Doctoral Thesis

An antibody-interferon gamma fusion protein for cancer therapy

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AN ANTIBODY-INTERFERON GAMMA FUSION PROTEIN FOR CANCER THERAPY

A dissertation submitted to the SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZURICH for the degree of DOCTOR OF NATURAL SCIENCES

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Zürich, June 2004
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1 SUMMARY
The majority of pharmacological approaches for the treatment of solid tumors suffer from poor selectivity, thus limiting dose escalation (i.e., the doses of drug which are required to kill tumor cells cause unacceptable toxicities to normal tissues). The situation is made more dramatic by the fact that the majority of anticancer drugs accumulate preferentially in normal tissues rather than in neoplastic sites, due to the irregular vasculature and to the high interstitial pressure of solid tumors.

The development of more selective anti-cancer drugs, with better discrimination between tumor cells and normal cells, is possibly the most important goal of modern anticancer research. One avenue towards the development of more selective, better anti-cancer drugs consists in the targeted delivery of bioactive molecules (drugs, cytokines, procoagulant factors, photosensitizers, radionuclides, etc.) to the tumor environment by means of binding molecules (e.g., human antibodies) specific for tumor-associated markers.

We chose to target the tumor neovasculature by targeting the extra domain B of fibronectin (EDB). EDB is one of the best characterized markers of angiogenesis and has been shown to selectively accumulate around new blood vessels in tumors and other pathologies, but to be absent in normal adult vasculature (with the exception of the female reproductive cycle, hair growth and wound healing).

In our laboratories, we have extensively characterized the human scFv antibody fragment L19 (scFv(19)), specific for the EDB domain. In addition to biodistribution studies in animal models, the radiolabeled scFv(L19) has been characterized in an immunoscintigraphy clinical trial in patients with cancer and a number of derivatives of scFv(L19), with a therapeutic potential, have been studied in animal models.

Among the bioactive molecules which can be fused to scFv(L19), interferon gamma (IFNγ) is a particularly attractive choice, as this cytokine is an approved biopharmaceutical in EU and USA, with a potential anti-cancer action which is limited by its toxicity to normal organs.

In this thesis, the following main issues have been addressed experimentally:

- The expression and in vitro characterization of mutants of murine interferon gamma (muIFNγ) fused to the scFv(L19) antibody fragment specific for the angiogenesis related protein EDB;
- The study of the targeting ability of the fusion protein L19-IFNγmut4 in tumor-bearing mice, examining the possible influence of interferon gamma receptors (IFNγRs) on the in vivo targeting efficiency;
A study of the therapeutic properties of L19-IFN\textsubscript{mut4} as a single agent or in combination with other immunocytokines and/or chemotherapy, on different subcutaneous and metastatic tumor models.

I could show that, among seven different mutants, in which the three cysteine residues of murine IFN\textsubscript{y} were either substituted by serines or completely removed, the engineering of a cysteine-free mutant of interferon \(\gamma\) (IFN\textsubscript{\(\gamma\)}), fused to the scFv(L19) antibody fragment (L19-IFN\textsubscript{\(\gamma\)}mut4) led to a properly expressed and active molecule. In biodistribution experiments performed on immunocompetent Sv129 and G129 (IFN\textsubscript{\(\gamma\)}R\textsuperscript{\(\gamma\)}T/+) mice bearing F9 subcutaneous tumors I could show that the tumor targeting performance is improved by > 4-fold in mice with a targeted deletion of IFN\textsubscript{\(\gamma\)}Rs suggesting an influence by the IFN\textsubscript{\(\gamma\)}Rs expressed on the surface of all cells (except red blood cells) on the antibody localization \textit{in vivo}.

Furthermore, I showed that L19-IFN\textsubscript{\(\gamma\)}mut4 displayed a potent anti-cancer therapeutic performance (remarkably better the untargeted IFN\textsubscript{\(\gamma\)}) in subcutaneous murine F9 tumors in Sv129 mice, but not in C51 and CT26 tumors in Balb/c mice. Moreover, L19-IFN\textsubscript{\(\gamma\)}mut4 was shown to strongly synergize with chemotherapy and with two other cytokines fused to the scFv(L19), interleukin 2 (IL2) and tumor necrosis factor \(\alpha\) (TNF\textsubscript{\(\alpha\)}) leading to a much better therapeutic outcome in the treated mice. Finally L19-IFN\textsubscript{\(\gamma\)}mut4, alone or in combination with L19-IL2, was able to cure F9 liver metastasis in Sv 129 mice, but not C51 lung metastasis in Balb/c mice.
1 ZUSAMMENFASSUNG

Die Entwicklung von selektiveren Krebsmedikamenten, die besser zwischen gesundem und malignem Gewebe unterscheiden können, ist sicherlich eines der wichtigsten Ziele in der modernen Krebsforschung. Eine Möglichkeit um eine selektivere und bessere Tumortherapie zu erreichen, ist die gezielte Anreicherung von bioaktiven Molekülen (chemische Wirkstoffe, Zytokine, Blutgerinnungsfaktoren, Photosensitizer, Radionuklide, usw.) am Tumorgewebe, durch bindende Moleküle (z.B. menschliche Antikörper), die spezifisch Tumor-assoziierte Antigene erkennen.

Unsere Wahl für ein Tumor-assoziiertes Antigen fiel auf die EDB Domäne von Fibronektin, einen Marker für neugebildete Blutgefäße. EDB ist einer der am Besten charakterisierten Marker für Angiogenese, und es konnte gezeigt werden, dass sich EDB selektiv um neugebildete Blutgefäße von Tumoren, und anderes pathologisches Gewebe anreichert. EDB ist abwesend in normalen Blutgefäßen (mit Ausnahme des weiblichen Menstruationszyklus, des Haarwuchses und der Wundheilung).

In unserem Labor wurde ein monoklonales Antikörper-Fragment scFv(L19) entwickelt, welches spezifisch EDB bindet. In Tiermodellen, sowie in einer ersten klinischen Studie an Krebspatienten, konnte dieses Antikörper-Fragment weitgehend charakterisiert werden. Unter den bioaktiven Molekülen, die mit dem scFv(L19) Antikörper-Fragment fusioniert werden können, ist Interferon gamma (IFNγ) ein interessanter Kandidat. Dieses Molekül ist ein zugelassenes Medikament in der EU und in den USA und besitzt potentielle anti-Tumor Eigenschaften, die aber durch seine hohe Toxizität für gesundes Gewebe limitiert sind.

In dieser Dissertation wurden die folgenden Punkte untersucht:

- Die Expression und die in vitro Charakterisierung von Mutanten von murinem Interferon gamma (muIFNγ) fusioniert mit dem scFv(L19) Antikörper-Fragment;
Die Anreicherung im Tumor von Fusionsprotein scFv(L19)-IFNγmut4 in Tumor-tragenden Mäusen, wobei ein möglicher Einfluss von Interferon gamma Rezeptoren (IFNγR) auf das Tumor-Anreicherungsverhalten in vivo analysiert wurde;

Die Anwendung von scFv(L19)-IFNγmut4 als Monotherapie oder in Kombination mit anderen Immunozytokinen und/oder Chemotherapie, in verschiedenen subkutanen und metastatischen Tumormodellen.

In meiner Dissertation konnte ich zeigen, dass von sieben verschiedenen Mutanten, in denen die drei Cysteine von dem murinen IFNγ entweder durch Serine ersetzt oder ganz entfernt wurden, eine Cystein-freie Mutante von IFNγ, fusioniert mit dem scFv(L19), zu einem gut zu exprimierenden und aktiven Molekül führt. In Biodistributions-Experimenten in immunokompetenten Sv129 und G129 (IFNγR−/−) Mäusen mit subkutanem Tumor, konnte ich nachweisen, dass die Anreicherung des Fusionsproteins scFv(L19)-IFNγmut4 im Tumor um einen Faktor > 4 besser ist, wenn der IFNγR deletiert ist. Dieses Resultat lässt auf einen Einfluss der IFNγR, die auf der Oberfläche aller Zelle exprimiert sind (mit Ausnahme der roten Blutkörperchen), auf die Antikörper-Lokalisierung in vivo schließen.

Außerdem zeigte die Applikation von scFv(L19)-IFNγmut4 eine starke therapeutische Wirkung in subkutanen F9 Tumoren in Sv129 Mäusen, aber keine Wirkung in C51 und CT26 Tumoren in Balb/c Mäusen. Eine bei weitem stärkere therapeutische Wirkung konnte erzielt werden, wenn scFv(L19)-IFNγmut4 in Kombination mit einem Chemotherapeutikum und zwei weiteren Fusionsproteinen, scFv(L19) mit Interleukin 2 (IL2) und Tumor necrosis factor α (TNFα) appliziert wurde. Auch F9 Lebermetastasen in Sv129 Mäusen konnten mit scFv(L19)-IFNγmut4 geheilt werden, während C51 Lungenmetastasen nicht auf die Therapie ansprachen.
2.1 TUMOR TARGETING

Cancer chemotherapy is based on the expectation that anti-cancer drugs will preferentially kill rapidly dividing tumor cells, rather than normal cells. Since a large portion of the tumor cells has to be killed in order to obtain and maintain a complete remission and unfortunately clinically used anti-cancer drugs have limited selectivity for the tumor, large doses of drugs are normally required, with significant toxicity towards proliferating non-malignant cells (Pratt et al., 1994). The development of more selective anti-cancer drugs, with better discrimination between tumor cells and normal cells, is possibly the most important goal of modern anticancer research.

One avenue towards the development of more selective, better anti-cancer drugs consists in the targeted delivery of bioactive molecules (drugs, cytokines, procoagulant factors, photosensitizers, radionuclides, etc.) to the tumor environment by means of binding molecules (e.g., human antibodies) specific for tumor-associated markers.

![Graph](image)

**Figure 2-1**: Tissue distribution of doxorubicin (µg/g of tissue) after a single i.v. injection of 4.5 mg/kg doxorubicin in tumor-bearing nude mice (adapted from Bosslet et al., 1998).

Even though the concept of a selective delivery of therapeutics to the tumor environment was first envisioned by Paul Ehrlich at the end of the 19th Century, several technologies had to be developed before this therapeutic strategy could become a reality. Indeed, the following considerations outline why discovery and validation of tumor-associated markers (as well as
the corresponding ligands) remain an important challenge for the development of better, targeted anticancer agents for the treatment of disseminated solid tumors:

-Most chemotherapeutic agents do not preferentially accumulate at the tumor site. Indeed, the dose of drug that reaches the tumor (normalized per gram of tissue) may be as little as 5-10% of the dose that accumulates in normal organs (Figure 2-1) (Bosslet et al., 1998). The high interstitial pressure and the irregular vasculature of the tumor account, in part, for the difficult uptake of drugs by tumor cells (Jain, 1987; Folli et al., 1993). On top of that, the activity of multidrug resistance proteins may further decrease drug uptake (Bradley et al., 1988).

-Recent advances in protein engineering have made it possible to generate high-affinity human antibodies against virtually any biomolecular target (Winter et al., 1994) (see 2.8.3). Furthermore, technologies are becoming available for the generation of high-affinity binding peptides (Collins et al., 2001), aptamers (Brody and Gold, 2000) and synthetic organic molecules (Shuker et al., 1996; Erlanson et al., 2003; Neri and Melkko, 2002; Melkko et al., 2004), which may be used as ligands for the development of targeted anti-cancer strategies.

-Ligand-based tumor targeting approaches (e.g., those based on high-affinity monoclonal antibody fragments) allow to achieve excellent ligand localization in the tumor environment, with tumor : organ ratios of > 10 :1 already few hours after intravenous injection (Buchegger et al., 1983; Adams, 1998; King et al., 1994; Wu et al., 1996; Birchler et al., 1999a; Demartis et al., 2001; Carnemolla et al., 2002; Borsi et al., 2002). The search for novel targets and ligands is just as important as the development of strategies which convert a ligand (capable of selective localization in the tumor environment) into a therapeutic agent which retains the selectivity for the tumor.

Until now, most of the efforts in the development of tumor targeting agents have focused on the targeting of markers located on the membrane of tumor cells (see 2.5.1). However, strategies aimed at the direct killing of individual tumor cells are difficult, since distant cells may be hardly accessible to ligands. Furthermore, the intrinsic genetic instability of cancer cells often results in heterogenous patterns of tumor marker expression. Consequently, markers which are selectively expressed around tumor blood vessels and in the tumor stroma may offer a number of potential advantages, such as better accessibility, stability and abundance. These issues will be addressed in more detail in the following chapters.
2.2 MONOCLONAL ANTIBODIES IN CANCER THERAPY

A quarter of a century after their discovery (Kohler and Milstein, 1975) monoclonal antibodies (mAbs) have become the most rapidly expanding class of pharmaceuticals for the treatment of many human diseases, including cancer.

In the 1980s first attempts to use murine mAbs in cancer were made (Miller et al., 1982). In 1986, the first American Food and Drug Administration (FDA) approved monoclonal antibody was released to treat patients: Muromonab-CD3 (Orthoclone® OKT3) to prevent or treat solid organ transplant rejection (Thistlethwaite et al., 1984; Smith, 1996). This initial success, led to a widespread commercial and academic interest, which however shriveled more rapidly then expected as soon as considerable toxicity (see 2.4.1), limited clinical efficacy and very high production costs together with low yields of antibody-producing cells from the fusion process turned out to be unexpected hurdles.

However, after almost 30 years of ups and downs today their therapeutic utility is finally being realized. Approximately 200 antibodies are in clinical trails and the FDA has approved several antibodies against cancer (Table 2-1), transplant rejection, rheumatoid arthritis, Crohn’s disease and antiviral prophylaxis.

The first anticancer therapeutic antibody, a chimeric anti-CD20 antibody for the treatment of non-Hodgkin's lymphoma (Rituxan®) (Reff et al., 1994), was approved by the FDA in 1997. After that, several other anticancer antibodies such as Tratuzumab (Herceptin®), a humanized anti-HER2/neu antibody for breast cancer and Alemtuzumab (Campath®) a humanized anti-CD52 antibody for B-cell chronic lymphocytic leukemia, have been approved so far. The last antibodies that got the FDA approval in February 2004 are Erbitux® and Avastin®, the latter being the first approved therapy designed to inhibit angiogenesis.

<table>
<thead>
<tr>
<th>Product</th>
<th>Target</th>
<th>Company</th>
<th>Therapeutic indication</th>
<th>FDA approved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mylotarg</td>
<td>humanized anti-CD33 mAb coupled to ozogamicin</td>
<td>Wyeth</td>
<td>Acute myelogenous Leukemia</td>
<td>2000</td>
</tr>
<tr>
<td>Campath</td>
<td>humanized anti-CD52 mAb</td>
<td>ILEX/Millenium Pharmaceuticals</td>
<td>Chronic lymphocytic leukemia</td>
<td>2001</td>
</tr>
<tr>
<td>Zevalin</td>
<td>$^{89}$Y labeled chimeric anti-CD20 mAb fragment (Fab)</td>
<td>IDEC Pharmaceuticals</td>
<td>Non-Hodkin’s lymphoma</td>
<td>2002</td>
</tr>
<tr>
<td>Erbitux</td>
<td>chimeric anti-EGFR mAb</td>
<td>ImClone Systems Incorporated</td>
<td>Colorectal cancer</td>
<td>2004</td>
</tr>
<tr>
<td>Product</td>
<td>Target</td>
<td>Company</td>
<td>Therapeutic indication</td>
<td>FDA approved</td>
</tr>
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<td>----------</td>
<td>---------------------------------</td>
<td>----------------------------------------------</td>
<td>-----------------------------------------------------</td>
<td>---------------</td>
</tr>
<tr>
<td>Avastin</td>
<td>humanized anti VEGF mAb</td>
<td>Genentech</td>
<td>Non-small-cell lung cancer/colorectal cancer</td>
<td>2004</td>
</tr>
<tr>
<td>Rituxan</td>
<td>chimeric anti-CD20 mAb fragment (Fab)</td>
<td>Genentech/ IDEC Pharmaceuticals</td>
<td>Non-Hodkin’s lymphoma, chronic lymphocytic leukemia</td>
<td>1997</td>
</tr>
<tr>
<td>Herceptin</td>
<td>humanized anti-HER2/neu mAb</td>
<td>Genentech</td>
<td>Metastatic breast cancer</td>
<td>1998</td>
</tr>
<tr>
<td>Bexar</td>
<td>$^{131}$I labeled anti-CD20 mAb</td>
<td>Corixa/GlaxoSmith-Kline</td>
<td>Non-Hodkin’s lymphoma</td>
<td>2003</td>
</tr>
</tbody>
</table>

Table 2-1: Monoclonal antibodies for cancer approved by the FDA. Source: [http://www.centerwatch.com/](http://www.centerwatch.com/)
2.3 MECHANISMS OF ACTION OF MONOCLONAL ANTIBODIES

Antibodies have traditionally been seen as glycoproteins that protect the body by blocking invasion by microbes. Nowadays, a number of potential mechanisms have been identified that allow mAbs to operate in vivo. Three principal mechanisms of action are known: blocking, targeting and signaling.

2.3.1 Blocking

The blocking activity of therapeutic antibodies is achieved by preventing cytokines, growth factors or other soluble mediators reaching their target receptors, which can be accomplished either by the antibody binding to the factor itself or to its receptors. Two good examples are in the use of infliximab, an anti-tumor necrosis factor α (TNFα) monoclonal antibody (Remicade®) for treating rheumatoid arthritis and Crohn’s disease (Maini et al., 1999; Sandborn and Hanauer, 1999) and bevacizumab (Avastin®), an anti-VEGF antibody approved by the FDA for the treatment of metastatic colorectal cancer in February of this year.

2.3.2 Targeting

Targeting means directing antibodies towards specific populations of cells. It is a versatile approach since antibodies can be engineered to carry different effector moieties such as enzymes, toxins, radionuclides, cytokines, or even DNA molecules to the target cells. Once the target cells are reached, the attached moiety can exert its effect. Examples of this clan of compounds include Mylotarg®, Zevalin® and Bexxar®. Since this thesis is based on the targeting ability of antibodies, these concepts will be discussed in more detail later (see 2.9 and 2.10).

