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ANTIBODY ENGINEERING: ADVANCES IN PHAGE DISPLAY TECHNOLOGY AND IN THE PRODUCTION OF THERAPEUTIC IMMUNOCYTOKINES

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To my family

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1 Summary

Antibody phage technology greatly facilitates the isolation of good-quality monoclonal antibodies to virtually any target antigen. Large combinatorial phage display libraries of human antibodies are routinely being used for the identification of antibody candidates for clinical applications. However, preclinical studies in rodents would benefit from the availability of good-quality single-pot mouse synthetic naïve antibody libraries, which at present are not available. Such libraries would be particularly useful for the generation of murine antibodies against self or highly conserved antigens or in case of highly toxic or deadly pathogenic antigens which do not allow animal immunization.

This thesis reports on the construction of a mouse antibody phage display library, containing over 1 billion antibody clones, based on the combinatorial mutagenesis of residues in the CDR3 loops of a given antibody scaffold. The library was shown to reliably yield good-quality antibodies towards all protein antigens used so far in selection experiments, including three tumor-associated antigens. The modular structure of the library facilitates a simple affinity-maturation procedure based on the combinatorial mutagenesis of CDR1 and CDR2 loops of the VH domain, which has led to the isolation of a high-affinity antibody [scFv(H7), Kd = 6 nM] specific to the EDB domain of fibronectin, a marker of angiogenesis. This single-pot antibody library may thus represent a useful source of binding specificities, facilitating preclinical studies in immunocompetent syngeneic mouse models of pathology.

Interleukin 12 (IL12) is a key mediator of innate and adaptive immunity. The immunomodulating and antiangiogenic functions of IL12 have provided the rationale for exploiting this cytokine as an anticancer agent in patients with advanced cancer, but its administration is typically associated with severe toxicity, hampering dose escalation to therapeutically-active regimens and the development as anti-cancer drug.

To overcome the clinical drawbacks associated to the administration of cytokines (and IL12 in particular) to patients, the use of "immunocytokines" (i.e., cytokines fused to antibodies or antibody fragments) has been proposed, with the aim to concentrate the immune stimulating activity at the site of disease while sparing normal tissues.

In this thesis I describe the design and construction of a heterodimeric immunocytokine F8-IL12, consisting of scFv(F8) (an antibody fragment, specific to the alternatively-spliced EDA domain of fibronectin) fused to both p35 and p40 subunits of human IL12. This immunocytokine could be stably expressed in mammalian cells and purified to homogeneity with full retention of cytokine activity. The resulting product exhibited an impressive tumor targeting performance in a mouse model of cancer.

1 Riassunto

La tecnologia del phage display di frammenti anticorpali ha reso possibile la generazione di anticorpi monoclonali contro potenzialmente qualsiasi antigene di interesse. Librerie combinatoriali di anticorpi umani, esposti sulla superficie di fagi, sono utlizzate di routine per l'identificazione di anticorpi a fini terapeutici.

Gli studi preclinici in roditori beneficerebbero di librerie sintetiche, naïve e funzionali di anticorpi murini che al giorno d'oggi non sono disponibili.

Tali librerie sarebbero particolarmente utili al fine di ottenere anticorpi murini contro antigeni self o conservati o in caso di antigeni tossici, gravementi patogenici o letali, caratteristiche che impediscono l'immunizzazione degli animali.

In questa tesi si descrive la costruzione di una libreria di phage display di anticorpi murini contenente oltre 1 miliardo di diversi cloni, basata sulla mutagenesi combinatoriale dei residui nei loop dei CDR3 di una data struttura anticorpale. La libreria si e' dimostrata un'ottima fonte di anticorpi contro tutti gli antigeni presi in esame, tra cui tre antigeni presenti esclusivamente a livello tumorale. La struttura modulare della libreria rende possibile una semplice procedura per l'aumento dell'affinita' mediante la mutagenesi combinatoriale dei loop del CDR1 e del CDR2 del dominio VH. Tale strategia ha cosentito l'isolamento di un anticorpo ad alta affinita' [scFv(H7), Kd = 6 nM] specifico per l'extra-dominio B della fibronectina, un antigene espresso nel processo di angiogenesi tumorale. La libreria potrebbe quindi divenire un'utile fonte di anticorpi in grado di permettere di effettuare studi preclinici in modelli murini singenici ed immunocompetenti di una data patologia.

L'interleuchina 12 (IL12) e' un mediatore chiave della risposta immunitaria innata e adattativa. Le funzioni immunomodulanti e antiangiogeniche della IL12 hanno motivato lo studio di tale citochina come agente per la terapia del cancro in pazienti con forme neoplastiche avanzate. Tuttavia la somministrazione di IL12 induce grave tossicita' nei pazienti, impedendo il raggiungimento della dose

terapeuticamente efficace e conseguentemente lo sviluppo come farmaco anticancro.

Al fine di superare i problemi derivanti dall'uso nei pazienti delle citochine (e dell'IL12 in particolare), e' stato proposto l'impiego di immunocitochine (citochine fuse ad anticorpi o frammenti anticorpali) con lo scopo di concentrare l'attivita' del sistema immunitario esclusivamente nella regione interessata dalla malattia.

In questa tesi descrivo il disegno e la costruzione di F8-IL12, una immunocitochina eterodimerica, composta da scFv(F8) (un frammento anticorpale, specifico per l'extra-dominio A della fibronectina, regione soggetta a splicing alternativo) fuso alle due subunita' dell'interleuchina 12 umana. Tale immunocitochina e' stata stabilmente espressa in cellule di mammifero e purificata all'omogeneita' mantenendo l'attivita' biologica. In experimenti preclinici in topi, questo nuovo prodotto farmaceutico ha mostrato di possedere straordinarie capacita' di accumulo selettivo a livello tumorale.

2 Introduction

2.1 Antibody-based targeting of disease

2.1.1 General concepts on antibodies

Antibodies are a class of glycoproteins belonging to the immunoglobulin super-family found in blood or other bodily fluids of vertebrates.

Five classes of immunoglobulins are known - IgM, IgD, IgG IgA and IgE - that differ for their constant region. IgGs are the most relevant class of antibodies for pharmaceutical applications (**Table 2.1**). The classic structure of an IgG consists of two identical light chains (25 kDa) and two identical heavy chains (50 kDa) covalently linked by a disulphide bridge between the two heavy chains and between heavy and light chains (**Figure 2.1**). Each heavy and light chain contains a variable domain (VH and VL respectively) of around 110 amino acids where the variability on the antibody structure is located. Within each variable domain, three hypervariable loops confer the binding properties and are defined as complementarity determining regions (CDRs).
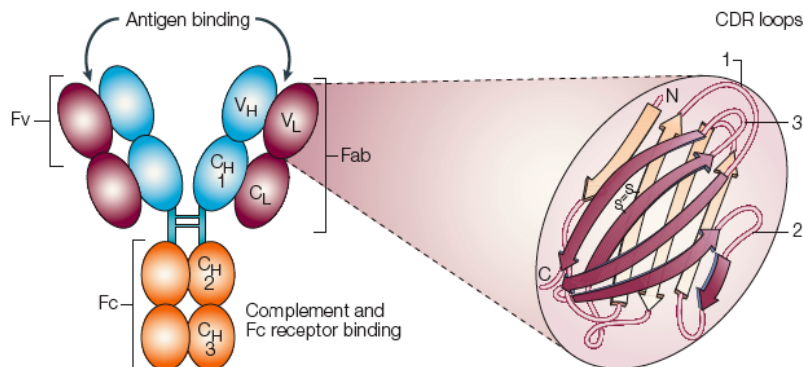
Figure 2.1. This figure shows the modular structure of an IgG. molecule. All immunoglobulin monomers are composed of two identical light (L) chains and two identical heavy (H) chains. Light chains are composed of one constant domain (C_L) and one variable domain (V_L), whereas heavy chains are composed of three constant domains (C_H1, C_H2 and C_H3) and one variable

domain (V_H). The heavy chains are covalently linked in the hinge region and the light chains are covalently linked to the heavy chain. The variable domains of both the heavy and light chains compose the antigen-binding part of the molecule, termed Fv. Within the variable domains there are three loops designated complementarity-determining regions (CDRs) 1, 2, and 3, which confer the highest diversity and define the specificity of antibody binding. Adapted from (Brekke and Sandlie 2003).

2.1.2 Mechanisms of action

In the last decades several monoclonal antibodies have been approved from FDA for the treatment of cancer and other diseases. Most monoclonal antibodies on the market are naked IgGs.

Naked IgGs have three main mechanisms of action which allow them to operate *in vivo*: neutralization, antibody-dependent cellular toxicity and complement activation.

The neutralization activity consists in the binding of the antibody to the target antigen and thereby blocking its biological function. Therapeutic applications include the neutralization of toxins (antiserum) or signaling molecules such as cytokines (e.g. Adalimumab**,** Remicade, Ustekinumab) or growth factors (e.g. Bevacizumab, Lucentis).

IgGs could also give rise to antibody-dependent cellular toxicity (ADCC). ADCC is the mechanism of cell-mediated immunity whereby the binding of antibodies on a target antigen expressed on a cell induces the lyses of the target cell. Classical ADCC is mediated by natural killer (NK) cells. NK cells expose on the cell membrane the FcγRIII, which recognizes the Fc portion of IgG. Once FcγRIII receptors bind to the Fc portion of antibodies bound to a cell surface antigen, NK cells release cytotoxic granules containing perforin and granzymes that enter the target cell and promote cell death by triggering apoptosis. ADCC is an important mechanism of action of therapeutic monoclonal antibodies, including trastuzumab and rituximab.

Antibodies (mainly IgM, IgG1 and IgG3) are able to trigger the classical pathway of complement activation: the starting event is the binding of C1q to the Fc region of antibodies bound to multiple sites of a cell surface (typically a pathogen). Subsequent activation of the complement cascade ultimately leads to three possible events: (i) direct killing of the cell via lysis (by creating pores on the membrane), (ii) opsonization and engulfment of the cell by phagocytes and (iii) uptake of the complement-coated-antigen by antigen presenting cells and its presentation to the adaptive immune system.

Antibodies and their derivatives can also be used as vehicle to concentrate a therapeutic agent specifically at the site of disease sparing healthy tissues. A targeted therapy is the ideal approach for the therapy of cancer where often potent drugs cannot be used at therapeutic relevant regimens because of their severe side effects. The targeted delivery of drugs by means of antibodies promises to enhance their therapeutic index and limit their side effects.
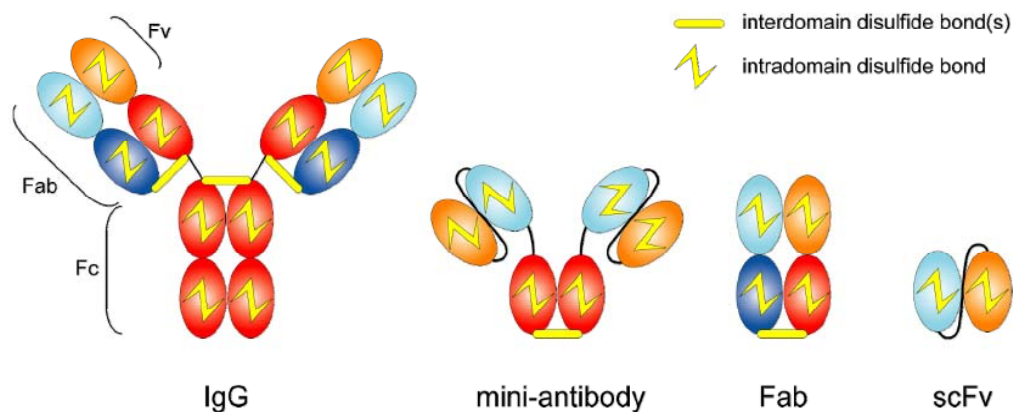
Figure 2.2. IgG and antibody fragments. IgG is a 150 kDa molecule comprising two variable fragments (Fv) which contain the antigen binding sites, and an Fc portion which mediated the effector function. The single-chain Fv (scFv) fragment, in which the variable domain of the heavy and light chains are joined by a polypeptide linker, is the smallest fragment which still contains the intact binding site. Whereas the Fab fragment, which naturally occur within the IgG molecule, is a monovalent fragment. The engineered mini-antibody format is a bivalent binding molecule obtained by fusing a scFv to a constant εCH4 domain of a human IgE

For tumor targeting applications the use of antibody fragments (*i.e.* scFv, diabody, or mini-antibody format; **Figure 2.2**), is preferable to full IgGs as they allow a better extravasation from the blood circulation and a superior tissue penetration. In comparison to naked IgG, antibody fragments *per se* do not mediate any therapeutic activity and need to be linked to bioactive agents such as cytokines, toxins, procoagulant factors, drugs with a cleavable linker, radionuclides and photosensitizers (**Figure 2.11**).

2.1.3   Therapeutic applications

Since their introduction, monoclonal antibodies have had a progressively increasing impact on different areas of medical diagnostics and therapeutics. Monoclonal antibodies have been approved for the treatment of cancer, inflammatory diseases, cardiovascular diseases, macular degeneration, transplant rejection, multiple sclerosis, and viral infection (**Table 2.1**). Most of these antibodies have the neutralization of their target or the induction of ADCC as mechanism of action. In the future, we may also see approval of antibody derivatives linked to a bioactive agent. These "immuno-derivatives" may offer the possibility to deliver a potent therapeutic agent that acts selectively at the site of the disease, sparing healthy tissues, and may considerably enhance the therapeutic index of the bioactive agent.

For the therapy of cancer the U.S. FDA approved a number of monoclonal antibodies (**Table 2.1**), some of which have become blockbusters in spite of their generally modest clinical benefit.

Bevacizumab is a humanized monoclonal antibody that targets and blocks the VEGF-A and was approved for the first-line treatment of metastatic colorectal cancer and as second line for other solid cancers. The neutralization of VEGF-A seemed to be a promising strategy as this molecule is supposed to be a key factor for the neoangiogenesis process required for tumor growth. Clinical studies showed that bevacizumab combined with chemotherapy can prolong life of

patients of 4-5 months compared to patients treated with standard chemotherapy (Hurwitz, Fehrenbacher et al. 2004)

Trastuzumab is a humanized monoclonal antibody approved for the therapy of metastatic breast cancer. The antibody targets the extracellular domain of the human epidermal growth factor receptor 2 (HER2), a protein over-expressed in ~20% of women with breast cancer. ADCC and arrest of cell proliferation by disrupture of receptor dimerization or downstream signaling pathways may be possible mechanisms of action *in vivo*, though they have been only demonstrated *in vitro* (Sarup, Johnson et al. 1991; Cooley, Burns et al. 1999).

Trastuzumab combined with chemotherapy increases response rates, time to progression, and survival (Cobleigh, Vogel et al. 1999). However, the majority of cancers that initially respond to trastuzumab begin to progress again within one year**.**

The discovery that signaling by the epidermal growth factor receptor (EGFR) plays a key role in tumorigenesis prompted efforts to target this receptor in anticancer therapy. Two different types of EGFR-targeted monoclonal antibodies were developed: first cetuximab, a chimeric monoclonal IgG1, and subsequently panitumumab a fully human IgG2. Both antibodies target the extracellular domain of the receptor, thereby inhibiting ligand-dependent EGFR signal transduction (Okamoto). Both therapeutics were approved for the treatment of patients with EGFR expressing KRAS wild-type metastatic colorectal cancer and head and neck cancer.

Clinical studies of cetuximab in combination with irinotecan showed tumor shrinking in 22.9% of patients and delayed tumor growth by 4 months. For patients who received cetuximab alone, the tumor response rate was 10.8% and tumor growth was delayed by 4 months (Alberts, Sinicrope et al. 2005).

Rituximab (Rituxan) is a chimeric monoclonal antibody against CD20, which is primarily found on the surface of B cells. Rituximab was approved for the treatment of certain lymphomas and some autoimmune disorders. In the treatment of lymphomas, Rituximab has significantly improved the response duration and time to disease progression (Van Oers, Hagenbeek et al. 2002).

Approved antibodies for therapeutic applications

Product name	Generic name	Target	Type	Indication	Year
Orthoclone OKT3	Muromonab	CD3	murine IgG2a	Transplant rejection	1986
OncoScint	Satumomab pendetide	TAG-72	murine IgG 111In	Colorectal and ovarian cancer	1991
ReoPro	Abciximab	GP IIb/IIIa	chimeric Fab	Cardiovascular	1994
CEA-Scan	Arcitumomab	CEA	murine Fab 99mTc	Coloretal cancer	1996
Prostascint	Capromab pendetide	PSMA	murine IgG1 111In	Prostata carcinoma	1996
Verluma	Nofetumomab	CD20	murine Fab 99mTc	Small cell lung cancer	1996
Zenapax	Daclizumab	CD25	humanized IgG1	Transplant rejection	1997
Rituxan	Rituximab	CD20	chimeric IgG1	B-cell lymphoma (Non-Hodgkin)	1997
Simulect	Basiliximab	CD25	chimeric IgG1	Transplant rejection	1998
Synagis	Palivizumab	RSV	humanized IgG1	RSV bronchiolitis	1998
Embrel	Etanercept		TNFR-Fc (CH2-CH3) of IgG1	Rheumatoid arthritis	1998
Remicade	Infliximab	TNF	chimeric IgG1	Rheumatoid arthritis; Crohns dis.	1998
Herceptin	Trastuzumab	HER2/neu	humanized IgG1	Breast cancer	1998
Mylotarg	Gemtuzumab	CD33	humanized IgG4 toxin-calicheamyci	Acute myeloid leukaemia	2000
MabCampath	Alemtuzumab	CD52	humanized IgG1	Chronic lymphatic leukaemia	2001
Zevalin	Ibritumomab	CD20	murine IgG1 90Y	B-cell lymphoma	2002
Humira	Adalimumab	TNF	human IgG1	Rheumatoid arthritis	2002
Xolair	Omalizumab	IgE	humanized IgG1	Asthma	2003
Bexxar	Tositumomab	CD20	murine IgG2a 131I	B-cell lymphoma	2003
Raptiva	Efalizumab	CD11a	human IgG1	Psoriasis	2003
Avastin	Bevacizumab	VEGF	humanized IgG1	Colorectal cancer	2004
Erbitux	Cetuximab	EGFR	chimeric IgG1	Colorectal cancer	2004
Tysabri	Natalizumab	alpha4 integrin	humanized IgG4	Multiple sclerosis	2004
Orencia	Abatacept		CTLa4e.c.-Fc (CH2-CH3) IgG1	Rheumatoid arthritis	2005
Lucentis	Ranibizumab	VEGF	humanized Fab fragment	Age related macular degeneration	2006
Vectibix	Panitumumab	EGFR	human IgG2	Metastatic colorectal cancer	2006
Soliris	Eculizumab	C5complement	humanized IgG 2/4kappa	Paroxysmal nocturnal hemoglobinuria	2007
Cimzia	Certolizumab PEGOL	TNF-alpha	humanized Fab fragment-PEG40kD	Crohn's disease	2008
Ilaris	Canakinumab	IL1b	human IgG1	Cryopyrin-Associated Periodic Syndro	2009
Arzerra	Ofatumumab	CD20	human IgG1	Chronic lymphocytic leukemia	2009
Simpioni	Golimumab	TNF-alpha	human IgG1	R.A., Ps.A., ankylosing spondylitis	2009
Stelara	Ustekinumab	p40 IL12/IL23	human IgG1	Psoriatic arthritis	2009
Actemra	Tocilizumab	IL6	humanized IgG1	Rheumatoid arthritis	2010

cancer
diagnostic
Receptors fused to Fc portions

Table 2.1. List of antibodies approved for therapy or diagnosis.

2.1.4 Immunogenicity of approved antibodies

The first murine monoclonal antibody approved from FDA, Muromonab-CD3 (orthoclone OKT3) for the prevention and treatment of solid organ transplant rejection (Smith 1996) was a scientific and commercial success. However it turned out that murine monoclonal antibodies lead to the development of human anti-mouse antibodies (HAMA) that cause rapid clearance and loss of efficacy of the therapeutic antibody and can induce severe allergic reactions. Moreover murine IgGs do not mediate ADCC and complement activation, thus limiting therapeutic applications.

In order to reduce the HAMA response, chimeric monoclonal antibodies were produced combining the murine variable regions of the immunoglobulin genes with constant domains of the human IgG. The use of chimeric antibodies substantially reduced the HAMA response but could not completely eliminate it. Although several chimeric antibodies achieved regulatory approval (**Table 2.1**), it became clear the need of a further reduction of murine sequences in the antibody scaffold in order to minimize the HAMA response. With the hope to solve this problem, humanized antibodies were generated. Humanized antibodies were constructed by grafting the CDRs of the heavy and light chains and a limited number of structural amino acids of the murine monoclonal antibody to the CDR-depleted human IgG scaffold. Although this process further reduced the HAMA response, in many cases, substantial additional antibody design procedures were needed to reestablish the required specificity and affinity of the original murine antibody (Presta, Lahr et al. 1993). In order to minimize the development of HAMA responses, the generation of fully human antibodies would be desirable. By means of phage display technology, it has been possible to obtain fully human antibodies that reached regulatory approval (Adalimumab). Fully human antibodies (e.g., Panitumumab) can be also generated by transgenic animals whose murine immunoglobulin loci have been replaced by the

entire human immunoglobulin loci determining a human antibody response in mice.

Immunogenicity problems, however, have not been totally solved. Adalimumab, a fully human antibody for the treatment of rheumatoid arthritis, was supposed to be less immunogenic than murine, chimeric and humanized monoclonal antibodies. Surprisingly it was observed to induce the formation of human anti-human antibodies (HAHA) in 12% of patients (Weinblatt, Keystone et al. 2003; van de Putte, Atkins et al. 2004). It is believed that the HAHA reactivity is either directed against non-human glycosylated sites on the surface of the antibody expressed in CHO cells or against the antigenic determinants in VL and VH regions of adalimumab that include the CDRs complementary determining regions (anti-idiotype antibodies).

The development of auto-antibodies in some cases can be related to degradation or aggregation of the antibody or to presence of impurities, such as toll-like receptor (TLR) ligands.

2.2 Antibody phage display technology

### 2.2.1 The phage display technology

Phage display is a powerful methodology that allows the selection of a particular phenotype (e.g., a ligand specific to a desired antigen) from repertoires of proteins displayed on bacteriophage. Phage display was first described in 1985 by Smith (Smith 1985), who presented the use of the non-lytic filamentous bacteriophage fd for the display of specific binding peptides on the phage coat. The power and applications of the methodology were further improved by the groups of Winter (McCafferty, Griffiths et al. 1990) and Wells (Lowman, Bass et al. 1991) who demonstrated the display of functional folded proteins on the phage surface (an antibody fragment and a hormone, respectively). Phage technology is based on the fact that a polypeptide (capable of performing a function, classically the specific binding to an antigen) can be displayed on the phage surface by inserting the gene coding for the polypeptide into the phage genome. Thus the phage particle physically links genotype to phenotype (**Figure 2.3**).
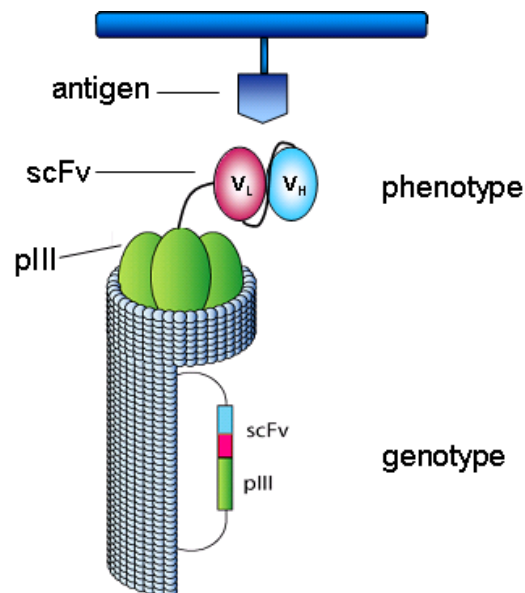
Figure 2.3. Phage displaying a binding protein: in this case a scFv antibody fragment as fusion protein of a minor coat protein pIII

It is possible to create large repertoires of phage (phage display libraries) in which the proteins displayed on each phage are slightly different from each other. If one is able to purify from this large phage repertoire a phage particle by virtue of the phenotype (e.g., the binding specificity) displayed on its surface, one also isolates the genetic information coding for the binding protein, and can amplify the corresponding phage by means of bacterial infection.

