, exposure to environmental carcinogens must be emphasised as a serious threat. There are several reports on the impact of environmental risk factors on the onset of the disease\(^1\text{-}^3\). As our environment is changing and we become more and more exposed to cancer causing agents, it can be concluded that we might face earlier onset of disease in the future.

Most solid cancers, despite some therapeutic progress, still represent a major medical concern. The rate of success with surgery is limited and relapses of tumors due to distant metastases are common. The standard clinical therapies have insufficient efficacy and compromise the patient’s life quality.

At present, cancer treatment mainly consists of a combination of surgery, radiotherapy and chemotherapy. The major limitation of successful chemotherapy is patient tolerability at effective doses. This toxicity is an intrinsic property of these agents, due to the fact that they rather unspecifically target DNA or microtubules. Therapeutic alternatives, which target clearly defined disease-relevant molecules, as well as compounds with better tolerability at effective doses, are slowly emerging. With a better understanding of the molecular bases of the pathophysiology of cancer, one can identify potential targets and aim anti-tumor therapies at the involved signalling pathways\(^4\). Anti-angiogenic and –lymphangiogenic therapies show promising new perspectives in specifically targeting the tumor without adverse side effects\(^5\text{-}^9\). Targeting lymphangiogenesis is described in more detail in 1.2 and 1.3.
1.2 Lymphangiogenesis

1.2.1 Metastatic spread via lymphatics

Lymphatic tumor spread to regional lymph nodes represents the most common pathway for initial tumor dissemination. The detection of lymphatic metastasis serves as an important prognostic indicator of tumor aggressiveness and frequently determines the choice of anti-tumoral therapy. Metastasis can be defined as the spread of cancer cells from the primary site to another organ, which is not directly connected to the primary. After overcoming the first vascular barrier, the cells have to survive in the circulation, attach to microvessels in the target organ, exit from these vessels and gain a foothold in the target tissue. In addition, the micrometastatic cells need to induce angiogenesis in the new target tissue, in order to form macrometastases.

Recent experimental and clinical data indicate that lymphangiogenic factors influence the spread of tumors. There are several reports of correlation between high vascular endothelial growth factor-C (VEGF-C) expression and metastasis via the lymphatics. VEGF-C as well as VEGF-D are ligands for VEGFR-3, which is a receptor shown to be involved in lymphangiogenesis and metastatic spread via the lymphatic system. Karpanen et al. implanted VEGF-C overexpressing MCF-7 breast carcinoma cells orthotopically in the fat pads of SCID mice and found hyperplastic lymphatic vessels in the periphery of these tumors. The same lymphatic vessels were often infiltrated by VEGF-C expressing tumor cells compared to none or only few in the lymphatic vessels of the control tumors (Fig. 1). Skobe et al. showed that tumor lymphangiogenesis, triggered by VEGF-C overexpressing breast cancer cells orthotopically implanted in nude mice, was highly correlated with the extent of lymph node and lung metastases. Recently it was shown in 56 cases of colorectal cancer that the frequency of lymph node metastasis in VEGF-C overexpressing patients (81.1%) was significantly higher than that in the negative group (42.1%) and it was concluded that VEGF-C may be a useful prognostic factor in colorectal carcinoma. It has also been shown that VEGF-D, the second ligand for VEGFR-3, can induce the formation of lymphatics within tumors, and that this leads to the spread of the
tumor to lymph nodes. In parallel, VEGF-D concomitantly promoted tumor angiogenesis and growth, suggesting that VEGF-D is not as specific as VEGF-C\textsuperscript{15}.

Inhibiting lymphangiogenesis is, as for angiogenesis, thought to be an effective therapeutic strategy to inhibit cancer growth because it can act on any tumor type, does not select for resistance of tumor cells and has little effect on normal tissue. Because VEGFR-3 is upregulated on lymphatic vessels in the vicinity of tumors, VEGFR-3 represents a promising target for inhibiting tumor-induced lymphangiogenesis and metastatic spread via the lymphatics\textsuperscript{17}. This upregulated expression leads to the increased proliferation of lymphatic endothelial cells\textsuperscript{18,19}, which most probably promotes the metastatic spread of tumor cells via the lymphatic vessels\textsuperscript{13,15,20,21}.

\textbf{Figure 1:} Schematic representation of a tumor showing the current understanding of the lymphatic system in tumors expressing low levels (left) versus high levels (right) of VEGF-C and –D. Lymphatic vessels are present in both the tumor margin and the peritumor tissue. It seems that VEGF-C/D increase the diameter and number of lymphatic vessels, thus providing increased surface area facilitating lymphatic metastasis\textsuperscript{16}. 

Endothelial cells from remnant lymphatics

Cancer cell
1.2.2 The lymphatic system

The lymphatic vasculature takes up fluid, cells and macromolecules from the interstitial space and recirculates them to the venous system, thus maintaining the interstitial fluid balance and enabling the clearance of macromolecules. It is also essential for immune surveillance and functions as a key route for tumor metastasis in many types of cancer\textsuperscript{22-24}. In 1902 Florence Sabin proposed that the lymphatic vasculature forms by budding of the lymph sacs at the anterior cardinal vein and subsequently the lymphatic vessels sprout. New insights into the field, obtained from lymphatic endothelial markers such as LYVE-1 and Prox1, support this theory\textsuperscript{25}. Lymphatics are composed of a single non-fenestrated endothelial cell layer, similar to blood vessels. Structurally some differences are found: for example, lymphatics generally posses a more irregular and wider lumen than do blood capillaries, have absent or poorly developed basal lamina and are not encircled by pericytes\textsuperscript{26}. The lymphatic system and the involved growth factors are extensively reviewed by Lohela \textit{et al}\textsuperscript{27}. Figure 2 illustrates the formation of the lymphatic system as a schematic representation of the lymphangiogenic process.

1.2.3 Lymphatics as a promising therapeutic target

Lymphangiogenesis is the formation of new lymphatics from pre-existing ones and has been identified as an essential process for growth and metastatic spread of tumor cells. Therefore, it is a promising target for anti-tumor therapy. The main barrier in cancer therapy is that cancer cells acquire drug resistance due to their genetic instability. Aiming therapy at genetically stable endothelial cells in the vicinity of tumors is thought to overcome this barrier. For a tumor to grow beyond the size of a few millimeters the recruitment of new blood (angiogenesis) and lymphatic vessels (lymph-angiogenesis) is required\textsuperscript{28}. This so called angiogenic switch is the required step for the transformation of benign to malignant tumors as shown in figure 3, where the red spots represent the angiogenesis occurring. Metastatic cells are only shed after the tumor has established its microcirculation\textsuperscript{29}. Tumors are angiogenesis-dependent because they need the blood vessels to provide nutrients and depend on lymphatic vessels for the removal of waste products from the interior regions of the neoplasm.
Anti-lymphangiogenic therapies, targeting the lymphatic endothelial cells, are promising for intra-tumoral drug delivery. Lymphangiogenesis-targeting therapy is a new and growing field, because until recently very little was known about the lymphatic system, due to the lack of specific markers for lymph vessels. To date four markers have been identified including vascular endothelial growth factor receptor-3 (VEGFR-3). LYVE-1 is a lymphatic endothelial receptor for the extracellular...
matrix/lymphatic fluid mucopolysaccharide hyaluronan. Prox1 is a homeobox gene product involved in regulating early lymphatic development and podoplanin is a glomerular podocyte membrane mucoprotein. However, recently VEGFR-3 has also been found to be upregulated on blood vasculature in the vicinity of tumors and even on tumor cells themselves (for more detail see section 1.2.5). Best results in lymphatic identification were obtained with co-staining of two or more of these markers, since the expression patterns do not coincide completely.

![Figure 3: The angiogenic switch: The progression from pre-angiogenic dysplasia to invasive carcinoma. The red spots represents blood vessels formed by angiogenesis.](image)

**1.2.4 Growth factors and their receptors**

The lymphatic and blood vascular systems are structurally distinct, but functionally interconnected and act in concert to maintain tissue homeostasis. Newly emerging blood vessels are leaky and need lymphatic growth to avoid tissue edema. Both lymph- and blood-angiogenesis are regulated by numerous growth factors, vascular endothelial cells and matrix proteins released from host stromal cells such as macrophages and mast cells\(^\text{10,32}\). The major regulators of angiogenesis and lymphangiogenesis are PLGF, VEGF-A, VEGF-B, VEGF-C, VEGF-D and VEGF-E which belong to the cysteine-knot protein family. The corresponding receptors VEGFR-1 (Flt-1), VEGFR-2 (Flk1/KDR) and VEGFR-3 (Flt4) are found on the surface of the endothelial cells lining blood and lymphatic vessels. Receptor tyrosine kinases (RTKs) are single-pass transmembrane receptors. VEGFR-1, -2 and -3 belong to the class III receptor kinases of the platelet-derived growth factor (PDGF) receptor subfamily\(^\text{33-35}\). They are characterised by seven extracellular immunoglobulin homology domains (Ig domains) and an intracellular tyrosine kinase domain split by a kinase insert, which is important for substrate recognition\(^\text{35}\). The amino acid
sequence of VEGFR-1 and -2, compared to VEGFR-3, is 30-40% identical in the extracellular domains while 80% similar in the tyrosine kinase domain\textsuperscript{36}. RTKs are essential components of signal transduction pathways that regulate proliferation, differentiation, migration and metabolism. Receptor activation occurs through ligand binding, which facilitates receptor dimerization and autophosphorylation of specific tyrosine residues in the cytoplasmic portion\textsuperscript{37}. VEGFR-2 has been found to be involved in angiogenesis, while VEGF-C and VEGF-D binding to VEGFR-3 induces lymphangiogenesis. In figure 4, signalling via these growth factors and their receptors is illustrated.

**Figure 4**: Several members of the VEGF gene family have been identified, including Placenta Growth Factor (PIGF), and VEGF-A to -E. Although structurally similar, these molecules display specific properties. These growth factors mediate their effect predominantly through 3 tyrosine kinase receptors, VEGFR-1, VEGFR-2 and VEGFR-3\textsuperscript{38}, as well by interaction with ancillary cell surface receptors and extracellular matrix receptors.
Inhibition of angiogenesis has been extensively investigated as a means of inhibiting tumor growth. Both targeting drugs towards the genetically stable endothelial cells lining the blood vessels as well as inhibiting VEGF-A signalling via VEGFR-2 have been investigated. Angiostatin and endostatin are known anti-angiogenesis drugs, specifically inhibiting endothelial cell proliferation, while Bevacizumab is an anti-VEGF antibody blocking the binding of the growth factor to its receptor. While the VEGF-VEGFR-2 pathway and angiogenesis have attracted a great deal of attention, very little is known about VEGFR-3 signaling and lymphangiogenesis. In recent years several researchers investigated the potential of this receptor as a target for anticancer therapy. VEGFR-3 was cloned from human erythroleukemia cells and placental cDNA libraries and the gene maps to region q33 of chromosome 5. The receptor is produced as 4.5- and 5.8-kb transcripts due to alternative splicing. The longer form encodes 65 additional amino acid residues and is the major form detected in tissues. After its biosynthesis, the 195-kDa receptor is proteolytically cleaved in the fifth Ig domain, but the resulting chains remain linked by disulfide bonds. The predicted molecular weight of the processed receptor is 143-kDa, but it migrates at 170-kDa on SDS-PAGE. The explanation for this is the N-linked glycosylation. VEGFR-3 is involved in blood vessel development during embryogenesis, and expression of the receptor starts at embryonic day (E) 8.5 of mouse development in all embryonic endothelial cells initially. Post day E8.5 VEGFR-3 expression is only seen on developing veins and lymphatics and not on arteries. Later on in development, the expression gradually becomes restricted to the lymphatic vessels. VEGFR-3 null mice die at day E9.5 due to cardiovascular failure. Vascular remodelling and maturation are abnormal in the larger vessels with defective lumens, which causes fluid accumulation in the pericardial cavity. Although VEGFR-3 is down-regulated after development, the receptor becomes upregulated in angiogenic blood vessels in breast cancer, in the lymphatic endothelium of metastatic lymph nodes, and in lymphangiomas, vascular skin tumors and Karposi’s sarcoma spindle cells. Interestingly a significant loss of the long but not the short isoform of VEGFR-3 has been observed in tumors compared with normal tissues ($P=0.02$ and $P=0.25$ respectively).
1.2.5 VEGFR-3 expression

As pointed out in chapter 1.2.4, for a long time VEGFR-3 was thought to be exclusively expressed on lymphatics in adults. But recent studies show the expression of the VEGFR-3 receptor on blood vessels in the vicinity of tumors in adults as well as on tumor cells themselves. In 1999, Valtola et al. demonstrated that VEGFR-3 expression is upregulated in the endothelium of angiogenic blood vessels in breast cancer. In the same year, Partanen et al. showed that although VEGFR-3 is specific for lymphatics in normal tissues, it is widely distributed: 82 benign, 31 borderline and 72 malignant vascular tumors were examined immunohistochemically using a monoclonal antibody to VEGFR-3. In normal tissue VEGFR-3 was only expressed on lymphatic endothelium but in both benign and malignant vascular tumors as well as in neovascularization of non-endothelial tumors widespread VEGFR-3 distribution was seen. Witmer et al. found that VEGF-C, VEGF–D and their receptor play a role in cell-to-cell signaling in adult blood vessel angiogenesis, suggesting that VEGFR-3 is recruited in VEGF-A driven angiogenesis. There are also reports on the correlation between VEGFR-3 expression on cancer cells and poorer overall survival. VEGF-C and VEGF–D are proteolytically processed, which regulates their biological activity: e.g. unprocessed VEGF-C binds to VEGFR-3, while the processed form binds to VEGFR-2. At the site of lymphatic sprouting, a paracrine expression pattern is seen for VEGFR-3 and VEGF-C, indicating a role for VEGF-C in the development of lymphatic vessels.

VEGFR-3 was initially expected to be an effective target for anti-cancer therapy since it was thought to be exclusively expressed on lymphatics in adults and only upregulated in the vicinity of tumors. Although it now becomes clear that the receptor is not restricted to lymphatic vessels, it still represents an excellent target, because it is only upregulated on endothelial cells (lymph and blood) in the vicinity of tumors as well as expressed on tumor cells. In addition, Kaushal et al. suggested that lymphangiogenic markers, such as VEGFR-3, are better targets for therapeutic intervention in advanced-stage disease, atleast for prostate cancer, because of the spatial expression pattern of these growth factors and their receptors. This might also be the case for other tumor types.
1.2.6 Peri- / intratumoral lymphatics and lymphangiogenesis

There is dispute about the existence of intratumoral lymphatic vessels. Most researchers claim that there are only peritumoral vessels, which enter the tumor mass to a maximal depth of 100 µm due to the high interstitial pressure. However, Skobe et al. demonstrated increased intratumoral lymphangiogenesis upon VEGF-C overexpression in breast cancer cells, which were orthotopically transplanted onto nude mice. Similarly, Hall et al. investigated 109 untreated patients with papillary thyroid carcinoma and found numerous intratumoral lymphatics. There was a significant association between the presence of intratumoral lymphatics and neck node metastasis. The formation of lymphatics within tumors was also found to be induced by VEGF-D, thus promoting metastatic spread of tumor cells to lymph nodes. However, researchers who support the existence of intratumoral lymphatics, found that these vessels appear to be functionally impaired. One dye uptake study showed that peripheral lymphatic vessels, but not intratumoral lymphatics were functional and the authors proposed that the lymphatic vessels at the tumor margin play a major role in the lymphatic spread of tumor cells. This hypothesis was verified by Padera et al., who showed that in the absence of functional intratumoral lymphatics, metastases were still found in the lymph vessels. This indicates that lymphatics in the tumor margin are sufficient for the metastatic spread to lymph nodes. High peritumoral lymphatic vessel number has been associated with lymph node metastasis in breast carcinomas. There are also differing opinions on whether these peri- or intratumoral lymphatics are remnants of pre-existing vessels or if neolymphangiogenesis occurs. Mandriota et al. showed in their Rip1Tag2 mouse model that increased expression of VEGF-C promotes lymphangiogenesis, which was seen around, but not within the islet architecture or cellular composition in the pancreas, supporting the occurrence of lymphangiogenesis at least in the periphery of tumors. The incidence of lymphangiogenesis has mainly been demonstrated in experimental murine tumors, but lymph vessel proliferation has also been shown in head and neck cancer from 70 untreated patients. The vessels were shown to be proliferating rather than trapped pre-existing lymphatics, because the morphology resembled immature rather than mature lymphatic vessels and because they contained dividing nuclei. Dadras et al. compared tumor samples from primary melanomas, which displayed early lymph node metastasis, with tumor samples of non-metastatic melanomas.
They found hot spots of proliferating intratumoral and peritumoral lymphatics in metastasizing tumors, concluding that tumor lymphangiogenesis is a novel prognostic indicator for the risk of lymph node metastasis in cutaneous melanoma\textsuperscript{61}. Furthermore it has been shown that lymphatic microvessel density and especially lymphovascular invasion are strongly associated with lymph node metastasis in breast cancer\textsuperscript{62}.

