Targeted delivery of immunomodulatory proteins: evaluation of novel antibody-chemokine and antibody-cytokine fusion proteins for cancer therapy

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Targeted delivery of immunomodulatory proteins: evaluation of novel antibody-chemokine and antibody-cytokine fusion proteins for cancer therapy

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presented by

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1 Summary

The antibody-mediated delivery of bioactive ‘payloads’ (e.g., drugs, growth factors, and cytokines) at the site of disease holds promises for the development of more selective and efficacious therapeutic agents. Conventional drugs usually exhibit poor selectivity between malignant and healthy tissues, resulting in drug-related toxicities that prevent dose escalation to a therapeutically active regime. ‘Targeting’ of payloads to diseased tissues, by disease homing antibodies used as ‘vehicles’, represents one of the most promising avenues for cancer therapy. Various classes of such ‘armed antibodies’ are being developed by pharmaceutical companies, the most advanced ones being antibody cytokine fusion proteins (‘immunocytokines’), antibody drug conjugates (ADCs), radiolabeled antibodies and bispecific antibodies.

In this thesis, antibody chemokine fusion proteins (‘immunochemokines’) have been evaluated as a novel class of armed antibodies for applications in cancer therapy. Ten chemokines with potential antitumoral activity were genetically fused to the F8 antibody, specific to the extra domain A (EDA) splice variant of fibronectin, a marker of angiogenesis. Expression was attempted in different mammalian expression systems and the resulting fusion proteins were purified to homogeneity, for subsequent biodistribution studies. Radioiodinated preparations of the immunochemokines which could be expressed were tested in quantitative biodistribution studies in tumor-bearing mice, but all fusion proteins failed to preferentially localize at the tumor site, while the unconjugated parental antibody displayed an excellent tumor to blood ratio. This thesis highlights expression, analytical and biodistribution challenges associated with the antibody-based in vivo delivery of chemokines to the tumor site. It also provides the first comprehensive report in this field and may facilitate future research efforts for the development of therapeutic immunochemokines.

Since antibody chemokine fusion proteins failed to preferentially accumulate at the tumor site, new members of the class of immunocytokines were investigated.
The potent pro-inflammatory murine cytokines IL1β and IL6 were fused either at the N-terminus or at the C-terminus of the monoclonal F8 antibody, used in diabody format. All immunocytokines retained the binding properties of the parental antibody and were homogenous after purification. Fusions with IL6 exhibited a preferential localization at the tumor site for both cytokine orientations, whereas IL1β fusions exhibited a worse tissue distribution profile, with lower tumor to organ ratios and a slower blood clearance. The fusion proteins with the cytokine payload at the C-terminus were studied in therapy experiments in immunocompetent mice bearing F9 tumors. Both F8-IL1β and F8-IL6 exhibited a <50% inhibition of tumor growth rate, which was substantially lower than the one previously reported for F8-TNF, a functionally related pro-inflammatory immunocytokine. This thesis shows that IL6 and IL1β can be efficiently delivered to the tumor neo-vasculature, but do not show an as potent antitumor effect as other immunocytokines. IL1β could be administered only at a very low dose due to toxic side effects. This study sheds light on the role of pro-inflammatory cytokines in boosting immunity against the tumor.

F8-IL13 is another novel immunocytokine cloned, expressed and tested for its in vivo performance in this thesis. The IL13 moiety was fused at the C-terminal extremity of the F8 antibody in diabody format. The resulting F8-IL13 immunocytokine retained the full binding properties of the parental antibody and cytokine bioactivity and showed a preferential accumulation at the tumor site. When used as single agent, F8-IL13 exhibited a strong inhibition of tumor growth rate in two models of cancer (F9 teratocarcinoma and Wehi-164), promoting an infiltration of various types of leukocytes into the neoplastic mass. This anticancer activity could be potentiated by combination with an immunocytokine based on the F8 antibody and murine IL12 (termed IL12-F8F8), leading to complete and long-lasting tumor eradications. Mice cured from Wehi-164 sarcomas acquired a durable protective antitumor immunity and selective depletion of immune cells revealed that the antitumor activity was mainly mediated by CD4+ T cells. This thesis shows that IL13 can be efficiently delivered to the tumor neo-vasculature and that it mediates a potent anticancer activity in two models of cancer. It may therefore represent an interesting candidate for further clinical development.
Within the scope of investigations aimed at the delivery of payloads with antiangiogenic function to the tumor site, two different versions of the trimeric TNF family member vascular endothelial growth inhibitor (VEGI) were fused to the F8 antibody, used in scFv format. The resulting immunocytokines were tested in \textit{in vivo} biodistribution experiments and the results were compared with biodistribution data of other TNF family members fused to antibodies. The study revealed that even payloads, which are structurally and functionally related, may exhibit dramatically different \textit{in vivo} pharmacokinetic behavior and tumor targeting performance.

The ability of antibodies to extravasate out of blood vessels is critical for therapeutic activity, since molecular targets for most diseases are located outside the endothelial lining. By performing detailed biodistribution studies with a novel Interleukin-9-armed antibody, we identified a clear correlation between N-glycan profiles and tumor targeting performance. Non-sialylated N-glycan structures negatively impact on pharmacokinetics and disease-homing activity, highlighting the importance of glycosylation control and characterization during process development.
Zusammenfassung


Im ersten Teil dieser Doktorarbeit wurde die neue Klasse der Antikörper-Chemokin Fusionsproteine, die sogenannten „Immunochemokine“, auf ihre Tauglichkeit für die Krebstherapie untersucht. Zehn Chemokine, welche gemäss Literaturangaben eine potentielle Antitumoraktivität haben könnten, wurden versucht als Fusionsproteine mit dem monoklonalen F8 Antikörper herzustellen. Dieser Antikörper erkennt eine Spleissisoform von Fibronectin, welche (fast) ausschliesslich bei neugebildeten Blutgefässen, wie sie in Tumorgewebe und bei inflammatorischen Krankheiten vorkommen, exprimiert wird. Somit sollte es mit diesem Antikörper theoretisch möglich sein, wie analog schon mit vielen Zytokinen gezeigt, Chemokine direkt zum Tumor zu transportieren. Allerdings stellte sich bereits die Expression der Fusionsproteine in Säugerzellen (CHO-S)

Da sich die Immunochemokine nicht im neoplastischen Gewebe anreicherten, wurden im zweiten Teil der Arbeit neue Vertreter der Immunozytokine bestehend aus dem F8 Antikörper und den entzündungsfördernden Zytokinen Interleukin-1β und Interleukin-6, kloniert, aufgereinigt und sowohl in vitro, als auch in vivo getestet. Beide Zytokine wurden jeweils N- und C-terminal an den Antikörper fusioniert. Die daraus resultierenden vier Konstrukte wurden in Biodistributionsstudien untersucht und alle waren in der Lage sich spezifisch im F9 Tumorgewebe anzureichern. Allerdings war die Selektivität gegenüber gesunden Organen bei den C-terminalen Fusionsproteinen besser, weshalb diese, genannt F8-IL1β und F8-IL6, für die darauffolgenden Therapiestudien verwendet wurden. Beide Immunozytokine zeigten einen Antitumoreffekt, allerdings war dieser bedeutend schwächer als beispielsweise der von F8-TNF, einem schon früher getesteten, verwandten Immunozytokin. Aus diesem Grund wurden F8-IL1β und F8-IL6 in der Folge auch nicht weiter entwickelt.

Bei F8-IL13 handelt es sich um ein aus dem F8 Antikörper und dem Zytokin Interleukin-13 bestehendes Immunozytokin, das im Rahmen meiner Arbeit hergestellt und charakterisiert wurde. Dieses Fusionsprotein zeigte eine starke Anreicherung im Tumor und auch einen sehr starken Antitumoreffekt in zwei murinen Krebsmodellen (F9 Teratokarzinom und WEHI164 Sarkom). In einer Kombinationstherapie zusammen mit IL12-F8F8, einem zuvor schon
beschriebenen Immunozytokin, konnten einige Mäuse geheilt werden. Eine erneute Injektion derselben Tumorzellen in geheilte Mäuse führte nicht zu erneutem Tumorwachstum, was zeigt, dass diese eine schützende Immunität gegen diese Krebszellen erlangt haben müssen. Eine Untersuchung des Antitumormechanismus lässt, entgegen allen bisherigen Erfahrungen mit anderen Immunozytokinen, darauf schliessen, dass der Effekt hauptsächlich von CD4+ T Zellen und nicht NK oder CD8+ T Zellen abhängig ist. Mit der vorliegenden Arbeit konnte also gezeigt werden, dass sich das Immunozytokin F8-IL13 spezifisch im Tumor anreichert, einen starken Antitumoreffekt aufweist und sich dessen klinische Weiterentwicklung dementsprechend in Betracht gezogen werden könnte.

Im Rahmen eines Projekts zum Vergleich der Tumorlokalisation von Immunozytokinen basierend auf dem F8 Antikörper und Zytokinen der Tumornekrose-Familie (TNF), wurden zwei sich in der Länge unterscheidende Versionen von F8-VEGI (vascular endothelial growth inhibitor) hergestellt, aufgereinigt und in vivo getestet. Der Vergleich mit andern Immunozytokinen bestehend aus anderen Zytokinen der Tumornekrose-Familie und dem F8 Antikörper zeigte, dass sich sogar die Tumorlokalisation von nah verwandten Immunozytokinen zum Teil drastisch unterscheiden kann.

3 Introduction

Despite substantial progress in conventional therapeutic modalities, such as surgery, radiotherapy or chemotherapy, cancer remains one of the leading causes of death in developed countries, second to cardiovascular diseases. Most people die due to the regrowth of metastasis or due to residual tumors, which could not be eliminated using conventional therapy methods. Thus, the selective delivery of bioactive payloads to the tumor mass by means of specific antibodies represents a therapy approach with great potential. Such ‘armed antibodies’ may allow the efficient targeting of disseminated tumors, with a potential to reduce off-target toxicities.

The mechanisms by which mutations give rise to neoplastic masses and confer an aggressive behavior have been extensively investigated, while the interactions of tumor cells with surrounding host cells and environment are less well understood. Tumor cells seem to be able to induce mechanisms to subvert host factors such as cytokines and chemotactic cytokines (chemokines) released in response to tissue injury and inflammatory reactions that may support tumor growth, invasion, metastasis and angiogenesis. In addition, the host’s immune system may fight tumor development. As a consequence, therapeutic approaches aimed at enhancing antitumor immunity against tumor cells which try to evade immune surveillance hold considerable potential. Immunomodulatory cytokines represent promising payloads for therapeutic intervention. Numerous cytokines have been investigated in clinical trials and some are clinically approved for cancer therapy. However, the majority of cytokine therapies fail due to dose limiting systemic toxicities. Potentially, this problem could be overcome by the antibody mediated delivery of cytokines to the neoplastic mass. This thesis describes the evaluation of various antibody cytokine and chemokine fusion proteins for cancer therapy in murine models of cancer. Chemokines are technically classified as a subfamily of cytokines. In this thesis, as ‘immunochemokines’ we refer to antibody chemokine fusion proteins. All other members of the cytokine family, fused to an antibody, are collectively referred to as ‘immunocytokines’.
3.1 Antibody structure

Antibodies, also known as immunoglobulins (Ig), are Y-shaped glycoproteins produced by B-cells and plasma cells, which specifically recognize (as part of the humoral immune system) foreign structures, known as antigens (ag), e.g. those located on intruding bacteria or viruses. The antibody-antigen interaction may lead the blockade and cross-linking of the target structure. Alternatively, antibodies may label target cells for phagocytosis and elimination by cellular components of the immune system.

Antibodies exist in a soluble form circulating in blood, or a membrane-bound form on the surface of B cells, referred to as the B cell receptor (BCR). Typically, Ig consist of two identical heavy chains (50 kDa) and two identical light chains (25 kDa), leading to a characteristic Y-shaped structure. Each heavy chain is covalently bound to a light chain by a disulfide bond. Heavy chains are covalently connected by two disulfide bonds at the so called hinge region, giving rise to a monomeric structure of 150 kDa. Depending on the sequence of the constant region, Ig are grouped into subclasses with different biological properties and localization; IgA (located in mucosal areas, preventing colonization by pathogens), IgD (serving as antigen receptor), IgE (allergen binding), IgG ('classical' antibody-based immunity against invading pathogens), and IgM (pentameric isoform, effective in complement activation). Furthermore, the IgG subclass is further divided into the four subgroups IgG1, IgG2, IgG3 and IgG4, each exhibiting different effector functions.

Immunoglobulin heavy and light chains are composed of a series of discrete protein domains with a similar fold, each about 110 amino acids long. The light chain contains two such domains while the heavy chain is made up by four immunoglobulin domains. Despite the common structure of all antibody isoforms, the amino-terminal domains (V-domains) of both heavy (VH) and light (VL) chain are highly variable and make up the V region or antibody-binding site of the antibody. The remaining domains are constant (C-domains). The heavy-chain C domains (CH) are numbered from the amino-terminus (CH1, CH2 and CH3) while the constant light chain domain is termed CL [Figure 1, left side].
While all immunoglobulin domains share a common β-barrel fold, there are distinct differences between V and C domains (1). The V domain is larger and contains an extra flexible loop. Three segments of particularly high variability, known as hypervariable regions (denoted HV1-3) or complementary-determining regions (denoted CDR1-3), can be identified in both the V_H and V_L domains [Figure 1, right side]. Because CDRs from both V_H and V_L domains contribute to the antigen-binding site, it is the combination of both chains that determines the final antigen specificity. This combinatorial diversity leads to millions of antibodies with slightly different binding pockets, allowing the recognition of virtually every (foreign) antigen structure. The C_H2 and C_H3 domains of both heavy chains form the so called Fc part of the Ig, which is N-glycosylated at a conserved asparagaine residue at position 297 in the C_H2 domain [Figure 1, left side and middle]. The N-linked glycan determines binding to various effector molecules of the immune system, such as the C1 complex of the complement system, and the interaction with Fc receptors. Immunoglobulin-like domains are present in a variety of other proteins of the immune system as well as proteins involved in cell-cell interactions in the nervous system and other tissues.

Figure 1. IgG structure. (left) Schematic representation of an Immunoglobulin G (IgG) molecule. (middle) Crystal structure of an IgG in surface representation (VH in dark blue; VL in light blue; constant domains in grey), residue N297 of the CH2 domain is glycosylated. (right) Crystal structure (structure file: igg1.pdb) of an IgG in ribbon representation, VH domain is enlarged and complementary-determining regions are indicated (CDR loops 1-3).
3.2 Antibody effector functions

There are four basic mechanisms by which antibodies may kill diseased cells: (I) the antibody's Fc-portion leads to the elimination of target cells via antibody dependent cellular cytotoxicity (ADCC); (II) complement dependent cytotoxicity (CDC) (2); (III) antibody binding can lead to functional neutralization and arresting of tumor cell proliferation (e.g., by blockade of the active site or by antigen sequestering and removal) or (IV) antibody binding can induce apoptosis or programmed cell death upon receptor binding.

The Fc domain of antibodies can activate ADCC through interactions with Fc receptors (FcyRs) on effector immune cells. Activation or inhibition of signaling through FcyRs occurs upon stimulation of immunoreceptor tyrosine-based activation motifs (ITAM) or immunoreceptor tyrosine-based inhibitory motifs (ITIM), respectively. There are three activating FcyRs (FcyRI, FcyRIIA and FcyRIIIA) and one inhibitory FcyR (FcyRIIB) known (3, 4). The main effector cells of ADCC are predominantly NK cells carrying FcyRIII (CD16) (3), although macrophages and granulocytes also mediate ADCC to a lesser extent. Cross-linking of activating FcyRs may then trigger lysis of the target cell through the release of cytokines, perforin and granzymes from cytolytic granules (2).

In case of CDC, the binding of an IgG or IgM antibody to certain cell surface antigens on the target cell can result in binding of C1q, a member of the complement system. This event triggers the complement cascade, a process of sequential proteolysis of proenzymes (zymogens) to generate proteolytic enzyme complexes, resulting in the formation of the membrane attack complex (MAC), which creates pores in the cell membrane leading to cell lysis. The complement cascade also leads to the activation of the C3 convertase, which triggers the production of C3b molecules upon C3 cleavage. Binding of C3b molecules, which act as opsonins, to complement receptors on phagocytes induces then the removal of the immune complexes [Figure 2].

Generally, IgG1 and IgG3 are more active in initiating ADCC, CDC and phagocytosis than IgG2 and IgG4, which interact only poorly with FcyR and
therefore act through inhibition or blockage mechanisms. Binding of an antibody to a surface receptor or ligand involved in a pathway important for apoptosis or survival and growth of tumor cells can lead to direct antibody-mediated cell killing.

**Figure 2.** Antibody effector functions. Antibodies are capable to induce target cell killing through various mechanisms: The Fc domain of intact antibodies can activate antibody dependent cellular cytotoxicity (ADCC) through interactions with Fc receptors (FcγIIIa) on effector immune cells, leading to the release of cytolytic proteins, such as granzymes and the pore forming perforin. In case of complement dependent cytotoxicity (CDC), the antibodies Fc part recruits C1q, the initiating member of the complement system, leading to cascades which result in either the formation of the membrane attack complex (MAC) or the interaction of C3b on the target cell with the C3b receptor on an effector cell, both mechanisms ultimately lead to death of the target cell. Further, binding of an antibody to a surface receptor or ligand involved in a pathway important for apoptosis (activating) or survival and growth (blocking) of tumor cells can lead to direct antibody-mediated cell killing. Cell debris of killed cells are then taken up by antigen presenting cells what triggers the release of antibodies specific to various tumor antigens as well as the activation of cytotoxic T lymphocytes (CTL) capable of killing antigen expressing tumor cells.