As an example, one can consider the selection of a binding specificity from repertoires of binders (**Figure 2.4**).
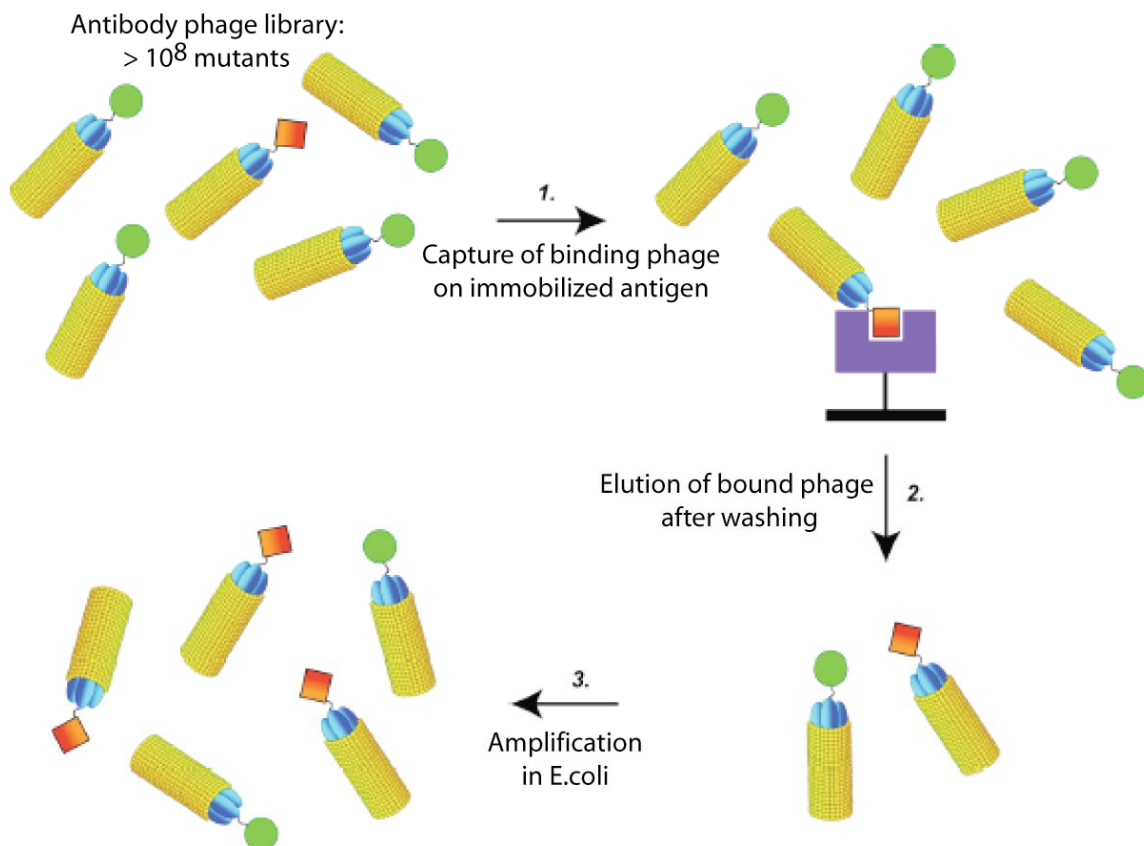
Figure 2.4. Selection of binding specificity from a phage display library. A library of proteins displayed on the phage surface is used as input for the selection. Phage displaying binding proteins are captured on immobilized target molecules, and after washing, bound phage can be eluted. This phage population is then propagated in *E.coli* cells and can be used for further rounds of selection.

The library on phage is panned against an antigen of interest. Unbound phage

are discarded whereas specifically binding phage are collected and amplified in bacteria. Several rounds of selection can be performed (in general 2-4 rounds using antibody phage libraries). As a result, even very rare phenotypes present in large repertoires can be selected and amplified from a background of phage carrying undesired phenotypes.

The possibility to amplify the selected phage in bacteria during biopanning experiments allows the enrichment of the pool of phage with the desired phenotype. Filamentous phage infect strains of *E.coli* that harbour the F conjugative episome by attaching to the tip of the F pilus and translocating the phage genome (a circular single-strand DNA molecule) into the bacterial cytoplasm. The genome is replicated involving both phage- and host-derived proteins, and packaged into elongated "filamentous" viral particles of roughly 6 nm in diameter and 900 nm in length (**Figure 2.5**).
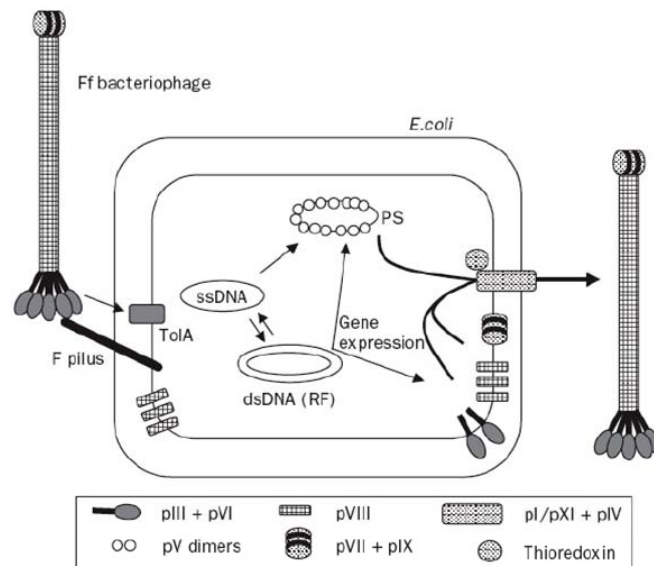
Figure 2.5. Life cycle of filamentous phage f1 (M13/fd). Sequential binding of pIII to the tip of the F-pilus and then the host Tol protein complex results in depolymerization of the phage coat proteins, their deposition in the cytoplasmic membrane (where they are available for reutilization), and entry of the ssDNA into the cytoplasm. The ssDNA is converted by host enzymes to a double-stranded reading frame (RF), the template for phage gene expression. Progeny ssDNA, coated by pV dimers (except for the packaging sequence hairpin (PS) that protrudes from one end), is the precursor of the virion. A multimeric complex that spans both membranes mediates conversion of the pV-ssDNA complex to virions and secretion of virions from the cell. This process involves removal of pV dimers and their replacement by the five coat proteins that

transiently reside in the cytoplasmic membrane. Adapted from "Phage display: a practical approach" Edited by Clackson T. and Lowman HB. Oxford University Press.

Filamentous phage particles are covered by several thousand copies of a small major coat protein (pVIII). Few copies of the minor coat proteins pIII and pVI are displayed at one extremity of the phage particle, while pVII and pIX are present at the opposite extremity. The minor coat protein pIII (the product of gene III), is displayed in 3-5 copies and mediates the adsorption of the phage to the bacterial pilus. Peptides and proteins have been displayed on phage as fusions with the coat proteins pIII (Smith 1985; Parmley and Smith 1988) or pVIII (Greenwood, Willis et al. 1991). Display of proteins encoded by a cDNA library as carboxy-terminal fusion with the minor coat protein pVI has also been described (Greenwood, Willis et al. 1991).

The first peptides and proteins were displayed on phage using phage vectors (essentially the phage genome with suitable cloning sites for pVIII or pIII fusions and an antibiotic resistance gene). Phage vectors carry all the genetic information necessary for the phage life cycle.

With pIII fusions in phage vectors, each pIII coat protein displayed on phage is fused with the heterologous polypeptide. Using phage vectors, most peptides and folded proteins can be displayed as pIII fusions, while only short peptides of 6-7 residues containing no cysteine give rise to functional phage when displayed as pVIII fusions (Iannolo, Minenkova et al. 1995).

Phagemids, a more popular type of vector for phage display, are plasmids that carry the gene III with appropriate cloning sites and a packaging signal (**Figure 2.6**; (Hoogenboom, Griffiths et al. 1991)).

For the production of functional phage particles, phagemid containing bacteria have to be superinfected with helper phage particles, which contain a complete phage genome. Phagemid vectors encoding the polypeptide-pIII fusion are preferentially packaged into the phage particles, because the typically used helper phage (M13K07 or VCS-M13) have a slightly defective origin of replication, which also serves as packaging signal.
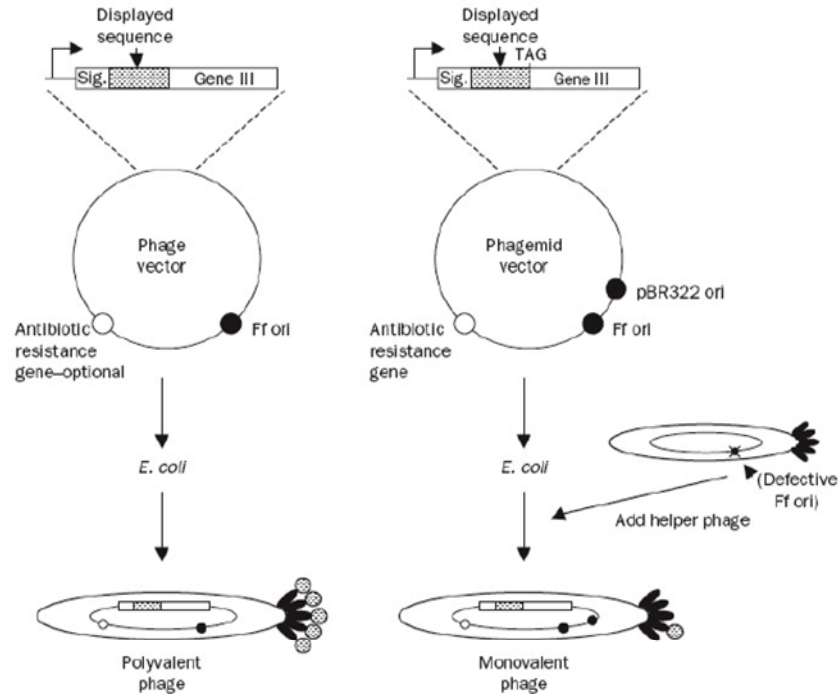
Figure 2.6. General scheme for phage display using phage or phagemid vectors. The difference between phage and phagemid vectors is illustrated for pIII display. Sequences for display are inserted between a secretion signal sequence (Sig.) and gene III. Both phage and phagemid vectors carry an Ff origin of replication to permit production of ssDNA and hence virions. Phagemid vectors also have a plasmid origin (here pBR322) and an antibiotic resistance marker to allow propagation as plasmids in *E .coli*. Phage vectors are also often modified with antibiotic resistance markers for convenience, as illustrated here. In many phagemid vectors, an amber stop codon (TAG) is interposed between the displayed sequence and gene III to allow soluble protein expression by transferring the vector into a non-*supE* suppressor strain. Adapted from "Phage display: a practical approach" Edited by Clackson T. and Lowman HB. Oxford University Press.

The resulting phage particles may incorporate either pIII derived from the helper phage or the polypeptide-pIII fusion, encoded by the phagemid. Depending on the type of phagemid, growth conditions used, and the nature of the polypeptide fused to pIII, ratios of polypeptide-pIII : pIII ranging between 1:9 and 1:10000 have been reported (Kristensen and Winter 1998). Furthermore, the proteolytic cleavage of protein-pIII fusions has been described, contributing to further elevated levels of wild type pIII (McCafferty, Griffiths et al. 1990). The number of displayed protein-pIII fusion per phage particle has important implications for

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selection experiments. Whereas phage particles obtained by using a phage vector are polyvalent (i.e. 3 to 5 identical polypeptides displayed on one phage particle), the use of phagemids often delivers monovalent protein-pIII display, which is instrumental for the isolation of high-affinity binders (e.g., for affinity maturation procedures). In reverse, polyvalent phage leads to the selection of lower affinity binders due to avidity effects. By performing superinfections with hyperphage, a phage which lacks gene III in the genome, polyvalent phage can still be generated with phagemids (Rondot, Koch et al. 2001).

2.2.2 Antibody phage display libraries

Antibody phage display technology is the display and use of repertoires of antibody fragments on the surface of bacteriophage. The filamentous phage surface constitutes a physical link between genotype and phenotype of the antibody, in the same way that surface immunoglobulins are linked to the B cells *in vivo*. The antibody fragments can be displayed as single chain Fv fragments, in which VH and VL domains of a full immunoglobulin are connected on the same polypeptide chain by a flexible polypeptide spacer or as Fab fragments where the association of the variable domains is stabilized by the first constant domain of the heavy chain and the first constant domain of the light chain (Better, Chang et al. 1988; Cabilly 1989).

ScFv fragments have a molecular weight of about 25 kDa and are not glycosylated. In a scFv fragment the order of the V domains may vary, with the VH domain at the N-terminus or at the C-terminus (Bird, Hardman et al. 1988; Huston, Levinson et al. 1988), whereby the linker length has to be adjusted for optimal spatial arrangement of the two V domains (Huston and Haber 1996). The most common format VH-$(Gly_4Ser)_3$-VL has been also used for the construction of various phage libraries (Clackson, Hoogenboom et al. 1991; Marks, Hoogenboom et al. 1991; Hoogenboom and Winter 1992)

By definition the term "antibody phage display library" refers to a collection of

recombinant phage which display an antibody fragment on their surface. The total number of different phage particles displaying each a unique antibody fragment in the repertoire defines the size of the library, which is a critical parameter for the success of antibody phage technology. The larger the library, the greater the chance of finding antibodies that bind to any given epitope, and the higher the affinity (Perelson and Oster 1979). The second key parameter which defines library performance is the diversity carried by the amino acid sequences of the antibodies in the library. As in the immune system, the antibodies of a phage display library may have a common scaffold, while diversity is inserted in the amino acid positions which determine the specificity of binding. CDR3 loops represent the antibody region in which diversity is mainly concentrated in nature. Therefore, also for synthetic libraries, the amino acid diversity is generally localized in CDR3 residues.

There are different ways to create diversity when building an antibody phage display library, which rely on the possibility to harvest VH and VL genes by PCR. Thanks to the extensive characterization of the V-genes and their flanking regions, several sets of "universal" PCR primers have been described for the cloning of human (Marks, Hoogenboom et al. 1991; Tomlinson, Walter et al. 1992), murine (also usable for rat) (Orlandi, Gussow et al. 1989; Clackson, Hoogenboom et al. 1991; Kettleborough, Saldanha et al. 1993; Orum, Andersen et al. 1993; Ridder, Schmitz et al. 1995), rabbit (Ridder, Schmitz et al. 1995), and chicken V-genes repertoires (Davies, Smith et al. 1995).

On the basis of the strategy followed to obtain diversity, antibody phage display libraries can be classified in "Immune repertoires" (antigen-biased), and "Single-pot" libraries (antigen-unbiased).

- Immune antibody phage display libraries
Immune antibody phage display libraries (Burton, Barbas et al. 1991; Clackson, Hoogenboom et al. 1991) take advantage of the diversity created *in vivo* by the

immune system: in this case the source of variable immunoglobulin genes are B-cells from an animal immunized with the antigen of interest or an immune patient. The resulting libraries are enriched in antigen-specific immunoglobulin domains, some of which have already been matured by the immune system, and may therefore yield high-affinity antibodies even when the library size is not spectacular (e.g., 10^7 clones). For example, Chester et al. (Chester, Robson et al. 1994) isolated from a murine immune library a well-behaved scFv fragment specific for the carcinoembryonic antigen (CEA) with a dissociation constant in the low nanomolar range. This scFv (MFE-23) has been shown to selectively target human tumors xenografted in nude mice (Verhaar, Chester et al. 1995). There are some disadvantages in isolating antibodies from immune repertoires. When the source of V genes is an immunized animal, the resulting antibodies are not human and therefore potentially immunogenic. Animal immunization and library construction are necessary for each individual antigen, making the whole procedure long and labour intensive.

However, the isolation of human anti-tumor antibodies from phage repertoires of antibodies derived from cancer patients immunized with autologous tumor cells (Cai and Garen 1995), or from their tumor-draining lymph nodes (Kettleborough, Ansell et al. 1994) is a powerful strategy for the isolation of novel tumor-associated binding specificities. We foresee that immune libraries, obtained by immunization against complex antigen mixtures and analyzed using efficient selection schemes (Hoogenboom 1997) and screening methodologies (e.g., high-throughput immunohistochemistry), will continue to be useful tools for the discovery of novel tumor markers.

- Single-pot libraries

Single-pot libraries contain virtually all possible binding specificities and are not biased for a particular antigen. They are cloned once, with the aim to reach a complexity > 10^8 clones and, if possible, > 10^9-10^{10} clones. The corresponding phage are stored frozen in aliquots (Neri, Pini et al. 1998) and can directly be used in panning experiments against a variety of different antigens. Typically,

when using pure antigen preparations, specific monoclonal antibodies are almost always isolated in 2-4 rounds of panning (5-10 days of work). In general, both library design and library size contribute to the performance of the library, and to the quality of the isolated antibodies. Larger libraries have a higher probability of containing high affinity antibodies (Griffiths, Williams et al. 1994). It is technically possible to make phage display libraries of complexity $>10^9$ using brute force electroporation, and $>10^{11}$ using combinatorial infection and cre-lox mediated recombination (Waterhouse, Griffiths et al. 1993; Griffiths, Williams et al. 1994). However, the combinatorial diversity that can in practice be explored in panning experiments is limited by several factors, including the solubility of phage particles (typically $\leq 10^{13}$ transforming units/ml), the efficiency of antibody display on phage, and the phage recovery yields in biopanning experiments (de Haard, Kazemier et al. 1998).

Single-pot libraries can be classified as naïve or synthetic.

- Naïve repertoires

In this case V-genes are isolated from unimmunized animals or human donors, and are combinatorially assembled to create large arrays of antibodies.

The murine naïve repertoire has been estimated to contain $<5 \times 10^8$ different B-lymphocytes, while the human repertoire may be a hundred to a thousand times bigger (Winter, Griffiths et al. 1994). This array of antibodies may be cloned as a "naïve" repertoire of rearranged genes, by harvesting the V genes from the IgM mRNA of B-cells isolated from peripheral blood lymphocytes (PBLs), bone marrow, or spleen cells.

Several naïve human antibody phage libraries have been cloned so far. The first library of Marks et al. (Marks, Hoogenboom et al. 1991) was made from the PBLs of two healthy human volunteers and has yielded several antibodies with different specificities.

While it is by now clear that high-affinity antibodies can easily be isolated from large naïve libraries if the corresponding pure antigen is available, potential disadvantages are (1) the lower affinity rescued when smaller repertoires are

used; (2) the time and effort needed to construct these libraries; (3) the largely unknown and uncontrolled content of the library; (4) the need to sequence the isolated antibodies and to design custom primers for affinity maturation strategies based on combinatorial mutagenesis of CDRs. Furthermore (5), it remains to be seen how well naïve libraries perform against self-antigens for which the immune system is tolerant.

- Synthetic repertoires

In synthetic repertoires, variability is entirely created outside the natural host. To construct a synthetic antibody library, V-genes are typically assembled by introducing randomized CDRs into germline V-gene segments (Hoogenboom and Winter 1992). The antibody residues in which synthetic diversity is concentrated are chosen to correspond to regions of natural sequence diversity of the primary antibody repertoire. Since the VH CDR3 is the most diverse loop, in composition, length and structure, it is usually chosen for partial or complete randomization.

The choice of the germline V-genes into which one can insert combinatorial diversity can greatly vary. The variable regions of human antibodies are assembled from 51 different VH germline genes (Chothia, Lesk et al. 1992) and 70 different functional VL segments (40 Vκ and 30 Vλ; (Tomlinson, Cox et al. 1995; Tomlinson, Walter et al. 1996; Ignatovich, Tomlinson et al. 1997). One can choose to use only one type of scaffold, based on qualities of the scaffold (Pini, Viti et al. 1998), or keep one of the heavy or light chains constant and use different scaffolds of the other one (Nissim, Hoogenboom et al. 1994), or take full advantage of the diversity of the scaffolds and combine the different heavy and light chains as much as possible (Griffiths, Williams et al. 1994).

Since not all of the different chain variants are equally well represented in the functional repertoire, there might be a disadvantage using such a great variation of scaffolds. Indeed, there is evidence that only a few germline V-genes dominate the functional repertoire (Kirkham, Mortari et al. 1992; Tomlinson, Cox et al. 1995). By using scaffolds that are not often represented among the binders,

library diversity would be wasted. To avoid this, one could consider constructing libraries with only one light chain and concentrate combinatorial diversity solely in the heavy chain (Nissim, Hoogenboom et al. 1994). This approach has proven to work well in practice and offers the possibility to affinity mature the binders by randomizing the light chain in a second step (Neri, Carnemolla et al. 1997).

One of the main advantages of synthetic antibody phage display libraries is that the content of the library (antibody structure, codon usage, knowledge of the antibody portions that are randomized and of those that are kept constant) is defined *a priori*. Moreover, since antibody genes have not undergone any immunological selection, the library is not biased against self antigens. Synthetic libraries have already allowed to isolation of good-quality antibodies against conserved antigens such as calmodulin (Griffiths, Williams et al. 1994), the EDA (Borsi, Castellani et al. 1998; Villa, Trachsel et al. 2008) and EDB domains of fibronectin (Carnemolla, Neri et al. 1996; Neri, Carnemolla et al. 1997; Pini, Viti et al. 1998) or against "difficult" antigens such as BiP (a molecular chaperone) (Nissim, Hoogenboom et al. 1994).

2.2.3   Murine antibodies and antibody libraries

Mouse monoclonal antibodies are routinely generated by means of hybridoma technology (Kohler and Milstein 1975). B lymphocytes of immunized animals are fused with immortalized myeloma cells producing hybrid cell lines (hybridomas) that able to secrete monoclonal antibodies and can be screened for the desired binding specificity.

The development of antibody-based therapeutics often requires the development and *in vivo* testing of antibody-based products in syngeneic preclinical settings (*e.g.*, murine antibodies in mouse models of pathology). Certain antigens are not immunogenic in rodents (Carnemolla, Leprini et al. 1992; Melkko and Neri 2003) (i.e. self proteins and conserved antigens) or cannot be used for immunization (lethal toxins or highly toxic proteins) that would allow the development of a B

lymphocytes population specific for the desired antigen necessary to generate hybridomas.

Current preclinical therapy studies involving human antibodies and antibody derivative therapeutics in mouse models are limited by the mouse anti-human antibody (MAHA) response. To minimize the MAHA response preclinical studies are limited to use of immunodeficient mouse strains or to short-time therapies.

To overcome this problem it would be useful to rely on good-quality mouse antibodies, as mice are often the standard animal model for the *in vivo* testing of novel antibody-based therapeutics.

A mouse synthetic antibody phage display library would thus fulfill the increasing need of mouse monoclonal antibodies that cannot be generated by hybridoma technology.

While several antibody libraries from immunized mice have been described so far (Clackson, Hoogenboom et al. 1991), there are only few reports of naïve single pot mouse antibody libraries (Gao, Huang et al. 1999; Okamoto, Mukai et al. 2004; Imai, Mukai et al. 2006), which have so far been used only for the isolation of few monoclonal antibodies. Such naïve libraries, based on the combinatorial assembly of VH - VL genes extracted from non immunized mice, have still an unsatisfactory performance towards self antigens and conserved antigens due to immunological tolerance. Therefore a naïve synthetic mouse antibody phage display library may overcome this limitation and allow the isolation of mouse antibodies against self and conserved antigens of pharmaceutical relevance (*i.e.* EDB of fibronectin). At present no library with these prerequisites is available.

## 2.2.4 Other selections methodologies

2.2.4.1 Yeast display

Yeast display of antibody fragments has demonstrated to be an efficient and productive methodology for directed evolution of scFv fragments for increased

affinity and thermal stability and for the display of naïve scFv and immune Fab libraries. A major advantage of yeast display is the possibility to characterize the binding properties, such as the affinity and epitope binding characteristics, of a clone without the need of subcloning, expression and purification of the antibody fragment. A further strength of yeast display is the compatibility with Fluorescent-Activated-Cell-Sorting (FACS). By means of FACS, one can isolate yeast clones of interest, based on their ability to bind fluorescently labelled antigen.