1.3 Tumor targeting

1.3.1 Antibodies as tumor targeting agents

Antibodies belong to the most versatile tools used today for characterisation and targeting of molecules in cells and in biological tissues. The development of phage display libraries encoding large repertoires of single chain antibodies, allows the rapid and efficient isolation of specific antibodies for almost any type of molecule\textsuperscript{63,64}. Antibodies have an enormous potential as therapeutic agents, but the specificity is crucial, because many tumor-associated antigens are also expressed on normal cells at lower levels. Molecules specifically expressed on tumor cells and/or stromal cells surrounding the tumor and in tumor-associated blood or lymphatic vessels represent suitable targets for antibody-based cancer therapeutics\textsuperscript{65}. The antibodies may serve to guide chemotherapeutic drugs, toxins, radionuclides or cytokines to tumors and could thus improve the efficacy and reduce the systemic toxicity of such cytotoxic agents\textsuperscript{63}. They can also be used on their own for antibody induced cytotoxicity. Whole antibodies (immunoglobulin G, IgG) can trigger antibody-dependent cellular cytotoxicity, but antibody fragments are favoured over IgG for targeting (discussed in more detail in chapter 3.2). For example, the major advantages of scFv antibodies are their excellent penetration into the tumor tissue, rapid clearance rates and significantly lower accumulation in normal organs\textsuperscript{66-68}.

1.3.2 Therapeutic inhibition of lymphangiogenesis

There are several reports on blocking lymphangiogenesis, either through blocking signalling with antibodies or by competing for ligand with soluble receptors. Some anti-VEGFR-3 antibodies have already been found to inhibit lymphangiogenesis and metastasis to regional lymph nodes\textsuperscript{5,69,70}. For example lymphangiogenesis was
blocked with a rat monoclonal antibody against mVEGFR-3, even in the presence of VEGF-C overexpressing breast carcinoma cells. Control tumors contained lymphatic vessels, while hardly any lymphatics were observed in tumors treated with anti-VEGFR-3 antibody as shown in figure 5. But even more importantly it was found that pre-existing lymphatics were unaffected, both morphologically and functionally even during a prolonged VEGFR-3 blockade.

Figure 5: Mice with orthotopic gastric tumors were either injected with control IgG or with anti-VEGFR-3 antibodies subcutaneously every 3 days between postoperative days 7 and 42. LYVE-1 positive vessels decreased dramatically upon VEGFR-3 inhibition (as reviewed in Takahashi et al.).

Other groups have also demonstrated the inhibition of lymphangiogenesis with anti-VEGFR-3 antibodies. Even fibroblast growth factor-2 induced lymphangiogenesis could be blocked with an anti-VEGFR-3-IgG, suggesting that VEGFR-3 can indirectly mediate lymphangiogenesis induced by other growth factors. More surprisingly, blocking VEGFR-3 with a novel monoclonal antibody suppressed tumor growth by inhibiting neo-angiogenesis of tumor-bearing tissues. This suggests that the presence of VEGFR-3 on vascular endothelial cells of tumor-bearing tissue is due to its involvement in maintaining the integrity of the endothelial lining during angiogenesis. The question arises whether anti-VEGFR-3 approaches might have a future role in anti-angiogenic therapies, since tumor blood vessels express VEGFR-3 and they might become VEGFR-3 dependent under anti-VEGF/VEGFR-2 therapy.

Inhibition of lymphatic vessel growth and metastatic spread via the lymphatics has also been achieved with an anti-VEGF-D antibody. VEGF-D overexpressing cells
Tumor targeting

were subcutaneously injected into SCID mice and analysed during 35 days. The anti-VEGF-D antibody blocks binding of this ligand to both its receptors VEGFR-2 and -3, while a control antibody showed no effect\textsuperscript{15}.

When VEGFR-3 transfected cells or primary dermal microvascular endothelial cells are stimulated with either soluble or immobilised extracellular matrix proteins, such as collagen or fibronectin, phosphorylation of the receptor is observed in absence of associated ligand. This phosphorylation was diminished by pre-treating the cells with blocking antibody against $\beta_1$-integrin\textsuperscript{74}. Collagen could induce migration of the cells to a limited extent and migration was dramatically enhanced by applying a gradient of VEGF-D, but the ligand for VEGFR-3 failed to induce cell migration in the absence of extracellular matrix proteins. This indicates that both $\beta_1$ integrin and VEGFR-3 signalling is required for cell migration\textsuperscript{74}. The fact that VEGFR-3 inhibition alone can inhibit tumor growth and metastasis holds promise that targeting this receptor with inhibitory molecules has a potential as new anti-tumor therapy.

Lymphangiogenesis and lymph node metastasis can also be inhibited by soluble VEGFR-3. VEGF-C induced metastatic spread was inhibited with a soluble VEGFR-3-Fc fusion protein, suggesting that VEGF-C facilitates the spread of tumor metastasis via the lymphatic vessels and that the interaction between VEGF-C and its receptor is needed for tumor shedding\textsuperscript{12}. He et al. showed that VEGF-C induced lymphatic sprouting and lymphatic metastasis could only be inhibited by a soluble VEGFR-3-Ig fusion protein in the first 2 to 3 weeks after tumor xeno-transplantation, before the main lymphatic vessel growth and lymph node metastasis takes place\textsuperscript{75}. The same group has also reported, that inhibiting VEGFR-3 signalling with a soluble fusion protein suppresses tumor lymphangiogenesis and metastasis to regional lymph nodes but not to the lungs\textsuperscript{70}.

\subsection*{1.3.3 Targeting strategies}

As described above, unmodified antibodies are already a valuable tool in cancer therapy, but they also have the potential as delivery agents. Figure 6 summarises the possible antibody targeting mechanisms used to date\textsuperscript{76}. For example tumor cell killing by antibody-dependent cellular cytotoxicity is triggered by the interaction of the Fc region of the antibody bound to a tumor cell with Fc$\gamma$ receptors on immune effector cells, such as neutrophils, macrophages or natural killer cells. Unfortunately
this method is not very specific. Direct arming on the other hand involves the
covalent linkage of toxins or radionuclides to the antibody, but this also often involves
high background toxicity, since the toxin is exposed. Pre-targeting of radionuclides
and prodrugs to tumors is particularly attractive since these methods have the
potential to reduce systemic toxicity of conventional radioimmunotherapy and
cytotoxic chemotherapy. The prodrug diffuses in the body upon injection, but is
ideally only activated at the tumor site following contact with the fusion protein, which
has localised and is retained at this site. The fourth method is indirect arming, for
example the use of immunoliposomes. Liposomes are particularly attractive targeting
vesicles since they are long-lived in vivo, can be loaded with large amounts of drugs
and can be targeted to tumors by the attachment of specific antibodies on their
surface. Liposomes are discussed in more detail in 1.3.4.

Figure 6: Strategies for the use of antibodies in anti-cancer therapy. a: antibody-dependent
cellular toxicity and/or complement dependent cytotoxicity, b: direct linking of toxins or
cytokines, c: attachment to surface of liposomes loaded with toxins or engineering bispecific
antibodies binding two different antigens and preloaded with toxins, d: pretargeting with
either radionuclides or prodrugs.
Antibodies against various targets have been selected and are now in advanced oncology trials. Examples include Avastin against VEGF, IMC-C225 recognising EGFR and Mylotarg specific for CD33. Another interesting target is VEGFR-2, which is overexpressed on the genetically stable blood endothelia during angiogenesis, either for delivery of cytotoxic drugs to the tumor or for blocking downstream signalling. The extracellular matrix has also been found to be a good target. Antibodies have been produced against the ED-B domain of the B-fibronectin isoform, which is only found in the stroma of neoplastic tissues and not in mature vessels. The transmembrane tyrosine kinase receptor HER-2 is upregulated in 30% of highly malignant breast and ovarian cancers. Antibodies against HER-2, such as Herceptin, can inhibit tumor growth and specific scFvs have been isolated by phage display. VEGFR-3 promises to be an effective target, since it is upregulated in the vicinity of tumors and thought not to be expressed elsewhere in the body.

1.3.4 Liposomes as target vesicles

Drug therapy is often limited by a narrow therapeutic index, i.e. the maximum tolerated dose of a drug is close to the minimum curative dose needed. One way to overcome this limitation is the use of drug carriers for active targeting of the tumor. The main advantages of particulate drug carriers are their high loading capacity and the fact that they entrap the drug, thus reducing toxic side effects on normal tissue as well as protecting the drug from enzymatic inactivation. There are different particulate drug carriers, such as polymeric nanoparticles, block copolymer micelles, dendrimers and liposomes, whereof the latter will be discussed in more detail below.

Liposomes are spherical lipid bilayers with a trapped aqueous phase. Hydrophilic drugs can be loaded inside the liposome, while hydrophobic substances can be integrated in the lipid bilayer, making liposomes a very attractive drug carrier. There are several different types of liposomal vesicles, for example multilamellar vesicles (MLV) ranging from 500-5000 nm in size, small unilamellar vesicles (SUV) of 20-200 nm in diameter and large unilamellar vesicles (LUV) of 200-800 nm. Liposomes can be specifically targeted to a certain site in the body by attaching antibodies to their
INTRODUCTION

Surface, and are thus named immunoliposomes (see 1.3.5). Liposomes are biologically attractive since they are biocompatible, meaning that since they are made up of the same molecules as living cell membranes (phospholipids), they are non-toxic to the organism and they are not likely to trigger a strong immune response\(^8^4\). Another advantage of using liposomes as drug carriers is that size, charge and surface properties can be altered by adding other components to the lipid mixture before preparation. Liposomes also provide a unique opportunity to deliver pharmaceuticals into cells and even inside cellular compartments\(^8^5\).

Liposomes were first discovered by electron microscopy in 1963 and were given their present name in 1968. Ryman was first to use liposomes as carriers for therapy of metabolic disorders in children\(^8^6\). However, it was not until the first sterically stabilised liposomes came to the market that they experienced a breakthrough. Conventional liposomes are very rapidly taken up by the reticuloendothelial system (RES) and their associated short circulation times prevent optimal delivery of the drug to the target site\(^8^7\). Surface coating with a hydrophilic carbohydrate polymer, usually a lipid derivative of polyethylene glycol (PEG), increases liposome circulation time several-fold while avoiding rapid RES uptake\(^8^5\). PEG is thought to provide protection from rapid clearance by forming a hydrophilic steric barrier, which shields the liposome from interaction with serum proteins (opsonisation) and cell surface receptors on macrophages\(^8^8,8^9\).

The liposome size also influences the circulation time. Examining liposome circulation of four different diameters between 82 and 154 nm showed that with larger liposome sizes, circulation time was drastically reduced\(^9^0\). It is therefore attractive to use small liposomes. However, the size still has to be optimised; liposomes need to be small enough to escape the Kupffer cells, but large enough to avoid passing through the sinusoidal fenestrae in the endothelial cells of the liver blood vessels to minimise liver uptake\(^8^4\). The most common size used is 100 nm\(^9^0,9^1\). The prolonged presence and small size of liposomes allows them to extravasate into sites where the vasculature is leaky, which is often the case near or within tumors. When using liposome encapsulated drugs more than ten times higher accumulation rates in the tumor are achieved compared to administrating the free drug\(^8^8,9^2-9^4\). Drug delivery is also superior with small liposomes, presumably because they are more readily ingested by cells\(^9^5\).
Drug delivery to solid tumors presents the challenge of limited drug penetration due to the high interstitial pressure within the tumor tissue. Liposomal drug formulations offer a solution to this problem. Sterically stabilised liposomes, with their long blood circulation times and their small size are excellent passive targeting agents. Such long circulating macromolecules preferentially extravasate from the leaky blood vessels and accumulate in the tumor tissue \(^{96,97}\). After extravasation, the liposome remains within the tumor stroma and eventually becomes subjected to enzymatic degradation and/or phagocytic attack, leading to the release of the drug. The next generation of drug carriers feature direct molecular targeting via antibody-mediated or other ligand-mediated interactions \(^{96}\).

### 1.3.5 Immunoliposomes

Drug delivery systems lacking specificity for a target may adversely affect surrounding tissue, continue to circulate downstream of the target site or suffer “washout” of the therapeutic component. It is therefore advantageous to exploit receptor molecules, which are differentially expressed on the surface of normal tissue, in order to deliver the liposomes to the precise site of disease. Targeting of liposomes can be achieved by attaching antibodies, or fragments thereof, or naturally occurring or synthetic low molecular weight ligands such as peptides, carbohydrates, glycoproteins, or receptor ligands to the surface of the vesicle \(^{82}\). Antibodies are ideal molecules for specific targeting of liposomes. Since the development of monoclonal antibody technology (Kohler and Milstein, 1975), specific antibodies of desired reactivity can be obtained in appreciable quantities. Unlike drug-immunoconjugates or immunotoxins, which deliver few molecules of drug or toxin per antibody molecule, immunoliposomes can be exploited to deliver thousands of molecules of a drug using few antibodies \(^{76,98}\). But since the affinity of an antibody varies, the number of targeting moieties attached to the surface of an immunoliposome needs to be optimised \(^{99}\). Several studies have investigated the optimal amount of targeting proteins and concluded that attaching too many molecules results in increased recognition by the RES \(^{100}\). Some researchers found 30-40 antibodies per liposome to be optimal \(^{101}\), while others state that they obtained best targeting with 100 molecules per liposome \(^{102}\). In addition to the targeting ligand, means of increasing the circulation lifetime of the liposome must be considered. Therefore, sterically stabilised liposomes are used for the production of immunoliposomes \(^{103}\). Covalent
binding of antibodies to the distal end of PEG does not appear to interfere with the binding ability of the antibody to its respective antigen. Furthermore, PEG still shields the liposome from opsonisation, leading to extended circulation half-lives. Another advantage of immunoliposomes is their potential for additive or synergistic effects of the signalling antibodies present on the liposome surface and the liposomal drug, entrapped in the liposome interior. Experimentally this has been shown in several animal xenogeneic studies of human cancers.

Originally, IgG were attached to the liposome surface, but immunoliposomes are now more frequently armed with antibody fragments such as Fab’s or scFvs with the scope of reducing RES uptake. Whole antibodies have the advantage of higher binding affinity, due to two binding domains on the same molecule, and stability than its fragments. The Fc domain of the IgG may trigger complement-mediated cytotoxicity and antibody-dependent cell-mediated cytotoxicity leading to apoptosis and cell death, which may be the reason for additive and synergistic responses seen between targeting antibodies and the liposomal drug. On the other hand, the use of antibody fragments reduces the immunogenicity of immunoliposomes, thus increasing their circulation time. Since antibody fragments help to reduce the uptake of immunoliposomes by tumor-associated macrophages, they might provide better penetration into solid tumors. The biodistribution of Fab’- versus mAb-immunoliposomes shows a six-fold increase in circulation half-life. This might be due to smaller antibody fragments usually showing faster localisation to disease sites and better penetration into tumor tissue. Due to their ease of production and decreased immunogenicity, scFv fragments might be even better candidates than Fab’ fragments.

Some new promising immunoliposome preparations include antiHer2/Neu ligand targeted liposomes (LTLs) against carcinoma cells, anti-CD19 LTLs to target malignant B cells, anti-GD₂ LTLs to treat neuroblastoma or melanoma and anti-β₁ integrin LTLs in metastatic lung tumors. A list of immunoliposomes is shown in Table 1. To date, two immunoliposome drugs have reached clinical trials: MCC-465, a PEG-immunoliposome encapsulating doxorubicin and targeted against cancerous stomach tissue with the F(ab’)_2 fragment of the human mAb GAH. Anti-HER2 (ErbB2) immunoliposomes have also entered the clinics. Different types of
anti-HER2 immunoliposomes are being assessed, either with Fab’ or scFv antibody fragments attached to the surface. The results look promising\textsuperscript{112,119}.

Coupling methods for the production of immunoliposomes should be simple, fast, efficient and reproducible, yielding stable, non-toxic bonds. The target recognition and binding specificity of the antibody as well as the drug loading efficiency should not be altered\textsuperscript{82}. The “classical” coupling methods in which antibodies are attached to the bilayer surface result in low numbers of attached antibody, probably due to sterical hindrance by PEG. By attaching the antibody to the PEG terminus, high coupling efficiencies can be achieved. The binding specificity can be retained by covalent coupling of the antibody in the right orientation, for example by coupling maleimide-derivatised antibodies to thiolated PEG or vice versa\textsuperscript{120}.