2.3.3 Signaling

The ability of mAbs to crosslink membrane receptors and generate intracellular signals is part of the mechanism by which they control tumor growth (Cragg et al., 1999). First evidence
came from the use of anti-idiotype antibodies in the treatment of B-cell lymphoma; the authors demonstrated that tumor regression correlated with the ability of the mAbs to induce intracellular signaling as measured by an increase in protein tyrosine phosphorylation (Vuist et al., 1994). MAbs in the treatment of solid tumors may also have a potential to affect signaling. One example is Trastuzumab (Herceptin®) which by binding to certain epitopes of Her2/neu receptor induces a number of signaling phenomena in vitro (Sarup et al., 1991; Hynes and Stern, 1994).
2.4 ENGINEERING MONOCLONAL ANTIBODIES

Despite the promising developments of the last years in the field of monoclonal antibodies, the efficacy of the approved clinical anticancer antibodies needs to be enhanced since cure is still rare. As an example the antigen of trastuzumab (Herceptin®), the Her2/neu receptor is overexpressed only in 25% to 30% of human breast cancers and, when administered alone, the response rate in patients is only 11% (Baselga, 2000). Nevertheless Herceptin® is today, in combination with other drugs, the most promising therapy approach used in the treatment of breast cancer. The same holds true for rituximab (Rituxan®), for which it was shown in a Phase II clinical trial that only about half of the patients responded (McLaughlin et al., 1998). Ways to improve the functioning and the efficacy of monoclonal antibodies, either by changing the structure in order to bypass the immunogenicity problem or by manipulating the Fc portion of an antibody in order to enhance the effector functions, will be discussed in the following chapters.

2.4.1 Immunogenicity

Many therapeutic applications for mAbs require repeated administration, especially for chronic diseases such as autoimmunity or cancer. Because mice are convenient for immunization and recognize most human antigens as foreign, mAbs against human targets with therapeutic potential have typically been of murine origin. However, murine mAbs have inherent disadvantages as human therapeutics. They require more frequent dosing to maintain a therapeutic level of mAb because of a shorter circulating half-life in humans than human antibodies (Weinstein et al., 1987; Zuckier et al., 1989). More critically, repeated administration of murine immunoglobulin creates the likelihood that the human immune system will recognize the mouse protein as foreign, generating a human anti-mouse antibody (HAMA) response (Schroff et al., 1985; Shawler et al., 1985). At best, a HAMA response will result in a rapid clearance of the murine antibody upon repeated administration, rendering the therapeutic useless. Worse, a HAMA response can cause a severe allergic reaction (Jaffers et al., 1986; Abramowicz et al., 1992; Choy et al., 1998).

The molecular basis for this reactivity is the binding of peptides derived from the breakdown of the antibody, to the HLA receptors expressed by the patient's immune cells. If these epitopes are recognized as "foreign" by the immune system, specific helper T lymphocytes...
are activated, which initiate and direct the formation of antibodies against the antibody. These antibodies can bind and neutralize the antibody, resulting in, as already described above, either decreased efficacy, total ineffectiveness of the drug or allergic reactions.

The incidence of immunogenicity is variable and depends upon the nature of the target antigen, the disease process being treated and the schedule of administration (the risk of immunogenicity may increase with the frequency of administration).

It is also possible that mAb immunogenicity can play some part in the therapeutic effect. Some studies with the anti-EpCAM monoclonal antibody (a mouse anti-colorectal cancer antibody), have suggested that a HAMA response might actually improve the outcome (Riethmuller et al., 1998).

2.4.1.1 Chimeric and humanized antibodies

To help to circumvent the HAMA response, chimeric and humanized antibodies were developed (Figure 2-2). Chimeric antibodies are formed by fusing DNA that combines the mouse variable region with the human constant region of an immunoglobulin (Boulianne et al., 1984; Rodwell, 1989) thus consisting of smaller amounts of foreign protein sequences compared to the fully mouse antibody.

To further decrease the amount of the murine protein sequence in synthetic antibodies, humanized antibodies were developed first by CDR grafting (Jones et al., 1986; Riechmann et al., 1988; Carter et al., 1992). This technique incorporates the murine protein sequence of the complementarity-determining region (CDRs) (sequence of an antibody that directly interacts with the antigen) directly into a human immunoglobulin framework.
Figure 2-2: Chimerization and humanization of an IgG antibody. The blue represents mouse sequences and the red human sequences. In a chimeric antibody, the mouse heavy- and light-chain variable region (V-region) sequences are joined onto human heavy-chain and light-chain constant regions (C-regions). In humanized antibodies, the mouse complementarity determining regions sequences (three from the heavy-chain V-region and three from the light-chain V-region) are grafted onto human V-region framework regions and expressed with human C regions (Clark, 2000).

In addition to the CDR grafting technique for humanization mentioned above fully human antibodies can be generated by immunization of transgenic mice expressing human antibodies (Lonberg et al., 1994; Jakobovits, 1998; Green, 1999; Bruggemann and Neuberger, 1996), by colony filter screening (Giovannoni et al., 2001), or by antibody phage technology (Winter et al., 1994; Low et al., 1996). These last two techniques will be discussed in detail in chapters 2.8.3 and 2.8.5.

In principle, however, anti-idiotypic response could still develop upon repeated administration of human antibodies and has indeed been documented in patients (Stephens et al., 1995; Isaacs et al., 1992).

2.4.2 Effector function

Apart from the general mechanisms of action of antibodies described above (see 2.3), another important feature, which leads to death of tumor cells, is the induction of antibody-dependent cellular toxicity (ADCC). When an antibody binds to a cell, the Fc portion of the immunoglobulin can be bound by an effector cell (lymphocyte, monocyte, macrophage, etc.), leading to destruction of the target cells (Steplewski et al., 1983).

A variety of parameters can affect ADCC reactions, including the type of effector cell and its level of activation, the isotype of the antibody and its glycosilation pattern, and properties of the target cell line such as its susceptibility to lysis.

The interaction of antibody and antigen can also produce cell death through complement-dependent cytotoxicity (CDC). In this case, the Fc portion of the antibody interacts with
complement, thereby activating the complement cascade and leading ultimately to complement-mediated lysis of the cell (Ballare et al., 1995).

These two mechanisms, namely ADCC and CDC are common effector functions, both making use of the Fc portion of an antibody.

Many mAb with tumor specificities cannot trigger above mentioned effector functions of sufficient potency to be clinically useful. One way to increase the ADCC is with co-administration of cytokines that increase the activity and number of circulating lymphocytes (Surfus et al., 1996; Frost et al., 1997).

2.4.2.1 Manipulation of the Fc portion of antibodies

The engineering of the glycosilation patterns of the Fc portion of an antibody by affecting their affinity for the three classes of FcγR is another approach to increase the biological activity of mAbs:

Umana and colleagues (Umana et al., 1999) engineered the glycosylation patterns of a chimeric IgG1 isotype by overexpression of glycosyltransferases in the host cells used for antibody production. They demonstrated that by optimizing antibody glycosylation, ADCC activity could dramatically and specifically be increased. Ongoing studies will address whether these antibodies have enhanced antitumor activity in vivo.

Alteration of the normal carbohydrate on human IgG1 via removal of the fucose moiety resulted in an up to 50-fold improved binding to human FcgRIII receptor and enhanced in vitro ADCC (Shields et al., 2002).

Otherwise manipulation of interactions with Fcγ receptors and with the complement component C1q by introducing mutations in different amino acid residues into the Fc portion of IgGs provides another potential for designing antibodies with novel and effective combinations of attributes (Idusogie et al., 2001; Armour et al., 2003).
2.5 TARGETS FOR MONOCLONAL ANTIBODIES

Selecting a suitable target is an essential first step in the design of any antibody based therapeutic. The antigens are abnormally expressed or over-expressed proteins or carbohydrates, called tumor-related antigens. The prerequisites for tumor targeting of an antigen are specificity, abundance and stability together with good accessibility for ligand molecules transported by the bloodstream. Antigens for tumor targeting can be grouped into three main categories, according to their localization in the tissue: antigens on the surface of tumors, antigens in the tumor-neovasculature and antigens in the extracellular matrix (ECM) surrounding the tumor.

![Figure 2-3: Schematic representation of characteristic tumor areas. Besides the tumor cells, the neovasculature represents an additional source of tumor-associated antigens. Moreover, components of the modified extracellular matrix can be considered valuable targets for antibody-based molecular intervention. Finally, necrotic areas are characteristic for many rapidly growing tumors and may represent another category of tumor antigens.](image)

2.5.1 Tumors

From chapter 2.3.1 one can learn that most of the antibodies approved by the FDA or in clinical trials are directed towards antigen on the surface of tumor cells. This is due to the fact that most of the research has been performed on tumor cells in culture. EpCAM (Armstrong and Eck, 2003; Went et al., 2004), HER2/neu (Hung and Lau, 1999), Carcinoembryonic antigen (CEA) (Duffy, 2001), placental alkaline phosphatase (PLAP) (Kosmas et al., 1998) are only a few examples. One limitation of this approach is the instability and plasticity of tumor genomes. Events such as partial or complete deletion of chromosomes, amplification of genes, translocations or rearrangements of chromosomes, and
simple mutations ensure efficient selection and overgrowth of drug-resistant tumor cells during and after chemotherapy. Moreover, reaching the tumor cells is not an easy task because of high interstitial pressure (Jain and Baxter, 1988), combined with the long distance to cover once extravasation occurred. Since necrotic regions are often located in the center of tumors, another approach is to target the necrotic areas in the tumor. Some groups exploited the presence of degenerating and necrotic cells within tumors by utilizing mAbs that were found to bind to abundant nuclear antigens, which are retained in permeable, dying cells (Epstein et al., 1988; Frankfurt et al., 1997).

2.5.2 Blood vessels

The tumor vasculature is an attractive target for therapy: a) it is easily accessible by blood-borne anticancer agents and most tumor cells rely on an intact vascular supply for their survival (Denekamp, 1982; Chaplin and Dougherty, 1999); b) a single vessel provides the nutrition for and facilitates removal of waste products of metabolism from hundreds or thousands of tumor cells; c) endothelial cells with a normal complement of chromosomes, given their relative genetic stability, should be far less prone to developing mechanisms of resistance than tumor cells (Kerbel, 1997), and therefore resistance to therapy is unlikely to occur.

2.5.3 Extracellular matrix

During tumor progression the microenvironment surrounding tumor cells undergoes extensive modification due to two main processes: a) degradation of the pre-existing EM by a number of proteolytic enzymes, which are produced, activated and/or induced by tumor cells, and b) neosynthesis of EM components, that in many cases are not present in the normal EM, by either neoplastic or stromal cells. These modifications generate a so-called “tumoral environment” which is different from the normal ECM and can therefore be used as a promising target for the delivery of therapeutic molecules.
Most of the known antigens on tumor neovascular and extracellular matrix are markers of angiogenesis. Before discussing some of them, the phenomenon of angiogenesis will be described.

2.6.1 The process of angiogenesis

Angiogenesis is the growth of new blood vessels from preexisting ones. It occurs primarily during embryogenesis as an essential process for the development of the vascular network of arteries, veins, arterioles, venules and capillary blood vessels that nourish and protect the body's tissues (Bischoff, 1995).

Once the vascular network is in place in the adult, the endothelial cells (ECs) lining the blood vessels are quiescent and angiogenesis is normally triggered only locally and transiently during some processes such as the female reproductive cycle, hair growth, wound healing and inflammation (Bischoff, 1995). Where required, endothelial cells secrete proteases to degrade the extracellular matrix (ECM), then migrate into the perivascular space, proliferate and align themselves to form new vessels (Figure 2-4). When sufficient angiogenesis has occurred, the endothelial cells become quiescent again and may regress if no longer needed. This transient triggering of angiogenesis relies on a balance between inhibitory controls and angiogenic inducers (Folkman, 1995a; Folkman, 1995b).

Figure 2-4: Model for the formation of new blood vessels from pre-existing ones. The extracellular matrix (ECM) is degraded by proteases secreted by the endothelial cells (EC). Endothelial cells then migrate into the perivascular space where they proliferate and align themselves to form a new vessel.
There are pathological conditions, however, in which this equilibrium is altered; blood vessels grow unabated and angiogenesis sustains the progression of the disease (Folkman, 1995a; Folkman, 1995; Folkman, 1995b). Angiogenesis is recognized to play a key role in a wide range of human disorders including cancer, rheumatoid arthritis, diabetic retinopathy, age-related macular degeneration and psoriasis.

2.6.2 Angiogenesis in cancer

Many tumors in humans persist in situ for months or even years without neovascularization, surviving as asymptomatic lesions rarely larger than 2 mm³ (Folkman, 1971; Folkman, 1972; Folkman, 1972). In these “prevascular” tumors, the high rate of tumor proliferation is balanced by a high rate of tumor cell death, probably caused by the low level of blood perfusion. Only when a group of tumor cells switches to the angiogenic phenotype the tumor mass expands and overtakes the rate of internal apoptosis by developing blood vessels. It is at this stage that the majority of tumors become clinically detectable and capable of invading surrounding tissues and metastasizing (Folkman, 1972; Blood and Zetter, 1990; Hanahan, 1998).

The angiogenic switch in pathological conditions occurs when the balance between inhibitors and stimulators of angiogenesis, which normally accounts for the absence of angiogenesis in adult tissues, is disturbed.

Modulators of angiogenesis are secreted by endothelial cells, tumor cells and by the surrounding stroma. Among proangiogenesis factors one should mention VEGF, angiogenin, epidermal growth factor and interleukin 8. Some well known antiangiogenesis factors are endostatin, angiostatin, thrombospondin and tissue inhibitors of metalloproteinases that prevent the tumors from increasing their size (Folkman, 2002).

The development of metastases is also dependent on angiogenesis (Folkman, 1995a). First, metastatic cells are not shed from a primary tumor until the tumor has become neovascularized. Second, once metastatic cells have colonized a target organ, they will again only grow to a metastasis of clinically detectable size if they can induce neovascularization. In some cases, the clinical pattern of metastasizing cancers seems to be related to the intensity of angiogenesis in their vascular bed and to the balance between angiogenesis promoters and inhibitors (Blood and Zetter, 1990; Folkman, 1995a).
2.6.3 Markers of angiogenesis

Angiogenic processes are regulated by a number of cell-surface and extracellular adhesion molecules. Molecules specifically expressed in tissues undergoing angiogenesis (Zetter, 1997), which comprise ECM and neovasculature described above, can be useful for the diagnosis and prognosis of angiogenesis related diseases and also as targets for the selective delivery of therapeutic agents. For this reason, the identification of new angiogenesis markers is the goal of many studies.

To date, only few good-quality markers of angiogenesis, either located on endothelial cells or in the modified sub-endothelial extracellular matrix, have been described and sufficiently characterized. The biggest problem with most of the so far known markers is their non-negligible expression in normal tissues, which may negatively affect imaging and therapeutic applications of ligands specific for these markers.

In the following pages, some of the most prominent markers of angiogenesis currently known will be briefly presented.

2.6.3.1 Integrins αvβ3 and αvβ5.

Some integrins, in particular αvβ3 and αvβ5, have been proposed both as markers for ligand-based targetind strategies and as functional mediators of angiogenesis in tumors and in ocular disorders (Brooks et al., 1994; Friedlander et al., 1995; Friedlander et al., 1996; Sipkins et al., 1998). Immunohistochemistry studies have shown that a number of normal tissues stain positive for the antigens, though to a lower extent as compared to tissues undergoing active angiogenesis (Max et al., 1997). A Phase I immunohistoscintigraphy clinical trial in 20 patients with cancer, using the radiolabeled humanized antibody Vitaxin, failed to image the tumor lesions in all but one patient (Posey et al., 2001).

Arginine, Glycine and Aspartate (RGD) – containing peptides, capable of high affinity binding to αvβ3 integrin, have been used successfully for the radio imaging of tumors in animal models (Haubner et al., 2001a; Haubner et al., 2001b). While good tumor to blood ratios were observed at early time points (1-2h), tumor to organ ratios were sometimes poor (particularly for colon, kidney, liver, lung).
2.6.3.2 Prostate-specific membrane antigen (PSMA)

This enzyme has originally been used as a serum marker for prostate cancer, providing a clinical prognostic information complementary to the one of other markers (Murphy et al., 1998; Murphy et al., 2000; Holmes, 2001). A number of reports have indicated a strong expression of PSMA around blood vessels in a wide variety of carcinomas (Liu et al., 1997; Chang et al., 1999). PSMA expression in blood vessels was also reported. An immunoscintigraphy clinical trial with radiolabeled mAb J591-DOTA-\(^{111}\)In (anti-PSMA antibody) showed a 15-90% reduction in mean tumor volume after a single dose. The median survival time increased 2-3 times relative to untreated controls (Vallabhajosula et al., 2004).

2.6.3.3 Endoglin (CD105)

The initial excitement about the potential of endoglin as a marker of angiogenesis (Wang et al., 1993) has been slowed down by later reports of significant expression of the antigen in a number of normal organs (Burrows et al., 1995; Balza et al., 2001). While most of the quantitative biodistribution results obtained with radiolabeled anti-endoglin antibodies in tumor-bearing mice were rather poor (Bredow et al., 2000), good imaging results in tumor-bearing dogs have also been reported (Fonsatti et al., 2000).

2.6.3.4 VEGF and VEGF-receptor complex

Vascular endothelial growth factor (VEGF; available in different forms) is one of the main mediators of the vascularization of solid tumors. In the tumor micro-environment, an up-regulation of both VEGF and its receptors occurs, leading to a high concentration of occupied receptors on tumor vascular endothelium. VEGF-receptor complexes were shown to be a specific target on tumor endothelium for antibodies in vivo. In a recent study, a monoclonal antibody (2C3) was shown to have anti-tumor activity against tumor xenografts in mice (Brekken et al., 2000). This antibody has also been shown to localize to tumor blood vessels by microscopic analysis, but quantitative biodistribution results have not yet been reported. Biodistribution studies in tumor-bearing mice with anti-VEGF antibodies or with VEGF itself
as a ligand for its receptors have been disappointing (Cooke et al., 2001; Halin et al., 2002). A humanized neutralizing antibody to VEGF (Avastin®, Genentech) has recently been approved by the FDA (see 2.2) (Ferrara, 2002).

2.6.3.5 CD44

CD44 is a cell adhesion receptor of great molecular heterogeneity due to alternative splicing and post-translational modifications. In spite of its widespread pattern of expression in blood cells and tissues, a monoclonal antibody to a CD44 variant has been reported to display spectacular tumor targeting results in tumor-bearing mice, with prominent perivascular accumulation (Wakai et al., 2000). At this time point, it is not clear if the excellent targeting results (% injected dose in tumor > 75%, 1 hour after injection in tumor-bearing mice) are due to a predominantly luminal pattern of expression of the antigen (Tsunoda et al., 1999).