In yeast display the a-agglutinin yeast adhesion receptor is used for the display of recombinant proteins on the surface of *Saccharomyces cerevisiae*. In *S. cerevisiae*, the a-agglutinin receptor acts as an adhesion molecule to stabilize cell-cell interactions and facilitate fusion between mating a and α haploid yeast cells. The receptor is composed of two proteins, Aga1 and Aga2: once Aga1 is secreted from the cell it becomes covalently attached to β-glucan in the extracellular matrix of the yeast cell wall. Aga2 binds to Aga1 through two disulfide bonds and after secretion remains attached to the cell through Aga1 (**Figure 2.7**). The display on the yeast surface of a recombinant protein profit by the association of Aga1 and Aga2 . The gene of interest is cloned into a vector as an in frame fusion with the AGA2 gene. Expression of both the Aga2 fusion protein from the vector and the Aga1 protein in the host strain is regulated by a tightly regulated promoter, GAL1. The use of this promoter allows the expansion of a scFv library 10^{10}-fold without any recognizable changes in either the percentage of antibody expression or the frequency of specific clones within the library . Upon induction with galactose, the Aga1 protein and Aga2-scFv fusion protein associate within the secretory pathway, and the epitope tagged scFv antibody is displayed on the cell surface at 10'000-100'000 copies per cell (**Figure 2.7**). ScFv antibody expression on the yeast cell surface can be monitored by flow cytometry with fluorescently labelled antibodies recognizing either the C-terminal c-myc or the N-terminal hemagglutinin (HA) epitope tags encoded by the display vector. The extracellular surface display of scFv by *S. cerevisiae* allows the detection of appropriately labelled antigen-antibody interactions by flow cytometry. The binding interactions between antigen and

scFv antibody are easily visualized by either direct or indirect fluorescent labelling of the antigen of interest. In a appropriate concentration range the fluorescent signal for antigen binding correlates to the affinity of the clone for this antigen. The use of yeast display of non-immune human scFv libraries is still limited as it has only been available since 2002. However, yeast display has some unique strengths as a platform for affinity reagent discovery and optimization. When screening a non-immune library for specific binders, enrichments of 10^9 can be achieved through multiple rounds of enrichment on a cell sorter, magnetic and/or flow cytometry based.
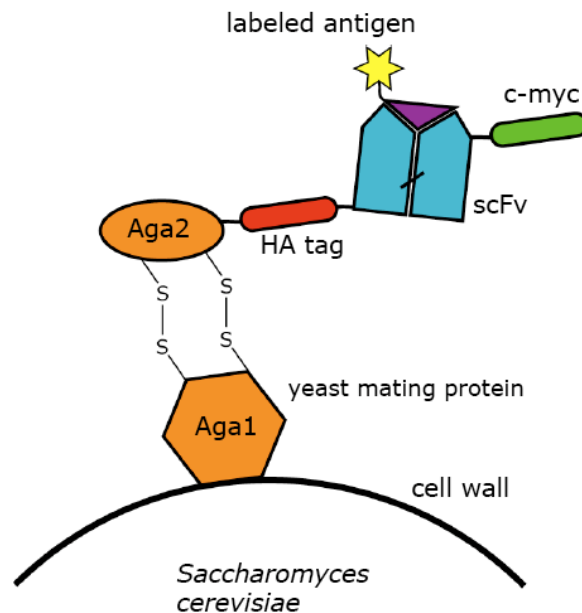
Figure 2.7. The scFv-Aga2 fusion protein surface expression system. Aga1 is bound to a cell wall glycan and connected by a disulfide bond to Aga2. The protein to be displayed is cloned in frame with the Aga2 gene. Using suitable antibodies, N-terminal hemagglutinin (HA) tag and C-terminal c-myc tag allow the monitoring of fusion protein expression. By addition of labelled antigen, yeast cells displaying antibody fragments binding the antigen can be isolated by affinity purification (e.g., biotinylated antigen) or FACS (e.g., fluorescently labelled antigen).

An additional advantage of yeast display is the ease of discriminating between clones with different affinities for the antigen on a flow cytometer during the

selection facilitating the isolation of higher affinity clones from lower affinity clones . Moreover yeast display selections are performed in solution, allowing the investigator to precisely control the concentration of antigen and establish a lower affinity threshold preventing the accumulation of low affinity clones, thereby facilitating clone characterization at the end of the selection. However, in cases where the antigen is not monovalent, strong avidity effects may come into play due to dense display of scFv on the cell wall of the yeast cell.

The characterization of binding clones is a time consuming and labor-intensive step in any antibody discovery process. Characterizations usually include: dissociation constant (K_D) determination, determination of off-rate (k_{off}) and of on-rate (k_{on}) constants and stability analysis. Yeast display is well suited for these analytical tasks, as the binding properties of multiple individually isolated scFv fragments can be rapidly and quantitatively determined directly on the yeast surface using flow cytometry.

Yeast surface display of scFv antibodies has also been successfully utilized to isolate higher affinity clones from small mutagenic libraries (1×10^{6} clones) created from a single antigen-binding scFv clone. These libraries are constructed by amplifying the parental scFv gene for affinity maturation using error-prone PCR incorporating three to seven point mutations per scFv. One type of selection of a mutagenized library is based on equilibrium antigen binding at defined concentration, usually at a concentration equal to the K_D of the parental clone. Selecting the brightest antigen-binding fraction of the population will often identify clones with increased affinity. Screening for slower off rates can also be performed. This requires saturating the antigen-binding sites and then allowing dissociation to occur in a large volume of buffer that does not contain antigen ("infinite dilution"). Yeast clones still binding biotinylated antigen are visualized on a flow cytometer and the clones retaining the highest degree of binding are sorted. Selections can also be focused on increasing the k_{on} rate constant of an antigen-scFv interaction by using shorter incubation times with a specific

concentration of antigen.

The directed evolution of an anti-CEA scFv antibody fragment with a 4-day monovalent dissociation half-time at 37°C was reported. The previously described scFv antibody fragment MFE-23 was first humanized by replacing 28 amino acid residues (hMFE-23) and then affinity matured by two rounds of mutagenesis and screening of yeast surface-displayed libraries. Several variants of hMFE-23 were isolated which showed 10-, 100-, and 1000-fold improvement in the off-rate over the original scFv. The biggest improvement corresponded to a half-life for binding to CEA of 4-7 days at 37° (versus 10 min for the parental antibody hMFE-23). This is the slowest reported dissociation rate constant engineered for an antibody against a protein antigen.
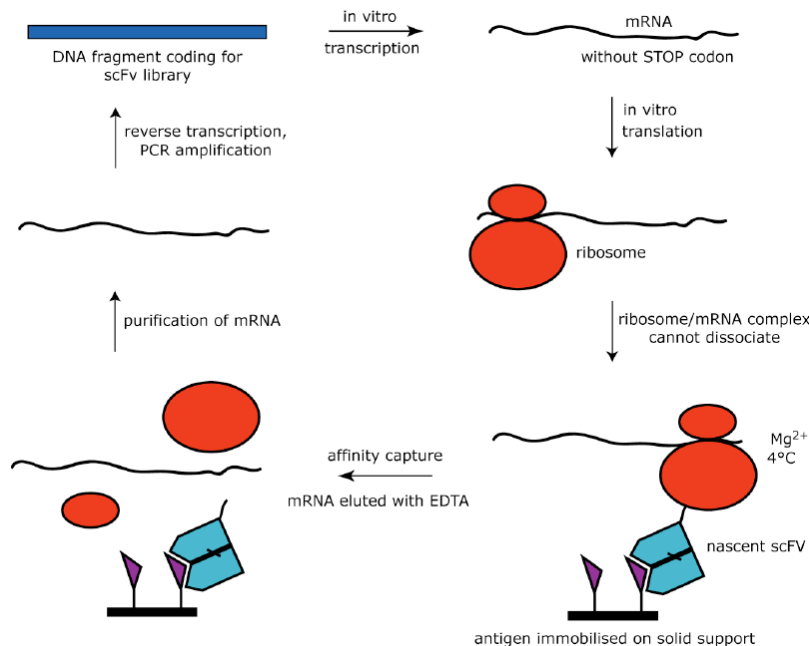

2.2.4.2 Ribosome display

The screening methodologies mentioned so far have some restrictions: library size is a limiting factor, due to cell transformation efficiency and the cloning of large libraries (≥109) can require a considerable amount of time and work. In order to circumvent these problems, fully in vitro selection techniques have been proposed.

In vitro display technologies combine two important benefits for identifying and optimizing ligands by evolutionary strategies. First, by obviating the need to transform cells in order to generate and select libraries, they allow higher library diversity. Second, by including PCR as an internal step in the procedure, they make PCR-based mutagenesis strategies convenient.

The concept underlying ribosome display was first described by Mattheakis *et. al* (Mattheakis, Bhatt et al. 1994). The key idea is to translate a library of mRNA molecules with a stoichiometric amount of ribosomes. The functional library size is limited by the quantity of *in vitro* transcription translation mixture used. There are two primary requirements for an efficient ribosome display. Firstly, it is necessary that the ribosome stalls on reaching the 3' end of the mRNA without dissociating. This was achieved by removing translation termination codons from

the mRNA (Hanes and Pluckthun 1997). This strategy also allows virtually all the full length translated protein to remain attached to the ribosome (Payvar and Schimke 1979). The second indispensable requirement is the correct folding of the protein while still attached to the ribosome in order to obtain satisfactory results in selections experiments. This can be achieved by introducing an unstructured tether or spacer region to the C-terminal end of a library of proteins, which is genetically encoded as a 3' end fusion to the DNA library. Another issue is the stability of the ternary complex (mRNA-ribosome-protein). Jermutus and co-workers could show that under appropriate experimental conditions, the complexes are stable for more than 10 days, allowing very extensive off-rate selections One unexpected advantage of these ternary complexes is that proteins displayed on the ribosome seem to be less aggregation-prone, expanding the range of proteins for which this technology can be applied.

After *in vitro* translation the ribosomal complexes are directly used for selection either on a ligand immobilized on a surface or in solution, with the bound ribosomal complexes subsequently being captured with, e.g. magnetic beads. The mRNA incorporated in the bound ribosomal complexes is eluted by addition of EDTA, purified, reverse-transcribed, and amplified by PCR. During the PCR step, the T7 promoter and the Shine-Dalgarno sequence are reintroduced by appropriate primers. Therefore the PCR product can be directly used for further selection cycles. Ribosome display is schematically depicted in **Figure 2.8**.

Figure 2.8. Schematic representation of the selection cycle of ribosome display. Linear DNA fragments coding for a protein library (here scFv antibody fragments) are transcribed *in vitro* and then purified before subsequent translation *in vitro*. After having reached the end of the mRNA translation, the ribosome is unable to dissociate from the mRNA because the stop codon is missing. The resulting ternary complex comprising the ribosome, mRNA and the nascent polypeptide can be stabilized by high concentrations of magnesium ions and at low temperature, therefore creating a stable linkage between the mRNA (genotype) and the encoded protein (phenotype). Ribosomes displaying a binding protein can be isolated by affinity selection on immobilized antigen, and the genetic information is amplified by reverse transcription and PCR after elution of selected mRNA molecules by addition of EDTA.

A large synthetic antibody library, HUCAL-1, of 2×10^9 independent members (Knappik, Ge et al. 2000), was used directly as the starting material for ribosome display selections (Hanes, Schaffitzel et al. 2000). This naïve library was applied for six rounds of selection using insulin as antigen. In three independent experiments, different scFv families with different framework combinations were isolated. Since the library was completely synthetic (Knappik, Ge et al. 2000), the starting scFv sequences were known and any mutation could be directly identified as being generated during the ribosome display procedure by non-

proofreading polymerases in the PCR steps. In summary, this procedure mimics to a certain degree the process of somatic hypermutation of antibodies during secondary immunization.

Ribosome display has been shown to work especially well for affinity maturation of scFv fragments. Two studies, here described, have been reported in which a given antibody was evolved to higher affinity. In both cases, off-rate selection combined with error-prone PCR was used.

An antibody fragment specific to fluorescein was evolved (Jermutus, Honegger et al. 2001) using selections in which the antibody-antigen complex needed to last up to 10 days, resulting in final dissociation constants of about 100 pM. The evolved scFv fragments all contained between 4 and 11 mutations, with the majority unlikely to be in contact with the antigen.

In another study the dissociation constant of a scFv fragment specific to a peptide from the transcription factor GCN4 was improved from 40 to 5 pM (Zahnd, Spinelli et al. 2004). In both cases libraries were generated with error-prone PCR and DNA shuffling, and selected for decreased off-rates.

ScFv antibody fragments have also been evolved for stability by ribosome display (Jermutus, Honegger et al. 2001) .

In a recent work, ribosome display was used in order to generate the tightest peptide-binding antibody reported to date. A single-chain Fv antibody fragment, showing a binding affinity of 1 pM to a peptide derived from the unstructured region of bovine PrP, was obtained by applying several rounds of directed evolution and off-rate selection with ribosome display using an antibody library generated from a single PrP binder with error-prone PCR and DNA-shuffling (Luginbuhl, Kanyo et al. 2006).

2.2.4.3 Iterative colony filter screening

Iterative colony filter screening is rapid methodology that allows the isolation of binding specificities from a large synthetic repertoire of human antibody fragments (Giovannoni, Viti et al. 2001). In this procedure a positional linkage

between the binding phenotype and the bacterial colony (therefore the genotype) is kept by two overlaid filters. Bacterial cells, expressing the library of antibody fragment, are spread and grown on a first master porous filter (membrane A, **Figure 2.9**). A second nitrocellulose filter (membrane B, **Figure 2.9**) is coated with the antigen of interest and transferred on a solid medium able to induce soluble antibody fragments expression. Placing the bacterial colony filter onto the antigen-coated filter allows the diffusion and binding of antibody fragments to the antigen immobilized on the nitrocellulose membrane. Detection of antibody fragment on the antigen filters and by overlaying it with the bacterial colony filter leads to the identification of clones displaying the desired binding phenotype.

This procedure can be iteratively reproduced until single clones are isolated. Typically two or three rounds of colony filter screening are applied in order to isolate monoclonal antibodies from big library repertoires. Iterative colony filter screening has been successfully used for the isolation of antibodies against EDB (Giovannoni, Viti et al. 2001) and EDA (Villa, Trachsel et al. 2008) domains of fibronectin.
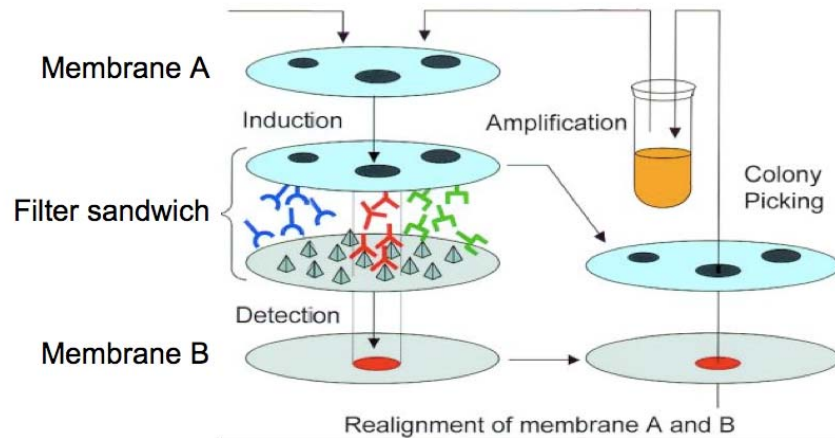
Figure 2.9. Schematic representation of the iterative colony filter screening method. Bacterial expressing a library of antibody fragments (i.e., scFv) are spread on a Durapore filter membrane A. On the filter, placed on a solid medium that allows the bacterial growth, colonies become visible after about 8h incubation at 37°C. Membrane B is a nitrocellulose filter coated with the antigen of interest. This second filter is placed onto a solid medium capable of inducing soluble antibody fragment expression. Once bacterial colonies are grown, membrane A is overlaid onto

membrane B, where after a few hours of incubation induced soluble antibody fragments are able to diffuse through membrane A and reach antigen coated membrane B. Membrane B is then developed for instance with colorimetric or electrochemoluminescence techniques in order to identify the position of the colonies expressing binding antibodies.

2.3   Vascular tumor targeting of interleukin 12

2.3.1   Antibody-based tumor vascular targeting

Most conventional pharmaceuticals in use for the treatment of cancer do not selectively accumulate in the tumor tissue, leading to poor efficacy and severe side effects. In general intravenously administered drugs equally distribute within the different organs and tissues of the body. The extravasation and accumulation of cytotoxic drugs in neoplastic lesions is especially poor as a consequence of the high interstitial pressure in the tumor environment, and the sluggish blood flow in the tumor neovasculature (Tozer, Ameer-Beg et al. 2005).

One promising approach to circumvent the disadvantages of conventional cancer therapy is based on the preferential delivery of a therapeutic agent to the tumor site by means of a binding molecule (for example human antibodies) specific for a tumor-associated marker (**Figure 2.10**). The selective targeting of a drug to the tumor environment will ultimately result in an increased local concentration at its site of action, while the host's healthy organs can be effectively spared. In most cases, this will lead to an improvement of the therapeutic index of the delivered pharmaceutical, that is, a higher efficacy with minimized side effects.
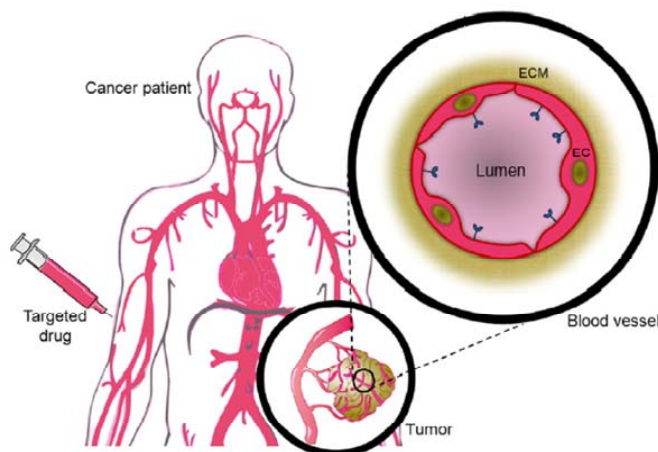
Figure 2.10. The concept of antibody-based tumor vascular targeting. The targeted compound, consisting of an antibody as a carrier molecule and an effector moiety, is applied intravenously

40

and homes to the tumor-specific vascular antigen, resulting in the accumulation of the pharmaceutical at the tumor site. Vascular antigen can be either expressed on the luminal surface of endothelial cells (EC) or in the perivascular extracellular matrix (ECM).

The favorable toxicity profile of site-specific therapeutics may open new avenues in cancer therapy, allowing the systemic administration of highly potent agents, which are currently either given at suboptimal doses or whose clinical application has been to date impeded by unacceptable toxicities (for example cytokines) when applied in an unmodified form.

Tumor vascular targeting is thus defined as the targeted delivery of a bioactive effector molecule (such as a drug, cytokine, toxin, procoagulant factor, radionuclide, or photosensitizer) to the tumor site by means of a binding molecule (typically a human antibody) specific to a tumor-associated vascular marker.

Due to their accessibility from the bloodstream and to the therapeutic options that they offer, vascular markers selectively expressed on tumor blood vessels seem to be ideally suited for antibody-based tumor-targeting strategies, opening a new avenue for the therapy of cancer.

Markers expressed on tumor neovasculature have the theoretical potential to meet all desired criteria for an efficient drug targeting approach. Vascular markers are accessible from the bloodstream to systemically administered agents. Endothelial cells and perivascular cells, that produce and secrete the vascular matrix proteins where tumor markers are generally found, are genetically more stable than tumor cells, therefore they bear a lower risk of drug resistance development. Moreover, a single vascular targeting agent could in principle be applicable to a wide range of different tumor types, because angiogenesis is a common feature of virtually all malignancies and tumor vessels in histologically distinct tumors express common markers.

Among all vascular tumor markers known so far, splice isoforms of fibronectin and tenascin are some of the most promising markers of tumor angiogenesis as they have been found in the stroma of the majority of human solid tumors (Brack, Silacci et al. 2006; Rybak, Roesli et al. 2007; Pedretti, Soltermann et al. 2009;

Schliemann, Wiedmer et al. 2009). Because of their abundant and selective expression at tumor site and limited presence in healthy organs they are excellent candidates for the antibody-based vascular tumor targeting.

Tenascins are extracellular matrix components primarily synthesized by cells in the connective tissue. Tenascin C is highly expressed during embryogenesis and is transiently expressed during organogenesis, while absent or much reduced in developed organs. Tenascin C reappears under pathological conditions caused by infections, inflammation or during tumorigenesis (Chiquet-Ehrismann and Chiquet 2003), where it is expressed by activated fibroblasts, endothelial cells or by cancer cells. Several isoforms of tenascin C can be generated, as a result of alternative splicing which may lead to the inclusion of (multiple) fibronectin-type III homology repeats in the protein, ranging from domain A1 to domain D (Borsi, Carnemolla et al. 1992; Carnemolla, Borsi et al. 1992). These large isoforms of tenascin C have been associated with tissue remodeling and with invasiveness of carcinomas (Jones and Jones 2000) and angiogenesis (Zagzag, Friedlander et al. 1995) as well as with wound healing. The large isoform of tenascin C is undetectable in most normal tissue making it an extremely interesting candidate for antibody-based tumor targeting.

Currently an antibody fragment scFv(F16) specific to the oncofetal domain A1 of tenascin C is investigated in the clinic as fusion protein with the human interleukin 2 for the therapy of breast cancer (Marlind, Kaspar et al. 2008).

Fibronectins are high molecular weight adhesive glycoproteins found in soluble form in plasma and other body fluids and in insoluble form in the extracellular matrix. Fibronectins play an essential role in several processes as in maintenance of normal cell morphology, cell adhesion, migration, hemostasys, thrombosis, wound healing, differentiation and proliferation.

By alternative splicing of the pre-mRNA and post-translational modifications, up to twenty isoforms of fibronectin are produced. Splicing occurs in three regions of the fibronectin gene. Two type-III exons known as EDA (extra-domain A) or EDB

(extra-domain B), can be either totally included or totally excluded. The other type-III repeats of fibronectin are coded by two exons. A third region, the variable V or IIICS, at the C-terminal of the molecule, may be spliced in several positions, giving rise to five potential variants (Schwarzbauer, Tamkun et al. 1983).

The alternatively-spliced EDB domain of fibronectin represents one of the best characterized markers of angiogenesis (Zardi, Carnemolla et al. 1987; Castellani, Viale et al. 1994; Kaczmarek, Castellani et al. 1994; Carnemolla, Neri et al. 1996; Castellani, Borsi et al. 2002; Kaspar, Zardi et al. 2006) .

EDB is virtually absent in normal adult tissues (exception made for the endometrium in the proliferative phase and some vessels of the ovaries), but is strongly expressed in tissue remodelling as in most aggressive solid cancer types, with a prominent vascular and/or stromal pattern of expression. The high-affinity human antibody L19 (Pini, Viti et al. 1998; Borsi, Balza et al. 2002) specific to EDB, has been shown to selectively localize around tumor neo-vascular structures in animal models of cancer (Borsi, Balza et al. 2002; Berndorff, Borkowski et al. 2005; Spaeth, Wyss et al. 2006; Tijink, Neri et al. 2006; Wyss, Spaeth et al. 2007) and in patients with cancer (Santimaria, Moscatelli et al. 2003; Sauer, Erba et al. 2009).

A mentioned above fibronectin contains a second alternatively spliced domain (EDA) (Muro, Caputi et al. 1999) which has been shown to display restricted pattern of expression in plasma and normal human tissues, while being over-expressed in the neo-vasculature and/or stroma structures of several aggressive solid tumors (Borsi, Castellani et al. 1998; Rybak, Roesli et al. 2007)

A high-affinity human antibody fragment, termed scFv(F8), specific to EDA was generated. Quantitative biodistribution studies of F8 exhibited an impressive tumor targeting performance (Villa, Trachsel et al. 2008).

2.3.2 Immunocytokines

Based on the evidence that impaired immune responses to tumors are due to tumor's inability to appropriately activate the immune system (Van Pel and Boon 1982), immunotherapy evolved rapidly as an alternative to the conventional therapies and immunoregulatory cytokines have been demonstrated to improve antitumor immune responses (Soiffer, Robertson et al. 1993; Rosenberg, Yang et al. 1998)

Cytokines are key mediators of innate and adaptive immunity. Physiologically cytokines exert their function as auto- or paracrine factors that reach high concentrations only in the close vicinity of the producing cell. Many cytokines has been used for therapeutic purposes in patients with advanced cancer, but their systemic administration is typically associated with severe toxicity, hampering dose escalation to therapeutically-active regimens and their development as anti-cancer drugs (Janeway`s Immunobiology);

To overcome these problems, the use of "immunocytokines" (i.e., cytokines fused to antibodies or antibody fragments) has been proposed, with the aim to concentrate the immune system stimulating activity at the site of disease while sparing normal tissues.

