Human antibody phage technology - isolation and characterization of monoclonal antibodies for the targeting of cancer and rheumatoid arthritis

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HUMAN ANTIBODY PHAGE TECHNOLOGY: ISOLATION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES FOR THE TARGETING OF CANCER AND RHEUMATOID ARTHRITIS

A dissertation submitted to attain the degree of

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(Dr. sc. ETH Zurich)

presented by

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1. Summary

Modern therapeutic approaches for the treatment of cancer and inflammatory diseases aim at the development of more selective drugs, that potentially act at the site of disease while minimizing side effects in normal tissues.

One avenue towards the development of more selective therapeutic agents consists in the targeted delivery of bioactive molecules (e.g., drugs, cytokines, photosensitizers, radionuclides, etc.) to a site of disease by means of binding molecules (e.g., human antibodies, peptides and small organic molecules) specific to accessible markers of disease. The selective accumulation of drugs at the site of disease may help spare normal tissues and will increase the therapeutic index of pharmaceutical drugs.

In order to generate a broad array of possible therapeutic targets, an extensive effort has been dedicated at the discovery of antigens which are overexpressed in cancer or in other pathological conditions. At present, it is possible to generate high affinity antibodies against virtually any biomolecular target. Antibody phage display technology represents a well established technology, which greatly facilitates the isolation of good-quality monoclonal antibodies. Indeed, large combinatorial phage display libraries of human antibodies are routinely being used for the identification of antibody candidates for clinical applications. Antibody-based therapy has become one of the most successful and important strategies for treating patients with hematological malignancies, solid tumors and inflammatory diseases.

The thesis consists of two main projects, with a focus on the generation and characterization at novel binding specificity for possible biomedical applications.
The first project aimed at the generation of high affinity antibodies against fibrin, an attractive target for antibody-based pharmacodelivery applications. Fibrin formation from its precursor fibrinogen is a pathological feature of thrombotic events, cancer and a wide range of inflammatory conditions. By contrast fibrin is practically undetectable in normal tissues.

The isolation of antibodies specific to fibrin, with no detectable binding to fibrinogen, is particularly challenging, as the two proteins differ only by the cleavage of two short peptidic fragments. We could generate fully human antibodies, with high binding affinity to fibrin and no detectable binding to fibrinogen, thanks to advances in antibody phage technology.

A first monoclonal antibody, named AP1, was isolated from the ETH-2-Gold library (Silacci, Brack et al. 2005) after two rounds of panning. Experiments performed on this clone confirmed that a K94E mutation in the VH domain was critical for high-affinity fibrin recognition. Based on this observation, we have constructed a “designed” antibody phage display library (containing 13 billion clones), from which a high-affinity human antibody specific to the N-terminal peptide of fibrin alpha chain (termed “AP2”) was isolated. The AP2 antibody has been shown to selectively stain fibrin in tissue sections, to inhibit fibrin clot formation and to selectively localize in vivo to tumors, which are rich in fibrin deposits. Furthermore, the AP2 antibody has been shown to selectively localize to inflamed joints in a mouse model of rheumatoid arthritis.

The second project focused on the identification and characterization of high affinity antibodies against follistatin-like 1 protein (FSTL1). For practical applications, antibodies with high-affinity for the antigen are generally preferable. We have isolated a first antibody clone (E8) and subsequently improved its affinity by constructing an
affinity maturation library (consisting of $1.3 \times 10^7$ individual antibody clones). From this library we could isolate the (A4) antibody, which had a dissociation constant $K_d = 8 \text{ nM}$ in scFv format. Due to challenges associated with the expression of the A4 antibody in small immunoprotein (SIP) format, the parental E8 antibody was used in order to perform immunohistochemistry analyses of rheumatoid arthritis specimens and for the in vivo targeting of arthritic lesions.

Finally, a side project is described in appendix 8.3.

We performed a comparative therapy study using two antibody-drug conjugates in SIP format. Specifically, we compared the monoclonal antibody Trastuzumab (an internalizing Ab), and F8 (a non-internalizing monoclonal antibody specific to EDA) (Villa, Trachsel et al. 2008). The antibodies were coupled to the maytansinoid DM1 via disulfide bonds and were tested in HER2+ SKOV3 ovarian cell cancer.
1. Riassunto

I moderni approcci terapeutici per il trattamento di tumori e malattie infiammatorie hanno come obiettivo principale lo sviluppo di farmaci più selettivi, in grado di agire esclusivamente sulla malattia e minimizzare gli effetti collaterali sui tessuti sani.

Una via verso lo sviluppo di agenti terapeutici più selettivi consiste nel “targeting” mirato di strutture patologiche per mezzo di composti bioattivi (es. potenti tossine, citochine, agenti fotosensibilizzanti, radionuclidi ecc...), sfruttando ligandi in grado di riconoscere specificatamente i marcatori di una data malattia (tali ligandi sono per esempio anticorpi umani, peptidi e piccolo molecole organiche). L’accumulo selettivo di farmaci al sito patologico potrebbe in tal modo aiutare a risparmiare tessuti sani ed accrescere l’indice terapeutico dei composti farmaceutici.

Si è dedicato uno sforzo intenso alla scoperta di antigeni che fossero iperespressi in cancro e altre condizioni patologiche, al fine di generare un’ampia gamma di possibili bersagli terapeutici. Oggi è idealmente possibile creare anticorpi ad alta affinità verso ogni tipo di bersaglio molecolare. A tale proposito, l’ “antibody phage display” rappresenta una tecnologia ormai ben consolidata, che facilita enormemente l’isolamento di anticorpi monoclonali di ottima qualità. Infatti, per la selezione di candidati per applicazioni cliniche, vengono abitualmente usate ampie librerie combinatoriali di anticorpi umani. La terapia basata sull’uso di anticorpi è divenuta una delle più importanti ed affermate strategie per il trattamento di pazienti con patologie ematologiche, tumori solidi e malattie infiammatorie.

La tesi è composta da due progetti principali che si focalizzano sulla generazione e sulla caratterizzazione di innovative specificità di legame per eventuali impieghi biomedici.
Il primo progetto mira a produrre anticorpi ad alta affinità contro fibrina, un bersaglio molto attrattivo per applicazioni di “pharmacodelivery” basate sull’uso di anticorpi. Infatti, la formazione di fibrina a partire dal suo precursore fibrinogeno è una caratteristica patologica di eventi trombotici, tumori e un’ampia varietà di condizioni infiammatorie pur risultando praticamente assente in tessuti normali.

Siccome le due proteine differiscono solamente nella presenza o meno di due corti frammenti proteici, l’isolamento di anticorpi specifici per la fibrina che non reagiscano con fibrinogeno è particolarmente difficile. Proprio grazie ai progressi nella tecnologia “phage display” siamo riusciti a creare tali anticorpi umani.

Un primo anticorpo monoclonale, chiamato AP1, è stato isolato dalla libreria ETH-2-Gold (Silacci, Brack et al. 2005) dopo due cicli del consueto protocollo. Gli esperimenti effettuati su tale clone hanno confermato che la mutazione K94E a livello del dominio VH (frammento variabile appartenente alla catena pesante dell’immunoglobulina) era critica per il riconoscimento ad alta affinità. Sulla base di tali osservazioni, abbiamo costruito una libreria di anticorpi (contenente 13 miliardi di cloni), dalla quale è stato isolato un anticorpo monoclonale ad alta affinità per il peptide N-terminale della catena alpha di fibrina (nominato AP2). L’anticorpo AP2 si è rivelato in grado di colorare selettivamente depositi di fibrina in fetine di tessuto, di inibire parzialmente la formazione del coagulo di fibrina e di localizzarsi selettivamente in vivo a livello di neoplasie dove fibrina appare essere abbondante.

Inoltre, si è provato che tale anticorpo AP2 è in grado di localizzarsi selettivamente a livello di articolazioni infiammate in modelli murini di artrite reumatoide.

Il secondo progetto si focalizza sull’identificazione e la caratterizzazione di anticorpi ad alta affinità contro “folistatin-like 1 protein” (FSTL1). Considerando che per applicazioni pratiche è generalmente preferibile disporre di anticorpi ad elevata affinità, abbiamo isolato un primo anticorpo (E8) e successivamente migliorato la sua affinità grazie alla
Costruzione di una libreria di anticorpi maturati per affinità (contenente $1.3 \times 10^7$ singoli clones). Da questa libreria è stato possibile isolare l'anticorpo A4, il quale presenta un valore di costante di dissociazione pari a 8 nM nel formato scFv (singola catena di frammento variabile). A causa delle difficoltà associate all'espressione dell'anticorpo A4 in formato SIP (piccola immunoproteina), si è optato per l'utilizzo dell'anticorpo progenitore E8 sia per l'analisi immunoistochimica di campioni di artrite reumatoide, sia per il “targeting” in vivo di lesioni artritiche.

Infine, nell'appendice 8.3 viene descritto un progetto collaterale. In tale progetto abbiamo effettuato uno studio comparativo utilizzando due coniugati anticorpo-farmaco nel formato SIP. Abbiamo comparato l'anticorpo monoclonale Trastuzumab (un anticorpo internalizzante) e F8 (un anticorpo non internalizzante specifico per EDA) \textsuperscript{(Villa, Trachsel et al. 2008)}. Gli anticorpi sono stati coniugati con il “maytansinoid” DM1 tramite legami di solfuro e testati su cellule SKOV3, un modello di tumore ovarico positivo per l'espressione dell'antigene HER2.
2. Introduction

2.1. Human antibodies as therapeutic agents

2.1.1. Antibody structure, characteristics and function

The terms antibody and immunoglobulin are interchangeable, indicating glycoprotein molecules produced by B cells and plasma cells in response to immunogens such as toxins, bacteria and viruses. The main features of antibody molecules is represented by their ability to bind specifically to a target molecule (most commonly a protein or a polysaccaridic antigen), recognizing a unique part of the antigen, called epitope. This mechanism of binding leads either to the direct neutralization of its target, or to the tagging of a microbe or of an infected cell for attack by other components of the immune system, such as leukocytes or the complement system.

An antibody in IgG format consists of two identical heavy chains (50 kDa) and two identical light chains (25 kDa), forming a typical Y-shaped monomeric structure of 150 kDa. This Y shaped molecule can also be split in three equal-size portions connected by a flexible tether: two “arms” containing the two antigen-binding fragments (Fab), and the “stem” of the Y shape, called crystallizable fragment (Fc). In the Fc region, at position 297, there is a site of N-linked glycosylation on the conserved asparagine residue. The two heavy chains interact with each other and with the light chains, by noncovalent interactions, which are further stabilized by disulfide bridges (one between heavy and light chain, two between the heavy chains, located in the so-called hinge region). An additional classification of the characteristic elements of an antibody discriminate between the more variable parts of the molecule compared to the conserved ones. The constant regions which are found in the heavy chain are named CH₁, CH₂, and CH₃, (the last two of them, forming the Fc part). A constant domain in the light chain is termed CL. The variable part of an IgG molecule, termed fragment
variable (Fv), consists of the variable domain of VH and VL, belonging respectively to the heavy and light chain. In any given immunoglobulin molecule, the two heavy chains and the two light chains are exactly the same, giving rise to an antibody molecule with two identical antigen-binding sites. The ability to simultaneously engage in a binding interaction with two identical antigens on a suitable structural arrangement (e.g. on the surface of a pathogen or on a polymeric support), may increase the functional affinity of interaction, by a mechanism which is called avidity (Janeway Charles A 2001).

Figure 2.1. Crystallographic structure of an intact IgG antibody and its schematic representation (Adapted from PDB 1IGT). The immunoglobulin (Ig) monomer is composed of two identical light chains and two identical heavy chains. Light chains are composed of a constant domain CL and a variable domain VL (pale pink and pale green), whereas heavy chains are composed of the three constant domains CH1, CH2, and CH3 and the variable domain VH (violet and grey). Two disulfide bridges link the two heavy chains in hinge region, and a single disulfide bridge attach the light chains to the heavy chain. The variable domains of both the heavy and light chains compose the antigen-binding site of the molecule. The Fc region is glycosylated at the conserved asparagine 297 residue.

The Fc region is also responsible of the classification of antibody molecules into the 5 different classes (or “isotypes”) of immunoglobulins: IgM, IgD, IgG, IgE, IgA. The
different sequences and domain arrangements within isotypes determine the functional activity of an antibody molecule (figure 2.2).

**Figure 2.2. Antibody isotypes, determined by constant region (Fc) (Adapted from Janeway's Immunobiology 8th edition).** The constant region of the heavy chain for each isotype is indicated in a different color. IgM and IgE lack the hinge region but they contain an extra heavy-chain domain. IgM and IgA are usually synthetized as multimers (pentamer and dimer respectively). The isotypes also differ in the distribution of N-linked carbohyfrate groups, shown as little gray points.

Antibodies are the key-factors of the humoral component of the immune system, existing in two forms: as receptor on the surface of the B cells (BCR) or as soluble and circulating molecules. Antibodies are present in blood and in the lymph, being able to distribute throughout the body, reaching virtually any site (including sites of inflammation), exception made for a few privileged structures (i.e. the brain, the eye vitreous) which are less reachable, due to physical and biological barriers. The primary function of an antibody is to recognize and tightly bind to a specific antigen. The
recognition of antigens by high affinity antibodies is generally described as a “lock and key” fit. On the tip of the Y-shaped antibody molecule the paratope is nicely complementary to one particular epitope on the cognate antigen. This specific recognition event is mediated by three hypervariable loops, located in each V domain, referred to as complementary determining regions (CDRs). In between the CDRs, the fairly well conserved portions of antibody sequence are called framework regions (FR) (Zhang, Berezov et al.), which are numbered from 1 to 4 according to their position. The CDRs are present on the light as well on the heavy chains, giving rise to a total of six “anchoring” portions for each binding site (figure 2.3).

![Diagram of antibody structure](image)

**Figure 2.3. Sequence variability in CDR regions of heavy chain V region. (Adapted from Janeway's Immunobiology 8th edition).** In the antibody molecule, the pairing of an heavy and a light chain brings together the hypervariable loops from each chain to create a single hypervariable surface, which forms the antigen binding site.

Antigen binding is one of the main functions of antibodies but frequently this process has no direct biological effect. Often, protective mechanisms also require secondary "effector functions" of antibodies, which are carried out by the Fc fragment. The Fc may trigger effector functions through interaction with Fc receptors (FcR) expressed on immune effector cells, such as macrophages, neutrophils, natural killer cells (Fcγ), mast cells, basophils and activated eosinophils (Fcε). These interactions may lead
either to direct phagocytosis of a pathogen or to antibody–dependent cellular cytotoxicity (ADCC). Alternatively the Fc region may bind to the C1q component of the complement system, initiating the so-called “classical” complement cascade, which may lead to the activation of phagocytes and to the formation of pores on the surface of pathogens. Finally, antibodies are also involved in an active transport process. A specific type of receptor, the neonatal Fc receptor (FcRn), has a special relevance for antibody presence in milk and for the transfer of protective humoral immunity from the mother to the baby. Additionally, thanks to the continuous and reversible interaction with FcRn, IgG molecules constantly shuttle between the bloodstream and endocytic vesicles.

2.1.2. Antibody formats and engineering

The field of antibody engineering has evolved rapidly in the last decade of the past century (Maynard and Georgiou 2000). Indeed, antibody products represent some of the most commonly used biopharmaceuticals for the therapy of cancer and of other relevant conditions, including chronic inflammatory diseases such as rheumatoid arthritis. The industrial development of antibody products features opportunities and challenges, which can be summarized in the following categories:

- Production costs
- Pharmacokinetic profiles
- Tissue penetration and retention at the site of disease
- Ability to trigger a sufficiently potent response against the disease of interest

**Production costs:** monoclonal antibodies (mAbs) are large and complex molecules, which typically need to be produced in a complex eukaryotic system (e.g., CHO cells), followed by extensive purification steps, all performed under Good Manufacturing Practice (GMP) conditions. On one hand, the use of living organisms for antibody
production results in complex manufacturing procedures and quality controls (e.g., absence of viral contaminants), which may lead to non-negligible production costs. On the other hand, modern cell line development and fermentation technologies routinely enable the production of antibodies in IgG format at yields > 1 gram/liter, resulting in costs of goods \(\sim 260-1500 \$/\text{gram} \) (depending on liter bioreactor capacity) at suitable industrial scales \cite{Werner2004, Farid2007}.

**Pharmacokinetic profiles:** intense research efforts in the field of antibody engineering were driven by the desire to create molecules with better disease-targeting properties and/or pharmacokinetic profiles. Several characteristics of mAbs can influence *in vivo* behavior, such as molecular size, shape, affinity and valence. A range of recombinant antibody formats which can be considered in terms of molecular weight and valence \((n)\). In general, antibody valence may vary from monomeric structures \((n = 1\), for Fab fragments), to decavalent structures \((n = 10\), in the case of IgM). Although antibodies are usually monospecific, meaning they contain at least two identical antigen-binding sites, recombinant antibodies have been engineered to display different antigen-binding sites, yielding “bispecific” antibodies. These macromolecules may bind to different epitopes on the same antigen or to distinct epitopes on different antigens. The single-chain variable domain antibody fragment (scFv), is one of the building block that has extensively been used in our laboratory, in which the heavy and light variable domains of an antibody molecule (VH and VL) are joined together by a peptide linker, typically containing 14 amino-acid residues \cite{HustonLevinson1988}. ScFv fragments can form stable oligomeric structures, which are not covalently bound. The formation of multimers appears to be determined primarily by the length of the linker joining the variable heavy and light domains. Shorter linkers between 5–12 amino acids result predominantly in the formation of dimers or diabodies, whereas a direct joining of VH and VL regions without a linker favors the formation of trimeric species \cite{IlladesKortt1997}. 
Figure 2.4. Structure of an IgG molecule and fragments derived from this structure by domain assembly. Here are shown the formats that are mentioned and/or that are relevant for my thesis.

An antibody format, which has been extensively studied by our laboratory and which is relevant for this thesis is the SIP (small immunoprotein) format (Borsi, Balza et al. 2002). This protein can be obtained by the fusion of a scFv fragment with the CH\textsubscript{4} domain of IgE, containing a cysteine residue at the C-terminal position. Antibodies in SIP format exist as stable homodimers, that can be chemically modified at the level of the C-terminal thiol groups without loss of immunoreactivity or tumor-targeting performance (Alonso, Palumbo et al. 2010).

ScFv-Fc fusion proteins are often considered as IgG substitutes, which enjoy a long circulatory half-life in blood, due to the interaction of the Fc moiety with FcRn receptors. Unlike conventional IgG's (which contain both heavy and light chains), antibodies in scFv format consist of a single polypeptide chain, which forms a disulfide-stabilized homodimer. Indeed, with the exception of monomeric scFv fragments, all other antibody formats generally used for biomedical applications (e.g., diabody, SIP and scFv-Fc) are bivalent and display a functional binding affinity (“avidity”) comparable to the one of the parental IgG molecule (figure 2.4).

When antibodies are used for the inhibition of target molecules or for the triggering of ADCC responses, the IgG format is generally preferred (Chames, Van Regenmortel et al. 2009), as it enjoys a slow clearance from blood circulation and an efficient localization at the site of
disease. For certain pharmaceutical applications, however, alternative antibody formats may be desirable. For example, Fab fragments are generally preferred when a rapid clearance from circulation is needed. This is the case for ReoPro®, a Fab fragment specific to glycoprotein IIb/IIIa receptor which is used for the inhibition of thrombotic events in individuals undergoing percutaneous coronary intervention (angioplasty with or without stent placement). Since the product works as a platelet aggregation inhibitor, a prolonged residence time of the antibody in blood would not be desirable, as it could lead to severe bleeding-associated complications.

The choice of a suitable antibody format is particularly relevant in the case of “armed” antibody products. For these pharmaceuticals, the antibody moiety serves as a vehicle for the selective pharmacodelivery of a suitable payload (e.g., drug, cytokine, radionuclide) at the site of disease. For certain payloads (e.g., radionuclides, cytokines) it is preferable to have antibodies with short clearance from blood, in order to minimize toxic effects on normal tissues (e.g., bone marrow toxicity (Steiner and Neri 2011)).

Antibodies in IgG format typically display a high liver uptake, due to the hepatobiliary clearance mechanism and to the FcRn-mediated recycling mechanisms. ScFvs and diabodies have a size below the renal filtration threshold and do not interact with FcRn. By contrast, larger antibody formats (e.g., SIPs, scFv-Fc and IgGs) are typically eliminated via the hepatobiliary route (Olafsen, Kenanova et al. 2005).

Tissue penetration and retention at the site of disease: the majority of targets for the pharmaceutical use of therapeutic antibodies is located in abluminal structures, requiring an extravasation of the antibody molecule and its diffusion into tissue. The ability of antibody products to reach their cognate target in vivo is crucially important for pharmaceutical activity. Research activities in the field of anti-cancer antibodies are
particularly suitable to illustrate the challenges associated with the relatively slow rate of extravasation of antibody molecules, as well as possible solutions to the problem (whose pharmaceutical implications are not limited to Oncology).

Over 85% of human cancers are represented by solid tumors. However, only 42% of the all antibody products approved for oncological applications, is used for the treatment of solid malignancies (Scott, Wolchok et al. 2012). The tumor mass is characterized by an irregular vasculature and by a high interstitial fluid pressure, making it difficult for antibodies to diffuse and penetrate into target tissues (Beckman, Weiner et al. 2007). Furthermore, the rate of diffusion is approximately inversely proportional to the molecular weight. Consequently, large macromolecules such as full IgG diffuse poorly, whereas small antibody fragments may be able to better penetrate into tissues.

The ability of antibodies to penetrate into the tumor mass depends also on their affinity to the target. Indeed, several studies have shown that very high binding affinities can be counter-productive for disease targeting, leading to a decreased performance in vivo (Adams, Schier et al. 2001). This effect, which is referred to as the “binding site barrier effect” (Fujimori, Covell et al. 1990), related to the possibility that high-affinity binders are trapped by antigen in perivascular cells, thus preventing diffusion into deeper structures. By contrast, antibodies with moderate affinity may be able to detach form the first antigen they encounter, thus reaching target cells which are located at a larger distance from blood vessels (Thurber, Schmidt et al. 2008, Schmidt and Wittrup 2009). Tissue penetration depends also on other factors, including antigen density, antibody internalization, as well as association and dissociation rates. The study of the interplay between antibody binding affinity and its in vivo performance remains an active area of pharmaceutical research.
Ability to trigger a sufficiently potent response against the disease of interest:
Major efforts in tuning antibody-based therapeutics for cancer have been focused in the modification of different domains of mAbs and mAb fragments to both influence target affinity and modify ADCC (antibody–dependent cellular cytotoxicity) or CDC (complement-dependent cytotoxicity).
For the IgG class of antibodies, the ADCC mechanism is governed by engagement of the Fc region with a family of receptors referred to as the Fcγ receptors (FcγRs). These receptors are expressed on a variety of immune cells, which are then activated, bursting an immune response. Since the formation of the complex Fc/ FcγR recruits the immune cells at the site of the targeted antigen, ADCC “on paper” is a very nice mechanism of action, as the antibody displays a toxic effect in vivo only when it is bound to the target cell at the site of disease. Nevertheless, there are situations in which the antibody alone is not able to trigger ADCC with sufficient potency for therapeutic intervention, leading to the necessity to manipulate the Fc variants to increase affinity and specificity towards the FcγRs. Point mutations and/or modified glycosylation have been investigated to efficaciously enhance effector functions.