2.6.3.6 Phosphatidyl serine phospholipids.

Phosphatidylserine phospholipids are normally located in the inner leaflet of the cell membrane and, therefore, not readily accessible to specific ligands. However, in cells undergoing apoptosis or under stress, they may become exposed in the outer cell membrane leaflet (Zachowski et al., 1989). Recently, Thorpe and colleagues have postulated that phosphatidylserine may serve as marker of angiogenesis for ligand-based vascular targeting applications, on the basis of binding studies with Annexin V and mAbs on endothelial cells undergoing oxidative stress. This hypothesis is supported by a fluorescence microscopic analysis of tumor targeting experiments with mAbs injected in tumor bearing mice (Ran et al., 2002). The potential of phosphatidylserine for vascular targeting applications remains to be tested by quantitative biodistribution studies. A potential concern comes from the surface exposure of phosphatidylserine in activated platelets (Bucki et al., 2001; Monroe et al., 2002).
2.6.3.7 Large isoforms of tenascin C

Tenascin C is a component of the extracellular matrix, which exists in a “small” isoform (devoid of extra-domains) or in a more tissue-restricted “large isoform”, which contains additional domains inserted by alternative splicing. Although expression of the large isoform of tenascin-C is detectable in certain normal tissues (e.g., at the interface between derma and epidermis), the protein is much more abundant in several aggressive tumors, with a prominent staining of the tumor stroma and of the tumor neo-vasculature (Borsi et al., 1992). Radiolabeled mAbs specific for the large tenascin isoform have been investigated in the clinic for several years, both in diagnostic and radioimmunotherapeutic applications (Riva, 1992; Bigner et al., 1998; Paganelli et al., 1999; Paganelli et al., 2001). Recently, it has been discovered that the extra-domain C within the large isoform features and even more restricted pattern of expression, is undetectable in normal human tissues, but expressed in aggressive tumors such as high-grade astrocytomas and lung cancers (Carnemolla et al., 1999), with a predominantly vascular staining pattern.

2.6.3.8 Magic roundabout

Magic roundabout (MR or ROBO4) belongs to the roundabout family, which contains several closely related genes (three in man) that were previously thought to be only present in neuronal tissue and involved in axon guidance. Roundabouts have five IgG and three fibronectin like extracellular domains. They are large transmembrane receptors for ligands known as slits. The discovery of an endothelial specific roundabout has been demonstrated by a combination of Northern blotting, in situ hybridisation and immunohistochemistry, confirming a highly restricted pattern of expression (Huminiecki et al., 2002). MR is highly expressed during embryonal development, but is absent from adult tissues except at sites of active angiogenesis, including tumors. A similar pattern of expression has also been found for delta4, an endothelial specific member of the delta family (Mailhos et al., 2001).
2.6.3.9 Others

Novel genes encoding markers on the tumor endothelial cells (TEM1, TEM5 and TEM8), discovered by means of the serial analysis of gene expression (SAGE) technique, should be added to the above list of angiogenesis markers (St Croix et al., 2000). However, many of these markers have failed to show a pattern of expression specific for tumor vasculature, and restricted enough to make of them valuable targets for therapeutic intervention. An exception is represented by the extra domain B of Fibronectin (EDB) which has been shown to selectively accumulate around new blood vessels in tumors and other models of pathological states, but to be absent in normal adult vasculature (see 2.7).
2.7 THE EDB DOMAIN OF FIBRONECTIN, A MARKER OF ANGIOGENESIS

2.7.1 Fibronectins

Fibronectins (FNs) are abundantly expressed, high-molecular weight adhesive glycoproteins present in vivo in the ECM and in body fluids (Hynes, 1990). FNs are involved in different biological processes including the establishment and maintenance of normal cell morphology, cell adhesion, migration, hemostasis, thrombosis, wound healing and oncogenic transformation (Hynes, 1990; Magnusson et al., 1998). They form dimers composed of two nearly identical 250-280 kDa subunits joined by a pair of disulfide bonds near their carboxyl termini (Alitalo and Vaheri, 1982; Yamada, 1983; Hynes, 1985; Ruoslahti, 1988). Each monomer is folded into a series of functionally distinct domains (Figure 2-5), consisting of smaller modules (type I-III), which are serially repeated (Leahy et al., 1996). These domains are resistant to proteolysis and contain binding sites for extracellular matrix proteins such as collagen and thrombospondin, cell-surface receptors such as integrins, circulating blood proteins such as fibrin, and glycosaminoglycans such as heparin and chondroitin sulphate.

FN is critically important in vertebrate development, as demonstrated by the early embryonic lethality of mice with targeted inactivation of the FN gene (George et al., 1993). Embryonic death is associated with widespread defects in mesoderm-derived structures, including absence of somites and notochord and developmental defects in the heart and vascular system.
Figure 2-5: Model of the domain structure of a human fibronectin monomer. A fibronectin monomer is made up of a series of repeating units of three different types (type I, type II, type III). Two disulfide bonds at their carboxyl termini join two monomers. Three repeats can be either inserted or omitted in the molecule by a mechanism of RNA alternative splicing: EDB, EDA, and IIICS.

2.7.2 Alternative splicing of fibronectins

Although FNs are the product of a single gene, the resulting protein can exist in multiple forms. Their polymorphism is due to alternative splicing patterns in three regions (IIICS, EDA and EDB) of the FN primary transcript as well as to post-translational modifications. The alternative splicing is regulated in a cell-, tissue-, and developmentally specific manner. In transformed cells and in malignancies, the splicing pattern of FN-pre-mRNA becomes altered (Castellani et al., 1986; Borsi et al., 1987; Zardi et al., 1987; Vartio et al., 1987; Carnemolla et al., 1989; Oyama et al., 1989; Oyama et al., 1990; Borsi et al., 1992; Castellani et al., 1994; Kaczmarek et al., 1994) leading to an increased expression of FN isoforms containing the IIICS, EDA and EDB sequences.

2.7.3 EDB

EDB has been discovered during experiments of proteolytic cleavage of FN molecules obtained from different sources. Borsi et al. (Borsi et al., 1985) reported that FN from tumor-derived or SV-40-transformed human cells contains, in the cell-binding domain 4 (Figure 2-5), a site sensitive to the proteolytic enzyme cathepsin-D, undetectable in FN from plasma and from normal human cells. This suggested a new splicing site and a transformed cell-specific splicing pattern leading to the expression of a new sequence in FN from transformed cells. In 1987, Zardi et al. demonstrated that a large percentage of FN molecules from
transformed human cells contain in the middle of the cell-binding domain 4 an additional peptide sequence (the EDB domain) containing a site sensitive to proteolytic enzymes (Zardi et al., 1987). Since then, immunohistochemical studies first with a murine monoclonal antibody binding to FN only in the presence of EDB (Carnemolla et al., 1989) and then with recombinant antibody fragments binding to EDB (Carnemolla et al., 1996), have allowed the identification of EDB as an angiogenesis-associated molecule.

In the FN isoform containing the EDB sequence (B-FN), EDB is inserted between the type III modules 7 and 8 (FN7 and FN8). It is a complete type III homology repeat composed of 91 amino acids and coded for by a single exon, which can be either completely included or completely omitted from the mature mRNA.

The 3D-structure of EDB has been solved by NMR spectroscopy in solution, and is characterized by two antiparallel $\beta$ sheets that form a $\beta$ sandwich (Figure 2-6). One $\beta$ sheet is formed by three $\beta$ strands (A, B and E), and the other by four $\beta$ strands (C, C', F and G). The two $\beta$ sheets enclose a hydrophobic core of 25 amino acid side chains. EDB is highly acidic and contains only two positively charged residues.

![Figure 2-6: Structure of the EDB domain of fibronectin. The two $\beta$ sheets formed by 3 and 4 $\beta$ strands, respectively, are colored in red and cyan. The $\beta$ strands are labeled A to G following the nomenclature used for other FN type III domains. For the individual $\beta$ strands, the first and last sequence positions are indicated. The N and C termini are indicated.](image)

The negatively charged residues are uniformly distributed over the protein surface, except for a solvent-exposed hydrophobic cluster formed by residues Ile35, Phe54, Ile78 and Leu80 (Fattorusso et al., 1999).
Figure 2-7: Immunohistochemical studies using a scFv fragment against the EDB domain of fibronectin. A) A section of glioblastoma multiforme showing staining of the typical pseudo-glomerulus vascular structures. Scale bar: 25 μm. B) Immunohistochemical staining of neovascular structures (large arrows) of the cornea in a rabbit model of ocular angiogenesis. A small arrow indicates the corneal epithelium. Scale bar: 50 μm.

EDB, with some very rare exceptions, is undetectable in normal adult tissues. It exhibits a much greater expression in foetal and tumor tissues as well as during wound healing (Zardi et al., 1987; Carnemolla et al., 1989; Ffrench-Constant et al., 1989; Laitinen et al., 1991). When EDB containing fibronectin is expressed, it accumulates around neovascular structures. This selective expression pattern has been extensively demonstrated in studies on many different tumor types, in particular on invasive ductal carcinoma (Kaczmarek et al., 1994) and brain tumors (Figure 2-7a) (Castellani et al., 1994), as well as in ocular angiogenesis (Figure 2-7b) (Birchlcr et al., 1999). Birchler et al. have recently shown that EDB is abundantly expressed around the neovascularature and in the stroma of the majority of malignant tumors of the head and neck (Birchlcr et al., 2003). In all these studies, new blood vessels have been found to be EDB positive, whereas the larger majority of mature vessels are negative for the antigen. Consequently EDB can be considered a marker of angiogenesis (Pujuguet et al., 1996; D'Ovidio et al., 1998; Karelina and Eisen, 1998; Midulla et al., 2000).

The EDB domain of fibronectin is also one of the few markers of angiogenesis, which has been extensively characterized and validated by biodistribution analysis in vivo (see 2.8) (Viti et al., 1999).

A specific role of the EDB domain of fibronectin in angiogenesis has not yet been identified. However, its high level of conservation in different species (identical in human, dog, rabbit, rat and mouse, 96% homology with chicken and 80% with Xenopus L.) together with its highly restricted pattern of expression may be indicative of a specific functional role.

To examine the function of the EDB segment in vivo, Fukuda and colleagues (Fukuda et al., 2002) generated mice lacking the EDB exon. They found out, that although EDB-containing FNs are highly expressed throughout early embryogenesis, EDB-deficient mice developed
normally and were fertile. Despite the absence of any significant phenotype observed \textit{in vivo}, however, fibroblasts obtained from EDB-deficient mice grew slowly \textit{in vitro} and deposited less FN in the pericellular matrix than fibroblasts from wild-type mice. These results indicated that expression of EDB-containing isoforms is dispensable during embryonic development, yet may play a modulating role in the growth of connective tissue cells via the FN matrix.
2.8 ANTIBODIES AGAINST THE EDB DOMAIN

Since EDB can be useful for the diagnosis of angiogenesis related diseases and also works as a target for the selective delivery of therapeutic agents, several antibodies recognizing EDB were produced. In the following sections, all kind of antibodies that have been made against EDB are listed and the different techniques used to produce them are briefly described.

2.8.1 Polyclonal Ab

Rabbit polyclonal antibodies to two different bacterial fusion proteins containing intact EDB segments, as well as to a synthetic 36 amino acid peptide from the center of the EDB segment, were produced.

Antibodies raised to each of these three immunogens recognized fusion proteins containing the EDB segment, but failed to recognize full length B-FNs produced by mammalian cells, suggesting that oligosaccharide linked to Asn1359 within the EDB segment, or potentially to other residues in FN, might interfere with antibody recognition of this segment. Consistent with this hypothesis, N-deglycosylation of recombinant full and partial length B-FNs permitted their specific recognition by the above-mentioned polyclonal antibodies (Peters et al., 1995).

2.8.2 Antibodies from hybridoma technology

With the development of the hybridoma technology over a quarter of a century ago (Kohler and Milstein, 1975), the ability to produce large quantities of well-characterized monoclonal antibodies revolutionized diagnostic and therapeutic medicine. However, there are certain biological limitations that have to be considered.

In addition to the problem of human anti-mouse antibody (HAMA) response (described in chapter 2.4.1), another problem stems from the difficulty to raise rodent or human mAbs against highly conserved antigens, especially against human self-antigens. In particular, when trying to raise antibodies against markers of angiogenesis, which very likely are also involved in normal physiological angiogenic processes (e.g., during wound healing or embryonal development), this approach might be unfeasible due to tolerance.
Due to these reasons the production of mAbs directly recognizing the EDB domain in B-FN has not been possible so far with the hybridoma technology.

2.8.2.1 BC1

BC1 was isolated against the human B-FN isoform (Carnemolla et al., 1989) and was widely used to study angiogenesis (Castellani et al., 1994). However, this antibody does not recognize the "inserted" EDB domain directly; it appears to recognize an epitope within the type III repeat 7, which is adjacent to EDB (Figure 2-5 on page 32). This epitope is cryptic in FN molecules lacking EDB and is unmasked in molecules containing this domain (Carnemolla et al., 1992).

Furthermore, the antibody is highly specific to human B-FN and does not recognize mouse or chicken B-FN, thus precluding the study of angiogenesis in animal models.

The tumor-targeting potential of BC-1 was tested against oncofetal fibronectin in nude mice bearing B-FN expressing human tumor implants, showing favorable tumor targeting potential (Mariani et al., 1997).

2.8.2.2 IST-6

IST-6 recognizes only FN molecules in which the EDB sequence is lacking (Carnemolla et al., 1992).

The two mAbs BC-1 and IST-6 clearly demonstrate that the presence of the EDB sequence within FN molecules generates a conformational modification in the central part of the molecules that unmaskes previously cryptic sequences and masks others.

2.8.3 Antibodies from phage libraries

Phage antibody technology (Smith, 1985; McCafferty et al., 1990; Winter et al., 1994) offers a solution to the limitations imposed by the HAMA and the tolerance problem: human antibody fragments can be isolated in a process that does not require immunization of humans, and antibody fragments can be made against both foreign and human self-antigens.
Phage display is currently the most widely used in vitro method for the selection of high-affinity recombinant antibody fragments. It is a powerful technology that allows the selection of a particular phenotype from large collections ("libraries") of proteins or peptides expressed on the surface of filamentous phage particles.

![Phage display diagram](image)

**Figure 2-8:** Phage displaying a binding protein. Schematic representation of a phage bound to an immobilized antigen via the protein displayed on its surface. The interior of the phage harbors the phage- or phagemid-vector in which the gene of the displayed protein is fused to 3' end of the filamentous phage gene pIII coat protein.

Phage display libraries of antibodies can be regarded as artificial immune systems mimicking the humoral arm of the immune response. The filamentous phage surface serves to physically link the genotype (i.e. the gene coding for the antibody) and phenotype of the antibody (i.e. its binding specificity) in the same way that surface immunoglobulins are linked to B cells in vivo. This is done by genetically fusing the gene coding for the antibody of interest to the gene of the minor coat protein pIII (Figure 2-8).

From these libraries, human antibodies against virtually any target can be obtained by passing the phage antibodies over immobilized antigen in a process called "panning". Phage are allowed to bind to the immobilized antigens, and non-bound phage are removed by washing. Bound phage are subsequently eluted from the target antigen and propagated in *E. coli* bacteria, thereby enriching the pool of phage with the desired phenotype. This cycle of binding, elution and propagation is repeated until phage with the desired specificity for the target are obtained.

### 2.8.4 Different antibody formats and their implication in targeting

Besides the entire IgG, other antibody formats have been investigated. The most common antibody format displayed on phage is the single-chain Fv fragment ("scFv"). ScFv fragments
(Bird et al., 1988; Huston et al., 1988) consist of a single polypeptide chain, comprising an antibody $V_H$ domain genetically linked by a flexible polypeptide linker to a $V_L$ domain, thereby preventing the $V_H$ and $V_L$ domains from falling apart. ScFv and Fv fragments are the smallest antibody fragments (about 27 kDa) that conserve the same binding affinity (although not avidity) as the parental immunoglobulin. The natural effector functions of antibodies described above are abolished in scFv fragments. In Fab fragments, the first constant domain of the heavy chain and the constant domain of the light chain stabilize the association of the $V_H$ and $V_L$ domain. Dimeric Fab fragments have been produced as well (Humphreys et al., 1997). Minibodies comprise two scFv-hinge-$C_{H3}$ chains covalently connected by disulfide bonds (Hu et al., 1996). A number of multivalent scFv-based structures has been engineered, including (scFv)$_2$ (Adams et al., 1993), diabodies (Holliger et al., 1993) and several others (Figure 2-9).

**Figure 2-9**: Different antibody formats commonly used for therapeutic applications. Fab and F(ab')$_2$ can be generated by papain or pepsin digestion, respectively, or by genetic engineering; all other forms are generated by genetic engineering. ScFv are composed of $V_H$ and $V_L$ linked together by polypeptide; monovalent scFv can be obtained by variation in length of the linker peptide. Minibodies comprise two scFv-hinge-$C_{H3}$ chains covalently connected by disulfide bonds (Presta, 2003).

Apart from already discussed antibody-independent characteristics of an antigen such as specificity, abundance, stability and accessibility (see 2.3) a number of factors determines the ability of an antibody-based molecule to target, penetrate and be retained in a tumor. Three other important antibody-dependent properties that have a direct effect on the success of tumor targeting are antibody affinity (and avidity), size and valence. They will be briefly discussed in the following section.
2.8.4.1 Affinity, valence and size - impact on tumor targeting

The interaction of an antibody with its antigen is described by its affinity, which is composed of the kinetic "on rate" and "off rate". Another important feature is the avidity. The affinity results from univalent interactions, but if two or more heads bind, a much more stable interaction is set up - the avidity effect. This can result in large increases in functional affinity but it depends critically on the density of the target antigen in relation to the spacing of the antibody heads. For this last reason, complete IgG, dimeric Fab or dimeric scFv fragments have higher avidity.

It is assumed that "the higher the affinity, the better the targeting". However it should be taken into consideration, that affinities above $10^{-9}$ M do not translate to increased tumor retention and that antibodies with an extremely high affinity will bind in an essentially irreversible manner to the first tumor antigen encountered, resulting in antibody localization only in the highly vascularized regions of the tumor and therefore hinder the ability of the antibodies to penetrate into the tumor from the vasculature (Fujimori et al., 1990; Jackson et al., 1998; Adams et al., 2001). Nevertheless, high affinities between $10^{-8}$ and $10^{-9}$ M showed best tumor accumulation (Adams et al., 1998) and are therefore desirable. They can be obtained either by engineering the antibody-binding site, or by augmenting the valence of an antibody by rendering it multivalent (see Figure 2-9).