The group of Reisfeld has pioneered the fusion of interleukin-2 (IL2) at the C-terminal end of the heavy chain of intact immunoglobulins and has brought these products to clinical trials in patients with cancer (Osenga, Hank et al. 2006). Our group has focused on the engineering of polypeptides consisting of individual cytokines fused to antibody fragments in scFv format, in order to reduce the circulatory half-life of these biopharmaceuticals to a minimum, while retaining their tumor homing properties (Neri and Bicknell 2005; Schliemann and Neri 2007). For pharmacodelivery applications, we have mainly focused on antibodies specific to splice isoforms of fibronectin and of tenascin-C, as these tumor targets are strongly expressed in the stroma and neo-vasculature of virtually all cancer types, while being undetectable in most normal adult tissues (Neri and Bicknell 2005; Brack, Silacci et al. 2006; Pedretti, Soltermann et al. 2009; Schliemann,

Wiedmer et al. 2009; Schwager, Kaspar et al. 2009). The antibody fragments scFv(L19) (specific to the alternatively-spliced EDB domain of fibronectin (Pini, Viti et al. 1998)), scFv(F8) (specific to the EDA domain of fibronectin (Villa, Trachsel et al. 2008)) and scFv(F16) (specific to the A1 domain of tenascin-C (Brack, Silacci et al. 2006)) have been genetically fused to several cytokines. Currently the immunocytokines L19-IL2 (Carnemolla, Borsi et al. 2002), L19-TNF (Borsi, Balza et al. 2003), F16-IL2 (Marlind, Kaspar et al. 2008) and F8-IL10 (Schwager, Kaspar et al. 2009) are being tested in Phase I and Phase II clinical trials. In addition, a number of other immunocytokines (IL12, IFNγ, GM-CSF, IL15 fused to antibody fragments) have been tested in preclinical models of cancer (Halin, Rondini et al. 2002; Halin, Gafner et al. 2003; Ebbinghaus, Ronca et al. 2005; Gafner, Trachsel et al. 2006; Rybak, Roesli et al. 2007; Wyss, Spaeth et al. 2007). In our experience the fusion of cytokines to a tumor targeting antibody fragment could significantly improve the therapeutic potency of the cytokine compared to the untargeted form in animal experiments (Halin, Rondini et al. 2002; Kaspar, Trachsel et al. 2007; Rybak, Roesli et al. 2007)
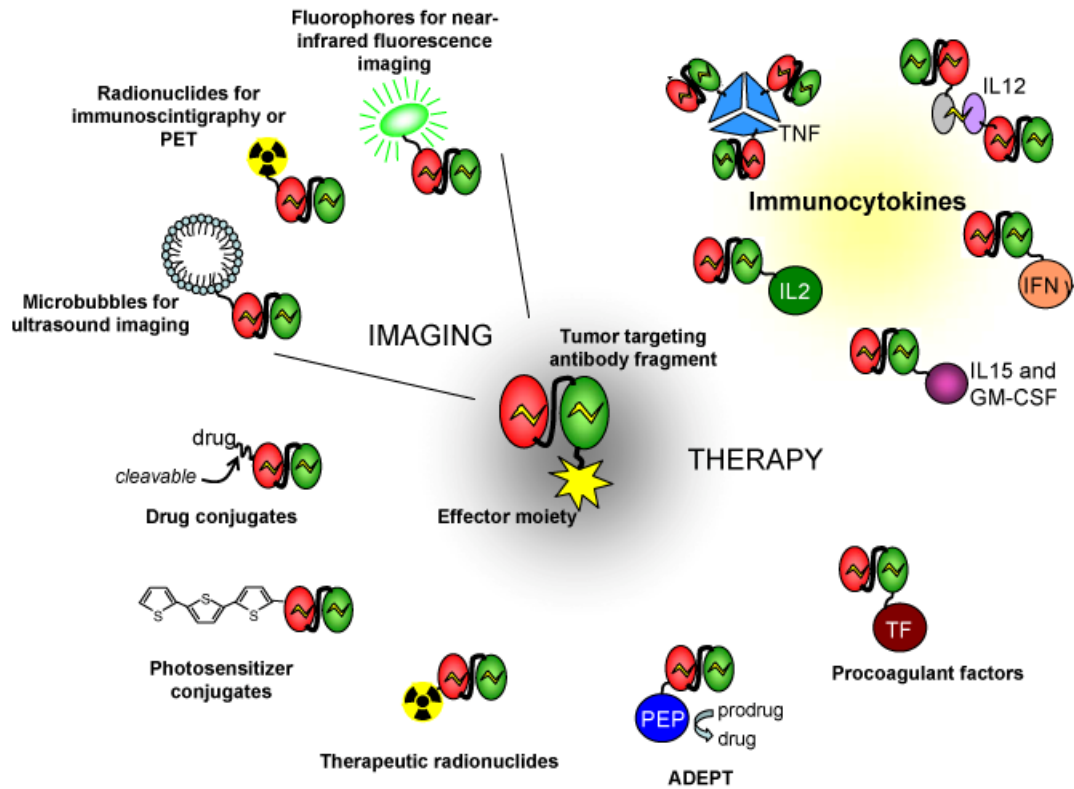
Figure 2.11: Scheme of scFv(L19) derivatives evaluated in pre-clinical model of cancer and in clinical studies. On the right upper part of the figure scFv(L19)-cytokines fusion proteins are grouped.

2.3.3 Interleukin 12

2.3.3.1 Structure and function

Interleukin 12 (IL12) is a heterodimeric cytokine, consisting of a p35 and a p40 subunits covalently linked by a disulfide bridge. The crystal structure of IL12 shows that the heterodimer p35-p40, named also p70, is similar to a class 1 cytokine-receptor complex in its architecture. The similarities of IL6 and its receptor to IL12 might suggest a possible evolution of this cytokine from a primordial cytokine of the IL-6 family and one of its receptors. IL23 and IL27, two other heterodimeric cytokines that are related to IL12, have been identified (Oppmann, Lesley et al. 2000; Pflanz, Timans et al. 2002) which indicates that

IL12 is the prototype member of a small family of heterodimeric cytokines. IL12 activity is mediated by the binding to a high affinity heterodimeric receptor, termed IL12R, that is composed of two subunits IL12Rβ1 and IL12Rβ2.

The binding of IL12 to its receptor leads to the activation of the Janus kinase (JAK)–STAT (signal transducer and activator of transcription) pathway of signal transduction.

IL12 is a key regulator of cell-mediated immune responses as it regulates the balance between Th1 and Th2 responses.

The more important functions of IL12 are the priming of the T helper 1 (Th1) immune responses and IFN-γ secretion by NK cells. *In vitro* studies in both human (Manetti, Parronchi et al. 1993) and murine (Schmitt, Hoehn et al. 1994) systems and *in vivo* studies in mice (Sypek, Chung et al. 1993; Magram, Connaughton et al. 1996) have confirmed that IL12 plays a critical role in the promotion of Th1 responses. IL12 generates the Th1 response in three modalities: (*i*) it promotes the differentiation of naïve T cells, during initial encounter with an antigen, into a population of Th1-cells capable of producing large amounts of IFN-γ following activation (Magram, Connaughton et al. 1996), (*ii*) it serves as a costimulus required for maximum secretion of IFN-γ by differentiated Th1 cells responding to a specific antigen (Murphy, Terres et al. 1994), and (*iii*) it stimulates the development of IFN-γ -producing Th1 cells from populations of resting memory T cells interacting with an antigen to which they have been previously exposed (Manetti, Parronchi et al. 1993). More recently IL12 was shown to play a role also in the differentiation of cytotoxic T lymphocytes (Kalinski, Hilkens et al. 1999; Curtsinger, Lins et al. 2003) and in the reactivation and survival of memory CD4+ T cells (Yoo, Cho et al. 2002). Moreover it potentiates the activity of cytotoxic T lymphocytes and lymphokine-activated killer cells.

IL12 directly stimulates early hematopoietic progenitor cells and synergizes with other hematopoietic growth factors in promoting proliferation and differentiation of bone marrow progenitors.

2.3.3.2 Anticancer properties and clinical studies

The immunomodulating and antiangiogenic functions of IL12 have provided the rationale for exploiting this cytokine as an anticancer agent in preclinical studies (Brunda, Luistro et al. 1993; Trinchieri 1997; Tsung, Meko et al. 1997; Rodolfo and Colombo 1999; Colombo and Trinchieri 2002). Brunda et al. have demonstrated in a number of murine tumor models the inhibition of established experimental pulmonary or hepatic metastases and a reduction in spontaneous metastases by treatment with murine IL12 (Brunda, Luistro et al. 1993). Mice bearing different subcutaneous tumor types treated with IL12 resulted in tumor growth inhibition, prolongation of survival, and, in some models, tumor regression. Interestingly, after depletion of TCD8+ cells the antitumor efficacy of IL12 was dramatically decreased while remained intact in TCD4+ depleted mice; these results may suggest that CD8+ cells are the critical cell type for mediating the antitumor activity of IL12 (Brunda, Luistro et al. 1993).

Some groups have shown that the anti-tumor activity of IL12 relies also on the inhibition of tumor neoangiogenesis (Voest, Kenyon et al. 1995).

Possible mechanisms are the cross-talk between the induced IFN-γ activated lymphocytes and endothelial cells, the reduction of vascular endothelial growth factor production by tumor cells (Dias, Boyd et al. 1998) observed also in several patients exposed to IL12 (Younes, Pro et al. 2004) and the reduction of metalloproteases production that play a role in matrix remodeling processes (Mitola, Strasly et al. 2003)

The encouraging preclinical data of IL12 as agent for cancer therapy suggested the possibility to extend the IL12-based treatment also in humans.

In spite of promising antitumor activity in preclinical studies, clinical trials revealed limited efficacy and extreme toxicity of systemic administration of IL12 at doses lower than 1 µg per kg per day, hampering dose escalation to therapeutically-active regimens (Atkins, Robertson et al. 1997; Bajetta, Del Vecchio et al. 1998; Motzer, Rakhit et al. 1998; Robertson, Cameron et al. 1999; Rook, Wood et al. 1999; Gollob, Mier et al. 2000; Mortarini, Borri et al. 2000; Motzer, Rakhit et al. 2001; Lenzi, Rosenblum et al. 2002; Portielje, Lamers et al.

2003; Weiss, O'Donnell et al. 2003; Wadler, Levy et al. 2004; van Herpen, van der Laak et al. 2005).

Not even the combination of IL12 with different kinds of vaccine (Lee, Wang et al. 2001; Cebon, Jager et al. 2003), IL2 (Gollob, Veenstra et al. 2003), interferon alfa (Alatrash, Hutson et al. 2004), trastuzumab (Parihar, Nadella et al. 2004) showed any relevant clinical benefit.

With the exception of cutaneous T-cell lymphoma variants (Rook, Wood et al. 1999), AIDS-related Kaposi sarcoma (Little, Pluda et al. 2006) and non–Hodgkin's lymphoma (Younes, Pro et al. 2004), where more than 20% of patients had a partial or complete response, efficacy was generally minimal, with an objective response rate ranging between 0% and 11%.

2.3.3.3 Antibody – interleukin 12 fusion proteins

Cytokines, as IL12, may be potent anticancer agents as they can enhance the capacity of the immune system to react against tumors. However in clinical studies exposure to IL12 was associated to unacceptable toxicity already at very low doses, preventing dose escalation to therapeutically active concentrations. Generally cytokines act locally in a paracrine or autocrine way and do not have any therapeutic action at distant sites. Therefore the systemic administration of an untargeted cytokine leads to an insufficient therapeutic concentration at the site of disease.

As IL12 and generally all cytokines do not preferentially accumulate at the tumor site following i.v. administration, the antibody-based targeted delivery of IL12 to the tumor environment seems to be a promising strategy for enhancing the therapeutic index and reducing side effects as lower doses are required.

Several antibody-IL12 fusion proteins for the therapy of cancer were reported.

Lo et al fused the IL12 to a fully humanized IgG (BC1) (Lo, Lan et al. 2007) targeting a cryptic epitope on the domain 7 of B fibronectin (Carnemolla, Leprini et al. 1992). By coupling IL12 to a full IgG a multifunctional cytokine fusion protein is obtained with a mass of about 300 kDa. This high molecular weight

impairs extravasation of the protein from the blood vessel into the extracellular matrix where the target protein is expressed and leads to a long half life with a slow clearance from the circulation. Phase I clinical trial of AS1409 (BC1-IL12) showed some clinical benefit at a MTD of 15 µg/kg. The clinical use of a such high dose, was not correlated to usual extreme side effects (the MTD of IL12 is 0.5 µg/kg) suggesting a partial loss of bioactivity of the cytokine by the fusion to the IgG. Further clinical trials will be needed to asses the real potential of this fusion protein.

The generation of a fully bioactive and efficient tumor targeting version of IL12 is not a trivial accomplishment. First a tumor targeting antibody must be available. Second, the fusion strategy of the IL12 to an antibody moiety must not alter the bioactive properties. Last, the fusion protein must selectively accumulate at the tumor site.

Preclinical potential of the antibody targeted delivery of IL12 at the tumor site has been reported and it was shown that fusions at N-terminus of IL12 can abrogate the cytokine bioactivity (Lieschke, Rao et al. 1997). Large molecular weight antibody-IL12 fusion proteins (>200 kDa) ((Gafner, Trachsel et al. 2006); Sommavilla R, Kaspar M, Neri D, unpublished data) generally abolish tumor extravasation, while the simple fusion of a single antibody moiety to the IL12 results in a poor tumor targeting performance (Halin, Rondini et al. 2002). Gafner et al could show that the fusion of the murine IL12 to an antibody fragment into the heterodimeric format scFv-p35/p40-scFV with a size of ~120 kDa combines perfect balance between extravasation and elimination from the blood, resulting in excellent tumor targeting performance (Gafner, Trachsel et al. 2006) (**Figure 2.12**). This complex heterodimeric protein was shown to have impressive therapeutic potentials and could serve as model for the creation of a fully human antibody-IL12 fusion protein.
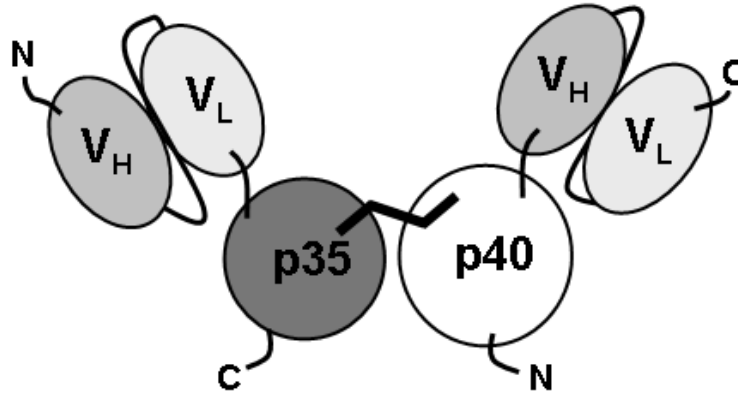
Figure 2.12. The schematic structure of the heterodimeric format scFv-p35/p40-scFv is depicted in the picture. The two subunits are covalently linked by a disulphide bridge between p35 and p40.

2.4 The aim of this thesis

Thanks to the pioneering work of Milsteins and Köhler, mouse monoclonal antibodies have been generated since 1975 via hybridoma technology (Kohler and Milstein 1975). No other technology was shown to yield good quality mouse antibodies so far.

Hybridoma technology has clear limitations when the antigen is not immunogenic (self and conserved proteins) or in case of highly toxic or deadly pathogenic antigens. Moreover the entire process, from immunization to the isolation of the desired hybridoma cell line, is very complex, laborious and expensive.

The mouse is the most frequent animal model for the in vivo testing of antibody-based therapeutics. Human antibodies and antibody derivatives studies in mouse models are limited by the mouse anti-human antibody (MAHA) response that force to use immuno-deficient mouse strains or to short-time therapies. Fully murine antibody-based products would allow long-term preclinical studies in syngeneic settings resulting in more significant preclinical results and in a better understanding of the immunological mechanisms of the therapeutic.

The aim of my thesis was the design and construction of a novel synthetic naïve mouse antibody phage display library from no prior art which could allow the isolation of fully murine monoclonal antibodies against virtually any desired antigen. The library could be especially useful for the generation of mouse monoclonal antibodies against conserved antigens, like the extra domain B of fibronectin where all immunization attempts failed (Carnemolla, Leprini et al. 1992; Peters, Trevithick et al. 1995).

IL12 is a highly potent and multifunctional cytokine acting as a key regulator of cell-mediated immune responses. The immunomodulating and antiangiogenic functions of IL12 have provided the rationale for exploiting this cytokine as anticancer agent. In spite of promising antitumor activity in preclinical studies, clinical trials with IL12 have shown limited efficacy and severe side effects in

most instances. The selective delivery of cytokines to the tumor site by means of suitable tumor targeting antibodies promises to enhance the potency of cytokines and reduce their side effects. Among all promising antibody-cytokine fusion proteins that have been produced in our laboratory, the IL12 fusion proteins showed impressive preclinical results (Halin, Rondini et al. 2002; Halin, Gafner et al. 2003; Gafner, Trachsel et al. 2006) without worsening of inflammatory processes (Trachsel, Kaspar et al. 2007).

The second aim of my thesis was the cloning, expression and characterization of a fully human heterodimeric immunocytokine for clinical use between the human IL12 and a tumor specific antibody scFv(F8). This project posed significant challenges since gene dosage studies were necessary for the optimal expression of the chosen heterodimeric format and since clinical-grade material should not contain the peptidic tags which were previously used to facilitate purification of heterodimeric proteins.

3 Design and construction of a naïve mouse antibody phage display library

3.1 Results

3.1.1 A novel approach for the generation of murine antibodies

There is a growing interest in the development of human monoclonal antibodies as therapeutics. Fully human monoclonal antibodies can be rapidly isolated from antibody phage display libraries to virtually any target antigen. However, preclinical studies in rodents are limited by the immunogenicity of human antibodies that induce mouse anti human antibody responses (HAMA). For this reason, immunodeficient mouse strains or short-time therapies are frequently used to evaluate human antibodies in rodent models of pathology. In order to perform more significant and reliable preclinical studies, one should execute therapy experiments in a syngeneic setting, such as the testing of murine antibodies in mouse models of pathology.

Murine monoclonal antibodies are routinely generated from mice using hybridoma technology. This approach has limitations when the antigen is highly conserved among species or is a self protein (resulting in low immunogenicity due to immunological tolerance), or in case of highly toxic or deadly pathogenic antigens. Antibody phage technology represents a valid alternative to overcome such problems. Phage display technology, not requiring immunization of the donor with antigen, avoids laborious laboratory work. Furthermore, production in *E.coli,* facilitates the genetic manipulation of antibodies. At present there is no functional synthetic naïve mouse antibody phage display library available.

For this reason, we aimed at designing and constructing a large mouse antibody phage display library, which would capitalize on previous experience of our lab in the construction of antibody libraries based on a single antibody scaffold with

combinatorial mutations at residues of the CDR3 loops of heavy and light chains (Pini, Viti et al. 1998; Silacci, Brack et al. 2005; Villa, Trachsel et al. 2008).

3.1.2 Design and construction of PHILOtop, a synthetic naïve mouse antibody phage display library

In order to generate a large, stable and highly diverse library of functional antibody fragments with similar biophysical properties, we chose to clone our synthetic antibody libraries in a phagemid vector of proven quality (Hoogenboom, Griffiths et al. 1991; Silacci, Brack et al. 2005) restricting sequence diversity to the CDR3 of VH and Vk domains, in which all other positions were kept constant (Pini, Viti et al. 1998). We chose to express antibody fragments in scFv format (Huston, Levinson et al. 1988). As this single polypeptide recombinant antibody format exhibits better expression yields and phage display properties compared to Fab fragments. The complete sequence of the library can be found in **Table 3.1**.

Variable heavy chain (VH)	Linker	Variable light chain (Vk)
QVQLQQPGAELVKPGASVKMS CKASGYTFTSYNMHWVKQTPG RGLEWIGAIYPGNGYTSYNQKF KGKATLTADKSSSTAYMQLSSL TSEDSAVYYCAR**XXXX(X)(X)**FD YWGQGTTVTVSS	GGGGSGGG GSGGGG	QIVLSQSPAILSASPGEKVTMTC RASSSVSYMHWFQQKPGSSPK PWIYATSNLASGVPVRFSGSGS GTSYSLTISRVEAEDAATYYCQQ **XXXX**P**X**TFGGGTKLEIKRTVAA

Table 3.1. Aminoacid sequence of PHILOtop library The amino acid sequence of the murine scFv antibody phage display library PHILOtop is represented in the table. The scFv is composed of a heavy chain, a linker and a variable chain; the randomized CDR3 regions are underlined.

The PHILOtop library design was based on the germline gene 186.2 for the VH (Bothwell, Paskind et al. 1981; Williams, Martinez et al. 2001; Chang and Mohan 2005) and on the Vk germline gene kv4-72 (Kirschbaum, Roschenthaler et al. 1999). The germline gene 186.2 was chosen as VH scaffold for library construction due to the predominant frequency of occurrence of this germline in

antibody responses in mice (Chang and Mohan 2005) suggesting that VH186.2 is a versatile scaffold able to bind a broad spectrum of antigens. As variable light chain, the Vk germline gene kv4-72 was chosen due to the fact that it is often found to pair with the VH186.
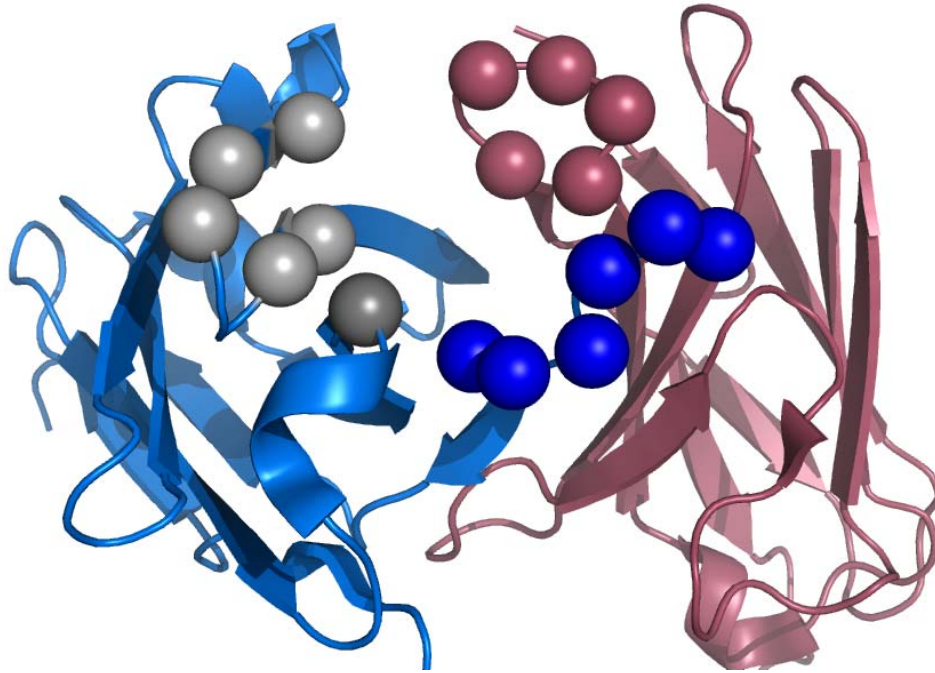
Figure 3.1. Representation of the scFv scaffold of the PHILOtop library. Heavy variable chain in blue: blue spheres CRD3 randomized residues of PHILOtop library, grey spheres CDR1 and CDR2 randomized residues in the affinity maturation library. In red the variable light chain: red spheres CDR3 randomized residues.
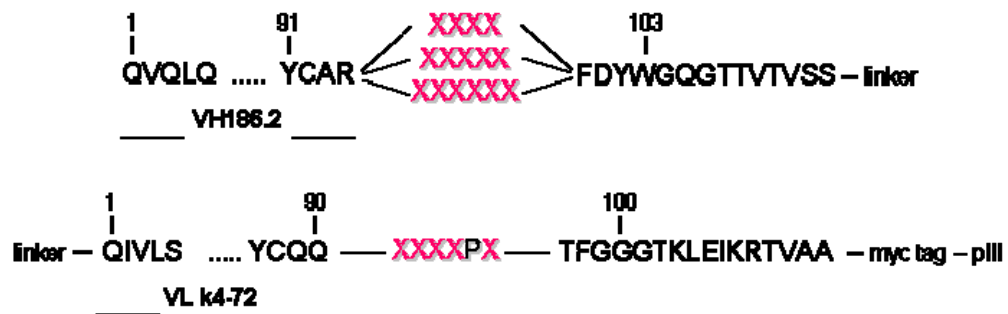
Figure 3.2. Design of the PHILOtop, scFv antibody phage display library, is depicted in the figure. Variable heavy and light domains are connected by a 14 aminoacid linker that allows the pairing of the two domains into a scFv format. Randomized positions in CDR3 of variable heavy and light chains are indicated in red.

The two germline genes, with only four amino acids mutated within the VH and the conserved germline gene sequence of the kv4-72, are contained in rituximab, a chimeric monoclonal antibody approved for the therapy of lymphoma and certain inflammatory diseases (Molina 2008).

A completely randomized sequence of four, five or six amino acids residues (followed by the conserved Phe-Asp-Tyr sequence) was appended to the VH germline segment giving rise to the three sub-libraries H4K, H5K and H6K, respectively. Five aminoacid positions were randomized in the CDR3 loop of the Vk domain (**Figure 3.2**).

The partially degenerate primers used for library construction can be found in **Table 3.6**. Library cloning yielded three sub-libraries (H4K, H5K and H6K) corresponding to the different length of the CDR3 loops of the VH domain, which contained a total of 1.5×10^9 individual clones (**Table 3.2**).

PHILOtop library

| sub-library	titer
H4K	5.6×10^8
H5K	3.2×10^8
H6K	6.2×10^8
total titer	1.5×10^9

Table 3.2. Titers of the three sub-libraries composing the PHILOtop library

PCR screening showed that 36/36 randomly picked clones from the library contained an insert of the correct size (**Figure 3.3 A**). A dot blot experiment revealed that approximately 90% of the analyzed library clones expressed soluble scFv fragments (**Figure 3.3 B**). Importantly, the majority of bacterial supernatants that scored positive with an anti-myc reagent could also be detected with Protein L (**Figure 3.4**).

The library was also tested for Protein A dot blot analysis but results were all negative.

In line with the good antibody expression data observed in the bacterial supernatants, a Western blot analysis of PHILOtop clones indicated that the band corresponding to pIII was approximately four times more intense than the scFv-pIII band (**Figure 3.3 C**), compatible with an average expression of one antibody fragment per phage particle.