### 3.3 Antibody-based cancer therapy

The first antibodies which were used for therapeutic applications were isolated from animals that had been immunized with the pathogen of interest. The
invention of hybridoma technology by Köhler and Milstein in 1975 (5), allowed for the first time the production of monoclonal antibodies (mAbs) specifically directed against a given antigen of interest (i.e., antibodies derived from a single B cell single of single specificity). Hybridoma technology triggered the development of antibody-based cancer therapy using mAbs specific to tumor-associated antigens as delivery vehicles. Antibodies represent ideal tools for tumor therapy, as they can be produced against virtually any target protein. In addition, they can be ‘armed’ with a variety of effector moieties. Targeted cancer therapy may lead to a preferential localization of an effector moiety (such as a naked antibody or an armed antibody) at the site of disease, thus potentiating therapeutic activity at the site of disease, while sparing healthy tissues [Figure 3]. By contrast, conventional chemotherapeutic drugs typically inhibit the proliferation of rapidly dividing cells. Therefore, not only tumor cells but also other fast proliferating cells (such as cells of the bone marrow, gastrointestinal tract and hair follicles) may be damaged by chemotherapy, preventing escalation to therapeutically active dose regimens.
**Figure 3.** Schematic comparison of targeted vs. untargeted drug delivery. Intravenous (i.v) administration of an untargeted drug, devoid of any specific tumor antigen binding moiety, distributes rapidly through the bloodstream from where it diffuses into tissues. Usually organ uptake is higher than tumor uptake (e.g., due to high interstitial pressure and active efflux mechanisms) and the untargeted drug gets cleared rapidly from the body including tumor mass. By contrast, a targeted drug selectively accumulates and remains at the tumor site, while unbound drug gets cleared rapidly from the body, leading to high tumor to organ ratios short time after administration. Usually a targeted drug can be administered at a lower dose than an untargeted drug and therefore spares healthy tissues and reduces side effects.

The first generation of mAbs was rather inefficient due their murine origin, resulting in shorter half-life, weak Fc receptor interaction and immunogenicity. Antibody engineering technologies allowed the development of less immunogenic chimeric, humanized and fully human mAbs with longer serum half-lifes and the ability to recruit effectors of the immune system [**Figure 4**]. Both, chimeric and humanized mAbs contain human Fc domains, but retain their target specificity by murine variable regions (chimeric) or grafted murine CDRs (humanized).

![Immunogenicity](image)

**Figure 4.** From murine to human antibodies. Murine sequences are shown in red and human sequences in blue. Light chains are depicted in light colors and heavy chains in dark colors.

Fully human antibodies can be produced by the immunization of transgenic mice, which carry the human IgG genes, leading to B cells secreting human antibodies which could then be used for hybridoma production (6). Alternatively, *in-vitro* antibody selection methods, such as phage display (7) or ribosome display (8), may allow the selection of fully human mAbs from combinatorial libraries. However, also humanized and fully human antibodies can elicit adverse events (9), such as immunogenic reactions, tumor lysis syndrome or other side-effects associated with the nature of the target antigen. The following Table shows a selection of mAbs currently approved for cancer therapy.
Table 1. Selection of currently approved mAbs for cancer therapy.

<table>
<thead>
<tr>
<th>Agent</th>
<th>Trade name</th>
<th>Target Antigen</th>
<th>Approved indication</th>
</tr>
</thead>
<tbody>
<tr>
<td>rituximab</td>
<td>Rituxan®, Mabthera®</td>
<td>CD20</td>
<td>CD20-positive NHL, CLL</td>
</tr>
<tr>
<td>trastuzumab</td>
<td>Herceptin®</td>
<td>ErbB2</td>
<td>ErbB2-positive breast and gastric cancer,</td>
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<tr>
<td>pertuzumab</td>
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<td>cetuximab</td>
<td>Erbitux®</td>
<td>EGFR</td>
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<td>panitumumab</td>
<td>Vectibix®</td>
<td>EGFR</td>
<td>Metastatic colon cancer, head and neck cancer</td>
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<tr>
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<td>Avastin®</td>
<td>VEGF-A</td>
<td>colon cancer, non-small lung cancer, glioblastoma, kidney cancer</td>
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<tr>
<td>ipilimumab</td>
<td>Yervoy®</td>
<td>CTLA-4</td>
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<td>alemtuzumab</td>
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<td>ofatumumab</td>
<td>Arzerra®</td>
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3.4 Antibody engineering

The pharmaceutical performance of an intact monoclonal antibody may be limited by an insufficient extravasation and dissociation into diseased tissue, due to its high molecular weight of 150 kD and the relatively high interstitial pressure at the site of disease. Furthermore, interaction with Fc receptors on healthy cells and the long circulation time may lead to suboptimal tissue distribution profiles for intact antibody products. Antibody engineering allows the construction of various antibody fragments, consisting of some of the domains of the parental IgG molecule. The variable domains for example, are the only ones which confer a binding specificity to the target antigen of choice. The smallest antibody format is the single-chain Fv (scFv) format of about 27 kDa size, which consists of the variable domains (V_H and V_L), typically connected by a 15 amino acid (aa) linker. The scFv format retains the binding affinity, but not avidity, of the parental antibody. The low molecular weight of this antibody format leads to a more rapid and more efficient diffusion from the bloodstream into the perivascular space (10, 11) compared to intact antibodies, but also to a more rapid clearance (>90% of the injected dose after one hour (12)) from the bloodstream (size below renal cut-
off) and reduced residence time at the target site due to its monovalent nature. A shorter linker (of 3-12 aa) between the \( V_H \) and \( V_L \) domains leads to the formation of a stable noncovalent scFv dimer, termed diabody (54 kDa), which has two antigen binding sites and therefore shows prolonged residence time at the tumor site due to avidity effects, while being rapidly cleared via the renal route. A number of other multivalent antibody formats of intermediate size, such as the small immune protein (SIP), F(\( ab' \))\(_2\) or scFv-Fc format, have been engineered to achieve a compromise between efficient targeting and acceptably fast clearance properties. The formats relevant for this thesis are shown in Figure 5.

**Figure 5.** Antibody formats relevant for this thesis. The smallest single-chain Fv (scFv) antibody fragment is shown on the left side. The noncovalent homodimeric and bivalent diabody format is shown in the middle, while the small immune protein (SIP) format is shown on the right side.

### 3.5 Armed antibodies

This section of the introduction has been adapted from the following publication:

**Hess C.*, Venetz D.*, Neri D.:** *Emerging classes of armed antibody therapeutics against cancer.* *MedChemComm* 2014, 5, 408-431 (*authors with equal contribution*)

Unmodified immunoglobulins may display an anticancer activity by a variety of mechanisms, including neutralization of cancer-promoting factors, induction of signaling events leading to apoptosis, complement activation and recruitment of Fcy receptor-positive leukocytes, which may eventually kill the tumor cell. A substantial therapeutic benefit has been demonstrated for the use of unmodified
antibodies in lymphoma, especially in combination with chemotherapy (13). In addition, the inhibition of immunoregulatory molecules expressed by immune cells, such as CTLA-4 or PD1 is gaining considerable attention for the treatment of immunogenic tumors such as metastatic melanoma. CTLA-4 blockade in melanoma patients mediates a modest percentage of objective responses (typically less than 10%), but substantially higher tumor shrinkage efficiency has been reported for PD1 blockers, especially when used in combination with anti-CTLA-4 antibodies (14). However, relatively low anti-tumor activities are normally observed when tumor-directed unmodified antibodies are used for the treatment of solid tumors. These limitations may be because of the rather inefficient antibody uptake into the neoplastic mass, a consequence of limited extravasation rates and the presence of perivascular tumor cells, which can form an ‘antigen barrier’. Tumor-induced immune tolerance may further compromise ADCC or CDC. Moreover, most cell-surface receptors expressed by malignant cells do not initiate programmed cell death or impair tumor cell growth upon antibody binding.

Monoclonal antibodies specific to tumor-associated antigens may also be used for pharmacodelivery applications. The best of these products may exhibit tumor-to-blood and tumor-to-organ ratios greater than 10 to 1 one or several days after injection, thus providing the basis for the targeted delivery of therapeutic payloads to malignant tissues. The antibody-mediated delivery of an effector moiety to the tumor site may lead to an improvement of the therapeutic index by selective killing of malignant cells at the site of disease while sparing healthy cells and tissues. However, the efficacy of such an armed antibody product highly depends in its biodistribution properties in vivo. It cannot be assumed a priori that an armed antibody retains the disease-homing properties of the parental antibody. The biophysical properties of the delivered payload moiety can dramatically reduce the extravasation rate of the parental antibody as reported for highly charged payloads (15), payloads of too high molecular weight (16) or payloads that interact with an abundantly expressed receptor (17). Furthermore, glycosylation patterns of a protein payload may increase the clearance rate of the antibody and therefore reduce its circulating half-life, while Fc receptor binding of an antibody in Ig format can increase the circulating half-life of an armed antibody. This can
lead to severe systemic side effects especially when very potent cytotoxic payloads are used.

Quantitative biodistribution studies, near-infrared dye imaging studies or small animal PET studies are optimal tools to assess the *in vivo* targeting properties of an antibody product in rodents, while SPECT and PET imaging is frequently used in human patients in order to get information about target receptor saturation, normal organ uptake or to study the optimal dose, schedule and disease indication for a patient.

So far, most approved therapeutic antibodies are in Ig format (1, 18), while only few antibody fragments are in the clinic, either as neutralizing agents (e.g. Lucentis®, ReoPro®) or as radiolabeled products for imaging purposes. **Figure 6** summarizes different classes of armed antibodies which were considered for cancer therapy.

**Figure 6.** Classes of armed antibodies considered for cancer therapy.
In the following, examples of antibodies used for the delivery of bioactive molecules, such as cytotoxic drugs, cytokines, radionuclides or a second antibody moiety to the tumor environment are described [Figure 7]. Antibodies armed with radionuclides or cytotoxic drugs are improving the therapeutic index of their bioactive payload. However, toxicities to clearance-related organs cannot be completely eliminated. Antibodies can also be armed with proinflammatory cytokines or additional antibody moieties in order to potentiate the immune response against the tumor and/or activate apoptotic pathways. This therapeutic strategy may involve cells of the adaptive and innate immune system and holds the promise of long-lasting protective immunity. Nonetheless, also these payloads may trigger undesired side effects, such as systemic immune activation and hypotension. The molecular format of armed antibodies determines pharmacokinetic and pharmacodynamic properties. The preferred antibody format should be chosen depending on the type of payload used and on the tumor-associated antigen chosen as target.

**Figure 7.** Schematic representation of the four most advanced classes of armed antibodies for cancer therapy applications.
3.5.1 Antibody-drug conjugates

3.5.1.1 Concept

ADCs represent an advanced class of armed antibodies, which is reflected by the fact that two such biopharmaceuticals are on the market and thirty ADC products are currently in clinical development (19). The concept of ADCs essentially relies on the use of antibodies for the disease-specific delivery of extremely potent cytotoxic agents. In order to spare healthy organs, the toxin should ideally not be active when antibody-bound and remain stably conjugated as long as the ADC circulates in blood. Once the ADC reaches its target in vivo, the toxic payload has to be released and may thus exert its toxic effect within the malignant cells. Internalization of the ADC/antigen complexes and subsequent drug-release mediated by lysosomal degradation represents the conventional approach for ADC development. Such drug-release mechanisms are, however, restricted to antigen-positive cells if the free drug is not able to diffuse across the cells’ membrane.

3.5.1.2 ADC formats

In addition to the antibody and its cognate antigen, also the linker and the drug, greatly influence ADC performance. In vivo, the therapeutic effect of an ADC depends on serum stability, antigen recognition and subsequent drug release mechanisms. All these processed can be engineered in order to achieve optimal therapeutic activity, as evidenced by the progress in this research area over the last two decades.

3.5.1.2.1 Intact antibodies vs. fragments

All clinical-stage ADCs make use of antibodies in full-length IgG format. Consequently, these ADCs exhibit long FcRn-mediated circulatory half-lives in
the range of one to two weeks, although drug conjugation may result in a more rapid clearance (20). Additionally, other interactions with Fc-receptors expressed by immune cells are also possible, depending on which IgG isotype is used. In contrast to other antibody-based therapeutics, Fc-mediated effector functions such as ADCC or CDC are not considered to be essential for the therapeutic activity of ADCs. Extensive research efforts have been devoted to the development of drug linkers with high plasma stabilities in order to prevent premature drug release in the blood and within non-disease organs.

Recently, also antibody fragments have been considered for the pharmacodelivery of cytotoxic payloads and their therapeutic potential has been investigated in animal models of cancer (21-24). Being smaller than intact immunoglobulins, antibody fragments could potentially extravasate more efficiently while exhibiting a more rapid blood clearance from non-disease organs, resulting in more favorable biodistribution profiles (25). Small bivalent antibody fragments, such as diabodies or small immune proteins (SIPs), are often the preferred formats for targeting applications because these preserve binding avidity and display longer tumor residence times.

3.5.1.3 Cytotoxic drug payloads

Initial proof-of-concept studies in the ADC field have focused on approved chemotherapeutic agents, such as doxorubicin, as cytotoxic payloads (26). These first-generation ADC products, however, performed poorly in the clinic, most likely because of insufficient drug potency (27). Subsequently, ADC development focused on the use of novel cytotoxic agents, which were orders of magnitude more potent than conventional chemotherapeutics. In fact, these agents are too toxic to be administered systemically in a non-conjugated form. This next generation of drug payloads has so far led to the market approval of three ADCs products, which are displayed in Figure 8. The majority ADCs currently in clinical development is based on these drugs, which are calicheamicins, auristatins, maytansinoids and derivatives thereof.
Figure 8: Chemical structures of cytotoxic drugs and linkers of the three approved ADC products Kadcyla® (a), Adcetris® (b) and Mylotarg® (c).

Calicheamicins bind to the DNA minor groove where they induce double-strand breaks leading to chromosomal disruption, ultimately resulting in programmed cell death (28), while the other two drugs interfere with microtubule organization and dynamics (29, 30).

Auristatins were originally derived from the naturally occurring pentapeptide dolastatin-10. Mono-methyl auristatin E (MMAE) and F (MMAF) are the most prominent dolastatin-10 derivatives, which are implemented in many current clinical-stage ADCs. These fully synthetic analogues differ from natural dolastatin-10 in terms of an N-terminal methyl group, which results in superior proteolytic stability. Additionally, MMAF features a C-terminal charged phenylalanine residue, which attenuates its cytotoxic activity compared to the uncharged MMAE (31). Maytansinoids such as DM1 and DM4 are derivatives of the natural product maytansine and represent two of the most widely used ADC payloads to date.
3.5.1.4 Conjugation strategies

Early attempts to conjugate cytotoxic drugs to monoclonal antibodies exploited the reactivity of solvent-accessible lysine residues, which are often found on the protein surface. The resulting ADCs may be heterogeneous in terms of drug-to-antibody ratio (DAR) and conjugation site occupancy, which could potentially lead to batch-to-batch variability, with implications on pharmacokinetic properties. Alternatively, cysteine residues involved in inter-chain disulfide bonds may be used for chemical modification strategies, since non-covalent domain interactions are sufficient to preserve the integrity of IgG molecules. Upon mild reduction, up to eight solvent-accessible cysteine residues may become susceptible to chemical reaction. However, subsequent conjugation reactions rarely go to completion, thus potentially contributing to variable DAR as well. Chromatographic separation and subsequent in vivo analysis of the different ADC species revealed that the DAR can be a key determinant for optimal therapeutic activity of ADCs (20, 32). Based on these studies, optimal DAR values are considered to be between 2 : 1 and 4 : 1, depending on the type of linker and drug used.

In order to overcome these problems, research efforts have been devoted to the development of methods for the site-specific coupling of drugs to antibodies. Current strategies are based on the introduction of non-natural amino acids, which can then be conveniently coupled to cytotoxic drugs using bio-orthogonal conjugation chemistries (33, 34). Site-specific introduction or substitutions of cysteine residues at positions not essential for antibody function represent another avenue (35). Emerging experimental results suggest that chemically defined ADCs may exhibit favorable pharmacokinetic and side effect profiles (35). Furthermore, solvent accessibility and charge environment of the conjugation site seem to be critical for tumor-specific drug release (36). These findings are in good agreement with our observations regarding antibody-drug spacer length (37). Site-specific antibody-drug coupling strategies are easier to perform with antibody fragments due to reduced molecular complexity. For example, thiol-containing drugs can easily be attached to the C-terminal cysteine of tumor
targeting antibody fragments in SIP format (21). Alternative methods exploit the distinctive reactivity of N-terminal cysteine residues, which may facilitate the site-selective coupling of drugs by means of a disulfide, amide or thiazolidine linkage (22). Recently, also solvent accessible tyrosine residues have been used for ADC production (38). The selective oxidation of vicinal diols in carbohydrate structures can also be considered for the coupling of drugs to antibodies, since the IgG molecule typically contains a single N-glycosylation site at Asn297 (39). Finally, trans-glutaminase-based conjugation strategies have also been investigated for ADC preparation (40-42).

3.5.1.5 Linker technologies

Specific review articles have extensively surveyed chemical strategies for linker design in ADC development (43). Ideally, drug linkers would preserve ADC integrity in circulation, while being efficiently cleaved at the site of disease. The first generation of ADC linkers contained acid-labile hydrazone functional groups, which decompose upon ADC/antigen internalization because of the gradual acidification of early endosomes. Many antibody-drug linkers are based on disulfide bonds, which can be reduced at the site of disease in the presence of cellular reducing agents such as glutathione or cysteine. These linkers may exhibit a shorter half-life compared to the long circulatory half-life of intact antibodies, potentially leading to premature drug-release. In some cases, protease-sensitive peptide linkers with superior plasma stabilities have been used to minimize premature drug release. Other ADCs, such as the recently approved trastuzumab-DM1 (T-DM1), feature a non-cleavable thioether-based linker. Interestingly, this linker remains conjugated to DM1 even after lysosomal degradation of trastuzumab and does not impair the therapeutic activity of the drug (44). In case of brentuximab vedotin, a valine-citrulline linker has been used. Upon cleavage by lysosomal proteases such as cathepsin B, the active auristatin drug is liberated by means of a self-immolating structure (45, 46). Either way, ADC linkers impose a considerable degree of complexity on the production
process and \textit{in vivo} behavior of this class of armed antibodies, providing the scientific rationale for the development of direct drug conjugation strategies.