The binding between IgG and FcγR or C1q (the first component of the complement cascade) depends on residues located in the hinge region and the CH2 domain. The group of L.G.Presta has extensively worked on the mapping of the binding site on human IgG1 for Fc and FcRn, showing that specific amino acid substitutions could lead to variants with improved binding capacity to the FcγRs (Armour, Clark et al. 1999, Idusogie, Presta et al. 2000, Shields, Namenuk et al. 2001).

In the niche of the antibodies related to complement activation, the relatively new company Genmab (founded in 1999), has done his entrance in the field with a competitive technology, termed HexaBody®: a broadly applicable antibody platform,
which allows the creation of potent therapeutics by inducing antibody hexamer formation (clusters of six antibodies). The HexaBody® platform builds on natural antibody biology and enhances complement-mediated cell killing, allowing antibodies with limited or absent CDC to be transformed into potent cytotoxic antibodies.

Another brilliant example of enhanced therapeutic activity for antibody through pharmacogenetic approach is represented by rituximab. Some clinical evidence based on genetic analysis of leukocyte receptor polymorphisms of cancer patients treated with anti-CD20 IgG1 rituximab has revealed that ADCC is one of the critical mechanisms responsible for the clinical efficacy of these therapeutic antibodies. Indeed, patients with FcγRs polymorphism exhibit higher objective response rates than otherwise similar patients lacking this polymorphism (Cartron, Dacheux et al. 2002, Weng and Levy 2003). An alternative strategy to enhance ADCC by mAb is to engineer production cell lines to tune the Fc glycosylation (Pablo Umaña; Glycart, Schlieren-Zürich, Switzerland). This research line has led to the approval of Gazyva® by Glycart/Roche/Genentech, indicated to treat patients with previously untreated chronic lymphocytic leukemia. Gazyva® is the first glycoengineered, type 2 anti-CD20 humanized mAb on the market and it is also the first drug with breakthrough therapy designation to received FDA approval. In line with these observations, also modulation of the fucosylation rate on therapeutic antibodies have been investigated: apparently IgG molecules fully lacking the core fucose of the Fc oligosaccharides have been found to exhibit much higher ADCC in humans than their fucosylated counterparts (Weng and Levy 2003).

2.1.3. Antibodies for therapeutic applications: an overview of clinically approved monoclonal antibodies

Since the approval of the first monoclonal antibody by the U.S. Food and Drug Administration (FDA) in 1986 (Smith 1996), 38 antibody products have received marketing
authorization for therapeutic applications [www.antibodysociety.org/news/approvedmabs.php]. It is believed that nearly 300 antibody products are currently in clinical development (Norman 2013). Sales for therapeutic antibodies were estimated to reach $24.6 billions in 2012 (Aggarwal 2014). Some of the most important antibody products are described below. For the complete list of approved monoclonal antibodies see table 2.1.

Rituximab (trade names Rituxan®, MabThera® and Zytux®, Biogen Idec/Genentech) became in 1997 the first monoclonal antibody to receive marketing authorization for oncological indications. The product was initially approved for the treatment of low grade B-cell lymphomas. Subsequently, it received marketing authorization for various oncological and non-oncological indications, including Chronic Lymphocytic Leukemia (CLL), Rheumatoid Arthritis (RA) in combination with methotrexate (in adult patients with moderately-to severely-active RA who have inadequate response to one or more TNF antagonist therapies), Granulomatosis with Polyangiitis (GPA) (Wegener’s Granulomatosis) and Microscopic Polyangiitis (MPA) with glucocorticoids [www.rituxan.com].

Adalimumab is currently the best-selling pharmaceutical product in the world, with sales in 2012 of $4.6 billions (Aggarwal 2014). This product, commercialized under the trade name Humira® (acronym of “human monoclonal antibody in rheumatoid arthritis) is a TNF-blocker, which has received marketing authorization for the treatment of many inflammatory conditions, including rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis, Crohn’s disease, ulcerative colitis, moderate to severe chronic psoriasis and juvenile idiopathic arthritis [www.humira.com]. Importantly, Adalimumab was the first phage-derived antibody to be approved for pharmaceutical applications.

Bevacizumab (trade name Avastin®, Genentech/Roche) is a humanized IgG1 that inhibits angiogenesis by blocking vascular endothelial growth factor A (VEGF-A).
Bevacizumab was initially approved in 2004 by the U.S. Food and Drug Administration (FDA) for the treatment of metastatic colon cancer in combination with chemotherapy (Los, Roodhart et al. 2007). Subsequently, the product has been approved for the treatment of certain forms of lung cancer, renal cancer, ovarian cancer, breast cancer and glioblastoma multiforme (www.avastin.com). However, the product rarely induces objective responses when used as single agent (Jubb and Harris 2010, Gilbert, Dignam et al. 2014) and its relatively modest therapeutic activity has been the cause of intense debate, which has also led to the lost of approval for breast cancer (Alberto J. Montero 2012). By contrast, VEGF-A appears to be a crucial contributor of other non-oncological disorders, such as angiogenesis-related conditions which may lead to blindness. Indeed, an affinity-matured Fab fragment derived from Avastin® (Lucentis®) has received marketing authorization for diabetic macular edema (Schliemann, Wiedmer et al.), an eye condition in people with diabetes that causes blurred vision, severe vision loss and sometimes blindness, neovascular (wet) age-related macular degeneration (wAMD), macular edema following retinal vein occlusion (RVO) (www.lucentis.com). Interestingly, Avastin® and Lucentis® appear to have a comparable therapeutic activity in this setting, as evidenced by comparative phase III clinical trial (ClinicalTrials.gov identifier NCT00593450) sponsored by the National Eye Institute (Sanchez-Pernaute, Filkova et al.) (Rosenfeld 2006).

Trastuzumab (trade name Herclon/Herceptin®, Genentech) is a humanized IgG1 antibody that binds to domain IV of the extracellular segment of the HER2/neu receptor (Cho, Mason et al. 2003), which is overexpressed on 20 – 30% of breast cancers. Trastuzumab received in 1998 FDA approval for use in women with metastatic breast cancer who have tumors that overexpress the HER2 protein, on the basis of the results of a phase III randomized clinical trial in 496 women (H0648g), which showed median overall survival of 25.1 months vs. 20.3 months and median time to disease progression of 7.4
months vs. 4.6 months compared with chemotherapy alone (Slamon, Leyland-Jones et al. 2001). The drug is indicated for treatment of patients both as first-line therapy in combination with paclitaxel chemotherapy and as a single agent for those who have received one or more chemotherapy regimens.

Herceptin® was the first therapeutic antibody targeted to a specific (HER2) cancer-related molecular marker to receive FDA approval. Subsequently, the product in combination with cisplatin and capecitabine or 5-fluorouracil, gained marketing authorization for the treatment of patients with HER2 overexpressing metastatic gastric or gastroesophageal junction adenocarcinoma, who have not received prior treatment for metastatic disease (www.herceptin.com). As only approximately 25% of patients with metastatic breast cancer are HER2-positive, patients must have a HER2 test to determine if their breast or stomach cancer is HER2 positive before using Herceptin.

Two are the methodologies employed: the HER2 fluorescence in-situ Hybridization (Richard G Margolese) and the HER2 Immunohistochemistry (IHC). There are distinct differences between stomach and breast cancers. The HER2 testing process (such as scoring and interpretation of results) is different for stomach and breast cancers. Though similar testing methods are used, improper testing procedures may impact the determination of HER2 status and subsequent treatment.

Side effects, as for other monoclonal antibodies, are some infusion-related toxicities like fever, rigors, chills, nausea and hypotension which are distinctive of the first infusion, but which are typically not seen in subsequent infusions.
<table>
<thead>
<tr>
<th>International non-proprietary name</th>
<th>Trade name</th>
<th>Type</th>
<th>Indication first approved</th>
<th>First EU (US) approval year</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muromonab-CD3</td>
<td>Orthoclone</td>
<td>Anti-CD3; Murine IgG2a</td>
<td>Reversal of kidney transplant rejection</td>
<td>1986* (1986#)</td>
</tr>
<tr>
<td>Abciximab</td>
<td>Reopro</td>
<td>Anti-GPIIb/IIIa; Chimeric IgG1 Fab</td>
<td>Prevention of blood clots in angioplasty</td>
<td>1995* (1994)</td>
</tr>
<tr>
<td>Rituximab</td>
<td>MabThera, Rituxan</td>
<td>Anti-CD20; Chimeric IgG1</td>
<td>Non-Hodgkin’s lymphoma</td>
<td>1998 (1997)</td>
</tr>
<tr>
<td>Daclizumab</td>
<td>Zenapax</td>
<td>Anti-IL2R; Humanized IgG1</td>
<td>Prevention of kidney transplant rejection</td>
<td>1999 (1997); #</td>
</tr>
<tr>
<td>Palivizumab</td>
<td>Synagis</td>
<td>Anti-RSV; Humanized IgG1</td>
<td>Prevention of respiratory syncytial virus infection</td>
<td>1999 (1999)</td>
</tr>
<tr>
<td>Infliximab</td>
<td>Remicade</td>
<td>Anti-TNF; Chimeric IgG1</td>
<td>Crohn disease</td>
<td>1999 (1998)</td>
</tr>
<tr>
<td>Trastuzumab</td>
<td>Herceptin</td>
<td>Anti-HER2; Humanized IgG1</td>
<td>Breast cancer</td>
<td>2000 (1998)</td>
</tr>
<tr>
<td>Gemtuzumab ogzomicin</td>
<td>Mylotarg</td>
<td>Anti-CD33; Humanized IgG4</td>
<td>Acute myeloid leukemia</td>
<td>NA (2000#)</td>
</tr>
<tr>
<td>Alemtuzumab</td>
<td>MabCampath, Campath-1H</td>
<td>Anti-TNF; Human IgG1</td>
<td>Rheumatoid arthritis</td>
<td>2001 (2001)</td>
</tr>
<tr>
<td>Adalimumab</td>
<td>Humira</td>
<td>Anti-TNF; Human IgG1</td>
<td>Rheumatoid arthritis</td>
<td>2003 (2002)</td>
</tr>
<tr>
<td>Tositumomab-I131</td>
<td>Bexxar</td>
<td>Anti-CD20; Murine IgG2a</td>
<td>Non-Hodgkin lymphoma</td>
<td>NA (2003)</td>
</tr>
<tr>
<td>Eflizumab</td>
<td>Rapliba</td>
<td>Anti-CD11a; Humanized IgG1</td>
<td>Psoriasis</td>
<td>2004 (2003); #</td>
</tr>
<tr>
<td>Cetuximab</td>
<td>Erbitux</td>
<td>Anti-EGFR; Chimeric IgG1</td>
<td>Colorectal cancer</td>
<td>2004 (2004)</td>
</tr>
<tr>
<td>Omalizumab</td>
<td>Xolair</td>
<td>Anti-IgE; Humanized IgG1</td>
<td>Asthma</td>
<td>2005 (2003)</td>
</tr>
<tr>
<td>Ranibizumab</td>
<td>Lucentis</td>
<td>Anti-VEGF; Humanized IgG1 Fab</td>
<td>Macular degeneration</td>
<td>2007 (2006)</td>
</tr>
<tr>
<td>Certolizumab pegol</td>
<td>Cimzia</td>
<td>Anti-TNF; Humanized Fab, pegylated</td>
<td>Crohn disease</td>
<td>2009 (2008)</td>
</tr>
<tr>
<td>Golimumab</td>
<td>Simponi</td>
<td>Anti-TNF; Human IgG1</td>
<td>Rheumatoid and psoriatic arthritis, ankylosing spondylitis</td>
<td>2009 (2009)</td>
</tr>
<tr>
<td>Canakinumab</td>
<td>Ilaris</td>
<td>Anti-I1b; Human IgG1</td>
<td>Muckle-Wells syndrome</td>
<td>2009 (2009)</td>
</tr>
<tr>
<td>Catumaxomab</td>
<td>Removab</td>
<td>Anti-EPCAM/CD3/Rat/mouse bispecific mAb</td>
<td>Malignant ascites</td>
<td>2009 (NA)</td>
</tr>
<tr>
<td>Ustekinumab</td>
<td>Stelara</td>
<td>Anti-IL12/23; Human IgG1</td>
<td>Psoriasis</td>
<td>2009 (2009)</td>
</tr>
<tr>
<td>Tocilizumab</td>
<td>RoActemra, Actemra</td>
<td>Anti-IL6R; Humanized IgG1</td>
<td>Rheumatoid arthritis</td>
<td>2009 (2010)</td>
</tr>
<tr>
<td>Ofatumumab</td>
<td>Arzerra</td>
<td>Anti-CD20; Human IgG1</td>
<td>Chronic lymphocytic leukemia</td>
<td>2010 (2009)</td>
</tr>
<tr>
<td>Belimumab</td>
<td>Benlysta</td>
<td>Anti-BLyS; Human IgG1</td>
<td>Systemic lupus erythematosus</td>
<td>2011 (2011)</td>
</tr>
<tr>
<td>Ilipilumab</td>
<td>Yervoy</td>
<td>Anti-CTLA-4; Human IgG1</td>
<td>Metastatic melanoma</td>
<td>2011 (2011)</td>
</tr>
<tr>
<td>Brentuximab vedolitin</td>
<td>Adcetris</td>
<td>Anti-CD30; Chimeric IgG1; immunoconjugate</td>
<td>Hodgkin lymphoma</td>
<td>2012 (2011)</td>
</tr>
<tr>
<td>Pertuzumab</td>
<td>Perjeta</td>
<td>Anti-HER2; Humanized IgG1</td>
<td>Breast Cancer</td>
<td>2013 (2012)</td>
</tr>
<tr>
<td>Raxibacumab (Pending)</td>
<td>Anti-B. anthrasis PA; Human IgG1</td>
<td>Breast cancer</td>
<td>2013 (2013)</td>
<td></td>
</tr>
<tr>
<td>Trastuzumab emtalansine</td>
<td>Kadcyla</td>
<td>Anti-HER2; Humanized IgG1; immunoconjugate</td>
<td>Breast cancer</td>
<td>2013 (2013)</td>
</tr>
<tr>
<td>Vedolizumab</td>
<td>Entyvio</td>
<td>Anti-alpha4beta7 integrin; Humanized IgG1</td>
<td>Ulcerative colitis, Crohn disease</td>
<td>2014 (2014)</td>
</tr>
</tbody>
</table>

*Country-specific approval; approved under concertation procedure; #Voluntarily withdrawn from market.

BlyS, B lymphocyte stimulator; C5, complement 5; CD, cluster of differentiation; CTLa-4, cytotoxic T lymphocyte antigen 4; EGFR, epidermal growth factor receptor; EPCAM, epithelial cell adhesion molecule; GP, glycoprotein; IL, interleukin; NA, not approved; PA, protective antigen; RANK-L, receptor activator of NFkb ligand; RSV, respiratory syncytial virus; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

Table 2.1. Therapeutic monoclonal antibodies approved in the European Union or United States. (Table adapted from “the antibody society” source).
2.1.4. Armed antibody products

For a number of indications, it may be attractive to use antibodies as "delivery vehicles" for suitable bioactive payloads, such as cytokines, growth factors, drugs and radionuclides. Paul Ehrlich, a German-Jewish physician who received the Nobel Prize in Physiology or Medicine in 1908, first postulated the idea of developing pharmaceutical agents as "magic bullets", which would selectively target the diseased cells, while sparing normal tissues. Ehrlich hypothesized that: "if a compound could be made that selectively targeted a disease-causing organism, then a toxin for that organism could be delivered along with the agent of selectivity. Hence, a "magic bullet" would be created that killed only the organism targeted." About one century after its original formulation, the concept of a "magic bullet" has become a pharmaceutical reality, with the approval of therapeutic antibody derivatives.

Most armed antibodies are currently in clinical development for oncological applications, but an increasing number of products is being considered for the treatment of chronic inflammatory conditions, such as rheumatoid arthritis and inflammatory bowel diseases. The concept of using armed antibodies for pharmaceutical applications relies on the ability of the immunoglobulin molecule to preferentially localize at the site of disease and to delivery suitable therapeutic payloads. In addition to cytotoxic drugs, cytokines and radionuclides, more "exotic" bioactive molecules can be considered (e.g., antibodies modified with point mutations and/or glycosilations, biotin-chelator-radionuclides, sterically stabilized immunoliposomes) or even a second antibody moiety, yielding bispecific products (Carter 2001) (Hess, Venetz et al. 2014).

Armed antibody products can be divided into two main categories. In the first one, the antibody is coupled to a payload (e.g., a drug or a radionuclide), capable of inferring direct damage to the target cell. In the second approach, a bispecific antibody product...
recruits leukocytes at the site of disease, which are ultimately responsible for the observed therapeutic effect.

Beyond these two families, it is worthy to mention an additional type of construct in which the antibody, in the format of a ScFv, is fused to an enzyme which convert the non-toxic prodrug into a potent cytotoxic drug only in the close proximity of the targeted cells. The antibody-directed enzyme prodrug therapy (ADEPT) is a brilliant example of antibody fusion with effector molecules (Carter 2001).

**Figure 2.5. Different types of armed antibodies (Adapted from Carter 2001)** Direct arming of antibodies entails their covalent linkage to killing machinery, such as radionuclides, toxins or small molecules. Alternatively, arming antibodies with cytokines is intended to create high intratumour concentrations of cytokines to stimulate the antitumour immune response (T cells, B cells or natural killer cells). Indirect arming of antibodies can be achieved by bispecific antibodies that bind to two different antigens: one on the tumor cell-surface and a second on an effector molecule. As example of pre-targeting strategy, an antibody-fragment–enzyme fusion protein (ADEPT) is depicted. The prodrug is administered and ideally converted to an active drug solely within the tumour.
2.1.5. Antibody-drug conjugates (ADC) for cancer therapy

An antibody-drug conjugate consists of three building blocks: the immunoglobulin moiety, which serves for the selective in vivo pharmacodelivery, the bioactive payloads, such as potent cytotoxic agents and the system of connection between the two structures, termed linker.

![Figure 2.6. Schematic representation of an antibody drug conjugate (ADC).](image)

ADC products may consist of antibodies in different formats. Furthermore, various types of linker-payload constructions can be considered.

First of all, the choice of the target antigen is of great importance for the development of an ADC. It is commonly believed, that the antibody internalization into the target cell, may represent an essential requirement for the efficacy of the compound (Chari 2008, Sievers and Senter 2013). After binding the target antigen, the ADC-antigen complex is typically internalized and transported to intracellular organelles where release of the attached drug can occur. Upon release, the cytotoxic drug can interfere with various cellular mechanisms, leading to cell death. However, recent work on an ADC that targets fibronectin in the tumor microenvironment, suggests that the internalization step might not be necessary (Bernardes, Casi et al. 2012, Perrino, Steiner et al. 2014).

Considering the three major components on an ADC, an ideal antibody has to exhibit high affinity and clear specificity for its target, in order to be efficiently transported at the tumor site and exploit its functions. (Pharmacokinetic properties of different antibody formats have been discussed in section 2.1.2). Subsequently, also the choice
of the linker plays an important role in the performance of an ADC. In fact, the connection between the antibody molecule and the drug has to be sufficiently stable in plasma in order to remain inactive while in circulation, preventing the release of cytotoxic agents prematurely and assuring the delivery of as much compound as possible at the neoplastic site. At the same time, the linker needs to allow rapid and efficient release of the cytotoxic drug in an active form inside the tumor cells (Noting 2013). Cleavable and noncleavable linkers can be used since both types have been proven to be safe in preclinical and clinical trials. Last but not least, also the choice of the drug marks represents an important point of the generation of an efficacious ADC. Nowadays, the field is focus on molecules with higher and higher potency, some of them interfere with the polymerization of the tubulin of the cell, causing the cell cycle arrest and subsequent apoptosis (i.e. auristatins, maytansinoids and cemadotin derivatives) (Jordan, Walker et al. 1998, Oroudjev, Lopus et al. 2010), some other are alkylating agents, they bind the DNA minor groove causing a permanent damage to the cell’s DNA, resulting in its final death (i.e. duocarmycin and calicheamicin) (Hinman, Hamann et al. 1993, Tercel, McManaway et al. 2013).

All three components (antibody, linker and payload) have a substantial influence on the performance of ADC products. For example, the number of drug molecules per antibody and their conjugation site may have a considerable impact on the ADC performance. For example, statistical drug coupling on surface-exposed lysine residues may result in variable antibody to drug ratios (“batch-to-batch variability”) and may influence pharmacokinetic properties (Chari 2008, Casi and Neri 2012, Sievers and Senter 2013). For these reasons, intense research efforts have been devoted to the development of chemically-defined, site-specific antibody-drug coupling strategies. In our laboratory, we have successfully used the mild reduction of the C-terminal disulfide bond of
antibodies in SIP format, generating two cysteine residues which can be chemically modified (Bernardes, Casi et al. 2012, Casi, Huguenin-Dezot et al. 2012).

Three ADC products have received marketing authorization: Kadcyla®, Adcetris® and Mylotarg® (this last product was voluntarily withdrawn from the market in 2010 in the U.S.A., while it was never approved in Europe). At least 30 ADCs are currently being investigated in clinical trials, accounting for approximately 15% of anticancer antibody-based pipeline in both hematological malignancies and solid tumor indications and outnumbering other forms of modified antibodies, such as bispecifics and immunocytokines. At least 17 ADC products entered the clinic in 2011 and 2012, which represents an increase compare to the 8 products which were studied in 2009 and 2010 (Mullard 2013). Interestingly, companies tend to work on a very broad range of tumor-associated antigens, which results in a minimal overlap of their respective pipelines (Mullard 2013). Furthermore, some companies use the ADC strategy for “saving” antibody products, which had previously failed in the clinic, when used as intact antibodies. This was the case, for example, for Seattle Genetics development of anti-CD30 antibodies and for the subsequent development of Adcetris®.
2.2. Antibody phage display technology

2.2.1. Methods for the isolation of human monoclonal antibodies

Currently, the main strategies for the isolation of fully human antibodies include the application of hybridoma technology in transgenic mice carrying the human immunoglobulin locus, the generation of monoclonal antibodies from single human B cells by cloning Ig genes and the use of combinatorial antibody libraries with suitable display technologies (Yamashita, Katakura et al. 2007).

The production of monoclonal antibodies was first made possible by discoveries of César Milstein and Georges J.F.Köhler in 1975 (Köhler and Milstein 1975). The two scientists were awarded the Nobel Prize for Medicine and Physiology in 1984 for their breakthrough invention. The technology is called hybridoma technology and relies on the possibility of forming hybrid cell lines (called hybridomas) by immortalizing a specific antibody-producing spleen B cell obtained from immunized mice by fusion with cancerous myeloma cells. By cloning individual cell lines at limiting dilution (i.e., one cell per well), monoclonal antibodies have a single amino acid sequence and display a single binding specificity. Hybridoma technology is relatively cumbersome to implement, requires immunization of animals and is time-consuming. However, it is possible to use complex antigens (e.g., cells) for immunization procedures and the hybridoma products are intact antibody molecules, typically in IgG format.

At the beginning of the ‘90s, “display technologies” were introduced, with the aim to avoid immunization procedures and, at the same time, to yield human antibodies which are not immunogenic in patients. Display technologies include in phage display, yeast display, bacterial display and ribosome display. The first three methods require the use of live cells (e.g., for antibody production and amplification), whereas ribosome
technology is fully performed \textit{in vitro}, without the use of cells.