Although there are several reports of improved therapeutic efficacy of immunoliposomes in the treatment of cancer, such liposomes have been slow in reaching the clinics. Finding the optimal ratio between targeting molecules on the liposome surface for specific accumulation at the disease site and avoiding recognition by the RES is very difficult. The majority of antibody-modified liposomes accumulate in the liver or the kidneys, which is usually a consequence of insufficient time for interaction of the target with the immunoliposome\textsuperscript{87}. Several strategies are being explored to improve the therapeutic index of immunoliposomes\textsuperscript{92,121}. For example, as described above, the use of small antibody fragments or genetically engineered antibodies as targeting moieties should reduce antibody immune response in patients. Phage display technology has helped to choose high affinity antibodies as well as internalizing ligands enabling fast uptake of the liposome at the tumor site, preventing wash out\textsuperscript{122,123}. Triggered drug release by use of pH-sensitive phospholipids, peptides and polymers might circumvent the uncontrolled leakage of the drug from the liposome\textsuperscript{124}. It is believed that immunoliposomes will perform best against targets on the vasculature or readily accessible sites nearby\textsuperscript{122,125}.
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Table 1: Ligand-targeted liposomes that were evaluated in vitro or in vivo for delivery of anticancer drugs

<table>
<thead>
<tr>
<th>Targeting agent</th>
<th>Cell surface target</th>
<th>Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibody, antiCD19</td>
<td>CD19</td>
<td>Namalwa hu-B-cell lymphoma</td>
</tr>
<tr>
<td>Antibody, antiCD19</td>
<td>CD19</td>
<td>Human multiple myeloma, ARH77 cell line</td>
</tr>
<tr>
<td>Antibody, recombinant human anti-HER2-Fab’ or scFv C6.5</td>
<td>HER2</td>
<td>HER2-overexpressing human breast cancer</td>
</tr>
<tr>
<td>Antibody, anti-hu CEA 21B2 and anti-hu CEA 21B2 Fab’</td>
<td>Human carcinoembryonic antigen, CEA</td>
<td>CEA-positive human gastric cancer, MKN45</td>
</tr>
<tr>
<td>Antibody, MRK16</td>
<td>P-glycoprotein</td>
<td>Human myelogenous leukemia, K562</td>
</tr>
<tr>
<td>Antibody, anti-β1-integrin Fab’</td>
<td>Human β1 integrins</td>
<td>Human non-small cell lung carcinoma</td>
</tr>
<tr>
<td>Antibody, CC52</td>
<td>CC531</td>
<td>Rat colon carcinoma</td>
</tr>
<tr>
<td>Antibody, anti-GD2 and anti-GD2 Fab’</td>
<td>GD2</td>
<td>Human neuroblastoma</td>
</tr>
<tr>
<td>Antibody, anti GD2</td>
<td>Distialoganglioside, GD2</td>
<td>Human melanoma</td>
</tr>
<tr>
<td>Antibody, anti-idiotypic mAb, S5A8</td>
<td>38C13</td>
<td>Murine B-cell lymphoma</td>
</tr>
<tr>
<td>Antibody, anti-human E-Selectin</td>
<td>E-Selectins</td>
<td>Activated human endothelial cells</td>
</tr>
<tr>
<td>Antibody, anti-ganglioside GM3 (DH2) or anti-Lea (SH1)</td>
<td>Carbohydrate, ganglioside (GM3): Lewis X (Lea)</td>
<td>B16BL6 mouse melanoma and HRT-18 human colorectal adenocarcinoma</td>
</tr>
<tr>
<td>Antibody, anti-ED-B scFv</td>
<td>B-fibronectin (ED-B domain)</td>
<td>Murine F9 teratocarcinoma</td>
</tr>
<tr>
<td>Peptides, antagonist G</td>
<td>Vasopressin</td>
<td>Human small cell lung cancer, H69</td>
</tr>
<tr>
<td>Peptides, vasoactive intestinal peptide (VIP-R)</td>
<td>VIP receptors</td>
<td>Rat breast cancer</td>
</tr>
<tr>
<td>Peptide, P-protein</td>
<td>Intercellular adhesion molecule-1 (ICAM-1)</td>
<td>Human M21 and A-375 melanoma</td>
</tr>
<tr>
<td>Synthetic peptide, cyclic peptide inhibitor CTHWG-FTLC (CTT)</td>
<td>Matrix metalloproteinases/gelatinases (MMP-2 and MMP-9)</td>
<td>U937 leukemia and HT1080 sarcoma</td>
</tr>
<tr>
<td>Angiogenic homing peptide, APRPG</td>
<td>Integrin, GPIIb–IIIa</td>
<td>Meth-A sarcoma</td>
</tr>
<tr>
<td>Vitamins, folate</td>
<td>Folate receptor</td>
<td>M109-R mouse carcinoma tumor</td>
</tr>
<tr>
<td>Vitamins, folate</td>
<td>Folate receptor</td>
<td>Murine acute myelogenous leukemia</td>
</tr>
<tr>
<td>Vitamins, folate</td>
<td>Folate receptor</td>
<td>Chinese hamster ovary and KG-1 human acute myelogenous leukemia cells</td>
</tr>
<tr>
<td>Transferrin</td>
<td>Transferrin receptor</td>
<td>Murine colon 26 and B16 melanoma</td>
</tr>
<tr>
<td>Transferrin</td>
<td>Transferrin receptor</td>
<td>MKN45P human gastric tumor</td>
</tr>
<tr>
<td>Transferrin</td>
<td>Transferrin receptor</td>
<td>C6 glioma</td>
</tr>
<tr>
<td>Carbohydrate, hyaluronic acid</td>
<td>Transferrin receptor</td>
<td>B16F10 murine melanoma</td>
</tr>
</tbody>
</table>
1.4 Aim of the study

The aim of this study was to isolate specific single chain antibodies to create a possible targeting vehicle aimed at the lymphatic vessels surrounding tumors as well as testing the potential of these antibodies as a diagnostic or therapeutic tool. To date, the lymphatic system and especially lymphangiogenesis have not been investigated in much detail, due to the lack of specific markers for the lymphatic endothelium. Thus there is a great need for diagnostic tools, such as antibodies. Using such specific antibodies to create targeting vesicles may circumvent toxic side-effects caused by administering an anti-cancer drug by itself, as well as providing more favorable pharmacokinetic properties.

The objective of this study was to obtain and evaluate the diagnostic and therapeutic potential of VEGFR-3 specific single chain antibodies and to construct scFv-immunoliposomes recognising the receptor on lymphatic endothelial cells in the vicinity of tumors as well as on tumor cells themselves. The hypothesis being that anti-VEGFR-3 single chain antibodies can be used diagnostically to visualise lymphatic vessels in immunohistochemistry as well as to target cytotoxic liposomes to tumors expressing the receptor and thus reducing the growth of that tumor, while having no toxic effect on normal tissue.
2 SELECTION AND PRODUCTION OF VEGFR-3 SPECIFIC SINGLE CHAIN ANTIBODIES

2.1 Abstract

Recombinant sVEGFR-3/Flt4 protein (ReliaTech GmbH, Germany) was used to select scFv antibodies from the ETH-2 phage display library. Three rounds of selection were carried out on human VEGFR-3 to obtain single chain antibodies specific against the human receptor (AFA2 and AFC5). Clones obtained after two rounds of selection against the hVEGFR-3 were used to carry out two further rounds of selection against the murine receptor to obtain cross specific single chain antibodies (AFF1 and AFA8). The specific clones were modified at their C-terminal end with three cysteins for coupling to liposomes and cloned into the Pichia pastoris expression vector pPICZαA for large scale production\textsuperscript{126}. The antibodies were produced in 6 liter cultures and then purified by Protein A chromatography, yielding typically 3-5 mg/L pure protein.

2.2 Introduction

Antibodies are among the most versatile tools used today for characterizing and targeting molecules in cells and in biological tissues. There are several ways of obtaining specific scFv antibodies against a certain antigen. One way is to modify a monoclonal antibody whose characteristics are known. Another approach is phage display technology. This technique is based on the display of scFvs on the surface of bacteriophages, which contain the corresponding gene. Synthetic single chain antibody repertoires, like the ETH-2 phage library, have reliably yielded good antibodies against a large variety of antigens\textsuperscript{64}. A great advantage of antibody libraries from large synthetic repertoires is that one library can be used for the selection of antibodies against any antigen. There are several different display technologies used to date, such as ribosome, yeast, bacterial and phage display, whereof phage display is still the most widely used in antibody research. It is a fairly simple method yielding several different antibodies against a certain antigen in one selection. The more rounds of selection are carried out the more specific the resulting antibodies are. Usually, three rounds of selection are used, which results in
specific clones, which can already be tested \textit{in vitro}. Another advantage of phage display is that one can easily create multi-selective antibodies, by carrying out the different rounds of selection against different antigens.

Protein production in the yeast system, such as \textit{Pichia pastoris}, is becoming more and more popular because it is more efficient and economical compared to bacterial and mammalian sources. Yeast cells grow faster and produce the protein of interest using a eukaryotic protein-synthesis pathway, enabling glycosylation. The costs for the media, equipment and infrastructure to culture yeast are also lower than those of mammalian cells. Usage of the yeast expression system enables the production of quantities needed for therapeutic purposes. Yields as high as 250 mg/L have been reported for production of scFvs in shaking flasks\textsuperscript{127}. Another advantage of this system is that it allows the extracellular production of the target protein when required, thus making the purification easier.

This chapter covers the selection of VEGFR-3 specific single chain antibodies from the ETH-2 library by phage display. These scFvs had to be modified at their C-terminal end for coupling to liposomes and cloned into the yeast expression vector pPICZ\textalpha{}A for high yield production. The obtained antibodies were purified by Protein A chromatography and yielded 3-5 mg/L pure protein.
2.3 Methods

2.3.1 Materials
Recombinant human VEGFR-3/Flt4 (purity >95%) expressed in insect cells, was purchased from RELIATech® GmbH, (Braunschweig, Germany). The ETH-2 phage library, a synthetic human antibody library, was a kind gift from Prof. D. Neri (Institute of Pharmaceutical Sciences, ETH Zürich, Switzerland). The construction of the library was previously described 128. E. Coli suppressor strain TG1 for propagation of phage particles were obtained from Invitrogen (Leek, the Netherlands). Helper phage VCS M13 for the rescue of phagemid libraries was purchased from Stratagene (Cedar Creek, TX). Antibiotics and isopropyl-β-D-thiogalctopyranoside (IPTG) were obtained from Invitrogen and Fermentas (Ontario, Canada), respectively. For detection of scFv Abs the murine M2 anti-Flag was obtained from Sigma and an alkaline phosphatase (AP)-conjugated goat anti-murine mAb was from Serotec (Oxford, UK). The ELISA plates were from Nunc, Denmark. The plasmid sequence of pCM3 for introduction of C-terminal cysteines was obtained from Dr. C. Marty 102. For sub-cloning XL10-Gold ultra competent E. Coli were purchased from Stratagene. The pPICZαA vector, the X-33 yeast strain, the 5’AOX1 and 3’AOX1 primers and the antibiotic zeocin for scFv production were from Invitrogen. Restriction endonucleases were from Roche Diagnostics Mannheim (Mannheim, Germany) and Life Technologies, Inc. (Gaithersburg, MD). All yeast culture media were from Becton Dickinson (Basel, Switzerland). All buffers and salts and other chemicals were purchased from GERBU (Gaiberg, Germany) and Fluka (Buchs, Switzerland).

2.3.2 Phage display selection
Immunotubes were coated with 25 µg/mL VEGFR-3-His (RELIATech®) at room temperature (RT) overnight. The phage display technology was carried out as previously described by F. Viti et al. (2000). Briefly, the phage library was allowed to react with the antigen on the immunotube for 1 h at RT. Tubes were stringently washed to remove unbound and weakly bound phages. Specific phages were eluted and transformed into the E. Coli strain TG1 for amplification using helper phage. The phages were harvested and used for subsequent selection rounds. In our case three rounds were carried out against hVEGFR-3 to obtain scFvs specific against the
human receptor. Two rounds of selection against hVEGFR-3 and subsequently two rounds against mVEGFR-3 were carried out to obtain cross-specific single chain antibodies.

After the selection rounds 50 clones were picked from a 2xTY agar plate (yeast extract tryptone medium, 1.5% (w/v) agar) containing 100 µg/mL ampicillin and 1% glucose and grown in a 96-well plate in 2xTY containing 100 µg/mL ampicillin and 1% glucose. Antibody production was induced with 1 mM IPTG for 16-24 h at 30°C. The supernatants of these clones were tested for scFv binding to immobilised VEGFR-3. A 96-well plate for enzyme-linked immunosorbent assay (ELISA) was coated with 100 µl of 10 µg/mL VEGFR-3, BSA or VEGF-His overnight at RT. The plate was subsequently washed with PBS (0.137 M NaCl, 2.68 mM KCl, 8.09 mM Na₂HPO₄, 1.76 mM KH₂PO₄, pH 7.4) and blocked with 2%MPBS (PBS + 2% (w/v) milk powder) for 2h at RT to prevent unspecific binding. Then, 80 µl scFv supernatant, 10 µl 10%MPBS and 15 µl 2%MPBS containing murine anti-FLAG tag mAb M2 (1:400 v/v) and alkaline phosphatase-conjugated goat anti-murine mAb (1:200 v/v) was added per well for 1h at RT. To remove weakly or unbound antibodies the plate was washed 10 times with TPBS (PBS + 0.1% Tween20) and subsequently 10x with PBS. The ELISA was then developed with the AP substrate NPP (3 mM 4-nitrophenyl phosphate, 50 mM NaHCO₃ and 50 mM MgCl₂) and measured at 410 nm.

2.3.3 Cloning into the yeast expression vector pPICZαA

The sequences of the selected scFvs were modified with cysteines for later coupling to liposomes and cloned into the yeast vector pPICZαA for extracellular expression of scFv in Pichia pastoris (X-33). The primers 0n and 3n used for PCR to modify the single chain antibodies with three cysteines at their C-terminal end were designed as described by C. Marty et al. ¹⁰². The modified scFv-DNA was cut with EcoR1 and Xba1 for ligation into the pre-cut pPICZαA vector and transformed into XL-10-Gold for DNA amplification. A MiniPrep was carried out and the DNA electroporated into the P. pastoris strain X-33 and plated on YPDS (Yeast Extract Peptone Dextrose medium containing 1 M sorbitol and 2% w/v agar) plates. Several clones were picked from the agar plates for selection of the highest expressor in 5 mL cultures.
2.3.4 scFv production in *Pichia pastoris*

Single clones of the high-expressers were picked and grown in 5 mL BMGY (buffered glycerol-complex medium: 1% yeast extract, 2% peptone, 100 mM potassium phosphate (pH 6.0), 1.34% yeast nitrogen base (YNB), 4x10^{-5}% Biotin and 1% Glycerol) overnight at 30°C and 250 rpm. This overnight culture was used to inoculate 1 L of BMGY in a 2 L baffled shaking flask and grown overnight at 30°C and 250 rpm. The culture was centrifuged at 2000 rpm for 15 min and the pellet resuspended in 1 L of BMMY (buffered methanol-complex medium, same composition as BMGY except 0.5% methanol instead of glycerol) for induction of protein expression. During further incubation at 27°C, 250 rpm for 48-72 h, cultures were supplemented with a final concentration of 0.5% (v/v) methanol every 12 h.

2.3.5 Purification by affinity chromatography

The scFv fragments contain a VH DP-47 germline segment and can therefore be purified on Protein A sepharose. The supernatant of the yeast cultures was concentrated to 100 mL and dialysed against 20 mM Tris, pH 8.0 overnight and loaded onto a 5 mL Protein A column. After washing the column with 20 mM glycine, pH 5.0, the protein was eluted from the column with 0.1 M glycine, pH 2.5. Fractions of 1 ml were collected and mixed with 100 µl 1 M Tris, pH 8.0 in the collection tubes to neutralise the pH. Pooled fractions were dialysed against PBS overnight at 4°C. The purity and activity of the scFvs was analysed by Coomassie blue staining and by Western blot analysis respectively, using the incorporated FLAG-tag for detection. The protein quantity was measured with a Nano Drop ND-1000 Spectrophotometer (Witec AG, Switzerland) using the extinction coefficient obtained from NTi software (Invitrogen Inc.).
2.4 Results

2.4.1 Selection of VEGFR-3 specific single chain antibodies
Here we show the results of the selection of hVEGFR-3 specific single chain antibodies as well as cross-specific murine/human reactive antibodies. The antibodies were selected from the synthetic human phage display library ETH-2. The objective for the selection is the need for lymphangiogenic markers as well as inhibitors.

After the last selection round 96 clones were picked from the plates and frozen for later use. Thereof, 50 hVEGFR-3 specific clones were tested and 14 showed specific binding to the human receptor by ELISA. Of these positive clones, 12 were further tested for binding to BSA, VEGF and plastic (Fig. 7a). The cross-reactive clones were analysed in the same way. Figure 7b shows the binding of the cross specific clones to the human and murine receptor.

![Graph showing absorbance values for different clones](image)

*Figure 7a: Binding of selected scFv clones to hVEGFR-3 (grey) on ELISA plate. Binding to plastic, BSA and VEGF-His was below the detection limit. The plate was coated overnight with the different antigens and incubated with scFv supernatant for 1h at RT. Binding visualised by colour reaction from AP incubated with NPP, measured at absorbance 410 nm. Binding to plastic, BSA and VEGF was below detectable limits.*
All scFvs were specific and were shown to bind to the receptor on transiently transfected HEK293T/hVEGFR-3 cells by immunofluorescence as described in more detail in 3.3.2. ScFvs AFA2 and AFC5 were selected for further characterization, since they showed the best binding to hVEGFR-3. The cross specific scFvs AFA8 and AFF1 showed strongest binding towards both receptors and were selected for further characterization. Although all clones obtained after selection were chosen under stringent conditions, it is often seen that only approximately one third shows specific binding when further analysed. When the clones are sequenced, mutations in the conserved regions are detected or identical clones are selected, which further reduces the number of specific clones.