\subsection*{3.5.1.6 Clinical trials}

\subsubsection*{3.5.1.6.1 Hematologic malignancies}

The first clinically efficacious ADCs were developed for the therapy of cancers of lymphoid or myeloid origin. These indications are characterized by circulating malignant blood cells, featuring a variety of cell-surface antigens, which are absent in healthy non-lymphoid organs but are easily accessible for intravenously administered drugs. ADCs intended for hematologic cancer therapy crucially rely on internalizing antigens in order to ensure tumor cell specificity. Additionally, the released drug should not be able to diffuse back through the plasma membrane where it could affect endothelia and non-malignant blood cells.

\textit{Mylotarg\textsuperscript{®} (gemtuzumab ozogamicin)} was the first ADC to receive marketing authorization. It was intended as a second-line treatment for patients with relapsed acute myeloid leukemia (AML). The ADC comprises a humanized anti-CD33 antibody of the IgG4 isotype, conjugated to a calicheamicin derivative via a composite hydrazine/disulfide linker as displayed in \textbf{Figure 8} (47). CD33 is expressed by leukemic cells in a subset of AML patients, but can also be found on the cell surface of normal myeloid progenitor cells and monocytes (48) as well as on potentially tumoricidal CD8\textsuperscript{+} T and NK cells (49). Initial clinical phase I/II trials, which led to accelerated approval of gemtuzumab ozogamicin, yielded impressive results with an objective response rate of around 30 \% (50). However, after an intravenously administered dose of 9 mg/m\textsuperscript{2}, signs of hepatotoxicity, i.e. hepatic veno-occlusive disease symptoms, became evident in some patients (51, 52). Subsequent clinical trials failed to confirm an added therapeutic benefit in combination with chemotherapy and seemed to increase lethal hepatotoxicity in some cases (53). For this reason, Pfizer voluntarily withdrew \textit{Mylotarg\textsuperscript{®}} from the market in 2010 after a request by the FDA.
The second marketed ADC, Adcetris® (brentuximab vedotin), was developed by Seattle Genetics. Adcetris® and has been approved in 2011 for two hematologic cancer indications, i.e. for the treatment of Hodgkin lymphoma patients after autologous stem cell transplantation and for anaplastic large-cell lymphoma (ALCL), which is refractory to standard chemotherapeutic care (54). Brentuximab vedotin consists of an anti-CD30 antibody, termed cAC10, conjugated to the auristatin MMAE via a protease-cleavable valine-citrulline peptide-linker (46).

Pivotal clinical trial results for Hodgkin lymphoma can be summarized as follows. After intravenous administration of 1.8 mg/kg brentuximab vedotin every three weeks, 75 % of the treated patients responded to the therapy of which 34 % experienced a complete response (55). The median progression-free survival was found to be 29 months. Also for a second indication, ALCL, Adcetris® provided a substantial benefit for patients compared to preexisting combination chemotherapy. Around 86 % of the patients achieved at least partial remissions, while 57 % of them experienced complete remissions, which lasted up to 14.6 months on average (56). The studies mentioned above revealed that brentuximab vedotin was well tolerated. Most current clinical-stage ADCs are intended for the treatment of B-cell malignancies, such as diffuse large B cell lymphoma (DLBCL) and follicular non-Hodgkin’s lymphoma (NHL). The most advanced ADC for hematological applications in clinical development is Pfizer’s CMC-544 (inotuzumab ozogamicin), which is being used for the treatment of NHL and DLBCL. In analogy to Mylotarg®, CMC-544 uses calicheamicin, conjugated via hydrazone linker to an anti-CD22 antibody (57). The maximum tolerated dose (MTD) of inotuzumab ozogamicin was determined to be 1.8 mg/m² in a phase I/II dose-escalation study (58). This dose resulted in an overall response rate of 68 % and 15 % for NHL and DLBCL patients, respectively. Reported side effects were thrombocytopenia, neutropenia, asthenia and nausea in both patient groups. SAR-3519 is another humanized anti-CD19 antibody conjugated to DM4 and represents an alternative ADC for the treatment of refractory or relapsed NHL or DLBCL. A phase I multidose-escalation study revealed overall responses in 74 % of the patients at an MTD of 160 mg/m² given every three weeks. According to
the study report, only minor hematologic and hepatic toxicities were observed (59).

3.5.1.6.2 Solid tumors

For solid tumor indications a wider set of tumor-associated antigens is being investigated. As for hematologic malignancies, most of the solid tumor antigens targeted by clinical-stage ADCs have been chosen based on their ability for ligand-induced internalization.

Kadcyla® (trastuzumab emtansine or T-DM1) is the first ADC approved for a solid cancer indication, i.e. the treatment of metastatic Her2-positive breast cancer. The ADC consists of trastuzumab (Herceptin®), a previously approved Her2-directed antibody, conjugated to the maytansinoid DM1 via a stable maleimide-based linker (60). In a randomized multi-center phase III clinical trial ultimately leading to the approval of the drug, T-DM1 was confirmed to be superior to the previous standard of care, i.e. combination chemotherapy with lapatinib and capecitabine (61). Progression-free survival was prolonged by a factor 1.5 compared to chemotherapy. The study also revealed complete remissions in 4% of the treated patients. Overall, 42.6% of the patients responded to T-DM1. The safety profile was also improved compared to chemotherapy, with adverse effects higher than grade 3 mainly manifested as transient thrombocytopenia and elevated serum levels of liver aminotransferases. Such events were observed in 41% of the treated patients at an administered dose of 3.6 mg/kg.

CR011-vcMMAE (glembatumumab vedotin) is also an ADC directed against a solid tumor antigen, the glycoprotein NMB, which is overexpressed in a variety of cancer types including metastatic melanoma and certain forms of breast cancer (62-64). Glembatumumab vedotin demonstrated its efficacy in phase I/II clinical trials while showing a favorable safety profile (65). Another promising ADC currently evaluated in phase II trials is PSMA-ADC developed by Progenics.
3.5.1.7 Preclinical research

Apart from novel conjugation and linker technologies, some new concepts for the antibody-based delivery of cytotoxic agents have been recently described. For example, ADCs have been developed against mutated forms of cell-surface receptors, which are exclusively expressed by cancerous cells. A prominent example for such a mutant target is epidermal growth factor receptor variant III (EGFRvIII), a truncated version of EGFR, which is frequently found in conditions such as glioblastoma multiforme or head-and-neck cancer (66, 67). Unlike EGFR, EGFRvIII is not expressed by normal cells, which could potentially confer an additional level of specificity to the corresponding AMG-595 product, currently investigated in Phase I studies.

More recently, our group could show that antigen internalization is not an obligatory requirement for the therapeutic efficacy of ADCs (21, 22). Proof-of-principle studies were performed using the clinical-stage antibody SIP(F8), which is directed against the extra-domain A of oncofetal fibronectin, an antigen expressed in the sub-endothelial extracellular matrix of a wide range of solid tumors and hematologic malignancies. This finding represents a novelty to the field and paves the way for future ADC development programs based on non-internalizing ADC targets. Although such strategies rely on extracellular drug release mechanisms, antigen-negative tumor cells could potentially be killed more efficiently by means of the so-called bystander effect, due to drug diffusion within the neoplastic mass.

3.5.2 Immunocytokines

3.5.2.1 Concept

Cytokines are a diverse group of soluble proteins capable of modulating immune responses against tumor cells. Most cytokines are pleiotropic, i.e. they can exert multiple, to a certain extent overlapping immunological functions. The inhibition
of cytokine activity, or the pharmacodelivery of cytokines to the tumor site, can profoundly affect the anti-tumor activity of leukocytes and other cell types, thus potentially leading either to a therapeutic effect or a worsening of the disease conditions.

Cancer therapy with immunostimulatory cytokines was initially established for interferon-α and interleukin-2 (IL2) and proved to be efficacious against immunogenic forms of cancer such as late-stage melanoma. However, systemic administration of cytokines rarely resulted in complete cancer cures (68, 69). On the contrary, systemic cytokine-based tumor therapy may give rise to severe dose-limiting toxicities, such as cytokine-release or vascular leak syndrome, already at doses lower than 1 mg per patient, preventing the escalation to therapeutically active dose regimens. A preferential accumulation of a cytokine in the tumor mass may lead to an enhanced anticancer activity compared to the untargeted cytokine, as suggested by preclinical and clinical therapy results (70). Antibody-based tumor targeting of cytokines provides a means to reduce the administered dose and consequently systemic side effects. Antibody-cytokine fusion proteins are generally referred to as ‘immunocytokines’ and are now finding increasingly broad applications for the treatment of cancer and other inflammatory conditions (70, 71).

3.5.2.2 Immunocytokine formats

Advances in protein engineering have allowed the generation of antibody fragments of different size and valence, directly influencing their in vivo targeting performance, as defined by tissue penetration, tumor accumulation and blood clearance. Many antibody-cytokine fusion proteins in different formats have been evaluated over the past years (72). Antibody formats range from scFv to full-length IgGs. The most widely used small bivalent formats include diabody, SIP, scFv-Fc (73). However, for certain cytokines such as homotrimeric tumor necrosis factor (TNF, formerly known as TNFα), the monomeric scFv fragment may be the preferred fusion partner, in order to preserve the natural oligomeric
state of the cytokine. Additionally, the antibody-fusion should not interfere with the cytokines’ receptor binding capability. If structural data of the cytokine/receptor complex is missing, it is essential to compare amino- and carboxy-terminal cytokine fusions in dedicated bioassays. Flexible glycine-serine linkers normally provide enough stability against proteolysis, are not immunogenic and do not interfere with cytokine function. Until now, immunocytokines that have advanced to anticancer clinical development programs are fused to antibodies in IgG, diabody or scFv format [see Figure 9], and were either based on Interleukin-2 (IL2), Interleukin-12 (IL12) or TNF.

3.5.2.3 Cytokine payloads

3.5.2.3.1 Interleukin 2

Interleukin-2 is a pleiotropic T-cell derived cytokine and a member of the common \( \gamma \)-chain family of cytokines. It occurs as a monomeric protein of 15.5 kDa, normally featuring an O-linked glycan when expressed in eukaryotic expression systems. The IL2 molecule can be a potent stimulator of the immune system, capable of activating NK cells, cytotoxic T cells and monocytes, but also immunosuppressive immune cells such as induced regulatory T cells (74, 75). Preclinical development of IL2-based products has been facilitated by the fact that this human cytokine cross-reacts with the murine receptor. However, immunogenic reactions associated with the use of human IL2 in the mouse \textit{de facto} limit preclinical treatment to a one-week interval. Clinical trials with unconjugated IL2 (Proleukin®), which has been approved for the treatment of metastatic melanoma and kidney cancer, revealed that vascular leak syndrome may represent the most common limiting toxicity (76). There are currently four IL2-based immunocytokines in clinical trials.
3.5.2.3.2 Tumor Necrosis Factor

TNF is a homotrimeric proinflammatory cytokine that belongs to the TNF superfamily and is a key regulator of inflammatory immune responses (77). It is mainly produced by activated monocytes as a 26 kDa trans-membrane protein that can be cleaved to a 17 kDa soluble TNF form. It is one of the most potent tumoricidal cytokines based on its ability to directly induce apoptosis and to promote inflammation at the tumor site (78). Substantial toxic side effects prevent systemic administration of TNF at doses above 300 µg. The clinical use of recombinant TNF has therefore been limited to loco-regional applications, e.g. for isolated limb perfusion with a combination of TNF and melphalan in soft tissue sarcoma and melanoma (79). As a cytokine-payload for vascular tumor targeting antibodies, TNF proved to have an acceptable safety profile.

3.5.2.3.3 Interleukin 12

The pro-inflammatory cytokine IL12 is a 74 kDa heterodimeric cytokine composed of the subunits p40 and p35, which are linked by a disulfide bond (80). IL12 is produced by antigen presenting cells and induces the production of other cytokines, such as interferon-γ. Thereby, it induces the proliferation of NK and T cells, the differentiation of T helper cells into the Th1 subtype as well as the activation of effector T cells and it plays therefore a central role in cancer and inflammation (81, 82). The anti-tumor activity of recombinant IL12 has been demonstrated in several animal models and was further evaluated in clinical trials in humans (83). Fatal toxicities upon systemic administration of recombinant IL12 were observed and prevented further clinical development as a single agent for cancer therapy (84). Two IL12 fusion proteins are currently being evaluated in clinical trials.
3.5.2.4 Clinical trials

To date, most clinically investigated immunocytokines have been developed for the treatment of solid tumors. However, recent translational studies confirmed that immunocytokines can also be efficacious against solid manifestations of hematologic malignancies (85, 86). Immunocytokine targets are either tumor cell surface receptors, such as epithelial cell adhesion molecule, GD2 disialoganglioside, EGFR or extracellular matrix proteins such as the extra-domains A or B (EDA or EDB) which occur in oncofetal splice-isoforms of fibronectin or the extra-domain A1 of tenascin-C (TnC A1). Additionally, immunocytokines have also been developed with antibodies directed against DNA/histone complexes for targeted necrosis therapy (87).

3.5.2.4.1 IL2-based immunocytokines

The first immunocytokines to enter clinical trials were based on humanized IgGs genetically fused to IL2. The fusion protein h14.18-IL2, directed against the GD2 disialoganglioside, was tested in adult melanoma patients as well as in pediatric neuroblastoma patients (88, 89). The MTD was determined to be 7.5 mg/m²/d. After one week, 58 % of the melanoma patients receiving h14.18-IL-2 experienced a stabilization of the disease but only 24 % of the patients were progression free 6 weeks after treatment. In a phase II study, only 22 % patients reached disease stabilization after 4-6 treatment cycles (at 4 mg/m²/d) of h14.18-IL2 and no objective responses were observed (89, 90).

In children with neuroblastoma the recommended dose of h14.18-IL2 was determined as 12 mg/m²/d for 3 days every 4 weeks. A phase II efficacy study was designed to test the relationship between tumor burden and potential response. In 54 % of the patients, a stable disease could be observed after six weeks. For the patients having a low tumor burden, a complete response rate of 22 % lasting between 9 and 35 months was observed (91). Further trials have been initiated to investigate this immunocytokine also for the treatment of patients suffering from metastatic melanoma. Symptoms of dose limiting toxicities were
mainly grade 2 and the similar as observed for recombinant IL2. Dose-limiting toxicities observed at the MTD included hypoxia, hypotension, and elevated serum level of liver transaminases (89).

The same dosing schedule as for hu14.18-IL2 was used in a phase I clinical trial testing the anti-EpCAM immunocytokine, huKS-IL2, in patients with hormone refractory prostate cancer. In this study, no objective responses were observed (92). There is currently no ongoing clinical trial with huKS-IL2.

Another IL2-based immunocytokine is NHS-IL2-LT, which consists of the chimeric NHS antibody in the full-IgG format targeting DNA/histone complexes in necrotic regions of tumors fused to a mutant version of IL2. This immunocytokine exhibits lower toxicity due to impaired activation of the intermediate-affinity IL2 receptors but not high-affinity IL2 receptors found on NK and T cells (93), leading to increased selectivity for activated T cells and reduced binding to endothelial cells. NHS-IL2-LT was tested in a phase I clinical dose-escalation study in patients with metastatic or locally advanced solid tumors (94). The MTD was found to be 0.6 mg/kg. A subgroup of the patients received in addition to NHS-IL2-LT, 300 mg/m² bolus injections of the alkylating agent cyclophosphamide for the depletion of regulatory T cells. Transient increases in lymphocyte, eosinophil and monocyte levels were reported. No tumor responses but prolonged disease stabilizations were observed.

The first fragment-based immunocytokine to enter clinical trials was L19-IL2. This compound is using the EDB-specific antibody L19 in the diabody format, resulting in high avidity and efficient tumor targeting in animal models of cancer (95). Because of its smaller size, the product exhibits a rapid clearance from the circulation in mouse (96) and in man (97, 98). L19-IL2 was first investigated in patients with metastatic renal cell carcinoma. A stabilization of the disease could be observed in 83 % of the patients The median progression-free survival was eight months (99).

Promising results have been reported for the combined use of L19-IL2 with dacarbazine in metastatic melanoma (97, 98). An objective response was
observed in 28% of the cases, which included a complete response in one patient who was still tumor free after 21 months. The median progression-free survival of pretreated melanoma patients with standard therapy is approximately 6 to 9 months. Sixteen of the 26 patients treated with L19-IL2 at the recommended dose were still alive after 12 months. Currently, a controlled phase IIb study with 90 metastatic melanoma patients is in progress.

F16-IL2 is a diabody-based immunocytokine directed against TnC A1. It is currently being tested in a phase I/II trial in patients with breast cancer, lung cancer or melanoma (100). Patients with non-small cell lung cancer and melanoma exhibited objective responses and long lasting disease stabilization in combination with paclitaxel (101, 102). Recently, it has been found that the A1 domain of tenascin-C is also expressed in the bone marrow of patients with AML and ALL. The successful treatment of a patient with AML-derived chloromas has been reported (86), suggesting a further clinical investigation IL2-based immunocytokines in hematologic cancer indications.

3.5.2.4.2 IL12-based immunocytokines

The TNT3-directed antibody NHS fused to the C-termini two human IL12 cytokine moieties constitutes the immunocytokine NHS-IL12, a biopharmaceutical currently being evaluated in a phase I trial. Furthermore, an EDB-specific immunocytokine consisting of the human IgG BC1 and IL12 is currently under clinical investigation. Results of a phase I study in melanoma and renal cell carcinoma patients have been published (103). At an MTD of 15 μg/kg weekly (3-5 fold molar increase compared to recombinant IL12), 6 out of 13 patients experienced a stable disease, two experienced a partial response lasting for 7 and 17 months. In general huBC1-IL12 was well tolerated with the majority of side effects being lower than grade two.
3.5.2.4.3 TNF-based immunocytokines

The human L19 antibody in scFv format fused to the pro-inflammatory cytokine TNF results in a stable, non-covalent trimetric L19-TNF immunocytokine (104). This compound is tested in clinical trials for systemic administration to patients with various advanced solid tumors (105) and isolated limb perfusion in patients with melanoma (106). In a study, L19-TNF plus melphalan (10 mg/L) was given to melanoma patients in the isolated limb perfusion setting. At doses of 325 μg or 650 μg of the fusion protein, corresponding to 3.125 % and 6.25 % of the approved TNF (Beromun®) dose, objective responses were observed in 86 and 89 % of the patients. In fifty percent (4 patients) of the high dose group a complete response lasting for 12 months was observed. No complete responses at 325 μg were reported, supporting the exploration of higher doses in a phase II trial. Only mild toxicities in very few of the patients were associated with L19-TNF in this setting. At present, L19-TNF is being investigated in a phase I/II clinical trial for the therapy of soft tissue sarcoma in combination with doxorubicin, on the basis of promising preclinical data (104, 107). In addition, the intraleSIONAL administration of L19-IL2 plus L19-TNF is being investigated in metastatic melanoma patients (108). Immunocytokines in clinical trials are shown in Figure 9.