**Phage display technology** will be described in detail in section 2.2.2.

**Yeast display technology** was pioneered by Professor K. Dane Wittrup, who first described the successful display of antibody fragments on the yeast cells, by fusion to cell wall components \citep{BoderWittrup1997}. In detail, the protein of interest can be displayed on the surface of a yeast cells by the fusion with Aga2p protein, which is a natural yeast component involved in cell-cell contact formation. Yeast display offers some distinctive advantages as a platform for affinity reagent discovery and optimization. For example, antibody folding is typically more efficient than in bacteria and levels of antibody expression on cells can be quantified by fluorescent-activated cell sorting (FACS). Selections can be performed in solution, using fluorescently-labeled antigen and detecting antigen-binding cells by FACS procedures. Libraries tend to be smaller compared to other display methodologies, as it is more difficult to transform yeast cells compared to bacteria. However, the precise control of selection procedures and the implementation of competitive selection schemes may facilitate the isolation of ultra-high affinity antibodies.

**Ribosome display technology** was first described by Mattheakis \textit{et al.} \citep{Mattheakis1994,Mattheakis1997}. The technology relies on the formation of a stable complex between a folded protein and the corresponding mRNA molecule which codes for it, using the ribosome as anchor. The complex can be stabilized by suitable salt concentrations, by working at low temperatures and by omitting stop codons in the mRNA molecule \citep{HanesPluckthun1997}. The size of ribosome display libraries can be very large (e.g., $10^{12}$ clones), as it is not limited by the efficiency of transformation of bacterial cells. Indeed, library size is only determined by the number of ribosomes and different mRNA molecules used \textit{in vitro} transcription/translation procedures. After each round of selection, the recovered mRNA molecules can be back-translated into the corresponding DNA fragments. If desired, random mutations can be easily introduce, by performing RT-PCR procedures
in suitable experimental conditions. Ribosome display has proven to be particularly suitable for the affinity maturation of antibody scFv fragments. In one study, an antibody specific to fluorescein was evolved to reach a dissociation constant of \( \sim 100 \) pM (Jermutus, Honegger et al. 2001). 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 et al. 2004). However, the practical implementation of ribosome display may be hindered by the difficulty of folding disulfide-containing proteins in \textit{in vitro} procedures. Furthermore, the technology is limited to antigens, which can be isolated and managed in the absence of contaminating RNases.

### 2.2.2. Antibody phage display technology

Phage display is a powerful technique for the study of protein-protein interactions and for the isolation of novel binding specificities. The technology is based on the use of bacteriophage (i.e., bacterial viruses) which "display" heterologous proteins on their surface, by means of a fusion with the minor coat protein pIII. As the gene coding for the heterologous protein needs to be incorporated in the phage genome, a phage display particle provides a direct physical linkage between genotype (i.e., the gene coding for a protein) and the corresponding phenotype (i.e., the properties associated with the protein, displayed on phage) (figure 2.7). The technology was first described in 1985 in a \textit{Science} publication of G.P. Smith (Smith 1985), who described the efficient display of peptides, capable of binding to a cognate monoclonal antibody, on the surface of a non-lytic filamentous bacteriophage (fd). Few years later, thanks to the efforts of the groups of Sir Gregory Winter (McCafferty, Griffiths et al. 1990) and of Prof. Jim Wells (Lowman, Bass et al. 1991), filamentous phage were first used to allow the functional display of folded proteins (antibody fragments or hormones, respectively) on their surface. As a result of this innovation, it was possible for the first time to display libraries of protein mutants (e.g., antibody libraries) on filamentous phage, allowing the selection and
amplification of desired binding specificities out of large combinatorial libraries.

Figure 2.7. Schematic representation of a phage displaying a binding protein. In this case the protein displayed is a scFv antibody fragment as fusion protein of a minor coat protein pIII.

The most common bacteriophages used for phage display applications are the non-lytic M13 strain, the lytic T4 and T7 phages, as well as λ phage. M13 has a cylindrical shape, of approximately 900 nm in length and 6-7 nm in diameter (Hoffmann-Berling, Marvin et al. 1963). It contains a single-stranded DNA genome, which encodes 11 genes, five of which are coated proteins. The most abundant coat protein is pVIII, present in approximately 3000 copies, covering the entire surface of the bacteriophage. At one extremity, pIII and pVI are displayed, while at the other extremity pVII and pIX are present. Usually, antibody fragments are fused to the N-terminus of the minor coat protein pIII (Figure 2.8.). The phages which are able to undergo lysogeny are known as temperate phages. They differ from lytic phages, in which the infected bacterial cells are broken and destroyed immediately after virion replication. In the case of lysogenic phages, the viral genome integrates in the host genome or is transmitted episomally, allowing a replication that does not kill the infected cell. Filamentous phages are released by secretion. The most extensively coat protein used for phage display applications is pIII,
which is present in three to five copies per phage particle and is involved in bacterial
infection. Antibodies were the first proteins to be successfully displayed on the surface
of phage (McCafferty, Griffiths et al. 1990). This result was achieved by fusing genes coding for
scFv fragments after the leader peptide sequence and upstream of gene III. Specifically, scFv fragments are cloned in frame with gene III and downstream of the
gene III signal sequence, which directs the translocation of the corresponding protein
to the bacterial periplasm. The VH and VL domains have to be stabilized by an
intramolecular disulfide bridges in order to fold correctly and pair, thus forming a
functional scFv (Skerra and Pluckthun 1988). Originally, phage vectors were used (McCafferty, Griffiths et
al. 1990), which carry all the genetic information required for the phage life cycle, plus a
suitable antibiotic resistance gene. Nowadays, however, phagemid vectors are more
commonly used, as they allow the construction of larger combinatorial libraries.
Phagemids are small plasmids that contain two additional genetic elements: the gene
III with suitable cloning sites and a phage packaging signal (Hoogenboom, Griffiths et al. 1991). The
lac Z promoter is frequently used to drive the expression of antibody-pIII fusion proteins
(Barbas, Kang et al. 1991). In order to regulate the activity of the lac Z promoter, its catabolic
repressor (glucose) is removed or depleted, leading to protein expression. Phagemid
vectors typically yield ‘monovalent’ phage particles, as they require the use of helper
phage (such as M13KO7 or VCS-M13) for phage particle productions, which may
incorporate pIII derived from the phagemid itself or from the helper phage. Usually,
helper phages have a slightly defective origin of replication (Vieira and Messing 1987), which
allows the preferential packaging of phagemid DNA over helper phage. When
considering the life cycle of a bacteriophage, it is possible to identify four main stages:
the infection of a bacterial cell, the replication of the viral genome inside the cell, the
assembly of new viral particles and eventually the release of multiple phage particles
from the host.
All the genetic information necessary for the phage life cycle is contained in the phage
vectors. The infection is mediated by the bacterial structure known as the “F pilus” which allows the contact between the plIII protein on the M13 phage and the TolA protein on the *E. coli* surface. Following this interaction, the phage genome is then transferred to the cytoplasm of the bacterial cell, where resident proteins convert the single stranded DNA genome into a double-stranded replicative form (“RF”). This DNA is used as a template for expression of the phage genes. Once inside the bacterial cell, the filamentous phage can initiate its reproductive cycle. The host cell will continue to survive and the virus will be produced in all of the cell’s offsprings. The secretion phase is the last step of the process, in which all coat proteins assemble, giving rise to a complete phage structure. The genome is packaged in single-stranded DNA form into a rod-shaped particle. All virion proteins, before assembly and extrusion, are transported to the cell periplasm.

![Schematic representation of M13 Bacteriophage](image)

**Figure 2.8. Schematic representation of M13 Bacteriophage.** In the legend are described the essential proteins.

A the key feature of antibody phage display technology consists in the implementation of “biopanning” technologies, in which phage libraries are incubated with a target
protein of interest, immobilized on a solid support. After a binding interaction has taken place, irrelevant phage particles devoid of specific binding activity are washed away, while those phages which remain attached to the antigen can be selectively eluted (e.g., at high pH values) and amplified by infection in bacteria (figure 2.9). As a result, a new phage population, enriched for antigen binders, can be generated and submitted to subsequent rounds of panning.

The binding interaction of antibodies with their cognate antigens may be mediated by electrostatic interactions, Van der Waals forces and hydrophobic interactions (Janeway Charles A 2001). Typically, a library containing > 10^9 antibody variants is biopanned against the antigen of interest, coated on a plastic surface (e.g., Petri dishes, microtiter plate well, immunotubes) or on magnetic beads. A good-quality library contains useful binding specificities against a broad range of diverse antigens (Winter, Griffiths et al. 1994). As passive adsorption of proteins on plastic may lead to partial denaturation and to the formation of non-functional products (Butler, Ni et al. 1992, Davies, Dawkes et al. 1994), alternative methods have been explored, in order to preserve the native conformation of the target antigen. The most broadly used biopanning method relies on the capture of biotinylated antigens on streptavidin-coated supports. The methodology allows a high density and uniform coating on a solid support. Moreover, lower amounts of antigen are needed, compared to the rather inefficient direct antigen coating on plastic (Butler, Ni et al. 1992, Davies, Dawkes et al. 1994). The use of paramagnetic streptavidin-coated microbeads allows a more precise control of the antigen concentration an efficient capture on magnetic supports, facilitating washing steps and antibody recovery (Hawkins, Russell et al. 1992, Schier, Bye et al. 1996).

Several washing steps are recommended, often in the presence of detergents, in order to reduce the recovery of non-selective phage particles. Phage particles are very stable, even after treatment with acids, bases or enzymes. They have the ability to remain infectious in a broad variety of selective elution protocols. Filamentous phages which are recovered at the end of biopanning procedures can infect E.coli, producing
additional phage particles. The cycle of panning can occur many times, thus facilitating the preferential enrichment of specific binders to the cognate target of interest. Usually, three or four rounds of panning are sufficient to select highly specific antibody clones. The resulting (polyclonal) phage pools (i.e., a mixture of all phages that bind to the antigen of choice) are tested by phage ELISA. After ELISA screening, selected candidates can be produced in large scale in *E.Coli* and used for further characterization. The large scale production of selected antibody fragments is possible because these proteins can be efficiently secreted into the bacterial periplasm and culture medium (Skerra and Pluckthun 1988).

**Figure 2.9. Selection Cycle.** 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 bacterial cultures after infection of *E.coli* cells and can be used for further rounds of selection.
2.2.3. Antibody phage display libraries

Phage display libraries of recombinant antibodies can be regarded as an artificial immune system, that reliably yields specific monoclonal antibody fragments in 1-2 weeks of experimental work, provided that a small amount of pure antigen is available (Winter, Griffiths et al. 1994). It is therefore possible to obtain specific recombinant antibodies against both foreign and self-antigens without immunization, starting from a panel of billions of different antibody clones.

In naturally occurring immunoglobulins, most of the amino acid diversity is concentrated in CDR3 regions. As a consequence, most artificial antibody phage display libraries (including those used in our group) feature a combinatorial mutagenesis of CDR3 positions (see section 2.1.1 and figure 2.3). There are different strategies for the construction of combinatorial antibody libraries. For example, families of VL and VH fragments can be PCR-amplified from B lymphocytes. Alternatively, synthetic libraries can be constructed, which contain combinatorial diversity at precisely defined amino acid positions. Based on the type of antibody genes which are used for phage library construction, human (Marks, Hoogenboom et al. 1991, Tomlinson, Walter et al. 1992), murine (Orlandi, Gussow et al. 1989, Clackson, Hoogenboom et al. 1991, Orum, Andersen et al. 1993, Kettleborough, Ansell et al. 1994), rabbit (Lang, Barbas et al. 1996) and chicken (Davies, Smith et al. 1995) antibody libraries have been described. Libraries can be “antigen-biased” if they derive from an immunized donor, or from an organism which has undergone negative selection. By contrast, synthetic libraries are typically not biased for certain antigens and are particularly useful for the isolation of binding specificities against conserved antigens (e.g., self antigens).

Immune libraries may be particularly useful if the donor has been exposed to certain relevant diseases. For example, immune libraries derived from cancer patients may favor the isolation of antibodies against tumor-associated antigens (Cai and Garen 1995). In most cases, however, synthetic libraries are preferable, as
they avoid the need for immunization and as they may allow the isolation of a broad range of useful binding specificities. One often refers to antigen-unbiased libraries as "single-pot libraries". The corresponding phages can be frozen in aliquots and used in biopanning experiments as needed (Neri, Pini et al. 1998). Highly specific monoclonal antibodies can be isolated in 2-4 rounds of panning, which correspond to 5-9 days of work. Preferably, very large combinatorial libraries (containing over $10^{10}$ clones) should be used. In practice, library performance is influenced by many factors, including library design, the choice of germline V-genes used, as well as methods employed for the functional display of antibodies on phage (Hoogenboom and Winter 1992). In this thesis, I used and/or developed the following large combinatorial libraries: ETH2-Gold library (3 billion antibody clones) (Silacci, Brack et al. 2005), Philo library (3 billion antibody clones) (Villa, Lovato et al. 2011), Philo Diamond library (40 billion antibody clones) (Weber, Bujak et al. 2014) and NITRO library (13 billion antibody clones) (Putelli, Kiefer et al. 2014). All four synthetic human antibodies libraries are based on antibodies in scFv format. This format exhibits higher expression yields and better phage display properties, compared to Fab fragments, which are larger and require the formation of a heterodimeric structure. ScFv’s are directly fused with the minor coat protein pIII, by cloning theis genes into the phagemid vector pHEN1 (Hoogenboom, Griffiths et al. 1991), which also appends a myc tag at the C-terminal extremity of the antibody molecule. All libraries mentioned above are based on the DP47 VH germline gene, while DPK22 and DPL16, respectively, were used as germline segments for the introduction of sequence variability in Vk and VL. VH and VL domains are connected via a flexible 14-amino acids linker, containing the Gly4SerGly4SerGly4 sequence (Huston, Levinson et al. 1988). The use of DP47 as VH segment offers advantages in terms of higher thermodynamic stability (Ewert, Huber et al. 2003) and protein A binding, which facilitates affinity purification procedures (Hoogenboom and Winter 1992). Sequence diversity is mainly confined to the CDR3 regions of both VH and VL, but diversity in CDR1 and
CDR2 loops can be easily introduced, by creating affinity-maturation libraries using combinatorial mutagenesis procedures with partially-degenerate primers (Brack, Silacci et al. 2006, Silacci, Brack et al. 2006, Villa, Trachsel et al. 2008).
2.3. Cancer

Cancer is a major public health problem in all parts of the world. In developed countries, cancer is one of the leading causes of death, second only to cardiovascular diseases (Siegel, Naishadham et al. 2012).

The term "cancer" describes a broad range of disorders characterized by uncontrolled cell growth, caused by a continuous accumulation of genetic mutations inside a cell population. Indeed, tumorigenesis is a multistep process, in which normal cells progressively acquire a malignant phenotype. Cancer cells share six essential pathologic changes in cell physiology, which collectively contribute to malignant growth: growth signals self-production, insensitivity to growth-inhibitory signals, evasion of programmed cell death (apoptosis), uncontrolled replicative capability, continued angiogenesis, and tissue invasion ability (metastasis formation) (Hanahan and Weinberg 2000). In addition, the ability of tumors to suppress the immune surveillance and to rely on an instructive extracellular environment are crucially important contributors for the disease (Hanahan and Weinberg 2011).

There are more than 100 distinct types of cancer, and a huge number of tumor-subtypes can be identified within specific organs. Classification of neoplastic lesions is based on the cell features to which tumor cells resemble, thus hinting at a possible origin for the malignancy. This classification includes cancers derived from epithelial cells (carcinoma), cancers arising from connective tissue (sarcoma), cancer arising from hematopoietic (blood-forming) cells (lymphoma and leukemia) and cancers derived from immature "precursor" cells or embryonic tissue (blastoma).

Concerning the epidemiology of the disease, it is recognized that the great majority of cancers, probably accounting for 90–95% of the cases, are due to environmental factors. The remaining 5–10% of neoplasms are due to inherited genetic features (Anand, Kunnumakkara et al. 2008).
2.3.1. Angiogenesis and vascular tumor targeting

Growth of solid tumors is always accompanied by neovascularization. The formation of new vessels is indispensable for the growth of neoplastic masses. At the same time, if neovascularization process is inhibited, most solid tumors stop growing (Folkman 1971). The new blood vessels are created in response to hypoxic environment and they facilitate malignant progression, by providing a supply of oxygen and nutrients (Folkman, Watson et al. 1989) and by facilitating a metastatic spread. Alternatively, some tumor cell preferentially use lymphatic vessels, in order to colonize distant sites in the body. Groups of metastatic cells may rest or, more often, may rapidly grow, forming clinically detectable lesions.

Angiogenesis is a common feature shared by virtually all aggressive cancer types. The selective targeting of antigens, located on newly-formed blood vessels, may represent an efficient strategy for the pharmacodelivery of bioactive payloads to various types of malignancies (Neri and Bicknell 2005). Angiogenesis plays a major role not only in cancer but also in inflammation-associated pathologies, like rheumatoid arthritis. Indeed, the entire process of angiogenesis resembles the outgrowth of capillary sprouts observed in fresh wounds or inflammation (Dvorak 1986).

Tumor blood vessels differ from normal vessels in terms of their architecture and properties. In particular, they grow in an unorganized fashion, they are bigger and more leaky. In addition, tumor blood vessels present an irregular shape, leading to an inefficient and irregular blood flow. As result of this anomalous architecture, the efficacy of anticancer treatments is frequently impaired by an insufficient delivery of cytotoxic agents to the neoplastic mass.
There are two fundamentally different approaches, which may interfere with tumor angiogenesis in vivo. On one hand, we may try to interfere with the growth of new blood vessels by inhibiting pro-angiogenesis factors. Alternatively, one may use selective ligands (in most cases, monoclonal antibodies) for the delivery of bioactive payloads at the site of disease. The first strategy is called “inhibition of angiogenesis”, while the second strategy is generally referred to as “vascular targeting”. Both therapy approaches may circumvent tumor resistance, since they target the normal endothelial cells, that are genetically more stable and less prone to mutation, compared to tumor cells (Boehm, Folkman et al. 1997).

Bavacizumab, a monoclonal antibody capable of blocking the action of various VEGF-A isoforms, is one of the most broadly used inhibitors of angiogenesis, which is approved for the treatment of a variety of certain types of malignancies (e.g., colorectal cancer, ovarian cancer, glioblastoma multiforme), frequently in combination with chemotherapy (see paragraph 2.1.3).

Instead, antibody-based vascular tumor targeting exploits the structural differences of the tumor vasculature compared to normal blood vessels. One could consider
delivering agents, that induce the collapse of pre-existing vessels, thus depriving the tumor of oxygen and nutrients (Neri and Bicknell 2005). Alternatively, antibodies could be used for a selective pharmacodelivery of immunomodulatory agents (e.g., pro-inflammatory cytokines) at the site of disease. Several markers associated with the neovasculature, but not present in normal blood vessels and healthy tissue, have been identified. In particular, part of my work focuses on alternatively-spliced domains of fibronectin, especially the EDA domain, which will be considered in the appendix, paragraph 8.3.1.
2.4. Rheumatoid arthritis

Rheumatoid arthritis (RA) is an autoimmune disease that causes chronic inflammation of the joints and other areas of the body. Indeed, RA is classified as an inflammatory polyarthritis because it involves many joints (affecting in particular the small joints in hands and feet, but virtually all of them can be involved). Almost 1% of the adult population in the industrialized world is affected by the disorder, with an onset typically between 30 and 50 years of age and an incidence of two to three times higher frequency in women than in men. RA leads to significant disability and a consequent reduction in life quality.

Unfortunately, even after years of extensive research in the field, the etiology of this serious illness still remains unknown, even if it is believed that it may result from a combination of genetic and environmental triggers. Nevertheless, some factors associated with an increased risk of RA development have been identified. Gender (women are more likely to develop rheumatoid arthritis than men are), age (rheumatoid arthritis can occur at any age, but it most commonly begins between the ages of 40 and 60), as well as family history and cigarettes smoke have been shown to act as predisposing factors.

Rheumatoid arthritis is an autoimmune disease. This means that the immune system attacks the body’s own tissues, in this specific case the synovium. Synovial membrane (also known as synovium or stratum synoviale) is the soft tissue found between the articular capsule (joint capsule) and the joint cavity of synovial joints. This thin membrane provides something like an inner tube, sealing the synovial fluid from the surrounding tissue. In normal conditions (as depicted on the left panel of figure 2.12),
this line is thin, whereas in pathological conditions (right panel of the figure 2.12) it is extremely swollen, thickened and irritated.

When an autoimmune reaction takes place, fluid builds up in the joints, causing pain and inflammation. Many cell populations are present in this fluid, including T cells, B cells, monocytes/macrophages, mast cells, dendritic cells and fibroblasts. T cells activate monocytes, macrophages and synovial fibroblasts, which then overproduce pro-inflammatory cytokines, mainly TNF, IL-1β and IL-6 \(^{(\text{Smolen and Steiner 2003})}\). In case of strong inflammation, an excess of synovial fluid weeping from inflamed synovium can provide a barrier to the diffusion of nutrients to the cartilage. The synovial cells may also use up nutrients so that the glucose level in the tissue is almost zero, inducing the destruction of the cartilage tissue and bone erosion.

![Figure 2.11. Comparison of a normal joint and rheumatoid arthritis joint.](image)

**Figure 2.11.** Comparison of a normal joint and rheumatoid arthritis joint. Left) In the healthy joint the thin synovial membrane lines the non-weight-bearing aspects of the joint. Right) In rheumatoid arthritis the synovial membrane becomes hyperplastic and infiltrated by chronic inflammatory cells. (Adapted from Proboost ®DIRECT ).
There is no cure for rheumatoid arthritis, but treatment can help reduce inflammation in the joints, relieve pain, prevent or slow joint damage and reduce disability. There are a number of medications available that can be used to help stop rheumatoid arthritis getting worse and reduce risk of further problems. These are often divided into two types of medication: “disease-modifying anti-rheumatic drugs (DMARDs)” and “biological treatments”.

DMARD drugs are particularly effective in easing symptoms of the condition and slowing down its progression. There are many different DMARDs that can be used, including methotrexate, leflunomide, hydroxychloroquine and sulfasalazine. Methotrexate is an antimetabolite and an antifolate drug, which acts by inhibiting the metabolism of folic acid. It is normally the first choice for the treatment of rheumatoid arthritis, often alongside another DMARD and a short-course of corticosteroids to relieve pain. It may also be combined with the biological treatments mentioned below.

Biological treatments represent a newer form of treatment for rheumatoid arthritis. They include chimeric monoclonal antibodies [infliximab (Remicade®, anti-TNF) and rituximab (Rituxan®, anti-CD20, see paragraph 2.1.3)], some humanized monoclonal antibodies [tocilizumab (RoActemra®, anti-IL6R), certolizumab (Cimzia®, anti-TNF); two human IgG1: adalimumab (Humira®, anti-TNF, see paragraph 2.1.3), golimumab (Simponi®, anti-TNF)], as well and the fusion protein etanercept (Enbrel®, TNF-inhibitor obtained by fusing the TNF receptor to the constant end of the IgG1 antibody). Biological drugs are administered by injection and they are usually taken in combination with methotrexate or another DMARD.
2.5. Fibrin

Fibrin (also called Factor Ia) is a fibrous, non-globular protein produced in response to bleeding and it is the major component of the blood clot.

In the healthy organism, fibrin is virtually undetectable. By contrast, fibrin is abundantly produced in a series of pathological conditions, including thrombus formation (Cilia La Corte, Philippou et al. 2011, Undas and Ariëns 2011), cancer (Dvorak, Dickersin et al. 1981, Dvorak 1986, Zacharski, Memoli et al. 1986, Yasunaga, Manabe et al. 2011) and rheumatoid arthritis (Sanchez-Pernaute, Flikova et al. 2013).