Multivalent antibodies will have a better affinity for the target, but an increase in size leads to worse tumor retention. Indeed, an inverse relationship exists between the impact of size on systemic clearance and tumor penetration on one hand and quantitative tumor retention on the other (Table 2-2). Small molecules (e.g., 27 kDa scFv) that are capable of relatively rapid diffusion into solid tumors undergo rapid renal clearance (because of their low molecular weight) (Maack et al., 1979) and hence are eliminated before significant tumor retention can occur. In contrast, large molecules (e.g., 150 kDa IgG) are not eliminated through the kidneys and therefore display a prolonged residence in circulation. A number of factors including heterogeneous tumor blood flow, heterogeneity of tumor vessel permeability and interstitial hypertension combines to form effective barriers to drug delivery (Jain, 1998). In this environment, smaller molecules exhibit a distinct advantage over larger molecules.
<table>
<thead>
<tr>
<th>Structure</th>
<th>Molecular weight (kDa)</th>
<th>Clearance</th>
<th>Tumor penetration</th>
<th>Tumor retention</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td>150</td>
<td>+</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>F (ab)2</td>
<td>100</td>
<td>++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Sc(Fv)2</td>
<td>54</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>scFv</td>
<td>27</td>
<td>++++</td>
<td>++++</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 2-2: Properties of different antibody formats.

Normally, in order to define the targeting properties of an antibody, biodistribution experiments in which the amount of antibody (in % injected dose/gram tissue) in different organs is compared, are performed.

Due to their size the IgG molecule show a relatively poor diffusion from the vasculature into and through the tumor. As already seen above, the smaller size of scFv fragments enables better tumor penetration but clears from the blood at much faster rate than intact IgG. The scFv with its faster clearance and lower affinity results in a lower percent-injected dose localizing in tumors when compared to the divalent IgG molecule. Figure 2-10 shows blood and tumor concentrations (% injected dose/gram tissue) at different time points of an IgG molecule compared to a scFv fragment. A hypothetical ideal targeting agent that would localize at high levels in the tumor and have a fast blood clearance is depicted in the last chart (Figure 2-10c).

Borsi and colleagues give more detailed information on this topic comparing different formats of L19, a scFv antibody fragment against the EDB domain of fibronectin (see 2.9.5).
Figure 2-10: Antibody localization in tumour and blood with typical uptake time courses for intact antibodies and sc(Fv) (Wong et al., 1999; Wu and Yazaki, 2000)

a) Targeting performance (tumor and blood levels versus time) of an antibody in the IgG format (molecular weight= 150KDa)
b) Levels in tumor and blood of a scFv fragment specific for a tumor antigen.
c) Ideal targeting agent: it should localize at high levels in the tumor and have a fast blood clearance.

After this excursion on antibody formats and their implications in targeting, the analysis of the different binding molecules for EDB will proceed in the coming sections.

2.8.4.2 CGS-1 and CGS-2

The scFv (CGS-1) and scFv (CGS-2) antibody fragments were isolated from a synthetic human antibody phage library, the Nissim library (Nissim et al., 1994). This library features antibodies with human V_{H} chains, which are randomized in the loop region of CDR3, linked to a human light chain. Precursors of CGS-1 and CGS-2 were subjected to subsequent affinity maturation, which involved randomization of positions in the CDR3 of the light chain variable domain. CGS-1 and CGS-2 bind to distinct epitopes on isolated EDB domains of human fibronectin, with binding affinities of 53 nM and 1.1 nM, respectively. Both antibodies also recognize EDB comprising fibronectin in tumoral tissue, as demonstrated in immunohistochemical studies (Carnemolla et al., 1996).

Additionally, CGS-1 and CGS-2 were reported to target tumors of F9 teratocarcinoma grafted mice and a partially dimeric scFv (CGS-1)_{2} has been shown to display better tumor-targeting properties than its monomeric counterpart (Neri et al., 1997).
The scFv (L19) is an affinity-matured version of scFv (E1), obtained by combinatorial mutagenesis of selected residues in the hypervariable loops of E1 (Pini et al., 1998). E1 is an antibody fragment that was isolated from an initial phage-display synthetic library containing $7 \times 10^8$ different scFv fragments (Pini et al., 1998) and that had been obtained by cloning of single VH (DP-47) and Vk (DPK-22) germline genes.

The initial library gave raise to an improved version, called ETH-2 library (Viti et al., 2000), for which three antibody germline gene segments, DP-47 for the heavy chain, DPK-22 and DPL-16 for the light chain were used and a large repertoire was produced by appending short CDR3 onto them (Viti et al., 2000).

Immunohistochemistry studies with tumor sections have demonstrated that both antibodies recognize B-FN in vascular structures (Pini et al., 1998).

The enhanced binding affinity of L19 is mainly due to a lower dissociation constant (0.054 nM, compared to 41 nM of E1). The enhanced binding affinity as well as valence (monomeric scFv vs. dimeric scFv) lead also to improved targeting of tumoral angiogenesis, as was shown in biodistribution studies in F9 murine teratocarcinoma bearing nude mice by Viti et al. (Viti et al., 1999).

### 2.8.5 Antibodies isolated by colony filter screening

Colony filter screening is a method for the rapid isolation of binding specificities from a lawn of confluent bacteria expressing a large synthetic repertoire of human antibody fragments in scFv configuration (Dreher et al., 1991; Skerra et al., 1991).

*E. coli* cells, expressing the library of antibody fragments, are grown on a porous master filter, in contact with a second filter coated with the antigen, onto which antibodies secreted by the bacteria are able to diffuse. Detection of antigen binding on the second filter allows the recovery of a number of *E. coli* cells, including those expressing the binding specificity of interest, which can be submitted to a second round of screening for the isolation of specific mAbs.
Using this methodology in an iterative fashion (Figure 2-11), a number of specifically binding antibodies of different amino acid sequence from an antibody library of $7 \times 10^8$ clones were recovered (Giovannoni et al., 2001).

### 2.8.5.1 ME4C

The antibody clone showing the strongest ELISA signal (ME4C) was further characterized. Its epitope on the EDB domain was mapped using the SPOT synthesis method, which uses a set of decapetides spanning the antigen sequence synthesized and anchored on cellulose membranes (Frank and Overwin, 1996).

ME4C binds to the EDB domain with a dissociation constant of 0.1 µM. It specifically stains new tumor blood vessels, as shown by immunohistochemical analysis on tumor sections of glioblastoma multiforme and on sections of human SKMEL-28 melanoma, grafted in nude mice (Giovannoni et al., 2001). For in vivo tumor-targeting applications, its affinity would have to be matured (Viti et al., 1999).
2.8.6 Non-peptidic ligands of EDB

For a number of applications it would be desirable to have a low-molecular weight binder specific for EDB. Such a compound would ideally be easier to produce in large amounts than an antibody, should not be immunogenic and could readily cross the endothelial cell layer which separates the blood stream from B-FN, which is typically located on the abluminal site of new blood vessels.

A library of 113 low-molecular weight organic compounds, containing both an aromatic moiety and at least one positive charge, was screened for binding to the EDB domain, using two-dimensional heteronuclear NMR-spectroscopy. One lead-compound, 2,2-diphenylethylamine, was found that binds specifically to the EDB domain, yet with dissociation constant in the millimolar range. The binding is drastically poorer than the one of other specific human mAbs that has recently been reported by our group (see 2.8.4.3 above) (Pini et al., 1998). The failure to derive comparably good binders with this method might be due to the relatively small size of the library (Scheuermann et al., 2003).
2.9 TARGETING EXPERIMENTS WITH ANTI-EDB ANTIBODY DERIVATIVES

The targeting results obtained with the scFv (L19) antibody fragment are impressive, considering that the target is a component of the modified extracellular matrix, located predominantly in the abluminal side of new blood vessels and that neo-vasculature represents only a small percentage of the total tumor mass.

2.9.1 Targeting with the radiohalogenated L19 antibody

L19 was stably labeled with iodine-125 and astatine-211 with full retention of immunoreactivity, using either the Iodogen method (Fraker and Speck, 1978) or a trimethylstannyl benzoate bifunctional derivative (Garg et al., 1989). Biodistribution of radiolabelled L19 antibody in mice bearing different types of tumor grafted subcutaneously, followed by ex vivo micro-autoradiographic analysis, revealed that radiohalogenated L19 rapidly localizes around tumor blood vessels, but not around normal vessels (Viti et al., 1999; Demartis et al., 2001).

Therapeutic radionuclides could in principle be delivered to the tumor neovasculature. In general, success of systemic radioimmunotherapy of solid tumors with radiolabeled antibodies has been limited by the relatively modest ratio of the radiation doses delivered to tumor and normal organs, respectively. However, alpha-emitting radionuclides, such as astatine-211, actinium-225 or bismuth-213, are particularly interesting in the context of vascular targeting (McDevitt et al., 2000). Alpha radiation is highly energetic and has a tissue penetration of approximately 50 μm, which matches the endothelial localization of vascular targeting antibodies. Because of their exquisite endothelial toxicity, vascular targeting antibodies, coupled to alpha-emitters, could be very useful to quantify the extent to which tumors are angiogenesis dependent.

2.9.2 Fluorophore-conjugated antibodies

Unlike radionuclides, fluorophores are not hazardous, are commercially available at low cost, can easily be conjugated to proteins, do not decay and can be detected within seconds using inexpensive equipment.
Birchler and colleagues (Birchler et al., 1999a; Birchler et al., 1999b) investigated whether B-FN is a specific marker of ocular angiogenesis and whether antibodies recognizing B-FN could selectively target ocular neovascular structures in vivo after systemic administration. Using immunophotodetection methods (Folli et al., 1994; Neri et al., 1997) it was demonstrated that L19 chemically coupled to the red fluorophore Cy5 selectively targets ocular angiogenesis upon intravenous injection. Fluorescence of growing ocular vessels was visible immediately after injection with L19 and persisted for at least two days. Subsequent ex vivo immunofluorescent microscopic analysis on cornea sections confirmed the localization of L19. Neri et al. (Neri et al., 1997) showed that the use of Cy7 is preferable to Cy5 because of better tissue penetration.

2.9.3 Immunoscintigraphy

In 1997, a pilot pharmacokinetic and immunoscintigraphic study with technetium-99m-labeled monoclonal antibody BC1 (see 2.8.2.1) in patients with brain tumors was performed. The results obtained from this study indicated a diagnostic potential of 99mTc-BC1 (Mariani et al., 1997).

Recently, first immunoscintigraphy trials for the imaging of primary tumors and metastatic lesions in cancer patients have started, using L19 labeled with the γ-emitter iodine-123 (Santimaria et al., 2003). The L19 antibody selectively targeted tumor lesions in aggressive types of lung cancer and colorectal cancer. The successful localization of the radiolabeled antibody in aggressive primary tumors and metastatic lesions warrants a number of diagnostic applications (Figure 2-12). Furthermore, the clinical development of therapeutic fusion proteins, in which L19 is engineered to deliver bioactive molecules to sites of angiogenesis, should gain momentum from these scintigraphic results (see 2.10).
2.9.4 Molecular properties that dictate the tumor-targeting performance of L19-derivatives

Different experiments performed in our laboratory, lead us to the conclusion that there are some molecular properties that dictate the tumor-targeting performance of L19-derivatives (see below).

2.9.4.1 Charge

TAT has been described as a protein with the ability to enter and leave cells by unconventional manner (classical cell entry mechanisms can be excluded), in a process termed protein transduction (Green and Loewenstein, 1988; Frankel and Pabo, 1988; Mann and Frankel, 1991). Consequently, our group investigated whether a site-specific conjugation of TAT peptides (containing the positively charged sequence RKKRRQRRR) to LI9 would result in improved crossing of endothelial barriers and, consequently, enhanced tumor uptake. Quantitative biodistribution analysis with the L19-Cys-TAT in murine F9 teratocarcinoma-bearing mice were performed. The results showed severely reduced tumor targeting performance compared to the unconjugated antibody (Niesner et al., 2002).

We have recently found that fusion of L19 to the proangiogenic cytokine VEGF_{165} (but not to the shorter isoforms VEGF_{121}) completely abolished the tumor-targeting property of the antibody (Halin et al., 2002). VEGF_{165} and VEGF_{121} differ by a positively charged heparin
binding peptidic moiety, which is inserted in the VEGF165 molecule by a mechanism of alternative splicing at the level of the pre-mRNA.

Aiming at improving the tumor to non-tumor ratio of radioactivity in immunoscintigraphy and radio-immunotherapy, pretargeting strategies have been studied (Grana et al., 2002). Usually, streptavidin or avidin is coupled to a tumor-targeting antibody, and as a second compound, radioactively labeled biotin is administered at a later time point.

In our group we aimed at introducing a calmodulin-peptide complex system as an alternative in order to solve the problem of immunogenicity induced by the (strept)avidin/biotin system (Neri et al., 1995).

A fusion protein consisting of L19 and calmodulin was developed and quantitative biodistribution studies in mice bearing the F9 murine teratocarcinoma were performed. L19-CaM is not taken up by the tumor when injected intravenously (Melkko et al., 2002). CaM is an unusually acidic protein, with a net charge of \(-24\) before Ca\(^{2+}\) binding.

A possible explanation for the inability of these fusion proteins to bind to EDB may lie in the predominantly abluminal localization of the target. More precisely, the addition of positively (such as TAT and VEGF165) and negatively (such as CaM) charged moieties may prevent the antibody extravasation from the blood vessel lumen, maybe by virtue of electrostatic interactions with endothelial cells and/or components of the extracellular matrix.

Systematic analysis of other derivatives of L19 recently produced in our laboratory suggests that antibody fusion proteins with extreme pi values (i.e. pI<5 and pI>9) may abrogate tumor targeting \textit{in vivo} (Melkko et al., 2002). These observations will need more quantitative investigations on the interplay between protein charge, size and vascular permeability.

2.9.4.2 Size

Our group had shown that targeted delivery of IL-12 to the modified ECM, mediated by fusion to L19, dramatically enhances the therapeutic index of this anticancer cytokine (see next chapter for more details) (Halin et al., 2002).

In order to improve the therapy results, Halin et al. (Halin et al., 2003) combined the therapeutic activity of L19-IL12 fusion protein with the one of TNF\(\alpha\), another cytokine with potent antitumor effects. A single L19 based antibody cytokine fusion protein (ILT) was created, comprising both the IL12 and the TNF\(\alpha\) molecule and expected to feature following advantages:
-Since TNFα forms a homotrimer, also the fusion protein should comprise three antibody molecules and thus be of high avidity;
-Due to the vasoactive properties of TNFα one would expect high levels of tumor uptake;
Surprisingly ILT, the produced cytokine fusion protein, which was shown to retain full IL-12 and TNFα activity in \textit{in vitro} assays, did not show any tumor accumulation in biodistribution studies and had no therapeutic activity in a subcutaneous murine tumor model.
The most reasonable explanation for the failure of this approach seems to be the large size of the fusion protein, which has a molecular weight of 340 kDa. Since ILT was shown to work in immunohistochemistry, it seems reasonable that the large molecular size of the fusion protein interferes with a rapid extravasation.

\subsection*{2.9.5 Comparison of different formats of L19}

Using the variable regions of L19, a human "small immunoprotein" (SIP) (comprising the variable regions of L19 and a CH4 domain of the human IgE) and a complete human IgG1 were constructed and expressed. Biodistribution studies in tumor-bearing mice to compare the blood clearance rate, \textit{in vivo} stability and performance in tumor-targeting of the 3 L19 formats (dimeric scFv \((\text{scFv})_2\), SIP and IgG1), were performed (Borsi et al., 2002). The accumulation of the different antibody formats in the tumors studied was a consequence of the clearance rate and \textit{in vivo} stability of the molecules. Using the SIP, the percent of antibody injected dose per gram of tissue (%ID/g) in tumors was 2-5 times higher than that of the \((\text{scFv})_2\), reaching a maximum 4-6 hr after injection. By contrast, the accumulation of IgG1 in tumors constantly rose during the experiments. However, due to its slow clearance, the tumor to blood ratio of the %ID/g after 144 hr was only about 3 compared to a ratio of 10 for the \((\text{scFv})_2\) and 70 for the SIP after the same period of time (see Table 2-3). The different \textit{in vivo} behavior of these 3 completely human L19 formats could be exploited for different diagnostic and/or therapeutic purposes, depending on clinical needs and disease.

\begin{table}[h]
\centering
\begin{tabular}{lcccccc}
\hline
 & \textbf{3 hrs} & \textbf{6 hrs} & \textbf{24 hrs} & \textbf{48 hrs} & \textbf{72 hrs} & \textbf{144 hrs} \\
\hline
\textbf{L19 (scFv)\textsubscript{2}} & & & & & & \\
Tumor & 2.47 ± 0.65 & 2.01 ± 0.72 & 1.62 ± 0.43 & 0.95 ± 0.14 & 0.68 ± 0.04 & 0.32 ± 0.14 \\
Blood & 1.45 ± 0.58 & 0.54 ± 0.12 & 0.10 ± 0.03 & 0.04 ± 0.01 & 0.03 ± 0.02 & 0.03 ± 0.01 \\
T/B ratio & 1.7 & 3.7 & 16.2 & 23.7 & 22.7 & 10.7 \\
\hline
\end{tabular}
\caption{Accumulation of different antibody formats in tumors.}
\end{table}
<table>
<thead>
<tr>
<th></th>
<th>3 hrs</th>
<th>6 hrs</th>
<th>24 hrs</th>
<th>48 hrs</th>
<th>72 hrs</th>
<th>144 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>L19 SIP</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor</td>
<td>5.23 ± 0.65</td>
<td>6.14 ± 2.23</td>
<td>4.20 ± 2.47</td>
<td>2.57 ± 0.31</td>
<td>2.33 ± 0.90</td>
<td>1.49 ± 0.65</td>
</tr>
<tr>
<td>Blood</td>
<td>9.82 ± 0.68</td>
<td>5.03 ± 0.52</td>
<td>1.39 ± 0.06</td>
<td>0.29 ± 0.04</td>
<td>0.08 ± 0.02</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>T/B ratio</td>
<td>0.5</td>
<td>1.2</td>
<td>3.0</td>
<td>8.9</td>
<td>29.1</td>
<td>74.5</td>
</tr>
<tr>
<td><strong>L19 IgG1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor</td>
<td>4.46 ± 0.08</td>
<td>5.39 ± 1.01</td>
<td>6.70 ± 2.10</td>
<td>7.80 ± 2.51</td>
<td>8.90 ± 2.52</td>
<td>11.22 ± 3.19</td>
</tr>
<tr>
<td>Blood</td>
<td>16.04 ± 0.81</td>
<td>12.02 ± 1.65</td>
<td>8.31 ± 1.77</td>
<td>5.12 ± 1.42</td>
<td>5.02 ± 3.81</td>
<td>4.87 ± 0.26</td>
</tr>
<tr>
<td>T/B ratio</td>
<td>0.3</td>
<td>0.4</td>
<td>0.8</td>
<td>1.5</td>
<td>1.8</td>
<td>2.3</td>
</tr>
</tbody>
</table>

Table 2-3: Biodistribution experiments of radiolabeled L19 (scFv)₂, L19 SIP, L19 IgG1 in SK-MEL-28 tumor bearing mice. The results are expressed as percent of antibody injected dose per gram of tissue (%ID/g). T/B ratio = Tumor-to-blood ratio (adapted from Borsi et al., 2002).
2.10 THERAPY EXPERIMENTS WITH ANTI-EDB ANTIBODY DERIVATIVES

2.10.1 Photosensitizers

One therapy approach features the targeted delivery of photosensitizers, i.e. molecules that upon irradiation and in the presence of oxygen release diffusible agents, such as toxic singlet oxygen or reactive radicals.