Fifteen randomly picked clones were sequenced, revealing that all amino acid sequences in the CDR3 regions of both heavy and light variable chains were diverse (**Table 3.3**). However sequence analysis showed that 2/15 clones contained frameshift mutations. These results were in agreement with the soluble antibody expression data presented in **Figure 3.3 B**.
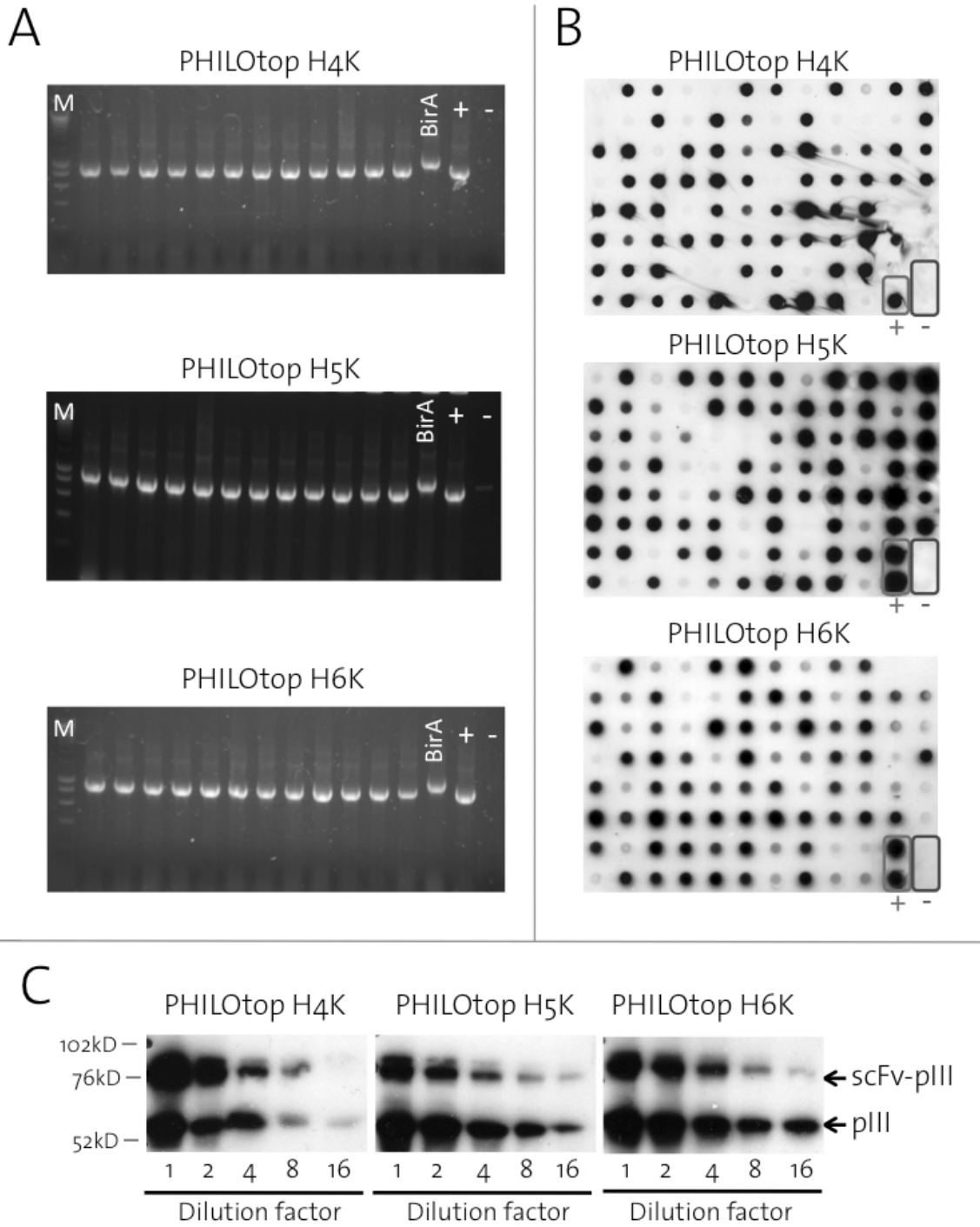
Figure 3.3. Characterization of the PHILOtop library. (**A**) PCR colony screening of 12 clones for each sub-library. As negative (-) control the PCR reaction mix was prepare omitting the template; as positive control (+) a scFv from the human antibody phage display library - ETH-2 Gold (Silacci, Brack et al. 2005)) was amplified; as internal control a BirA insert (1200bp) of a pHEN1

vector was amplified. All the tested clones showed an insert with the correct size of approximately 1000 bp. (**B**) Dot blot analysis of 92 induced supernatants of individual library clones for each of the three sub-libraries. The soluble scFv fragments were detected with the anti-myc-tag mAb 9E10. As positive control (+) a clone from the ETH-2 Gold library was expressed; as negative control (-) only 2xYT was blotted on the membrane. Approximately 90% of the clones express a detectable amount of soluble sc-Fv fragment. (**C**) Western blot analysis to evaluate the efficiency of the display of the scFv-pIII fusion protein on the surface if the phage particle. Different amounts of purified phage of each sub-library were analyzed. The protein pIII and the fusion protein scFv-pIII were detected with a monoclonal anti-pIII antibody.
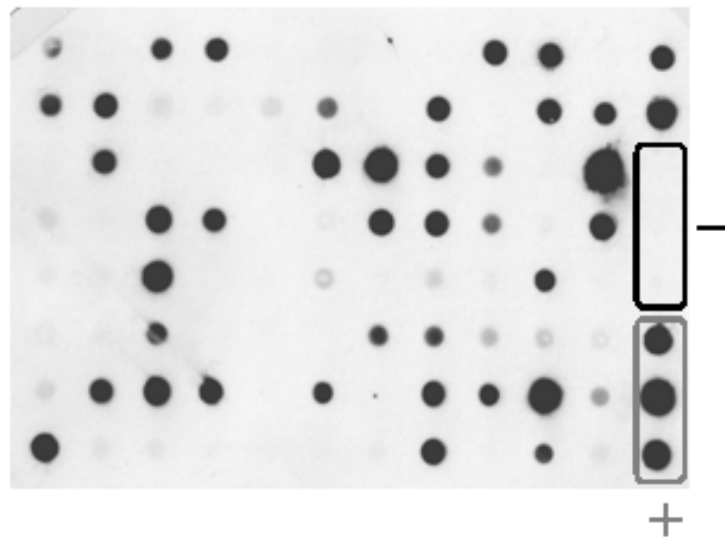
Figure 3.4. Dot blot analysis of supernatants of individual clones from PHILOtop library. The soluble scFv fragments were detected with Protein L-HRP conjugate. As positive control (+) clones from the ETH-2 Gold library were used; as negative control (-) only 2xYT was blotted on the membrane. The majority of antibody clones from PHILOtop library were able to bind to Protein L.

PHILOtop		
Clone	VH CDR3	Vk CDR3
PHILOtop H4K-1	T H S G	P M Q D P S
PHILOtop H4K-2	A V V L	Q R F N P R
PHILOtop H4K-3	M K E L	G R R Q P W
PHILOtop H4K-4	P M P S	S I T I P D
PHILOtop H4K-5	Y D V G	N S N T P P
PHILOtop H5K-1	N T L R H	H Y P P P A
PHILOtop H5K-2	S T L L C	H T G H P R
PHILOtop H5K-3	Y L H F E	S A V S P R
PHILOtop H5K-4	S L L L Y	E A E P P A
PHILOtop H5K-5	M A I *	N S H H P S
PHILOtop H6K-1	T D V L M T	W W L A P Q
PHILOtop H6K-2	C G P V R M	S N P E P S
PHILOtop H6K-3	W R I K S L	V L L T P N
PHILOtop H6K-4	P H M Y C T	T T S S P K
PHILOtop H6K-5	Q R K D G I	V A N E P *

* Nucleotide missing in the CDR3 (frame shift)

Table 3.3. Sequence diversity of VH and VL CDR of 15 randomly picked clones from PHILOtop sub-libraries.

The functionality of the PHILOtop library was tested performing selections on immunotubes against a panel of antigens. It was possible to isolate specific antibody clones against all the tested antigens (**Table 3.4**), including splice isoforms of fibronectin and of tenascin-C as biomedically-relevant tumor-associated antigens (Neri and Bicknell 2005).

Antigen	Positive clones/ screened clones	Rounds of panning
α2-Macroglobulin	59/94	3
Glutathione S-transferase	26/94	3
Transferrin	30/94	2
BCD domain of tenascin C	84/94	2
Hemoglobin	31/94	2
7B89 domain of fibronectin	51/94	2
11A12 domain of fibronectin	23/94	3

Table 3.4. The PHILOtop library was biopanned against several different antigens. The ratio between "positive antibody clones/screened antibody clones" and the number of rounds performed for each antigen are shown in this table.

For the selections towards fibronectin splice isoforms, larger recombinant proteins containing the EDA or EDB domains were used in analogy to what previously described by our group (Carnemolla, Neri et al. 1996; Borsi, Castellani et al. 1998), yielding at least 2 and 10 different sequences which were also capable of specific recognition of the recombinant EDA and EDB domains, respectively (**Table 3.5**). Sequence analysis of individual selected clones revealed substantial aminoacid diversity in the CDR3 regions of selected clones, with certain consensus preferences for antibody clones specific to the EDB domain of fibronectin and to transferrin (**Table 3.5**).

Antigen	Clone	V_HCDR3	V_κCDR3
α2-macroglobulin	C6, C9, F4, F6, G6	S D A G V	T S K E P F
Glutathione S-transferase	A4, B2, F8, G10	H S R G T	G S G L P R
	C9	R T H L G K	Q S S H P I
Transferrin	A5	G S H L K R	T R L I P Y
	B1, D3	N Q R R T H	S R W D P H
	C8	G T R H I	S T R P P I
	H10	Q K K R K K	S Q T V P T
BCD domain of Tenascin C	A11	N R I R K P	S H L L P L
	C1	R Q A Y R S	N M A P P F
	C9	N S R Y L V	R K A F P L
	D1	R N Q L G F	P P G Y P F
	G1	R R Q A R	N G L L P L
Hemoglobin	C11, D9	Q T S N L L	F S F L P T
	F11	Q P A Y K T	G S I L P L
	H3	Q F R K L L	F T R V P S
	H11	A S G F T	P R G A P T
7B89 domain of Fibronectin	A9	S T H R H R	S T Q R P Y
	A12	A S H R N L	T R T L P V
	C9	G L S R H I	S Y K M P P
	C12	A S S R H R	S A T T P F
	D9	W R P K R G	A V M H P L
	D10	Y R V R H A	T T D Y P T
	E2	H R A Q H G	Y S T A P I
	E11	G G H R A	R E M A P L
	G2	G R P R R S	T N T G P L
	G7	G N L R K P	A A R Y P T
11A12 domain of Fibronectin	B10, B11, C8, D1, D2, D5, E1, FT, G9	G S T H G	T M K T P S
	D9	G S T H G	G A I R P Q

Table 3.5. VH and Vk CDR3 randomized positions of PHILOtop selected antibody clones. The conserved proline residue of the Vk CDR3 is underlined. Single amino acid codes are used according to standard IUPAC nomenclature.

3.1.3 Isolation, characterization and affinity maturation of murine monoclonal antibodies specific to the extra domain B of fibronectin
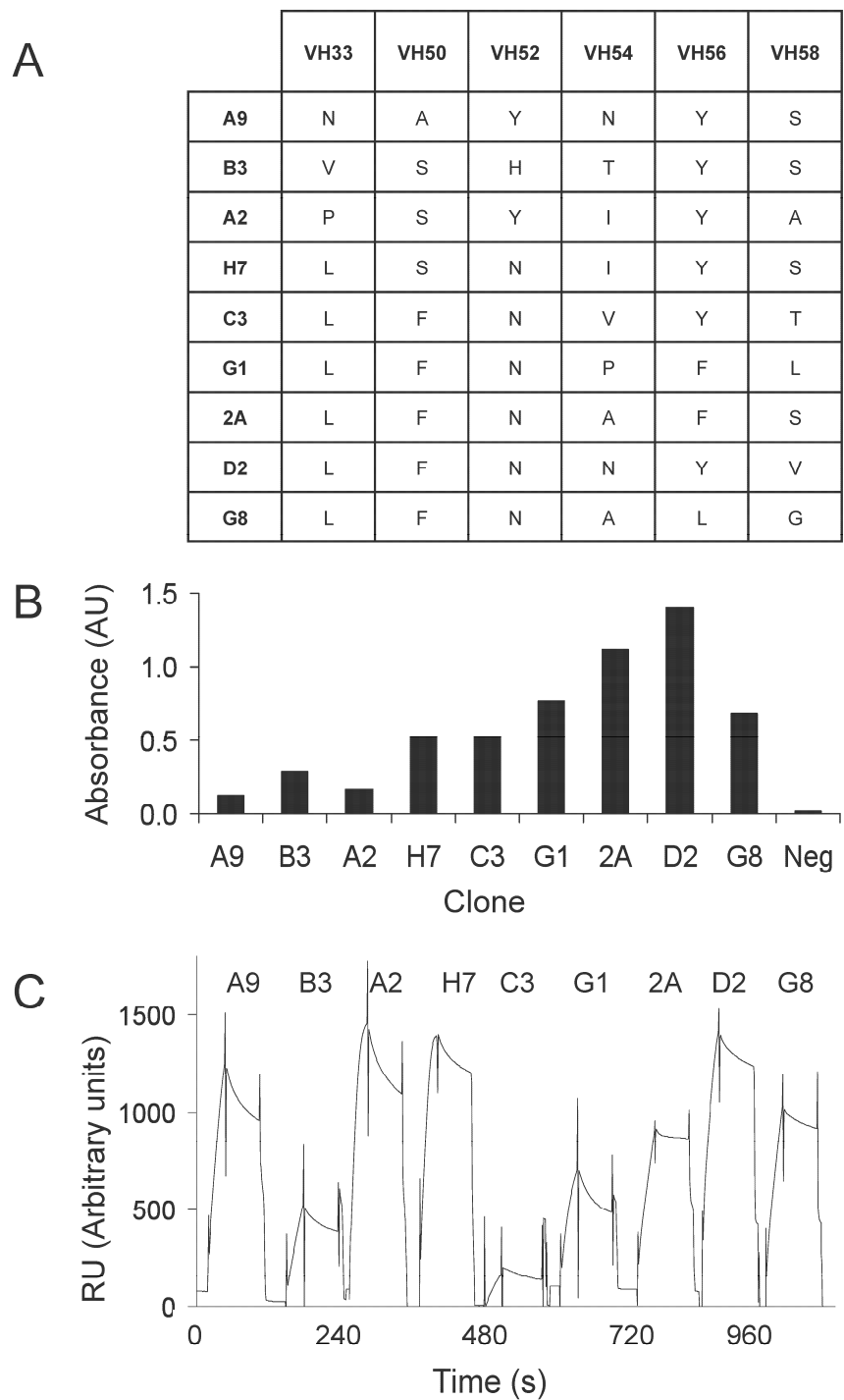
We successfully isolated murine monoclonal antibodies against the EDB domain of fibronectin, a conserved tumor associated antigen for which there has been a series of documented failures *i.e.* the attempt to generate murine antibodies by hybridoma technology (Peters, Trevithick et al. 1995)

One anti-EDB antibody named scFv(A9) was produced in *E.coli*, purified to homogeneity and analyzed both by BIAcore and by immunohistochemistry. BIAcore analysis revealed a dissociation constant (K_D) of 100 nM (k_{off} = 1.1 x 10^{-1} s^{-1}; k_{on} = 1.1 x 10^6 M^{-1} s^{-1}) (**Figure 3.7 A, B**). Immunohistochemistry experiments performed on frozen tumor sections confirmed the ability of the scFv(A9) clone to recognize the cognate antigen with good affinity and in tumor neo-vascular structures (**Figure 3.7 E**). Finally, we tested the possibility of affinity-maturing the A9 antibody by combinatorial mutagenesis of residues in the CDR1 (residue 33) and CDR2 loops (residues 50, 52, 54, 56, 58) of the VH domain. The six amino acid residues randomized in the affinity maturation library were chosen for their high degree of variability in naturally-occurring antibodies, as shown in **Figure 3.5**, suggesting a probable relevance in predicting a contact with the antigen (Chang and Mohan 2005).

An affinity-maturation library containing 2.2 x 10^7 clones was constructed starting from the anti-EDB antibody A9. One round of selection on immobilized antigen and a BIAcore analysis (**Figure 3.6 C**) of the clones exhibiting the strongest ELISA signals (**Figure 3.6 B**) led to the identification of scFv(H7), an antibody fragment with a K_D = 6 nM (k_{off} = 1.0 x 10^{-3} s^{-1}; k_{on} = 1.6 x 10^5 M^{-1} s^{-1}; **Figure 3.7 C, D**). All clones in analysis showed a CDR1 and CDR2 sequence diversity (**Figure 3.6 A**) from the parental clone A9 and exhibited certain consensus preferences both in CDR1 and CDR2 randomized residues (VH33 L; VH50 S/F; VH52 N; VH56 aromatic residue)
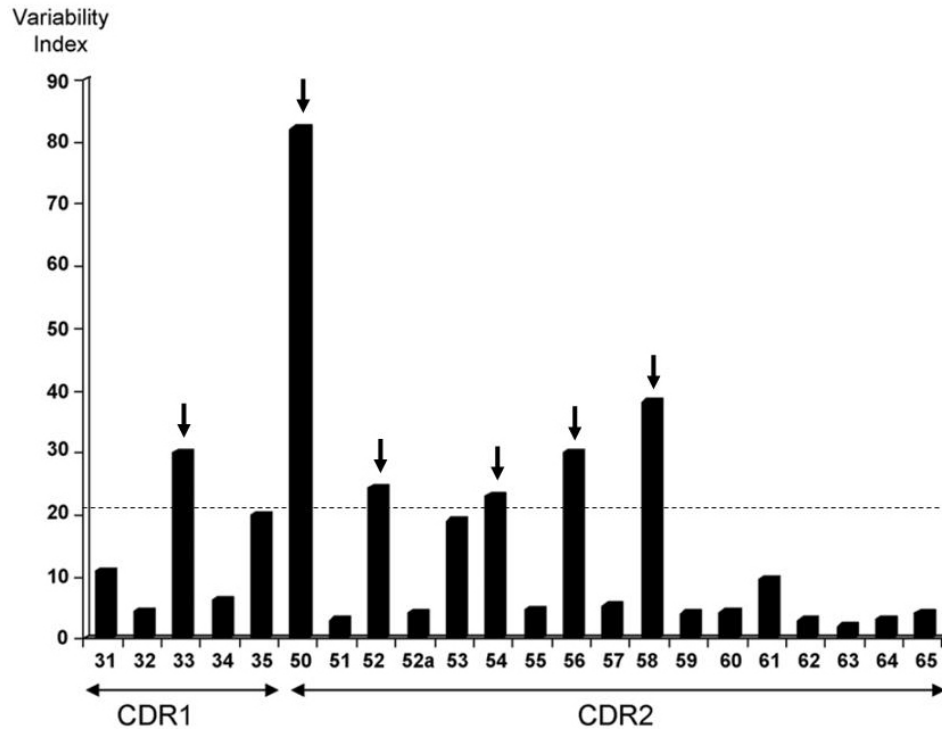
Figure 3.5. The amino acid usage in the CDR1 and CDR2 regions of the *VH1/J558* germlines family, in which the VH186.2 gene is one of the predominants, is depicted in the figure. Aminoacid residues VH33, VH 50, VH52, VH54, VH56, VH58 show the highest variability index and have been chosen for randomization in the affinity maturation library of scFv(A9). Adapted from Chang and Mohan 2005.

A

	VH33	VH50	VH52	VH54	VH56	VH58
A9	N	A	Y	N	Y	S
B3	V	S	H	T	Y	S
A2	P	S	Y	I	Y	A
H7	L	S	N	I	Y	S
C3	L	F	N	V	Y	T
G1	L	F	N	P	F	L
2A	L	F	N	A	F	S
D2	L	F	N	N	Y	V
G8	L	F	N	A	L	G

Figure 3.6. Analysis of eight affinity matured clones against EDB. A9 is the parental clone of the affinity maturation library. H7 is a 6nM affinity matured clone. Table A shows the sequence of randomized aminoacid residues in CDR1 and CDR2 of VH of the clones. Panel B shows the ELISA signal of the eight affinity matured clones. Negative control: 2xYT medium. Panel C: Biacore analysis on supernatants of the eight affinity matured clones.
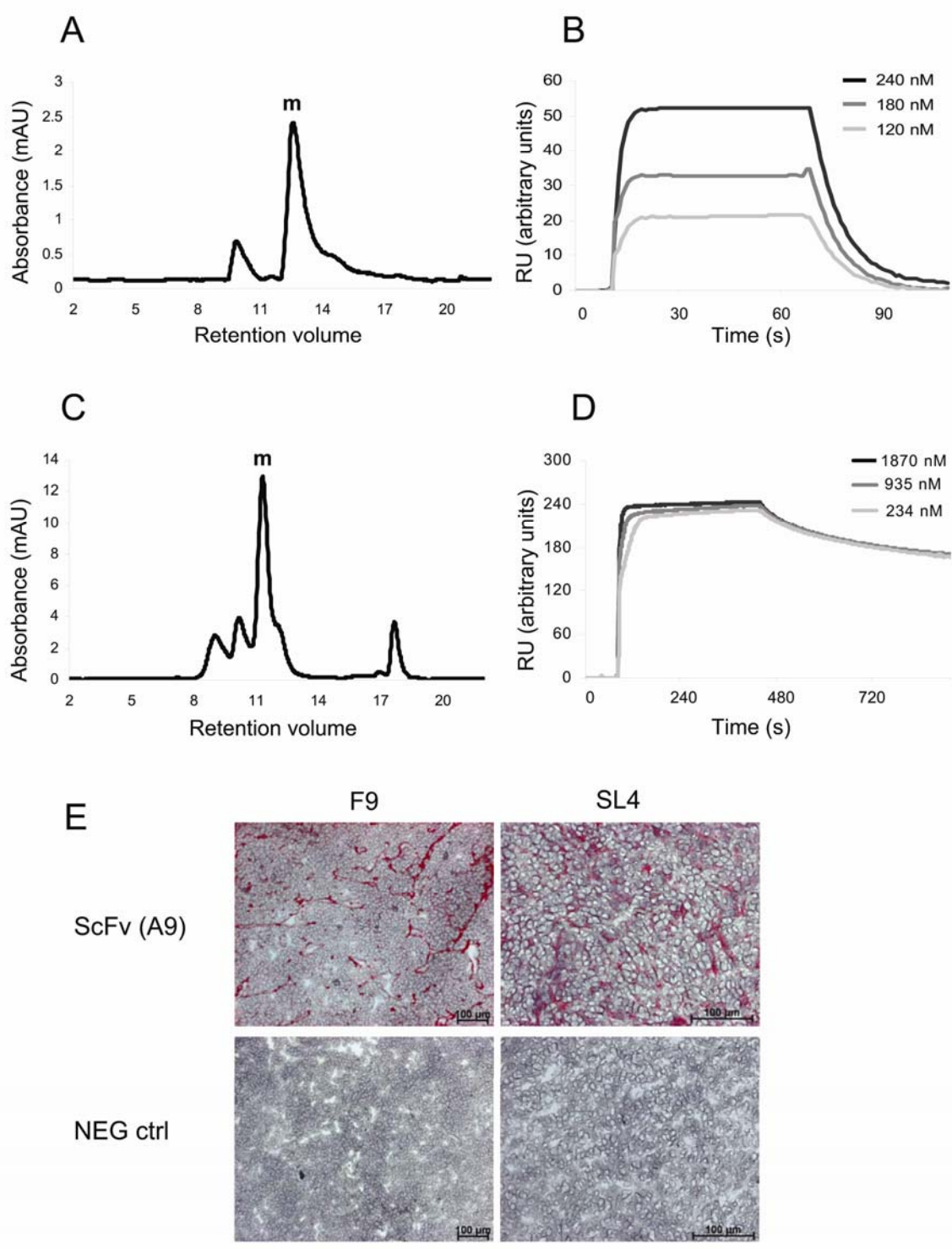
Figure 3.7. Anti-7B89 domain of fibronectin antibody. (**A**) Size-exclusion chromatography profile of the purified scFv(A9). The retention volume (mL) of the major peak corresponds to the

monomeric form (m) of the scFv fragment: this fraction was collected and used for BIAcore measurements. (**B**) BIAcore analysis of the binding of the scFv(A9) to the 7B89 domain of fibronectin. Different concentration of purified scFv(A9) were injected and the kinetic constant were calculated with the BIAevaluation 3.1 software. (**C**) Size-exclusion chromatography profile of the purified scFv(H7). The retention volume (mL) of the major peak corresponds to the monomeric form (m) of the scFv fragment: this fraction was collected and used for BIAcore measurements. (**D**) BIAcore analysis of the binding of the scFv(H7) to the 7B89 domain of fibronectin. Different concentration of purified scFv(H7) were injected and the kinetic constant were calculated with the BIAevaluation 3.1 software. (**E**) Immunohistochemical analysis on F9 murine teratocarcinoma and SL4 murine colon carcinoma sections. The scFv(A9) stains the vascular structure in red. Negative control: ScFv(A9) was omitted. Scale bars: 100 µm.