To date, the function of fibrin has been extensively studied because of its role in certain rare hereditary disorders involving deficiency or abnormal production of fibrinogen, its precursor. More recently, the function of the protein has been investigated also in other pathological conditions, where in principle fibrin might be considered as a target for pharmacodelivery applications.

2.5.1. Fibrin formation from fibrinogen precursor

When blood clots, the soluble plasma glycoprotein fibrinogen is cleaved by thrombin at the level of its alpha- and beta-chains, yielding a fibrin monomer, which spontaneously polymerizes to form a gel (Pacella, Hui et al. 1983). Fibrin differs from fibrinogen by the cleavage and removal of two short fibrinopeptides (16 and 14 amino acids in length for the alpha- and beta-chain, respectively), which result in new positively charged termini for the two chains (figure 2.12).

The proteolytic removal of two short peptides leaves unaltered the rest of the fibrin molecule, which still shows the 98% similarity in the final structure when compared with the precursor (Doolittle 1973). This explains why the generation of high affinity antibodies specific to fibrin and with no detectable binding to fibrinogen represents a formidable chemical challenge. Indeed, in order to be useful for biomedical applications, a fibrin-
specific antibody needs to display no detectable binding affinity towards fibrinogen, which is present at 1.5 – 5.5 mg/ml concentrations in the blood plasma.

Haber and collaborators have pioneered the generation of polyclonal and monoclonal antibodies against fibrin (Hui, Haber et al. 1983, Pacella, Hui et al. 1983, Hui, Haber et al. 1986). One murine antibody (59D8), specific to the beta-chain of fibrin, was used for in vivo targeting application. A $^{111}$In-labeled Fab fragment of 59D8 was studied *in vitro* and in animal models (rabbit and dog), in order to evaluate its potential for the imaging of thrombi and emboli (Knight, Maurer et al. 1988). The antibody was successfully fused to tissue plasminogen activator and to hirudin, with the aim to achieve anti-thrombotic and thrombolytic activities in vivo, which were superior to the ones of the non-targeted payloads (Runge, Harker et al. 1996, Bode, Hanson et al. 1997).

**Figure 2.12.** Amino terminus of alpha and beta-chains of human fibrinogen and fibrin, respectively before and after thrombin cleavage. FBP A and FBP B are shown on the left side of the thrombin cleavage site while decapeptides representing the newly exposed alpha- and beta-chains of human fibrin are shown to the right.
2.5.2. The role of fibrin in cancer

Every solid tumor consists of at least two different components: the malignant cells themselves and the stroma in which they are embedded.

The stroma facilitates the growth of tumor cells, providing the nourishment for the neoplastic mass, in the form of vascular supply. Moreover, the stroma may create a physical and biochemical barrier, which inhibits immunologic reactions against the tumor cells, limiting the access of inflammatory cells at the site of disease (Dvorak, Senger et al. 1983). The tumor stroma is composed of four main different elements: new blood vessels, connective tissue, inflammatory cells and the fibrin-gel matrix. This last component was discovered relatively late (Dvorak, Senger et al. 1983), even if the association between cancer and clotting had been established long time before (Donati 1984). Fibrin deposition is consistently an early event in solid tumors and precedes new blood vessel in-growth. Furthermore, it has been demonstrated that fibrin gels play a pivotal role in the induction of an angiogenic response, even in absence of tumor cells or platelets (Dvorak, Harvey et al. 1987). Neoplastic vessels are hyper-permeable, leading to a persistent leakage of fibrinogen, which, once transformed into fibrin, rapidly coagulates and cross-links in the extracellular space. Not all the fibrin present in the stroma is long-lived. Fibrin can be rapidly degraded by tumor-secreted plasminogen activators. In tumors (different from wounds, in which normal vascular permeability is restored within few days after injury), the cycle of leakage-deposit-degradation is continuous, leading to a constant formation of fibrin deposits. Taken together, all these findings underline the strong correlation between fibrin formation and tumor growth. As a consequence, fibrin could be considered as a target for biomedical applications, either as antigen for pharmacodelivery strategies or as a functional protein for the development of inhibitors.
2.5.3. The role of fibrin in rheumatoid arthritis

The thickening of a synovial membrane as a consequence of joint inflammation is often accompanied by neovascularization. Fibrin deposition is directly associated with the production of a new vessels and is a prominent finding in the synovium of patients with rheumatoid arthritis (RA). Macrophages have been shown to play a role in fibrin formation, together with a variety of procoagulant and anticoagulant molecules (Weinberg, Pippen et al. 1991). It has been postulated that the role of fibrin may extend beyond the mere function as a mesh, acting as provisional matrix for the attachment of endothelial cells. It has been hypothesized that fibrin may display functional properties, which are essential for the maintenance of the disease (Sanchez-Pernaute, Filkova et al. 2013).

As fibrin is virtually undetectable in the normal healthy adult, but is virtually always present in conditions of extensive tissue remodeling, it may be considered as a suitable target for the generation of monoclonal antibodies. In the context of RA, antibody-based fusion proteins could be considered as a tool for the inhibition of inflammatory process at the site of disease (Campbell, Lowe et al. 2011).
2.6. FSTL1

2.6.1. The role of FSTL1 in rheumatoid arthritis

Follistatin-like 1 (FSL1) is also known as FSTL1, FRP, Flik, and TSC-36. It is a 35 kDa extracellular glycoprotein belonging to the BM-40/SPARC/Osteonectin family. FSTL1 is overexpressed in human arthritis. Its serum and synovial fluid levels correlate with severity of rheumatoid arthritis in patients. The high levels reflect not only joint disease but also degree of inflammation and tissue degradation (Li, Wang et al. 2011).

Several groups have investigated the mechanism of action of the protein but so far its function is still poorly understood. Indeed, it is not sure whether the protein has an exacerbating (Clutter, Wilson et al. 2009, Chaly, Marinov et al. 2012) or preventing role (Tanaka, Ozaki et al. 2003) on the development of the disease. Nevertheless, there is a common and firm belief in the use of FSTL1 as potential target for the treatment of certain forms of arthritis and its possible application for monitoring the course of the disease. Interestingly, mature human FSTL1 shares respectively 94% and 95% identity with mouse and rat, which ensures that potential monoclonal antibody candidates react fast equally well with the human and the murine antigen.
2.7. Aim of the thesis

The aim of this thesis was to isolate high affinity, fully human monoclonal antibodies specific to over-expressed targets in cancer and rheumatoid arthritis, using Phage Display technology. We then proceeded with the characterization of the antibody clones by *in vitro* microscopic analysis, immunohistochemistry and *in vivo* biodistribution experiments.
3. Results

The results on targeting approach using Fibrin and FSTL1 as antigens are described in this chapter.

Appendix 8.3. is dedicated to a side project on the development and comparison of different of ADC molecules.
3.1. A fibrin-specific monoclonal antibody from a designed phage display library inhibits clot formation and localizes to tumors in vivo

3.1.1. Selections of antibody phage libraries against fibrin peptides

In order to mimic the structural features of the N-terminal portion of fibrin, we designed a C-terminally biotinylated peptide, containing the first ten residues of fibrin alpha-chain (GPRVVERHQS; Figure 2.12). This peptide was used as antigen in various experimental conditions in phage selections using the three different and well characterized libraries available in our laboratory (Silacci, Brack et al. 2005, Villa, Lovato et al. 2011, Weber, Bujak et al. 2014), using capture protocols based on streptavidin and avidin (see paragraph 6.1.1). Relatively few binders (in scFv antibody format) were isolated, compared to the standard performance of the antibody library, possibly reflecting the chemical challenge in the isolation of fibrin-specific reagents. Surprisingly, the antibody clone (termed AP1, isolated from the ETH-2-Gold library), which gave the best ELISA results, exhibited a mutation of the positively charged lysine residue at position 94 into a negatively charged glutamic acid (figure 3.1). The AP1 antibody strongly bound in ELISA to GPRVVERHQS and to its shorter N-terminal variant (GPRVV), but did not react with fibrinogen, avidin, streptavidin and with N-acetylated versions of the peptides (figure 3.2A). The full N-terminal peptide and its shorter version did not exhibit significant statistical difference between each other but they are both significantly different when compared with the other peptides, according to the Bonferroni’s multiple comparison test (appendix 8.4.1). The role of the K94E mutation for fibrin binding was confirmed by back-mutation of the glutamate residue into the original lysine amino acid, which was accompanied with an almost complete loss of fibrin binding activity in ELISA (figure 3.2B), (test of statistical significance in appendix 8.4.2). Surface plasmon resonance analysis on a BIAcore instrument revealed that monomeric scFv(AP1) bound to fibrin with a Kd = 7 µM, while no binding was detectable for fibrinogen (figure 3.2C).
Figure 3.1. Amino acid sequence of the AP1 antibody in scFv format (VH, linker and VL domain). The scFv(AP1) antibody fragment carries a specific mutation at position 94 of VH (shown in red), resulting from a mutation in the original ETH-2-Gold library. The asterisk indicates residues, which had been combinatorially mutated in that library.

Figure 3.2. Specificity of the anti-fibrin AP1 antibody. (A) The selected anti-fibrin scFv(AP1) antibody fragment was tested in an ELISA experiment, using different antigens coated on a Maxisorp microtiter plate. (B) Binding properties of scFv(AP1) and of a mutant, containing the germline lysine residue a position 94 (“DP47WT”), analyzed by ELISA on biotinylated fibrin-derived peptide. (C) Biacore analysis of a monomeric preparation of scFv(AP1), tested on a microsensor chip, coated with biotinylated fibrin-derived peptide or with fibrinogen. Both experiments A and B were performed at identical antibody concentrations, using the anti-myc-tag 9E10 monoclonal antibody as detection reagent.
3.1.2. Design and construction of the NITRO library

In order to obtain anti-fibrin antibodies with higher affinity, we constructed an antibody library based on scFv fragments, which featured the K94E substitution in the DP47 VH germline gene segment (Tomlinson, Walter et al. 1992) and the combinatorial mutagenesis of residues in the CDR3 loops of VH and VL domains (figure 3.3).

**Figure 3.3. Construction of a designed affinity maturation library (termed NITRO).** The DPK22 (Vκ) and DPL16 (Vl) backbone are represented in turquoise and the DP47 (VH) in purple. Residues subject to random mutations are DP47 CDR3 positions 95, 96, 97, 98, 99, and 100, DPK22 CDR3 positions 91, 92, 93, 94 and 96 and DPL16 CDR3 positions 91, 92, 93, 94, 95 and 96. On the right side, the structure of the scFv fragment is based on the structure of protein, data base file 8FAB (Brookhaven Protein Data Bank) using the Molsoft program. The residues are numbered according to (Tomlinson, Cox et al. 1995).

In analogy to the design of the ETH-2-Gold library, the light chain was based either on a Vκ (DPK22; Cox, Tomlinson et al. 1994) or a Vl (DPL16; Marks, Hoogenboom et al. 1991) gene segment. The library (termed “NITRO”) was constructed in full analogy to other synthetic antibody libraries previously constructed by our group (Silacci, Brack et al. 2005, Villa, Lovato et al. 2011, Weber, Bujak et al. 2014).

After randomization of CDR3 regions by PCR using partially degenerate oligonucleotides (see “NITRO library construction”, appendix 8.2), the resulting VH and
VL segments were assembled by PCR. A total of 49.8 µg of insert was ligated into a double digested phagemid vector pHEN1 and electroporated into freshly prepared electrocompetent *E.coli* TG1 cells (figure 3.4). Four different sublibraries (DPL16-1, DPL16-2, DPK22-1 and DPK22-2) containing a total of 1.3x10^10 individual clones were obtained (table 3.1).

**Figure 3.4. Library cloning strategy.** Mutations were introduced in the CDR3 regions by PCR using partially degenerate primers. Genes are indicated as rectangles and CDRs as numbered boxes within the rectangle. The VH and VL segments were then assembled by PCR and cloned into the pHEN1 vector. Primers used in the amplification and assembly are listed in appendix 8.2 (“NITRO library construction”).
Table 3.1. Titors of the ETH-2-Gold library. The titers were deduced from colony counting by plating of dilution series after series of electroporated bacteria.

<table>
<thead>
<tr>
<th>Sublibrary</th>
<th>Number of individual antibody clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPL16-1</td>
<td>$1.48 \times 10^9$</td>
</tr>
<tr>
<td>DPL16-2</td>
<td>$1.11 \times 10^{10}$</td>
</tr>
<tr>
<td>DPK22-1</td>
<td>$1.77 \times 10^8$</td>
</tr>
<tr>
<td>DPK22-2</td>
<td>$7.47 \times 10^7$</td>
</tr>
<tr>
<td>NITRO (total)</td>
<td>$1.28 \times 10^{10}$</td>
</tr>
</tbody>
</table>

3.1.3. Characterization of the NITRO library

The quality and functionality of the library were assessed by PCR colony screening, dot blot analysis, DNA sequencing and test selections against standard antigens. A PCR analysis of 44 library clones revealed that 43/44 bacterial colonies contained an insert of the expected size (figure 3.5).

![Figure 3.5. PCR colony screening](image)

About 98% (43 out of 44) of all tested clones could be shown to carry a scFv of correct length.
A dot blot analysis of 372 clones (93 for each sub-library) revealed that >90% of the analyzed antibody clones expressed soluble scFv fragments (figure 3.6), in analogy to other synthetic antibody phage libraries previously produced by our group (Silacci, Brack et al. 2005).

**Figure 3.6. Dot blot analysis.** 372 bacterial supernatants were screened from randomly picked clones (93 for each sublibrary, based on DPK22 or DPL16 germline genes). Soluble scFv fragments were detected using the anti-myc-tag 9E10 antibody. Culture medium was used as negative control (see corner bottom right Dpk22-1 plate).

Sequencing of 20 randomly picked clones revealed a correct sequence for all antibodies, without frameshift mutations. In addition, the sequencing proved that all amino acid sequences in the CDR3 regions of both heavy and light variable chains were diverse (table 3.2).
Table 3.2. Amino acid sequences of the CDR3 regions of 8 out of 20 randomly selected scFv fragments. The 8 clones were a representative example for all the possible types of randomization that were introduced in the library (i.e., 4, 5 or 6 randomized residues in CDR3 VH and five different randomization variants for CDR3 VL). Positions that are mutated are written in bold character. Single amino acid codes are used according to standard IUPAC nomenclature.

Selections against Tenascin-C (TnC) as a control antigen were performed. After 2 rounds of panning, hits could both be generated with the ETH2-Gold library as well as with the N-terminal library. The number of 39 positive clones from the ETH2-Gold library was in line with the typical results obtained from previous selections performed in the laboratory. The ETH2-Gold library thus outperformed the N-terminal library, which gave 18 positive clones (figure 3.7).

**Figure 3.7. Characterization of the NITRO library.** Selections against Tenascin-C antigen comparing ETH-2-Gold and NITRO library.
3.1.4. Isolation and biochemical characterization of the AP2 antibody

Antibody selections from the NITRO library against fibrin were performed as previously described for the ETH-2-Gold library (see paragraph 6.1.1) and yielded 23/94 strongly positive clones after two rounds of selection (figure 3.8).

**Figure 3.8. Characterization of the NITRO library.** Selections against peptide of N-terminal alpha chain of fibrin, comparing ETH-2-Gold and NITRO library.

One of these antibodies (termed AP2; see sequence figure 3.9), which had exhibited the most promising binding results in ELISA and surface plasmon resonance investigations (data not shown), was characterized in more detail.

**Figure 3.9. Amino acid sequence of the AP2 antibody in scFv format (VH, linker and VL domain).** The scFv(AP2) antibody fragment carries a specific mutation at position 94 of VH resulting from the mutation inserted in the NITRO library. The asterisk indicates residues, which had been combinatorially mutated in that library.
First, the AP2 antibody was expressed in mammalian cells in scFv format, leading to a homogenous protein preparation as confirmed by SDS-PAGE analysis. Nevertheless, the product exhibited the co-existence of a monomeric and non-covalent homodimeric species (diabody). The antibody bound tightly to fibrin peptides immobilized on a BIAcore microsensor chip. Monomeric preparations of scFv(AP2) allowed the determination of a dissociation constant to fibrin (Kd = 44 nM considering the concentration on 3 µM, which shows a variation in factor 2 when compared with the other three concentrations applied) (see asterisk, figure 3.10 A). The AP2 antibody was then produced in small immune protein (SIP) format, showing clear bands of pure product in SDS analysis, as well as intact binding capacity in both ELISA and SPR experiment (figure 3.10 B). As last format of expression, a fully human IgG was produced, leading to a homogenous protein preparation even if the formation of some aggregates was observed, as revealed by gel filtration analysis. (figure 3.10 C) All three antibody formats exhibited a preferential binding to fibrin-derived peptides in ELISA assays, compared to fibrinogen and to acetylated peptides (figure 3.10 D-F). The full N-terminal peptide and its shorter version did not exhibit significant statistical difference between each other but they are both significantly different when compared with the other peptides, according to the Bonferroni’s multiple comparison test (test of statistical significance for AP2 Scfv format in appendix 8.4.3).

In terms of protein production, the three formats showed very different yield of expression. The average yields obtained by a transient gene expression (TGE), were 3 mg/L, 0.3 mg/L and 25 mg/L for scFv, SIP and IgG format respectively. The SIP format was chosen for further experiments. In order to overcome the problem of low production yield, a monoclonal stable cell line was established, resulting in 11 mg/L yield of purified SIP(AP2) protein.
Figure 3.10. Characterization of the AP2 antibody in three formats. The AP2 antibody was cloned and expressed in mammalian cells in scFv (A), SIP (B) and IgG (C) formats. For all three preparations, a SDS-PAGE, gel-filtration (on S75 or S200 column) and BIAcore characterization is provided, as well as a schematic representation of the domain structure of the recombinant antibody. Lane 1: molecular markers; Lane 2: non-reducing conditions; Lane 3: reducing conditions. BIAcore experiments were performed on a microsensor chip, coated with biotinylated fibrin-derived peptide. For the AP2 ScFv monomeric fraction, the Kd value equal to 44 nM refers to the curve indicated with the asterisk (*). (D,E,F) ELISA confirmation of AP2 binding specificity towards various fibrin-derived peptides and fibrinogen. Streptavidin (SA) was used as negative control.
3.1.5. Fibrin clot isolation

In order to test the ability of the AP2 antibody to inhibit fibrin clot formation, a water solution of fibrin was treated with thrombin, in the presence or absence of variable concentrations of scFv(AP2). A blue dye was used to better visualize the clotting results and facilitate the pipetting of residual supernatant to a separate microtiter plate, whose spectrophotometric readout allowed the plotting of % inhibition of clotting, at various concentrations of scFv(AP2) (figure 3.11). At the highest antibody concentration tested (18 µM), a 60% inhibition of fibrin clotting could be observed. By contrast, only a minimal inhibition was observed using an antibody of irrelevant specificity as negative control.

Figure 3.11. Inhibition of fibrin clot formation by the AP2 antibody. A purified preparation of scFv(AP2) was used at various concentrations to inhibit the process of clot formation, triggered by addition of thrombin (10U/ml) to a fibrinogen solution (2 mg/ml), upon 30 min. incubation at RT. In the positive control, the AP2 antibody was omitted, while in the negative control no thrombin was added. An antibody in scFv format, specific to FSTL1, was used as negative control (indicated in the figure as “irr”=irrelevant antibody).
3.1.6. Immunofluorescence analysis of subcutaneously-grafted tumors

The AP2 antibody was used in SIP format to stain normal murine organs and subcutaneously grafted tumors. The presence of the εCH4 domain of IgE in the SIP structure allowed a specific antibody detection using anti-human IgE reagents. CD31 was co-stained in immunofluorescence procedures, in order to reveal blood vessels in the tissue sections. All normal organs tested (heart, kidney, liver, spleen, uterus and placenta) did not exhibit a detectable staining with SIP(AP2) and SIP(KSF), a hen egg lysozyme-specific monoclonal antibody used as negative control. By contrast, some of tumor sections exhibited an intense staining using the AP2 antibody (but not with the KSF antibody) around vascular structures, identified by CD31 co-staining (figure 3.12).
Figure 3.12. Immunofluorescence analysis of tumor sections and healthy tissues. (A) Immunofluorescence analysis of healthy organ sections, stained using SIP(AP2) or SIP(KSF) as negative control. All sections were co-stained, using an anti-CD31 antibody (Jemal, Bray et al.). All organs were of murine origin, except for placenta (human). (B) A similar immunofluorescence analysis was performed on sections of human and murine tumors, grafted subcutaneously in mice. Scale bar= 20 µm. Each slice is one representative example out of three sections analyzed.
3.1.7. Biodistribution analysis of subcutaneously-grafted tumors

Motivated by the immunofluorescence results, a radioiodinated preparation of the SIP(AP2) antibody was injected intravenously into mice bearing either F9 tumors or SKRC-52 tumors (for both, n = 4). Twenty-four hours after injection, mice were sacrificed, organs were weighed and radioactively counted. Biodistribution results, expressed as percent injected dose per gram of tissue or body fluid (%ID/g), revealed that a preferential accumulation could be observed in F9 teratocarcinomas (which had exhibited the most intense staining with the AP2 antibody in immunofluorescence analysis), but not in SKRC-52 tumors (figure 3.13). Tumor:organ ratios were > 4:1, with the exception of kidney and blood, which exhibited 2.9% and 2.1 %ID/g values, respectively, at this time point. In the same animal model, antibodies of irrelevant specificity in the mouse (e.g., the HyHEL-10 antibody, specific to hen egg lysozyme), used in SIP format, typically exhibit tumor:blood ratios of 1 – 1.5 at 24 h after injection (Villa, Trachsel et al. 2008, Pfaffen, Frey et al. 2010).

(Figure 3.13. Biodistribution studies in tumor-bearing mice. Biodistribution study performed using $^{125}$I-labelled SIP(AP2) antibody in 129SvEv mice bearing F9 tumors (A) and BALB/C nude mice bearing SKRC-52 tumors. Results are expressed as percent of the injected dose of radioalabeled antibody per gram of tissue (%ID/g) ± standard error, 24 h after a single i.v. injection of the radiolabeled protein (20 µg per mouse). Four mice were used for each group.)
3.2. A human monoclonal antibody specific to fibrin selectively localizes to inflamed joints in mice with arthritis

3.2.1. Staining of perivascular structures in specimens of rheumatoid arthritis

Sections of arthritic paws derived from mice with collagen-induced arthritis were stained with the AP2 antibody or with KSF (both antibodies were used in SIP format). CD31 was co-stained in immunofluorescence procedures, in order to reveal blood vessels in the tissue sections. Inflamed tissues exhibited an intense staining around vascular structures with the AP2 antibody (figure 3.14), while no staining was detectable when using the KSF antibody at identical concentrations.

Figure 3.14. Immunofluorescence staining of inflamed murine arthritic paw sections. AP2 and KSF antibodies are displayed in green (i,v). Vessels are stained in red using an anti-CD31 antibody (ii,vi). The overlay of red and green fluorescence is presented in iii and vii. Nuclei were stained with DAPI (iv,viii). Scale bar = 100 µm. Each slice is one representative example out of three sections analyzed.
3.2.2. Targeting of arthritic lesions *in vivo*

The *in vivo* targeting properties of AP2 were investigated in mice with collagen-induced arthritis, using autoradiographic imaging procedures. Radioiodinated preparations of SIP(AP2) and SIP(KSF) were injected intravenously into arthritic mice. Twenty-four hours after injection, mice were sacrificed, paws were photographed and exposed to a phosphorimaging plate. A preferential accumulation of the AP2 antibody was visible in inflamed paws and toes, which increased with the severity of inflammation at the site of disease indicated with a score from 0 to 4 (for details about the scoring system used see paragraph 6.2.1). By contrast, a substantially reduced uptake in inflamed joints was observed for the KSF antibody, which varied little between score 0 and 4 (figure 3.15).