Birchler and coworkers (Birchler et al., 1999b) have reported in a rabbit model of ocular angiogenesis that L19, chemically coupled to a photosensitizer, mediates the complete and selective occlusion of ocular neovasculature after irradiation with red light. This process was accompanied by apoptosis of the corresponding endothelial cells.

To further evaluate whether B-FN could be used as a therapeutic target for specific antibody-photosensitizer immunoconjugates, the presence of EDB in surgically excised human choroidal neovascular membranes (CNVM) was investigated. B-FN was detected by the human recombinant antibody, CGS-1 (see above), and compared with immunostaining for endothelial cells with factor VIII-related antigen. Nicolò and colleagues (Nicolo et al., 2003) found that the extradomain B is abundantly expressed in CNVMs, but its expression is more restricted in eyes harboring no apparent choroidal neovascularization.

For applications in oncology, this strategy may be limited by low accessibility of the malignant tissue to an external light source.

2.10.2 L19-TF

The approach to cancer therapy based on the antibody-directed targeting of the human coagulation-inducing protein Tissue Factor (TF) to tumor vasculature has first been proposed by Thorpe et al. (Ran et al., 1998; Huang et al., 1997). It was based on the concept that thrombosis of tumor vessels may stop the supply of nutrients and oxygen to tumor cells, thereby causing their death.

A fusion protein consisting of L19 fused to the extracellular domain of Tissue Factor was produced in our laboratory.
It was shown that L19-TF selectively targets tumor blood vessels in vivo and that it mediates the complete and selective infarction of three different types of solid tumors in mice. At the highest doses administered, complete tumor eradication was observed in 30% of the mice treated, without apparent side effects (Nilsson et al., 2001).

2.10.3 Anti-EDB scFv antibody modified liposomes

Liposomes are widely used as delivery systems with a broad spectrum of agents including chemotherapeutics, imaging agents, antigens, lipids and DNA (Mastrobattista et al., 1999). Targeting of liposomes to sites of drug action is achieved by attachment of specific antibodies or antibody fragments to their surface (de Kruif et al., 1996; Hansen et al., 1995; Schwendener et al., 1990). Marty and colleagues (Marty et al., 2002) prepared and tested small unilamellar liposomes derivatized with modified CGS-1 single chain antibody fragments in biodistribution and therapy experiments in mice bearing F9 teratocarcinoma tumors.

Radioactively labeled single chain antibody fragments-liposomes accumulated in the tumors at 2-3-fold higher concentrations during the first 2 h after i.v. injection as compared to unmodified liposomes. Animals treated i.v. with single chain antibody fragments-liposomes containing the new cytotoxic agent 2'-deoxy-5-fluorouridylyl-N4-octadecyl-1—D-arabinofuranosylcytosine showed a reduction of tumor growth by 62-90% determined on days 5 and 8, respectively (Marty et al., 2002).

2.10.4 Immunocytokines

This section presents a general overview of the tumor immune response and the involvement of cytokines in this phenomenon.

The potential ability of the immune system to eliminate or contain cancer has been recognized for decades. In principle, the immune system can recognize cancer cells for two reasons (Smyth et al., 2001): (1) the self-perpetuating and invasive phenotype of cancer cells depends on altered or overexpressed proteins that are distinct from the proteins of most normal cells; (2) tumor growth can stress or damage the surrounding tissues, generating a "danger" signal that stimulates tumor antigen presentation to the immune system, resulting in
an anti-tumor immune response. After “recognition”, two types of immune response may be
directed against cancer cells. The innate, or antigen non-specific response is generally
composed of natural killer (NK) cells, γδT cells (important component of the innate immune
system recognizing allo- and/or self-antigens upon cell infection), stress or transformation,
granulocytes, and macrophages, which recognize stressed cells and clear them from the
tissue. In addition, dendritic cells (DC) in the vicinity of the stressed cells are activated to
present antigens in the stressed environment on their major histocompatibility complex
(MHC) molecules and travel to the draining lymph nodes to initiate the adaptive, or antigen-
specific response. Within the lymph nodes, the now mature DC stimulate antigen-specific T
cells to proliferate and acquire effector functions. In addition, antigen-specific B cells are
triggered to proliferate and produce antibodies against the tumor. Effector T cells, both CD4⁺
helper cells and CD8⁺ cytotoxic T lymphocytes (CTL), return to the tumor site and either
attack the tumor directly or express cytokines and stimulatory cell-surface molecules that
trigger other cells to kill the tumor cells (Figure 13).
The innate and adaptive immune responses are closely linked and mutually reinforcing. In
particular, the type of adaptive immune response is strongly influenced by the cytokines
produced by innate immune cells (Belardelli and Ferrantini, 2002). More precisely, cytokines
produced by cells of the innate defense system play an essential role in influencing the
immune response towards protective antitumor immunity. These cytokines might act as first
“danger” signals in alerting the immune system. By promoting the differentiation and
activation of dendritic cells, antigen presentation and T-cell-mediated immune responses,
these cytokines could be powerful natural adjuvants for the development of cancer strategies.
Figure 2-13: Schematic and simplified representation of the events occurring in the course of an immune response against tumor cells. Dendritic cells (DCs) act as sentinel cells for monitoring tissue damage and modulating the immune response. a) "Danger" signals (e.g. cytokines or heat shock proteins) can act on DC precursors and promote DC differentiation and activity. Tumor-associated antigens (TAAs) are captured by DCs. b) cytokines can promote DC differentiation and activity by multiple mechanisms including the up-regulation of expression of costimulatory signals, which favor the physical interaction of DCs with T cells. c) Then, DC can acquire a highly activated mature phenotype and migrate to the lymph nodes, where the TAA-derived peptides are presented to CD4+ and CD8+ T cells in the context of MHCI or MHCIi molecules, respectively. The activation of B cells (not shown) is also likely to occur. d) CD4+ and CD8+ T cells can migrate to the tumor site where the antitumor attack relies on both, cellular and humoral responses (adapted from Belardelli and Ferrantini, 2002).

Although these and other types of immune responses are in fact observed, the immune system is nevertheless unable to control tumor growth and spread for a number of reasons (Drake and Pardoll, 2002). First, the relevant innate immune cells or T cells may not be mobilized against the tumor until it is too well established to be controlled (Perales et al., 2002). Second, tumor-specific T cells and/or DC may encounter tumor antigens in a context that renders them unresponsive (Vicari et al., 2002). Third, the immune effector cells may have difficulty penetrating the tumor in sufficient numbers to control growth (Mukai et al., 1999). Fourth, the effectors that do infiltrate the tumor may not be able to expand or carry out their functions because the tumors no longer present the relevant antigen (Pettit et al., 2000) or they express suppressive factors, including IL-6 (Frassanito et al., 2001) and IL-10 (Salazar-Onfray, 1999). Fifth, the tumors may have become resistant to immune attack via upregulation of anti-apoptotic mechanisms (O'Connell et al., 1996).
In order to enhance the insufficient anti-tumor response to cancer the targeted delivery of anti-cancer cytokines to the tumor environment appears to be a particularly promising strategy since systemic delivery of cytokines is very often associated with strong toxicity. A number of cytokines are either approved drugs or are in advanced clinical trials, yet these biopharmaceuticals do not typically localize efficiently in solid tumors, and manifest their therapeutic potential at the expense of severe side effects. Expression of cytokine genes close to tumors, either by transducing the tumor cells (Hurford et al., 1995) or by delivering cytokine-expressing cells to tumor sites (Soiffer et al., 1998), demonstrate that a sufficient concentration of cytokine in the tumor can effectively stimulate the host immune system to identify and destroy tumor cells in both animal models and patients. Moreover, direct injection of cytokines at the tumor site (Maas et al., 1989; Akporiaye and Hersh, 1999) and the slow local release of cytokines from polymeric beds implanted close to the tumor (Nguyen et al., 2001) confirmed the value of therapeutic approaches relying on the targeted delivery of suitable cytokines to solid tumors. As cancer is often a disseminated disease, it appears that the most straightforward avenue for the targeted delivery of cytokines to tumors should rely on the fusion of cytokines to a suitable binding molecule (in most cases, an antibody or an antibody fragment). These so-called immunocytokines are designed to create a preferential accumulation of the cytokines at the tumor site, thus improving the therapeutic index of these biopharmaceuticals (Harvill and Morrison, 1995; Hornick et al., 1997; Gillies et al., 1998). In turn, this may lead to a more efficient activation of a specific anti-tumor response, or allow the administration of lower doses and limit toxicities to normal organs.

The earliest immunocytokines contained the coding sequence for a cytokine inserted in frame downstream of an immunoglobulin heavy chain coding sequence (Gillies et al., 1992; Gillies et al., 1991). The heavy chain-cytokine fusion gene was cotransfected with the appropriate light chain gene into a myeloma cell line for subsequent expression and purification. Several variants of this basic structure are possible. The cytokine may be inserted at the N-terminus of either the heavy chain or the light chain, provided that antigen binding is retained. Heterodimeric cytokines may be incorporated into immunocytokines by fusing one of the subunits to the heavy chain and coexpressing the other subunit as a free molecule. It is also possible to make fusion proteins from two cytokines and attach the bifunctional fusion protein to an antibody heavy chain (Gillies et al., 2002). The demonstration that single chain Fv (scFv) molecules can have appropriate binding activity in vitro as well as in vivo has prompted their incorporation into fusion proteins with cytokines.
The most relevant studies performed in our laboratories in the field of immunocytokines, namely the scFv(L19) fused to different cytokines, are described below.

2.10.4.1 IL12-L19

A fusion protein in which the murine p40 and p35 domains of IL-12 were sequentially fused to the N-terminus of L19 (IL12–L19) was produced. This fusion protein was found to have a potent antitumor activity, markedly superior to that of untargeted IL-12, in a mouse lung-metastasis model and in two different subcutaneous tumor models established in immunocompetent mice. Moreover, in mice treated with IL12–L19, the tumor environment became infiltrated with macrophages, lymphocyte-activated killer (LAK) cells, NK cells, and T lymphocytes. The targeted delivery of IL-12 to B-FN also resulted in a marked increase in IFNγ, both in the tumor mass and in the blood (Halin et al., 2002).

2.10.4.2 L19-IL2

Carnemolla and colleagues (Carnemolla et al., 2002) showed that a fusion protein between the C-terminus of L19 and interleukin 2 (L19-IL2) mediates the selective delivery and concentration of IL-2 to tumor vasculature, thereby leading to a dramatic enhancement of the therapeutic properties of the cytokine. Immunohistochemical analysis of tumor infiltrates demonstrated a significantly higher number of T lymphocytes, natural killer cells, and macrophages, as well as increased IFNγ accumulation, in tumors from animals treated with L19-IL2 compared to tumors from control groups (Borsi et al., 2002).

2.10.4.3 L19-TNFα

The systemic administration of large, therapeutically effective doses of TNFα was discontinued for the unacceptably high levels of systemic toxicity. Loco-regional therapies, on the other hand, such as “isolated limb perfusion” (ILP) (Lienard et al., 1992), allow reaching therapeutic concentrations of TNFα. In patients with in transit melanoma
metastases, local perfusions with TNFα, especially in combination with IFNγ and Melphalan (Alkeran®), resulted in improved tumor response rates. Moreover, ILP with Melphalan and TNFα resulted in limb salvage in soft-tissue sarcoma patients (Eggermont et al., 1996). Consequently, this application of TNFα was approved for use by the European Agency for the Evaluation of Medicinal Products (EMEA) (Eggermont, 2000). Very recently, the American College of Surgeons (NCI) approved a Phase III clinical trial using Melphalan with or without TNFα for the ILP of melanomas.

A number of approaches are presently under investigation to improve the therapeutic effects and to reduce the toxic side effects of systemic TNFα (Gillies et al., 1993; Barbara et al., 1994; Pietersz et al., 1997; Moro et al., 1997; van der Veen et al., 1998; Curnis et al., 2000; Cooke et al., 2002; Curnis et al., 2002). Borsi and colleagues (Borsi et al., 2002) described very recently the production and antitumor activity of the L19-TNFα fusion protein. This fusion protein was found to have an antitumor activity markedly superior to that of the untargeted TNFα in syngeneic mouse tumor models. Furthermore, its antitumor activity was dramatically increased when used in combination with Melphalan or with L19-IL2. These findings indicate that the targeted delivery of TNFα to the subendothelial ECM opens the possibility for reconsideration of a systemic use of TNFα in tumor therapy (Borsi et al., 2003).

As already mentioned in chapter 2.9.4.2, a triple fusion proteins of L19 antibody with IL-12 and tumor necrosis factor TNFα (ILT) was generated in our laboratory (Halin et al., 2003). Since ILT, which proved to be fully active in in vitro assays, failed to show tumor accumulation in biodistribution studies and displayed a modest therapeutic activity in a syngeneic tumor model, combinations of the two antibody-cytokine fusion proteins, IL12-L19 and L19-TNFα, were investigated. This combination displayed potent synergistic anticancer activity and led to the eradication of F9 teratocarcinomas grafted in immunocompetent mice. When cured mice were rechallenged with tumor cells, a delayed onset of tumor growth was observed, indicating the induction of a partial antitumor vaccination effect (Halin et al., 2003).
2.11 AIM OF THE THESIS

As described in-depth in the previous section, our group has previously reported the anticancer properties of scFv(L19) fused to interleukin 2 (IL2) (Carnemolla et al., 2002), to IL12 (Halin et al., 2002a) and to TNFα (Borsi et al., 2003), as well as the combination of L19-IL12 with L19-TNFα (Halin et al., 2003) and of L19-TNFα with cytotoxic agents (Borsi et al., 2003). To summarize, these fusion proteins were shown to retain both antibody and cytokine functions and displayed superior anti-cancer activities, compared to equivalent amounts of free cytokines (and antibody). Interestingly, antibody fusions with IL2 or TNFα exhibited an improved tumor targeting performance, compared to the parental antibody. By contrast, fusion of scFv(L19) with IL12 or VEGF led to lower accumulation in the tumor (Halin et al., 2002a; Halin et al., 2002b). The molecular determinants for this different tumor targeting behavior are understood only in part (Melkko et al., 2002; Niesner et al., 2002; Khawli et al., 2003; Halin et al., 2003).

In this thesis, I describe the cloning, expression and characterization of a fusion protein between scFv(L19) and different mutants of murine interferon γ (IFNγ).

IFNγ is considered to be a pleiotropic cytokine that plays a central role in promoting innate and adaptive mechanisms of host defense (Farrar and Schreiber, 1993; Boehm et al., 1997). More precisely, IFNγ is able to directly inhibit tumor cell growth, to generate an anti-tumor cellular immune response by directly stimulating CD8+ cytotoxic T-lymphocytes (CTLs) (Chen et al., 1986; Maraskovsky et al., 1989), natural killer (NK) cells (Djeu et al., 1982) and macrophages (Collart et al., 1986; Nathan and Hibbs, 1991), as well as enhancing antigen presentation through increased MHC class I and II expression (Rosa et al., 1986).

Upon interaction with target cells, NK and T cells express IFNγ that exerts its biologic effects by interacting with an IFNγ receptor that is ubiquitously expressed on nearly all cells (Bach et al., 1997). IFNγ is also upregulated by certain cytokines, particularly interleukin 12 (IL12).

In different murine tumor models, the genetic transfer of IFNγ resulted in significant inhibition or abrogation of the growth of genetically modified tumor cells (Gansbacher et al., 1990; Watanabe et al., 1989; Hock et al., 1993; Yanagihara et al., 1994). However, the clinical responses observed so far, have not been very promising. For example, in a phase II/III clinical trial in patients with metastatic melanoma the previously identified potent effects on immunomodulation did not translate into therapeutic activity (Schiller et al., 1996),
and in patients with metastatic renal cell carcinoma no significant difference was seen in patients receiving IFNγ compared to placebo (Gleave et al., 1998; Small et al., 1998).

By now, human recombinant IFNγ is approved in the U.S.A. and in Europe for reducing the frequency and severity of serious infections associated with chronic granulomatous disease (CGD) and for delaying the time to disease progression in patients with severe, malignant osteopetrosis (ACTIMMUNE®). Furthermore it is in clinical trials for the treatment of idiopathic pulmonary fibrosis (IPF) (Raghu et al., 2004) and ovarian cancer (Windbichler et al., 2000). Severe systemic toxicity (Kurzrock et al., 1985) and short pharmacological half-life certainly play a role in the limited effectiveness of systemic applications of IFNγ in patients with cancer.

The anti-cancer properties of one L19-IFNγ mutant, as a single therapeutic agent or in combination with chemotherapy or other immunocytokines are then analyzed. The therapeutic properties of L19-IFNγ have been studied in a number of different animal models.
3 RESULTS
3.1 ENGINEERING OF L19-IFNγ MUTANTS

3.1.1 Cloning and expression

The human antibody fragment scFv(L19), preceded by a secretion sequence (Li et al., 1997) required for secretion of recombinant proteins, was genetically fused to the murine IFNγ (muIFNγ) with a 9 amino-acid linker between the C-terminus of the scFv(L19) and the N-terminus of IFNγ and cloned into the HindIII / NotI restriction sites of the pcDNA3.1 mammalian expression vector, leading to vector pCE10 (Figure 3-1).

Attempts to express this fusion protein in stably transfected HEK cells led to protein preparations which could be purified on EDB antigen resin, which migrated correctly in SDS-PAGE gels in reducing conditions, but not in non-reducing conditions, thus indicating the formation of misfolded, disulfide-stabilized aggregates. In order to circumvent these folding problems, seven IFNγ mutants were fused to scFv(L19). The three cysteine residues of IFNγ were substituted by serines either individually (vectors pDG1-3) or simultaneously (pDG4), or were removed (pDG5-7) (Table 3-1). A His₆Tag was also appended at the C-terminus of the fusion protein to facilitate immunodetection.

Figure 3-1: Cloning and engineering of L19-IFNγ fusion proteins. Schematic representation of a pcDNA3.1 vector containing the essential elements of the L19-IFNγ fusion proteins. The muIFNγ moiety was fused to the C-terminus of the scFv(L19) antibody fragment by a 9 amino acid linker (SGSAGGSGG). The secretion sequence at the N-terminus is required for secretion of recombinant proteins and the His₆Tag at the C-terminus of muIFNγ was used for detection of the fusion protein.
Table 3-1: Schematic view of amino acid mutations of the different L19-IFNγ fusion proteins. Codons for cysteine in the muIFNγ gene at position 1, 3 and 136 were either mutated to code for serine, individually (vectors pDG1-3) or simultaneously (pDG4), or completely removed (pDG5-7).