![Immunocytokines in clinical trials](image)

**Figure 9.** Immunocytokines in clinical trials. Immunocytokines based on full-length IgG antibodies (a) and based on antibody fragments (b).
3.5.2.5 Preclinical research

The preclinical and clinical development of immunocytokines poses some challenges. First, if the target antigen and/or the cytokine receptor differ substantially between species, it is not possible to use the same product in mouse and man. The use of xenograft models of human cancer, which rely on the use of immunocompromised mice, may not allow to completely assess the immunomodulatory properties of an antibody-cytokine fusion protein. Furthermore, cytokines may have a different biology and activity in different species. Nonetheless, a systematic preclinical evaluation of the therapeutic potential of different cytokines still holds the promise of safer and more efficacious immunocytokines. Our group has recently investigated Interleukin-4 as a cytokine payload of the vascular targeting antibody F8 in different mouse model of cancer. The obtained results suggest that F8-IL4 may be broadly efficacious against various cancer types (109). Interleukin-15 represents another interesting cytokine payload. A fusion protein termed RLI consisting of the soluble form of the human IL15Rα-shushi domain linked to human IL15 showed superior biological activities than IL-15 in vitro and stronger anti-tumor activity in mice (110). A novel immunocytokine based on RLI fused to the antibody targeting the GD2 disialoganglioside has demonstrated its potency in two mouse cancer models (111).

3.5.2.6 Combination studies

Several preclinical studies have explored the combinatorial effect of immunocytokines with other therapeutic agents, such as cytotoxic drugs (100, 112-114), monoclonal antibodies (85) or other cytokines (115, 116), sometimes with spectacular results, leading to complete cures, which cannot be achieved with other therapeutic modalities. These results provide encouragement to continue preclinical and clinical developments in the field. Typically,
immunocytokines and conventional chemotherapeutic agents do not exhibit overlapping limiting toxicities, thus facilitating their use in combination at the recommended dose. Recently, the immunocytokine L19-IL2 has been shown to eradicate cancer in combination with CTLA-4 blockade or with L19-TNF (108). However, translesional tumor remissions have not been reported yet. Synergistic therapeutic effects leading to significant tumor growth retardation were observed for combination therapies of F8-IL2 and sunitinib in mouse models of renal cell carcinoma (117). Complete tumor eradication was observed when L19-IL2 and rituximab were combined for the treatment of B-cell lymphoma (85). The combination of L19-TNF and melphalan, or F8-TNF and doxorubicin, eradicated murine sarcomas and made mice resistant to subsequent challenges with tumor cells (104, 107). The combination of L19-TNF and L19-IL2 was curative in a mouse model of neuroblastoma, which could not be cured by either of the two products when used as single agent (118). Combination therapies of KS-IL2 and F8-IL2 with paclitaxel showed an enhancement of the targeted delivery of IL2 to the tumor upon prior injection of paclitaxel as well as synergistic anticancer effects in different mouse models of cancer (113, 119). Moreover, better anti-tumor efficacy and survival of KS-IL2 treatment was observed after radiofrequency ablation, compared to mice treated with RFA or KS-IL2 alone (120).

3.5.3 Radioimmunoconjugates

3.5.3.1 Conventional radioimmunotherapy

Radioimmunotherapy (RIT) relies on the use tumor-targeting antibodies to selectively deliver cytotoxic radiation doses to neoplastic cells. To achieve this, a suitable radionuclide is covalently attached to the antibody molecule or binds to a chelating agent coupled to the antibody. Depending on the type of radionuclide used, the resulting radioimmunoconjugate can either be used for image diagnostics or therapy, or for a combination of them (so-called ‘theranostic’ applications). The most advanced antibody-based imaging technique to date is
immuno-positron emission tomography (immuno-PET). It combines the high resolution and sensitivity of PET with the specificity of monoclonal antibodies, providing detailed information about antibody localization in the body at multiple time points. The combination of imaging procedures (for dosimetric calculations and patient selection) with the delivery of therapeutic radionuclides makes RIT one of the few areas of cancer research, for which treatment outcome can largely be predicted on the basis of imaging data. Immuno-PET procedures are not only useful for the development of RIT products, but their value is also increasingly being recognized for the ‘precision medicine’ clinical development of other types of antibody-based products (121).

Antibodies labeled with cytotoxic radionuclides represent a relatively old class of cancer therapeutics. The first radioimmunoconjugates were based on ‘immunogenic’ polyclonal antibodies of animal origin coupled to β-emitting radionuclides (122). Meanwhile, it has become clear that either fully human or humanized antibody products should preferably be used for RIT applications, as their non-immunogenic nature allows repeated administrations of the product to patients (123). Radiolabeled monoclonal antibodies have been extensively used for imaging and therapy applications (124, 125), showing the potential but also challenges and limitations associated with radioimmunotherapy.

A major determinant for the success of targeted radiation therapy is the intrinsic radiosensitivity of tumor cells (126). In general, the eradication of solid tumors needs much higher radiation doses compared to hematological malignancies, due to their higher intrinsic radioresistance (127). The slow extravasation of antibodies, combined with elevated interstitial pressure and hypoxia at the tumor site, can limit the selectivity and the total radiation dose which radiolabeled antibodies can deliver to solid tumors in vivo (128). Targeting the tumor-associated extracellular matrix may help overcome some of these limitations, as these components are typically more abundant, more accessible and more stable, compared to antigens on the cell membrane.

A general limitation of radioimmunotherapy is given by radiation dose, which can be tolerated by the bone marrow, the most radiosensitive tissue in the body. Less
conventional types of radionuclides, such as those emitting α-particles or Auger electrons, may allow the development of radiopharmaceuticals with improved therapeutic index. Additionally, virtually 100 % of the injected radiation dose will at some stage pass through clearance-associated organs, i.e. the kidneys or the liver. Solid tumors may still receive a higher radiation dose compared to kidney and liver, if the radiolabeled antibody exhibits a long residence time in the neoplastic mass, but a relatively low dose rate may negatively affect therapeutic outcome.

3.5.3.2 Pretargeting

Attempts have been made to improve the biodistribution properties of radiolabelled antibodies, which display acceptable tumor-to-organ ratios only at later time points, using a two-step strategy known as ‘pretargeting’. Typically, a bispecific antibody is injected and allowed to bind to its tumor-associated antigen, while being cleared from other tissues. At a later time point, a radiolabeled small molecule is injected, which is specifically recognized as a hapten by the pretargeted bispecific antibody. Because of its small size, the radiolabeled molecule readily diffuses into the tumor mass and is retained by the antibody. At the same time, it is rapidly eliminated from the rest of the body via the renal route. As a consequence, much higher radiation doses can be delivered to the tumor while systemic side effects are reduced and restricted to clearance organ-associated toxicities.

3.5.3.3 Radioimmunoconjugate formats

The only two approved radioimmunotherapy products are both based on murine antibodies in IgG format, specific to the CD20 antigen. ⁹⁰⁹Y-ibritumomab tiuxetan (Zevalin®) and ¹³¹I tositumomab (Bexxar®) are indicated for relapsed or refractory NHL [see Figure 10 ]. Bone marrow toxicity represents the main limitation for dose-escalations of IgG-based radioimmunoconjugates alongside with radiation-
induced clearance organ damage. Engineered antibody fragments such as scFv, diabodies or SIPS have also been investigated and compared in quantitative biodistribution studies, showing that smaller formats are cleared rapidly via the renal route, while larger formats are cleared via the hepatobiliary route (129, 130). In mice, the SIP format was shown to exhibit superior biodistribution properties and better RIT performance, compared to the use of the same antibody in scFv or IgG format (129, 131).

Figure 10. Schematic representation of the approved radioimmunoconjugates Zevalin® (a) and Bexxar® (b).

3.5.3.3.1 Radiolabeling

There are two well-established methods for the chemical coupling of radionuclides to targeting antibodies. First, radioisotopes of iodine can be selectively reacted with aromatic residues, in the presence of a mild oxidative agent, such as iodogen or chloramine-T. Second, radioactive metals can form very stable coordination complexes with chelating agents such as diethylenetriaminepentaacetic acid (DTPA) or macrocyclic tetraazacyclododecane-tetraacetic acid (DOTA), which can be coupled to the amino group of lysines, to modified carbohydrates or to cysteine side chains.
3.5.3.3.2 Radionuclide payloads

The majority of clinical RIT studies to date use β-emitting radionuclides. Conventional beta-emitting radionuclides, such as $^{90}$Y, $^{131}$I and $^{177}$Lu, deposit their energy within 1 to 10 mm from the site of antibody localization, thus potentially enabling a ‘crossfire effect’, which could kill proximal antigen-negative cells (132). The therapeutic performance of RIT depends on several parameters apart from the intrinsic radiosensitivity of different cell types. The radiation doses delivered to the tumor relative to other organs the as well as physical parameters such as the radionuclide-specific dose rate are other key determinants for clinical efficacy and safety of RIT.

As cures for solid tumors have rarely been reported using RIT applications, researchers have started to investigate whether more energetic α-emitting radionuclides may increase therapeutic efficiency of radioimmunoconjugates. The availability and the rather short half-life of α emitters have so far limited their use for product development. Only few radioisotopes, namely $^{149}$Tb, $^{211}$At, $^{213}$Bi, $^{225}$Ac, $^{213}$Ra and $^{227}$Th, are of potential clinical interest for targeted α radionuclide therapy. $^{213}$Bi was the first α-emitter to be tested in clinical trials with encouraging results (132). Solid tumor indications for antibodies labeled with α-emitters are breast cancer (133), metastatic melanoma (134) and glioma (135, 136). Unfortunately, the short half-life of $^{211}$At and $^{213}$Bi prevented central radiolabeling procedures and product storage prior to clinical use, hindering multicenter clinical studies and a broader use of these RIT products. The recent availability of reliable procedures for the antibody radiolabeling with the long-lived $^{225}$Ac and $^{227}$Th have stimulated a renewed interest in the development of therapeutic biopharmaceuticals based on α-emitting radionuclides (137, 138).

The therapeutic potential of α-emitters has been demonstrated in patients with bone-metastatic prostate cancer, on the basis of clinical studies with Alpharadin® ($^{223}$Ra-chloride). $^{233}$Ra, which acts as calcium mimetic and targets regions of osteoblastic metastases has been approved for this indication.
In addition to $\alpha$ and $\beta$-emitting radionuclides, Auger electron emitters, such as $^{125}$I and $^{111}$In, are also possible radionuclides for RIT. Auger electrons are low energy electrons which are emitted instead of an x-ray after electron capture by an excited ion. Most of the energy is emitted in a sphere of several nanometers around the decay site. Auger electron emitters have not been widely used so far, both because of the radioprotection measures required for their clinical use and because they need to be delivered to the intracellular compartments of tumor cells in order to exhibit maximal efficacy. This poses stringent requirements on the use of antibodies with internalization properties and the capability of targeting all tumor cells within a neoplastic mass (139).

### 3.5.3.4 Clinical trials

#### 3.5.3.4.1 Hematologic malignancies

Lymphomas are more radiosensitive than other forms of cancer (140). The approval and clinical success of the intact anti-CD20 antibody Rituximab\textsuperscript{®} stimulated the investigation of the therapeutic performance of radiolabeled anti-CD20 products. Both commercially available products, Zevalin\textsuperscript{®} and Bexxar\textsuperscript{®}, are based on murine antibodies specific to CD20. In the clinic, $^{90}$Y-ibritumomab prolonged the median progression free survival of NHL patients by two years (141). However, due to the murine origin of the antibody moiety, repeated dosing with $^{90}$Y-ibritumomab led to the development of antibody drug antibodies. The superiority of the above-mentioned radioimmunoconjugates over rituximab-based combination therapy procedures (which represents the standard of care for NHL) has not yet been demonstrated. For targets also expressed within normal organs, as it is the case of CD20 in the spleen and on healthy B lymphocytes, pre-dosing with unlabeled antibody can significantly improve tumor targeting of the radiolabeled antibody by saturating the ‘easily accessible’ binding sites with unlabeled antibody before the labeled antibody is injected (142).
The perception that hematological malignancies may be more susceptible to RIT than solid tumors, is reinforced by the experience accumulated using a radiolabeled antibody in SIP format (L19, specific to EDB of fibronectin), which has been administered to patients with different tumor types. For $^{131}$I-SIP(L19), reported objective responses were almost exclusively observed in lymphoma patients (123, 143).

3.5.3.4.2 Solid cancers

It is reasonable to question whether the systemic application of monoclonal antibodies or antibody fragments will ever be able to deliver radiation doses that are high enough to mediate major anticancer objective responses, with acceptable toxicities. There is evidence that the largest radiation uptake levels are achieved in small microscopic lesions (144). RIT of large solid tumor lesions remains a challenge because these neoplastic tissues are intrinsically more radioresistant, i.e. the absorbed doses needed for cell death are higher than for lymphomas (140). Most clinical reports on solid tumor RIT with $^{131}$I- or $^{90}$Y-labeled antibodies state that the tumor radiation doses did not exceed 30-40 Gy. The doses needed for solid tumor eradication are probably in the 50-100 Gy range, as suggested by RIT studies in which objective responses were observed and based on experience with external beam radiation therapy of neoplastic lesions (even though the radiobiology of RIT and external beam radiation is different) (145, 146).

Early reports on RIT of solid cancer suggested the applicability of this therapeutic approach in colorectal cancer (CRC), a malignancy that is normally incurable once it has reached the metastatic stage (147). Investigations in these indications were stimulated by the fact that the carcinoembryonic antigen (CEA) has long been known to be abundantly expressed in epithelial tumors of the digestive system (148). Other CRC-associated antigens, such as A33 (149), EpCAM (150) and TAG72 (151), have been targeted with radiolabeled antibodies in clinical studies. However, with only few objective responses reported so far, the overall
clinical results were disappointing. PSMA is not only the target of clinical-stage ADCs, but is also investigated in radio-immunotherapy. Promising results in men with androgen-independent metastatic prostate cancer were obtained in phase I and phase II studies with $^{177}$Lu-labeled J591, a humanized monoclonal antibody in IgG format, directed against the extracellular domain of PSMA (152). The most studied target in glioma is tenascin-C since it is known to be upregulated in high grade glioma but not in normal brain tissues (153). Several studies with radiolabeled antibodies directed against this antigen were performed (154). Intratumoral injections of $^{131}$I-BC2, a murine antibody, in 10 patients with recurrent glioblastoma multiforme, led to a patient with a complete tumor remission, 3 partial responses and disease stabilization in 5 patients. All were relapse-free for longer than 11 months. For studies performed in patients with recurrent tumors (155, 156) and in patients with newly diagnosed malignant glioma (157) similar encouraging results were obtained. As radioimmunoconjugates can deliver high radiation doses to small tumors (158, 159), it would be conceivable to use RIT in the minimal residual disease setting (160). Promising results were initially reported for intraperitoneal administration of RIT in patients with advanced ovarian cancer after surgery and chemotherapy (161, 162). The only agent to be investigated in controlled phase III clinical studies was $^{90}$Y-labeled murine HMFG1 directed towards the trans-membrane protein mucin-1, in patients with epithelial ovarian cancer who had experienced a complete response after surgery and chemotherapy. However, this radioimmunoconjugate did not extend survival or time to relapse after a single intraperitoneal administration of $^{90}$Y-muHMFG1 (163).

Several studies with the $^{90}$Y- and $^{177}$Lu-labeled CC49 antibody directed against tumor associated glycoprotein 72 have been conducted (162). Other antibodies, such as trastuzumab directed against HER-2/neu (164) or pertuzumab directed against the dimerization domain of HER-2 (165), were radiolabeled and clinically evaluated with encouraging results. The use of α-emitting radionuclides may renew interest in locoregional RIT procedures for the treatment of ovarian cancer (166). A pretargeting approach in patients with medullary thyroid carcinoma who received a bispecific antibody directed against CEA and DTPA-indium, followed
4 days later by a $^{131}$I-labeled bivalent hapten a long-term disease stabilization and a significantly longer survival in high-risk patients was reported (167) In spite of these promising results, the efficient targeting of large tumor masses may still be limiting efficacy.

The high energy transfer rate and short tissue penetration of α-emitters provide a strong rationale for the antibody-based delivery of α-emitters to the tumor neovasculature, resulting in a highly selective killing of endothelial cells (168). Promising in vivo tumor targeting results have been shown for several antibodies targeting neovascularization, some of which are currently in clinical use for RIT (143) or imaging (121).