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<tr>
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<td>SIP(KSF)</td>
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**Figure 3.15. Autoradiography image in mice with collagen-induced arthritis.** Arthritic mice (*n* = 3) were injected intravenously with 20 µg of SIP(AP2) or SIP(KSF), labeled with $^{125}$I. Twenty-four hours after injection, mice were sacrificed and individual paws were imaged. Next to each autoradiographic image, a photograph of the corresponding paw is shown, documenting paw swelling. The two red arrows indicate the two toes inflamed. Numbers represent the swelling score, as defined in the section 6.2.1.
Accumulation of the two SIP antibodies in various organs was evaluated in biodistribution experiment, which was performed by conventional weighing and radioactivity counting procedures.

Figure 3.16. Biodistribution study in mice with collagen-induced arthritis. Biodistribution study performed using $^{125}\text{I}$-labelled SIP(AP2) antibody and SIP(KSF) antibody in arthritic mice. Results are expressed as percent of the injected dose of radioalabeled antibody per gram of tissue (%ID/g) ± standard error, 24 h after a single i.v. injection of the radiolabeled protein (20 µg per mouse). Three mice were used for each group.
3.3. High affinity monoclonal antibodies against FSTL1 isolated by phage display technology

3.3.1. Expression, purification and quality control of FSTL1

Recombinant follistatin-like 1 protein (FSTL1) was expressed in HEK293 cells from pCEP4–based expression vector and purified on Nickel-sepharose resin via the histidine-tag appended at the C-terminus of the protein. The expression level was estimated to be about 18 mg/L. The quality and the concentration of the purified protein were checked by SDS-PAGE (figure 3.17A) and size exclusion chromatography (figure 3.17B). In SDS-PAGE analysis, a smeared protein band (about 50 kDa) was visible indicating that FSTL1 was highly glycosylated. After treatment with PNGase, a clear single band of 35 kDa was detectable (figure 3.17A, line 4). Size exclusion chromatography analysis showed that the protein was eluted as a single peak.

Figure 3.17. Characterization of FSTL1 protein. A) SDS-Page analysis; Lane 1: molecular markers; Lane 2: non-reducing conditions; Lane 3: reducing conditions; Lane 4:FSTL1+PNGase; Lane 5: PNGase. B) Gel filtration on S200 column.
Mass spectrometry analysis allowed the identification of 42 peptides, with excellent average of the full sequence and identification of 95 % purity (figure 3.18).

![Peptide Identification Diagram](image)

**Figure 3.18. MS analysis of FSTL1 protein.** Sequence coverage using Trypsin and Chymotrypsin enzymes. The gray box represents the whole sequence, the blue and the red bars correspond to the peptide identified with digestion of chymotrypsin and trypsin respectively.

### 3.3.2. Isolation of recombinant antibodies specific to FSTL1

The purified FSTL1 antigen was used in different selections and specific recombinant antibodies were selected from the three libraries available in our laboratory (Silacci, Brack et al. 2005, Villa, Lovato et al. 2011, Weber, Bujak et al. 2014). Panning experiments were performed with both, biotinylated or unbiotinylated protein, following standard protocols (for details see paragraph 6.1.1).

### 3.3.3. Characterization of scFv(E8)

Preliminary real-time interaction studies with a BIAcore 3000 instrument revealed that the E8 antibody clone (sequence shown in figure 3.19), displayed the slowest dissociation from the antigen, among all antibody clones tested (data not shown). Therefore the scFv(E8) was produced in larger scale in bacteria cells and characterized by SDS-PAGE (figure 3.20A), size exclusion chromatography (figure 3.20B) and BIAcore analysis (figure 3.20C).
Figure 3.19. Amino acid sequence of the E8 antibody in scFv format (VH, linker and VL domain). The scFv(E8) antibody resulted from the Philo Diamond library. The asterisk indicates the CDR3 residues. The position 52 (mutated in the Philo Diamond library compared with the ETH-2-Gold library) is indicated in green.

![Amino acid sequence of E8 scFv](https://via.placeholder.com/150)

The antibody was produced in scFv format. The E8 antibody was then produced in scFv-Fc format, showing single bands of pure product in SDS analysis, as well as binding in a SPR experiment (figure 3.21). E8 was also expressed and produced in SIP format, leading to a homogenous protein preparation even if the formation of some degradation product was observed, as revealed by gel filtration analysis (figure 3.22).
**Figure 3.21. Characterization of scFv-Fc(E8).** A) SDS-Page analysis; Lane 1: molecular markers; Lane 2: non-reducing conditions; Lane 3: reducing conditions. B) Gel filtration on S200 column. C) BLAcore binding experiment performed on a microsensor chip, coated with FSTL1 protein.

**Figure 3.22. Characterization of SIP(E8).** A) SDS-Page analysis; Lane 1: molecular markers; Lane 2: non-reducing conditions; Lane 3: reducing conditions. B) Gel filtration on S200 column. C) BLAcore binding experiment performed on a microsensor chip, coated with FSTL1 protein.

### 3.3.4. Construction and characterization of an affinity maturation library

The scFv fragments isolated directly from the libraries in use in our laboratory typically show dissociation constants (Kd) in the range between $10^{-6}$ and $10^{-8}$ M. These affinities are normally sufficient for use as research reagents (e.g. ELISA, Western Blot, etc.), but often suboptimal for therapeutic applications. Further mutagenesis in the CDRs of the selected binder and phage display selection allow the isolation of recombinant antibodies with improved affinity (Neri, Carmemolla et al. 1997, Pini, Spreafico et al. 1997).

The affinity maturation of scFv(E8) was performed by combinatorial mutagenesis of...
CDR1 residues in the both VH and VL chain. A new antibody repertoire, in which positions 31, 32, 33 of the VH CDR1 and positions 31,31b and 32 of the VL CDR1 of the scFv(E8) were combinatorially mutated, was constructed using the cloning strategy shown in Figure 3.23. The corresponding primers are listed in appendix 8.2.

**Figure 3.23. Library cloning strategy of affinity maturation.** Mutations were introduced in the VH CDR1 and VL CDR1 regions by two PCR amplifications using partially degenerate primers. Genes are indicated as rectangles and CDRs as numbered boxes within the rectangle. The VH (in which positions 31, 32 and 33 of the CDR1) and the VL (in which positions 31, 31b and 32) were mutated were assembled by PCR and cloned into the pHEN1 vector. Primers used in the amplification and assembly are listed in Appendix 8.2 (see E8 affinity maturation library construction).
A repertoire of $1.3 \times 10^7$ individual antibody clones was obtained by ligating 5 µg of combinatorially mutated insert in 6.6 µg of double digested pHEN1 vector, and electroporation into freshly prepared electrocompetent E.coli TG1 cells. Even if the size of the library was slightly under the expectations for an affinity maturation library, we decided to characterize it and to proceed with selection experiments.

3.3.5. Characterization of the scFv(E8) affinity maturation library

The quality and functionality of the library were assessed by PCR colony screening. A PCR analysis of 20 library clones revealed that 16/20 bacterial colonies contained an insert of the expected size (figure 3.24).

![Figure 3.24. PCR colony screening.](image)

About 80% (16 out of 20) of all tested clones could be shown to carry a scFv of correct length.

Sequencing of 10 randomly picked clones revealed a correct sequence for all antibodies, without frameshift mutations. The clones were randomly picked and sequenced, revealing that variability in the amino acid sequences in the CDR1 regions of both heavy and light variable chains was achieved (table 3.3).
Characterization of the scFv(E8) affinity maturation library. Amino acid sequences of the CDR1 regions of 10 randomly selected scFv fragments. The 10 clones were a representative example for possible types of randomization that were introduced in the library. Single amino acid codes are used according to standard IUPAC nomenclature.

Antibody selections from the scFv(E8) affinity maturation library against FSTL1 were performed as previously described for the ETH-2-Gold library (see paragraph 6.1.1) and yielded 12/94 strongly positive clones after two rounds of selection (data not shown). One of these antibodies (termed A4; see sequence Figure 3.25), which had exhibited the most promising binding results in ELISA and surface plasmon resonance investigations (data not shown), was characterized in more detail.

<table>
<thead>
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<th>Clone</th>
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Table 3.3. Characterization of the scFv(E8) affinity maturation library. Amino acid sequences of the CDR1 regions of 10 randomly selected scFv fragments. The 10 clones were a representative example for possible types of randomization that were introduced in the library. Single amino acid codes are used according to standard IUPAC nomenclature.

Figure 3.25. Amino acid sequence of the A4 antibody in scFv format (VH, linker and VL domain). The asterisk indicates the CDR1 residues, which had been combinatorially mutated in that library in both VH and VL. In this particular clone only two residues got changed and they are displayed in orange. The position 52 (mutated in the Philo Diamond library compared with the ETH-2-Gold library) is indicated in green.
3.3.6. Isolation and biochemical characterization of the A4 antibody

First, the A4 antibody was expressed in bacteria cells in scFv format. The scFv(A4) showed a size exclusion chromatography profile very similar to the one of the parental clone, scFv(E8) (see Figure 3.20). At micromolar concentration, scFv(A4) was mainly present in its monomeric form (mass = 27 kDa), but some aggregates at higher molecular weight were also visible, comprising most probably also the corresponding non-covalent homodimer (mass = 54 kDa). Affinity measurements were performed by real-time interaction analysis on a low density antigen-coated Biacore chip. A monomeric preparation of the scFv(A4) was applied at different concentrations (Figure 3.26C) and kinetic constants were calculated, using the BIAevaluation3.1 software, from the experimental sensograms. This analysis revealed a dissociation constant (Kd) of 8 nM. Thus, the affinity of scFv(A4) for FSTL1 was improved by a factor 80 compared to the affinity of the parental scFv(E8).

Figure 3.26. Characterization of scFv(A4). A) SDS-PAGE analysis; Lane 1: molecular markers; Lane 2: non-reducing conditions; Lane 3: reducing conditions. B) Gel filtration on S75 column. C) Biacore binding experiment performed on a microsensor chip, coated with FSTL1 protein. Kd considered at 50 nM concentration, as indicated by the asterisk (*).

The A4 antibody was cloned into pcDNA3.1.(+) vector in SIP format and transfected into mammalian CHO cells. Unfortunately, the antibody in SIP format could not be expressed. Therefore, we decided to use the parental antibody SIP(E8) for further investigations.
3.3.7. Staining of perivascular structures in specimens of rheumatoid arthritis

Immunohistochemistry analysis revealed that the SIP(E8) preferentially stains perivascular structures in specimens of rheumatoid arthritis (figure 3.27). This experiment suggests that it may be possible to perform *in vivo* experiments in a mouse model to validate the performance of our antibody as targeting agent.

![Image of immunofluorescence staining](image)

**Figure 3.27. Immunofluorescence staining of inflamed murine arthritic paw sections.** F8 (used as positive control), KSF (used as negative control) and E8 antibodies are displayed in red. All antibodies were used in SIP format. Vessels are stained in green using an anti-CD31 antibody. Scale bar = 50 μm.

3.3.8. Targeting of arthritic lesions *in vivo*

The *in vivo* targeting properties of SIP(E8) were investigated in mice with collagen-induced arthritis, using autoradiographic imaging procedures. Radioiodinated preparations of SIP(E8), SIP(KSF) and SIP(F8) were injected intravenously into arthritic mice (5μg for each protein, corresponding to 4uCi). Twenty-four hours after injection, mice were sacrificed, paws were photographed and exposed to a phosphorimaging plate. The SIP(F8) antibody showed the highest uptake in inflamed paws, while the arthritis targeting performance of SIP(KSF) was substantially lower. For SIP(E8), the uptake at sites of inflammation was less efficient compared to the SIP(F8) antibody, specific to the alternatively-spliced EDA domain of fibronectin. Nevertheless, a difference in uptake between non-inflamed and inflamed paws could be observed.
A biodistribution analysis was also performed in parallel to evaluate the accumulation in different organs (figure 3.28).

Figure 3.28. Analysis of the targeting properties of antibodies in SIP format in the collagen-induced model of rheumatoid arthritis, using radioiodinated protein preparations. For each antibody, the autoradiographic phosphorimage data are shown in the left side of the figure, while on the right side values corresponding to the percentage of injected dose per gram of tissue (%ID/g) are reported. The numbers above the paws and above the columns indicate the arthritic score. E8 (in blue) showed an increased accumulation in the arthritic paws compared to non-inflamed joints. F8 (in red) showed the highest accumulation at the site of disease, with values up to 7%ID/g. KSF (in green), used as negative control, did not show any accumulation neither in the arthritic paws nor in the non-inflamed joints. One mouse, injected with F8 (light red) and KSF (light green), had never received collagen immunization and served as negative control. In the figure, paws are labeled according to their position; front right (FR), front left (FL), back right (BR) and back left (BL).
4. Discussion and Outlook

In the thesis we describe the selection of high affinity binders against a synthetic peptide, which mimics the sequence of the N-terminal fibrin alpha chain. The N-terminal regions of fibrin protein, revealed after cleavage of fibrinogen by thrombin, are the only structures unique to fibrin when compared to its precursor molecule. Therefore, the isolation of antibodies specific to fibrin, which do not cross-react with fibrinogen, constitutes a chemical challenge due to the similarity between the two structures. While, the selection of antibody-phage libraries against large proteins represents by now a well established process, the isolation of antibodies against small peptides is more complex. For this reason, relevant modifications to conventional selection-protocol were adopted to circumvent the difficulties in performing phage display against small molecules, such as peptides.

Some positive clones were obtained from selections performed against biotinylated peptides. Based on the analysis of the best candidates isolated from ETH-2-Gold library (among those the leading candidate, named AP1), we discovered that a K94E mutation in the VH domain of human antibodies is crucial for high-affinity fibrin recognition. Capitalizing on this discovery, we have constructed a “designed” antibody phage display library (containing 13 billion clones), in which CDR3 regions of both VH and VL were combinatorially mutated and the specific mutation K94E was introduced. As shown in this thesis, the NITRO library fulfills the criteria of an efficient library, being highly functional, as >90% of randomly selected clones expressed the corresponding antibody.

In addition to size and innovative design, also the robustness of a library represents an important pre-requisite for most practical applications. A phage display library can be defined robust, if it allows the isolation of specific binders against a broad variety of
antigens. With the hypothesis of making use of NITRO library for the selection of antibodies against a pool of N-terminal peptide antigens, we have tested the library against a panel of peptides involved in cancer, viral infections and neurodegenerative disorders (i.e. those derived from Angiostatin, Endostatin, GPCR 37, KS-HHV-8-GPCR and beta amyloid protein). The results of these selections did not meet our expectations, since no strongly positive antibodies could be isolated from the NITRO library (data not shown). These data suggest that the K94E mutation may not serve as a “general” anchoring point for the N-terminus of any peptidic sequence, but may work particularly well in the case of anti-fibrin antibodies. We define NITRO as a “designed” phage display library, since the K94E mutation, which was kept constant during library construction, was a “key” element for the isolation of high-affinity anti-fibrin binders. Unlike conventional affinity maturation strategies (in which a first binder is isolated from a combinatorial library and subsequently additional residues are mutated, which were not randomized in the original library), the new designed library featured a combinatorial mutagenesis of residues in the CDR3 loops of VH and VL (i.e., the same residues which were mutated in the ETH2-GOLD library, from which the AP1 antibody was isolated).

A high-affinity human antibody specific to the N-terminal peptide of fibrin alpha chain (termed “AP2”) was isolated, with better characteristics compared to the parental AP1. ScFv(AP2) was shown to specifically recognize the cognate antigen in ELISA assays. The Kd dissociation constant was measured by real-time interaction analysis on a BIAcore 3000 instrument, yielding a value of Kd = 44 nM. The versatility of the antibody was confirmed by the successful expression in different antibody formats, including scFv, SIP and IgG format. In all cases, a homogenous protein preparation could be observed in reducing SDS-PAGE analysis. The AP2 antibody in scFv format exhibited the simultaneous presence of a monomeric and of a non-covalent homodimeric
species (diabody), while some aggregates were observed for the antibody in IgG format, as revealed by gel filtration analysis. For the SIP format, a monoclonal stable cell line was established (data not shown), to overcome the limitation of the transient gene expression, which often results in low yield of produced protein. The average yield for the batches purified from the monoclonal cell line was around 11mg/L, which represents an improvement of a factor 30 compared to the yield obtained from TGE-procedures.

The AP2 antibody was tested in experiments for the inhibition of fibrin clot formation. Already at concentration of 18 µM, AP2 scFv was able to inhibit at least 60% of fibrin polymerization. In addition, the AP2 antibody has been shown to selectively stain fibrin in tissue sections. Our immunofluorescence findings revealed that different tumors exhibit variable levels of fibrin accumulation, typically with a perivascular staining pattern. The antibody nicely stained two tumor models, and they were therefore chosen to evaluate its tumor targeting performance in vivo.

Nude mice bearing SKRC52 human renal carcinoma xenograft and in immunocompetent mice bearing F9 murine teratocarcinoma have been used to perform biodistribution experiment. This analysis revealed that SIP(AP2) could preferentially localize to F9 tumors, while the same product did not show any accumulation at the SKRC52 tumor site 24 hr after intravenous injection. The fact that the AP2 antibody did not preferentially accumulate in this tumor model may be explained in different ways. On one hand, it is possible that F9 tumors may exhibit better targeting performance, compared to SKRC52 tumors, because of higher antigen expression and/or vascular permeability. Alternatively, one could postulate that other parameters (e.g., concentration of dehalogenases, capable of a selective removal of iodine-125 from the radioiodinated antibody product) may impact on biodistribution results. Indeed, it has
previously been reported that anti-CAIX antibodies exhibit worse biodistribution results in the SKRC52 model, compared to two other models of renal cell carcinoma (van Schaijk, Oosterwijk et al. 2005). Further investigations with different tumor models may help understand whether the promising tumor targeting findings observed for the F9 tumors may be extended to other types of cancer.

The biodistribution results obtained in both tumor models showed a significant antibody uptake in the kidney. This organ might be involved in the formation of some fibrin-complexes. Fibrin deposits in the glomeruli and fibrin/fibrinogen degradation products (FDP) in blood and urine has previously been regarded as a consequence of renal diseases (Tishkov, Tschoukanov et al. 1982).

A final part of this project provided an experimental evidence that rich fibrin deposits are present in inflamed joints from mice with rheumatoid arthritis and that these deposits could be targeted by the AP2 antibody.

The thesis describes, for the first time, the abundant deposition of fibrin in sub-endothelial structures associated with blood vessels in inflamed paws, derived from mice with collagen-induced arthritis. SIP(AP2) was shown to selectively stain perivascular structures in specimens of rheumatoid arthritis while no staining was detectable when using the KSF antibody at identical concentrations. Encouraged by these preliminary results, we aimed at targeting arthritic lesions in vivo. A preferential accumulation of the AP2 antibody was visible in inflamed paws and toes, with a targeting performance which increased proportionally with the severity of inflammation at the site of disease. By contrast, the negative control KSF antibody showed almost no uptake neither in the non-inflamed paws, nor in the heavily inflamed ones.

When the two antibodies were studied in biodistribution experiments, no preferential accumulation in other organs was detectable. However, blood and kidney values were slightly higher compared to other organs.
In the context of RA, it may also be worth exploring the possibility of arming the AP2 antibody with suitable payloads, such as anti-inflammatory cytokines (i.e. IL4 or IL10). These payloads have been very efficacious in RA targeting experiments when coupled to the disease-targeting F8 antibody (Schwager, Kaspar et al. 2009, Hemmerle, Doll et al. 2014).

In the second project, I described the isolation of high affinity human monoclonal antibodies specific to FSTL1. One clone (E8) was chosen as leading candidate for further characterization and was submitted to an affinity maturation procedure by the construction and screening of a combinatorial library derived from the E8 sequence. The residues chosen for randomization are found to frequently contact the antigen in the known three-dimensional structures of antibody-antigen complexes. Moreover, in natural antibodies these residues are frequently found to be diverse and to be subjected to somatic hypermutation (Tomlinson, Walter et al. 1992).

The library was tested in panning experiments against recombinant preparations of FSTL1 and allowed the isolation of the A4 clone. It is generally believed that the VL domain contributes less than the VH domain to antigen binding. The affinity-matured clones contained mutations exclusively in the CDR1 of the VH domain, while the CDR1 of VL was identical to the one of the parental antibody. Kinetic measurements of the A4 antibody indicate that the affinity maturation improved the association constant by a factor 80 compared to the parental scFv(E8) antibody.

Unfortunately, the A4 antibody could not be expressed in SIP format, due to expression problems which are sometimes encountered with some of our antibody clones. For this reason, the targeting properties in a mice model of RA, were assessed using SIP(E8) parental antibody. Immunohistochemistry analysis revealed that SIP(E8) stained perivascular structures in specimens of rheumatoid arthritis. However, the lower level of affinity of the antibody could negatively impact on targeting performance.
We investigated the ability of the antibodies to preferentially localize to the site of RA in vivo using radioiodinated protein preparations and autoradiographic detection methods, as well as organ counting. SIP(F8) antibody showed the highest uptake in inflamed paws, reaching a value of 6 %ID/g for one paw with score 3, 24h after i.v. injection. The targeting values of SIP(KSF) were substantially lower, confirming that specific antigen recognition is important for an active targeting process. Regarding SIP(E8), the uptake at sites of inflammation was less efficient compared to SIP(F8), but a difference between non-inflamed and inflamed paws could be clearly observed. For both SIP(E8) and SIP(F8) antibodies, radioactivity counting of the organs revealed also a substantial accumulation in spleen and intestine for SIP(F8), and kidney and blood for SIP(E8)).

FSTL1 was one of the target antigens which were used to validate the performance of the PHILO Diamond antibody phage library. A panel of more than 15 proteins were successfully used to screen the library. In all cases, specific monoclonal antibodies could be isolated (Weber, Bujak et al. 2014).
5. Conclusions

Biopharmaceuticals are continuously growing in importance, among the various classes of pharmaceutical agents and the sales of biologics continue to grow (Aggarwal 2014). Monoclonal antibodies and their derivatives represent the largest class of biopharmaceutical products, maintain their ranking as the highest selling drugs. Thus, there is a considerable biomedical interest in the identification of antigens, which are not expressed in healthy organs, but are abundantly found in different pathological conditions, as these markers may be used as targets for the development of antibody products.

In principle, fibrin could be considered as a target for pharmacodelivery applications. Fibrin formation from fibrinogen is a rare process in the healthy organism, but is a pathological feature of thrombotic events, cancer and a wide range of inflammatory conditions. Fibrin differs from fibrinogen only by the proteolytic cleavage of two short peptides resulting in only about 2% difference between the two sequences. Therefore, the isolation of fully human antibodies, with high binding affinity to fibrin and no detectable binding to fibrinogen, represented a formidable biochemical challenge.

In this thesis, we report the isolation of high-affinity antibodies against fibrin using phage display technologies. To our knowledge, only one report has been published so far on the selection of antibody phage display libraries against fibrin. The study yielded one scFv fragment with a weak, but specific, binding affinity to the whole fibrin clot (Yan, Ko et al. 2004).