3.2 Discussion

Based on our previous experience with synthetic antibody libraries (Pini, Viti et al. 1998; Viti, Nilsson et al. 2000; Silacci, Brack et al. 2005) we here described the design, construction and characterization of a large synthetic naïve mouse antibody phage display library containing 1.5 billions antibody clones.

The library, termed PHILOtop, largely fulfils the quality requisites as percentage of clones carrying the full-length insert, bacterial expression of the soluble scFv fragments and display of the scFv-pIII fusion protein on the phage particle. The library was shown to reliably yield good-quality antibodies towards all protein antigens used so far in selection experiments. As an illustrative example of the practical performance of the PHILOtop library, we presented details on the isolation of a specific monoclonal antibody to the EDB domain of fibronectin, a marker of angiogenesis (Carnemolla, Neri et al. 1996), showing the ability of the antibody to recognize the native antigen in ELISA, real-time interaction analysis in a BIAcore 3000 instrument, as well as in sections of F9 teratocarcinoma. One of the anti-EDB clones isolated from the PHILOtop library (scFv(A9)) was affinity-matured by combinatorial mutagenesis of CDR1 and CDR2 loops of VH domain, leading to clone H7, which displays a 6 nM dissociation constant to the antigen and which is now being used in our laboratory for the production of fully murine therapeutic immunocytokines.

To our knowledge, only constructions of non-immune scFv phage display libraries from spleen and bone marrow of unimmunized mice were described so far. However cloning of antibody gene from non-immune donors is inefficient: this due to the fact that their RT-PCR primers sets, PCR conditions and efficiency of subcloning for construction of a antibody gene library cannot encompass all the antibody diversity (Okamoto, Mukai et al. 2004).

Monoclonal antibodies are routinely generated from mice or rats using hybridoma technology: this approach has clear limitations when the antigen is highly conserved among species (resulting in low immunogenicity), or in case of highly

toxic or deadly pathogenic antigens. Antibody phage technology represents a valid alternative to overcome such problems. Moreover phage display technology, not requiring immunization of the donor with antigen, avoids much laborious laboratory work. Furthermore production in *E.coli* facilitates further genetic manipulation of antibodies.

Current preclinical therapy studies involving human antibodies and antibody derivative therapeutics in mouse models are limited by the mouse anti-human antibody (MAHA) response. To overcome this problem, such studies are limited to use of immuno-deficient mouse strains or to short-time therapies: a mouse antibody phage display library would fulfil the increasing need of mouse monoclonal antibodies. For instance, antibody clones selected from the PHILOtop could be affinity matured and fused to murine cytokines allowing the use of syngeneic mouse models of disease: the use of fully murine immunocytokines will enable more significant therapy studies.

The PHILOtop library design is compatible with affinity maturation strategies, based on library construction by combinatorial mutagenesis of residues in CDR1 and/or CDR2 loops (**Figure 3.6**). An affinity maturation strategy consisting in the randomization of VH CDR1 and VH CDR2 residues with the greatest degree of variability in J558 family mouse antibodies (**Figure 3.5**)(Chang and Mohan 2005) led to the isolation of an antibody clone with a 16 fold increased affinity (**Figure 3.6 and 3.7**). CDR1 and or CDR2 combinatorial mutagenesis is an affinity maturation strategy which have extensively been used by our group in the past (Pini, Viti et al. 1998; Brack, Silacci et al. 2006; Villa, Trachsel et al. 2008) yielding the L19, F16 and F8 antibodies, whose derivatives are currently being investigated in multiple clinical trials (Santimaria, Moscatelli et al. 2003; Sauer, Erba et al. 2009).

We anticipate that the PHILOtop library may provide a useful complement to the many human antibody phage display libraries described so far. We mainly foresee applications for preclinical research activities, when fully murine antibodies are needed for the *in vivo* implementation of biomedical strategies in mouse models of pathology. In particular, the PHILOtop library has solved an old

problem of our lab, namely the isolation of mouse monoclonal antibodies to extra-domains of fibronectin and of tenascin-C which can be used for the cloning and *in vivo* testing of fully murine immunocytokines for therapeutic applications in rodents (Carnemolla, Borsi et al. 2002; Halin, Rondini et al. 2002; Gafner, Trachsel et al. 2006; Schliemann, Palumbo et al. 2009)

3.3 Material and methods

Growth media, helper phage, and general procedures used for library selections and screening procedures were essentially as described by (Viti, Nilsson et al. 2000).

Unless stated otherwise, chemicals were purchased from Sigma-Aldrich (Buchs, Switzerland).

Library construction and cloning

Synthetic genes (GenScript Corporation; NJ, USA) were used as templates for the PCR amplification of the variable heavy chain (VH) gene 186.2 (Bothwell, Paskind et al. 1981; Williams, Martinez et al. 2001; Chang and Mohan 2005) and for the amplification of the light variable chains (Vk) gene kv4-72 (Kirschbaum, Roschenthaler et al. 1999). The amplified fragments were used for the construction in the scFv format of the PHILOtop library. the linker $Gly_4SerGly_4SerGly_4$ was introduced between the heavy and the light variable chain amplified genes (**Table 3.1** and **Figure 3.2**). The resulting scFv segments were used for library construction as follows.

Antibody residues are numbered according to (Chothia and Lesk 1987; Tomlinson, Cox et al. 1995) and are indicated in **Figure 3.2**. Sequence variability in the variable heavy chains component of the libraries was introduced by PCR using partially degenerated primers (**Table 3.6** and **Figure 3.2**), in a process that generate random mutations at position 95-99 of the VH CDR3. The variable light chain components of the libraries were generated in a similar fashion, introducing random mutations at position 91, 92, 93, 94 and 96 in the Vk CDR3 (**Table 3.6** and **Figure 3.2**). VH/Vk combinations were assembled in scFv format by PCR assembly, using gel purified VH and Vk segments as templates. The assembled VH/Vk fragments were doubly-digested with NcoI/NotI (New England Biolabs; MA, USA) and cloned (T4 DNA ligase, New England Biolabs; MA, USA) into NcoI/NotI-digested pHEN1 phagemid vector (Hoogenboom, Griffiths et al. 1991).

The resulting ligation product was electroporated into electrocompetent *Escherichia coli* TG1 cells according to Viti et al. (Viti, Nilsson et al. 2000). The library was electroporated on three different days, thereby obtaining three different sub-libraries, named PHILOtop H4K, H5K and H6K. The sub-libraries were stored as glycerol stocks, rescued and used for phage production according to standard protocols (Viti, Nilsson et al. 2000).

PHILOtop library construction primers		
a	LMB3long	5'-CAGGAAACAGCTATGACCATGATTAC-3'
b1	186_CDR304ba	5'-GTCCCTTGGCCCCAGTAGTCAAAMNNMNNMNNMNNTCTTGCACAATAATAGACCGCA-3'
b2	186_CDR305ba	5'-GTCCCTTGGCCCCAGTAGTCAAAMNNMNNMNNMNNMNNTCTTGCACAATAATAGACCGCA-3'
b3	186_CDR306ba	5'-GTCCCTTGGCCCCAGTAGTCAAAMNNMNNMNNMNNMNNMNNTCTTGCACAATAATAGACCGCA-3'
c	186_CDR3fo	5'-TTTGACTACTGGGGCCAAGGGAC-3'
d	kv4-72_CDR3ba	5'-CGAGCTTGGTCCCCCCTCCGAACGTMNNCGGMNNMNNMNNMNNCTGCTGGCAGTAATAAGTGGC-3'
e	kv4-72_FR4NotIba	5'-TTTTCCTTTTGCGGCCGCTGCAGCCACAGTCCGTTTGATCTCGAGCTTGGTCCCCCCTCC-3'
	fdseqlong	5'-GACGTTAGTAAATGAATTTTCTGTATGAGG-3'
PHILOtop affinity maturation library primers		
a	LMB3long	5'-CAGGAAACAGCTATGACCATGATTAC-3'
f	CDR1VH 1aa ba	5'-GTGTCTGCTTTACCCAGTGCATMNNGTAGCTGGTGAAGGTGTAGC-3'
g	CDR2 VH 5aa fo	5'-CAGGGGTCTGGAATGGATCGGGNNMATTNNMCCTGGANNMGGTNNMACTNNMTACAATCAGAAGTTCAA GGGCAAG -3'
h	Ext FR1/2 ba	5'-CCGATCCATTCCAGACCCCTGCCAGGTGTCTGCTTTACCCAGTG -3'
	fdseqlong	5'-GACGTTAGTAAATGAATTTTCTGTATGAGG-3'

M and N are defined according to the IUPAC nomenclature (M=A/C, N=A/C/G/T)

Table 3.6. Primers for the construction of the PHILOtop library and the affinity maturation library

Library characterization

A total of 36 clones, 12 for each sub-library, were tested by PCR screening using the primers LMB3long and fdseqlong (**Table 3.6**) and the REDTaq ReadyMix (Sigma) to verify the correct size of the insert. For all the libraries fifteen clones (five for each sub-library) were selected at random and sequenced (Big Dye Terminator v1.1 Cycle Sequencing kit; ABI PRISM 310 Genetic Analyzer; Applied Biosystems, Foster City, CA, USA) to check for the absence of frameshifts and pervasive contamination. The percentage of clones expressing soluble antibody fragments was determined by dot blot analysis of bacterial supernatants (ELIFA system; Perbio, Lausanne, Switzerland) using anti-myc mouse mAb 9E10 (Marks, Hoogenboom et al. 1991). and anti-mouse horseradish peroxidase

immunoglobulins (Sigma-Aldrich) as detecting reagents or Protein L-horseradish peroxidase (Pierce; Rockford, IL, USA). Peroxidase activity was detected using the ECL plus Western blotting detection system (Amersham Biosciences). The display of the scFv-pIII fusion protein on the phage surface was evaluated by Western blot. Different amounts of purified phage were loaded on a SDS gel and then transferred to nitrocellulose membrane (Protran BA 85; Schleicher & Schuell, Dassel Germany). As detecting reagents anti-pIII mouse mAb (MoBiTec; Göttingen, Germany) and anti-mouse horseradish peroxidase immunoglobulins (Sigma-Aldrich) were used. Peroxidase activity was detected using the ECL plus Western blotting detection system (Amersham Biosciences).

Construction of the affinity maturation library

Antibody fragment scFv(A9) specific to the extra-domain B (EDB) of fibronectin was isolated from the PHILOtop library. The affinity maturation library was cloned by introducing sequence variability in the CDR1 and CDR2 of heavy chain. Mutations at position 33 of the VH CDR1 and 50, 52, 54, 56, 58 of the VH CDR2 were introduced by PCR using partially degenerated primers (**Table 3.6**).

First, two fragments were obtained by PCR on the parental clone plasmid as template, using primer pairs a/f and g/fdseqlong (**Table 3.6**). After gel-purification of both segments, a/f amplified segment was PCR amplified using primers pair a/h and gel-purified. The fragments obtained by PCR using a/h and g/fdseqlong primers were assembled by PCR and further amplified using primers a/fdseqlong. The VH-VL combinations were doubly digested with NcoI/NotI and cloned into the NcoI/NotI-digested expression vector pHEN1. The resulting ligation mixture was purified and electroporated into fresh electrocompetent *E. coli* TG-1 cells. Electrocompetent *E. coli* TG-1 cells were prepared by washing the cells twice with 1 mM HEPES / 5% glycerol and twice with 10% glycerol in water. Finally, cells were resuspended in 10% glycerol. Electroporated cells were spread on 2xYT-agar plates (16 g/l bacto-tryptone, 10 g/l bacto-yeast extract, 5 g/l NaCl pH 7.4 – 15 g/l agar – 100 µg/ml ampicillin, 1% glucose) and incubated

at 30 ºC overnight. On the next day, cells were rescued with 2xYT-10% glycerol from the plates, snap-frozen in liquid nitrogen and stored at -80 ºC.

Antigens

Human α-2-Macroglobulin was purchased by BIODESIGN International (MILAN ANALYTICA AG, La Roche, Switzerland). Glutathione-s-transferase (GST) was expressed and purified from the GST Gene Fusion Vector pGEX-4-T2 (Amersham Biosciences) according to the manufacturer's instructions. Human apo-transferrin as well as bovine Hemoglobin were purchased by Sigma-Aldrich. The recombinant extra-domain A (EDA) containing human fibronectin fragment (termed 11A12) (Borsi, Castellani et al. 1998); the recombinant EDB containing human fibronectin fragment (termed 7B89) (Carnemolla, Neri et al. 1996); and the recombinant domain C of human tenascin-C (termed BCD) (Silacci, Brack et al. 2006) were expressed and purified as previously described.

Library selection on immunotubes

All selections were performed using recombinant or commercially available antigens with purity >90%.

Immunotubes (Nunc; Wiesbaden, Germany) were coated with antigen at a concentration of 50 µg/ml in PBS, over-night at room temperature. Immunotubes were then rinsed with PBS and blocked for 2 hours at room temperature with 2% MPBS. After rinsing with PBS, >10^{12} phage particles in 2% MPBS were added to the immunotubes. The immunotubes were first incubated on a shaker for 30 minutes and then for 90 minutes standing upright at room temperature. Unbound phage were washed away by rinsing the immunotubes ten times with PBS 0.1% Tween 20 and ten times with PBS. The bound phages were eluted by means of incubation of 1 ml 100 mM triethylamine for 5 minutes. Triethylamine was neutralized by adding 0.5 ml 1 M Tris-HCl pH 7.4. The eluted phage were used for the infection of exponentially growing *E.coli* TG1, for 40 min at 37°C. Dilution series of bacteria were then plated on small 2xYT agar plates, 100 µg/ml ampicillin (Applichem; Darmstadt, Germany), 0.1% glucose (2xYT-Amp-Glu), and

incubated at 30°C overnight to determine the titer of the eluted phage. The remaining phage-infected bacteria were centrifuged for 10 min at 3300 g and 4°C. The pellet was resuspended in 0.5 ml 2xYT, spread on a large 2xYT-Amp-Glu agar plate and incubated at 30°C overnight. The following day the bacteria were rescued from the large plate using 5 ml 10% glycerol 2xYT by means of a sterile glass loop. The rescued bacteria were stored at -80°C.

The rescued bacteria were used to inoculate 50 ml 2xYT-Amp-Glu (initial $OD_{600\ nm}$ between 0.05 and 0.1) and the culture was grown at 37°C and 200 rpm until $OD_{600\ nm}$ 0.4 – 0.5. Of this culture, 10 ml ($4 – 5 \times 10^8$ bacteria/ml) were infected with 100 µl helper phage VCS-M13 (1×10^{12} phage) at 37°C for 40 min. The bacteria were then centrifuged for 10 min at 3300 g and 4°C, the pellet resuspended in 100 ml 2xYT-Amp-Glu including kanamycin 33 µg/ml (2xYT-Amp-Kan-Glu) and the culture incubated at 30°C overnight. The overnight culture was centrifuged for 30 min at 3300 g and 4°C. The supernatant was transferred to new bottles and 10 ml 20% PEG/2.5 M NaCl was added for each 40 ml of supernatant. The mixture was incubated on ice for 40 min and then centrifuged for 30 min at 3300 g and 4°C. The pellet was resuspended in 40 ml sterile H_2O and 10 ml PEG/NaCl was added. The mixture was again incubated on ice for 40 min and then centrifuged for 30 min at 3300 g and 4°C. The pellet containing the precipitated phage was resuspended in 2 ml sterile PBS containing 15% glycerol. In order to remove cell debris, the solution was then centrifuged for 3 min at 15000 g and the pellet was discarded. Phage used on the same day for a further round of panning were kept on ice; otherwise they were stored at -20°C. More details about selection protocols can be found in (Viti, Nilsson et al. 2000).

Elisa screening

Bacterial supernatants containing scFv fragments were screened for binding to antigen by ELISA essentially as described in (Viti, Nilsson et al. 2000) Individual colonies were inoculated in 180 µl 2xYT-Amp-Glu in 96-well plates (Nunclon Surface, Nunc). The plates were incubated for 3 hours at 37°C in a shaker incubator. The cells were then induced with isopropyl-thiogalactopyranoside

(IPTG; Applichem) at a final concentration of 1 mM, and grown overnight at 30°C. The bacterial supernatants assayed were tested in ELISA experiment as described in (Marks, Hoogenboom et al. 1991) using the anti-myc tag 9E10 mAb and anti-mouse horseradish peroxidase immunoglobulins.

7B89 affinity resin preparation

Recombinant 7B89 of human fibronectin was conjugated to sepharose beads as follows: 1.5 mg of CNBr-sepharose (GE-Healthcare) beads were resuspended in cold 1 mM HCl resulting in approximately 5 ml of resin after swelling. The resin was washed several times with cold 1 mM HCl (200 ml / g resin) and 100 mM $NaHCO_3$, 500 mM NaCl, pH 8.2 (coupling buffer, 20 ml / g resin) followed by addition of 7B89 (5 mg / ml resin) dissolved in coupling buffer. After incubation overnight at 4°C, the resin was washed again several times with coupling buffer (minimal 5 column volumes) and free reactive groups on the beads were quenched by addition of 100 mM Tris / HCl, pH 8.9. After blocking for 2 hours at room temperature, the beads were washed repeatedly with alternating buffers (100 mM sodium aceteate, 500 mM NaCl, pH4, and 100 mM Tris / HCl, 500 mM NaCl, pH 8). The resin was washed with PBS, resuspended in PBS, 0.1% sodium azide and stored at 4°C.

Expression and purification of scFv antibody fragments

ScFv(A9) and scFv(H7) antibody fragments from a selected bacterial clone were produced by inoculating a single fresh colony in 10 ml 2xYT medium, 100 μg/ml ampicillin, and 1% glucose. This preculture was grown overnight at 37°C to an $OD_{600nm} > 0.9$ and then diluted 1:100 in 1 L of 2xYT medium, 100 μg/ml ampicillin, and 0.1% glucose and grown at 37°C to an $OD_{600nm} = 0.4$. The protein expression was then induced by the addition of IPTG (final concentration 1mM) and grown at 30°C overnight. The scFv fragment was purified by affinity chromatography using a 7B89 coupled resin (see *Antigens* and *7B89 affinity resin preparation)*

Size-exclusion chromatography

Size-exclusion chromatography of purified scFv(A9) and ScFv(H7), at a concentration of 2.3×10^{-6} M and 1.6×10^{-5} M respectively, was performed on a ÄKTA FPLC system using the Superdex 75 column (Amersham Biosciences).

Surface Plasmon resonance experiments

For affinity measurements and kinetic constant determination, monomeric form of scFv(A9) and scFv(H7) were isolated by size exclusion chromatography. Serial two-fold dilutions of the antibody fractions were then analyzed by surface plasmon resonance (BIAcore 3000 system) using a low-density coated chip. CM5 chip (BIAcore) was prepared by covalent coupling the recombinant 7B89, at a concentration of 2.5×10^{-7} M. 20 µl of monomeric antibody were injected using the kinject command at a flow of 20 µl/min. The binding curves were analysed with the BIAevaluation 3.2 software. As negative control, an ETH-2 GOLD library (Silacci, Brack et al. 2005) derived antibody fragment (scFv(9A) specific to the fluorescent dye Cy5), which did not display any detectable affinity to 7B89, was used.

Immunohistochemistry

mmunohistochemistry with scFv(A9) fragment was performed essentially as described by (Brack, Silacci et al. 2006). Tissues of a thickness of 10 μm were treated with ice-cold acetone, rehydrated in TBS (50mM Tris, 100mM NaCl, pH 7.4), blocked with fetal bovine serum (Invitrogen) and then incubated with 10 μg/ml of purified scFv together with the biotinylated 9E10 anti-myc antibody (5 μg/ml). Bound antibody was detected using streptavidin:biotinylated alkaline phosphatase complex (Biospa, Milano, Italy) and subsequent staining reaction with Fast-Red TR (Sigma). Hematoxylin solution (Sigma) was used for counterstaining.

4 Expression, engineering and characterization of the tumor-targeting heterodimeric immunocytokine F8-IL12

4.1 Results

4.1.1 Optimization and stable transfection of cells expressing F8-IL12

The heterodimeric protein scFv(F8)-p35/p40-scFv(F8) consists of two polypeptides joined together by a disulfide bond between C74 of p35 and C177 of p40 (Yoon, Johnston et al. 2000) (**Figure 4.1 B**). The C-terminal fusion of scFv(F8) with the p40 subunit is recommended, since the N-terminus of this subunit should not be chemically modified in order to retain maximal biological activity (Lieschke, Rao et al. 1997)
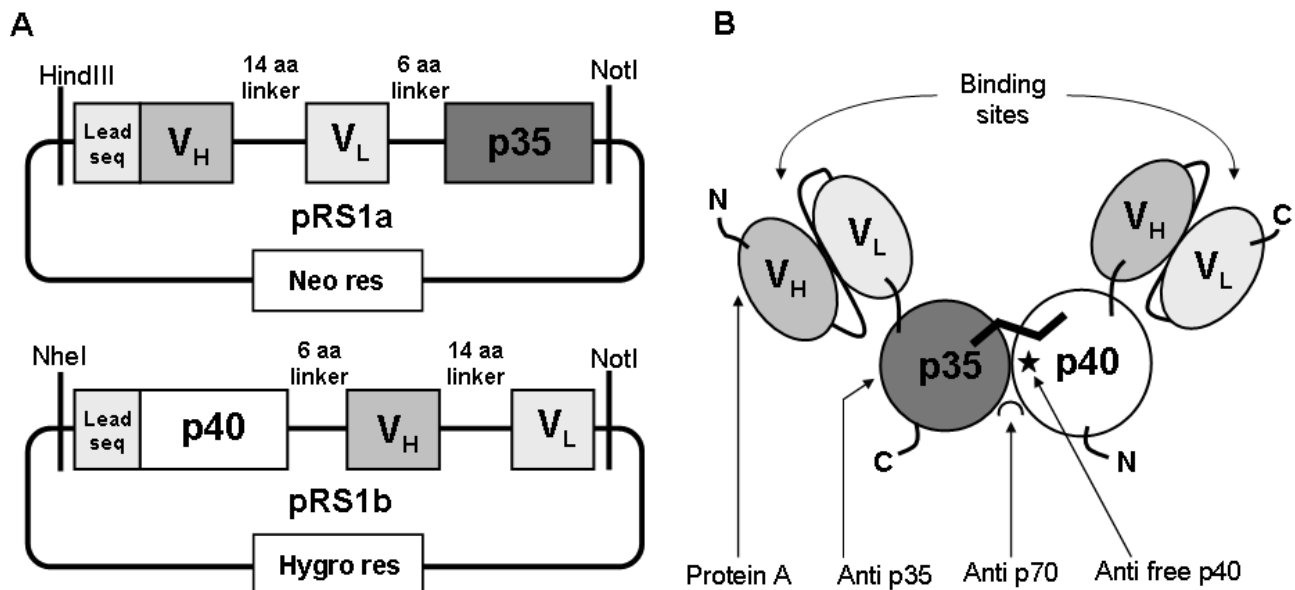

Figure 4.1. Structure of F8-IL12. (A) Schematic representation of the cloning strategy of the F8-IL12 fusion protein. **(B)** Protein structure and binding sites of antigen, Protein A, anti-p35 antibody, anti-p70 antibody and anti-p40 antibody.

For immunochemical detection, the scFv moiety can be detected with a recombinant fibronectin fragment containing the EDA domain (Villa, Trachsel et al. 2008) and with Protein A (Hoogenboom and Winter 1992; Silacci, Brack et al.

2005). The p35 subunit is recognized by monoclonal antibody "affinity purified anti-human IL-12, p35" (irrespective of whether it is free or paired with p40 or p35) and the p40 subunit by monoclonal antibody "affinity purified anti-human IL-12, p40" (only when it is not associated with p35) (**Figure 4.1 B**). Furthermore, the heterodimeric moiety p70, consisting of the disulfide-linked p35 and p40, can be detected using monoclonal antibody "anti-human IL-12 p70 antibody".