3.5.3.5 Preclinical research

A significantly prolonged survival after tumor therapy with a $^{131}$I-labeled folate receptor-specific dimeric human antibody fragment ($^{131}$I-AFRA-DFM5.3) has been shown in a mouse model of ovarian cancer. These preclinical studies provide the basis for the rationale design of therapeutic treatments of ovarian cancer patients with a radiolabeled anti-folate receptor antibody (169). New internalizing human antibodies termed ESC11 and ESC14, which bind to human and murine fibroblast activation protein, a serine protease that has emerged as a promising target for cancer therapy, were labeled with $^{177}$Lu and found to extend mouse survival and delay growth of established tumors (170). Recently, two new radioimmunoconjugates based on Herceptin® have been tested in mice bearing SKOV-3 xenografts. The α-particle-emitting $^{227}$Th-trastuzumab delayed tumor growth and prolonged survival of mice compared with the β-emitting radioimmunoconjugate $^{177}$Lu-trastuzumab when administered at the same absorbed radiation dose (171).
Combining RIT with classical chemotherapy has attracted recent interest, in spite of the fact that the two therapeutic modalities risk to simultaneously impact on the bone marrow, thus leading to unacceptable myelotoxicity. A humanized mucin-1 targeting $^{90}$Y-labeled antibody ($^{90}$Y-clivatuzumab tetraxetan) administered in combination with gemcitabine in pancreatic carcinoma patients resulted in 63% of the patients in a partial response or stable disease (172). The combination of an anti-CEA antibody and an anti-angiogenic agent has been investigated in patients with gastrointestinal carcinomas (173). A humanized, $^{90}$Y-labeled antibody that targets a mucin antigen ($^{90}$Y-clivatuzumab tetraxetan) was administered in combination with gemcitabine in patients with pancreatic ductal carcinoma with good results (172). The $^{131}$I-labeled anti-CEA antibody A5B7 in combination with combrestatin-A4-phosphate, a vascular disrupting agent, has been tested in patients with gastrointestinal carcinomas, although dose-limiting myelo-suppression occurred (173).

In general, RIT procedures seem to be most suitable for combination with agents that do not impact on the bone marrow. A synergistic therapeutic benefit has been reported in preclinical studies for the combination of RIT with the intact monoclonal antibody Erbitux® (131). Positive results were obtained in a phase I study evaluating ibritumomab tiuxetan (Zevalin®) in combination with bortezomib (Velcade®) in relapsed/refractory mantle cell and low grade B-cell NHL with 42% of the patients experiencing a complete response (174).

The field of radioimmunotherapy has exhibited promising results for the treatment of lymphomas, but has so far failed to demonstrate therapeutic potential in the case of solid tumors. The marketing authorization of Zevalin® and Bexxar®, for the treatment of hematological malignancies, represents a success for the field, despite the fact that these products are not broadly applicable and are seldom used in the clinic. Promising results for the treatment of MRD have not been confirmed at the level of randomized phase III clinical studies.
3.5.4 Bispecific antibodies

3.5.4.1 Concepts

Bispecific antibodies are a special class of armed antibodies because the payload, which is responsible for therapeutic activity, is represented by a second antibody moiety. The engineering of antibody products with dual specificities may open a broad range of different therapeutic strategies for the treatment of solid and hematological cancers. The field has made considerable progress over the past two decades. In particular, bispecific antibodies capable of recruiting cytotoxic T lymphocytes to tumor cells have proven to be clinically efficacious in certain settings. Other strategies for bispecific antibody therapy may include the simultaneous binding to pairs of tumor-associated antigens on the same cancer cell, the inhibition of two soluble cancer-promoting factors, as well as the recruitment of leukocytes other than T lymphocytes to the neoplastic site.

3.5.4.1.1 Immune cell recruitment

The anti-tumor activity of unmodified antibodies may depend on Fc-effector functions such as ADCC and CDC. The corresponding Fcy-receptors are expressed by a variety of immune cells such as macrophages and NK cells, which are known to mediate anti-tumor responses. However, there is evidence that cytotoxic T cells feature the highest tumor cell-killing potential within our immune system. Paradoxically, T cells do not express Fcy receptors and can therefore not participate in conventional ADCC. The use of engineered bispecific antibodies, which are capable of simultaneous binding to a tumor cell-surface antigen and the T cell co-receptor CD3, represents a clinically validated strategy to recruit T cells to cancer cells (175). When cytotoxic T cells are brought into close proximity to malignant cells, cancer-specific cytolysis may occur. The recruitment of CD3-positive cells is at the basis of the therapeutic strategy employed by Removab®, the only market approved bispecific antibody and by
other bispecific products, which are currently evaluated in advanced clinical trials mainly for hematological applications. Various antibody formats may be considered for T cell recruitment, some of them are displayed in Figure 11. The mechanism of action of bispecific antibodies is not fully understood, but it is likely to involve the generation of an immunological cytolytic synapse between a cytotoxic cell and its target cell (176). Bispecific antibodies cross-link their cognate antigens, bringing the corresponding cells into close contact, ultimately inducing the release of cytotoxic granules. Remarkably, bispecific antibodies initiate these events in the absence other T cell co-stimulatory signals, which are normally needed to potentiate TCR/MHC signaling.

Figure 11. Schematic representation of clinically investigated bispecific antibody formats. Bispecific full-length IgG antibodies (a) and fragment-based antibodies (b). Disulfide bonds are indicated with asterisks.

3.5.4.1.2 Dual-inhibition and -targeting

The progression of many cancer types is driven to a varying degree by growth factors such as epidermal growth factor (EGF), insulin-like growth factor (IGF) or platelet-derived growth factor. In addition, cancer growth and metastatic spread
are promoted by the formation of new blood vessels (angiogenesis), a process mediated by various proteins, including vascular endothelial growth factor (VEGF). However, the antibody-mediated blockade of individual cancer associated proteins rarely results in long-lasting tumor remissions. Consequently, the simultaneous inhibition of multiple mediators of the disease or their cognate receptors has become an emerging strategy in oncology research (177-179). Such approaches could potentially expand the therapeutic applicability of blocking antibodies since the expression of cancer-associated factors is extremely variable among different cancer types and patients. Dual-targeting antibodies could also have the potential to improve the tumor targeting specificity of antibody-based therapeutics. This could either be achieved by using biparatopic antibodies, i.e. bispecific antibodies recognizing two adjacent epitopes on the same target molecule (180, 181) or by the use of antibodies with two different cancer antigen specificities (182).

3.5.4.2 Formats

3.5.4.2.1 IgG-based bispecifics

Early attempts to generate bispecific antibodies have relied on the fusion of two different hybridoma cell lines, resulting in so-called ‘hybrid hybridomas’ or ‘quadromas’ (183). The resulting co-expression of two antibodies within the same cell leads to random association of heavy and light chains and consequently to low production yields of the anticipated functional bispecific antibody (184). These limitations have initially prevented the preclinical development of IgG-based bispecific antibodies. In an attempt to improve the production of more homogenous bispecific antibodies, a first major breakthrough occurred in 1995 with the so-called TriomAb® technology (185). By fusing two hybridoma cell lines with distinct IgG-subtypes from different species, i.e. mouse IgG2a and rat IgG2b, species-restricted heavy/light chain pairing could be achieved. The resulting bispecific TriomAbs could be easily separated from the parental IgGs, which permitted their large-scale production. Surprisingly, these molecules were found
to exhibit special biological properties as compared to conventional IgGs. The Fc-portions of TriomAbs preferentially bind to activating FcγRI and FcγRIII but not to inhibitory FcγRIIb receptors. As a consequence, these molecules proved to be capable of activating dendritic cells, inducing NK cell-mediated ADCC and of promoting phagocytosis by macrophages more efficiently than IgGs (186, 187). TriomAb technology has been validated in several clinical trials and has led to the market-approval of Removab® (catumaxomab), but patients typically receive only few loco-regional injections of the product.

Several research groups have explored alternative methods for the generation of human bispecific antibodies. A summary of clinically investigated bispecific IgG formats is displayed in Figure 11. An alternative method for generating bispecific human IgGs is known as the ‘knobs-into-holes’ approach. It relies on the structure-based re-engineering of Fc-domains, with reciprocal mutations in the CH3 domains, favoring correct heterodimerization of two different half-antibodies (188, 189). For targets also expressed within normal organs, as it is the case of CD20 in the spleen and on healthy B lymphocytes, pre-dosing with unlabeled antibody can significantly improve tumor targeting of the radiolabeled antibody by saturating the ‘easily accessible’ binding sites with unlabeled antibody before the labeled antibody is injected (142). The development of such domain ‘crossover’ mutants, termed CrossMabs, may be generally applicable and should not require further sequence optimization. A different way of generating dual-specificity antibody products is to evolve hypervariable loops of variable antibody domains by phage display, with the aim to generate binding sites capable of recognizing multiple antigens (190). It has been shown that such ‘two-in-one’ antibodies can exhibit superior therapeutic activities (179, 191). Bispecific multivalent antibodies can also be produced by the genetic incorporation of heterodimerization domains into two different antibodies. An example for this kind of bispecific is given by Immunomedics’ Dock-and-Lock® (DNL) platform, which relies on the natural docking domains of protein kinase A (PKA) and its A-kinase anchoring protein (192).
3.5.4.2.2 Bispecific fragments

To date, close to fifty different multispecific antibody formats have been reported (193), the majority of which is based on the intact IgG format. However, small antibody fragments are favored for a variety of applications, which do not necessarily rely on Fc-mediated effector functions. Bivalent bispecific fragments directed against CD3 and a tumor cell surface marker seem to be particularly suited for redirecting T cells to lymphoma and leukemia cells. In vitro, there is experimental evidence that fragment-based bispecifics may be superior to full-IgG bispecifics in terms cytolytic activity (194). The most prominent immune cell-recruiting molecules are the bispecific T cell engagers (BiTEs®). These molecules consist of two different scFvs fused in tandem via a flexible linker (195). Encouraged by impressive clinical results of the most advanced BiTE, which is termed blinatumomab and features specificities for CD19xCD3, other BiTE-like compounds have recently advanced to clinical programs. An example of such an alternative is the dual-affinity retargeting (DART®) platform of MacroGenics. Structurally, DARTs differ from BiTEs, because they are based on a disulfide-stabilized diabody format (196). A third format used for bispecific antibody development is the tandem diabody (TandAb®). These products are composed of two different diabodies genetically fused by distinct flexible linkers, leading to the non-covalent association of the diabody chains and to the formation of a bispecific tetravalent molecule. The resulting constructs can bind to both targets with high avidity resulting in slower dissociation from the tumor cells and their target leukocytes (197). This format may be advantageous for solid tumor targeting applications because armed cytotoxic cells may require more time to encounter their target cells within the malignant tissue. However, to date, definite comparative in vivo evaluations of TandAbs, BiTEs or DARTs are missing (198, 199).
3.5.4.3 Clinical trials

3.5.4.3.1 Hematologic malignancies

The engagement of cytotoxic T cells and malignant blood cells via small bispecific antibody fragment represents the most advanced therapeutic application of bispecific antibodies. Blinatumomab (MT103), a BiTE directed against CD19xCD3 is currently investigated in phase II clinical trials. Prolonged infusion of very low doses of this compound can result in complete clearance of different types of malignant blood cells from the circulation and lymphoid organs (200, 201). In a phase I/II clinical trial, patients suffering from follicular and mantle cell NHL or chronic lymphocytic leukemia (CLL) responded strongly to blinatumomab therapy. An advanced clinical stage bispecific antibody for hematologic cancers is the TriomAb Bi20 (Lymphomun® or FBTA05) whose specificities are directed against CD3 and CD20, a cell-surface antigen expressed by most malignant B cells. Bi20 has already been investigated as a treatment option in clinical phase I/II trials, together with donor-lymphocyte infusions for relapsed CLL and NHL patients, which did not respond to previous allogeneic stem cell transplantation (202, 203).

3.5.4.3.2 Solid tumors

So far, only Removab® (catumaxomab), has received marketing authorization for solid tumor indications. Catumaxomab is a trifunctional bispecific immunoglobulin with a tumor cell-targeting specificity for endothelial cell adhesion molecule (EpCAM) and a T cell-engaging moiety specific for CD3. The third functionality is given by the Fc-portion, which is of the chimeric mouse/rat IgG2a/b-isotype and therefore primarily binds to activating Fcγ-receptors. Catumaxomab is indicated for the treatment for malignant ascites and is administered intraperitoneally. Malignant ascites is a common manifestation of various advanced cancer types for which parenthesis, i.e. peritoneal fluid drainage represented the only treatment option prior Removab® approval. Upon intraperitoneal administration,
95% of the treated patients exhibited prolonged puncture-free survival compared to parenthesis alone, in a combined phase I/II study. The therapeutic benefit of catumaxomab has later been confirmed in a randomized phase II/III multicenter clinical trial, which led to the approval of the drug (204). The clinical-stage BiTE MT110, which is directed against EpCAM and CD3, proved to be efficacious in several preclinical tumor-xenograft mouse models, which was surprising since the anti-CD3 specificity is not cross-reactive with mouse CD3. These experiments suggest that MT110 is able to reactivate exhausted or anergic human T cells still present within the xenografted tumors (205).

Bispecific T cell recruiting antibodies have also been developed with the second specificity directed against Her2. For example, Rexomun® (ertumaxomab), a TriomAb which also contains a mouse/rat IgGa/b Fc-portion, demonstrated clinical efficacy against Her2-positive malignant ascites when administered analogously to catumaxomab (206). Ertumaxomab is currently investigated in a phase II dose-escalation study for metastatic breast cancer, after safety and efficacy have been confirmed in phase I (207).

Immune cell retargeting represents the most advanced therapeutic application of bispecific antibodies, which is reflected by their clinical success. In contrast, most dual targeting or blocking antibodies have not yet reached the clinical stage. A notable example of such a compound is MEHD7945A, a fully human bispecific IgG1 antibody whose variable domains are capable of recognizing both, EGFR (Her1) and Her3 (179).

3.5.4.4 Preclinical research

Although progress has been made in terms of production methods and formats, the field of bispecific antibody research continuously produces innovation. Recently, the co-expression of two different half-antibodies assembled via the knobs-into-holes system has been shown to work in a co-culture of two bacterial strains (208). Although this production method could be very cost-effective, the
resulting bispecific IgG will lack N-linked glycans within the Fc-domains which are known to be important for ADCC. Another recent bispecific format is based on the natural occurrence of bispecific IgG4 antibodies. Such antibodies are transiently formed in vivo by a mechanism known as Fab-arm exchange, which has become a well-understood phenomenon (209, 210). Inspired by these mechanisms, researchers have recently succeeded in realizing Fab-arm exchange in vitro by introducing two complementary point mutations within the heavy chain CH3 domains of two separately produced IgG1 half-antibodies (211). Under controlled reducing conditions, two such half-antibodies can be assembled into the desired bispecific IgG1 antibody.

Dual action antibodies, i.e. bispecific blocking or targeting agents, offer a vast number of specificity-combinations, all of which require preclinical studies in order to develop more broadly efficacious antibody-based therapeutics. The dual targeting/blocking approach is challenged by conventional combination therapy comprising individual therapeutic agents. Obviously, clinical development of a single bispecific antibody is simpler and more economic, as it involves the investigation of only a single product. However, such products do not permit individual dose adjustments, which could be advantageous in light of the high variability of most cancer types.

3.5.5 Comparison of the four main avenues of armed antibody therapeutics

Various strategies using armed antibodies have been pursued for the treatment of solid cancers and hematologic malignancies. A direct evaluation of the relative advantages and weaknesses of the technologies presented above is difficult, since direct comparative studies are missing. Over time, products reaching marketing authorization will provide information about the indications, for which a certain class of armed antibody products may be most efficacious. Certain general considerations can still be made on the basis of extensive preclinical and clinical research activities of the past two decades. Table 2 represents an attempt
to summarize and to compare the four main classes of armed antibody for cancer therapy application.

Radiolabeled antibodies have so far demonstrated only limited efficacy for the treatment of solid tumors, but were found to be extremely active for the treatment of lymphomas, possibly the most radiosensitive forms of cancer. It is thus reasonable that this class of armed antibody therapeutics may be first developed for hematological indications. Antibody-drug conjugates represent a class of products, which is attracting considerable investments and has led to approved biopharmaceuticals. This type of armed antibodies would greatly benefit from a better understanding of the tumor targeting properties of ADCs, ideally thanks to the use of immuno-PET methodologies and dosimetric studies. Little is known about the amount of product, which reaches the tumor cells in vivo and the microscopic localization within the neoplastic mass. It has also been proposed that ADC internalization may play a less significant role, than what was originally thought. As more ADCs featuring cytotoxic drugs with different biological activities, e.g. DNA damaging agents and microtubule poisons will reach clinical trials, it will be possible to learn more about the therapeutic potential of ADCs for different types of solid tumors.

<table>
<thead>
<tr>
<th>Clinical-stage payloads</th>
<th>Radiolabeled antibodies</th>
<th>Antibody-Drug Conjugates</th>
<th>Immuno-cytokines</th>
<th>Bispecific Antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td>90Y, 131I, 133Lu</td>
<td>MMAE/F, DM1/4, calicheamicins</td>
<td>IL2, TNF</td>
<td>CD3, CD16a</td>
<td></td>
</tr>
<tr>
<td>Solid tumor targets</td>
<td>CEA, PSMA, TnC A1, Her2</td>
<td>Her2, many others</td>
<td>EDA, EDB, TnC A1</td>
<td>EpCAM, Her2, CEA, PSMA</td>
</tr>
<tr>
<td>Hematologic targets</td>
<td>CD20, EDB</td>
<td>CD33, CD33, CD22</td>
<td>EDA, EDB, TnC A1</td>
<td>CD19, CD20</td>
</tr>
<tr>
<td>Efficacy in cancer patients</td>
<td>lymphoma, MRO</td>
<td>hematologic &amp; breast cancer</td>
<td>melanoma, renal cell carcinoma</td>
<td>lymphoma, rare indications, MRO</td>
</tr>
<tr>
<td>Biodistribution data</td>
<td>Mouse models and patients (theranostic agents)</td>
<td>mouse models</td>
<td>mouse models and patients</td>
<td>few reports in mice</td>
</tr>
<tr>
<td>Molecular Formats</td>
<td>mainly IgGs</td>
<td>mainly IgGs</td>
<td>IgGs and fragments</td>
<td>IgGs and fragments</td>
</tr>
<tr>
<td>Clearance-related toxicity</td>
<td>direct</td>
<td>direct</td>
<td>indirect (vasoactivity)</td>
<td>indirect (vasoactivity)</td>
</tr>
<tr>
<td>Haematotoxicity</td>
<td>Lymphopenia and thrombocytopenia</td>
<td>reversible thrombocytopenia</td>
<td>Not main toxicity</td>
<td>Lymphopenia (cytokine storm possible)</td>
</tr>
<tr>
<td>Bystander effect</td>
<td>strong</td>
<td>target, linker &amp; drug dependent</td>
<td>Immune-mediated</td>
<td>possibly</td>
</tr>
<tr>
<td>Antigen internalization</td>
<td>not required</td>
<td>hematologic cancers</td>
<td>counterproductive</td>
<td>counterproductive</td>
</tr>
<tr>
<td>Solid tumor penetration</td>
<td>target dependent</td>
<td>target dependent</td>
<td>Not required in case of vascular targeting antibodies</td>
<td>target dependent</td>
</tr>
<tr>
<td>Immunogenicity</td>
<td>strong if rodent antibodies are used</td>
<td>Still being investigated</td>
<td>Low (species dependent)</td>
<td>species dependent</td>
</tr>
<tr>
<td>Chemotherapy compatibility</td>
<td>poor (overlapping toxicities)</td>
<td>poor (overlapping toxicities)</td>
<td>good (non-overlapping toxicities)</td>
<td>probably good (non-overlapping toxicities)</td>
</tr>
</tbody>
</table>

Table 2. Comparison of the four main avenues for armed antibody therapeutics for cancer.
The development of immunocytokines is facilitated by the ease of expression of these armed antibodies as fusion proteins, without the need for subsequent chemical modification. Multiple payloads with different immuno-regulatory properties can be considered and a wealth of biodistribution data has been collected in animals. Initial preclinical studies have exhibited very promising results, with the ability to eradicate cancer in a number of difficult models in mice, which cannot be cured by conventional chemotherapy. However, there is no certainty that the same cytokine will exhibit a comparable therapeutic activity in species as different as mouse and man. Clinical studies have so far mainly been focused on IL2 and on TNF. Long-lasting cures of cancer patients were reported only rarely so far.