### 2.4.2 scFv production

The expression of scFvs by different clones was first tested on a small scale and analysed by Western blot, prior to choosing the best scFv producer for large scale production. Often, one experiences that clones which produced specific scFv in the 96 well plate, do not produce any protein in larger volume batches, which further reduces the number of specific clones. In figure 8, scFv production over time of two different AFC5 expressing clones in 5 mL BMGY is shown. By using a maximal end
concentration of 0.5 % MeOH for induction, reaching toxic levels during protein production can be avoided. Carrying out the production in baffled flasks provides the essential aeration needed.

![Figure 8: AFC5 single chain production in Pichia pastoris. Lane 1: Marker, lane 2: positive control, lanes 3 + 7: growth stage in BMGY of two different AFC5 clones, lanes 4 – 6 and 8 – 10: MeOH induced production after 24h, 33h and 48h. Antibody production was carried out in 5 mL Falcon Snap Cap tubes at 30°C, 150 rpm.]

2.4.3 Protein purification

The expression of the scFvs as extracellular protein is optimal since in the yeast system hardly any other protein is secreted\(^{129}\). Figure 9 shows the purification profile of AFA2, which had been produced in 6 x 1 L BMGY/BMMY in 2 L baffled flasks and concentrated to 100 mL for purification. Since the scFvs from the ETH-2 library contain a DP47-sequence, they can easily be purified by Protein-A chromatography. Purification over Protein A is a very simple and reliable system resulting in 99% pure protein. A yield as high as 3-5 mg/L of pure scFv, was obtained with 72 h of expression.

To determine if the antibody exists as a monomer or forms dimers due to the cysteins, the scFvs were run on SDS-PAGE under reducing and non-reducing conditions and then stained by Coomassie. In figure 10A it is visible that the scFvs predominantly exist in the monomeric form. This was further demonstrated by size exclusion chromatography, where approximately 70% monomer was seen. Comparison to a standard curve suggests that the monomer, 30 kDa, should elute at approx. 16-17 mL and the dimer at 14-15 mL, which represents the major peaks on the chromatogram (Fig 10B).
Figure 9: Analysis of scFv purification. Reducing SDS-PAGE gel (Coomassie blue stain) with probes taken at different timepoints during the purification of P. pastoris supernatant over Protein A. Lanes 1-4: flowthrough fractions sampled during the loading of the column. Lane 5: the pure eluted scFv AFA2.

Figure 10: A: SDS-PAGE gel (Coomassie blue stain) comparing band height under reducing (2-4) and non-reducing conditions (6-8) of scFv A1cys (2 + 6), AFA8 (3 + 7) and AFC5 (4 + 8); B: HPLC analysis of scFv AFC5. Red line: 260 nm, blue line: 280 nm, and peak 16.41 representing the monomer while peak 14.16 represents the dimer.
2.5 Discussion

To date phage display is the most widely used display technology in antibody research. This fairly simple method can yield several different antibodies directed against a certain antigen in one selection round. To increase the specificity of the obtained antibodies, several rounds of selection against the same antigen can be carried out, or if a cross-specific antibody is desired, selection rounds can be directed against different antigens. Here, four VEGFR-3 specific single chain antibodies selected from the ETH-2 phage display library are described. Two thereof being cross-reactive, meaning that they recognise both the human and murine receptor. The advantage of cross-specific antibodies is the possibility to use them both pre-clinically in mouse xenogeneic tumor models and in the clinics, without having to humanise them. The number of selective clones obtained from a library varies, since the same clone can be selected, mutations in the variable domain can occur and even unspecific clones can be selected, despite of stringent conditions. From 90 selected clones, 50 were tested for specificity to VEGFR-3 by ELISA and 12 thereof were chosen for further testing by immunofluorescence (described in more detail in 3.3.4), whereof all were specific. Usually, one ends up with one or two specific antibodies at the end of all selection rounds (personal communication Dr. Alma Rubio-Demirovic). The clones showing best binding to the receptor were cloned into pPICZ\(\alpha\)A for production in the yeast \(P.\ pastoris\). Protein production in yeast is desirable since it is a eukaryotic system enabling glycosylation. A further advantage is secretion into the extracellular space, which facilitates protein purification. Four clones produced scFvs in high amounts and were therefore chosen for large scale production. Pure protein (>95\%) were obtained at quantities of 3-5 mg/L. Yields as high as 250 mg/L have been reported for scFv production in shaking flasks\(^{64}\).
3 IN VITRO AND IN VIVO CHARACTERISATION OF SPECIFIC SINGLE CHAIN ANTIBODIES

3.1 Abstract

We could show specific binding of our scFvs in vitro on recombinant VEGFR-3, as well as in cell lysates made from receptor expressing transiently transfected HEK 293T cells or of naturally VEGFR-3 expressing U937 tumor cells. Specific antibody binding was seen at concentrations as low as 1 nM. In comparison, commercially available anti-human VEGFR-3 IgG antibody is used at 3nM for ELISA. First, binding properties of the obtained antibodies were characterised by testing the specific interaction between the single chain antibodies and the purified receptors by ELISA, resulting in saturating binding curves. Pre-incubating the antibodies with either murine or human VEGFR-3 proved the specific binding of AFA2 and AFC5 to the human receptor, while it showed cross-specific binding of AFF1 and AFA8 to murine and human VEGFR-3. Since antibody affinity appears to play a significant role in tumor targeting efficiency, it was important to confirm that our scFvs are highly selective for VEGFR-3. The $K_d$ values obtained for AFA2 and AFC5, 10.6 nM and 8.9 nM respectively, are in the same range as reported for other scFvs from the same library\textsuperscript{130}. Other researchers have reported $K_d$ values of 3.9 nM and 143 nM for their scFv antibodies\textsuperscript{130,131}. The single chain antibodies were further tested for specific binding on transfected receptors expressed on HEK 293T cells by immunofluorescence, which showed membrane bound antibodies. The binding was also visualised on naturally occurring receptors on U937 cells by flow cytometry. Specific binding to VEGFR-3 was confirmed in all cell lines by Western blot analysis on cell lysates. The scFv binding to VEGFR-3 derived from U937 cell lysates confirms the findings of Schoppmann et al.\textsuperscript{132}, who showed that this erythroleukemia cell line naturally expresses VEGFR-3. Once specific binding had been shown by immunofluorescence, the possible diagnostic potential of these antibodies was tested by investigating their usefulness in immunohistochemistry. On sections of neonatal human foreskin AFC5 staining overlaped with LYVE-1 staining, while the unspecific scFv A1cys did not show any overlap. Compared to LYVE-1 the antibody AFC5 did not recognise all cells surrounding a lymphatic vessel, but in addition recognised vessels, which were not visualised by LYVE-1.
In addition we investigated if the scFvs can inhibit binding of VEGF-C to its receptor and thus have a therapeutic potential. Using competitive ELISA, it was shown that AFC5 inhibits ligand-receptor interaction, but unfortunately not the cross-specific scFv AFA8. It was therefore further tested whether AFC5 could inhibit receptor function, i.e., proliferation and migration of human lymphatic endothelial cells in vitro. Inducing these cells with a variant VEGF-C (hVEGF-C156S), which only binds VEGFR-3, we could show concentration dependent functional inhibition of the receptor. Comparing the migration of cells incubated with AFC5/hVEGF-C156S versus unspecific scFv A1cys/hVEGF-C156S showed a significant difference \((P=0.0036)\) as well as when compared to the PBS/hVEGF-C156S control \((P=0.0119)\). Proliferation of these cells by hVEGF-C156S was also inhibited by AFC5 compared to unspecific A1cys/hVEGF-C156S \((P=0.0334)\) and PBS/hVEGF-C156S \((P=0.0174)\). These results are very promising for the therapeutic usefulness of this antibody. The in vivo binding properties of AFC5 were investigated by a biodistribution experiment on female SV129 mice bearing either F9 or F9/VEGFR-3 s.c. tumors. The pharmacokinetic properties of AFC5 were compared to unspecific single chain antibody A1cys, which confirmed the specific targeting of AFC5 to VEGFR-3 expressing tumor cells. Tumor accumulation was further visualised by single photon emission computed tomography (SPECT). In summary, our scFvs show a higher specificity than expected for small single chain antibodies.

3.2 Introduction

Tumors need to recruit blood and lymphatic vessels to grow above a size of a few millimetres. Lymphangiogenesis, the formation of new lymphatics from pre-existing ones has been identified as an essential process for growth and metastatic spread of tumor cells. VEGFR-3 is involved in lymphangiogenesis and is upregulated on endothelial cells in the vicinity of tumors as well as on tumor cells themselves, thus representing an interesting target for anti-lymphangiogenic therapy.

Inhibition of lymphangiogenesis can be achieved by either blocking receptor function or by targeting cytotoxic drugs to the site of action. Antibodies are well established targeting molecules and are considered as interesting tools for imaging and therapy. Many different monoclonal antibodies have been raised over the years and tested in
vivo. However, several limitations to their usefulness in the clinics have been recognised. One problem is that the patient generates an immuneresponse towards monoclonal antibody from other species. One approach to overcome this hurdle is to generate human monoclonal or chimeric antibodies. Such molecules are thought to be less immunogenic than the original antibodies. Another way to minimise the immune response is to reduce the size of the molecule. By enzymatic removal of the Fc part of the IgG, a F(ab)2 antibody fragment is obtained. Single chain antibodies are the smallest fragment to contain the original antigen binding sites. They consist of the variable heavy (VH) and variable light (VL) chain, joined by a flexible linker.

Figure 11 illustrates the relationship between IgG, F(ab)2 and scFv antibody fragments.

![Figure 11: Overview of antibodies and antibody fragments: A) IgG (Mr: 150 kDa); B) F(ab)2 fragment (Mr: 100 kDa)(Fab' being one part of this structure); C) scFv (Mr: 30kDa)](image)

However, the immune response of whole antibodies is not the only in vivo obstacle. IgG molecules have practical limitations for the clinics due to their large size (150 kDa), which leads to slow clearance from the blood resulting in significant exposure to normal tissue. In comparison, the small size (30 kDa) of scFv antibodies leads to extremely rapid plasma and whole body clearance. These pharmacokinetic properties are positive with respect to normal tissue exposure but also result in a reduced percentage of injected dose/g of scFv accumulation in tumors compared to whole antibodies. Additional problems associated with using whole antibodies include inefficient penetration into tumor tissue and general systemic toxicity at the high doses required to deliver sufficient antibody to the site of action. Single chain
antibodies have been shown to penetrate tumors more efficiently than whole antibodies or Fab' fragments \textsuperscript{134}. This is probably due to their small size and results in tumor : normal tissue ratios comparable or even greater than for other Ig forms \textsuperscript{135}. Disadvantages of using scFvs however, include their decreased stability, short half-life, and in some cases, decreased affinity to the antigen \textsuperscript{135,136}.

The scFvs obtained against VEGFR-3 have proven to bind as specifically to the receptor as commercially available anti-VEGFR-3 IgG. In this chapter we show the specificity of scFv binding to VEGFR-3 by various means \textit{in vitro}, using naturally expressed or transfected receptor. \textit{In vivo} studies were carried out to evaluate the targeting properties of the antibodies as well as their potential to inhibit receptor function. For targeting studies, a syngeneic mouse model was used. Here, one needs to take into account that such a model does not represent a tumor in its native state, especially when using transfected cell lines. But still they are a valuable tool for initial proof of principle studies.
3.3 Methods

3.3.1 Materials and animals
All buffers and salts and other chemicals were purchased from GERBU (Gaiberg, Germany) and Fluka (Buchs, Switzerland). For detection of scFv antibodies the murine M2 anti-Flag antibody was obtained from Sigma, the alkaline phosphatase (AP)-conjugated goat anti-murine mAb from Serotec (Oxford, UK) and the FITC-conjugated goat anti-murine mAb from Southern Biotechnology Associates (Birmingham, USA). The HEK 293T cells were obtained from Dr. F. Wurm (EPFL, Switzerland), the U937 cell line from ATCC CRL-1593 (USA) and the F9 cells were from ECACC Nr. 85061803 (UK). The DNA for the full length hVEGFR-3 was a kind gift from Dr. D. Birnbaum (IPC, France). F9 and HEK 293T cells were grown in DMEM (Dulbecco Modified Eagle’s Medium) without non-essential amino acids and pyruvate, supplemented with 10% FBS (Fetal Bovine Serum), 100 U/mL penicillin, 100 µg/mL streptomycin, 100 U/mL nystatin. U937 cells were maintained in RPMI 1640 supplemented as above. The hVEGF-C protein used for the competitive ELISA was constructed and produced in our laboratory by S. Arn and the processed hVEGF-C for the migration and proliferation assays was purchased from R&D. 

125Iodine was purchased from Amersham Biosciences Europe GmbH (Otelfingen, Switzerland). The SV129 mice used for the biodistribution were bred at Charles River (Sulzfeld, Germany) and the double transgenic Rip1Tag2-VEGF-C mice were a kind gift from Dr. G. Christofori (University of Basel, Switzerland).

3.3.2 Cloning of pCDNA/hVEGFR-3D1-7TM and transient transfection of HEK 293T
The domains 1-7 and the transmembrane domain of VEGFR-3 were cloned into a pCDNA 3.1+ vector by the PCR subcloning method (pCDNA/VEGFR-3D1-7TM)\textsuperscript{137}. The pCDNA vector contains a neomycin resistance to enable stable transfections. HEK 293T cells were transiently transfected with 5 µg pCDNA/hVEGFR-3D1-7TM using the calcium phosphate method\textsuperscript{138}. Briefly, the DNA was suspended in solution A (250 mM CaCl\textsubscript{2}) and then mixed by vortex with an equal amount of solution B (140 mM NaCl, 50 mM HEPES, 1.5 mM Na\textsubscript{2}HPO\textsubscript{4}, pH 7.05) and left to stand at RT for 1 min. Thereafter the mix was added to the cells and evenly suspended in medium.
The cells were incubated at 37°C, 5% CO₂ for 4 h before replacing the medium and incubating the cells for a further 24 h.

3.3.3 Western blot and ELISA

Cell lysis samples of transfected HEK 293T and U937 cells were prepared by resuspending cell pellets in 6xSDS loading buffer and heating at 95°C for 10 min prior to three cycles of freeze-thawing. Samples were run on SDS-PAGE and subsequently blotted onto Polyvinylidene Difluoride (PVDF) membrane. To check for cross-specificity, the scFvs (1 nM) were incubated with a 4x molar excess of antigen for 30 min at RT before incubation with the PVDF-membrane. The blot was subsequently reacted with the anti-Flag M2 (15 µg/mL) and the goat anti-murine-alkaline phosphatase antibody (70 ng/mL) for 45 min at RT. Signal was developed with colour substrates 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt and nitro-blue tetrazolium chloride (BCIP/NBT) for alkaline phosphatase.

Nunc ELISA plates with 96 wells were coated with 100 µL of 10 µg/mL (38.5 nM) antigen in PBS overnight at RT. Plates were washed three times with PBS and then blocked with 200 µL 2% MPBS for 2 h at RT. Thereafter 100 µL of the scFvs (at concentrations ranging from 0.1-5 nM), secondary antibody and alkaline phosphatase in 2% milk-PBS were added and incubated for 45-60 min at RT. The plates were washed with TPBS and PBS prior to adding the substrate NPP and the absorbance read at 410 nm.

3.3.4 Immunofluorescence on HEK293T/VEGFR-3 cells

Coverslips were placed in 6 cm plates and coated with poly-L-lysine-A (Sigma-Aldrich, Switzerland) for 5 min at room temperature and left to dry for 30 min. 293T cells (1x10⁶ cells well⁻¹) were plated and cultured for 24 h at 37°C, 5% CO₂. Transient transfection with pCDNA/hVEGF-3D1-7TM was carried out by the calcium-phosphate method as described in 3.3.2. Washed coverslips were incubated with 3 nM scFv for 1 h at 4°C. Coverslips were washed again and incubated with anti-Flag M2 antibody (15 µg/mL) for 1 h at 4°C. The washing step was repeated and the coverslips incubated with goat anti-murine-FITC antibody (fluorescein isothiocyanate) (20 µg/mL) for 1 h at 4°C. The washed coverslips were fixed in 3.7% formaldehyde for 30 min at RT prior to mounting on slides with gelvatol and left to dry in the dark.
overnight. Coverslips were viewed with an Axiophot microscope under aqueous conditions and 40 x magnifications.

### 3.3.5 Flow cytometry on U937 cells

U937 cells were grown for 48-72 h (37°C, 5% CO₂). Per tube 1x10⁶ cells were added and washed in buffer. Cells were incubated with scFvs and secondary antibodies anti-Flag-M2 (30 µg/mL) and FITC-conjugated anti-murine-antibody (2 µg/mL) sequentially for 30 min on ice. The washed cells were fixed in 3.7% formaldehyde and analysed by FACS-Scan flow cytometer (Becton Dickinson, Mountain View, CA) and quantified using the CellQuest software (Becton Dickinson). Buffer for flow cytometry consists of PBS with 2 % FBS and 0.02 % NaN₃. All experiments were carried out in triplicates.