The research activity described in this thesis was inspired by the pioneering work of Prof. Ed Haber and collaborators at Harvard University, who used anti-fibrin antibodies for pharmacodelivery applications in the cardiovascular disease field (Bode, Hanson et al. 1997).
The advantages of our approach and our reagents relates to the possibility to generate fully human monoclonal antibodies and to monitor (and improve) binding affinity and selectivity during the process. The availability of reagents, which are not immunogenic in patients, should allow the implementation and clinical translation of pharmacodelivery strategies, based on fibrin targeting antibodies. Moreover, the N-terminal portion of fibrin, recognized by the AP2 antibody, is conserved across species, thus facilitating preclinical and clinical development activities without the need to generate novel affinity reagents. The AP2 antibody may serve as modular building block for the generation of a novel class of targeted biopharmaceutical agents.

A fibrin deposition is a rare event in the healthy adult, we believe that the AP2 antibody (or similar antibodies) may facilitate the *in vivo* targeting of cancer and rheumatoid arthritis.

In the thesis, I also generated human monoclonal antibodies specific to FSTL1. This protein is an attractive target for certain inflammatory conditions, being strongly expressed at the site of rheumatoid arthritis. Even though the biodistribution results obtained with the E8 antibody were clearly worse than the ones obtained with the anti-EDA F8 antibody, it is possible that higher affinity reagents specific to FSTL1 may one day be used for pharmacodelivery applications.
6. Materials and Methods

Material used for phage display selection and library construction

Growth media and plates:
2xTY liquid medium: 16g/l bacto-tryptone, 10g/l bacto-yeast extract, 5g/l NaCl pH 7.4.
Sterilized by autoclaving.
2xTY Amp(Kana)-Glu-Agar plates: add to 2xTY medium 15g/l agar, autoclave, add glucose and antibiotic only when a temperature of 50°C has been reached, pour into plates. 1% (w/V) Glucose, 33 µg/ml Kanamycin100 and/or µg/ml Ampicillin.

Buffers:
Phosphate buffered saline: 50 mM phosphate, 100 mM NaCl, pH 7.4; (PBS).
Milk-Phosphate buffered saline : PBS plus X% (w/v) dried skimmed milk; (% MPBS).

Bacterial strain:
TG1 (K12, D(lac-pro ), supE , thi , hsdD5 /F’traD36 , proA+B+, laqIq, lacZDM15 )

Helper phage:
VCSM13 (Stratagene)

Antibody phage display libraries:
The ETH-2-Gold, Philo and the Philo Diamond phage display libraries, which were used for antibody selections, has previously been described, respectively in (Silacci, Brack et al. 2005), (Villa, Lovato et al. 2011) and (Weber, Bujak et al. 2014).

A list of all primers described in this thesis can be found in appendix 8.2.

6.1. AP2 antibody inhibits clot formation and localizes to tumors in vivo

6.1.1. Selection of fibrin-specific antibodies by phage display
Fibrinogen was obtained from CALBIOCHEM (Merck Millipore, Darmstadt Germany).
For biochemical experiments, 20 mg of Fibrinogen were dissolved in 10 ml of filtered
phosphate buffered saline (PBS).

Peptides were purchased from Biomatik (Wilmington, Delaware, 19809 USA). Purity was >98% as assessed by HPLC, with MS confirmation of molecular mass. Peptides were resuspended in water (0.4mg/ml) and the corresponding stock solution was aliquoted and stored at -20°C.

Biotinylated peptides were immobilized on streptavidin-coated strip (Roche) at a final concentration of 18 µM in PBS (30 min. at 37°C). The wells were rinsed three times with PBS and blocked with 200 µl 4% (w/v) skimmed milk in PBS (MPBS) at room temperature for 2 hr, then rinsed again three times with PBS. The antibody phage display library was then added to the wells, submitted to gentle shaking for 30 min. at room temperature, followed by 90 min. standing. The wells were rinsed 10 times with PBS-0.1% Tween 20 and 10 times with PBS. As a last step, phage were eluted from the wells by adding 100 µl/well of 100 mM triethylamine water solution, under gentle shaking for 5 min at room temperature. Eluted phage was blocked with 1 M Tris-HCl solution (pH 7.4) and added to 10 ml of exponentially growing TG1 E.coli cells, which were then incubated for 30 min. at 37°C. A small amount of the culture was used for titer determination, plating dilution series of phage-infected bacteria on 2xTY-Amp-Glu plates and counting the number of colonies after an overnight incubation at 37°C. The remaining infected TG1 cells were spun down at 3300g for 10 min. The resulting bacterial pellet was resuspended in 0.5-1 ml 2xTY, spread on a large 2xTY-Amp-Glu plate, and incubated at 30°C overnight, until colonies were visible. The following day, 10 ml 2xTY-Amp-Glu-15% glycerol were added to the large agar plate and bacteria were gently loosened with a glass spreader, until a homogenous suspension was obtained. A portion of the recovered bacteria was added to 50 ml of 2xTY-Amp-Glu medium, until an OD of 0.1 was reached. The culture was allowed to grow at 37°C until an OD 0.4-0.5 was obtained. At that point, 10 ml of culture were infected with VCS-M13 helper phage at a ratio of 20:1 (phage:bacteria). The infected bacteria were spun
down at 3300g for 10 min. and the pellet was gently resuspended in 100 ml of 2xTY-
Amp, supplemented with 33.3 µg/ml of kanamycin, and allowed to grow at 30°C
overnight. The following day, phage were precipitated using a PEG solution (20% PEG
in 2.5 M NaCl) in a ratio 1:5 (v/v) with supernatant or water, respectively for the first or
the second step. The procedure was repeated twice. The phage were spun down at
3300 for 30 min. at 4°C and kept on ice in between every centrifuge-cycle. Eventually,
the pellet was resuspended in 2 ml of PBS 10% glycerol. A portion of these phages
was used for other rounds of panning and the remaining aliquot was stored at -20°C.
Two rounds of panning were performed before the bacterial supernatants, containing
recombinant antibody fragments, were screened by ELISA as previously described,
using the 9E10 antibody for the detection of myc-tagged scFv fragments\textsuperscript{\textregistered} (Viti, Nilsson et al.\textsuperscript{2000}). For ELISA detection biotinylated peptides were immobilized on Avidin-coated
Maxisorp (Nunc) (O/N at 4°C, 50 µl/ml) at a final concentration of 10 µM in PBS (30
min. at 37°C).

6.1.2. NITRO antibody library construction
The NITRO phage display library was designed based on the observation that a K94E
substitution facilitates antibody binding to fibrinopeptides. In addition to this amino acid
substitution, residues in the CDR3 loops of VH and VL domains were combinatorially
mutated, using the oligonucleotides listed in appendix 8.2 ("NITRO library
construction").

DPL16 and DPK22 antibody germline genes (Marks, Hoogenboom et al. 1991, Tomlinson, Walter et al. 1992,
Cox, Tomlinson et al. 1994) were used as templates for the PCR-based assembly of the library,
using the strategy depicted in figure 3.4. ScFv fragments in the library contained a
hydrophilic GGGGSGGGSGGGG linker between VH and VL, in full analogy to clones
of the ETH-2-Gold library (Silacci, Brack et al. 2005). Sequence diversity was created by
combinatorial mutagenesis at positions 95–98 of the VH. Similarly, positions 91, 92, 93,
94 and 96 in DPK22-based VL domains, or at positions 91–96 in DPL16-based VL domains, were combinatorially mutated using partially-degenerate oligonucleotides. In full analogy to what had previously been described for the ETH-2-Gold library (Silacci, Brack et al. 2005), VH and VL domains were PCR-assembled using gel-purified VH and VL segments as templates. The resulting genes, coding for combinatorially-mutated scFv fragments, were doubly-digested with *Ncol/NotI* and cloned into the pHEN1 phagemid vector (Hoogenboom, Griffiths et al. 1991). The resulting ligation product was electroporated into electrocompetent Escherichia coli TG1 cells according to previously described procedures (Viti, Nilsson et al. 2000). The library was electroporated on four different days, thereby obtaining four different sublibraries (two for the DPK22 portion and two for the DPL16 portion), yielding the following sub-library sizes: DPK22-1, 1.77 x 10^8 antibody clones; DPK22-2, 7.47 x 10^7 antibody clones; DPL16-1, 1.48 x 10^9 antibody clones and DPL16-2, 1.11 x 10^10 antibody clones (cumulative library dimension: about 13 billion clones). The NITRO library was stored as bacterial glycerol stocks, which were rescued and used for phage production as previously described (Viti, Nilsson et al. 2000).

6.1.3. NITRO antibody library characterization and use
The percentage of library clones capable of expressing soluble antibody fragments was determined by dot blot analysis of bacterial supernatants using anti-myc mouse mAb 9E10 (Silacci, Brack et al. 2005) and anti-mouse horseradish peroxidase immunoglobulins (Sigma-Aldrich; Buchs, Switzerland. Peroxidase activity was detected using the ECL Prime Western blotting detection system (GE Healthcare, Amersham Biosciences).

In total, 44 clones were tested by PCR screening procedures, using primers LMB3long and fdseqlong (appendix 8.2), in order to assess the presence of inserts of correct size in the vector. Cycling parameters: 94°C-5 min, (94°C-1 min, 53°C-1 min, 72°C-1min)28 , 72°C-10 min. In addition, 20 clones were selected at random and sequenced by GATC biotech (GATC Biotech AG (Headquarter) European Genome and
Diagnostics Centre, Constance, Germany), in order to check for the absence of frame shifts and of pervasive contamination. Prior to analysis, the DNA was eluted with NE elution buffer (5mM Tris/HCl, pH 8.5) from NucleoSpin Gel and PCR clean-up kit (Macherey-Nagel REF.740609.250). Eight illustrative sequences are shown in table 3.2. Antibody phage selections were performed as previously described for the ETH-2-Gold library (see section 6.1.1). The sequence of the AP2 antibody can be found in figure 3.9.

6.1.4. Expression of scFv antibody fragments in mammalian cells

The DNA of scFv fragments was digested and extracted from the original pHEN1 vector and ligated into a pcDNA 3.1.(+) vector in order to allow the transfection in mammalian cells. The resulting plasmid was transfected in CHO cells (Chinese Hamster Ovary cells). The day before transfection, cells were diluted down to 2 million cells per mL using fresh Power-CHO medium (Lonza, REF. BE 12-771Q). Immediately prior to transfection, cells were counted and their concentration was adjusted to 2 million cells per mL in Pro-CHO medium (Lonza, REF. BE 12029Q), that had previously been warmed up to 37 °C. The cells, resuspended in the new media, were transferred into a sterile bottle. Plasmid DNA (1.25 µg per million cells) was dissolved in 150 mM water solution of NaCl (1/40 of the total volume of transfection), followed by addition of a solution of 3.75 µg of polyethylenimine per million of cells, dissolved in 150 mM NaCl solution. The DNA-PEI mixture was allowed to incubate for 10’ at RT and was added to CHO cells, which were incubated for 3 h in a shaking cell culture incubator at 37 °C at the shaking speed of 100 RPM. After 3 h, Power-CHO medium was added, supplemented with 8 mM Ultraglutamine (200 mM, LONZA), HT Supplement (50X, GIBCO) and Antibiotic antimycotic (100X, GIBCO), resulting in a final cell concentration in the medium of one million cells per mL. The cells were then incubated for 6 days in a shaking cell culture incubator at 31 °C and 100 RPM. On day
6 after transfection, the antibody fragments were purified from the CHO cell culture supernatant by affinity chromatography, using Protein A Sepharose™ Fast Flow resin (Amersham Biosciences). The Protein A sepharose resin was first equilibrated with PBS and then the supernatant was applied to it. The resin was then washed with PBS 100mM NaCl, 0.1% Tween 20, 0.5mM EDTA. Next, the column was washed with PBS 500mM NaCl, 0.5M EDTA. (For both buffers the volume used was approximately half of the volume of supernatant applied). The scFv fragments were eluted in 1 ml fractions with 100mM triethylamine. Triethylamine was neutralized by adding 0.5 ml 1 M Tris/HCl pH 7.4 solution to each 1 ml fraction. The binding to protein A is conferred by the VH domain, which is based on the DP47 germline gene (Hoogenboom, Marks et al. 1992).

6.1.5. Expression of antibodies in SIP format

The Small Immune Protein (SIP) format provides certain advantages for in vivo targeting applications (Borsi, Balza et al. 2002). Cloning of the AP2 antibody into the SIP format was performed using the pcDNA 3.1(+) vector, as previously described, genetically fusing the scFv fragment at the N-terminus of a human εCH4 domain of the secretory isoform S2 of human IgE (Borsi, Balza et al. 2002). This domain promotes the formation of homodimers, that are further stabilized by disulfide bonds between the C-terminal cysteine residues, resulting in a 75 kDa bivalent miniantibody. (For the primers used see list in appendix 8.2 “Dpl16 SIP reformatting”).

The resulting plasmid was transfected in Chinese Hamster Ovary cells following a procedure which was identical to the one described above for scFv fragments. Antibodies in SIP format were purified from the cell culture supernatant by affinity chromatography using Protein A Sepharose™ Fast Flow resin (Amersham Biosciences) as previously described in 6.1.4.
6.1.6. Expression of antibodies in IgG format

The VL region of the AP2 antibody was amplified by PCR using primers 094-LC16-1PCR-SF and DPL16-Hpal-rev (see appendix 8.2). The PCR product was double-digested with SacI and AvrII and ligated into the corresponding sites of the pMM137-KSF-IgG vector (kindly provided by Philochem AG), giving rise to the vector pMM137-AP2-LC. In a second step, the VH region of the A2 antibody was PCR-amplified using primers 044-LS-1PCR-F and DP47-XhoI-rev (see appendix 8.2). The resulting amplicon was digested with PvuII-XhoI and inserted into the corresponding sites of the pMM137-A2-LC giving rise to the pMM137-AP2-IgG (figure 6.1).

For the production of the AP2 antibody in IgG format, suspension adapted CHO-S cells (Invitrogen, Zug, Switzerland) were seeded at 5 x 10^6 cells/mL in 200 mL ProCHO4 (Lonza, Vervier, Belgium) medium supplemented with 4mM Ultraglutamine (Lonza, Vervier, Belgium). For transient gene expression procedures, 600 µg of plasmid DNA and 3 mL of PEI (1 mg/mL in water at pH 7; Polysciences, Eppelheim, Germany) were sequentially added to the cells. The transfected cultures were maintained at 31°C in shaking incubator (INFORS-HT, Basel, Switzerland) with 5% CO₂ and with agitation at 120 rpm. Cells were fed with 4g/L glucose (SIGMA) and 4 g/L Hypep 4601 ™ (SIGMA) on days 3 and 5 post transfection. The supernatant of transfected cells was harvested by centrifugation at day 7 and processed for antibody purification on protein A resin, as previously described (section 6.1.4).
**Figure 6.1. IgG reformatting.** A 2-step procedure for the IgG reformatting is applied. In the first step the AP2 VL fragment is inserted into pMM-137 leading to the pMM-137 AP2-LC vector. The resulted vector is then re-digested and the AP2 VH fragment is inserted, leading to the complete AP2 IgG vector.
6.1.7. Size-exclusion chromatography SEC of purified scFv, SIP and IgG formats

The various formats of the AP2 antibody were analyzed by size-exclusion chromatography on an ÄKTA FPLC system. A Superdex 75 column (Amersham Biosciences) was used for scFv(AP2), while a Superdex 200 column was used for SIP(AP2) and for IgG(AP2).

6.1.8. Surface plasmon resonance experiments

Purified antibody preparations were analyzed by surface plasmon resonance using a BIAcore 3000 instrument (BIAcore AB, Uppsala, Sweden). Real-time interaction analysis was performed on a CM5 microsensor chip, coated with avidin (2800 RUs), to which biotinylated peptides (1 µM concentration, leading to 28 RUs of coating) were added, prior to the analysis of antibody binding. On a reference flow cell line no antigen was coated, in order to allow background subtraction using the running buffer (0.01% NaN₃, 0.05% Surfactant P20 10%v/v, PBS). Freshly prepared monomeric fractions scFv(AP1) and scFv(AP2) were used immediately after size-exclusion chromatography for BIAcore analysis, in order to minimize avidity artifacts due to diabody formation. All data were analyzed using the BIAevaluation Software (version 3.2).

6.1.9. Fibrin clot inhibition assay

A thrombin-catalyzed fibrin clot formation assay was performed in presence or absence of antibody fragments. ScFv(AP2) and scFv(E8), an antibody specific to FSTL1 protein, dissolved in PBS, were mixed at various concentrations with fibrinogen (CALBIOCHEM, Merck Millipore, Darmstadt Germany; 2mg/ml) and with thrombin (Sigma-Aldrich; 10U/ml) for 20 minutes at 37 °C. In a negative control experiment, thrombin was omitted. Inhibition of fibrin clot formation was measured on the basis of the liquid volume collected from each well in the fibrin clot experiments at various
antibody concentrations, considering that in the absence of thrombin no clot formation is observed, while in the absence of inhibitory antibody all solution is converted into a gel (i.e., no supernatant is left).

6.1.10. Immunofluorescence staining of murine healthy organs and of subcutaneous tumors

Tissue sections of a thickness of 10 µm were cut in a cryostat, placed on Superfrost® Plus glass slides (Menzel Gläser; Braunschweig, Germany), air dried, fixed with ice-cold acetone at RT for 10 min and used immediately or stored at -80°C if not. Tissue sections were then rehydrated with PBS and blocked with 20% FBS in PBS. Purified anti-fibrin SIP(AP2) antibody were applied to sections at a concentration of 2 µg/mL in 3% bovine serum albumin (BSA). As negative control, SIP(KSF), specific to hen egg lysozyme, was used. Antibodies in SIP format were detected with rabbit anti-human-IgE IgG (Dako), followed by incubation with Alexa Fluor 488 donkey anti-rabbit IgG antibody (Molecular Probes). Blood vessels were stained with a rat anti-mouse CD31 antibody (Invitrogen), followed by donkey Alexa Fluor 594 anti-rat IgG antibody (Molecular Probes). Nuclei were counterstained with DAPI (BD Bioscience; data not shown in the Figures). All commercial binding reagents were diluted according to the manufacturer’s recommendation in 3% BSA solution. A rinsing step with PBS was performed between all incubation steps. Slides were mounted with Fluorescent mounting medium (Dako) and analyzed with a Zeiss AxioVision 4.7 image analysis software (Carl Zeiss AG).

6.1.11. Tumor models

The F9 murine teratocarcinoma cells (CRL-1720, ATCC), were grown in tissue flasks coated with 0.1% gelatin in Dulbecco’s Modified Eagle’s medium (DMEM 1X,
REF.41965-039 GIBCO® supplemented with 10% fetal calf serum (FCS, Invitrogen) and antibiotic-antimycotic (AA, Invitrogen) and maintained at 37°C in an atmosphere of 5% CO₂.

The SKRC52 human renal carcinoma cells, were maintained in RPMI medium (RPMI, REF.21875-034 GIBCO®) supplemented with 10% fetal calf serum (FCS, Invitrogen) and antibiotic-antimycotic (AA, Invitrogen) at 37 °C in an atmosphere of 5% CO₂.

Female mice received subcutaneous injections of 20 x 10⁶ F9 cells and 9 x 10⁶ SKRC52 cells in the right flank. The F9 tumors were allowed to grow for 5-7 days and SKRC52 tumors were allowed to grow for about 2 weeks, then the mice were used for biodistribution experiment.

6.1.12. Biodistribution experiment in F9 and SKRC52 tumor bearing mice

The in vivo targeting performance of the AP2 antibody was assessed by quantitative biodistribution studies as described before (Pasche, Woytschak et al. 2011). SIP(AP2) was radioiodinated with ¹²⁵I (PerkinElmer) and injected into the lateral tail vein of immunocompetent 129SvEv mice (Charles River Laboratories, USA) bearing subcutaneously grafted F9 tumors or immunocompromised BALBc/nude mice Charles (River Laboratories, USA) bearing subcuteous SKRC52 xenograft tumors; in all cases 4 mice per group. F9 murine teratocarcinoma and SKRC52 human renal cell carcinoma bearing mice were injected i.v. with 20 µg of immunoreactive SIP fragment. Mice were sacrificed 24h after injection, organs were excised, weighed and radioactivity was measured using a Packard Cobra γ counter. Radioactivity was expressed as percent of injected dose per gram of tissue [%ID/g +/- SEM (standard error of the mean)].
6.2. AP2 antibody localizes to inflamed joints in mice with arthritis

6.2.1. Mouse model of collagen-induced arthritis
Male DBA/1J mice (8 weeks old) were immunized by two subcutaneous injections at the base of the tail. Mice were injected with 0.05 ml emulsion of bovine type II collagen emulsified in Complete Freund’s Adjuvant (Hooke Laboratories, Lawrence, MA, USA) at day 0 and with 0.04 ml bovine collagen/Complete Freund’s Adjuvant (Hooke Laboratories, Lawrence, MA, USA), three weeks later, as booster injection. After the booster injection, mice were inspected daily and disease was monitored using the following scoring system: 0 = normal; 1 = one toe inflamed and swollen; 2 = more than one toe, but not entire paw, inflamed and swollen or mild swelling of entire paw; 3 = entire paw inflamed and swollen; 4 = very inflamed and swollen paw (adapted from Hooke Laboratories; Hooke Kit™ Bovine Collagen/CFA Emulsion (EK-0220)). The swelling of affected paws was monitored daily, using a caliper under isoflurane anesthesia. All animal experiments were performed in agreement with Swiss ethical regulations. Ethical approval for all experiments was given by the state veterinary office (reference number 208/2010; Veterinäramt des Kantons Zürich, Zürich, Switzerland).

6.2.2. Immunofluorescence staining of inflamed paws in a murine model of collagen-induced arthritis
Tissue sections of a thickness of 10 µm were cut in a cryostat, placed on Superfrost Plus glass slides (Menzel Gläser; Braunschweig, Germany), air dried, fixed with ice-cold acetone at RT for 10 min and used immediately or stored at -80°C if not. Tissue sections were then rehydrated with PBS and blocked with 20% FBS in PBS. Purified anti-fibrin SIP(AP2) antibody were applied to sections at a concentration of 2 µg/mL in 3% bovine serum albumin (BSA). As negative control, SIP(KSF), specific to hen egg
lysozyme, was used in identical conditions. Antibodies in SIP format were detected with rabbit anti-human-IgE IgG (Dako), followed by incubation with Alexa Fluor 488 donkey anti-rabbit IgG antibody (Molecular Probes). Blood vessels were stained with a rat anti-mouse CD31 antibody (Invitrogen), followed by donkey Alexa Fluor 594 anti-rat IgG antibody (Molecular Probes). Nuclei were counterstained with DAPI (BD Bioscience). A rinsing step with PBS was performed between all incubation steps. Slides were mounted with Fluorescent mounting medium (Dako) and analyzed with a Zeiss AxioVision 4.7 image analysis software (Carl Zeiss AG).

6.2.3. Autoradiographic analysis

DBA/1J mice (8 weeks old, about 20-22 g, three mice per group) with arthritis were administered 20 µg of radioiodinated antibody by tail-vein injection in 0.3 ml saline and sacrificed with carbon dioxide (CO2) exposition and neck dislocation 24 h later. In order to evaluate the localization of these compounds at the inflamed sites, the four paws were cut and exposed for 16 hours to a BAS-IP MS-2025 phosphorimaging plate (Fujifilm, Tokyo, Japan). Images were acquired using a phosphorimager system BAS 5000 Bio-imaging device (Fujifilm, Tokyo, Japan) and analyzed with the software AIDA image analyzer v.450.