The genes coding for the two chains, featuring a 14-aminoacid linker between VH and VL domain, and a 6-aminoacid linker between scFv and IL12 subunit, were assembled by PCR and cloned into the pcDNA3.1 expression vectors carrying different antibiotic resistance genes, yielding plasmids pRS1a and pRS1b (**Figure 4.1 A** and **Table 4.1**). In order to investigate the effect of gene dosage on the correct assembly and production of F8-IL12, Chinese hamster ovary (CHO) cells were stably co-transfected with pRS1a and pRS1b used at different ratios (**Figure 4.2**). The highest protein expression (as assessed by ELISA with Protein-A-based detection) and the highest titer of heterodimeric protein (as assessed with the anti-human IL-12 p70 antibody) were observed at a pRS1a/pRS1b ratio of 3/7 (**Figure 4.2**). These transfectants were then used for the isolation and characterization of monoclonal cell lines.
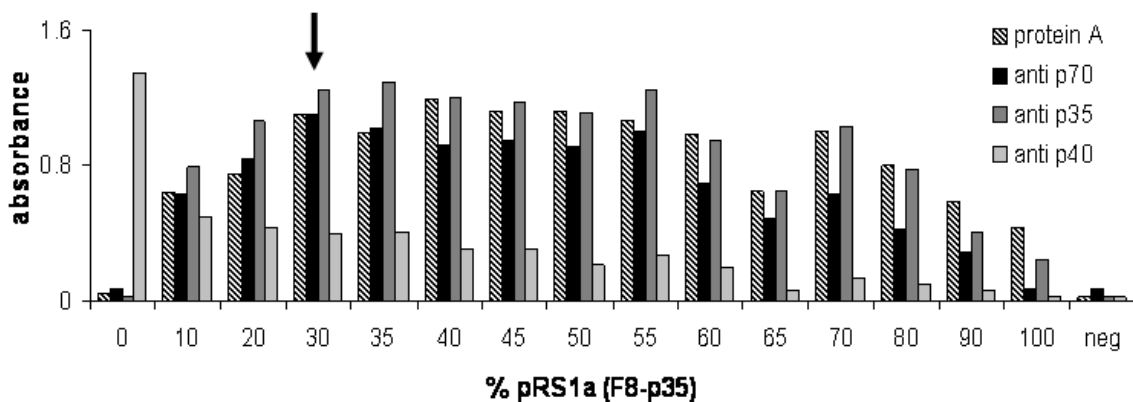
Figure 4.2. ELISA on supernatants of co-transfected CHO-S cells with pRS1a and pRS1b at different ratios. The x axis shows the percentage of pRS1a (F8-p35 plasmid) used. The arrow indicates the highest titer of heterodimeric protein (anti-human IL-12 p70 antibody) that was observed at a pRS1a/pRS1b ratio of 3/7.

Figure 4.3 A shows the results of an ELISA characterization of culture cell supernatants derived from different monoclonal cell lines expressing F8-IL12, which retained the high-titer formation of heterodimer observed at the polyclonal level. Clone G11 was used for further subcloning steps and for stability studies. **Figure 4.3 B** shows that 26/26 daughter clones derived from G11 stably produced F8-IL12, as assessed by anti-p70 ELISA. Subclone 26 was used for cell bank production according to GMP guidelines (ICH guidelines Q5B, Q5C, Q5D).
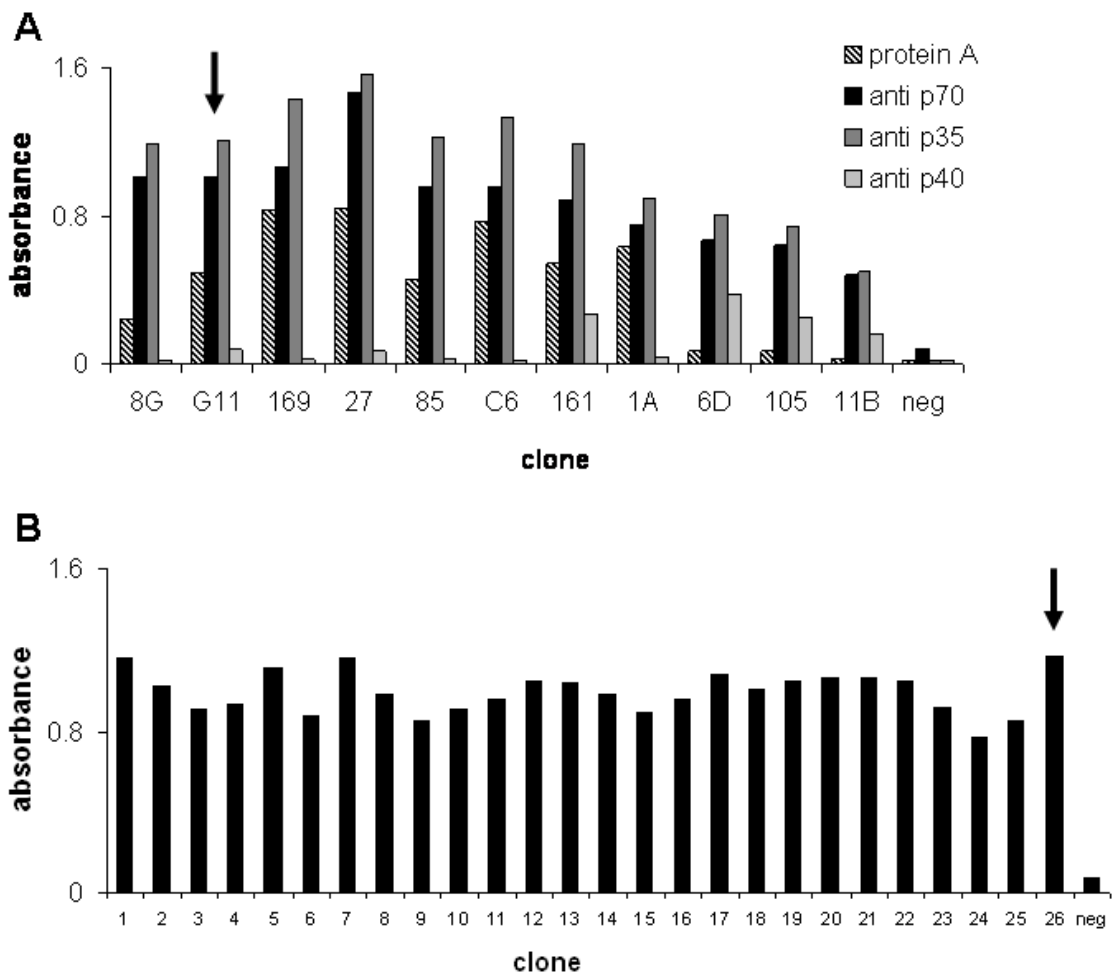
Figure 4.3. Monoclonal cell lines. (**A**) ELISA on 11 monoclonal cell lines supernatants using four different detection systems. The arrow indicates clone G11 chosen for subcloning (**B**) ELISA on 26 monoclonal cell lines supernatants with an anti-human IL-12 p70 antibody that recognizes the heterodimeric protein. The arrow indicates clone 26 that was used for large scale production of F8-IL12.

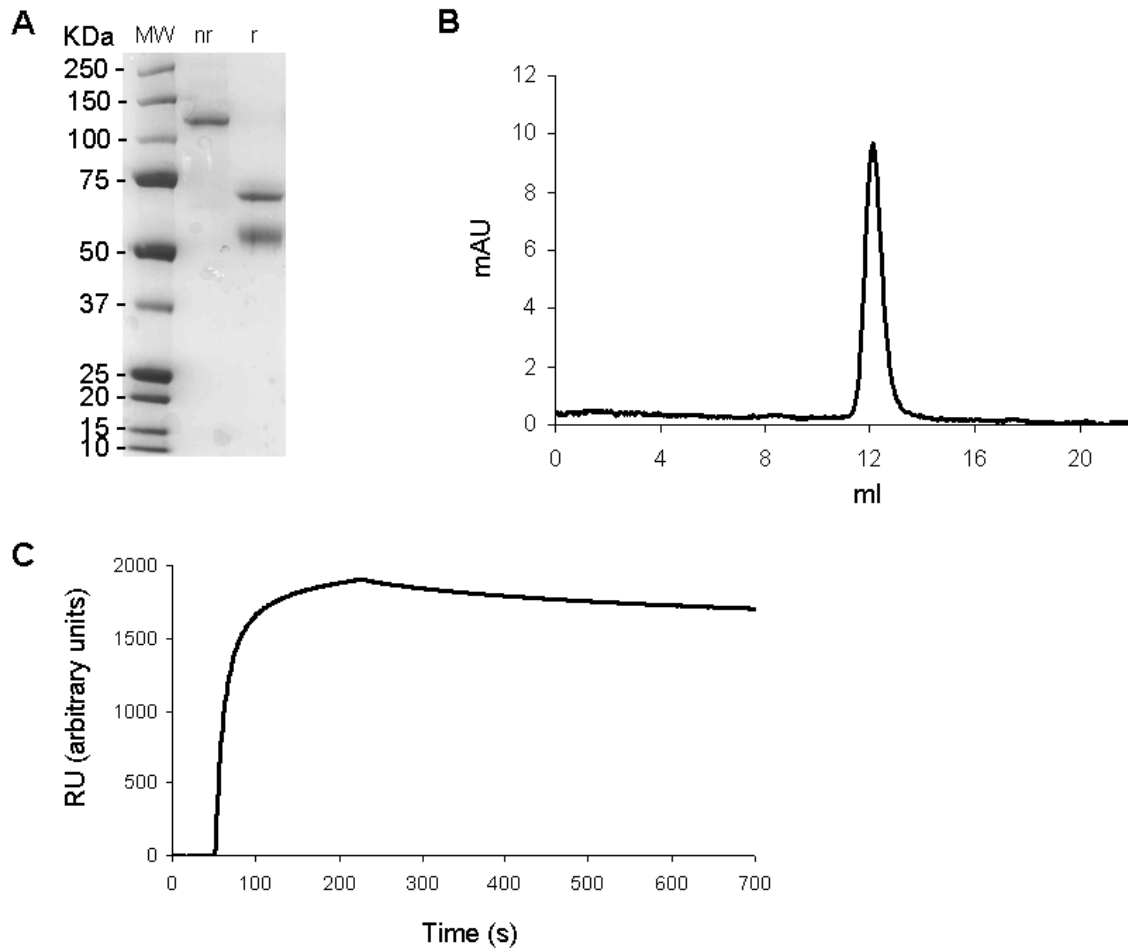## 4.1.2 Production and characterization of F8-IL12

The stable clone 26, driving F8-IL12 expression was used for large scale culture in bioreactor (see also Materials and Methods). A 7 days fed batch fermentation process was found to be compatible with the production of F8-IL12. The cell culture was interrupted after 7 days as the cell viability was drastically decreased (<65%) and no increase in total protein yield could be observed from the previous 24 h measurement (**Figure 4.4**)
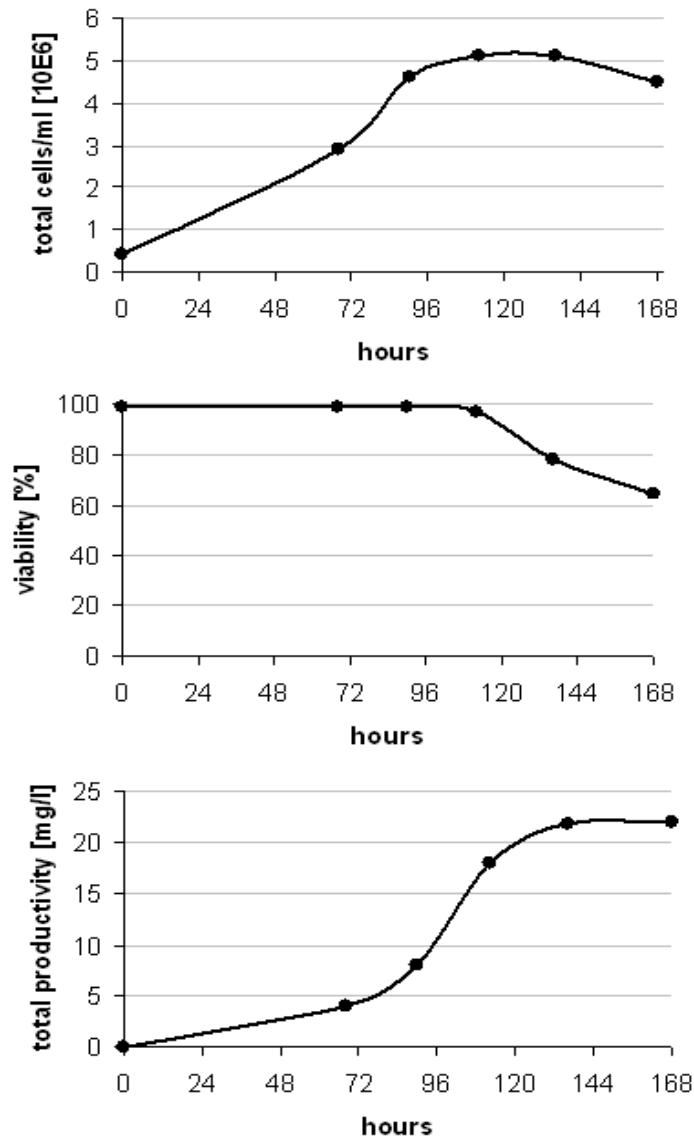

Figure 4.4. Large scale production in bioreactor. Upper panel shows cell growth, middle panel viability and lower panel productivity over time.

At the end of the fermentation, the culture supernatant was recovered by cell filtration on vacuum filtration flasks (Stericups, Millipore) and purified by Protein A chromatography. The resulting product was further purified by ion exchange chromatography and size exclusion chromatography, yielding the final product after a sterile filtration step with an overall yield of 7 mg/liter. This yield is satisfactory, in consideration of the complex expression of heterodimeric immunocytokines and of the fact that the maximal tolerated dose of IL12 in patients is 0.5 μg/kg of body weight which would correspond to approximately 70 μg of immunocytokine per patient and administration (yielding 100 doses of fusion protein per liter production).

The resulting purified F8-IL12 was characterized by SDS-PAGE analysis, confirming the presence of a single band of apparent molecular weight equal to 120 kDa in non-reducing conditions and of two bands of approx. 55 and 65 kDa in reducing conditions, as expected (**Figure 4.5 A**). The product eluted as a single peak in size-exclusion chromatography on a Superdex-200 column, with no evidence of protein aggregation (**Figure 4.5 B**). The heterodimeric F8-IL12 exhibited a high functional affinity towards the cognate antigen, with flat dissociation profile (apparent k_{off} = 3,3 x 10^{-4} s^{-1}) as assessed by real time interaction analysis on a BIAcore 3000 instrument (**Figure 4.5 C**).

Fig. 4.5. Characterization of F8-IL12. (**A**) SDS-PAGE analysis of the protein under non reducing and reducing conditions. (**B**) Gel filtration analysis reveals one single peak when analyzed under native conditions for the heterodimer F8-IL12. (**C**) Surface Plasmon Resonance analysis shows flat dissociation phase of F8-IL12 (Biacore 3000, flow 20ul/min, F8-IL12 5×10^{-7}M).
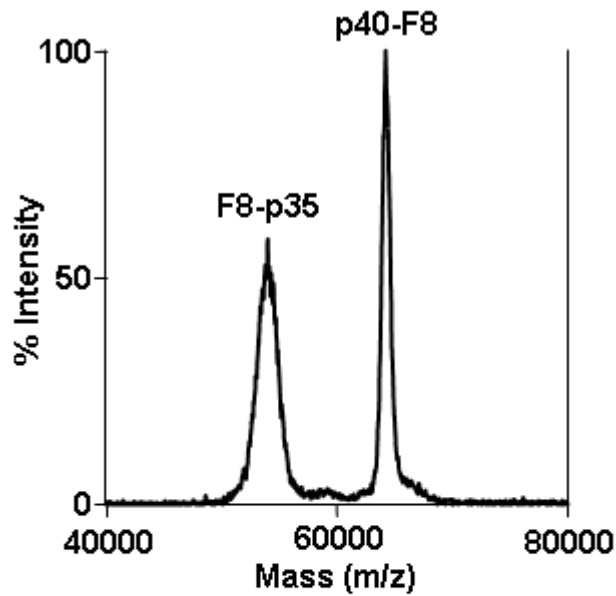
	Mass AA	Mass MS
F8-p35	48299Da	53969Da ±88Da
p40-F8	60454Da	64246Da ±46Da

Fig. 4.6. Mass spectrometric analysis of F8-IL12. Following reduction of the heterodimer F8-IL12, the two subunits F8-p35 and p40-F8 were identified by MALDI-TOF mass spectrometry. The determined mass of both subunits (Mass MS) was considerably higher than the mass calculated according to the amino acid composition (Mass AA). This difference is due to glycosylation of the two subunits.

A mass spectrometric characterization on a 4800 MALDI-TOF/TOF analyzer revealed that the two subunits F8-p35 and p40-F8 of the heterodimer F8-IL12 have molecular weights of 53969 ± 88 Da and 64246 ± 46 Da, respectively (**Figure 4.6**). We experimentally detected an increased molecular weight for both subunits [**Figure 4.6**] compared to the calculated molecular mass of the amino acid sequence [**Figure 4.6 and Table 4.1**], in agreement with the previously reported complex glycosylation patterns of unmodified IL12 (Carra, Gerosa et al. 2000)

In order to confirm that the engineering of the p70 moiety into scFv-p35/p40-scFv format (**Figure 4.1**) did not impair the biological activity of IL12, we compared the immunostimulatory properties of recombinant human IL12 and of F8-IL12 in a lymphocyte proliferation assay. The two proteins exhibited superimposable mitogenic profiles, when plotted on an equimolar basis, with a half-maximal activity at 600 pM IL12 equivalents (**Figure 4.7**).
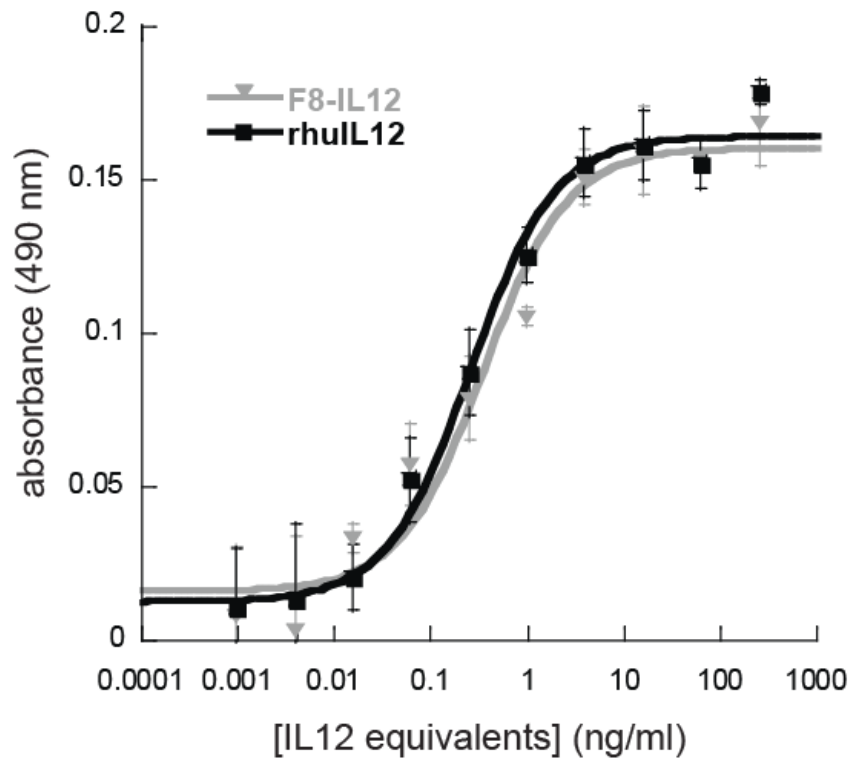
Fig. 4.7. Bioactivity assay. Comparison of the lymphoproliferative activity of rhuIL12 (in black) and F8-IL12 (in grey)

Finally, we investigated the tumor targeting properties of F8-IL12 by performing a quantitative biodistribution analysis in mice bearing F9 tumors (Villa, Trachsel et al. 2008) with a radioiodinated protein preparation. Cohorts of 4 mice were sacrificed 24 and 48 hours after intravenous injection and the resulting organs and body fluids were weighed and counted. F8-IL12 selectively accumulated in the tumor, with percent injected dose per gram (%ID/g) values of 22 and 15 at the two time points, respectively ([**Figure 4.8 A**). When correcting for tumor growth during the experiment (F9 tumors have a doubling time of 40 hours; (Tarli, Balza et al. 1999)). one observes a stable accumulation of immunocytokine in the tumor at high levels (approx. 35 % ID/g) which is consistent with the inert nature of EDA-containing fibronectin fibers (**Figure 4.8 B**). Furthermore an *ex vivo* microautoradiography of tumors from biodistribution revealed selective accumulation of the radiolabeled F8-IL12 protein around tumor vessels (**Figure 4.8 C**).
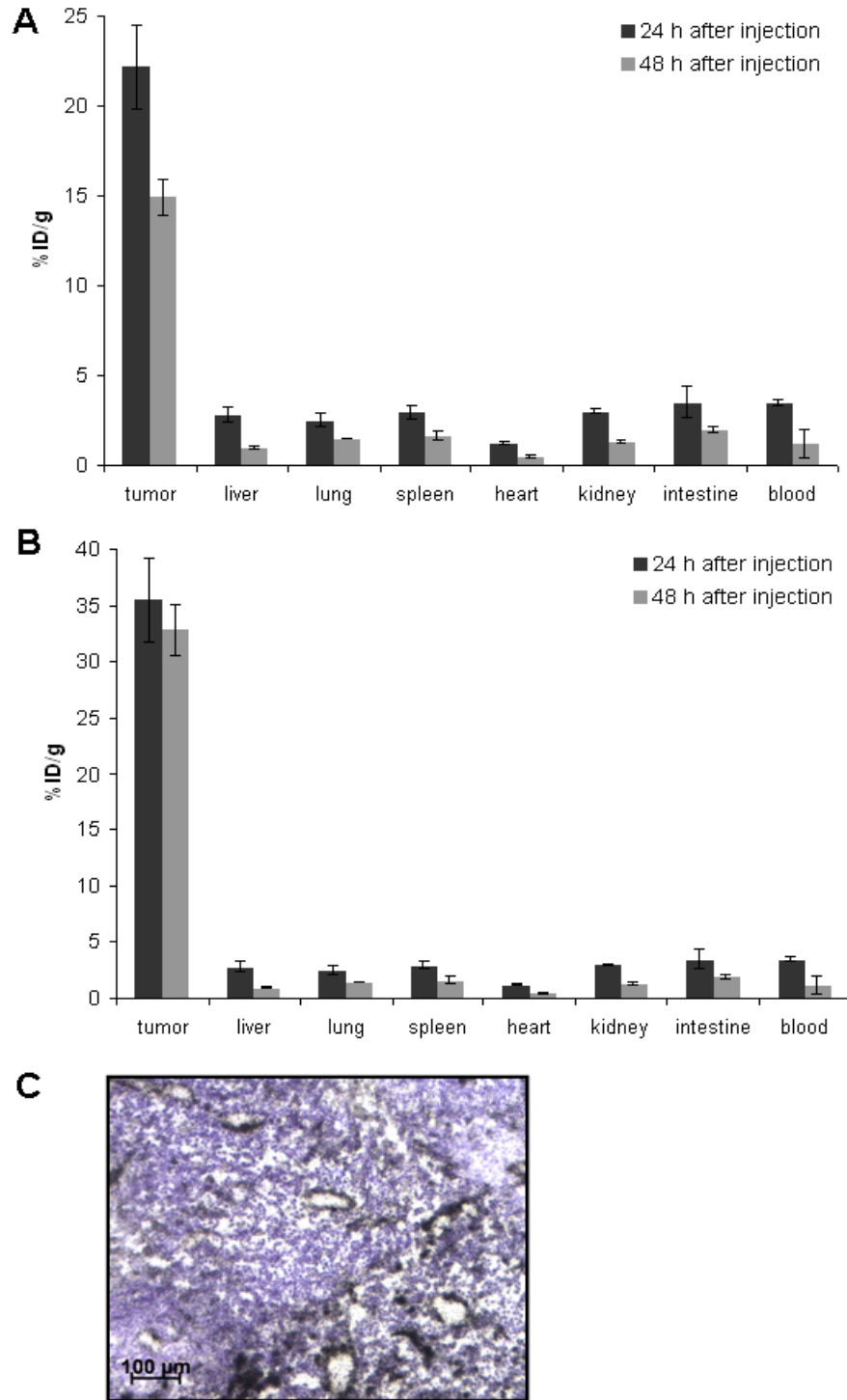
Fig. 4.8. Biodistribution studies. **(A)** F9 tumor bearing mice were injected intravenously with 125I-labeled F8-IL12 and sacrificed 24 or 48 hours after injection. (**B**) Tumor values are corrected for tumor growth during the experiment (F9 tumors have a doubling time of 40 hours). (**C**) An *ex vivo* microautoradiography shows selective accumulation of 125I-labeled F8-IL12 around tumor vessels.

4.2 Discussion

In this thesis, we have described the cloning, expression and characterization of a novel fully human immunocytokine, suitable for clinical development programs in patients with cancer. Compared to monomeric immunocytokines, the fusion of heterodimeric cytokines to a full antibody or antibody fragment presents several biotechnological challenges. First, multiple formats can be considered for the construction of fusion proteins, which do not exhibit identical *in vivo* biodistribution properties (Gafner, Trachsel et al. 2006). Moreover, vector design and gene dosage effects influence the stability of the resulting stably-transfected cell lines and product quality.