### 3.3.6 Determination of $K_d$

The dissociation constant was measured in solution as described before¹³⁹. Briefly: Varying concentrations of antigen (0–10 µg/mL) were incubated in solution with 1 nM scFv at RT overnight. A 96 well plate was coated with 10 µg/mL antigen at RT overnight. All solutions were made in PBS containing 0.02% BSA. The coated plate was washed 3x with PBS and blocked with 200 µL 2% MPBS at RT for 2 h. The preincubated solutions were added to the coated plate and incubated at RT for 45 min. The washed plate was thereafter incubated with secondary antibody anti-Flag M2 (15 µg/mL) and goat anti-murine-alkaline phosphatase (70 ng/mL) for additional 45 min at RT. The plate was washed 10x with PBS-Tween and 10x with PBS prior to addition of 100 µL NPP. The colour reaction was allowed to develop at 37°C for up to 5 h. All experiments were carried out in triplicates.

### 3.3.7 Competitive ELISA

Nunc plates with 96 wells were coated with 100 µL hVEGF-C-His (produced by S. Arn) at a concentration of 2 µg/mL (ca. 43 nM) overnight at RT. Both specific single chain antibodies AFA8 and AFC5 and the unspecific scFv A1cys were pre-incubated in a concentration gradient ranging from 0-16 nM with 54 nM hVEGFR-3-His for 1 h at RT. After blocking the plate with 2 %MPBS for 2 h at RT, the plate was washed with PBS and incubated with the preincubated scFv-hVEGFR-3 mix for 2 h at RT.
Then, the plate was washed again with PBS before incubating with an antibody mix containing the anti-Flag M2 (15 µg/mL) and the goat anti-murine-alkaline phosphatase antibody (70 ng/mL) for 1 h at RT. Finally, the plate was washed 5 x PBS before incubating with fresh NPP and measuring the absorbance at 410 nm.

### 3.3.8 Migration assay

The bottom side of floroblok plates (Falcon) with a pore size of 8 µm were incubated with fibronectin (10 µg/mL) for 60 min at RT in a closed hood, preventing evaporation. After carefully removal of the fibronectin, the plate was blocked with 100 µg/mL BSA for 60 min at RT in a closed hood. BSA was carefully removed and 1000 ng/mL hVEGF-C156S (variant which only recognises and binds VEGFR-3) (R&D Systems, USA) in 0.5 mL EBM (Endothelial Basal Medium) with 0.2% BSA was added to the bottom plate. Human lymphatic endothelial cells (HLECs) isolated from neonatal human foreskins (as described in Kajiya et al. 2005) were trypsinised and resuspended in EBM / 0.2% BSA to 1x10⁶ cells/mL. The scFvs A1cys and AFC5 were added to 100 µL cells at concentrations ranging from 25 nM to 100 nM and incubated for 5 min at RT prior to addition to the top insert of the plate. The plate was incubated for 3 h at 37°C, 5% CO₂. In 25 µL DMSO 50 µg calcein AM (Molecular Probes) was dissolved and mixed with 13 mL PBS and 13 µL Hoechst 33342 stain (Molecular Probes, USA). Calcein solution (0.5 mL) was added per well on a new 24 well plate and the insert with the membranes added and stained for 10 min at 37°C, 5% CO₂, before the migration was measured in a fluorometer (SpectraMax Gemini EM, Molecular Devises, USA; excitation 485 nm, emission 538 nm). To visualise migration under a microscope the cells were fixed for 30 min in the dark with 4% paraformaldehyde (PFA) in PBS and stored at 4°C.

### 3.3.9 Proliferation of lymphatic endothelial cells

A 96 well plate was coated with 10 µg/mL fibronectin for 30 min. HLECs were trypsinised and resuspended to 10’000 cells/mL before plating out at 1500 cells per well in EBM containing 20% FBS. After 24 h at 37°C, the medium was changed to 2% FBS for further 24 h incubation. Medium was removed and 150 µL 2% FBS medium containing 1000 ng/mL hVEGF-C156S and scFv was added and incubated for 72 h at 37°C. The wells were washed twice with PBS before adding 4-
methylumbelliferyl heptanoate (MUH) (0.1 mg/mL) in PBS and incubating the plate for 1 h at 37°C. The fluoresence produced was measured on a fluorometer with bottom reading function (SpectraMax Gemini EM, Molecular Devices, USA; 355 nm excitation, 450 nm emission).

3.3.10 Production of a stable cell line expressing VEGFR-3
The murine teratocarcinoma cell line F9 (5 x 10^5 cells) was transfected with 5 μg either circular or linearised pCDNA vector containing domains 1-7 and the transmembrane domain of hVEGFR-3 (pCDNA/hVEGF-3D1-7TM) by the calcium-phosphate method as described in 3.3.2. After 4 h incubation, fresh medium was added and cells were incubated for 24 h at 37°C, 5% CO₂. After 24 h the cells were split and incubated with 1.5 mg/ml G418 sulfate to select for neomycin resistant clones. At day 10 subclones were picked by detachting with 5 μl trypsin. Subcloning was carried out six times over 4 months until an almost completely transfected population was obtained. The transfection stability was evaluated by injecting the subclones s.c. in SV 129 mice and collecting the tumors after 10-14 days. The tumors were put through a sterile metal grid to obtain a single cell suspension and the expression of the receptor was analysed on a Western blot (cell lysates) and by flow cytometry.

3.3.11 Immunocytochemistry and immunohistochemistry
Immunocytochemistry on transfected F9 tumors was carried out in the laboratory of Prof. B. Odermatt, Departement of Pathology at the University Hospital Zürich (Switzerland). F9 and F9/VEGFR-3 cells were grown to confluency on culture slides overnight. The FBS was eliminated from the medium by washing twice with PBS and air drying the slides in a laminar flow hood for 3 h. The cells were fixed for 10 min in anhydrous 100% acetone and finally dried for another hour to eliminate any residual acetone prior to freezing the slides wrapped in aluminium foil at -80°C. The F9 and F9/VEGFR-3 cultureslides were stained with scFvs A1cys and AFC5.

Immunohistochemistry on human neonatal foreskin freeze-sections was carried out in the laboratory of Prof. Detmar, Departement of Pharmaceutical Sciences, ETH Zürich (Switzerland). Slides were incubated in acetone at -20°C prior to 80% (v/v) methanol at 4°C and finally PBS at RT for 5 min. The sections were blocked in 12% (v/v) PBS-BSA for one hour at RT. The antibodies (A1cys, AFC5, podoplanin and
LYVE-1) were left to interact with the sections at 4°C overnight. Between antibody incubations the slides were washed three times 8 min at RT. The slides were incubated further with anti-Flag M2 and subsequently with ALEXA-labelled tertiary antibodies (Molecular Probes) and Hoechst 33342 stain for 1 h at RT. Finally, slides were washed again with PBS before a final washing step in 10 mM Tris (pH 8.8).

### 3.3.12 Radioactive labelling of scFvs

For biodistribution analysis the scFv was labelled with $^{125}$Iodine. ScFv (150 µg) was mixed with PBS in an Iodogen-50-tube in a maximum volume of 300 µL. On ice 3 µL $^{125}$I (0.7 mCi) was added to the mix and left to incubate for 15 min. PBS/0.5% BSA (200 µL) was added and the whole mix was loaded on a pre-equilibrated PD-10 column (PBS/0.5% BSA). Fractions (0.5 mL) were collected and the radioactivity measured. The labelled scFv was contained in 2-2.5 mL and had an activity of 50-60 µCi/mL.

For SPECT analysis AFC5 was labelled with $^{131}$I, because higher activity is needed for detection. In PBS 1 mg of scFv was mixed with 10 µL $^{131}$I (5 mCi) on ice and incubated as described above. The labelled scFv was contained in 2 mL with an activity of 917 µCi/mL.

### 3.3.13 Biodistribution of iodine labelled scFvs in tumor bearing mice

Stably transfected murine F9 teratocarcinoma (F9/VEGFR-3) cells were maintained in DMEM medium without non-essential amino acids and pyruvate, supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin and 100 U/mL nystatin. The cells were harvested and resuspended in PBS. Female SV 129 mice were injected s.c. on the side of the back with $10^7$ cells in 50 µl. After 10-14 days, when the tumors had reached diameters of 0.5 – 1.5 cm, each mouse was injected with 5 µg scFv containing $1 \times 10^7$ cpm $^{125}$I in 100 µl PBS. At 5, 15, 30, 60, 120, 240, 360 min and 24 h after injection the animals were anaesthetised with Isofluorane and immediately sacrificed for the removal of blood, heart, liver, kidneys, spleen and tumor. Three animals were used for each time point. The organs were weighed and the radioactivity was measured using a gamma counter.

For SPECT (single proton emission computed tomography) analysis of biodistribution, F9 and F9/VEGFR-3 cells were grown as above and $0.5 \times 10^7$ cells in
50 µl were injected s.c. at shoulder height on the back of female SV 129 mice. After 10 days, when tumors had reached diameters of approximately 1.5 cm, each mouse was injected with 100 µg scFv labeled with 150 µCi $^{131}$I in 200 µl PBS. At 120 min after injection the animals were sacrificed in a CO$_2$ chamber and the scFv biodistribution analysed post mortem with a X-SPECT™ (Gamma Medica Inc.). SPECT data was acquired and reconstructed using the LumaGEM software (version 5.407 lum_10). CT data was acquired with the Gamma Medica™ (X-Ray CT-system) software and reconstructed by the Cobra software (version 4.5.1, detector size: pix 1024 x 1024, acquired projections: 256 and smoothing filter 1). Fusion of SPECT- and CT-data was performed by IDL Virtual Machine™ (version 6.0) software and images were generated with Amira™ (version 3.1.1) software.

### 3.3.14 Lymphangiogenesis mouse model

This experiment was carried out by Lucie Kopfstein in the laboratory of Prof. Gerhard Christofori, University Basel. Briefly, double transgenic Rip1Tag2 mice overexpressing VEGF-C were injected i.p. daily over three weeks with 0.5 mg AFA8 (approx. 25 mg/kg) recognizing mVEGFR-3. As a negative control, mice were injected with 100 µL PBS or 0.5 mg unspecific scFv A1cys. Paraffin sections of the pancreatic tumors were made and immunohistochemistry staining blood and lymphatic vessels was carried out. Tumor incidence, tumor volume and tumor associated LYVE-1 positive vessels were analysed.
3.4 Results

3.4.1 Characterisation of scFvs in vitro
Pre-incubating the scFvs (AFA2/AFC5) with the human receptor prior to incubating with immobilised antigen on a Western blot resulted in inhibition of binding of scFv to VEGFR-3. When the scFvs were incubated with murine antigen, they were still available for binding to the immobilised receptor, which means that these two scFvs are highly specific against human VEGFR-3 and show no cross-reactivity to the murine receptor (Fig. 12). The two bands seen, corresponding to VEGFR-3, are consistent with previous observations that a 175-kDa precursor of VEGFR-3 matures to a 195-kDa form, which is then proteolytically cleaved into a 125-kDa fragment\textsuperscript{132,140}. The protein runs higher due to glycosylation.

![Western blot](image)

*Figure 12: Western blot by using scFvs preincubated with antigen in solution. A SDS gel was loaded with cell lysate samples of transfected HEK293T/hVEGFR-3 (1.) and U937 cells (2.). Lane A: Binding of scFv AFA2 pre-incubated with the murine receptor. Lane B: Binding of the scFv AFA2 pre-incubated with a four time molar excess of hVEGFR-3. Lane C: Control, binding of non-pre-incubated scFv.*

Incubating varying concentrations of scFvs (AFA2/AFC5) with a constant amount of hVEGFR-3 resulted in a binding curve, showing that more than 5 nM scFv is needed to saturate 1 µg of immobilised human antigen (Fig. 13). This indicates a relatively high affinity for the scFv antibody. In the same way, binding of scFvs AFA8 and AFF1 to both human and murine VEGFR-3 was confirmed by ELISA and Western blot.
Figure 13: Binding of scFvs AFA2 (♦) and AFC5 (▼) to immobilised VEGFR-3 (10 µg/mL). (■) specific scFv (AFA2/AFC5) binding on plastic. Binding of the unspecific scFv A1cys to the receptor was below the detection limit.

The affinity of scFv AFA2 and AFC5 was determined by calculating the dissociation constant ($K_d$). Incubating varying concentrations of antigen with scFv in solution prior to addition to a pre-coated plate enables calculation of the concentration of free antibody, free antigen and antibody-antigen complex. For scFv AFA2 a Kd of 10.64 nM was obtained, which is in the expected range for a single chain antibody generated from the ETH-2 library\textsuperscript{64}, the Kd of 8.9 nM for AFC5 (Fig. 14) confirms the slightly higher affinity of this scFv seen also in the other assays.

Figure 14: Calculated dissociation constants for scFvs AFA2 and AFC5 on human VEGFR-3. Scatchard binding plots of scFv to receptor were calculated from the binding data according Friguet et al.\textsuperscript{139}, where $\nu$ is the fraction of bound scFv and $\alpha$ the concentration of free antigen at equilibrium. The total concentration of scFv was $10^{-9}$M. The experiment was carried out in triplicates.
Transfected HEK 293T/hVEGFR-3 cells exposed to varying concentrations (1-10 nM) of scFv showed that only 1 nM scFv is required to visualise binding to the receptor (Fig. 15). The scFvs showed no background binding to untransfected cells, nor does an unspecific single chain antibody (A1cys) show any binding to transfected cells (Fig. 16).

![Figure 15: Immunofluorescence showing scFv binding to VEGFR-3 on transiently transfected HEK 293T cells. (A) secondary antibodies anti-flag M2 and anti-murine-FITC (B-E) scFv AFA2 ranging from 1-10 nM (G-J) scFv AFC5 ranging from 1-10 nM, (F) unspecific scFv A1(164 nM)](image1)

![Figure 16: Visualisation of scFv binding to membrane-bound receptor on transiently transfected HEK 293T/hVEGFR-3 cells (A) Secondary antibodies anti-flag M2 and anti-murine-FITC on transfected cells. (B) scFv AFC5 on VEGFR-3 negative cells. (C) Unspecific scFv A1cys on VEGFR-3 positive cells. (D) Cross-reactive scFv AFA8 on HEK 293T/hVEGFR-3 cells. (E) scFv AFC5 on HEK 293T/hVEGFR-3 cells. (F) Cross-reactive scFv AFF1 on hVEGFR-3 transfected cells.)](image2)
The diagnostic potential of AFC5 was investigated by immunohistochemistry on human neonatal foreskin section. The unspecific scFv A1cys was used as a negative control and the binding of AFC5 was compared to LYVE-1 and podoplanin. No co-staining was seen on LYVE-1 positive lymphatic vessels with A1cys (Fig. 17-B1). AFC5 recognised cells on the LYVE-1 positive lymphatics, but did not stain the whole vessel as seen in Figure 17-D1 and –D2. However in addition, scFv AFC5 recognised LYVE-1 negative vessels (Fig. 17-D2). It would be interesting to carry out triple stainings with antibodies against CD31 and for example podoplanin to investigate what these other vessels are.

Figure 17: Immunohistochemistry on human neonatal foreskin sections. A and C: Hoechst staining of cell nuclei. B: Overlay of LYVE-1 (green) staining of lymphatic endothelial cells and unspecific A1cys binding (red), B1: enlargement of a lymphatic vessel not showing any overlap in staining. D: Overlay of LYVE1 (green) staining of lymphatic endothelial cells and scFv AFC5 binding (red) to VEGFR-3 on certain cells. D1 and D2: enlargement of stained vessels, white arrows indicate regions with overlapping staining.

Binding of scFvs AFA2, AFC5, AFF1 and AFA8 to human VEGFR-3 positive erythroleukemia cells (U937) was seen as an increase in fluorescence in comparison to the autofluorescence of U937 cells, as shown in Figure 18 for AFC5. This shift indicates the specific binding of the scFvs to the naturally expressed receptor on the surface of these cells. Discrepancies in the degree of shift, when repeating the
Inhibiting lymphangiogenesis is possible, if signalling via the receptor is blocked. One possible way of accomplishing this is blocking binding of the ligands VEGF-C and VEGF-D to the receptor. To check if the specific scFvs AFA8 and AFC5 block hVEGF-C binding to hVEGFR-3, a competitive ELISA was carried out (Fig. 19). As a negative control the unspecific single chain A1cys was used in parallel and as a positive control the plate was also coated with hVEGFR-3 to prove that the scFv samples used for the assay were functional. Unfortunately, AFA8 did not inhibit the binding, which also to some extent explains the results obtained with the lymphangiogenesis mouse model in 3.4.3. Speculations on the reasons for this result are discussed in chapter 3.5. The specific single chain AFC5 strongly inhibits the binding of soluble VEGFR-3 to immobilised VEGF-C, making this antibody a useful tool for both therapeutic and targeting strategies.