For all fusion proteins, termed L19-IFNγ, L19-IFNγmut1-7 (Table 3-1), expression yields (judged by EDB affinity chromatography) ranged between 0.5 - 2 mg of purified fusion protein per liter of culture supernatant. Only L19-IFNγmut4 and L19-IFNγ mut7 showed a comparable electrophoretic behavior (43 kDalton) in reducing and non-reducing conditions in SDS-PAGE (Figure 3-2).

Figure 3-2: SDS-PAGE analysis of purified L19-IFNγ mutants under reducing (even lanes) and non-reducing (odd lanes) conditions. M: molecular weight markers; lanes 1, 2: L19-IFNγ non mutated; lanes 3, 4: L19-IFNγ mut1; lanes 5, 6: L19-IFNγ mut2; lanes 7, 8: L19-IFNγ mut3; lanes 9, 10: L19-IFNγ mut4; lanes 11, 12: L19-IFNγ mut5; lanes 13, 14: L19-IFNγ mut6; lanes 15, 16: L19-IFNγ mut7. The highlighted lanes 9 and 10 show the L19-IFNγ mut4 which was selected for further investigations.
3.1.2 Characterization

Among the fusion proteins, L19-IFNγmut4 (in which all three IFNγ cysteine residues had been mutated to serines) showed the best performance in size-exclusion chromatography and biodistribution (see 3.1.2.2), giving a single peak corresponding to the stable non-covalent homodimeric form of the fusion protein (Figure 3-3). This mutant was thus selected for further investigations.

![Figure 3-3: Size-exclusion chromatography of purified L19-IFNγ mut4 (Superdex 200). The retention volume (ml) of the peak corresponds to the position of the non-covalent homodimeric form of the fusion protein.](image)

3.1.2.1 Activity assay

As expected, the non-covalent homodimer was shown to be active in a biological assay based on the IFNγ cell growth inhibition of C51 adenocarcinoma cells. In equimolar terms, the biological activity of L19-IFNγmut4 was higher than the one all other mutants (data not shown), but slightly lower compared to recombinant murine IFNγ (Figure 3-4). The original, non-mutated form of L19-IFNγ displayed no detectable activity in this assay.
Figure 3-4: The antiproliferative activity of IFNγ was tested *in vitro* for the different fusion proteins (only data of the non mutated L19-IFNγ and L19-IFNγ mut4 are shown). The different fusion proteins and recombinant IFNγ as a positive control were added at increasing concentrations (1, 10, 100, 1000 ng/ml) to C51 adenocarcinoma cells and after 5 days, viable cells were counted. The experiment was performed in triplicate and results were expressed as the ratio between the number of viable cells divided by the number of untreated cells.

3.1.2.2 Biodistribution experiments

In a first biodistribution experiment performed on Sv129 immunocompetent mice bearing F9 tumors, radioiodinated L19-IFNγmut4 showed the highest tumor accumulation as compared to the other mutants (Figure 3-5 A and data not shown). The ratio of the %ID/g of tumor and blood (T/B) at 24 hours post-injection was approx. 3 : 1. In a biodistribution experiment performed on nude mice (which are devoid of T-cells), tumor uptake was higher (1.1%ID/g) as compared to Sv129 mice and at 24 hours the tumor to organ ratio was approx. 5 :1 (data not shown). In interferony receptor (IFNγR) knockout mice (G129) the tumor accumulation was about 4%ID/g of tumor with a T/B ratio of approximately 7 demonstrating a better targeting performance in the absence of IFNγ-Rs (Figure 3-5 B).
**Figure 3-5:** Biodistribution analysis of L19-IFNγmut4 in two different mouse models. **A:** F9 teratocarcinoma bearing immunocompetent Sv129 mice. **B:** F9 teratocarcinoma bearing G129 mice (IFNγ-R knockout mice). All mice (n=4) were injected with ~5 μg of ¹²⁵I-labeled L19-IFNγmut4 and sacrificed after 24 hours. Targeting results are expressed as percent injected dose per gram of tissue (%ID/g).
3.2 THERAPEUTIC PROPERTIES OF L19-IFN\(\gamma\) MUT 4

3.2.1 L19-IFN\(\gamma\) mut 4 as a single therapeutic agent

3.2.1.1 Effects on immunocompetent Sv129 mice bearing subcutaneously grafted F9 teratocarcinoma tumors

A first set of experiments on immunocompetent Sv129 mice, grafted with F9 teratocarcinoma tumor cells, was performed in order to find a suitable dose and time schedule for injections (Figure 3-6). Injected doses are expressed as equivalent of IFN\(\gamma\) contained in the L19-IFN\(\gamma\) mut4 fusion protein used in therapy experiments (i.e. 1 \(\mu\)g IFN\(\gamma\) equals 2.6 \(\mu\)g L19-IFN\(\gamma\)mut4).

In the first experiment, groups of mice (n=4) were injected i.v. with 20, 2 and 0.2 \(\mu\)g equivalent IFN\(\gamma\) of L19-IFN\(\gamma\) mut4 at day 4 after tumor implantation, when solid tumor masses started to be visible. Injections were repeated three times, every 72 hours (closed arrows). In an attempt to inject a fourth time, one mouse died and the other animals showed signs of toxicity (Figure 3-6A).
Figure 3-6: A: Dose escalation study of L19-IFNγ mut4 on F9 teratocarcinoma tumor growth in Sv129 mice. Four days after tumor implantation mice were separated in four different groups (n=4) and injected i.v. every 72 h either with 20 μg (open squares), 2 μg (open triangles), 0.2 μg (open diamonds) IFNγ of L19-IFNγ mut4 or with saline (filled triangles). Injections were continued as long as compatible with the side effects. Tumor volume is expressed in mm³ (mean ± SE) and arrows indicate the days of treatment. B: Effects of administration at different time points of 20 μg equivalent of L19-IFNγ mut4 on F9 teratocarcinoma tumor growth in Sv129 mice. Five days after tumor cell implantation mice were grouped (n=5) and i.v. injected either every 72 h (open squares and filled arrows) or every 24 h (open diamonds and open arrows) with 20 μg equivalent of L19-IFNγ mut4. In both groups, injections were repeated three times. A saline group (filled triangles) was included.

Once the most suitable dose was set, administration schedule was tested in another experimental setting: L19-IFNγmut4 was given i.v. either three times every third day (closed arrows) or on three consecutive days (open arrows) starting at day 5 after tumor implantation (Figure 3-6B). Markedly stronger therapeutic benefit was obtained with the administration schedule three times every 72 hours.

In order to assess toxicity of the treatment, body weight of all mice was monitored daily for every therapy experiment. After the third injection a transient weight loss up to 10% was observed in all mice belonging to the same treatment group (Figure 3-7).
Figure 3-7: Toxicity in mice treated with 20μg equivalent of L19-IFNγmut4 in a therapy experiment on Sv129 mice. Body weight of all animals was monitored daily and expressed as percent weight changes. Arrows indicate the days of treatment.

The therapeutic properties of L19-IFNγmut4 were then compared to a scFv antibody fragment of irrelevant specificity fused to IFNγmut4 (HyHEL10-IFNγmut4, specific to hen egg lysozyme, but not to mouse lysozyme). HyHEL10-IFNγmut4 displayed only minor therapeutic effects, compared to L19-IFNγmut4 (Figure 3-8A). When tumors in the control group reached a volume of 2000mm³ all animals were sacrificed, tumors excised and pictures were taken (Figure 3-8B).

Figure 3-8: A: F9 teratocarcinoma tumor growth curves after i.v. treatment of groups of 5 mice with 20 μg IFNγ of L19-IFNγmut4 (open squares), 20 μg IFNγ of HyHEL10-IFNγmut4 (filled squares) and saline (filled triangles). Tumor volume is expressed in mm³ (mean ± SE) and arrows indicate the days of treatment. B: F9 teratocarcinoma tumors. Tumors of all mice groups were excised at day 12 after tumor cell implantation.

The most relevant results of an immunohistochemical analysis of the excised tumors are summarized in Figure 3-9, Figure 3-10 and Figure 3-11. These findings include: a) no difference in cellular appearance in treated and untreated tumors and correlation of necrosis
with the size of the tumor (Figure 3-9); b) higher microvessel density (MVD) in L19-IFNγmut4 treated tumors (Figure 3-9); c) increased infiltration of CD4+ T-lymphocytes, natural killer cells (NK), macrophages and granulocytes in L19-IFNγmut4 treated tumors, compared to controls (Figure 3-10 and Figure 3-11); d) no change in the infiltration of CD8+ positive cells (Figure 3-10).

Figure 3-9: Immunohistochemical analysis of the excised tumors. A: Histological analysis of tumors and microvessel density (MVD) in L19-IFNγ treated vs. untreated (PBS) tumor sections. 4μm cryostat sections of the frozen tumor tissue were stained with H&E for detection of cellular appearance and with anti-CD31 antibody for analysis of MVD. B: Extent of necrosis compared to entire tumor surface in tumors treated with L19-IFNγmut4, HyHEL10-IFNγmut4 and saline. The whole tumor area (in mm²) compared to the area of necrosis is depicted.
Figure 3-10: Immunohistochemical analysis of the lymphocytic infiltration in L19-IFNy treated vs. untreated (PBS) tumor sections. Anti-CD4 and anti-α/β T-cell receptor (TCR) antibodies were used to detect infiltration of lymphocytes in tumor tissue. Quantitative evaluation of immunohistochemical analysis is graphically represented on the right.

Figure 3-11: Immunohistochemical analysis of granulocyte, macrophage, dendritic cell and NK cell infiltrations in L19-IFNy treated vs. untreated (PBS) tumor sections. On the right side of the figure the corresponding quantitative evaluation of the immunohistochemical analysis is graphically represented.
3.2.1.2 **Effects on immunocompetent Balb/c mice bearing subcutaneously grafted C51 colon adenocarcinoma and CT26 colon carcinoma tumors**

In contrast to the promising results obtained with F9 tumors, no therapeutic benefit could be obtained by administration of L19-IFNγmut4 to Balb/c mice bearing two different syngenic tumor models (C51 colon adenocarcinoma or CT26 colon carcinoma) (Figure 3-12A and Figure 3-12B). L19-IFNγmut4 treatment slightly accelerated tumor growth, consistent with previous reports implicating a role of IFNγ in the down-regulation of tumor antigens on CT26 cells (Beatty and Paterson, 2000). These data were confirmed in a L19-IFNγmut4/L19-IL2 combination therapy experiment with the C51 colon adenocarcinoma subcutaneous tumor model (see Figure 3-20 on page 81).

**Figure 3-12**: L19-IFNγmut4 therapy effects on Balb/c mice bearing C51 and CT26 syngenic tumors. A: Effects of L19-IFNγmut4 on C51 colon carcinoma tumor growth in Balb/c mice. Three days after tumor cell implantation mice were separated in 4 different groups (n=5) and injected i.v. three times every 72 h either with 20 μg (open squares), 2 μg (open triangles), 0.2 μg (open circles) equivalents of L19-IFNγmut4 or with saline (filled triangles). B: Effects of L19-IFNγmut4 on CT26 colon carcinoma tumor growth in Balb/c mice. Five days after tumor cell implantation mice were separated in 2 different groups (n=4) and injected i.v. every 72 h either with 20 μg (open squares) equivalents of L19-IFNγmut4 or with saline (filled triangles).
3.2.2 L19-IFNγ mut 4 as therapeutic agent in metastasis models

3.2.2.1 Effects on F9 liver metastasis in immunocompetent Sv129 mice

Intravenous injections of $2 \times 10^5$ F9 teratocarcinoma cells (adapted to grow in cell culture flasks without 0.1% gelatine; kindly provided by Dario Rusciano; Sifi, Sicily, Italy) in the tail vein of male Sv129 mice led to liver metastasis.

In a first experimental setting, eighteen Sv129 mice were injected i.v. with $2 \times 10^5$ F9 teratocarcinoma cells. Seven days after i.v. tumor cell injection nine mice were treated with i.v. injections of 20 µg IFNγ of L19-IFNγmut4 every third day; the remaining nine mice received no treatment. Three weeks after the tumor cell injections, all mice were sacrificed, livers were extracted and analyzed for metastatic foci. A picture of the livers at the end of the experiment can be seen in Figure 3-13 (Figure 3-13).

Figure 3-13: A: Picture of F9 liver metastasis in Sv129 mice treated with i.v. injections of 20 µg IFNγ of L19-IFNγmut4 every third day starting seven days after tumor implantation. The control group received no treatment. Mice were sacrificed after three weeks and livers were extracted. B: Picture of one sacrificed mouse with liver metastasis at higher magnification.
No therapeutic benefit could be achieved in the treated animals as compared to the control group (Figure 3-13). However, when therapy on liver metastasis using a combination of L19-IFN\textsubscript{mut4} and L19-IL2, L19-IFN\textsubscript{mut4} or L19-IL2 alone was started one day after i.v. tumor cell injection (instead of seven days after tumor cell injection) all treated mice (except for one treated with L19-IL2 alone, which had a single metastatic focus) were completely cured, in contrast to mice treated with saline (Figure 3-14). It is worth mentioning, that the amount of L19-IFN\textsubscript{y} injected was the same as in the previous metastasis therapy experiment (i.e. 20 \textmu g IFN\textsubscript{y} of L19-IFN\textsubscript{mut4}).

**Figure 3-14:** One day after i.v. F9 tumor cell injection mice were grouped into 4 different cages (n=6) and treatment started with i.v. injections of 20 \textmu g IFN\textsubscript{y} of L19-IFN\textsubscript{mut4}, 3\mu g IL2 of L19-IL2 (i.e. 1 \mu g IL2 = 3 \mu g L19-IL2), or the combination of L19-IFN\textsubscript{mut4} and L19-IL2 every third days. The control group received no treatment. Mice were sacrificed after 17 days and livers were extracted. On the right side a representative picture of two extracted livers, one without treatment and the other treated with L19-IFN\textsubscript{y}, is shown.

Strong expression of EDB in metastatic foci of the liver was confirmed by immunohistochemistry (Figure 3-15).
3.2.2.2 Effects on C51 lung metastasis in Balb/c mice

Ten Balb/c mice were injected i.v. with $10^5$ C51 colon carcinoma cells (Halin et al., 2002a). Seven days after tumor implantation five mice were treated with i.v. injections of 20 $\mu$g IFN$\gamma$ of L19-IFN$\gamma$mut4 every third day; the remaining five mice received no treatment. Three weeks after the tumor cell injections, all mice were sacrificed and analyzed for lung metastasis by taking pictures and weighing the metastases. As in the subcutaneous C51 tumor model, no therapeutic benefit could be achieved in any of the treated animals (Figure 3-16) although EDB expression in C51 lung metastasis was previously confirmed (Halin et al., 2002a).
Figure 3-16: Mice lungs with C51 metastasis after i.v. treatment with 20 μg IFNγ of L19-IFNγmut4 every third day. The control group received no treatment. The chart represents the average weight (mg) of metastasis of treated vs. untreated lungs.

3.2.3 Combination therapy

In the last therapy studies on subcutaneous F9 tumors in Sv129 mice, we tested whether chemotherapy and/or combination with other L19-based immunocytokines could improve the therapeutic performance of L19-IFNγmut4. The immunocytokines were L19-IL2 (Carnemolla et al., 2002; Carnemolla et al., 2002) and L19-TNFα (Borsi et al., 2003).

3.2.3.1 L19-IFNmut4 and doxorubicin on Sv129 mice bearing F9 tumors

Five days after tumor implantation, when tumors had reached a size of about 50 mm³ tumors, mice were grouped (n=4) in 4 different cages and therapy was started by injecting i.v. 20μg equivalent IFNγ of L19-IFNγmut4 alone, doxorubicin alone or the combination of L19-IFNγmut4 and doxorubicin. Another group received only saline treatment. Doxorubicin was given i.p. three times, three hours after immunocytokine injection at a dose of 100μg.
Figure 3-17A displays the outcome of the treatment modalities, represented as tumor volume plot. Three i.p. injections of 100 μg doxorubicin alone at days 5, 8 and 11 led to a tumor growth stasis. In the combination treatment, the tumor at day 12 started to shrink. However, this doxorubicin treatment schedule, either in combination with L19-IFNγ mut4 or alone was lethal for all mice tested, and significant toxicity was observed already after the second doxorubicin injection (> 15% weight loss) (Figure 3-17B).

**Figure 3-17:**

A: F9 tumor volume plot after i.v. treatment of 4 groups of 4 mice treated with the combination L19-IFNγmut4 and doxorubicin (cross), L19-IFNγmut4 alone (open squares), doxorubicin alone (filled circle) and saline (filled triangles). Twenty μg equivalents IFNγ were injected three times every 72 h (black arrows), starting at day 5 after tumor implantation. Three i.p. injection of 100 μg doxorubicin were given ~3 hours after each L19-IFNγmut4 injection. B: Percent weight changes plot of treated mice. Injections are indicated by black arrows.
3.2.3.2 Combination therapy of L19-IFNγmut4, L19-IL2 and doxorubicin

3.2.3.2.1 Sv129 mice

The next experiment was designed to test whether chemotherapy and/or combination with another L19-based immunocytokine (L19-IL2) could improve the therapeutic performance of L19-IFNγmut4. Five days after F9 tumor implantation in Sv129 mice, when tumors had reached a size of about 50 mm³ tumors, mice were grouped (n=6) in 8 different cages and therapy was started by injecting i.v. the two immunocytokines either alone or in combination (20μg equivalent IFNγ and 10 μg equivalent IL2). In addition, in order to test synergistic effects with chemotherapy, one single i.p. dose of 150μg of doxorubicin was given on day 6 to groups of mice injected with the two immunocytokines either alone or in combination. Thus the total doxorubicin dose was lowered compared to three injections of 100μg in the previous experiment which turned out to be lethal for all mice tested (see Figure 3-17). Furthermore, one group of mice received a saline treatment and one group was given only doxorubicin. All fusion proteins were injected three times every 72 hours, according to the previously defined injection schedule (see Figure 3-6 on page 68).