The field of bispecific antibodies may be the most versatile of all classes of armed antibody products, since a huge variety of formats and of antibody combinations could be considered. Information on the tumor targeting performance of bispecific antibodies is rare, as only few quantitative biodistribution studies have been reported (212, 213). Binding affinities and kinetics for the individual targets may greatly affect the performance of bispecific antibodies, thus offering an opportunity and a challenge for product development, at the same time.

The molecular format of armed antibody products can have a profound influence on their therapeutic window and should therefore be chosen with respect to the target antigen and payload-related toxicities. Target accessibility is probably the most crucial aspect determining therapeutic efficacy of armed antibodies. So far, most investigated targets are cell-surface proteins, which are more or less specific for a given cancer type. While these targets are relatively easy to access on hematologic cancer cells, they are rarely targeted with the same efficiency in solid cancer indications. The targeting of tumor-specific sub-endothelial matrix proteins represents therefore an alternative, also because their expression is not restricted to a single cancer type. In fact, oncofetal splice isoforms of fibronectin and tenascin-C occur in most aggressive tumor types including solid manifestations of lymphomas and leukemia. Armed antibodies for vascular tumor targeting are in principle amenable to all kinds of bioactive payloads maybe with the exception of T cells recruiting antibody moieties. Vascular targeting of solid
tumors does not rely on long plasma half-life because small bivalent antibody fragments display favorable biodistribution profiles. Indeed, high tumor uptake values relative to other organs can be reached after 24h. By contrast, IgG-based antibodies have been used to target internalizing tumor cell-surface antigens in order to ensure maximal in vivo activity. From a patients’ perspective, the use of full-IgGs seems attractive because such compounds have to be injected rather infrequently. However, Fc-mediated interaction in organs not affected by the disease could potentially explain some of the clinically-observed systemic toxicities which have been reported for armed antibodies.

Protein-based products devoid of cytotoxic moieties, such as immunocytokines or bispecific antibodies, typically exhibit non-overlapping side-effect profiles when co-administered with chemotherapeutic agents and should therefore be more compatible with combination regimens. By contrast, ADCs and radioimmunoconjugates exert their cytotoxicity by direct interference with cell proliferation, in a similar way as conventional chemotherapeutics.

3.6 Extra domain A of fibronectin and the F8 antibody

The glycoprotein Fibronectin (Fn) is one of the most abundant proteins in blood and tissues. It exists either as soluble dimers in plasma or as cross-linked cellular Fn in the extracellular matrix (ECM) (214). Each Fn molecule is composed of repeated arrays of type I and type II domains, but depending on alternative splicing events at three different sites, the type III domains EDA and EDB as well as the connecting segment (IIICS) can be present or absent, leading to multiple splice isoforms of this protein originating from one and the same gene [Figure 12]. Under normal physiologic conditions, neither EDA nor EDB are detectable in normal adult tissues (exceptions: placenta, endometrium and ovaries (215)). However, during tissue remodeling and angiogenesis both type III domains become abundantly expressed in the subendothelial matrix of newly formed blood vessels. EDB is inserted between domains 7 and 8, while EDA is inserted between domains 11 and 12 of the high molecular weight Fn protein (216).
Phage display technology has allowed the isolation of fully human high affinity antibodies against both type III domains: the L19 antibody against EDB and the F8 antibody against EDA (215, 217). The tumor targeting properties of both antibodies have been assessed in various mouse models of cancer and inflammation, as well as in cancer patients (71). For this thesis only the F8 antibody is of relevance.

It may be advantageous to target an antigen expressed in the extracellular matrix at sites of active angiogenesis and tissue remodeling compared to antigens expressed on the surface of diseased cells, since components of the ECM are usually more stable and tumor cells can’t evade therapy by down regulating the target antigens. Furthermore, antigens of the ECM are more abundant and accessible than cell surface antigens and certain alternatively-spliced ECM proteins (including EDA of fibronectin) are over-expressed in a variety of malignancies, thus offering a broad coverage for various indications (218).

Figure 12. Schematic representation of the fibronectin structure including the type III domains EDA and EDB as well as the connecting segment IIICS. For visualization the crystal structure of the EDA domain (pdb structure file: 1J8K.pdb) and a generic antibody in IgG format (pdb structure file: igg1.pdb) is shown.

3.7 Angiogenesis and vascular targeting

Antibodies used for targeted drug delivery to the tumor site can bind a variety of target antigens expressed either on cancer cells, the tumor stroma or the tumor
vasculature. However, cancer cells rarely express tumor associated antigens that are truly specific. Usually, tumor antigens are expressed at higher levels on cancer cells compared to healthy cells, but the presence of antigen in normal tissues may hinder a selective tumor targeting and may lead to severe side effects, respectively. Furthermore, not all cancers show an upregulation of the same tumor antigens and there is even evidence that different cells within a tumor mass show different target expression patterns (219). This feature complicates the development of efficient tumor targeting strategies. Often, tumor associated antigens are not present under normal physiologic conditions, but may be expressed under certain circumstances, such as tissue remodeling. Vascular targeting, defined as the targeted delivery of therapeutic agents to newly formed blood vessels within the neoplastic mass, may reflect an alternative to the antibody-based targeting of tumor cells. Angiogenesis is defined as the growth of new blood vessels from pre-existing vasculature. It is a fundamental, highly regulated process for normal physiologic functions, such as wound healing, embryogenesis or reproduction. However, many pathologic conditions such as arthritis and cancer are associated with persistent and uncontrolled angiogenesis. Targeting the neovasculature is beneficial due to the inherent accessibility to systemically administered drugs and the fact that neovascularization is a common feature of most types of cancer of a certain size. Neovascularization is a response to a hypoxic environment, since simple diffusion of nutrients and oxygen is insufficient once the tumor mass reaches a critical size of 2-3 mm³. Tumors lacking an adequate vascularization can’t grow beyond this size and cells undergo apoptosis (220). Small tumors may persist for a long time in the body without growing. There is a balance between cell proliferation and cell death during the so-called prevascular phase. As soon as the tumor mass switches from the prevascular to the vascular phase (‘angiogenic switch’), the tumor mass can grow rapidly, as a result of the malignant transformation of the lesion (221). Under physiologic conditions angiogenesis is a tightly controlled process. Within the tumor, angiogenic sprout of new blood vessels is characterized by an imbalance between proangiogenic and antiangiogenic signals. Tumor cells respond to low oxygen conditions by the upregulation of hypoxia inducible factor (HIF-1α), which leads to the upregulation
of proangiogenic proteins e.g. VEGF, VEGF receptors, platelet derived growth factors and nitric oxide synthase, resulting in the breakdown of the basement membrane, remodeling of the ECM and finally in sprouting of new blood vessels. The morphology and function of newly formed tumor blood vessels differs from normal blood vessels. They are leakier, more disordered, dilated and irregularly shaped, what leads to an irregular blood flow and a higher interstitial pressure within the tumor (222), ultimately hampering drug uptake by the tumor mass.

Vascular structures or structures of the subendothelial extracellular matrix may be superior targets compared to antigens expressed on tumor cells, since they are genetically more stable and less prone to antigen down-regulation. Direct targeting of tumor neovasculature may aim at the destruction of tumor blood vessels and tumor eradication by starving out tumor cells. Alternatively, antibody-based pharmacodelivery strategies directed to antigens located in the subendothelial matrix, such as the EDA of fibronectin, not only offer the possibility to deliver highly cytotoxic agents but also immunomodulatory proteins, such as cytokines, to the tumor site in order to evoke an immune reaction against tumor cells [Figure 13]. Markers on the luminal side of endothelial cells may be the most easily accessible targets for i.v. administered drugs. However these antigens are often only weakly expressed, thus allowing only low doses of the drug to accumulate on the tumor. By contrast, components of the perivascular ECM are often abundantly expressed making them superior targets for pharmacodelivery strategies. There are several markers associated with neovascularization (e.g., Fn splice isoforms, Tn-C splice isoforms, PSMA, CD44 splice isoforms, integrins). The EDA-containing splice isoform of fibronectin is probably one of the best characterized markers of angiogenesis so far and represents the target antigen used for this thesis.
Figure 13. EDA targeted anticancer therapy. An EDA targeting antibody product is injected i.v. into a cancer patient. The antibody distributes throughout the body and finds its target antigen (EDA of fibronectin) after extravasation (black arrows) in the subendothelial matrix of newly formed blood vessels and the delivered effector moiety (e.g., a cytokine) can locally exert its anticancer effect.

3.8 Cytokines and cancer

Cytokines are a large and diverse family of intracellular signaling peptides. There is no unified classification system and they are variously identified by numeric order of discovery (IL1 to IL35), by their primary cell of origin (monokine = monocyte derivation; lymphokine = lymphocyte derivation), by functional activity, by their kinetic and functional role in inflammatory responses or, most recently, by structural homologies or their receptor structures, respectively [Figure 14].

Cytokine superfamilies, such as colony-stimulating factors (CSF), interferons, chemokines, transforming growth factors (TGF) and tumor necrosis factors, share sequence similarity and exhibit homology and some promiscuity in their reciprocal receptor systems, but they do not exhibit functional similarity.
Cytokines represent a central part of the communication pathway between various immune cells that regulate fundamental processes, such as cell survival, differentiation, growth or the trafficking of cells. Therefore a dysfunction in a cytokine signaling pathway can result in malignant transformation. Cytokines have pivotal effect on the carcinogenic process. They can be involved in the activation of a tumor suppressing immune response, but they can also induce and promote an inflammatory response, tumor growth, invasion and metastasis. Tumor, stromal and immune cells within the tumor release an array of cytokines resulting in an inflammatory immune response which often accompanies tumor growth. However, the cytokine environment is probably in rapid change during tumor development. IFNα, TNF, IL2 and IL12 were among the first clinically investigated cytokines. These proteins all have shown potential for tumor therapy,
despite their different biological functions. There is increasing evidence that cytokines are key mediators in the molecular communication between cells within the tumor microenvironment (224). The investigation of the role of cytokines in the tumor environment represents an emerging research area with an important impact on the clinical diagnosis and treatment of cancer.

The induction of angiogenesis is an important step for most solid tumors, but especially for carcinomas (221). Once the angiogenic switch is activated, tumors start to grow rapidly. Cytokines play an important role for the activation of this angiogenic switch, since they stimulate endothelial cells to migrate to the ECM where they proliferate and form vascular buds, from which then capillaries are built. Stimulated by angiogenic cytokines released by the tumor cells, endothelial cells proliferate rapidly and give rise to morphologically abnormal, large and broad vessels without pericytes and smooth muscle cells as well as a discontinuous and leaky basement membrane. Tumor angiogenesis is regulated by a balance of proangiogenic and antiangiogenic factors. Most of these factors are cytokines, which are classified in direct acting and indirect acting based on their effect on endothelial cells. VEGF is one of the most potent stimulators of angiogenesis. It stimulates endothelial cell proliferation, migration and differentiation (225) and has the capability to induce the production of nitric oxide resulting in vasodilatation and increased blood flow. VEGF has been shown to be a survival factor for endothelial cells and to maintain the viability of the endothelium by inducing antiapoptotic factors in endothelial cells. Fibroblast growth factor is another cytokine with an important role in angiogenesis. It is involved in the regulation of the proliferation, migration and differentiation of various cell types, including endothelial cells and in remodeling of the ECM. Chemokines as a subfamily of cytokines have been recognized as critical mediators of the inflammatory response by regulating recruitment of various immune cells, but they have been shown to regulate not only the migration, proliferation and survival of immune cells but also a variety of other cells types. These proteins regulate various biological processes, including angiogenesis and germ, stem and epithelial cell migration. Deregulated expression and activity of several chemokine signaling pathways have been implicated in cancer.
progression. The tumor surrounding stroma also shows significant changes in chemokine expression of certain chemokines, correlating with tumor size and lymph node invasion (226).

Transgenic mouse models, which were developed in the 80s (227), represent meaningful tools for the study of gain-of-function malignant cytokine dysfunctions, while the development of the knockout mouse technology allowed the investigation of loss-of-function genotypes. Such murine models of cancer may serve as tools for the study of cytokine function in a malignant setup and to test experimental therapeutic interventions. As a consequence of these studies, the role of many cytokines in a malignant setting became clearer. However, there is still a significant gap in our knowledge of what cytokines and the immune system contribute to each phase of tumor development.

In the following paragraphs, the role of chemotactic cytokines (chemokines) and cytokines IL1β, IL6, IL9, IL13 and VEGI in cancer is discussed. The following part of the introduction as well as the Results and discussion parts of this thesis were adapted from the following publications:


3.8.1 Chemotactic cytokines (chemokines) as payload

Chemokines are the largest subfamily of cytokines, consisting of structurally related chemotactic cytokines of small molecular weight that are acting in gradients to promote directional migration of leukocytes (228) [Figure 15].

![Diagram of chemokine action](image_url)

**Figure 15.** Chemokines act in gradients to promote the directional migration of chemotactic cells. An inflammatory stimulus leads to the expression of selectins by the endothelium. Binding to their receptors on the chemotactic cell initiates rolling, activation and adhesion to the vessel’s luminal wall. Integrin interaction leads to a firm adhesion and arrest of the cell allowing transmigration along a chemokine gradient. Chemokines guide the chemotactic cells and modulate the surface expression of adhesion molecules during the whole process.

Initially, chemokines were defined as soluble proteins regulating directional migration of leukocytes during inflammation. However, chemokine function extends to many cell types, including most human neoplastic cells. Some
chemokines can have additional activities including HIV inhibitory activity, tumor-promoting or -inhibitory activity, angiogenic or angiostatic activity or the ability to modulate gene expression, phagocyte activation and cell differentiation.

Based on the relative position of the conserved N-terminal cysteine residues they are classified into CXC, CC, CX3C or C chemokines. They mediate their signals through G-protein coupled receptors (GPCRs) and are both pleiotropic and redundant in their effects. Internalization and signal transduction is mediated by a number of adapter proteins which dynamically interact with the GPCRs and form a ‘chemosynapse’ that is vital for directional sensing and polarization of the chemotactic cell (229-231).

Chemokines contain disulfide bonds, which stabilize the overall protein conformation and share a common structure [Figure 16]. Many chemokines form dimers and higher-order oligomers in solution or upon binding to glycosaminoglycans (GAGs). Despite highly variable sequence homologies, all chemokines share remarkably conserved tertiary structures, consisting of a disordered N-terminus of 6-10 amino acids, which functions as key signaling domain (232). They undergo post-translational proteolysis by various proteases (233-241), leading to truncated products with different affinities and specificities for their receptors and therefore different biological properties.

**Figure 16.** Typical chemokine topology. The N-terminal loop is responsible for receptor recognition and its structure is restrained by two disulfide bonds (depicted in yellow). It is followed by the short $3_{10}$ helix (shown in ribbon representation) and a three stranded anti-parallel $\beta$-sheet. The C-terminal $\alpha$-helix folds over the $\beta$-sheet and helps stabilizing the overall structure (pdb file used: 1RTN).
Only few reports on antibody-chemokine fusion proteins (‘immunochemokines’) have been published so far (242-244). Several reports have indicated that the induction of certain chemokines at the tumor site may lead to a therapeutic benefit. Promising preclinical antitumor effects have been reported for CCL19 and CCL21 (245-249). These promising preclinical results have led to the execution of a Phase I clinical study in melanoma using CCL21-transduced dendritic cells (DCs) (250).

Complete tumor rejection associated with CD8+ and T cell infiltration could be shown with CXCL11 overexpressing tumor cells (251). Similarly, mice treated with CCL17 or CCL5 in combination with granulocyte macrophage colony-stimulating factor (GM-CSF) in a WEHI3B tumor model showed significantly higher survival rates than untreated mice and mice injected with GM-CSF-transduced cells alone (252). Furthermore, CCL20 overexpression in established murine tumors suppressed tumor growth by attraction of DCs and activation of tumor-specific cytotoxic T cells (253).

Tumors depend on neoangiogenesis for growth and survival (254). Many cancer cells secrete proangiogenic factors acting directly on endothelials cells, but there is evidence that also chemokines play an important role by attracting monocyte-derived cells to the site of disease (255). As a general rule, CXC chemokines regulate angiogenesis either positively or negatively depending on the presence or absence of the ELR (Glu-Leu-Arg) motif in their N-terminus (256). Members of the CXC family with the ELR motif are angiogenic, while ELR negative CXC chemokines, are angiostatic (257). CXCL9 overexpression resulted in the inhibition of non-small-cell lung carcinoma tumor growth and metastasis (258). CXCL10 inhibits CXCL8 and bFGF-induced angiogenesis (259). Furthermore, CXCL11 inhibits angiogenesis in a murine model of pulmonary fibrosis (260).