*Figure 18: Specific binding of scFv AFC5 to VEGFR-3 on naturally expressing U937 cells seen as shift in fluorescence compared to the autofluorescence of the cells measured by flow cytometry. Cells (1x10^6) were incubated with 5 nM scFv on ice and fluorescently labelled with a secondary antibody for visualisation of binding.*
Since AFC5 prevents binding of hVEGF-C to hVEGFR-3 as shown in ELISA, we wanted to clarify whether this also has functional consequences. Until recently it has not been possible to investigate therapeutic effects on lymphatics in vitro, since isolated lymphatic endothelial cells (LEC) were not available. The discovery of specific lymphatic markers has made it possible to select for these cells. To test the therapeutic potential of AFC5, migration and proliferation assays were carried out on LEC isolated from human neonatal foreskin in collaboration with the institute of Pharmaceutical Sciences, ETH Zürich. To be able to only investigate VEGFR-3 activated migration and proliferation, a mutated form of hVEGF-C was used. When Cys156 is exchanged for serine the resulting VEGF-C156S selectively activates only VEGFR-3, even after further proteolytic processing. In the migration assay (Fig. 20), the PBS controls suggest that hVEGF-C156S is not able to stimulate migration of these cells. Although, when comparing the wells incubated with scFv AFC5 to the PBS control, the difference in migration seen with the Student's t-test is statistically significant ($P= 0.0119$). When comparing specific and unspecific scFv at the same concentration the difference in migration is even more dramatic ($P=0.0036$). However, these are only preliminary results and will be optimised further.
Figure 20: Analysis of migration of lymphatic endothelial cells stimulated with VEGF-C156S and incubated with unspecific A1cys or specific AFC5 scFv at concentrations ranging from 25-100 nM (* P < 0.05, ** P < 0.01).

Similar results were obtained when investigating the inhibition of hVEGF-C156S induced proliferation (Fig. 21). Incubated during 72 h hVEGF-C156S was able to induce proliferation of HLEC as seen in the PBS controls. When comparing cell proliferation in the wells incubated with scFv AFC5 to the PBS controls, a significant difference was observed (P=0.0174) as well as when compared to the unspecific scFv at the same concentration (P=0.034). These are also preliminary results and the assay will be further optimised.

Figure 21: Analysis of proliferation of lymphatic endothelial cells stimulated with hVEGF-C156S and incubated with unspecific A1cys or specific AFC5 scFv at concentrations ranging from 25-100 nM (* P < 0.05).
3.4.2 Generation of stably transfected F9 teratocarcinoma cells (F9/VEGFR-3)

VEGFR-3 positive cells were obtained by transfecting with either linearised or circular DNA, although the clones from the circular DNA produced more receptor positive cells. After six rounds of subcloning, a culture with approximately 70 % cells expressing the receptor was obtained. The number of receptors on the surface of these cells was too low for immunofluorescence to be carried out, but with flow cytometry a shift in fluorescence could be seen, when the cells were incubated with specific scFvs (Fig. 22a). By immunocytochemistry the specific binding of scFv AFC5 to VEGFR-3 on these cells could be demonstrated by comparing with the staining by the unspecific scFv A1cys (Fig 22b). VEGFR-3 expression was thus visualised as red staining of the cells (Fig 22c). Not all cells were stained red, which confirms the partial transfection of this cell line, as also seen by flow cytometry.

Figure 22: A) VEGFR-3 expression on a subclone of F9/VEGFR-3 (subclone 3b-4) seen as shift in fluorescence due to specific binding of AFC5 to the cell surface, compared to the autofluorescence of the cell. Cells (1x10^6) were incubated with 5 nM scFv on ice and fluorescently labelled with a secondary antibody for visualisation of binding. VEGFR-3 expression visualised by immunocytochemistry on F9/VEGFR-3 (3b-4) incubated with unspecific scFv A1cys (B) or anti-VEGFR-3 scFv AFC5 (C). Red colour shows VEGFR-3 expression stained by binding of antibody to the receptor.

F9/VEGFR-3 cells were injected s.c. into the back of female SV 129 mice and let grow for 10-14 days, until a tumor diameter of 1-1.5 cm was reached. Tumors were excised and a single cell suspension was made. These single cells were used to
analyse receptor expression by flow cytometry as well as by preparing cell lysates for Western blot analysis (Fig. 23). Interestingly, no receptor was detected in the *ex vivo* cell lysates of the F9/VEGFR-3 2b-3 subculture by Western blot, although a shift in fluorescence was seen with these cells by flow cytometry, which suggests that the cells are still expressing the receptor. No explanation was found for this discrepancy.

Figure 23: VEGFR-3 expression visualised by Western blot, using the scFv AFC5. Lane 1: Marker, lane 2: cell lysate from negative cells (F9), lane 3: cell lysate from subclone 2b-3 grown in vitro, lane 4: cell lysate of subclone 2b-3, *ex vivo*, lane 5: cell lysate subclone 3b-4 grown in vitro, lane 6: cell lysate of subclone 3b-4 *ex vivo*.

### 3.4.3 *In vivo* properties of scFvs

The biodistribution of specific versus unspecific scFvs in female SV 129 mice bearing F9/VEGFR-3 or F9 teratocarcinoma tumors was evaluated. For detection the scFvs were labelled with $^{125}$Iodine (3.3.12., page 48). F9 tumors were used because they are highly vascularised and do not invade the surrounding organs.

The distribution of the scFvs was analysed by removal of tumor, heart, liver, kidney, spleen and blood at given time points, followed by measuring the radioactivity. Table 2 summarises the biodistribution of the scFvs at different time points.

The scFv AFC5 accumulated in a specific manner in the tumor, showing a typical fast blood clearance. Already 1 h after injection, 50% of the scFv had been cleared from the blood, and 2.5 % injected dose (%ID) per organ had accumulated in the tumor, with 2.75 % ID still remaining 4 h after injection. AFC5 rapidly accumulated in the kidney (18 % ID, 15 min after injection) but showed fast clearance. The
unspecific scFv A1cys showed similar blood clearance, but showed only a temporary accumulation in the tumor. After 30 min 1.75 %ID had accumulated in the tumor, but 3 h after injection only 0.5 %ID remained.

In mice bearing the wildtype F9 tumor the scFv AFC5 showed a similar tumor accumulation pattern as the unspecific scFv A1cys in the transfected tumors. One hour after injection only 1% ID had accumulated in the tumor and after 3 h only 0.5 % ID remained. The different accumulation patterns are illustrated in figure 24. Since the single chain AFC5 accumulated and remained in the tumor at higher levels than the controls, the tumor to blood ratio (Fig. 25) was higher, although all single chain antibodies showed similar blood clearance patterns.

![Figure 24](image-url)

*Figure 24: Comparison of single chain antibody blood elimination (A, C, E) and tumor accumulation (B, D, F) over time, expressed as % injected dose (%ID) per organ. (A+B) AFC5 in F9/VEGFR-3 s.c. bearing mice, (C+D) A1cys in F9/VEGFR-3 s.c. bearing mice and (E+F) AFC5 in F9 s.c. bearing mice. Three mice per group were injected i.v. with $^{125}$I labelled single chain antibodies and sacrificed at different time points.*
Table 2: Organ distribution of specific (AFC5) versus unspecific scFv (A1cys) over time. Three mice bearing s.c. F9 or F9/Flt4 tumors per group were injected with $^{125}\text{I}$ labelled scFv and killed after different time points. Results are expressed as % of injected radioactive dose (%ID). Heart and spleen showed insignificant accumulation (< 0.4 %ID) of the single chain antibodies (data not shown).

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**Figure 25:** Comparison of tumor to blood ratios of, (x) unspecific scFv A1 on transfected tumors, (▲)scFv AFC5 on negative F9 tumors and (▼) the specific scFv AFC5 on transfected F9/Flt4 tumors at different time points. Three mice per group bearing s.c. F9 or F9/Flt4 tumors were injected with $^{125}$I labelled single chain antibodies and killed at different time points. Results are expressed as the ratio of % injected dose of radioactivity per organ.

To visualise the biodistribution of AFC5 in SV129 mice bearing either no tumor, F9 control tumor or the receptor expressing F9/VEGFR-3 tumor, a SPECT analysis was carried out. The tumors were positioned on the back of the mice so that they do not shield any other organ. The scFv was labelled with $^{131}$I and injected i.v. in the tail vein of the mice. After 2 h the mice were sacrificed by CO$_2$ and analysed post mortem. In the mouse without tumor all the scFv accumulated in the thyroid gland and the stomach, as expected for $^{131}$I. This is convincingly illustrated in Figure 26-B. When comparing Figure 26-E and 26-H, one sees some unspecific and insignificant accumulation of AFC5 in the F9 tumor, as compared to the accumulation in the F9/VEGFR-3 tumor. Figure 26-H visualises the specific tumor accumulation of AFC5.
Figure 26: SPECT analysis of $^{131}$I-AFC5 biodistribution in SV129 female mice. Mice were either injected with $0.5 \times 10^7$ F9 tumor cells s.c. or with stably transfected F9/VEGFR-3 cells. Mice without tumor (A-C) were compared to non-receptor expressing tumors (D-F) as well as VEGFR-3 positive tumors (G-I).

In collaboration with Prof. G. Christofori at the University of Basel, we tested if the scFv AFA8 can block the formation of new lymphatic vessels in a lymphangiogenesis mouse model. Although AFA8 was not able to block ligand binding to the human receptor in vitro, we analysed if this scFv could block receptor function in a double transgenic mouse model overexpressing VEGF-C (Rip1Tag2/VEGF-C), with pancreatic β-cell tumors surrounded by an extensive network of lymphatics. Lymphangiogenesis inhibition in these mice was unfortunately insignificant (Fig. 27). Only a reduction of 21% in tumor associated LYVE-1 positive vessels could be achieved in comparison to the controls (37.56% positive vessels with controls vs. 30.63% with specific AFA8 scFv). Reasons for these findings are discussed in chapter 3.5.
Figure 27: Comparison of LYVE-1 positive vessel association with tumors. (1) % of tumors with no association to LYVE-1 positive cells. (2) % of tumors with less than 50% of tumor circumference associated with LYVE-1 positive cells. (3) % of tumors with more than 50% of tumor circumference associated with LYVE-1 positive cells. (4) % of tumors completely surrounded by LYVE-1 positive cells. Experiment was carried out with 6 mice per group (pooled controls, PBS, unspecific scFv A1cys, anti-VEGFR-3 scFv AFA8).
3.5 Discussion

In this chapter we described how the specificity of scFv binding to VEGFR-3 was evaluated. As described in chapter 2, one can produce antibodies against almost any antigen by phage display technology, but the obtained antibodies will vary in binding specificity against the target. Therefore, it is important to verify the specific targeting properties of an antibody against its antigen. We could demonstrate specific binding of our scFvs in vitro on both recombinant and purified VEGFR-3, as well as in cell lysates made from receptors expressed on HEK 293T and of naturally VEGFR-3 expressing U937 cells. Specific binding was seen at concentrations as low as 1 nM.

In comparison, commercially available anti-human VEGFR-3 IgG antibody is used at 3nM for ELISA. Pre-incubation experiments showed that the initially obtained scFvs, AFA2 and AFC5, are not cross-specific against mVEGFR-3. Therefore, additional phage display selection rounds were carried out to obtain cross-reactive scFv antibodies. The new scFvs AFA8 and AFF1 were shown to exert cross-reactivity to both the human and murine receptor, which makes them a valuable tool for investigating VEGFR-3 expression.

Since antibody affinity plays a determining role in tumor targeting efficiency, it was important to confirm that our scFvs are highly selective for VEGFR-3. The $K_d$ values for AFA2 and AFC5, 10.6 nM and 8.9 nM respectively, are in the same range as those reported for other scFvs from the same library$^{127}$. Other researchers have reported $K_d$ values of 3.9 nM and 143 nM for their scFv antibodies $^{130,131}$. Tachibana et al. report $K_d$ values for their Fab' fragments ranging from 3-20 nM and Borrebaeck et al. compared their Fab' and scFv fragments with 6 nM and 25 nM respectively $^{141,142}$. Dissociation constants of 10-0.1 nM have been reported for several therapeutic antibodies $^{143}$. The $K_d$ values obtained for AFA2 and AFC5 are at the lower end of the scale when compared to these other reported values and therefore indicate high affinity of these two scFvs for VEGFR-3.

It was also essential to confirm the specific binding of the scFvs to the native receptor as shown with the erythroleukemia cell-line U937. The scFv binding to the receptor in U937 cell lysates confirms the findings of Schoppmann et al.$^{132}$, who showed that this cell line endogenously expresses VEGFR-3. Binding was
determined by flow cytometry as shown in Figure 18. A shift in cell-fluorescence was seen, when the cells were incubated with specific scFvs, but not with unspecific single-chain antibodies. Discrepancies in the degree of the shift, when repeating the experiment, are thought to be due to differential receptor expression depending on the growth stage of the cells. This behaviour in receptor expression has been seen for other cell lines as well (personal communication Prof. Dr. G. Christofori).

The diagnostic potential was evaluated by immunohistochemistry on neonatal human foreskin sections. The scFv AFC5 was able to recognise VEGFR-3 on cells in lymphatic vessels, as seen by double staining with LYVE-1. The secondary antibody for AFC5 (anti-Flag M2) showed high background staining, which would need to be optimised in future experiments. Summarised, our scFvs show a higher specificity in vitro than expected for small single chain antibodies and hold promise as a diagnostic tool.

The Rip1Tag2 VEGF-C overexpressing lymphangiogenesis mouse model was used to test if the scFvs can block receptor signalling and thus inhibit lymphangiogenesis. The scFv AFA8 was chosen since it recognises mVEGFR-3. Unfortunately this scFv did not block VEGF-C binding to the receptor, which was later confirmed by competitive ELISA. A small trend was visible, but the fact that fewer lymphatic vessels were seen surrounding the tumor was not significant. The scFv recognition site on the receptor must therefore be located too far away from the VEGF-C binding site to interfere. This system would be excellent for a proof of principle and it would therefore be interesting to test other selected cross-specific scFv in this mouse model.

Both AFA8 and AFC5 were tested by competitive ELISA whether they could block hVEGF-C binding to hVEGFR-3. VEGF-C was immobilised on ELISA plates while scFv and receptor were pre-incubated before being added. AFC5 blocked VEGF-C binding to the receptor efficiently at low concentrations. We were also interested to see whether co-incubating scFv with VEGF-C prior to addition to the immobilised receptor, would give the same results, however this experiment did not work. This was either due to kinetic properties, because VEGF-C has a higher affinity for the receptor than the scFv, or the binding sites on the receptor were not accessible for either protein. This could be tested by repeating the experiment and detecting VEGF-
C by an anti-VEGF-C antibody. To functionally test inhibition of ligand to receptor interaction, migration and proliferation assays were carried out. A variant hVEGF-C (hVEGF-C156S), which only recognises VEGFR-3 and does not bind VEGFR-2, was used to stimulate migration and proliferation. This enables one to specifically monitor VEGFR-3 signalling. In the preliminary experiments scFv AFC5 significantly inhibited migration in comparison to the PBS control ($P=0.0119$) and the unspecific scFv A1cys ($P=0.0036$). Interestingly, the controls showed no further induction of migration by hVEGF-C156S, presumably due to the presence of endogenous growth factor produced by the cells. VEGF-C is a less effective stimulant of migration than VEGF-A (personal communication Dr. C. Halin) and therefore the 3 h incubation time for the migration may have been too short. The same concentration of ligand induced proliferation of the same cells over 72 h and inhibition of receptor function by AFC5 was statistically significant in comparison to the PBS control ($P=0.0174$) and the unspecific scFv ($P=0.0334$). The proliferation results are preliminary and will need to be confirmed. To date few reports exist on the potential of single chain antibodies to inhibit migration and proliferation of cells. One example is the scFv described by Jimenez et al., which inhibited VEGF-C induced migration in bovine aortic endothelial cells\textsuperscript{144}.

In vivo properties of the scFvs were tested in biodistribution studies using a syngeneic mouse model with stably transfected F9/VEGFR-3 cells. The stability of VEGFR-3 expression in the transfected cells, after culturing for 14 days in vivo, had been tested previously and confirmed by Western blot analysis and flow cytometry. However, these cells display low receptor expression both in vitro and in vivo, as seen by flow cytometry and immunocytochemistry.

The specific scFv AFC5 was rapidly eliminated from the blood and accumulated in the tumor in a specific manner, resulting in 2.4 %ID ($\pm 0.9$) per organ after 60min and 2.8 %ID ($\pm 1.8$) still remaining in the tumor after 240min. The unspecific scFv A1cys showed similar blood elimination properties, but accumulated to 1.7 %ID ($\pm 0.7$) in the tumor after 30min, and was eliminated at a fast rate with only 0.5 %ID ($\pm 0.3$) remaining after 180min. The tumor accumulation pattern of A1cys is typical for an unspecific antibody. A similar distribution pattern was also found for scFv AFC5 in mice with control F9 tumors lacking VEGFR-3. The biodistribution pattern was successfully visualised by SPECT, very effectively showing specific tumor
accumulation of AFC5 in receptor expressing tumors compared to F9 tumors (see Fig. 26). Accumulation of AFC5 in VEGFR-3 expressing tumors is specific and long-lasting, which makes this scFv a promising delivery agent. The rather low tumor accumulation might be due to the low receptor expression on the transfected cells. A more suitable mouse model would be needed to confirm these results.
4 PREPARATION AND CHARACTERISATION OF IMMUNOLIPOSOMES

4.1 Abstract

The goal of this project was to target VEGFR-3 for tumor directed drug delivery using immunoliposomes. For this purpose the generated scFvs were modified on their C-terminal end with three cysteines as described in 2.3.3. These cysteines are needed for scFv-coupling to the liposome surface.