6.2.4. Biodistribution experiment in collagen-induced arthritis bearing mice

For biodistribution procedure see paragraph 6.1.12.

Mouse strain used: DBA/1J mice.

Mice (n= 3) were injected i.v. with 20 µg of immunoreactive SIP fragment.
6.3. High affinity monoclonal antibodies against FSTL1 protein
isolated from phage display libraries

6.3.1. Expression and purification of FSTL1 antigen

Synthetic gene was purchased from Eurofins MWG Operon, cloned into pCEP4 vector (Qiagen), expressed in mammalian HEK293 cells (CRL-1573, ATCC) and purified on a Nickel-sepharose column (Qiagen) by means of the (His)$_6$-tag appended at the C-terminus of this protein.

6.3.2. Expression and characterization of E8 antibody in scFv, scFv-Fc and SIP formats

ScFv(E8) fragment was produced by inoculating a single fresh colony in 10 ml 2xTY medium, 100 µg/ml ampicillin and 5% glucose. This preculture was grown for at least 6 hours (or alternatively also overnight) at 37°C to an OD$_{600nm}$ > 0.9 and then diluted 1:100 in 800 ml 2xTY medium, 100 µg/ml ampicillin and 0.1% glucose and grown at 37°C to give OD$_{600nm}$ = 0.4. The culture was then induced by the addition of IPTG (final concentration 1 mM) and grown at 30°C overnight. The day after bacterial cells were centrifuged in a SLA-3000 rotor at 10'800 g for 20 min and the antibody-containing supernatant was collected. The supernatant was filtered (0.22 µm filter, TPP) and the scFv fragment was purified from the bacterial supernatant by affinity chromatography using Protein A Sepharose™ Fast Flow resin (Amersham Biosciences).

The cloning of the E8 antibody into Fc format was performed using the pcDNA 3.1.(+) vector, as previously described, genetically fusing the scFv fragment to a domain consisting in CH2 and CH3 segments. This domain promotes the formation of homodimers, resulting in a 110 kDa bivalent antibody. (For the primers used see list in appendix 8.2, “ScFv-Fc reformatting(Dpl16)”). The resulting plasmid was transfected in Chinese Hamster Ovary cells following a procedure which was identical to the one
described for scFv fragments (paragraph 6.1.4). Antibodies in scFv-Fc format are purified from the cell culture supernatant by affinity chromatography using Protein A Sepharose™ Fast Flow resin (Amersham Biosciences) as previously described in 6.1.4. For the SIP format see paragraph 6.1.5.

For size-exclusion chromatography SEC: a Superdex 75 column (Amersham Biosciences) was used for scFv(E8) and scFv(A4), while a Superdex 200 column was used for SIP(E8) and for ScFv-Fc(E8).

Purified antibody preparations were analyzed by surface plasmon resonance using a BIAcore 3000 instrument (BIAcore AB, Uppsala, Sweden). Real-time interaction analysis was performed on a SA microsensor chip, coated with FSTL1 protein (1.4 µM concentration, leading to 1800 RUs of coating). On a reference flow cell line no antigen was coated, in order to allow background subtraction using the running buffer (0.01% NaN₃, 0.05% Surfactant P20 10%v/v, PBS). Freshly prepared monomeric fractions scFv(E8) and scFv(A4) were used immediately after size-exclusion chromatography for BIAcore analysis, in order to minimize avidity artifacts due to diabody formation. All data were analyzed using the BIAevaluation Software (version 3.2).

6.3.3. Affinity maturation library construction

The scFv(E8) clone was used as template for the construction of the library. Sequence variability in the VH CDR1 and CDR2 of the library was introduced by PCR using partially degenerate primers (figure 3.23 and appendix 8.2 “E8 affinity maturation library construction”), in a process that generates random mutations at positions 31-33 of the VH CDR1 and at positions 31, 31b and 32 of the VL CDR1. VH-VL combinations were assembled in scFv format by PCR assembly. The assembled VH-VL fragments were doubly-digested with Ncol/NotI and cloned into Ncol/NotI-digested pHEN1 phagemid vector. The resulting ligation product was electroporated into
electrocompetent *E. coli* TG1 cells according to (Viti, Nilsson et al. 2000), giving rise to a library containing $1.3 \times 10^7$ individual antibody clones.

As for the NITRO library, affinity maturation libraries were stored as glycerol stocks, rescued and used for phage production according to standard protocols (Viti, Nilsson et al. 2000).

6.3.4. Affinity maturation library characterization and use

In order to assess the presence of inserts of correct size in the vector, a total of 20 clones were tested by PCR screening procedures, using primers LMB3long and fdseqlong (appendix 8.2). In order to assess the presence of a correct sequence, without frameshift mutations, 10 clones were selected at random and sequenced by GATC biotech (GATC Biotech AG (Headquarter) European Genome and Diagnostics Centre, Constance, Germany). For details of the procedures see paragraph 6.1.3.

Antibody phage selections were performed as previously described for the ETH-2-Gold library (see section 6.1.1). The sequence of the A4 antibody can be found in figure 3.25.

6.3.5. Immunofluorescence staining of murine arthritic specimens

For immunofluorescence staining procedure see 6.1.10.

The only difference consists in the antibodies applied. Purified anti-FSTL1 SIP(E8), anti-EDA SIP(F8) as positive control and anti-<em>hen</em> egg lysozyme SIP(KSF) as negative control, were applied to sections at a concentration of 2 µg/mL in 3% bovine serum albumin (BSA). Antibodies in SIP format were detected with rabbit anti-human-IgE IgG (Dako), followed by incubation with Alexa Fluor 594 anti-rabbit IgG antibody (Molecular Probes). Blood vessels were stained with a rat anti-mouse CD31 antibody (Invitrogen), followed by Alexa Fluor 488 anti-rat IgG antibody (Molecular Probes).
6.3.6. Autoradiographic analysis and biodistribution evaluation

DBA/1J mice (8 weeks old, about 20-22 g, three mice per group) with arthritis were administered 5 µg of radioiodinated antibody (4 µCi) by tail-vein injection in 0.2 ml saline and sacrificed with carbon dioxide (CO₂) exposition and neck dislocation 24 h later. For details about the autoradiographic analysis see paragraph 6.2.3.

For biodistribution procedure see paragraph 6.1.12.

Mouse strain used: DBA/1J mice.

Mice (n= 3) were injected i.v. with 5 µg of immunoreactive SIP fragment.
7. References


Hayes, A. (February 22, 2013). "New late-stage breast cancer treatment approved." CNN.


Robinson, P. J., et al. (2014). "Obesity is associated with a poorer prognosis in women with hormone receptor positive breast cancer." *Maturitas.*


Shields, R. L., et al. (2001). "High resolution mapping of the binding site on human IgG1 for Fc gamma RI, Fc gamma RII, Fc gamma RIII, and FcRn and design of IgG1 variants with improved binding to the Fc gamma R." J Biol Chem 276(9): 6591-6604.


8. Appendix

8.1. List of abbreviation

°C: Celsius degree

%ID/g: Percentage of injected dose per gram

ADC: Antibody-drug conjugate

ADCC: Antibody-dependent cellular cytotoxicity

ADEPT: Antibody-directed enzyme prodrug therapy

ATCC: American type culture collection

CDR: Complementarity-determining regions

CH: Constant heavy chain

CHO: Chinese hamster ovary cell line

CL: Constant light chain

CV: Column volume

Db: Diabody

DMARD: disease-modifying anti-rheumatic drugs

DMEM: Dulbecco’s Modified Eagle Medium

DMF: Dimethylformamide

DNA: Deoxyribonucleic acid

ECM: Extracellular matrix

EDA: Extra domain A of fibronectin

EDB: Extra domain B of fibronectin

EDTA: ethylenediaminetetra acetic acid

EGF: Epidermal growth factor

ELISA: Enzyme-linked immunosorbent assay

Fab: Fragment antigen-binding portion of an antibody

FBS: Fetal bovine serum
Fc: Crystallizable fragment of Ig molecule
FcR: Fc receptor
FcRn: Neonatal Fc receptor
FDA: U.S. Food and Drug Administration
FPLC: Fast protein liquid chromatography
FSTL: follistatin-like 1 protein
Fv: Variable fragment of Ig molecule
GSH: reduced glutathione
HER2: human epidermal growth factor receptor 2
HPLC: High-performance liquid chromatography
HRMS: High-resolution mass spectrometry
i.v.: Intravenous
IC50: Half maximal inhibitory concentration
Ig: Immunoglobulin
kDa: kiloDalton
Kd: Dissociation constant
mAb Monoclonal antibodies (mAbs)
MBC: Metatstatic breast cancer
MPBS: Milk-PBS
MWCO: Molecular weight cut off
NHS: N-hydroxysuccinimide
NMR: Nuclear magnetic resonance
PBS: Phosphate buffered saline
PCR: Polymerase chain reaction
PEG: Polyethylene glycol
REF: reference/catalog number
RT: Room temperature
RU: Resonance units
s.c.: Subcutaneous
scFv: Single-chain variable fragment
SDS: Sodium dodecyl sulfate
SDS-PAGE: Sodium dodecylsulfate polyacrylamide gel electrophoresis
SEC: Size-exclusion chromatography
SIP: Small immonoprotein
SMCC: succinimidyl trans-4-(maleimidylmethyl)cyclohexane-1-carboxylate linker
TEA: Triethylamine
TCEP-HCl Tris-(2-carboxyethyl)phosphine hydrochloride
TFA: Trifluoroacetic acid
TnC: Tenascin-C
VH: Variable heavy chain
VL: variable light chain
8.2. List of primers

**NITRO library construction:**

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<th>Step</th>
<th>Primer Name</th>
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<td>LMB-long</td>
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<td>b1)</td>
<td>DP47CDR3ba1</td>
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<td>b2)</td>
<td>DP47CDR3ba2</td>
<td>5' TCC CTG GCC CGA GTA GTC AAA MINN MINN MINN MINN TTC CGC ACA GTA ATA TAC GGC GTT 3'</td>
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<td>DP47CDR3ba3</td>
<td>5' TCC CTG GCC CGA GTA GTC AAA MINN MINN MINN MINN TTC CGC ACA GTA ATA TAC GGC GTT 3'</td>
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<td>c)</td>
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<tr>
<td>d1)</td>
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<td>f)</td>
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<td>g)</td>
<td>Dpl1StopMobba</td>
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**AP2 IgG reformatting:**

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<td>5'-GTCTGAGCTCACTCAGGAC 3'</td>
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<td>DPL16-HPaI-rev</td>
<td>5'-CAGGCTAGGAGTCACTTTGTCC 3'</td>
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<tr>
<td>044-LS-1PCR-F</td>
<td>5'-GGTGAGCTTGGAGTCTGGG 3'</td>
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<td>DP47Xhol-rev</td>
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**SIP reformatting (Dpl16):**

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<tr>
<td>CH4-VL-Ba&lt;</td>
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<tr>
<td>VL-CH4-Fo&gt;</td>
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<tr>
<td>Not-Stop-CH4-Ba&lt;</td>
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**SIP reformatting (Herceptin):**

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<tr>
<td>CH4-VL-Ba&lt;</td>
<td>5'-GCACGCACGCGCCGCGCGCGCAGCCTCAGCCGACCCACTTAGTCTGCTCTGGGCTGATGTCTCTGGGCC 3'</td>
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<tr>
<td>VL-CH4-Fo&gt;</td>
<td>5'-CAAGGCAGGAGGGAGGAGGTAGGGTACTTCCGGAGGCTGGGCTGATGTCTCTGGGCTGATGTCTCTGGGCC 3'</td>
</tr>
<tr>
<td>Not-Stop-CH4-Ba&lt;</td>
<td>5'-TTTTTGTTTTGCGCGCGGGTCTGACCGCCACCCCTCTCGATGACTC 3'</td>
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**ScFv-Fc reformatting (Dpl16):**

**LsDP47 Fo>:**  
5’ TCCTCTGTTCCCTCGCTGCTGCTACAGGTGTGCACTCGGAGGCGAGCTTTGGAGTCTGG 3’

**Hinge-DPL16-Ba<:** 5’ GTTTTGTCAACAGATTGGGCTCGCTAGCGAGCTTTGGGTCGCTCCC 3’

**Hinge-CH2-Fo>:** 5’ GAGCCAAATCTTGTGACAAACTCACACATGCCACCCACGCTGCCCAGCACC 3’

**Not-Stop-CH3-Ba<:** 5’ TTTTCTTTTGGGCGCTTTATTTACCGGGAGACAGGGAGGAGG 3’

**Hind-LSDp47-Fo>:**  
5’ CCAAGCTGTGCCATGGGCTGGAGCCTCCTCTGCTCTGCTGCTGCTGC 3’

**E8 affinity maturation library construction:**

a) LMB3long

f) Fdseqlong

b) DP47-CDR1-Ba<: 5’- TGGTCCGCCAGCTCCAG -3’

c) DP47-CDR1-Fo>: 5’- CTGGAGGCTTGGGGCCCGCTACGCTCATMNNMNNMNGCTAAAGGTGAATCC -3’

d) DPL16-CDR1-Ba<: 5’- TGGTACCAGCAAGCCAGG -3’

e) DPL16-CDR1-Fo>: 5’- TCCTGGCTTCTGCTGGTCTGACGACGCTTGGCMNNMNNMNTCTGAGGCTGTCT -3’
8.3. Arming antibodies with the maytansinoid DM1 drug

8.3.1. Introduction

EDA domain of fibronectin

Fibronectin is a high-molecular weight (~440 kDa) glycoprotein of the extracellular matrix. It exists as a protein dimer, consisting of two identical monomer units linked by disulfide bonds (Pankov and Yamada 2002). Two different types of fibronectin are present in vertebrates: the soluble plasma fibronectin (produced in the liver by hepatocytes) and the insoluble cellular fibronectin, which is a major component of the extracellular matrix (ECM). The fibronectin protein is produced from a single gene, but alternative splicing of its pre-mRNA leads to the creation of several isoforms. In the corresponding gene three different regions are present, which may generate splice variants, by the insertion of additional Type III domains (figure 8.1). In cellular fibronectin, one or both of the "extra" type III modules (EDA and EDB) may be present, but these domains are virtually absent in plasma fibronectin. Indeed, in healthy conditions, EDA and EDB are virtually absent in healthy adult tissues (with the exceptions of placenta, the endometrium in the proliferative phase and some vessels in the ovaries; (Zardi, Carnemolla et al. 1987, Schwager, Villa et al. 2011). By contrast, alternative splicing events are a characteristic feature of fibronectin in conditions of tissue remodeling and angiogenesis. The corresponding protein variants are particularly abundant in the subendothelial ECM of tumor blood vessels (Rybak, Roesli et al. 2007, Villa, Trachsel et al. 2008, Schliemann, Wiedmer et al. 2009, Gutbrodt, Casi et al. 2014).

The EDA domain of fibronectin has a restricted pattern of expression in plasma and normal human tissues. However, the majority of aggressive murine and human tumors is strikingly positive for the antigen (Borsi, Castellani et al. 1998, Rybak, Roesli et al. 2007, Schliemann, Wiedmer et al. 2009, Schwager, Villa et al. 2011). In our laboratory, a fully human high affinity antibody against
EDA (termed F8), was isolated in collaboration with Philochem AG \textsuperscript{(Villa, Trachsel et al. 2008)}. The ability of the F8 antibody to selectively localize to tumor blood vessels has been extensively validated in biodistribution studies \textsuperscript{(Villa, Trachsel et al. 2008)}. A number of different payloads have been coupled to the F8 antibody, in order to test the therapeutic activity of the corresponding product \textsuperscript{(Villa, Trachsel et al. 2008, Steiner, Gutbrodt et al. 2013, Gutbrodt, Casi et al. 2014, Hemmerle and Neri 2014, Hess and Neri 2014, Perrino, Steiner et al. 2014, Pretto, Elia et al. 2014)}.

The EDA domain of fibronectin is highly conserved from mouse to man (96% amino acid identity). The F8 antibody cross-reacts with both antigens with the same affinity, thus facilitating the use of F8 derivatives both in mouse models of disease and in patients. Our group has recently shown that both angiogenesis and oncofetal fibronectin expression are not only restricted to oncology. Indeed, highly debilitating conditions such as endometriosis \textsuperscript{(Schwager, Bootz et al. 2011)} and rheumatoid arthritis \textsuperscript{(Schwager, Kaspar et al. 2009, Hemmerle, Doll et al. 2014)} strongly react with the F8 antibody in immunohistochemical analysis.

\textbf{Figure 8.1. Schematic representation of the fibronectin domain structure.} The three different splicing forms (Type I, II, and III) are illustrated as well the F8 antibody recognizing the EDA domain. (Figure adapted from \textsuperscript{(Neri and Bicknell 2005)})
HER2

HER2 (human epidermal growth factor receptor 2, also termed HER2/neu or ERBB2) is a gene located at the long arm of human chromosome 17, coding for a 185 kDa transmembrane glycoprotein possessing intrinsic protein tyrosine kinase activity. HER2 is a member of the epidermal growth factor receptor (EGFR/ERBB) family, which includes HER1, HER2, HER3 and HER4 receptors. The large modular glycoprotein consists of three different parts: an extracellular domain, a single transmembrane region and an intracellular cytoplasmatic tyrosin kinase, which is flanked by non-catalytic regulatory regions (Yamamoto, Ikawa et al. 1986) (figure 8.2). Homo/heterodimerization of the receptors takes place after the transformation from a closed, inert conformation, to an open, active one. This transformation is accomplished upon ligand-binding on the extracellular domain and at least 12 ligands are known to bind mammalian ERBBs, including epithelial growth factors (EGF) (Riese and Stern 1998) and neuregulins (Falls 2003). ERBB2 (termed “orphan” receptor) is the only member that does not bind known ligands. As a consequence, ERBB2 is always in an active conformation, and for this reason appears to be the major signaling partner for other ERBB receptors, by forming heteromeric complexes with the other members of the family (Garrett, McKern et al. 2003). Dimerization results in the autophosphorylation of tyrosine residues within the cytoplasmic domain of the receptors and initiates a variety of signaling pathways.

Amplification, overexpression and inappropriate activation of this oncogene is associated with the development and severity of many types of cancers (i.e. ovarian, stomach, and aggressive forms of uterine cancer) (Careen K. Tang 1998). In particular, the protein is found in approximately 20-30% of breast tumors and HER2 expression correlates with aggressiveness of the disease and poor prognosis (Slamon, Clark et al. 1987).
Figure 8.2. ErbB tyrosine kinase receptors family (figure adapted from (Zhang, Berezov et al. 2007)). ErbB2 receptor (represented in green) is the only receptor of the family with a constant open conformation, which makes it the preferential binding partner for dimeric complexes formation.

Breast cancer: an overview

Breast cancer is the second most common cancer in the world and, by far, the most frequent cancer among women, with an estimated 1.67 million new cancer cases diagnosed in 2012 (25% of all cancers). Breast tumor ranks as the fifth cause of death from cancer overall (522,000 deaths) and while it is the most frequent cause of cancer death in women in less developed regions (324,000 deaths, 14.3% of total), it is now the second cause of cancer death in more developed regions (198,000 deaths, 15.4%) after lung cancer (Ferlay J 2012).

Risk factors may be divided into “non-preventable” and “preventable” ones. The first group includes gender (men have a much lower risk of developing breast cancer than women, representing the 1% of the total of malignancies diagnosed in the developed countries) (Richard G Margolese 2000), age (the risk of getting breast cancer increases with age)
heredity (two autosomal dominant genes, BRCA1 and BRCA2, account for most of the cases of familial breast cancer) and prior cancer history (people who have previously been diagnosed with breast, ovarian, uterine, or bowel cancer have a higher risk of developing breast cancer in the future). The “preventable” risk factors include environmental factors and lifestyle habits. Those aspects might be even more important than the influence of genetic factors. Indeed, diet (high fat diet might have a negative incidence on the development of the disease whereas brassica vegetables intake seems to be inversely related with the appearance of the malignancy) (Sieri 2014), obesity and lack of exercise (Robinson, Bell et al. 2014) as well as tobacco smoking (Johnson, Miller et al. 2011), alcohol intake (Lu, Ni et al. 2014) and exposure to pollution seem to play a central role in the development of the disease. Although many epidemiological risk factors have been identified, the cause of any individual breast cancer is most often unknowable.

Almost one third of the diagnosed breast tumors shows an overexpression of the HER2 protein, making the protein an extremely important biomarker and target for therapy of breast cancer patients.

**Trastuzumab emtansine**

Trastuzumab emtansine (trade name Kadcyla®, T-DM1) is an antibody-drug conjugate consisting of the monoclonal antibody trastuzumab (Herceptin®) linked to the cytotoxic agent mertansine (DM1) with a non-cleavable SMCC linker (LoRusso, Weiss et al. 2011). The drug is conjugated to trastuzumab by lysine side chains, with an average of 3.5 maytansinoid molecules per antibody (figure 8.3). The mechanism of action takes advantage of the target specificity against HER2 positive (HER2+) cells given by the trastuzumab monoclonal antibody unit (see section 2.1.3 “Antibodies for therapeutic applications: an overview of clinically approved monoclonal antibodies”) and the
extreme powerful toxicity of DM1. The maytansine molecules are macrolides of the ansamycin-type, which inhibit the assembly of microtubules by binding to tubulin at the rhizoxin binding site, inducing microtubule disassembly and disrupting mitosis. The accepted mechanism of action foresees internalization, followed by antibody digestion in the lysosome, leaving the drug and linker connected to a lysyl group. Once internalized, proteolytic degradation of the linker releases both trastuzumab and the active metabolite, the maleimidomethyl cyclohexane-1-carboxylate (MCC)-emtansine. MCC-emtansine contains both positive and negative charges and therefore does not readily cross plasma membranes, maintaining intracellular concentrations.

Trastuzumab emtansine was developed by Genentech, a subsidiary group of Roche, and is manufactured by Lonza (Franklin 24 Jul 2014). FDA approved marketing on February 22, 2013 based on the results of the EMILIA study (ClinicalTrials.gov identifier NCT00829166). The EMILIA clinical trial was an international, phase III, randomized, open-label study comparing Kadcyla® alone to lapatinib (a tyrosine kinase inhibitor) in combination with capecitabine (a prodrug of 5-fluorouracil) in 991 people with HER2+ locally advanced breast cancer or metastatic breast cancer (MBC) who had previously been treated with trastuzumab and a taxane chemotherapy. As result T-DM1 significantly prolonged progression-free and overall survival with less toxicity than lapatinib plus capecitabine (median overall survival: 30.9 months vs. 25.1 months) (Verma, Miles et al. 2012). With a $9,800 monthly price tag, a typical course of Kadcyla® (just over nine months) is estimated to cost $94,000 (Hayes February 22, 2013). Four other important phase III clinical trials are currently ongoing. In the TH3RESA clinical trial (ClinicalTrials.gov identifier NCT01419197), trastuzumab emtansine is compared with the treatment of the physician’s choice in metastatic breast cancer patients who previously received two regimens of HER2-directed therapy. From preliminary data TH3RESA demonstrates that T-DM1 offers statistically significant and clinically meaningful improvement in delaying disease progression compared to a treatment of
physician’s choice. In the MARIANNE clinical trial (ClinicalTrials.gov identifier NCT01120184), trastuzumab emtansine in combination with pertuzumab/pertuzumab placebo is compared in a first-line setting to trastuzumab plus a taxane. In the KAITLIN study (ClinicalTrials.gov identifier NCT01966471), trastuzumab emtansine plus pertuzumab following anthracyclines is compared with trastuzumab plus pertuzumab and a taxane following anthracyclines as adjuvant therapy in patients with operable HER2+ primary breast cancer. In the KATHERINE study (ClinicalTrials.gov identifier NCT01772472), trastuzumab emtansine is evaluated versus trastuzumab as adjuvant therapy in patients with HER2+ breast cancer who have residual tumor in the breast or axillary lymph nodes following preoperative therapy.