We used the strategy of Gafner et al (2006) for the cloning of the heterodimeric cytokine IL12 to the tumor-targeting antibody fragment scFv(F8). The resulting immunocytokine consists of the disulfide-linked IL12 subunits p35 and p40, each joined to a scFv fragment. In contrast to the cloning strategy of Gafner et al, we omitted all C-terminal peptide tags, an essential requirement for compatibility with current GMP guidelines and subsequent clinical development of this biopharmaceutical. In spite of the complex cloning strategy with two independent vectors, the absence of peptidic tags for affinity purification and the need for cell line stability over multiple passages, we were able to generate a clone expressing the F8-IL12 heterodimer at 7 mg/l. These yields are compatible with clinical development programs, in consideration of the fact that the Maximal Tolerated Dose of IL12 in cancer patients is as low as 0.5 µg per kilogram of body weight. The fusion protein retained its full bioactivity as shown by *in vitro* proliferation assay and kept its high binding affinity to the cognate antigen, the alternatively spliced extradomain A of fibronectin. Biodistribution studies revealed an impressive tumor uptake of F8-IL12, with excellent tumor to organ ratio. Microroutoradiography confirmed the selective accumulation of F8-IL12 at vascular structures of the tumor.

Our group has generated and investigated in clinical trials the human monoclonal antibodies F8, L19 and F16 (Pini, Viti et al. 1998; Brack, Silacci et al. 2006; Villa,

Trachsel et al. 2008) specific to the alternatively spliced EDA and EDB domains of fibronectin, and to the alternatively spliced A1 domain of tenascin-C, respectively. The tumor targeting properties of these antibodies have been extensively studied in animal models of pathology (Carnemolla, Borsi et al. 2002; Brack, Silacci et al. 2006; Kaspar, Trachsel et al. 2007; Marlind, Kaspar et al. 2008; Villa, Trachsel et al. 2008). In addition, a radioiodinated version of the L19 antibody in scFv format (Santimaria, Moscatelli et al. 2003) and in SIP format (Sauer, Erba et al. 2009) have been studied in imaging and radioimmunotherapy clinical trials in patients with cancer. Similar studies are currently being conducted also for SIP(F16) and SIP(F8).

The clinical-stage L19, F8, and F16 antibodies have been used as modular building blocks for the construction of immunocytokines based on IL2 (Carnemolla, Borsi et al. 2002; Marlind, Kaspar et al. 2008), IIL10 (Trachsel, Bootz et al. 2007; Schwager, Kaspar et al. 2009), IL12 (Halin, Rondini et al. 2002; Gafner, Trachsel et al. 2006), TNF (Borsi, Balza et al. 2003), IFN-γ (Ebbinghaus, Ronca et al. 2005), IL15, GM-CSF (Kaspar, Trachsel et al. 2007) and other cytokines.

Interleukin-12-based immunocytokines appear to display potent anti-tumor effects, without worsening inflammation in animal models of chronic autoimmune diseases (Trachsel, Kaspar et al. 2007).

At present, the immunocytokines L19-IL2, F16-IL2, L19-TNF and F8-IL10 are being investigated in multiple clinical trials at 18 European Centers. The development activities for F8-IL12 described in this article represent the basis for the future clinical studies of this product. Investigations in patients with cancer are particularly encouraged by the excellent biodistribution results observed with F8-IL12 (**Figure 4.8**) and by the fact that the EDA domain of fibronectin is virtually undetectable in normal adult organs, while being strongly expressed in the majority of aggressive solid tumors and lymphomas (Pedretti, Rancic et al.; Rybak, Roesli et al. 2007; Villa, Trachsel et al. 2008; Schliemann, Wiedmer et al. 2009)

4.3 Material and methods

Cloning of F8-p35

For the cloning of F8-p35, the scFv(F8), containing a NheI restriction site and a leader sequence (Li, Pedraza et al. 1997) at the 5`, was amplified by PCR with primer RS9fo (5'-ctagctagcgtcgaccatgggctggagcctgat-3`) and NP5ba (5`-atcagcgcttcctttgatttccaccttggtcccttg-3`) which appends a part of the human p35 sequence. Human p35 gene was isolated from a human cDNA library (Human immune system MTC panel; Clontech, US) and was amplified by PCR with primer NP6fo (5'-gtggaaatcaaaggaagcgctgatggaggtagaaac-3') that appends an overlapping sequence to the VL of scFv(F8) and RS12ba (5`-atagtttagcggccgctcattaggaagcattcagatagctcatcac-3') which contains two stop codons and a NotI restriction site at the 3`. The scFv(F8) antibody sequence and the human p35 sequence were PCR assembled. The double digested NheI-NotI assembly product was cloned into the mammalian cell expression vector pcDNA3.1(+) (Invitrogen, Switzerland) containing the resistence for neomycin.

Cloning of p40-F8

For the cloning of p40-F8, the human p40 gene and its leader sequence was isolated from a human cDNA library (Human immune system MTC panel; Clontech, US) and was PCR amplified with primer KS5fo (5'-cccaagcttatgtgtcaccagcagttggtcatc-3`) that anneals on the leader sequence and introduces a HindIII restriction site at the 5` and RS8ba (5'-cccagactccaacagctgcacctcacctccatcagcgcttccactgca-3') that contains an overlapping sequence with the scFv(F8). The scFv(F8) was amplified with primers RS3 (5'-gaggtgcagctgttggagtctggg-3') which partially overlaps with the sequence of human p40 and NP7 (5'cctttgcggccgctcactatttgatttccaccttggtcccttg-3') that introduces two stop codons and a NotI restriction site. Human p40 and the scFv(F8) sequences were PCR assembled and double digested with NheI and NotI restriction enzymes. The digestion product was cloned into the mammalian cell expression vector pcDNA3.1(+) (Invitrogen, Switzerland)

containing the resistance for hygromycin. All PCR reactions were performed using the High Fidelity Taq Polymerase (Roche Diagnostics, Germany).

Nucleotide sequence of F8-p35

```
GCTAGCGTCGACCATGGGCTGGAGCCTGATCCTCCTGTTCCTCGTCGCTGTGGCTACAGGTAAGGGGCTCACAGTAGCAGG
CTTGAGGTCTGGACATATATATGGGTGACAATGACATCCACTTTGCCTTTCTCTCCACAGGTGTGCACTCGGAGGTGCAGCT
GTTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTCCCTGAGACTCTCCTGTGCAGCCTCTGGATTCACCTTTAGCCT
GTTTACGATGAGCTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTCTCAGCTATTAGTGGTAGTGGTGGTAGCA
CATACTACGCAGACTCCGTGAAGGGCCGGTTCACCATCTCCAGAGACAATTCCAAGAACACGCTGTATCTGCAAATGAACAG
CCTGAGAGCCGAGGACACGGCCGTATATTACTGTGCGAAAAGTACTCATTTGTATCTTTTTGACTACTGGGGCCAGGGAACC
CTGGTCACCGTCTCGAGTGGTGGAGGCGGTTCAGGCGGAGGTGGCTCTGGCGGTGGCGGAGAAATTGTGTTGACGCAGTC
TCCAGGCACCCTGTCTTTGTCTCCAGGGGAAAGAGCCACCCTCTCCTGCAGGGCCAGTCAGAGTGTTAGCATGCCGTTTTTA
GCCTGGTACCAGCAGAAACCTGGCCAGGCTCCCAGGCTCCTCATCTATGGTGCATCCAGCAGGGCCACTGGCATCCCAGAC
AGGTTCAGTGGCAGTGGGTCTGGGACAGACTTCACTCTCACCATCAGCAGACTGGAGCCTGAAGATTTTGCAGTGTATTACT
GTCAGCAGATGCGTGGTCGGCCGCCGACGTTCGGCCAAGGGACCAAGGTGGAAATCAAAGGAAGCGCTGATGGAGGTAGA
AACCTCCCCGTGGCCACTCCAGACCCAGGAATGTTCCCATGCCTTCACCACTCCCAAAACCTGCTGAGGGCCGTCAGCAAC
ATGCTCCAGAAGGCCAGACAAACTCTAGAATTTTACCCTTGCACTTCTGAAGAGATTGATCATGAAGATATCACAAAAGATAA
AACCAGCACAGTGGAGGCCTGTTTACCATTGGAATTAACCAAGAATGAGAGTTGCCTAAATTCCAGAGAGACCTCTTTCATAA
CTAATGGGAGTTGCCTGGCCTCCAGAAAGACCTCTTTTTATGATGGCCCTGTGCCTTAGTAGTATTTATGAAGACTTGAAGATG
TACCAGGTGGAGTTCAAGACCATGAATGCAAAGCTTCTGATGGATCCTAAGAGGCAGATCTTTCTAGATCAAAACATGCTGG
CAGTTATTGATGAGCTGATGCAGGCCCTGAATTTCAACAGTGAGACTGTGCCACAAAAATCCTCCCTTGAAGAACCGGATTTT
TATAAAACTAAAATCAAGCTCTGCATACTTCTTCATGCTTTCAGAATTCGGGCAGTGACTATTGATAGAGTGATGAGCTATCTG
AATGCTTCCTAATGAGCGGCCGC
```

Aminoacid sequence of F8-p35

```
EVQLLESGGGLVQPGGSLRLSCAASGFTFSLFTMSWVRQAPGKGLEWVSAISGSGGSTYYADSVKGRFTISRDNS
KNTLYLQMNSLRAEDTAVYYCAKSTHLYLFDYWGQGTLVTVSSGGGGSGGGGSGGGGEIVLTQSPGTLSLSPGE
RATLSCRASQSVSMPFLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQMR
GRPPTFGQGTKVEIKGSADGGRNLPVATPDPGMFPCLHHSQNLLRAVSNMLQKARQTLEFYPCTSEEIDHEDITKD
KTSTVEACLPLELTKNESCLNSRETSFITNGSCLASRKTSFMMALCLSSIYEDLKMYQVEFKTMNAKLLMDPKRQIFL
DQNMLAVIDELMQALNFNSETVPQKSSLEEPDFYKTKIKLCILLHAFRIRAVTIDRVMSYLNAS
```

Nucleotide sequence of p40-F8

```
AAGCTTATGTGTCACCAGCAGTTGGTCATCTCTTGGTTTTCCCTGGTTTTTCTGGCATCTCCCCTCGTGGCCATATGGGAACT
GAAGAAAGATGTTTATGTCGTAGAATTGGATTGGTATCCGGATGCCCCTGGAGAAATGGTGGTCCTCACCTGTGACACCCCT
GAAGAAGATGGTATCACCTGGACCTTGGACCAGAGCAGTGAGGTCTTAGGCTCTGGCAAAACCCTGACCATCCAAGTCAAA
GAGTTTGGAGATGCTGGCCAGTACACCTGTCACAAAGGAGGCGAGGTTCTAAGCCATTCGCTCCTGCTGCTTCACAAAAAGG
AAGATGGAATTTGGTCCACTGATATTTTAAAGGACCAGAAAGAACCCAAAAATAAGACCTTTCTAAGATGCGAGGCCAAGAAT
TATTCTGGACGTTTCACCTGCTGGTGGCTGACGACAATCAGTACTGATTTGACATTCAGTGTCAAAAGCAGCAGAGGCTCTTC
TGACCCCCAAGGGGTGACGTGCGGAGCTGCTACACTCTCTGCAGAGAGAGTCAGAGGGGACAACAAGGAGTATGAGTACTC
AGTGGAGTGCCAGGAGGACAGTGCCTGCCCAGCTGCTTCTGAGGAGAGTCTGCCCATTGAGGTCATGGTGGATGCCGTTCACA
AGCTCAAGTATGAAAACTACACCAGCAGCTTCTTCATCAGGGACATCATCAAACCTGACCCACCCAAGAACTTGCAGCTGAA
GCCATTAAAGAATTCTCGGCAGGTGGAGGTCAGCTGGGAGTACCCTGACACCTGGAGTACTCCACATTCCTACTTCTCCCTG
ACATTCTGCGTTCAGGTCCAGGGCAAGAGCAAGAGAGAAAGAAAGATAGAGTCTTCACGGACAAGACCTCAGCCACGGTC
ATCTGCCGCAAAAATGCCAGCATTAGCGTGCGGGCCCAGGACCGCTACTATAGCTCATCTTGGAGCGAATGGGCATCTGTG
CCCTGCAGTGGAAGCGCTGATGGAGGTGAGGTGCAGCTGTTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTCCCT
GAGACTCTCCTGTGCAGCCTCTGGATTCACCTTTAGCCTGTTTACGATGAGCTGGGTCCGCCAGGCTCCAGGGAAGGGGGCT
GGAGTGGGTCTCAGCTATTAGTGGTAGTGGTGGTAGCACATACTACGCAGACTCCGTGAAGGGCCGGTTCACCATCTCCAG
AGACAATTCCAAGAACACGCTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCCGTATATTACTGTGCGAAAAGT
ACTCATTTGTATCTTTTTGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCGAGTGGTGGAGGCGGTTCAGGCGGAGGT
GGCTCTGGCGGTGGCGGAGAAATTGTGTTGACGCAGTCTCCAGGCACCCTGTCTTTGTCTCCAGGGGAAAGAGCCACCCTC
TCCTGCAGGGCCAGTCAGAGTGTTAGCATGCCGTTTTTAGCCTGGTACCAGCAGAAACCTGGCCAGGCTCCCAGGCTCCTC
ATCTATGGTGCATCCAGCAGGGCCACTGGCATCCCAGACAGGTTCAGTGGCAGTGGGTCTGGGACAGACTTCACTCTCACC
ATCAGCAGACTGGAGCCTGAAGATTTTGCAGTGTATTACTGTCAGCAGATGCGTGGTCGGCCGCCGACGTTCGGCCAAGGG
ACCAAGGTGGAAATCAAATAGTGAGCGGCCGC
```

Aminoacid sequence of p40-F8

IWELKKDVYVVELDWYPDAPGEMVVLTCDTPEEDGITWTLDQSSEVLGSGKTLTIQVKEFGDAGQYTCHKGGEVL
SHSLLLLHKKEDGIWSTDILKDQKEPKNKTFLRCEAKNYSGRFTCWWLTTISTDLTFSVKSSRGSSDPQGVTCGAA
TLSAERVRGDNKEYEYSVECQEDSACPAAEESLPIEVMVDAVHKLKYENYTSSFFIRDIIKPDPPKNLQLKPLKNSR
QVEVSWEYPDTWSTPHSYFSLTFCVQVQGKSKREKKDRVFTDKTSATVICRKNASISVRAQDRYYSSSWSEWAS
VPCSGSADGGEVQLLESGGGLVQPGGSLRLSCAASGFTFSLFTMSWVRQAPGKGLEWVSAISGSGGSTYYADSV
KGRFTISRDNSKNTLYLQMNSLRAEDTAVYYCAKSTHLYLFDYWGQGTLVTVSSGGGGSGGGGSGGGGEIVLTQ
SPGTLSLSPGERATLSCRASQSVSMPFLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGSGSGTDFTLTISRLEPE
DFAVYYCQQMRGRPPTFGQGTKVEIK

Table 4.1. Nucleotidic and aminoacid sequence of scFv(F8)-p35 and p40-scFv(F8) are represented in the table.

Cell culture

Adherent CHO-S cells were cultured in RPMI 1640 (Gibco, Switzerland) containing 10% FCS (Invitrogen, Switzerland) and incubated at 37ºC and 5% CO_2. Suspension CHO-S cells were cultivated in PowerCHO-2 CD (Lonza, Switzerland) containing 8mM ultraglutamine (Lonza, Belgium) incubated at 37 °C and 5% CO_2. When necessary G418 (Calbiochem, Germany) and Hygromicin (Invivogen, Switzerland) were added to the medium at a concentration of 0.5 mg/ml.

Generation of a cell line expressing F8-IL12

Adherent CHO-S cells were electroporated using an Amaxa nucleofector according to the manufacturer's protocol. Different ratios of plasmids F8-p35 / p40-F8 were used to find the best conditions to maximize the expression of the heretodimer. Three days after electroporation, stably-transfected cells were selected in presence of G418 0.5 mg/ml (Calbiochem, Germany) and 0.5 mg/ml hygromycin (Invivogen, Switzerland). Monoclonal cell lines were obtained by serial dilutions and screened for expression of the fusion protein by ELISA.

Large scale culture of F8-IL12 in bioreactor

Stably-transfected suspension CHO-S cells driving F8-IL12 expression were grown in PowerCHO-2 CD containing 8mM ultraglutamine, 0.5 mg/ml G418 and 0.5 mg/ml Hygromicin. The cell culture inoculum for the 3 liters Labfors bioreactor

(Infors AG, Switzerland) was obtained starting from a static culture of the cells of the master cell bank. The cell culture was expanded in roller bottles untill cell density reached $2\text{-}3\text{x}10^6$ cells/ml. The fed batch fermentation process was used for the production of F8-IL12. Cells were seeded at $5\text{x}10^5$ cells/ml in the bioreactor. Fixed cell culture parameters of F8-IL12 in the bioreactor were defined as 37 °C temperature, 50% pO_2, 130 rpm helical stirring, pH 7.25. Glucose concentration was monitored and manual feed were applied to bring its concentration up to 2 g/l. In the exponential and plateau growth phase (72h - 144h) feeds of fresh medium supplemented with ultraglutamine were introduced to maintain cell vitality. In order to monitor the total protein productivity, 100 ml supernatant samples were daily collected and affinity purified on HiTrap Protein A resin (GE Healthcare, Sweden).

Purification of F8-IL12

The fusion protein could be purified from cell culture medium by protein A affinity chromatography, as there is a staphylococcal protein A binding site present on most VH3 domains (Sasso, Silverman et al. 1991; Hoogenboom and Winter 1992; Silacci, Brack et al. 2005) Ion exchange chromatography (Ceramic hyper D; PALL, USA) and preparative size exclusion chromatography on a HiLoad 26/60 Superdex 200 size exclusion column (GE Healthcare, Sweden) of the eluted protein A preparation allowed the isolation of the F8-IL12 heterodimer from a mix containing also aggregates and polymers.

Elisa of supernatants

ELISA experiments on the conditioned culture media were performed according to Carnemolla et al. (Carnemolla, Neri et al. 1996) To detect different clones expressing F8-IL12, the biotinylated 11-EDA-12 domain of fibronectin, which includes the epitope recognized by scFv(F8), was immobilized on a streptavidin coated plate (StreptaWell, Roche Applied Bioscience, Germany). Four different detection systems were used and allowed the evaluation of the expression of the different components of the fusion protein. Horseradish peroxidase conjugated

Protein A (GE Healthcare, Sweden), diluted according to the manufacturer's recommendations, was used as agent to detect the VH domain of the fusion protein. In order to detect the heterodimer p70, the p35 subunit and the free p40 subunit, three different mouse monoclonal antibodies were used ("affinity purified anti-human IL-12, p35" (eBioscience, Germany); "affinity purified anti-human IL-12, p40" (eBioscience, Germany); "anti-human IL-12 p70 antibody" (R&D Systems, Germany)) and detected with a goat anti-muose IgG peroxidase conjugate (Sigma-Aldrich, USA). In all cases, the immunoreactivity with the immobilized antigen was detected using the substrate BluePOD (Roche Diagnostics, Germany) for peroxidase, and photometric absorbance at 405 nm was measured.

Bioactivity assay

The biological activity of F8-IL12 was determined by a T-cell proliferation assay (Gately, Chizzonite et al. 2001) Freshly isolated human peripheral blood mononuclear cells (PBMC) were cultured immediately after isolation with 25 µg/ml mitogen Phytohemagglutinin-M (Roche Diagnostics, Germany) for 3 days. Cells were diluted 1:2 by adding equal volume of supplement medium and further cultivated with 50 I.U./ml of human Interleukin-2 (Roche Diagnostics, Germany). After 24 hr cells were seeded in 96-well plates at a density of 2×10^4 cells/well in 200 µl of medium containing serial dilutions of either F8-IL12 or commercially available, recombinant, human IL12 as standard (R&D Systems, Germany) or culture medium as a negative control. After 48 h, 20 µl Cell Titer 96 Aqueous One Solution (Promega, USA) was added to each well. The plate was incubated for 4 h and absorbance was read at 490 nm. The experiment was performed in triplicate.

Biodistribution studies

The *in vivo* targeting performance of F8-IL12 was evaluated by biodistribution analysis as described before (Carnemolla, Borsi et al. 2002) Briefly, purified F8-IL12 was radioiodinated and injected into the tail vein of immunocompetent

129SvEv mice bearing s.c. implanted F9 murine teratocarcinoma. Mice were sacrificed 24 h or 48 h after injection of the fusion protein (8 µg, 7 µCi/mouse). Organs were weighed and radioactivity was counted with a Packard Cobra gamma counter. Radioactivity content of representative organs was expressed as the percentage of the injected dose per gram of tissue (%ID/g).

Microautoradiography

Twenty-four hours after the tail injection of radiolabeled F8-IL12, mice were sacrificed and tumors were embedded in paraffin. Ten µm sections were cut and fixed with paraformaldehyde. Sections were then coated with NBT KODAK autoradiography emulsion (KODAK, US), dried and stored at 4°C in the dark for approximately three weeks. The autoradiography emulsions were developed (Developer D-19, KODAK, France) and fixed (EASTMAN Fixer, KODAK France). Finally, slides were rinsed with deionized water and counterstained with hematoxilin (SIGMA, Switzerland).

Mass spectrometry

Following reduction of F8-IL12 with 10 mM TCEP for 30 min at room temperature, the solution was desalted and concentrated using C_4 microcolumns (OMIX™ tips, Varian Inc., Paolo Alto, CA, USA) according to the manufacturer's guidelines. The reduced and desalted protein was mixed with sinapinic acid (20 mg/ml in 70% ACN, 0.1% TFA) and spotted on a MALDI (matrix assisted laser desorption/ionization) target plate. The mass spectrometric analysis was carried out using a 4800 MALDI-TOF/TOF Analyzer (Applied Biosystems, Foster City, CA, USA). All spectra were acquired in the linear mode in a mass range of 10'000 to 100'000 m/z with a solid-state laser (355 nm) at a laser repetition rate of 200 Hz. A total of 1500 laser shots were summed for each spectra. Spectra were further processed using the Data Explorer software (Applied Biosystems).

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6 Curriculum Vitae

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Personal details

Date of birth	6th May 1982
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Education

Nov. 2006 to present	**Ph.D thesis in the group of Prof. Dr. Dario Neri**, Swiss Federal Institute of Technology - Zurich (ETHZ) and Philochem AG, Switzerland Title of the thesis: "*Antibody engineering: advances in phage display technology and in the production of therapeutic immunocytokines*".
Oct. 2004 to Oct. 2006	**M.Sc. in Pharmaceutical Biotechnologies** Summa cum Laude Title of the thesis: "*Novel immunocytokines for vascular tumour targeting*". Faculty of Pharmac. Sc., University of Padua, Italy.

Academic advisor: Prof. Dr. Dario Neri and Dr. Patrizia de Laureto Polverino.

Mar. 2006 to Oct. 2006 **Erasmus fellowship** at Swiss Federal Institute of Technology – Zurich (ETHZ)
Master Thesis in the research group of Prof. Dr. Dario Neri

Sep. 2001 to Sep. 2004 **B.Sc. in Health Biotechnologies**
Title of the thesis: "*Use of calcium-sensitive photoprotein aequorin for the study of calcium homeostasis in starfish oocytes*".
Faculties of Medicine and Pharmac. Sc., University of Padua, Italy. Academic advisor: Prof. Ernesto Carafoli and Dr. Marisa Brini.

Sep. 1996 to June 2001 **High school** "Liceo Scientifico G. Galilei" -Belluno, Italy

Teaching and relevant work experience

Two months internship at Philogen S.p.A. (Siena, Italy) - a biotech company specialized in GMP grade antibodies production (2009).

Speaker at the "Third Experimental Course on Antibody Phage Technology", Swiss Federal Institute of Technology – Zurich (ETH) (2008)

Supervision of one master thesis student (2007)

Languages

Italian	Native speaker
English	Fluent
German	Intermediate level
French	Basic

Patents and publications

Sommavilla R, Villa A, Lovato V (2009). US patent application 61/221,913 "Murine Antibody Display Library"

Sommavilla R, Valeria L, Villa A, Sgier D, Neri D (2010). "*Design and construction of a naïve mouse antibody phage display library*". Journal of Immunological Methods; 352 (1-2):31-43.

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Mårlind J, Kaspar M, Trachsel E, **Sommavilla R**, Hindle S, Bacci C, Giovannoni L, Neri D (2008). "*Antibody-mediated delivery of interleukin-2 to the stroma of breast cancer strongly enhances the potency of chemotherapy*". Clinical Cancer Research; 14 (20) 6515-24.

Villa A, Trachsel E, Kaspar M, Schliemann C, **Sommavilla R**, Rybak JN, Roesli C, Borsi L, Neri D (2008). "*A high-affinity human monoclonal antibody specific to the alternatively spliced EDA domain of fibronectin efficiently targets tumor neo-vasculature in vivo*". International Journal of Cancer; 122 (11): 2405-13.

Voluntary activities

2005-present: Member ofDoctors with Africa (CUAMM), Padua, Italy.
CUAMM is the largest Italian non-governmental organization to improve and safeguard the health of African populations.

2005: Speaker at Radio Sherwood – Northeastern Italy broadcast

2001-1998 Voluntary fireman – Protezione Civile del Veneto, Italy

Hobbies

Volleyball, swimming, diving, fencing, hiking, climbing, skiing.

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