Collectively, these preclinical findings provide a strong rationale for the implementation of therapeutic strategies, aiming at the pharmacodelivery of certain chemokines to neoplastic sites in vivo. However, while cytokines have extensively been studied as partners for the preparation of antibody-based fusion proteins with an impressive therapeutic potential for cancer (71, 73) and other
diseases (261, 262), there have been only few reports related to the implementation of tumor-targeting strategies with immunochemokines.

Human CCL5 (RANTES) was fused to the N-terminus of the heavy chain of an IgG3 antibody specific to the tumor associated antigen HER2/neu. A selective in vivo localization of the fusion protein to HER2/neu-expressing xenografted tumors in SCID mice has been reported, although no biodistribution data has been shown (263). Specific binding of RANTES.her2.IgG3 fusion protein to HER2/neu Ag expressed on EL4 cells and on SKBR3 breast cancer cells could be shown (264).

Epstein and colleagues described the production of a recombinant immunochemokine, consisting of murine liver-expression chemokine (LEC)/CCL16 fused to the murine antibody chTNT-3 in IgG format, which targets DNA in necrotic regions of tumors (265). In biodistribution studies with this antibody-chemokine fusion protein, a biologic half-life of 3 hours and a tumor uptake of 2.4% injected dose/g, have been reported (244). The product, administered at a daily dose (5 injections) of 20 µg/mouse, mediated a 37-55% reduction in tumor growth rate in immunocompetent mice bearing (MAD109, COLON26 and RENCA) tumors.

In a third report featuring the use of a recombinant murine antibody fragment specific to human acidic isoferritin in scFv format, the fusion protein mCXCL10-scFv could be expressed (242, 266) and the immunochemokine retained its antibody binding specificity and chemokine function, but was not tested in vivo for biological activity and for therapeutic applications.

This thesis describes the first comprehensive attempt to generate and characterize several antibody-chemokine fusions for tumor targeting applications. As delivery vehicle, we chose the human monoclonal antibody F8 in diabody or scFv format (215), which recognizes the alternatively-spliced EDA of fibronectin (267) with identical affinity in mouse and man. The tumor targeting performance of the F8 antibody alone or fused to several different cytokines has previously been studied using radioiodinated protein preparations by quantitative
biodistribution analysis in tumor-bearing mice, revealing a selective accumulation on neoplastic masses, with excellent tumor to organ ratios (268-270). The F8 antibody was fused to ten different murine chemokines [Table 3] the expression of the corresponding fusion proteins by transient gene expression in mammalian cells was attempted to ultimately test the in vivo applicability of immunochemokines.

<table>
<thead>
<tr>
<th>Class</th>
<th>Name</th>
<th>Alternative name</th>
<th>Receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CXC-Chemokines</strong></td>
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</tr>
<tr>
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</tr>
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<td>ITIP</td>
<td></td>
<td>CXCR3</td>
</tr>
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<td>CCL21</td>
<td>SLC</td>
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Table 3. Selected chemokines for the attempted expression as fusion proteins. Five chemokines belong to the CXC-class and five to the CC-class of chemokines, which all share a common structure. Names, alternative names and the cognate receptors are listed. CXCL11/10 is a chimeric chemokine consisting of the N-terminal part of CXCL11 and the C-terminal part of CXCL10.

3.8.2 Interleukin 1β and Interleukin 6 as payloads

Some recombinant cytokines (e.g., IL2, TNF, IFNα, IFNβ, IFNγ, GM-CSF) have received marketing authorization for applications in oncology, infectious disease and autoimmunity. The systemic administration of certain pro-inflammatory cytokines (e.g., IL2) may confer a potent antitumoral activity, which can be curative in a small portion of patients with metastatic melanoma or renal cell carcinoma (271, 272) in young patients, who are fit enough to tolerate this toxic
therapeutic procedure. For most other indications, however, recombinant cytokines rarely mediate objective responses (68, 69) and may cause serious adverse events already at low doses, thereby preventing the escalation to therapeutically active dose regimens. For these reasons, tumor-targeting antibodies have been considered as vehicles for pharmacodelivery applications, with the aim to improve the therapeutic index of the corresponding immunomodulatory payload (273-275). Immunocytokines are being developed, both clinically and preclinically, for the treatment of cancer and certain inflammatory diseases (70, 71).

The F8 antibody-based targeting of the sub-endothelial ECM in tumors is particularly attractive, because immunocytokines typically remain bound to their abundant and stable antigen for several days, thus having the opportunity to interact with in-transit receptor-positive leukocytes and to modulate immunity at the site of disease.

Many cytokines (including GM-CSF, IFNα, IFNγ, IL2, IL4, IL7, IL10, IL12, IL15, IL17, IL18, TNF, CD40L, FasL, TRAIL, LiGHT, VEGI, lymphotoxin α, lymphotoxin β and lymphotoxin α1/β2) have already been fused to tumor-targeting antibodies specific to splice variants of fibronectin (71, 109, 276). Fusions with IL2, IL4, IL12 and TNF were potently active against cancer as single agents and the activity could be further improved in combination therapy modalities (277-279), while the other cytokines showed either a modest therapeutic benefit or no benefit at all. Similarly, TNF-based immunocytokines display a potent anticancer activity against certain tumor types (especially sarcomas) (104, 278, 280).

High levels of TNF in plasma have been associated with poor prognosis for various tumor types and preclinical studies have shown that TNF can promote cancer development and dissemination (281). TNF is one of the main mediators of acute and chronic inflammation (281) and is able to induce the expression of IL1β and IL6 (282, 283). IL1, IL6 and TNF are three related examples of multifunctional cytokines involved in the regulation of the immune response, hematopoiesis, and inflammation, with partially overlapping functions. Like TNF, the other two cytokines can have pro- and anti-inflammatory functions, making
them a double-edged sword for cancer therapy. Unlike TNF, however, IL1β and IL6 have not previously been fused to antibodies and studied for pharmacodelivery applications.

The IL1 family comprises eleven proteins, whereof the two agonists IL1α and IL1β were discovered first and are therefore the most studied members. While IL1α is membrane-bound, IL1β is secreted and therefore can act systemically. IL1 family cytokines bind to the IL1-receptor, which exists in two different forms. Type I receptor is primarily responsible for transmitting the inflammatory effects of IL1, while type II receptor may, together with IL1-receptor antagonist (IL1RA), suppress the IL1 activity. IL1β is a pluripotent cytokine involved in normal physiological processes but is also secreted under pathological conditions (e.g., in autoimmune diseases). Recombinant human IL1β (rhIL1β) was evaluated in a phase I clinical trial in patients with metastatic or non-resectable solid tumors (284). Four dose levels up to 200 ng/kg of rhIL1β were evaluated. The major toxicities were chills, rigors, headache, fatigue, and hypotension. IL1β exhibited extensive hematological effects but its usefulness in clinical practice is limited by the extensive toxic side effects at all tested dose levels, a problem that could be overcome with a targeted delivery to the site of disease.

As for TNF, there are contradictory reports about the effect of IL1β on the growth of tumor cells. Some (more recent) studies have shown stimulatory effects, whereas other (older) investigations have reported an inhibitory activity. IL1β is significantly linked to poor prognosis for patients with esophageal cancer and may be a promising molecular target for therapeutic intervention for esophageal squamous cell carcinoma (285). The IL1 gene is frequently expressed in metastases from patients with several types of human cancers (286). However, there are reports showing anticancer activity of IL1β (287-289). Intraperitoneal administration of rhIL1β led to dose dependent effects on tumor growth in nude mice bearing human ovarian tumor xenografts. At low dose levels (10 ng/day) a promoting effect on micrometastatic peritoneal implants could be observed, while at 1 µg/day an anti-tumor effect was reported (290), in keeping with more recent observations (291). The targeted delivery of TNF to the tumor neo-vasculature has been reported to have an even more complex effect, with tumor growth
inhibition at ultra-low doses and at the maximal tolerated dose, but lower effect in the intermediate dose range (292).

IL6 is a pleiotropic cytokine involved in the host immune defense mechanism and the modulation of growth and differentiation of cells as well as the regulation of metabolic, regenerative, and neural processes. IL6 can be produced by various cell types, including tumor cells. IL6 (and IL6 type cytokines except IL31) share a common receptor and signal transducer, termed gp130. IL6 initially binds to the membrane-bound IL6-receptor (α-receptor) and this complex then subsequently dimerizes with gp130 leading to intracellular signaling events.

In mice injected with IL6, no treatment-related deaths were observed at doses up to 50 μg per injection (maximum tested dose) and no signs of toxicity were reported. Unlike IL2, which was also investigated in the same study, IL6 did not induce vascular leak syndrome (282). IL6 possesses multiple biological functions affecting a broad range of cells, including those directly involved in immune responses. The systemic use of this cytokine led to a reduction in the number of micro-metastases in four syngeneic mouse models of cancer (282). In addition, tumor regressions and even cures rates were observed, when IL6 was administered in combination with sub-therapeutic doses of TNF (293). However, reports on IL6 as being a tumor-promoting agent are predominant in the scientific literature. IL6 has been reported to promote the growth of tumor cells in malignant melanoma, renal cell carcinoma multiple myeloma and cervical cancer. Increased serum IL6 concentrations in patients are associated with advanced tumor stages (e.g., prostate cancer, breast cancer, ovarian cancer, multiple myeloma, non-small cell lung carcinoma, colorectal cancer, renal cell carcinoma) and short survival (294). Therefore, preclinical and clinical studies are currently investigating anti-IL6 therapy as a potential anticancer strategy (294).

In view of the contrasting tumor-promoting and tumor-inhibitory functions attributed to the two cytokines, it would be important to learn whether IL1β and IL6 can be fused to antibodies and delivered to neoplastic sites, thus observing the effect of the corresponding biopharmaceuticals on the tumor mass. In this thesis, the expression and in vivo evaluation of IL1β- and IL6-based
immunocytokines is described. Therefore, both cytokines were fused both to the N- and to the C-terminus of the F8 antibody. Targeting performance of all four immunocytokines was evaluated by quantitative biodistribution experiments in 129/SvEv mice bearing F9 teratocarcinoma tumors. The best-performing immunocytokine formats were then used for therapy studies in the same immunogenic mouse model of cancer.

3.8.3 Interleukin 13 as payload

Interleukin-13 is a pleiotropic cytokine, mainly produced by activated Th2 cells and closely related to IL4. IL13 contributes to the regulation of IgE class switching, eosinophilic inflammation, mucus secretion, airway hyper-responsiveness, gastrointestinal parasite expulsion and tissue remodeling and fibrosis. It mediates a variety of different effects on many cell types including B cells, natural killer cells, endothelial cells and fibroblasts, while several cell types beside activated Th2 cells, such as natural killer T cells, dendritic cells, mast cells and macrophages, can produce IL13 (295, 296). Further, this cytokine has anti-inflammatory effects on monocytes, inhibiting the release of pro-inflammatory cytokines. Together with IL4 and IL10, IL13 contributes to the development of a Th2-dependent humoral response. However, unlike IL4, IL13 does not affect T cells due to the lack of expression of functional IL13 receptors by this cell population (297, 298). Additionally, some unique effector functions distinguish this cytokine from IL4. For example, IL4 inhibits IFNγ production in large granular lymphocytes and promotes a Th2 response while IL13 does not inhibit IFNγ production but can synergize with IL2 and can have, similar to IL12, a direct effect on IFNγ synthesis and may therefore promote also a cellular Th1 response (299). IL13 mediates signal transduction, like IL4, through Janus kinases and phosphorylation of STAT6 [Figure 17]. However, IL13 can also signal through IL13Rα2 in a STAT6-independent manner leading to the activation of the TGFβ1 promoter resulting in TGFβ production, inflammation and fibrosis. The primary receptor chains of IL13 are IL13Rα1 and IL13Rα2 but only IL13Rα2 chain binds IL13 with high affinity and is overexpressed in many human cancers (e.g. RCC,
SCC, Kaposi’s sarcoma, glioma) (300). The IL13 receptor does not include the common y chain and therefore some of the downstream IL4 signaling events are not induced by either IL4 or IL13 binding to the IL13 receptor.

IL13 can have profound effects on tumor cell growth and can interact directly with cancer cells, inhibiting their proliferation in a concentration-dependent manner (301-303). Mice injected with transfected IL13-secreting P815 mastocytoma cells rejected the tumors and developed a systemic long lasting antitumor immunity mainly by recruiting infiltrating neutrophils and macrophages. Antibody-based depletion of T-lymphocytes had shown that IL13-mediated tumor rejection was not mediated solely by T cells, but also requires an indirect nonspecific tumor defense mechanisms, such as the action of NK cells or the infiltration of neutrophils and macrophages in HeLa tumors (304). However, in weakly immunogenic 3LL lung carcinoma, IL13 expression did not induce long-term antitumor protection, while IFNγ did (305). Recently also direct IL13-mediated cell killing via induction of an apoptotic pathway in cancer cells has been proposed (306), making this cytokine an interesting payload for tumor targeted therapy.
Figure 17. IL4 and IL13 signal pathways. The primary receptor chains of IL13 are IL13Rα1 and IL13Rα2. IL13 mediates signal transduction, like IL4, through Janus kinases and phosphorylation of STAT6. The role of IL13Rα2 is not clear yet, this receptor exists as a soluble and a membrane bound version.

In this thesis, the production, characterization and antitumor effects mediated by the fusion of murine IL13 to the F8 antibody is described. The F8-IL13 fusion protein selectively localized to tumors in vivo and potently inhibited tumor growth in two syngeneic immunocompetent models of cancer.

3.8.4 Vascular endothelial growth inhibitor as payload

VEGI (also called TNFSF15 or TLA1) was discovered in 1999 (307) and is abundantly expressed in endothelial cells where it acts as autocrine factor to induce apoptosis. Its expression is inducible by TNF and IL1α and it binds to TNFRSF25 receptor and TNFRSF21/DR6, a decoy receptor. VEGI has been claimed to inhibit endothelial cell proliferation, making it an interesting payload for the F8 antibody mediated delivery to neovascular structures within the tumor mass. VEGI exists in three different isoforms of 174, 192 and 251 aa length. For this thesis the 192 aa murine isoform has been used.

Our group has described the in vitro characterization and comparative quantitative biodistribution analysis of 8 antibody-cytokine fusion proteins based on murine CD40L, TRAIL, FasL, VEGI (TLA1), LiGHT (HVEML), lymphotoxin alpha (LTα), lymphotoxin beta (LTβ) and lymphotoxin alpha2/beta1 (LTα1β2) (276). During my thesis I cloned and expressed the two TNF based immunocytokines F8-VEGI (192aa) and a truncated version termed F8-VEGItrunc (N-terminal 28aa absent) for cancer therapy.

The tumor necrosis factor superfamily consists of more than 20 membrane bound or secreted ligands with a common trimeric structure, which mediate a broad range of biological activities ranging from tumor cell apoptosis, sepsis and cachexia to the maintenance of lymphatic tissues and organogenesis. Each ligand specifically recognizes one or more cell surface receptors of the
corresponding TNF receptor (TNFR) family. Receptor binding triggers an intracellular signal cascade, which can result in cell death or in activation of inflammation (308-310). VEGI can activate NF-kappaB and MAP kinases.

Severe dose limiting toxicities of recombinant TNF observed in anticancer therapy studies (311) stimulated the development of several TNF superfamily based immunocytokines (312). The fusion of TNF to the L19 antibody is currently in clinical investigation for cancer therapy (104, 280, 313).

The experiments, which were performed in immunocompetent F9 tumor-bearing mice using radioiodinated protein preparations, aimed at assessing the cancer targeting properties of closely related fusion proteins, based on the F8 antibody in scFv format (314). The tumor-targeting properties of the F8 antibody (314) and of the corresponding fusion protein with murine TNF (104, 315) have previously been reported. Surprisingly, it was found that the seven new immunocytokines based on structurally related members of the TNF superfamily differed substantially in terms of biodistribution properties in tumor-bearing mice.

3.8.5 IL9 as payload

Interleukin-9 (IL9) has been discovered in 1988 (316, 317). It is a 14.2kDa monomeric soluble glycoprotein with pleiotropic functions (318) and belongs to the common gamma receptor cytokine family. Several other members of this family (IL2, IL4, IL7, IL15, and IL21) have already been investigated for cancer therapy. We focused our attention on murine interleukin-9 (mIL9) as a therapeutic payload, based on recent reports on its potent immune cell-driven anti-tumor activity (319, 320).

Most biopharmaceuticals, including antibodies, feature post-translational modifications such as N-linked glycans and therefore rely on mammalian cell expression systems (321). Antibodies, used in a human immunoglobulin G (IgG) format, contain structurally distinct N-linked glycans at conserved positions within the Fc region. Depending on the IgG-subtype, Fc-glycosylation has been
recognized to have a profound effect on the activation of immune cells (322, 323). Indeed, the first glycoengineered antibody product obinutuzumab (Gazyva®) has recently been approved for Non-Hodgkin lymphoma and diffuse large B-cell lymphoma. The impact of protein glycosylation on pharmacokinetics has been extensively studied for glycoprotein hormones, including the prominent examples of recombinant erythropoietin and its glycoengineered derivative Darbepoetin alfa (324). The introduction of additional N-glycosylation motifs into the peptide sequence of erythropoietin resulted in increased serum half-lives (325). Glycosylation also dictates the serum half-life of glycoprotein drugs, capable of neonatal Fc receptor-mediated recycling, as shown for the systemic TNF inhibitor Lenercept, a fusion protein consisting of an IgG1 Fc portion and the extracellular p55 TNF receptor domain (326). However, quantitative studies investigating the impact of protein glycosylation on disease-homing properties of therapeutic proteins are rare.

Exploiting their exquisite target selectivity and ability to localize at sites of disease, there is an emerging trend to monoclonal antibodies as pharmacodelivery vehicles, thus moving from intact antibodies towards armed antibody products (327, 328). The attachment of therapeutic payloads to a targeting antibody can be accomplished either by chemical conjugation in the case of small molecules or by genetic fusion of protein domains (328). The fusion of bioactive protein payloads (e.g. cytokines) may lead to additional O- or N-glycans in the resulting armed antibody when expressed in eukaryotic cell expression systems.