**Figure 8.3. Structure of trastuzumab emtansine (Kadcyla®). Adapted from** (Krop, Beeram et al. 2010). On the left side, the DM1 drug, in the center, the [N-maleimidomethyl)cyclohexane-1-carboxylate (MCC) linker and on the right side, trastuzumab antibody. An average of 3.5 DM1 molecules are conjugated to the Fc region of trastuzumab.
8.3.2. Results

We have prepared three antibody drug conjugates consisting of the potent maytansinoid drug DM1 and three different antibody moieties: Herceptin (abbreviated with Herc), F8 and KSF, all in SIP format.

Disulfide-linked ADCs were produced using a site-specific conjugation strategy (Bernardes, Casi et al. 2012). Specifically, the C-terminal disulfide was reduced with TCEP reagent and the free cysteine was reacted with Ellman’s reagent. The subsequent addition of thiol-containing DM1 yielded a homogeneous mixed disulfide-linked ADC (see section 8.3.3 “Antibody-DM1 conjugates preparation”). Conjugation reactions proceeded with high conversion (>95%). All conjugation steps were monitored by SDS-PAGE in non-reducing conditions as readout for conjugation quality (figure 8.4).

Figure 8.4. Gel analysis of thiol derivatives of maytansinoid coupled to SIP(F8) antibody, the negative control antibody SIP(KSF) and the SIP(Herc) antibody. Upon reduction or modification with Ellman’s reagent, the monomeric fragments are no longer joined covalently and migrate differently (~39 kDa) from the covalently linked, homodimeric unmodified SIP fragments (~75 kDa)
Size-exclusion chromatography analysis showed a homogeneous profile, with a retention time corresponding to the expected homodimeric species (figure 8.5) and LC-MS analysis confirmed the predicted molecular weight (figure 8.6).

**Figure 8.5. Analytical characterization of SIP(F8)-SS-DM1, SIP(KSF)-SS-DM1 and SIP(Herc)-SS-DM1.** Gel-filtration analysis of purified ADCs. The peaks eluting at a retention volume of ~14/15 mL correspond to the noncovalent homodimeric form of the ADCs.

**Figure 8.6. Analytical characterization of SIP(F8)-SS-DM1, SIP(KSF)-SS-DM1 and SIP(Herc)-SS-DM1.** LC-MS spectra of purified ADCs: deconvoluted and ion series (inset). In the red circle is indicated the mass measured.
In order to choose the most suitable mouse tumor models, a cytotoxicity assay was performed with the three ADCs produced. We found that the MCF7 cells are less sensitive to treatment with SIP(Herc)-SS-DM1 (IC50 values of $4 \times 10^{-8}$ M), compared to SKRB3 cells (IC50 value of $80 \times 10^{-9}$ M) and to SKOV3 (IC50 value of $88 \times 10^{-9}$ M). For the SIP(F8)-SS-DM1 conjugate, IC50 values were respectively of $87 \times 10^{-9}$ M, $44 \times 10^{-9}$ M and $189 \times 10^{-9}$ M for SKOV3 cells, SKRB3 cells and MCF7 cells. For the SIP(KSF)-SS-DM1 conjugate, IC50 values were respectively of $214 \times 10^{-9}$ M, $16 \times 10^{-9}$ M and $71 \times 10^{-9}$ M for SKOV3 cells, SKRB3 cells and MCF7 cells (figure 8.7).

**Figure 8.7. Cytotoxicity assay of methylated drugs in three different cell lines.** The in vitro response to SIP(F8)-SS-DM1, SIP(KSF)-SS-DM1 and SIP(Herc)-SS-DM1 in the HER2-expressing cell lines: an ovarian carcinoma line, SKOV3 (HER2 2+) and two breast carcinoma lines, SKRB3 (HER2 3+) and MCF7 (normal HER2 expression).

In order to evaluate the antigens expression in the SKOV3 tumor model, we performed an immunohistochemistry analysis on frozen xenograft sections. Both SIP(F8) and SIP(Herc) did show a clear staining, confirming the presence of their targets (respectively EDA and HER2), while no staining was detectable when using the KSF antibody at identical concentrations.
Figure 8.8. Immunofluorescence staining of SKOV3 tumor sections. F8, Herceptin and KSF (used as negative control) are displayed in green. All antibodies were used in SIP format. Scale bar = 50 µm. Each slice is one representative example out of three sections analyzed.

We performed a quantitative biodistribution experiment, in immunocompromised Balb/c nude mice bearing subcutaneous SKOV3 tumors, using the DM1 conjugates. Analysis of %ID/g of tissue 24 hours after intravenous administration revealed a preferential accumulation of F8-DM1 at the tumor site, when compared with Herc-DM1 conjugate for which no accumulation was observed. In the case of the F8-DM1 conjugate, tumor: blood ratio was 5.09 while for the Herc-DM1 conjugate was 2.04.

Figure 8.9. Biodistribution study. A single dose of 7 mg/kg of radioiodinated SIP(F8) and SIP(Herc) antibodies, both conjugated with DM1, was injected intravenously into Balb/c nude mice bearing SKOV3 tumors. 24h after, mice were sacrificed, organs excised and radioactivity counted. Results are expressed as percent of injected dose per gram of tissue (%ID/g) ± SEM. Values tumor: blood ratio were respectively 5.09 and 2.04 for SIP(F8)-SS-DM1 and SIP(Herc)-SS-DM1.
We tested the therapeutic activity of the ADCs based on DM1, in immunocompromised Balb/c nude mice bearing subcutaneous SKOV3 tumors. When the tumors reached a size of 80-120 mm$^3$, seven intravenous doses of 7 mg/kg every 72 hours were administered.

In a previous study performed by Elena Perrino in our laboratory, SIP(F8)-SS-DM1 was administered at the same concentration in the F9 tumor model (Perrino, Steiner et al. 2014). In that setting, the F8-based ADC product mediated a complete and long-lasting tumor eradication, with three out of five mice which were cured (i.e., remained tumor-free for > 300 days), whereas tumors in the remaining two mice started to regrow after day 20. In contrast, in the SKOV3 tumor model neither SIP(F8)-SS-DM1 nor SIP(Herc)-SS-DM1 were able to show a substantial delay in tumor growth.

Figure 8.10. Therapeutic results of maytansinoid drug conjugates in a ovarian adenocarcinoma model. Immunocompromised female Balb/c nude mice bearing subcutaneous SKOV3 adenocarcinoma cells, were treated intravenously with 7 mg/kg of SIP(F8)-SS-DM1, SIP(KSF)-SS-DM1 and SIP(Herc)-SS-DM1 (5 mice per group). Vehicle is PBS containing 10 % DMA and 5 % sucrose. Treatment started 4 weeks after tumor implantation (when tumors reached a size of 80 - 120 mm$^3$) and was performed every 72 hours for a period of 19 days for a total of 7 injections (arrows). Mice were sacrificed at day 63. A) Data represents mean tumor volumes ± standard deviation. B) Body weight of mice as an indicator of toxicity was monitored daily and is plotted as percentage of initial weight.
An ex vivo immunofluorescence analysis of tumor sections following a single intravenous administration (7 mg/kg) of the ADCs products, confirmed the selective accumulation of SIP(F8)-SS-DM1 and SIP(Herc)-SS-DM1 in the sub-endothelial extracellular matrix, while no selective accumulation was observed for the KSF-based ADC product.

Figure 8.11. Immunofluorescence analysis performed on SKOV3 xenograft after a single i.v. injection of SIP(F8)-SS-DM1 (i,v), SIP(Herc)-SS-DM1 (ii,vi) and SIP(KSF)-SS-DM1 (iii,vii). In the top panels (i-iv) is shown the overlay staining. Antibodies are displayed in green, vessels are stained in red using an anti-CD31 antibody and nuclei are stained with DAPI. In panel iv, a section of untreated tumor was used as negative control. In the bottom panels (v-vii) is displayed only the green staining, to better visualize the binding properties of the antibodies. Scale bar = 20 µm. Each slice is one representative example out of three sections analyzed.
8.3.3. Materials and methods

Cloning, expression and protein in vitro characterization

The complete sequence of F8 antibody, the isolation of KSF antibody, as well as the cloning, the expression, the characterization of the two antibodies in SIP format have been previously been described (Villa, Trachsel et al. 2008). The amino acid sequence of the scFv sequence of Herceptin was designed based on trastuzumab sequence, available on DrugBank web page (www.drugbank.ca/drugs/DB00072(BIOD00098,BTD00098)) and optimized for Homo Sapiens by Eurofins MWG Operon (Ebersberg, Germany). The gene was originally provided in pEX-A vector, double digested into pcDNA3.1.+ using HindIII and XhoI restriction enzymes. The resulting scFv fragment was then reformatted and expressed in SIP format, using the primers listed in appendix 8.2 (Herceptin SIP reformatting).

Antibody-DM1 conjugates preparation

Protein solutions were thawed and filtered with a 0.22 µm filter (99722, TPP) and the concentration (0.4 - 0.5 mg/ml) was determined by measuring the UV absorbance at 280 nm (Nanodrop 2000c, Thermo Scientific). Polypropylene round-bottom tubes (352059, BDFalcon®) filled with the protein solution were placed inside a Schlenk flask and degassed by 3 alternating rounds of vacuum and argon flow. First, the antibody was reduced with 30 equivalents of 0.1 M tris (2-carboxyethyl)phosphine hydrochloride (TCEP•HCl, AB121644, ABCR) in PBS, the solution was briefly mixed and the protein was incubated at 4°C overnight (ideally 16h), under argon atmosphere. The next day the solution was modified with 2,500 equivalents over antibody monomer of the Ellman reagent (5,5′-Dithiobis(2-nitrobenzoic acid), D8130 Sigma-Aldrich). Due to the poor solubility of the drug, the antibody–Ellman conjugate was purified in PBS, containing 5% sucrose (w/v; AppliChem) and 10% N,N-dimethylacetamide (DMA; Acros Organics). 10 equivalents of thiol drug DM1 (obtained from Concortis Biosystem, Corp.)
(San Diego, CA, U.S.), over antibody monomer were then weighed into a plastic vial and dissolved in DMA immediately before addition to the purified antibody-Ellman conjugate. The solution was stirred vigorously at RT and after 5 minutes the reaction was quenched with the addition of 500 equivalents (relative to the antibody monomer) of iodoacetamide (GERPN6203, Sigma-Aldrich). For the purification of maytansinoid conjugates, a HiPrep® desalting column (GE Healthcare) was equilibrated with PBS containing 5 % sucrose (weight/volume) and 10 % N,N-dimethylacetamide (DMA). The concentrated reaction mixture (10 ml) was injected onto the pre-equilibrated column at a flow rate of 2 mL/min and eluted over 3 column volumes (CV; 1 CV= 53 mL). Fractions of 1 mL of the eluting conjugate were collected manually, with a typical recovery of 75%.

![SIP Diagram](image)

**Figure 8.12. SIPantibody-DM1 conjugation mechanism.** Site-specific modification of C-terminal cysteines of a SIP molecule via mixed disulfide formation with a potent thiol drug. (Adapted from Bernardes, Steiner et al. 2013)

**ADCs Characterization**

All ADCs were analyzed by SDS–PAGE (Invitrogen), size-exclusion chromatography (Superdex200 10/300GL; GE Healthcare), and protein mass spectrometry. The binding properties of the SIP(Herc)-SS-DM1 was analyzed by surface plasmon resonance (BIACore 3000 System; GE Healthcare) on HER2-coated CM5 sensor chip.
Figure 8.13. Biacore analysis of purified SIP(Herc)-SS-DM1 towards HER2 protein. The binding capacity of the antibody as ADC in SIP format was proved to be preserved.

Cytotoxicity assay

A cryo-stock of MCF-7 was kindly provided by Paul Scherrer Institute (PSI, 5232 Villigen, Switzerland). The cells were grown in DMEM, High glucose medium (41695-039, Gibco) supplemented with 2mM ultraglutamine (BE17-605E/U1, Lonza), 1X Antibiotic-antimycotic 100x (15240-062, Gibco), 10% FBS (10270-106, Gibco) and sodium pyruvate 100mM solution (11360-039, Gibco). A cryo-stock of SK-RB-3 was kindly provided by Philochem AG (Otelfingen, Switzerland). The cells were grown in RPMI 1640 medium, Glutamax (61870-044, Gibco) supplemented with 1x MEM non-essential amino acid solution, 110x (11140-035, Gibco), 1x HEPES solution sodium salt, 1M (051M8606, Sigma-Aldrich), supplemented with 2mM ultraglutamine, 1X antibiotic-antimycotic 100x, 10% FBS and sodium pyruvate 100mM solution (11360-039, Gibco). SKOV3 cells were grown in McCoy’s 5A medium (M8403, Sigma-Aldrich) supplemented with 10% FBS, 1x antibiotic-antimycotic and 2mM ultraglutamine. All cells were cultured at 37°C, 5% CO₂. To test the ideal concentration of cells for the cytotoxicity assay, four different concentrations (15000, 10000, 5000 and 2500 cells/well) were plated in a 96 wells plate (Flat bottom, TPP, 92096). Cells were
resuspended in 100 µl of corresponding medium, incubated at 37°C, 5% CO2 and checked after 24h, 48h, 72h and 96h. The day before the assay, cells were seeded into 96-well plates at the concentration of 8000/well, 9000/well and 8000/well in 100 ml of corresponding medium, respectively for MCF-7, SKBR3 and SKOV3 cells line. After 24h, time in which the cells were allowed to adhere, drugs were diluted in medium and added to the cells at different concentrations (every concentration measurement was performed in triplicate). Three days after the addition of the drugs, viable cells were quantified in a CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (MTS, G5430, Promega). From the absorbance measured at 490 nm using the VERSAmax plate reader, the % of cell viability was calculated with the equation:

\[
\text{Viability} \% = 100 \times \frac{\text{Average (abs) cells and drug} - \text{Average (abs) medium}}{\text{Average (abs) cells and medium} - \text{Average (abs) medium}}
\]

IC50 values were determined by fitting a sigmoidal curve to the data using KaleidaGraph v 4.1.2 (Synergy Software). Sigmoidal curves were derived from this equation:

\[
\frac{m1 + m2}{1 + \left(\frac{MO}{m3}\right)^{m4}}
\]

where m1 is the zero lower plateau, m2 is the top plateau (100% viability), m3 is the IC50 value and m4 is the geometrical curve bending.

Immunofluorescence study of SKOV3 tumor model

In order to confirm the expression of the antigens (EDA and HER2) in this tumor model, an immunofluorescence analysis was performed (see section 6.1.10).

Animals and tumor models

Eight to ten-weeks-old female mice Balb/c nude mice received subcutaneous injections
of 5 x 10^6 SKOV3 cells plus Matrigel (used 1:4 v/v) in the right flank. The xenograft tumors were allowed to grow for 4 weeks, then the mice were used for therapy and biodistribution experiment.

**Biodistribution study**

The *in vivo* targeting performance of the antibody–DM1 conjugates was assessed by quantitative biodistribution studies as previously described (Pasche, Woytschak et al. 2011). SIP(F8)-SS-DM1 and SIP(Herc)-SS-DM1 antibodies were radioiodinated with ^125^I (PerkinElmer) and injected into the lateral tail vein of immunocompromised Balb/c nude mice, bearing subcutaneously grafted SKOV3 xenograft (3 mice/group) at the dose of 7 mg/kg (i.e. 177 nmol of ADC per kilogram of body weight of animal, corresponding to 130 µg of drug per kilogram of body weight of animal, ~2.6 µg per injection per mouse). Mice were sacrificed 24h after injection, organs were excised, weighed and radioactivity was measured using a Packard Cobra γ counter. Radioactivity was expressed as percent of injected dose per gram of tissue [%ID/g +/- SEM (standard error of the mean)].

**Immunofluorescence studies of treated tumors**

For *ex vivo* detection of the localization of SIP(F8)-SS-DM1 and SIP(Herc)-SS-DM1, a microscopic analysis was performed. Immunocompromised Balb/c nude mice, bearing subcutaneously grafted SKOV3 xenograft tumors, were treated with a single injection of 7 mg/kg of the SIP(F8)-SS-DM1 and SIP(Herc)-SS-DM1 or of the control SIP(KSF)-SS-DM1 conjugate (dose in analogy to the therapy experiment) and sacrificed 24 hours after the injection. Tumors were excised, embedded in cryo-embedding medium (Thermo Scientific) and cryostat sections (10 µm) were stained using the following antibodies: rabbit anti-human IgE (Dako Cytomation), to detect the antibodies, and rat
anti-mouse CD31 (BD Biosciences) to detect endothelial cells. Anti-rabbit IgG-Alexa Fluor 488 (Molecular Probes by Life Technologies) and anti-rat IgG-Alexa Fluor 594 (Molecular Probes by Life Technologies) were used as secondary reagents for microscopic detection.

**Therapy study**

When tumors were clearly palpable, 4 weeks after subcutaneous tumor implantation, and the tumors typically exhibited a size of 80 to 120 mm$^3$, mice were randomly grouped ($n = 5$) and injected intravenously into the lateral tail vein. The dose of antibody–DM1 conjugates was 7 mg/kg (i.e., 177 nmol of ADC/kg of body weight of animal, corresponding to 130 µg of drug/kg of body weight of animal, ~2.6 µg/injection/mouse) and mice were injected 7 times, in intervals of 72 hours. The body weight of mice was monitored daily and tumor volumes were measured daily with a digital caliper (volume = length x width$^2$ x 0.5). Results are expressed as volume in mm$^3$ ± SEM. Therapy was stopped at day 63.
8.3.4. Conclusion, discussion and outlook

Antibody-drug conjugates (ADCs) represent an innovative class of biopharmaceuticals, which aim at achieving a site-specific delivery of cytotoxic agents at the tumor site. To date, many ADC molecules in clinical development rely on antibody internalization in the target cells for drug delivery. In our laboratory promising results were achieved coupling the F8 antibody (a non internalizing antibody, specific to the alternatively spliced EDA domain of fibronectin) with the DM1 drug, which led to complete tumor eradications in immunocompetent mice bearing F9 teratocarcinoma (Perrino, Steiner et al. 2014). These findings are of therapeutic significance, since they represent the first example, in which anticancer curative results were obtained with non-internalizing antibody-drug conjugates. The demonstration that cancer cures can be obtained without antibody internalization, by the targeted delivery of a suitable disulfide-linked ADC to the sub-endothelial extracellular matrix in solid tumors, suggests that the selective delivery of a high concentration of a potent cytotoxic agent to the abluminal compartment of tumor neo-vasculature, may be able to mediate an extensive damage to the whole neoplastic mass.

The previous characterization of F8-based ADC products in the F9 model prompted us to test the performance of SIP(F8)-SS-DM1 conjugate in other tumor models. We decided to compare our own ADC products with similar products, directed against internalizing antigens. SIP(Herc)-SS-DM1 is the SIP version of T-DM1 (Kadcyla®, see section 8.3.1 “Trastuzumab emtansine”), an internalizing antibody, approved for the treatment of HER2-positive, late-stage (metastatic) breast cancer. Both F8 and Herc antibodies were used in Small Immune Protein (SIP) format, in order to facilitate C-terminal coupling strategies, while achieving favorable tumor:organs ratios \textit{in vivo}. All three ADCs needed for the project were successfully produced and further
characterized. *In vitro* experiments were performed to evaluate the effective toxicity on those compounds in different cell lines expressing HER2. According to the cytotoxicity assay results, SKRB3 cell line would have been the most suitable one for therapy experiment, being more sensitive to both drugs, SIP(F8)-SS-DM1 and SIP(Herc)-SS-DM1. However, the implantation of SKRB3 human breast cancer cell line proved to be difficult. Consequently we focused on another HER2 positive tumor cell line, the SKOV3 human ovarian carcinoma, which has previously been used to evaluate the tumor targeting performance of anti-HER2 antibodies. In order to investigate whether a correlation between *in vitro* sensitivity of tumor cells and *in vivo* performance could be observed, we tested the therapeutic activity of SIP(F8)-SS-DM1 and SIP(Herc)-SS-DM1 in mice bearing subcutaneously-grafted SKOV3 tumors. Immunohistochemistry analysis performed on these tumor specimens confirmed a high expression level for both target proteins, the HER2 receptor and EDA domain of fibronectin. However, surprisingly, in a quantitative biodistribution analysis performed in immunocompromised Balb/c nude mice bearing subcutaneous SKOV3 tumors, analysis of percentage injected dose per gram of tissue 24 hours after intravenous administration revealed a preferential accumulation of SIP(F8)-SS-DM1 at the tumor site, while SIP(Herc)-SS-DM1 conjugate failed to preferentially target tumors *in vivo*. The tumor:blood ratio for the SIP(F8)-SS-DM1 conjugate was 5:1 at 24, compared to the value of 2:1 observed for SIP(Herc)-SS-DM1. In addition to this experiment, also an *ex vivo* immunohistochemical analysis was performed, confirming the selective accumulation of SIP(F8)-SS-DM1 and SIP(Herc)-SS-DM1 in the sub-endothelial extracellular matrix of tumor blood vessels. Albeit SIP(F8)-SS-DM1 showed favorable tumor staining characteristics and a preferential accumulation at the tumor site, the product failed to show any therapeutic activity *in vivo*. Mice were sacrificed at day 63 of the therapy, because of failure in the achievement of a significant statistical difference.
between SIP(F8)-SS-DM1 or SIP(Herc)-SS-DM1 groups and the negative controls SIP(KSF)-SS-DM1 and buffer groups.

A possible explanation for the different \textit{in vivo} performance of the two ADC products in different tumor models could be related to the different tumor environment in F9 and SKOV3 tumors. F9 cells divide rapidly and may display a higher death rate, releasing more reducing agents into the extracellular space, thus triggering the release of more DM1 from the ADC product at the tumor site. When considering the Herceptin-DM1 conjugate, it is known from a Phase II trial of 837 patients with ovarian cancer treated with trastuzumab, that only 7\% of the 47 ERBB2-positive patients responded to treatment, making the antibody not a suitable option for ovarian cancer patients (Bookman, Darcy et al. 2003). However, the antibody seems to work in immunocompromised nude mice model, showing growth inhibitory activity (Faraejian, Zweemer et al. 2011), which makes it a good candidate for comparing therapy experiments in mice. The fact that in our setting the ADC was not able to induce the same effect might be due to the change in the format used (SIP instead as full IgG).

To conclude, the type of the tumor model chosen for therapy studies and more in general \textit{in vivo} studies, seems to influence prominently the final outcome of the experiment. Translating those results in the clinic may suggest that, in addition to important features (i.e. drug, linker and antibody moiety), also the patient sub-populations to be enrolled have to be considered in the evaluation of the potential success of an ADC molecule in cancer therapy.
8.4. Statistical studies

8.4.1. AP1 binding specificity

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8.4.2. AP1 DP47 mut vs AP1 DP47 WT

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8.4.3. AP2 binding specificity

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Putelli A., Kiefer J.D., Zadory M., Matasci M., Neri D.

A Fibrin-Specific Monoclonal Antibody from a Designed Phage Display Library Inhibits Clot Formation and Localizes to Tumors In Vivo.
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