Liposomes were prepared using the extruder method to obtain small unilamellar vesicles with mean diameters of 100 nm. All liposomes contained 100 mol% (hydrogenated) soya phosphatidylcholine ((H)SPC), 20 mol% cholesterol and 7 mol% 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine(-PEG2000) (DPPE(-PEG)). For immunofluorescence and flow cytometry the liposomes were labelled endogenously with the fluorescent dye 3,3'-diocta-decyloxacarbo-cyanine perchlorate (DiO). The liposomes were further modified with sulfosuccinimidyl 4-(N-maleimimidomethyl)cyclohexane-1-1-carboxylate (sulfo-SMCC) to introduce maleimide groups for the reaction with thiol groups present on the C-terminal end of the single chain antibodies. The antibodies were reduced and the monomers coupled to the liposomes. According to previous work approximately 200 single chain antibodies can be coupled to the surface of 100 nm liposomes. For cytotoxicity studies the liposomes were loaded with cytotoxic drugs, either N⁴-octadecyl-1-β-D-arabinofuranosylcytosine-(5'-5')-3'-C-ethinylcytidine (ETC-NOAC) or 2'-deoxy-5-fluorouridyl-N⁴-octadecyl-1-β-D-arabinofuranosylcytosine (5FdU-NOAC). Target cell binding of these anti-VEGFR-3 scFv-immunoliposomes was studied in vitro. Both AFA8- and AFC5-immunoliposomes showed specific binding to either VEGFR-3 transfected HEK 293T cells as shown by immunofluorescence or to U937 cells naturally expressing the receptor as demonstrated by flow cytometry. Cytotoxic immunoliposomes were tested for their specific drug targeting properties on receptor negative F9 cells or stably transfected F9/VEGFR-3. The specific immunoliposomes showed 30% specific killing of VEGFR-3 positive cells compared to 0-10% killing seen with unspecific immunoliposomes and control liposomes. The weak cytotoxicity exerted by the immunoliposomes on the F9/VEGFR-3 cells is probably due to slow release of drug from the liposomes. Because the receptor on the transfected cells is
not functional, no receptor mediated internalisation of the liposome is expected to take place, which is needed for cytotoxic action. Biodistribution studies showed very fast blood elimination of the immunoliposomes compared to control liposomes. This might be due to both a non-optimal mouse model as well as increased RES uptake due to the recognition of the bulky scFv-$^{99}$Tc complex on the surface or the rather high number of scFv antibodies on the liposomes.

4.2 Introduction

To date, liposomes are being used to deliver chemotherapeutics, anti-angiogenic drugs and anti-sense DNAs to targets in tumors and their vasculature. Homogeneous unilamellar vesicles ranging in size from 50 to 200 nm are most commonly used. The preparation of such SUV is simple, by mixing the components in an organic solvent which then is evaporated. The thin film which results, is resuspended in buffer by agitation and/or sonication. At this stage in the process MLVs are formed, which are too large and heterogenous for medical applications. There are several methods for producing SUVs from MLVs, e.g. sonication, filter extrusion, homogenization or detergent dialysis. The most common being the extruder method, where the liposome suspension is extruded at high pressure through a polycarbonate filter with a defined pore size.

Sterically stabilised liposomes are produced to evade the host immune system. Attaching PEG to the surface of the liposome forms a protective layer around the vesicle, protecting it from recognition by the RES. Pegylated liposomes show increased circulation times in vivo, compared to conventional liposomes (for more details see 1.3.4).

To achieve specific in vivo homing to defined targets in the body, liposomes may be coated with whole IgG or antibody fragments recognising epitopes expressed on target cells. A disadvantage of IgG-immunoliposomes is their rapid uptake by cells of the RES, possibly mediated by Fc receptors interacting with the constant region of the antibody molecule. The latter obstacle being minimised by the use of Fab' or scFv fragments not containing the Fc portion of the IgG. In addition single chain antibodies are promising targeting tools for coupling to liposomes because of their
small size. Whereas immunoliposomes with whole IgG antibodies or Fab’ fragments have been tested over the years\textsuperscript{122,148}, not much is known about immunoliposomes with scFvs\textsuperscript{101}. It is expected that such immunoliposomes will more easily evade the host immune system\textsuperscript{120,149}.

ScFv-immunoliposomes that have been evaluated for tumor drug delivery include anti-HER2-liposomes tested on HER-2 overexpressing breast cancer cells and anti-ED-B-liposomes, which target the ED-B domain of B-fibronectin expressed in the extracellular matrix of tumors. Anti-HER2 immunoliposomes have recently entered clinics and show promising results\textsuperscript{112,119}.

When administering liposomes or immunoliposomes subcutaneously they get taken up by draining lymphatic capillaries and become actively captured by macrophages in the regional lymph nodes\textsuperscript{150}. Subcutaneous or intra-lymphatic administration of VEGFR-3 specific immunoliposomes could thus be used to eliminate lymph node metastasis. Furthermore, administering labelled VEGFR-3 specific immunoliposomes s.c. could be used diagnostically to visualise receptor expressing metastatic tumor cells.

In this chapter the evaluation of the targeting properties of anti-VEGFR-3 scFv-immunoliposomes \textit{in vitro} by immunofluorescence and flow cytometry is described. Once specific targeting was established, the liposomes were loaded with the drugs ETC-NOAC or 5FdU-NOAC and tested for drug delivery and cytotoxic effects on F9/VEGFR-3 cells. Cytotoxicity levels were rather low, probably due to the model system using transfected cells. \textit{In vivo} targeting was also evaluated, but unfortunately did not show promising results. The anti-VEGFR-3-immunoliposomes will need to be optimised further and a more suitable tumor cell line is required.
4.3 Methods

4.3.1 Materials
All chemicals were obtained from Fluka (Buchs, Switzerland) unless stated otherwise. Phospholipon 90H (hydrogenated soy phosphatidylcholine, HSPC) was purchased from Nattermann Phospholipid GmbH (Köln, Germany). Soy phosphatidylcholine (SPC) was purchased from L. Meyer, Sugro AG, Basel. 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE-PEG-2000) was from Sygena Ltd. (Liestal, Switzerland). 3,3'-dioctadecyloxacarbocyanine perchlorate (DiO) was obtained from Molecular Probes (Leiden, The Netherlands). Sulfocuccinimidyl-4-((N-maleimidomethyl) cyclohexane-1-carboxylate (Sulfo-SMCC) was from Pierce (Lausanne, Switzerland). Cysteine was purchased from Sigma-Aldrich, Buchs (Switzerland).

4.3.2 Liposome production
Small unilamellar liposomes were prepared by mixing (H)SPC:Cholesterol:DPPE(-PEG)-NH₂ at a molar ratio of 1:0.2:0.07 in MeOH : CH₂Cl₂. The organic solvent was evaporated in a rotary evaporator at 40°C for 30min. The lipid film was resuspended in HBSE buffer (10 mM HEPES, 150 mM NaCl, 0.1 mM EDTA, pH 7.5) using glassbeads and sonication. Liposomes with a diameter of 100 nm were prepared with a Lipex™ extruder (Lipex Biomembranes Inc., Vancouver, Canada), by pushing the lipid mix through 400 nm, 200 nm and finally 100 nm size filters. Size and stability of the liposomes were analysed with a particle sizer (Nicomp Model 370, Santa Barbara, USA). For the preparation of cytotoxic liposomes 5 mg/ml of the cytotoxic drugs ETC-NOAC or 5-FdU-NOAC were added to the organic lipid mix. For the preparation of fluorescent liposomes DiO was added to the lipid mix at a molar ratio of 0.004 compared to HSPC.

4.3.3 scFv coupling
The liposomes were incubated with sulfo-SMCC at a molar-ratio of amino- to maleimide groups of 1:2.5 for 3 h at RT under agitation. Excess sulfo-SMCC was removed by dialysis against HBSE at 4°C overnight. After reduction of the scFv dimer (4 mg/mL) in HBSE with a 2 mM final concentration of tributylphosphine (TBP) for 4 h at RT, sulfo-SMCC modified liposomes in HBSE were added and incubated
for 72 h at 4°C under nitrogen atmosphere. Modified liposomes and non-reacted scFv were separated on a Ficoll™ PM70 (Amersham Biosciences, Sweden) gradient by ultracentrifugation (16 h, 35'000 rpm, 4°C) and subsequently dialysed against PBS.

4.3.4 Flow cytometry of α-VEGFR-3-scFv-(PEG-)immunoliposomes on U937 cells

U937 cells were grown for 48-72 h in RPMI medium supplemented with 10% FBS and antibiotics. Per 5 mL polystyrene round bottom tube 1x10^6 cells were added and washed in buffer. Cells were incubated with the different liposomes for 60 min at 37°C. The washed cells were fixed in 3.7% formaldehyde and analysed in a FACS-Scan instrument (argon laser 488/ emission 530). The buffer for flow cytometry consisted of PBS with 2 % Fetal Bovine Serum (FBS) and 0.02 % NaN₃. All experiments were carried out in triplicate.

4.3.5 Immunofluorescence of α-VEGFR-3-scFv-(PEG-) immunoliposomes on HEK293T/VEGFR-3 cells

HEK 293T cells were cultured and transfected as described in 3.3.3. Washed coverslips were incubated with DiO-liposomes for 1 h at 37°C. The washed coverslips were fixed in 3.7% formaldehyde for 30 min at room temperature prior to mounting on slides with gelvatol and left to dry in the dark overnight. Coverslips were viewed with an Axiophot microscope under aqueous conditions and 40 x magnifications.

4.3.6 Cytotoxicity assay on F9/VEGFR-3 tumor cells

Black 96-well microplates were coated with 0.1 % gelatine prior to incubating with approximately 7500 F9 or F9/Flt4 cells per well. The cells were left to attach and grow overnight at 37°C, 5% CO₂. Various concentrations of cytotoxic and control liposomes in medium were added to the cells in triplicates and allowed to interact for 30, 60 or 120 min at 37°C, 5% CO₂. The liposomes were washed off and the cells incubated with fresh medium overnight. After 24 h an in vitro resazurin based cytotoxicity assay was carried out, to visualise the metabolic activity of the cells.
To check for specific binding of immunoliposomes the cells were preincubated with 1 µM unspecific or specific scFv prior to addition of 100 µM ETC-NOAC-immunoliposomes.

To test cytotoxicity of ETC-NOAC the free drug was incubated with F9/VEGFR-3 cells at concentrations ranging from 0-5000 µM for 30 min at 37°C and left to recover for 24 h in fresh medium.

4.3.7 Biodistribution of $^{99}$Tc-labeled scFv-PEG-immunoliposomes in tumor bearing mice

Liposomes were prepared as described in 4.3.2 but additionally containing 5 mg/mL MT-23 chelator for radioactive labelling. This chelator is a lipophilic dihexadecyl derivative of the $^{99m}$Tc(I)-tricarbonyl complex, prepared in the laboratory of Prof. Schibli, ETH Zürich. The pegylated SPC liposomes were either coupled with cysteine or scFv AFC5 as described in 4.3.3. The liposomes were then labeled by coupling $^{99}$Tc to the chelator in the membrane and separated on a Sephadex column. The radiolabeled liposomes were injected (20-30 µCi/mouse) i.v. in F9/hVEGFR-3 bearing mice 14 days post tumor cell application and the mice were anaesthetised with Isoflurane and immediately sacrificed for the removal of blood, heart, liver, kidney, spleen and tumor. Three animals were used for each time point (4 h and 24 h). The organs were weighed and the radioactivity measured.
4.4 Results

4.4.1 Characterisation of immunoliposomes \textit{in vitro}

The targeting properties of the DiO-liposomes were analysed by both immunofluorescence on transiently transfected HEK 293T/VEGFR-3 cells as well as by flow cytometry on endogenously VEGFR-3 expressing U937 cells. Low but insignificant background binding of the unspecific immunoliposomes A1cys-DiO-liposome was seen, compared to the specific binding of AFA8-DiO- and AFC5-DiO-liposomes. Unspecific cell binding of all liposomes was also tested on non-transfected HEK 293T cells. The results are summarised in Figure 28. The specific binding to individual cells is better visualised in Figure 29.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure28.png}
\caption{Comparison of the binding of DiO-liposomes on transiently transfected HEK 293T/VEGFR-3 cells. A-D represents unspecific background binding on non-transfected HEK 293T cells and E-H represents transiently transfected HEK 293T/VEGFR-3 cells. A + E: control liposomes, B + F: unspecific A1-DiO-liposomes, C + G: specific AFA8-DiO-liposomes, D + H: specific AFC5-DiO-liposomes. Cells on coverslips were incubated with DiO-liposomes for 1 h at 37°C.}
\end{figure}
Figure 29: Visualisation of liposome binding to membrane-bound receptor on transfected cells. Transiently transfected HEK 293T/hVEGFR-3 cells cultured 24 h on polylysine coated coverslips were incubated with DiO-liposomes for 1h at 37°C. (A) Control liposomes (B) Unspecific immunoliposomes (C) Specific scFvAFA2-immunoliposomes (D) Specific scFvAFC5-immunoliposomes on transfected cells.

By flow cytometry considerable high background binding of unspecific A1cys-DiO-liposomes was seen on U937 cells. This cannot be due to unspecific antibody binding to the cells, since A1cys on its own did not bind. A possible explanation could be that the scFv enhances unspecific liposome attachment to the cell surface. A slight shift in fluorescence was also seen with control liposomes (data not shown), but still the fluorescent intensity of the cells bound with specific DiO-liposomes was greater than both controls, as visualised in figure 30. More liposomes bound to U937 cells when incubated at 37°C in comparison to 4°C, indicating possibly receptor mediated endocytosis.
Figure 30: Comparison of the shift in fluorescence of U937 cells with bound liposomes seen by flow cytometry. Red: autofluorescence of U937 cells, green: A1-DiO-liposomes bound unspecifically to U937 cells and black: shift in fluorescence due to specific binding of scFv to U937 cells (A: AFA8, B: AFC5). Cells were incubated with single chain antibody for 1 h at 37°C.

4.4.2 Cytotoxicity on F9/VEGFR-3 tumor cells in vitro

Incubating cells for 30-120 min with cytotoxic liposomes resulted in cell killing by specific immunoliposomes of VEGFR-3 positive cells. Incubating F9/VEGFR-3 with specific immunoliposomes containing either ETC-NOAC (Fig. 31) or 5FdU-NOAC (Fig. 32) resulted in a 30% reduction of viable cells. The fact that only 30% of the cells were killed could either be due to there being fewer receptors present on the transfected cells than on naturally expressing cells or, more likely, that the transfected receptor is not functionally active and therefore cannot internalise the liposomes, which is required for cytotoxicity.

Interestingly, we saw higher cytotoxicity on the receptor negative F9 cells with all four cytotoxic liposomes (Fig. 33). That no difference was seen between the VEGFR-3-specific and the control liposomes on these cells proved that there is no receptor-binding, but only unspecific attachment. The reason for the higher cytotoxicity might be that the stable transfection with prolonged G418 incubation renders the F9/VEGFR-3 cells less susceptible to cytotoxic drugs. To test the cytotoxic action of ETC-NOAC, transfected cells were incubated with free drug for 30 min and survival was analysed by the resazurin based viability assay 24 h later. An IC$_{50}$ of approximately 100 µM was obtained, again suggesting that the low cytotoxicity is due to the non-functional receptor (Fig. 34).
Figure 31: Cytotoxicity assay on F9/VEGFR-3 cells, incubated with control liposomes (■), ETC-NOAC-liposomes (▲), unspecific A1-ETC-NOAC-liposomes (▼), specific AFA8-ETC-NOAC-liposomes (◊) and specific AFC5-ETC-NOAC-liposomes (○). The experiment was carried out in triplicates.

Figure 32: Cytotoxicity assay on F9/VEGFR-3 cells, incubated with control liposomes (■), 5FdU-NOAC-liposomes (▲), unspecific A1-5FdU-NOAC-liposomes (▼) and specific AFC5-5FdU-NOAC-liposomes (○). The experiment was carried out in triplicates.
Figure 33: Cytotoxicity assay on receptor-negative F9 cells, incubated with control liposomes (■), ETC-NOAC-liposomes (▲), unspecific A1-ETC-NOAC-liposomes (▼), specific AFA8-ETC-NOAC-liposomes (◊) and specific AFC5-ETC-NOAC-liposomes (○). The experiment was carried out in triplicates.

Figure 34: Cytotoxicity of free ETC-NOAC on F9/VEGFR-3 cells. The experiment was carried out in triplicates.

Pre-incubating cells with specific or unspecific single chain antibodies prior to addition of liposomes was used to confirm the specific binding of the immunoliposomes (Fig. 35). Pre-incubation with either specific or unspecific scFvs had no effect on the binding of unspecific immunoliposomes, showing that the cytotoxicity observed with these control liposomes is unspecific. Binding of the
specific immunoliposomes although, was reduced significantly by pre-incubating with specific scFvs ($P=0.0096$), while pre-incubating with the unspecific scFv had no effect. These results prove that these liposomes specifically bind the receptor on the cells.