Figure 3-18 displays the outcome of the treatment modalities, either as tumor volume plot (Figure 3-18A) or as Kaplan-Meier curve (Figure 3-18B). Significant tumor growth retardation could be achieved in the group of the triple combination (IFNγmut4, L19-IL2 and doxorubicin). IFNγmut4 and L19-IL2 together were clearly more effective than the treatment of the two single fusion proteins, with or without chemotherapy. However, the two immunocytokines were well tolerated only if administered in combination with doxorubicin. In the combined IFNγmut4 + L19-IL2 treatment group (black squares), 2/6 mice died immediately after the third injection, while the four surviving mice did not show weight loss. The reasons for this heterogeneity are at present unclear. High-dose treatment with doxorubicin alone (150 μg i.p.) displayed only a minimal retardation of tumor growth.
Figure 3-18: A: F9 tumor volume plot after i.v. treatment of 8 groups of 6 mice treated with L19-IFNmut4, L19-IL2 and doxorubicin, L19-IFNmut4, L19-IL2, L19-IFNmut4, L19-IL2, doxorubicin, L19-IFNmut4 and doxorubicin, L19-IL2 and doxorubicin and saline. L19-IFNmut4 and L19-IL2 (20 μg equivalents IFNγ and 10 μg equivalents IL2) were injected three times every 72 h (filled arrows), starting at day 5 after tumor implantation. One single i.p. injection of 150 μg doxorubicin was given at day 6 after tumor implantation (open arrow). B: Kaplan-Meier analysis of combination therapy of L19-IFNmut4, L19-IL2 and doxorubicin (for detailed description see legend 18A). 1200mm³ tumor volume was set as an artificial threshold for the survival curve of mice (see Methods).

Figure 3-19 illustrates the tumors of the combination therapy of Figure 3-18 at day 11 after tumor cell implantation.
Day 11 after tumor implantation

**Figure 3-19**: Pictures of tumor bearing mice after 11 days of treatment with the combination L19-IFN\textsubscript{mut4}, L19-IL2 and doxorubicin in comparison to the saline control.

### 3.2.3.2 Balb/c mice

Since L19-IFN\textsubscript{mut4} did not show any therapeutic benefit on Balb/c mice in both tumor models used (see Figure 3-12 on page 72), we tested whether a better therapeutic effect could have been achieved in combination with doxorubicin and L19-IL2.

IFN\textsubscript{mut4} treatment confirmed the previous findings of slightly accelerated tumor growth. The same held true for L19-IL2 (which was used at suboptimal doses, i.e. at a concentration lower than the one published by Carnemolla and colleagues (Carnemolla et al., 2002), more precisely at 3 \mu g equivalent of L19-IL2, corresponding to 9 \mu g fusion protein) and for the combination of IFN\textsubscript{mut4} together with L19-IL2. The only treatment group leading to some therapeutic benefit was the combination group with doxorubicin, clearly demonstrating that doxorubicin contributed to the observed tumor growth retardation (Figure 3-20).
Figure 3-20: C51 tumor volume plot after i.v. treatment of 5 groups of 6 mice treated with L19-IFNγmut4, L19-IL2 and doxorubicin (filled circles), L19-IFNγmut4, L19-IL2 (filled squares), L19-IFNγmut4 (dotted line, open squares), L19-IL2 (dotted line, open diamonds) and saline (filled diamonds). L19-IFNγmut4 and L19-IL2 (20 μg equivalents IFNγ and 3 μg equivalents IL2) were injected three times every 72 h (filled arrows), starting at day 5 after tumor implantation. One single i.p. injection of 150 μg doxorubicin was given at day 6 after tumor implantation (open arrow).

3.2.3.3 Preliminary combination therapy of L19-IFNγmut4, L19-TNFα on Sv129 mice bearing F9 tumors

In a last preliminary therapy study on subcutaneous F9 tumors in Sv129 mice, we investigated whether L19-TNFα (Borsi et al., 2003) could enhance the therapeutic performance of L19-IFNγmut4. Five days after tumor implantation, mice were grouped (n≥3) into 4 different cages and therapy was started by injecting i.v. the two immunocytokines either alone or in combination (20μg equivalent IFNγ and 2 μg of L19-TNF fusion protein). According to previous studies showing great toxicity of TNFα, L19-TNFα was injected only once (2 μg) while L19-IFNγmut4 was injected three times every 72 hours, according to the previously defined injection schedule (see Figure 3-6 on page 68).

Significant tumor growth retardation could be achieved in the group of the double combination (IFNγmut4, L19-TNFα) (Figure 3-21). IFNγmut4 and L19-TNFα together were more effective than the treatment of the two single fusion proteins.
Figure 3-21: F9 teratocarcinoma tumor growth curves after i.v. treatment of 20 μg IFNγ of L19-IFNγmut4, given three times every 72 hours starting at day 5 after tumor implantation. One single i.v. injection of 2 μg L19-TNFα fusion protein was given at day 5 after tumor implantation together with L19-IFNγmut4. In the L19-IFNγmut4 treatment group 2 mice were used which explains the absence of error bars.
4 DISCUSSION
Only a careful mutational analysis, in which all IFNγ cysteine residues were mutated to serines or deleted, allowed the expression of functional fusion proteins without formation of disulfide-stabilized covalent aggregates. We have encountered similar problems when trying to fuse scFv(L19) to other proteins rich in cysteine residues (data not shown), in spite of the use of a mammalian cell expression system.

The engineered L19-IFNγmut4 fusion protein fulfilled all characteristics required for preclinical studies, i.e. purity assessed by gel electrophoresis, homogeneity in a size-exclusion chromatography analysis, excellent immunoreactivity and cytokine activity. Cytokine activity was slightly decreased compared to recombinant IFNγ. This might be due to a steric hindrance induced by the fusion of IFNγ to the scFv(L19). The original, non-mutated form of L19-IFNγ displayed no detectable activity in this assay supporting the assumption of the formation of misfolded, disulfide-stabilized aggregates.

In a first biodistribution study, performed with radioiodinated L19-IFNγmut4 in immunocompetent Sv129 mice the blood clearance resulted to be approx. 5-fold faster and the tumor uptake lower (0.8%ID/g), compared to SIP(L19), a homodimeric scFv(L19)-based miniantibody of comparable molecular weight (Borsi et al., 2002). Notably, a high uptake of the immunocytokine was observed in the liver (0.7 % injected dose per gram), consistent with previous reports of biodistribution studies performed with recombinant murine IFNγ (Gonias et al., 1988).

We then compared biodistribution studies, performed with radioiodinated L19-IFNγmut4 in immunocompetent, nude and IFNγR knock-out mice. The data revealed a clearly enhanced tumor targeting in mice lacking IFNγ-R, strongly suggesting that at least part of the fusion protein is sequestered in vivo before binding to the EDB antigen. These findings will most probably have implications in the design, monitoring and outcome of clinical trials with immunocytokines, since therapeutic performance is crucially related to the amount and location of the cytokine receptors involved. Our results also suggest that immunocytokines may display different tumor targeting abilities in different animal species, depending on the relative stoichiometric ratios between administered dose and accessible receptors. In the case of IFNγ, the IFNγR is ubiquitously expressed, with the exception of red blood cells. One way to circumvent this "receptor trapping", could be a "two step therapy" approach in which, before injecting the therapeutic cytokine, receptor inactivating binders (e.g. antibodies or small organic molecules) are injected with the aim to transiently block the cytokine receptors.
In our study, L19-IFNγmut4 exhibited a more potent anti-cancer activity with F9 tumors (subcutaneous and metastatic), as compared to C51 tumors. It is worth noting that the pattern of EDB expression is different in the two systems (predominantly vascular in the first case, diffuse and partly intracellular in the second case; L. Tarli, unpublished data), and that the L19 antibody accumulates more efficiently in F9 tumors. It remains to be seen in other experimental systems to which extent tumor targeting efficiency correlates with the therapeutic performance of immunocytokines.

The influence of different mouse strains in IFNγ therapy should not be underestimated. Recently it has also been observed that patients with production or signaling deficiencies may respond differently to IFNγ therapies (Newport et al.; 1996 Jouanguy et al., 1999).

Moreover, the different therapeutic outcome in the two tumor models, is consistent with several reports of experimental studies in mice that indicate that IFNγ can induce marked antitumor effects in some tumor models, but no effect or even an increase in tumor metastasis in other models (reviewed in Ferrantini and Belardelli, 2000).

The CT26 tumor model turned out to be an IFNγ insensitive tumor, confirming data that claim that IFNγ can promote tumor escape in CT26 colon carcinoma by down-regulating the protein expression of the endogenous antigen gp70, which has been reported to be the immunodominant antigen of CT26 (Beatty and Patterson, 2000).

These findings of an IFNγ dependent decrease in tumor immunogenicity, were demonstrated by another group (Morel et al., 2000). They showed that induction of the immunoproteasome by IFNγ results in less efficient processing of some tumor antigens, allowing for evasion of recognition by CTL.

The data of this thesis demonstrate that IFNγ fusion to the scFv(L19) tumor targeting antibody fragment could not revert this phenotype.

Epstein and coworkers had previously described the construction of a fusion protein between chimeric IgG-TNT-3 (specific to a histone marker abundant in necrotic tumor areas) and murine interferon-γ (Mizokami et al., 2003). The electrophoretic analysis presented in the article does not allow to assess whether high-order covalent aggregates were formed in non-reducing conditions. The biodistribution of the fusion protein was studied at late time points (3 and 7 days post-injection) as a result of the slower blood clearance of IgGs and their derivatives. Tumor to organ ratios were comparable to the ones of the intact antibody, but the fusion protein exhibited a faster clearance profile.
Several studies showed that the combination of different cytokine based immunotherapies may result in synergistic antitumor activity. As an example, synergy in antiproliferative effects by treatment with a combination of TNFα and IFNγ on tumor cells has been reported (Williamson et al., 1983).

Therapeutic activity can be additionally enhanced by co-administration of doxorubicin, which is one of the most effective anti-cancer drugs (Pratt, 1994) and more importantly, in contrast to many other cytotoxic drugs, has been reported to selectively enhance the murine host defense system (Ehrke et al., 1982). The combination of IFNγ with doxorubicin was assessed in vitro and in vivo in murine tumor models leading to promising data (Eppstein et al., 1988). Moreover, IFNγ and IL2 synergistically stimulate the immune system by activating a variety of immune effector cells and can lead to a significantly augmented therapeutic activity by the co-administration of doxorubicin (Lumsden et al., 1992).

Two approaches can be considered for the delivery of two synergistic cytokines to the tumor environment: a) the combination of two fusion proteins, in which an antibody is fused to individual cytokines; b) the construction of a triple fusion protein, comprising the antibody and the two cytokines (Gillies et al., 2002). In this work we decided to combine two separate fusion proteins since expression and tumor targeting of smaller molecules is easier (Halin et al., 2003). Moreover the appropriate dose to increase the synergistic effects can be freely chosen for each cytokine.

The best therapeutic results were obtained when L19-IFNγmut4 was combined with other L19-based immunocytokines (L19-IL2, L19-TNFα) and/or with doxorubicin. F9 tumor-bearing mice treated with a triple combination (L19-IFNγmut4+L19-IL2+doxorubicin) exhibited a substantial tumor growth delay, while high-dose doxorubicin chemotherapy (1 x 150 μg) was ineffective in the same animal model. The improved efficacy of this triple agent regime may be due to reduced impact of doxorubicin induced myelosuppression, thereby allowing for better maintenance of the host's immune functions. The mouse weight loss with the combination treatment never exceeded 5% and was lower than in the doxorubicin treatment group (10%). At higher doxorubicin doses (2 x 100 μg), an anti-tumor effect could be observed, but toxicities became unacceptably high (> 10% weight loss) and led to death in all mice tested.

Although weight loss in the L19-IFNγmut4+L19-IL2 combination treatment never exceeded 5%, 2/6 mice died immediately after the third injection. It is possible that doxorubicin
decreases the activity of the effector cells of the immune system strongly, activated by the combination of the two cytokines, thus avoiding onset of toxicity. Treatment with L19-IFNγmut4+L19-TNFα of mice bearing F9 tumors also resulted in significant tumor growth retardation. This synergy is due, at least in part, to the vasoactive properties of TNFα (Folli et al., 1993; Khawli et al., 1994), which enhance antibody delivery to tumor antigen. This was previously shown by the combination of L19-TNFα with L19-IL2 (Borsi et al., 2003) and with IL12-L19 (Halin et al., 2003).

In summary, we have shown that L19-IFNγmut4 is a novel anti-cancer agent, which is efficacious in some models of aggressive tumor and whose activity can be boosted by combination with other treatment modalities, without excessive cumulative toxicities. The ability of the L19 antibody to target tumors in patients with cancer has been demonstrated in immunoscintigraphy clinical studies (Santimaria et al., 2003). For applications in humans, L19-IFNγ fusion proteins are not likely to require mutation studies, as human L19-IFNγ (unlike murine L19-IFNγ) does not contain cysteine residues. Clinical trials will be needed to elucidate whether receptor trapping effects, which reduce tumor targeting efficiency in mice, may limit the therapeutic potential of a fully human L19-IFNγ fusion protein.
5 MATERIAL AND METHODS
5.1 CELL LINES, GENES AND COMMON REAGENTS

The tumor cell lines used were F9 murine teratocarcinoma (Berstine et al., 1973), C51 murine colon adenocarcinoma (Corbett et al., 1975), and CT26 murine colon carcinoma (Wang et al., 1995; Berstine et al., 1973).

For mammalian cell transfection the human embryonic kidney (HEK293) cell line was used (ATCC; Rockville, MD, USA).

Except for CT26 murine colon carcinoma cells, which were cultured in RPMI-1640, all cell lines were cultured in DMEM containing 10% FCS and incubated at 37°C in 5% CO₂.

scFv (L19) and scFv (HyHel10) have been described elsewhere (Viti et al., 1999; Smith-Gill et al., 1984).

The cDNAs for the mulIFNγ was obtained from (ATCC). Recombinant murine IFNγ was purchased from Merck (Darmstadt, Germany). Doxorubicin was purchased from Sigma (Buchs, Switzerland). Anti-His₆-Tag antibody was obtained from Sigma (Buchs, Switzerland). EDB was produced in bacteria, conjugated to Sepharose 4B (Amersham Pharmacia Biotech; Dübendorf, Switzerland) following the manufacturer’s instructions and used to immunopurify the below mentioned fusion proteins, except from HyHel10-IFNγ mut 4, which was purified on a Sepharose 4B resin (Amersham Pharmacia Biotech; Dübendorf, Switzerland) conjugated to lysozyme.

5.2 CLONING OF L19-IFNγ MUTANTS AND HYHEL10-IFNγMUT4

The mulIFNγ gene was amplified by polymerase chain reaction (PCR) using the following primers, which appended a 9 amino-acid linker (SGSAGGSGGG) at its N-terminus and the His₆-tag at its C-terminus:

-IFNγHisNotIfo (S'-ttt teg ttt ggc ggc tca tta atg atg gta atg gca gcg act cct ttg cct-3');

-IFNγlinker9ba (5'-tcc gga teg gcg gga ggt tea ggt gga tgt tac tgc cac ggc aca gtc-3').

Site-specific mutations of the mulIFNγ gene were generated by PCR as follows: change pos.1 (IFNγmut1): IFNγHisNotIfo, Mut1IFNγlin9ba (5'-tcc gga tcc gcg gga ggt tca gtt gga tgt tac tgc cac ggc aca gtc att g- 3'); change pos.3 (IFNγmut2): IFNγHisNotIfo, Mut31IFNγlin9ba (5'-tcc gga tcc gcg gga ggt tca gtt tgt atg tta ctc cca cgg cac aat cat tgt gtc gtc gtc gtc gtc- 3'); change pos.136 (IFNγmut3): Mut136IFNγHisNotIfo (5'-ttc ttc ttc ggg cgg ctc tta ttc atg atg atg gta atg gga ggc act cct ttg cct ctc- 3'), IFNγlinker9ba; change pos.1, 3, 136 (IFNγmut4):
Mut136IFNγHisNotIfo, Mut1-3IFNylin9ba (5'-tcc gga tcc gec gga ggt tca ggt gga tct tcc cac ggc aca gtc att gaa- 3'); remove 1, 2, 3 (IFNγmut5): IFNγHisNotIfo, w/o-3IFNylinker9ba (5’-tcc gga tcc gec gga ggt tca ggt gga cac ggc aca gtc att gaa agc- 3’); remove 136 (IFNγmut6): IFNylinker9ba, w/o136IFNyHisNotIfo (5’-ttt tcc ttt tgc ggc cgc tca tta atg gtg atg gtg atg ggc act cct ttt ccc ctt cct g- 3’); remove 1, 3, 136 (IFNγmut7): w/o-3IFNylinker9ba, w/o136IFNyHisNotIfo.

After PCR-amplification with the above-mentioned primer combinations the non-mutated muIFNγ and the seven mutants of muIFNγ were PCR-assembled with the scFv(L19), which in turn was amplified using the primers FRT-CMVfo (5’-cgc aaa tgg gcg gta ggc gtg-3’) and L19linkerfo (5’-cgc gga tcc gga ttt gat ttc cac ctt ggt ccc ttg-3’) from pCH34, a pcDNA3.1 derivative. The DNA of a signal peptide was co-amplified with the scFv(L19).

HyHEL10-IFNγmut4 was obtained by PCR assembly of IFNγmut4 and scFv(HyHEL10), which was amplified using the primers FRT-CMVfo and HH10lin9fo (5’-ttc ace tag acc acc cgc gga tcc gga ttt tat ttc cag ctt ggt gcc ccc tc- 3’) from pCE1 (a derivative of pcDNA3.1).

The assembled non-mutated L19-IFNγ, the L19-IFNγ mutants and HyHel10-IFNγmut4 genes were cloned into the HindIII and NotI restriction sites of the mammalian cell expression vector pcDNA3.1(+) (Invitrogen; Basel, Switzerland) yielding vectors pCE10 (L19-IFNγ), pcDG1-7 (L19-IFNγ mut1-7) and pCE17 (HyHel10-IFNγ mut4).

DNA primers were purchased from Microsynth (Balgach, Switzerland) and restriction enzymes from Qbiogene (Basel, Switzerland).

5.3 EXPRESSION AND PURIFICATION OF L19-IFNγ, L19-IFNγ MUTANTS 1-7 AND HYHEL10-IFNγMUT4

HEK 293 cells were stably transfected with the previously described plasmids (pCE10, pcDG1-7 and pCE17) and selection was carried out in the presence of G418 (0.5 g/l). Clones of G418-resistant cells were screened for expression of the fusion protein by ELISA, using recombinant EDB domain of human fibronectin or lysozyme as antigens and an anti-His6Tag antibody (Sigma, Buchs, Switzerland) for detection. The fusion proteins were purified from cell culture medium by affinity chromatography over antigen columns as described previously (Neri et al., 1996; Tarli et al., 1999). The size of the fusion proteins was analyzed under reducing and non-reducing conditions on SDS-PAGE as well as under native
conditions by FPLC gel filtration on a Superdex S-200 (Amersham Pharmacia Biotech; Dübendorf, Switzerland).

5.4 IFNγ ACTIVITY ASSAY

The antiproliferative activity of IFNγ was tested in vitro for the different fusion proteins. C51 adenocarcinoma cells were seeded into 24-well plates at a concentration of 1.5x10^4 cells/well in 1ml of DMEM, 10%FCS. After 6-8 hours the different mutants of L19-IFNγ fusion protein and recombinant IFNγ were added at increasing concentrations (1, 10, 100, 1000 ng/ml). At day 5, viable cells were counted using Trypan Blue staining. The experiment was performed in triplicate and results were expressed as the ratio between the number of viable cells divided by the cell number of an untreated control culture.

5.5 EXPRESSION AND PURIFICATION OF L19-IL2 AND L19-TNFα

L19-IL2 and L19-TNFα were expressed in sp2/0 myeloma cells and HEK293 cells, respectively. Both fusion proteins were then purified from cell culture medium by affinity chromatography over antigen columns as described previously (Neri et al., 1996; Tarli et al., 1999), dialyzed in saline and aliquoted. L19-IL2 was flash frozen in liquid nitrogen and stored at −80°C while L19-TNFα was kept at 4°C. The size of the fusion proteins was analyzed on SDS-PAGE and by FPLC gel filtration on Superdex S-200 (Amersham Pharmacia Biotech; Dübendorf, Switzerland).

5.6 BIODISTRIBUTION EXPERIMENTS

The in vivo targeting performance was evaluated by biodistribution analysis as described by (Tarli et al., 1999). Briefly, purified L19-IFNγmut4, 5, 6 and HH101IFNγmut4 (according to the experiments performed) were radioiodinated and ~5 µg of each fusion protein (~6 µCi) were injected into the tail vein of immunocompetent Sv129 mice, nude mice or G129 (IFNγRγ) mice bearing s.c. implanted F9 murine teratocarcinoma. Mice were sacrificed 24 hours after injection, organs were weighed and radioactivity was counted with a Packard Cobra Gamma counter (model B5002, S/N 104385).

The radioactivity content of representative organs was expressed as the percentage of the injected dose per gram of tissue (%ID/g).
5.7 TUMOR MOUSE MODELS

Tumor-bearing mice were obtained by injecting subcutaneously (s.c.) $10^6$ F9 murine teratocarcinoma cells in 12-14-week-old female Sv129 mice, $10^6$ C51 murine colon adenocarcinoma and $10^4$ CT26 colon carcinoma cells in 12-14-week-old female BALB/c mice, respectively. Tumor cell were always injected in a total volume of 100 μl in cell culture medium (0% FCS).

Normally 4-5 days after tumor cell implantation, mice were grouped ($n \geq 4$) and injected intravenously (i.v.) in the lateral tail vein with saline, with HyHell10-IFNγmut4 or with L19-IFNγmut4 at different doses and time points diluted in a volume of 200 μl of saline (see figure legends and more detailed explanation for combination therapies below).

Mice were monitored daily and tumor growth was measured with a caliper using the formula: 
Volume = length x width$^2$ x π/6. Animals were sacrificed when tumors reached a volume greater than 2000 mm$^3$. Tumor sizes are shown as mean ± SE. As our Animal License (Veterinäramt des Kantons Zürich, Bew. 83/2002) forced us to sacrifice mice with tumor loads > 10% body weight, an artificial threshold of 1200 mm$^3$ was used for the construction of the Kaplan-Meier survival curve. Mice were purchased from Taconic, Denmark.

5.8 COMBINATION THERAPY EXPERIMENTS

5.8.1 L19-IFNγmut4 and doxorubicin

For the combination therapy with L19-IFNγmut4 and doxorubicin, 20μg equivalent IFNγ of L19-IFNγmut4 (i.e. 1 μg IFNγ = 2.6 μg L19-IFNγmut4) were injected i.v. at days 5, 8 and 11 after tumor implantation. Doxorubicin was administered intraperitonealy (i.p.) three hours after each immunocytokines injection, at a dose of 150 μg/mouse in 100 μl 0.9% saline solution.

5.8.2 L19-IFNγmut4, L19-IL2 and doxorubicin

For the combination therapy study with L19-IL2, 20μg equivalent IFNγ of L19-IFNγmut4 and 10 μg equivalent (or 3 μg in the combination study on Balb/c mice bearing C51 tumors)
IL2 of L19-IL2 (i.e. 1 µg IL2 = 3 µg L19-IFNγmut4), respectively, were mixed prior to i.v. injections, which were carried out at days 5, 8 and 11 after tumor implantation. The day after the first injection of the fusion protein mix (day 6 after tumor implantation), 150 µg/mouse in 100 µl 0.9% saline solution of doxorubicin were injected intraperitonealy (i.p.).

5.8.3 L19-IFNγmut4 and L19-TNFα

For the combination therapy study with L19-TNFα, 20µg equivalent IFNγ of L19-IFNγmut4 were injected i.v. at days 5, 8 and 11 after tumor implantation. Two µg of the fusion protein L19-TNFα diluted in a volume of 200 µl of PBS were injected once at day 5 after tumor implantation.

5.9 METASTASIS MODELS

5.9.1 Lung metastasis

Female Balb/c mice were injected i.v. with 10^5 cells C51 murine colon adenocarcinoma cells (Halin et al., 2002a).

After 7 days, mice were divided into two groups (n=5) and injected i.v. with either PBS or 20µg equivalent IFNγ of L19-IFNγmut4 four times every third day. Mice were killed after three weeks; the lungs were removed, fixed in PBS containing 3% formaldehyde, and examined with a Zeiss stereomicroscope (Jena, Germany). Metastases were then removed with tweezers and the weight determined immediately after removal (to avoid drying).

5.9.2 Liver metastasis

Male Sv129 mice were injected i.v. with 2x10^5 F9DR murine colon adenocarcinoma cells (adapted to grow on uncoated plastic flasks; kindly provided by Dario Rusciano, Sifi, Catania, Italy).

In the first experimental setting, 7 days after tumor implantation, mice were divided into two groups (n=9) and injected i.v. with either PBS or 20µg equivalent IFNγ of L19-IFNγmut4 four times every third day. Mice were killed after three weeks; the livers were excised and
pictures were taken. Metastatic livers were then snap-frozen in liquid nitrogen for immunohistochemistry.

In another experiment, therapy was started on day 1 after tumor implantation; L19-IFNγmut4 therapy was administered as described above, but two groups receiving L19-IL2 alone (3μg equivalent IL2 of L19-IL2) or in combination with L19-IFNγmut4 were added.

5.10 QUANTITATIVE ANALYSIS OF NECROTIC TUMOR AREA

An H&E section along the largest diameter of the tumor was scanned at low power using the computer aided image analysis system Quantimet 600 and Qwin software (Leica, Germany). The total tumor area and necrotic tumor area were subsequently marked by the examiner on the screen. The areas were calculated by the image analysis system and the ratio tumor necrosis area / total tumor area was given in percent.

5.11 IMMUNOHISTOCHEMISTRY

For immunohistochemical analysis of the lymphocytic infiltration and vascularization tumors were excised and immediately after tumor resection snap-frozen in liquid nitrogen. 4μm cryostat sections of the frozen tumor tissue samples were fixed in ice-cold acetone for 15 min and subjected to immunohistochemistry. The following primary antibodies were used to differentiate tumor infiltrating leucocytes:
rat anti-mouse CD8a/Ly2 (T-helper / inducer, clone 53-6.7); rat anti-mouse CD4/L3T4 (T-suppressor / cytotoxic, clone H129.19); rat anti-mouse CD11b/Mac-1α chain (granulocytes, macrophages, natural killer cells, clone M1/70); rat anti-mouse CD19 (B-cells, clone 1D3); rat anti-mouse CD45/LCA/Ly5 (all cells of hematopoietic origin, clone 30-F11); rat anti-mouse CD31 (PECAM-1, endothelial cells, clone MEC 13.3, all from BD Pharmingen Int.); biotinylated hamster anti-mouse α/β T-cell receptor (clone H57-597); rat anti-mouse CD45R/B220 (B-cells, activated T-cells, non-MHC-restricted lytically active lymphokine-activated killer cells, clone RA3-6B2) (both from Southern Biotechnology Associates, Inc.); and biotinylated mouse anti-mouse CD161b/c/NK1.1 (natural killer cells, clone PK136, from Serotec Int.).

Immunohistochemical staining was performed on an AutostainerPlus (DakoCytomation, Hamburg, Germany) using the DAKO ChemMate™ Detection Kit (AP/Red) (DakoCytomation, Hamburg, Germany) as recommended by the manufacturer. Biotinylated
mouse anti-rat IgG2b and IgG1/2a (BD Pharmingen Int.) were used as secondary antibodies for detection of unlabeled primary antibodies.

5.12 QUANTITATIVE EVALUATION OF LYMPHOCYTIC INFILTRATION

**CD11b, CD45 and a/b TCR:** Quantitative evaluation of immunohistochemical results was carried out by computer aided image analysis using the image processing and analysis system Quantimet 600 and the Qwin software (Leica, Germany). First a red colour threshold was interactively set by the examiner for each antibody to select the red stained immunopositive area. All pixels in the image that meet the defined threshold were transferred into a binary image and analyzed. For each tumor specimen the immunostained area was detected in 5 randomly selected measurement areas (if possible mainly from the tumor periphery, each 0.19mm²) and was expressed in % of the whole measurement area. For each animal group the mean value of all measurement results was calculated.

**CD4, CD8a, CD19, CD45R/B220 and NK1.1:** For each tumor specimen the immunostained cells were counted by the examiner in 5 randomly selected 200 x fields (if possible mainly from the tumor periphery). For each animal group the mean value of all counts was calculated.

5.13 ASSESSMENT OF MICROVESSEL DENSITY

Microvessel density (MVD) was assessed according to the criteria of Weidner et al. (Weidner et al., 1991) after immunostaining for CD31. Intratumoral MVD was evaluated by light microscopic scanning at low power of tumoral areas containing the most capillaries and small venules. The microvessel were counted by the examiner in 5 randomly selected 200 x fields. For each animal group the mean value of all counts was calculated.
6 APPENDIX (OTHER EXPERIMENTAL PROTOCOLS)
6.1 CLONING OF MIP3α -L19

pCE15, which is a derivative of the mammalian cell expression vector pcDNA3.1 (+) (Invitrogen, Basel, Switzerland) was used for cloning. MuMIP3α was amplified using the primer muLARCEcoRIback (5’-ccg gaa ttc atg gcc tgc ggt ggc aag cgt-3’), which anneals to the endogenous secretion sequence of MIP3α and appends to its 5’ end a restriction site for the endonuclease EcoRI and linkermuLARCfo (5’-ctc acc tcc agc gct tcc cat ctt gac tct tag gct gag g-3’), which introduces a 6 amino acid linker (GSADGG).

The sequence of L19, which was cloned after the linker (at the C-terminus of MIP3α), was amplified using primers LinkL19back (5’-gga age get gat gga ggt gag gtg cag ctg ttg gag tc-3’), which appends to the 5’ end of L19 the complementary DNA sequence of the amino acid linker GSADGG, and the primerL19forHisA6 (5’-ttt tcc ttt tgc ggc cgc tea tta atg gtg atg gtg atg atg atg atg ttt gat ttc cac ctt ggt ccc-3”), introducing two stop codons as well as a His6 tag.

The MIP3α sequence and the L19 sequence were then PCR assembled and cloned into the Hind III and NotI restriction sites of pcDNA3.1(+).

6.2 CLONING OF L19-IL1β

The vector used for cloning was pCE11, which is a derivative of the mammalian cell expression vector pcDNA3.1 (+) (Invitrogen, Basel, Switzerland), in which the genes for a secretion sequence [Li et al., 1997], required for the secretion of the recombinant proteins and the sequence of scFvL19 was introduced. The gene coding for human IL1β, was cloned between at the C-terminus of the antibody fragment sequence and a 5 amino acid linker (SGSAG) by PCR assembly; a His6 Tag was appended at the C-terminus of the human IL1β sequence.

Following primers were used: FRT-CMVfo (5’-cgcaaatgggcggtaggcgtg-3’), L19linkerfo (5’-tcc cgc gga tcc gga ttt gat ttc cac ctg ggt ccc ttg-3’) to amplify the scFvL19, IL1 β linkerba (5’-aaa tcc gga tcc gcc gga gca cct gta cga tca ctg aac tgc-3’) and IL1 β forHisNotI (5’-ttt tcc ttt tgc ggc cgc tea tta atg gtg atg atg atg atg atg ttt gat ttc cac ccc cat ggt gaa g-3’) to amplify the extracellular domain of IL1β from a human cDNA library.

The assembled L19-IL1β was cloned into the Nhe III and NotI restriction sites of pcDNA3.1(+).
DNA primers were purchased from Microsynth (Balgach, Switzerland) and restriction enzymes were purchased from Qbiogene (Basel, Switzerland).

6.3 IL1β ACTIVITY ASSAY

Based on the ability of IL-1 β to induce IL-8 in fibroblasts. NDHF cells were seeded into 24-well plates and at 90-100% confluency L19-IL1β was added. After 24 hrs at 37°C presence of IL-8 in the supernatants was analyzed with a commercial human IL-8 ELISA kit (Pierce, Lausanne, Switzerland).

6.4 CLONING OF MIP3β-L19

PCE14 which is a derivative of the mammalian cell expression vector pcDNA3.1 (+) (Invitrogen, Basel, Switzerland) was used for cloning. MuMIP3β was amplified from PT7T3D-PacI (ATCC; Rockville, MD) using the primer ELCEcoRback(5’-ccg gaa ttc atg gcc ccc cgt gtg ace cca-3’) which anneals to the endogenous secretion sequence of MIP3β and appends to its 5’ end a restriction site for the endonuclease EcoRI and linkerELCfo (5’-ctc ace tec ate age get tec aga cac agg get cet tct ggt cgt-3’) which introduces a 6 amino acid linker (GSADGG).

The sequence of L19, which was cloned after the linker (at the C-terminus of MIP3β), was amplified using primers linkL19back (5’-gga age get gat gga ggt gag gtg cag ctg ttg gag tc-3’), which appends to the 5’ end of L19 the complementary DNA sequence of the amino acid linker GSADGG, and the primerL19forHisNotI (5’-ttt tcc ttt tgc ggc cgc tca tta atg atg atg atg atg ttt gat ttc cac ctt ggt ccc-3’), introducing two stop codons as well as a His6 tag.

The MIP3β sequence and the L19 sequence were then PCR assembled and cloned into the HindIII and NotI restriction sites of pcDNA3.1(+).

6.5 CLONING OF L19-TRAIL

The cDNA for the extracellular domain of huTRAIL was amplified from a human cDNA library (Clontech, BD Biosciences, Basel, Switzerland).

PCE4 which is a derivative of the mammalian cell expression vector pcDNA3.1 (+) (Invitrogen, Basel, Switzerland) was used for cloning. The cDNA for the extracellular domain of huTRAIL was amplified from a human cDNA library (Clontech, BD Biosciences, Basel, Switzerland) using the primer TRAILba (5’-gtg
aga gaa aga ggt cct cag aga g-3') which anneals to the extracellular domain of TRAIL at the 5' end and TRAIL for NotI (5'-ttt ttc ttt tgc ggc cgc tta aac taa aaa gcc ccc gaa aaa ac- 3') which introduces a stop codon and a restriction site for the endonuclease NotI. The sequence of L19 with the signal sequence and a (Ser4Gly)3 linker at its C-terminus, was amplified using primers FRT-CMVf0 (S'-cgc aaa tgg gcg gta ggc gtg-3'), and TRAILlinkf0 (S'-ctc tct gag gac etc ttt etc tea cgc cgc tgg acg atg age cg-3") which contains a part of the (Ser4Gly)3 linker and the 5' of TRAIL. The L19 as well as the TRAIL sequence were then PCR assembled and cloned into the Hind III and NotI restriction sites of pcDNA3.1(+).

6.6 CLONING OF RANTES-L19

PCE13 which is a derivative of the mammalian cell expression vector pcDNA3.1 (+) (Invitrogen, Basel, Switzerland) was used for cloning. MuRANTES was amplified from PT7T3D-Pac-RANTES (ATCC; Rockville, MD) using the primer RANTESEcoRIback (5'–ccg gaa ttc atg aag ate tct gca get gcc–3') which anneals to the endogenous secretion sequence of RANTES and appends to its 5' end a restriction site for the endonuclease EcoRI and LinkerRANTESfo (5'– etc ace tec ate age get tec get cat etc caa ata gtt gat gta–3') which introduces a 6 amino acid linker (GSADGG). The sequence of L19, which was cloned after the linker (at the C-terminus of RANTES), was amplified using primers LinkL19back (5'-gga age get gat gga ggt gag gtg cag ctg ttg gag tc-3'), which appends to the 5' end of L19 the complementary DNA sequence of the amino acid linker GSADGG, and the primerL19forHisMsf (5'-ttt ttc ttt tgc ggc cgc tca tta atg gtt atg atg gtt gtt gat ttc cac ctt ggt ccc–3'), introducing two stop codons as well as a His6 tag The RANTES sequence and the L19 sequence were then PCR assembled and cloned into the Hind III and NotI restriction sites of pcDNA3.1(+).

6.7 CLONING OF L19-FASL

The cDNA for the extracellular domain of FasL was amplified from a mouse cDNA library (Clontech, BD Biosciences, Basel, Switzerland). PCE16 which is a derivative of the mammalian cell expression vector pcDNA3.1 (+) (Invitrogen, Basel, Switzerland) was used for cloning. The cDNA for the extracellular domain of TRAIL was amplified from a mouse cDNA library (Clontech, BD Biosciences, Basel, Switzerland) using the primer FasLba (5'-caa ata gcc aac
ccc agt aca cc -3’) which anneals to the extracellular domain of FasL at the 5’end and FasLforHisNotI (5’-ttt tcc ttt tgc gcc cgc tca tta atg gta gta gta gta gta aag ctt ata caa gcc gaa aca ggc gat c-3’) which introduces two stop codons and a restriction site for the endonuclease NotI.

The sequence of LI9 with the signal sequence and a (Ser₄Gly)₃ linker at its C-terminus, was amplified using primers FRT-CMVfo (5’-cgc aaa tgg gcg gta ggc gtg-3’), and FasLlinkfo (5’-ggg tgt act ggg gtt ggc tat ttg cgc gat gga cga tga ggc g-3’) which contains a part of the (Ser₄Gly)₃ linker and the 5’ of FasL.

The L19 as well as the TRAIL sequence were then PCR assembled and cloned into the Nhel and NotI restriction sites of pcDNA3.1(+).
7 REFERENCES


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