![Figure 35: Cytotoxicity assay on F9/hVEGFR-3 cells, preincubated with specific or unspecific scFv prior to addition of 100 µM ETC-NOAC liposomes. A) 100 µM scFv-AFC5-ETC-NOAC liposomes, B) 100 µM control A1cys-ETC-NOAC liposomes, C) preincubated with scFv AFC5, D) preincubated with control scFv. The experiment was carried out in triplicates (** $P < 0.01$).](image)

4.4.3 Biodistribution of labelled scFv-PEG-immunoliposomes in tumor bearing mice

Injecting $^{99}$Tc-labeled liposomes i.v. into F9/hVEGFR-3 tumor bearing mice resulted in very low tumor accumulation for both control liposomes and targeted liposomes, as shown in figure 36. The immunoliposomes were eliminated at surprisingly fast rates from the blood, with only 5% ID remaining 4 h after injection. The control liposomes on the other hand showed prolonged circulation times. Interestingly immunoliposomes kept on accumulating in the tumor up to 24 h post injection. Although this increase was not significant, it was also seen in a repeated experiment (data not shown).
Figure 36: Comparison of biodistribution pattern of untargeted Cys-PEG-liposomes (A) versus specifically targeted scFv AFC5-PEG-liposomes (B) expressed as % injected dose (%ID) in F9/VEGFR-3 s.c. tumor bearing mice. Three mice per group were injected i.v. with $^{99}$Tc labelled liposomes and killed at different time points.
4.5 Discussion

As described in chapter one of this thesis, liposomes are promising drug delivery agents. They are made up of naturally occurring lipids, thus avoiding clearance by the immune system and they can be loaded with any kind of drug. Sterically stabilising a liposome further prolongs its blood circulation time, thus therefore we used pegylated liposomes for this study. Single chain antibody fragments were coupled to the liposomes, since scFv-immunoliposomes have promising in vivo properties. The number of scFv molecules attached to the surface for optimal binding and long circulation times, has been optimised in a previous work. The immunoliposomes used in this study were produced according to these recommendations, attaching approximately 100 scFv molecules per 100 nm diameter liposome.

The specific targeting of AFA8- and AFC5-liposomes was confirmed by immunofluorescence on transiently transfected HEK 293T cells. The binding properties were compared to pegylated control liposomes as well as to liposomes coupled with an unspecific scFv. Binding was also evaluated on non-transfected cells, lacking the receptor (see Fig. 27+28). In the same way, specific binding was verified by flow cytometry. With this method quite high background binding was seen with the unspecific scFv-liposomes. The reasons for this finding are not known, since the unspecific scFv A1cys on its own does not bind to U937 cells. Increased liposome binding to U937 cells was observed when the incubation step was carried out at 37°C compared to 4°C on ice. This indicates that receptor mediated endocytosis takes place, which is an essential first step in many therapeutic approaches using antibody targeted vehicles, including immunotoxins, immunoliposomes, antibody-drug conjugates and antibody targeted gene delivery.

To test the in vitro drug delivery potential of these scFv-liposomes, they were loaded with either ETC-NOAC or 5-FdU-NOAC, two lipophilic cytotoxic drugs, and incubated with stably transfected F9/VEGFR-3 cells. Unfortunately, only 30% of the cells could be killed specifically by the cytotoxic immunoliposomes in comparison to control or unspecific liposomes. It was investigated if this result could be due to intrinsic drug resistance of these stably transfected cells as a result of their prolonged selection by
G418. This was not the case, since free ETC-NOAC could efficiently kill these cells at similar concentrations as encapsulated in the liposomes. Therefore, we conclude that the low cytotoxicity found is a result of the lack of receptor mediated endocytosis, since the cells used, do not contain a functional receptor\textsuperscript{153}. Unfortunately, no substitute adherent tumor cells, expressing the functional VEGF receptor-3, were available. Nevertheless, the experiment provides a proof of principle, since no specific cell killing was seen when untransfected F9 cells were used. All four liposome types behaved similarly on these control cells. The specificity of the cytotoxic activity was further established by pre-incubating the cells with either specific or non-specific scFv prior to incubation with the liposomes. Specific scFv blocked the binding of the AFC5-liposomes to the cells, while this was not the case with the control antibody (see figure 32). In a study published by Pagnan \textit{et al.} immunoliposomes were incubated with the cells for 2 h, which is longer than what was done in this study. They also left the cells to respond to the internalised drug for 72 h compared to the 24 h used here\textsuperscript{117}. Although such parameters are mostly drug-related, it would be interesting to analyse if this would have an effect on our system.

Since immunoliposomes showed low cytotoxicity \textit{in vitro} on the transfected cells, a more suitable mouse model is needed before the therapeutic potential of these cytotoxic liposomes can be examined \textit{in vivo}. However, since the liposomes showed specific binding \textit{in vitro}, a biodistribution experiment was carried out. Unfortunately, immunoliposomes were cleared from the blood almost completely within 4 h (shorter time points not analysed). This rapid elimination from the blood stream is very uncharacteristic for pegylated liposomes, even if antibodies are coupled to their surface. It was hypothesised that the radioisotope used, blocks the scFv from binding to the receptor, but this was ruled out by testing \textsuperscript{99}Tc-immunoliposome binding to F9/VEGFR-3 cells \textit{in vitro}. Another possibility is, that by scaling up liposome production and coupling with \textsuperscript{99}Tc, too many scFvs were coupled to the liposome and the immunoliposomes were more easily taken up by the RES. Therefore immunoliposome production for radioisotope labelling would need to be optimised. In addition the tumors might not have expressed the receptor at concentrations needed for specific recognition and binding by immunoliposomes. But all the above possibilities cannot account for the fast elimination of the immunoliposomes from the body. After 4 h only 30\% of injected dose remained in the mouse. It would therefore
be plausible to conclude that the $^{99}$Tc-immunoliposome complex was not stable *in vivo*, although *in vitro* stability had been verified. This could be investigated by loading the liposomes with another radioisotope or a fluorescent dye and analyse for co-localisation.
5 Conclusions and Outlook

The recent discovery of the lymphatic endothelial receptor VEGFR-3, and the elucidation of its expression pattern, presents an excellent target for selective tumor therapy. In this research project we therefore investigated the capability of selected VEGFR-3 scFv antibody fragments to target and block VEGFR-3 function.

The single chain antibodies selected here have high affinity for VEGFR-3 as was repeatedly confirmed by various in vitro experiments described in chapter 3. The antibodies therefore have a promising diagnostic potential, which was further verified by immunohistochemistry on human neonatal foreskin sections. Thus, the scFvs hold promise as diagnostic reagents for immunohistochemistry on vascularised tumor sections, for example, to identify VEGFR-3 expressing tumors. Identifying VEGFR-3 positive tumors would be of great aid in the development of clinical applications.

Inhibiting the function of VEGFR-3 has been shown to inhibit lymphangiogenesis and the metastatic spread of tumor cells via lymphatic vessels. We could show that the single chain antibody AFC5 has therapeutic competency, as seen in the preliminary experiments presented in this thesis, where the scFv was able to inhibit the function of VEGFR-3. By blocking the interaction of VEGF-C with its receptor, AFC5 could significantly inhibit both migration and proliferation of human lymphatic endothelial cells. The next goal would be to test if inhibition of receptor function by scFv AFC5 hinders lymphangiogenesis. Suitable models for testing this hypothesis include, for example, the chorioallantoic membrane (CAM) assay\(^26\), where VEGF-C has been shown to induce lymphatic proliferation. Other interesting ways of studying the capability of the scFvs to inhibit lymphatic vessel formation are metastases or orthotopic mouse models including the recently described *Xenopus laevis* tadpole model\(^154\). Since lymphatics are becoming identified as the primary route for metastatic spread, the need for lymphangiogenic inhibitors increases.

On top of its in vitro applicability, AFC5 shows specific tumor accumulation, which suggests that this antibody could be used favourably for in vivo applications. Since the tumor mouse model used in this study was not appropriate, an even higher tumor accumulation could be achieved when using a cell line expressing the functional receptor. With such a tumor cell line, one could additionally investigate the therapeutic potential of the single chain antibody in vivo. It would be of great interest
to evaluate if functional inhibition of VEGFR-3 would result in inhibition of tumor growth.

Besides testing cross-specific scFvs for binding to a recombinant murine receptor, as was shown in this study, it would be attractive to produce a tumor cell line expressing a functional murine VEGFR-3 for further experiments. As described above for AFC5, such a tumor cell line could be used to characterise these antibodies both in vitro and in vivo. Further, it would be appealing to analyse additional cross-specific scFv clones obtained in our screening for their ability to block human and/or murine ligand binding to the respective receptor. Single chain antibodies capable to inhibit this interaction could then be tested in lymphangiogenesis mouse models for their usefulness. For example, the Rip1Tag2 mouse model is a good proof of concept tool to study the inhibition of new lymphvessel formation.

Specific antibodies like AFA8 and AFC5 are ideal candidates for the production of immunoliposomes for targeted drug delivery. To analyse if the antibodies retain their specificity and binding capacity to the receptor after coupling to liposomes, the ability of the immunoliposomes to target VEGFR-3 positive cells was verified using immunofluorescence and flow cytometry on HEK293T/VEGFR-3 and U937 cells, respectively. For cytotoxicity and in vivo targeting studies a stably transfected F9/VEGFR-3 cell line was created. However, since a kinase inactive receptor is expressed on these transfected cells, cytotoxic immunoliposomes were not able to kill the cells efficiently. In addition to the advantages mentioned in the previous paragraph a tumor cell line endogenously expressing the human receptor would also be a valuable tool for evaluating whether VEGFR-3 specific cytotoxic immunoliposomes are effective. With such a cell line, the cytotoxic effects of immunoliposomes could be verified in vitro and in vivo and immunoliposome formulations could be further optimised.

Looking at the literature to date and the results obtained in this study we come to the conclusion that combination therapies are the most promising way to fight cancer and future experiments should reflect this. For example, combinations of anti-haemangiogenic and anti-lymphangiogenic therapies as well as conventional cytotoxic drugs promise to effectively inhibit both tumor growth and metastatic
spread. Because tumor blood vessels may become VEGFR-3 dependent under anti-VEGF/VEGFR-2 therapy, angiogenesis and lymphangiogenesis represent ideal targets for combination therapy\textsuperscript{73}. Another prospect would be to load immunoliposomes against the same molecular target with different cytotoxic drugs, thus evaluating the additive or synergistic effects of these drugs.

The aim of this study was to select and produce VEGFR-3 specific single chain antibodies. Their potential as diagnostic tools was confirmed by immunohistochemistry and SPECT analysis. The scFv AFC5 is able to inhibit receptor function on human lymphatic endothelial cells \textit{in vitro}. \textit{In vivo} the scFv shows specific tumor accumulation and is retained in the tumor for several hours. With these properties, this antibody shows all the features to become a valuable diagnostic and therapeutic tool for blocking the formation of new lymphatic vessels.
### 6 ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCIP</td>
<td>5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt</td>
</tr>
<tr>
<td>BMGY</td>
<td>buffered glycerol-complex medium</td>
</tr>
<tr>
<td>BMMY</td>
<td>buffered methanol-complex medium</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>DiO</td>
<td>3,3'-dioctadecyloxacarbocyanine perchlorate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco modified eagle’s medium</td>
</tr>
<tr>
<td>DPPE</td>
<td>1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine</td>
</tr>
<tr>
<td>EBM</td>
<td>endothelial basal medium</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ETC-NOAC</td>
<td>N$_4$-octadecyl-1-β-D-arabino-furanosyl-cytosine-(5′-5′)-3′-C-ethinylcytidine</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence activated cell sorting</td>
</tr>
<tr>
<td>5-FdU-NOAC</td>
<td>2′-deoxy-5-fluorouridylyl-N$_4$-octadecyl-1-β-D-arabino-furanosylcytosine</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>G418</td>
<td>geneticin antibiotic</td>
</tr>
<tr>
<td>HLEC</td>
<td>human lymphatic endothelial cells</td>
</tr>
<tr>
<td>HSPC</td>
<td>hydrogenated soy phosphatidylcholine</td>
</tr>
<tr>
<td>ID</td>
<td>injected dose</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-beta-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>K$_d$</td>
<td>dissociation constant</td>
</tr>
<tr>
<td>LEC</td>
<td>lymphatic endothelial cells</td>
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<tr>
<td>LTL</td>
<td>ligand targeted liposome</td>
</tr>
<tr>
<td>LUV</td>
<td>large unilamellar vesicle</td>
</tr>
<tr>
<td>MLV</td>
<td>multilamellar large vesicle</td>
</tr>
<tr>
<td>MUH</td>
<td>4-methylumbelliferyl heptanoate</td>
</tr>
<tr>
<td>NBT</td>
<td>nitro-blue tetrazolium chloride</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet derived growth factor</td>
</tr>
<tr>
<td>PEG</td>
<td>poly-ethylene glycol</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene fluoride</td>
</tr>
<tr>
<td>RES</td>
<td>reticuloendothelial system</td>
</tr>
<tr>
<td>RPMI 1640</td>
<td>Roswell park memorial institute medium 1640</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>RTK</td>
<td>receptor tyrosine kinase</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SPC</td>
<td>soy phosphatidylcholine</td>
</tr>
<tr>
<td>SPECT</td>
<td>single proton emission computed tomography</td>
</tr>
<tr>
<td>sulfo-SMCC</td>
<td>sulfocuccinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate</td>
</tr>
<tr>
<td>SUV</td>
<td>small unilamellar vesicle</td>
</tr>
<tr>
<td>scFv</td>
<td>single chain Fv antibody fragment</td>
</tr>
<tr>
<td>TBP</td>
<td>tributylphosphate</td>
</tr>
<tr>
<td>TY</td>
<td>trypton yeast extract</td>
</tr>
<tr>
<td>VEGFR-3</td>
<td>vascular endothelial growth factor receptor-3</td>
</tr>
<tr>
<td>YNB</td>
<td>yeast nitrogen base</td>
</tr>
</tbody>
</table>
7 REFERENCES


43. Pajusola, K. *et al.* Signalling properties of FLT4, a proteolytically processed receptor tyrosine kinase related to two VEGF receptors. *Oncogene.* 9, 3545-3555 (1994).


93. Northfelt, D. W. et al. Doxorubicin encapsulated in liposomes containing surface-bound polyethylene glycol: pharmacokinetics, tumor localization, and


Ref Type: Thesis/Dissertation


8  APPENDIX

8.1 List of Publications

8.1.1 Publications


Rhodococcus opacus *PWD4*. Applied and Environmental Microbiology, Vol. 67, No. 6, 2829-2832


### 8.1.2 Posters

Fjällman A., Zeisberger S., Schwendener R.A.. Immunoliposomal Inhibition of tumor induced angiogenesis: FoKo Meeting; March 2002; Villigen-PSI (Switzerland).


Fjällman A., Ballmer-Hofer K., Schwendener R.A.. VEGF-Receptor-3 specific immunoliposomes for targeted drug delivery; 35th Annual meeting of USGEB/USSBE; April 2003; Kongresszentrum Davos (Switzerland).


Fjällman A., Ballmer-Hofer K., Schwendener R.A.. VEGF-Receptor-3 specific immunoliposomes for targeted drug delivery. 36th Annual meeting of USGEB/USSBE; Februar 2004; University of Fribourg (Switzerland).


8.1.3 Oral presentations

Vascular endothelial growth factor-3 specific immunoliposomes for targeted drug delivery; Doktorandentag des Departements Pharmazie; April 2004; ETH Zürich (Switzerland).
8.2 Curriculum Vitae

Surname: Zehnder- Fjällman
Name: Ann Helen Margrith
Date of birth: 10.5.1975
Place of birth: Uppsala, Sweden
Family status: married
Nationality: Swedish, Swiss

1980-82: Preschool, Lusaka, Zambia
1982-87: Primary school, Uppsala, Sweden
1987-88: Primary school, Guntershausen, Switzerland
1988-92: High school, Baden, Switzerland
1992-93: Gymnasium, Baden, Switzerland

3/1994-2/97: Studies of Biochemistry and Microbiology, School of Science and Technology, Department of Biological Sciences, University of Waikato, Hamilton, New Zealand


2/1998: Bachelor of Science and Technology (BSc(Tech))

3/1998-12/99: Masters thesis: Confectionary made from honey with high antibacterial activity for the protection of dental health (Prof. Peter Molan, Honey Research Unit, University of Waikato, New Zealand)
8/1998-11/99: Teaching assistant in Genetics, Department of Biological Sciences, University of Waikato, New Zealand

3/1999-6/99: Teaching assistant in Cellular and Molecular Biology, Department of Biological Sciences, University of Waikato, New Zealand

4/2000: Masters of Science (MSc, with 1st Class Honours)

1/2000-6/00: Post graduate position in Genetic Toxicology, Novartis Pharma Inc., Basel, Switzerland

7/2000-12/01: Microbiological research assistant, Department of Biotechnology, ETH, Zürich, Switzerland

since 1/2002: PhD position at the Paul Scherrer Institute, Molecular Cell Biology group and the Institute of Pharmaceutical Sciences, Department of Chemistry and Applied Biosciences, ETH, Zürich, Switzerland
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