Most antibody-mediated pharmacodelivery approaches rely on extravasation of the biopharmaceutical product in order to diffuse into tissues and reach the site of disease. Glycans present on the therapeutic protein can modulate this process by different glycan-receptor interactions. For example, hepatocytes express the asialoglycoprotein receptor with specificity for non-sialylated proteins with terminally exposed galactose residues (329). Another receptor involved in glycoprotein homeostasis, primarily expressed by macrophages and dendritic cells, is the mannose receptor, recognizing terminal mannose or N-
acetylglucosamine (330). Certain glycan epitopes are also known to be immunogenic and can lead to anti-drug antibody responses in humans (331).

Our group has extensively worked on the production and in vivo characterization of armed antibody products directed against splice isoforms of extra-cellular matrix components (215, 267). The F8 antibody has been used for the pharmacodelivery of drugs, radionuclides and cytokines to various types of disease lesions (328, 332). Small bivalent antibody fragments without Fc portion, may be preferred for the delivery of highly potent payloads, as they are rapidly cleared from circulation while exhibiting favorable biodistribution profiles (327). This thesis describes that variations in N-linked glycan structures, present on the IL9 moiety of different F8-based diabody fusion protein preparations, led to dramatic changes in tumor targeting efficiencies, as revealed by quantitative biodistribution analysis.

3.9 Aim of the thesis

Armed antibodies are gaining increasing attention for their use in cancer therapy. The targeted delivery of drugs represents a promising strategy to improve the therapeutic window of bioactive payloads. A variety of effector moieties has already been explored for their use as payloads [Figure 6]. So far, there are only very few reports about the antibody mediated delivery of chemokines to the site of disease and there is no clinical data available for this novel class of armed antibodies. The aim of the first part of my thesis was to evaluate antibody chemokine fusion proteins for anticancer therapy. Therefore several chemokines with potential anticancer efficacy should be selected, cloned and produced as F8, specific for the EDA splice isoform of fibronectin, antibody fusion proteins. In particular, I focused my work on the following chemokines: CCL5, CCL17, CCL19, CCL20, CCL21, CXCL4, CXCL9, CXCL10, CXCL11 and a chimeric CXCL10/CXCL11 chemokine. From the literature it was already known that expressing chemokines was not trivial, therefore at first instance the aim was to produce this difficult-to-express proteins as antibody fusion proteins at acceptable levels and purity to investigate their in vitro and in vivo behavior. A
very crucial point in the development of novel therapeutic armed antibody products is the ability of the antibody to recognize its cognate antigen \textit{in vivo} with high affinity and to be able to specifically localize at the site of disease. This feature can be investigated by performing biodistribution studies with radiiodinated protein preparations, as regularly done in our group. Only if the antibody fusion protein shows high and selective accumulation at the site of disease, further investigation in preclinical disease models can be considered. The ultimate aim of the chemokine project was to figure out whether antibody chemokine fusion proteins could be expressed and would be able to target tumors \textit{in vivo}. In case of successful targeting, we would have to investigate whether they exhibit a therapeutic effect in murine models of cancer.

In the second part of my thesis, novel immunocytokines should be evaluated for their use as cancer therapeutics. To date, only a few cytokines are approved as anticancer drugs despite encouraging results in animal models. However, complete responses are rarely seen when cytokines are systemically administered and serious side effects can be observed \textit{in vivo} already at low doses, which prevent escalation to therapeutically effective concentrations. The targeted delivery of these immunostimulatory proteins can lead to an improved therapeutic index and to more potent therapeutic efficacy with acceptable toxicities. New immunocytokines based on Interleukin 1\(\beta\), Interleukin 6, Interleukin 9, Interleukin 13 and the TNF family member vascular endothelial growth inhibitor should be cloned, expressed and purified to homogeneity and their \textit{in vivo} targeting performance should be investigated fused to the F8 antibody. Immunocytokines exhibiting specific accumulation at the tumor site should be further investigated for their therapeutic potential in murine models of cancer as single agents and eventually in combination therapy modalities. Whenever possible, the mechanism of action of the new products should be studied by immunofluorescence microscopy of leukocyte infiltration at the tumor site, measurement of cytokine levels in the tumors and \textit{in vivo} depletion of lymphocytes.
4 Results

4.1 Evaluation of antibody-chemokine fusion proteins for tumor-targeting applications

This section has been adapted from the following publication:


4.1.1 Results

Since chemokines typically require an intact N-terminus for biological activity, we fused ten murine chemokines at the N-terminal extremity of the F8 antibody in diabody format (270) and expressed the corresponding immunochemokines in CHO-S cells using a transient gene expression methodology (333) [Figure 18a]. The chemokines CCL5, CCL17, CCL19, CCL20, CCL21, CXCL4, CXCL9, CXCL10, CXCL11 and the chimeric chemokine ITIP were chosen as possible payloads, on the basis of previous reports on their potential anticancer activity. As positive control for tumor targeting applications, the F8 antibody was used in SIP format [Figure 18b]. This recombinant antibody format has previously been shown to display biodistribution properties in tumor-bearing mice similar to the ones of F8 in diabody format (270). Furthermore, the homobivalent SIP(F8) antibody has a molecular weight of about 80 kDa, which is comparable to the one of non-covalent homobivalent immunochemokines in diabody format [Figure 18b]. Unfortunately, the yield and purity of all ten immunochemokines after protein A purification was unsatisfactory, in sharp contrast to our previous experience with F8-based immunocytokines (71) and with SIP(F8) [Figure 18c].
Figure 18. Cloning and expression of chemokine-F8 fusion proteins. The F8 antibody is specific to EDA of human and murine fibronectin. (a) Schematic representation of the expression vectors. (b) Schematic representation of chemokine-F8(diabody), chemokine-F8(scFv) and SIP(F8) proteins. (c) SDS-PAGE analysis of protein A purified chemokine-F8(diabody) test expressions and the SIP(F8) antibody used as control in biodistribution studies. M = molecular marker (kDa); NR = non reducing; R = reducing.
In transient gene expression experiments, CXCL10-F8 exhibited the presence of a fragment with the size of the F8 diabody, suggesting that proteolytic degradation had taken place [Figure 18c]. Since CXCL10 is known to bind to heparin, we repeated the expression of CXCL10-F8 adding heparin to the expression medium, in order to protect the fusion protein from the action of proteases. In this case, after protein A purification, 2 mg of protein could be recovered per liter of medium, but only ~ 30 % showed the correct molecular weight of the fusion protein, as revealed by SDS-PAGE and by MS analysis (data not shown). An attempt to recover the intact CXCL10-F8 (diabody) fusion protein by ion exchange chromatography led to unsatisfactory purity and yields of the desired fusion product which were not compatible with the execution of in vivo experiments. Therefore another fusion protein with F8 antibody in scFv format was cloned and expressed in CHO-S cells with heparin added to the medium [Figure 21a]. In this format ~ 0.4 mg/L of the desired product could be recovered after ion exchange purification and dialysis against PBS. A Western blot analysis of the CXCL10-F8 expression showed that the fusion protein is stable in the culture supernatant and that the addition of heparin can prevent the formation of higher molecular weight disulfide-bonded oligomers [Figure 19a]. In a small scale test-expression the fusion protein could not be observed in the flow-through and wash fractions and did not elute from a protein A column, suggesting that the immunochemokine may aggregate and/or precipitate when reaching high local concentrations in the affinity chromatography procedure or may be degraded during purification procedure [Figure 19b].
Figure 19. (a) Western blot analysis of non-processed CXCL10-F8(scFv) showing the expression at day 1 (D1), day 3 (D3), day 4 (D4); - = addition of heparin (100 mg/l), + = no heparin added to the expression medium. Detection was done with biotinylated anti-mCXCL10 antibody and streptavidin-HRP. (b) Western blot analysis of CXCL10-F8(scFv) purification showing the expression (supernatant) at day 1 (D1), day 2 (D2), day 3 (D3), flow through (FT), wash 1 (W1), wash 2 (W2) and the elution (E) after protein A affinity chromatography. Detection was done with biotinylated anti-mCXCL10 antibody and streptavidin-HRP.

Immunochemokines based on CC-Chemokines CCL19, CCL20 and CCL21 displayed the presence of the correct molecular species in transient gene expression and protein A purification, although with extensive contaminations and/or proteolytic degradation [Figure 18c]. We repeated the experiment in larger scale (up to 1 liter) and shorter (3 days) expression times. After protein A and IEC purification [Figure 20d], all three immunochemokines revealed a band of the expected size in SDS-PAGE analysis [Figure 20a], but also the presence of glycosylation for CCL19-F8, which was confirmed by mass spectrometric analysis [Figure 22]. In addition, CCL19-F8 and CCL21-F8 were characterized by native PAGE analysis, revealing the presence of non-covalent higher order oligomers [Figure 20b].

Attempts to characterize the proteins on Superdex S200 gel-filtration columns failed, in analogy to our previous experience with recombinant chemokines (e.g., CCL5; data not shown). However, the fusion proteins could be recovered from shorter columns, such as PD10 desalting columns (GE Healthcare) (e.g. after
radiolabeling). Radioiodinated preparations of the four immunochemokines revealed a full retention of immunoreactivity, as assessed by affinity chromatography, which was comparable to the one of the parental antibody in SIP format [Figure 20c]. BIAcore analysis of all CC-immunochemokines [Figure 20e] and the CXC-immunochemokine [Figure 21e] on EDA-coated chips confirmed the ability of the fusion proteins to recognize their antigen with kinetic profiles which were comparable to the ones of the parental antibody in diabody format (270). The chemotactic activity of CCL19- and CCL21-based immunochemokines was confirmed in a DC transwell migration assay, in which the fusion proteins exhibited an activity which was comparable to the one of recombinant murine CCL21 from a commercial source [Figure 20f]. All four immunochemokines were able to specifically bind to the cognate antigen EDA and did not react with structurally-related proteins (e.g., the BCD recombinant fragment of tenascin-C, containing three Type-III fibronectin homology repeats) as evidenced by ELISA experiments performed at various concentrations [Figure 20g and Figure 21d].
Figure 20. Expression and characterization of CCL20-F8 = (1); CCL21-F8 = (2); CCL21-F8 = (3). (a) SDS-PAGE analysis of protein A and ion exchange chromatography purified preparations of (1), (2) and (3). (b) Native PAGE analysis of (1) and (3). (c) Immunoreactivity (%) of 125I-labeled preparations of CCL19-F8, CCL20-F8, CCL21-F8 and SIP(F8) in EDA affinity chromatography. Values expressed as percentage of eluted radiodinated protein after protein A affinity chromatography of the total amount applied on the resin. (d) Ion exchange chromatography of CCL19-F8, CCL20-F8 and CCL21-F8 in 20 mM NaP buffer, pH 6.8; Adsorption at 280 nm is depicted as black line; conductivity is depicted as grey dashed line. (e) BIACore analysis of 1 μM (1), 150 nM (2), and 250 nM (3). (f) ELISA on EDA of CCL19-F8, CCL20-F8, and CCL21-F8. (g) Absorbance at 450 nm for ELISA on Tn-C with different concentrations of CCL19-F8, CCL20-F8, and CCL21-F8.
(2) and 100 nM (3) on an EDA-coated sensor chip. (f) CCL19 and CCL21 chemotactic assay with LPS-matured dendritic cells in transwell plates (3-5μm, Corning®). CCL19-F8 and CCL21-F8 were added at 100 nM concentration to the lower transwell chamber, while cells were added to the upper transwell chamber. The number of migrated cells could be monitored by fluorescence-activated cell sorting (Canto) due to their YFP-expression. Recombinant murine CCL21 served as positive control. Values expressed as percentage migrated cells in comparison to the cells present in the 100% control well. (g) Comparative ELISA of CCL19-F8, CCL20-F8 and CCL21-F8 on EDA and Tenascin-C coated surfaces at concentrations of 250 nM, 50 nM and 10 nM. Pos. = 250 nM SIP(F8) positive control; neg. = PBS buffer negative control.

Figure 21. CXCL10-F8(scFv) expression, purification and in vivo characterization. (a) SDS-PAGE analysis of CXCL10-F8(scFv) with addition of heparin. (b) Ion exchange chromatography purification of CXCL10-F8(scFv) in 20 mM NaP buffer, pH 6.8, absorbance (280 nm) black line, conductivity (mS/cm) dashed, grey line. Fraction
numbers are indicated below.  

(c) SDS-PAGE analysis of ion exchange chromatography fractions, M = molecular marker (kDa), numbers correspond to the fractions numbers in (b), fraction 19 = CXCL10-F8(scFv).  

(d) ELISA of CXCL10-F8(scFv) on immobilized EDA antigen at concentrations of 400 nM, 100 nM and 25 nM, detection of the chemokine moiety was done with a biotinylated anti-mCXCL10 antibody and HRP-conjugated streptavidin, neg. 1 = no primary antibody, neg. 2 = no secondary antibody, neg. 3 = no primary and secondary antibody.  

(e) BIAcore analysis of 400 nM CXCL10-F8(scFv) on an EDA-coated sensor chip.  

(f) Biodistribution study of radioiodinated CXCL10-F8(scFv). Immunocompetent 129/SvEv mice bearing subcutaneous F9 teratocarcinoma tumors were injected i.v. with 10 μg radiolabeled protein (n = 5). Mice were sacrificed after 24 h. Organs were excised and radioactivity counted, expressing results as percent of injected dose per gram of tissue (%ID/g ± SE).  

(g) Immunocompetent 129/SvEv mice (n = 4) bearing subcutaneous F9 teratocarcinoma tumors were injected daily (i.t.) with 10 μg CXCL10-F8(scFv) (dissolved in PBS) or PBS as control on five consecutive days (black arrows). Tumor growth over time was monitored.

**Figure 22.** Mass spectrometry analysis of CCL5-F8 = (1), CCL19-F8 = (2), CCL20-F8 = (3), CCL21-F8 = (4) and CXCL10-F8(scFv) = (5) fusion proteins. Calculated masses: 33503.6 Da for (1), 34844.2 Da for (2), 33584.9 Da for (3), 37682.4 Da for (4) and 34872.5 Da for (5).

Radioiodinated preparations of SIP(F8), CCL19-F8, CCL20-F8, CCL21-F8 and CXCL10-F8(scFv) were injected (10 μg and 6-12 μCi per mouse) into the tail vein
of immunocompetent mice bearing subcutaneously-grafted murine F9 tumors. A biodistribution analysis, performed 24h after injection, revealed that the parental SIP(F8) antibody was able to selectively localize at the tumor site, with 10% injected dose per gram (%ID/g) in the tumor and a tumor to blood ratio of 29 to 1 at this time point. By contrast, all four immunochemokines failed to preferentially accumulate in the neoplastic lesion [Figure 21f and Figure 23a]. Analysis of biodistribution of CCL19-F8 20 min after injection revealed that the majority of the fusion protein was in the liver, suggesting the presence of an active hepatobiliary excretion process [Figure 23b]. Interestingly, pre-administration of 30 µg of unlabeled CCL19-F8 prior to the administration of radiolabeled CCL19-F8 (10 µg per mouse) led to a partial rescue of tumor targeting properties, with 0.5 %ID/g in the tumor at 24 h and a tumor to blood ratio of 5 [Figure 23c].

![Figure 23](image)

**Figure 23.** Biodistribution studies of CCL-chemokine-F8 fusion proteins. (a) Biodistribution studies of radioiodinated preparations of CCL19-F8, CCL20-F8 and CCL21-F8 compared to SIP(F8). 129/SvEv mice bearing subcutaneous F9 teratocarcinoma tumors were injected i.v. with 10 µg radiolabeled protein.
Immunocompetent 129Sv mice injected with $^{125}$I-SIP (F8) (black bar, $n = 5$) or with radioiodinated chemokine-F8 preparations (1-3) (gray bars, $n = 5$). Mice were sacrificed after 24 hours. Organs were excised and radioactivity counted, expressing results as percent of injected dose per gram of tissue (%ID/g ± SE). (b) 129/SvEv mice bearing subcutaneous F9 teratocarcinoma were injected i.v. with 10 μg radiolabeled CCL19-F8. Mice were sacrificed after 20 min and organs were excised and radioactivity counted (c) 129/SvEv mice bearing subcutaneous F9 teratocarcinoma were injected (i.v.) with 30 μg unlabeled CCL19-F8 followed (15-20 min later) by 10 μg of radioiodinated CCL19-F8 (light grey). Biodistribution of 10 μg of radioiodinated CCL19-F8 is depicted as comparison (dark, grey). Mice were sacrificed after 24 h, organs were excised and radioactivity counted.

The expression of CCL5-F8 was very weak and the available amount of protein was not sufficient for the entire characterization of this fusion protein [Figure 24]. The purity after protein A chromatography was acceptable and the fusion protein was able to bind to its cognate antigen, but also this immunochemokine did not reach its target antigen in vivo.

**Figure 24.** (a) SDS-PAGE analysis of CCL5-F8 expressed in CHO-S cells. (b) ELISA of CCL5-F8 (displayed in grey) and diabody(F8) (displayed in black) on immobilized EDA antigen at concentrations between 200 nM and 0.4 nM. (c) Biodistribution study of radioiodinated CCL5-F8. Immunocompetent 129Sv mice bearing subcutaneous F9 teratocarcinoma were injected i.v. with 10 μg radiolabeled protein ($n = 5$). Mice were sacrificed after 24 h. Organs were excised and radioactivity counted, expressing results...
as percent of injected dose per gram of tissue (%ID/g ± SE). (d) Mass spectrometry of CCL5-F8, confirming the correct size.

Despite the failure of selective tumor accumulation, CXCL10-F8(scFv) was tested in vivo in F9 teratocarcinoma bearing 129/SvEv mice. Four injections of ten micrograms of CXCL10-F8(scFv) were administered i.t. and the tumor growth was compared to a control group injected with PBS. A modest but significant (p = 0.0404; n = 4 mice per group) tumor growth inhibition could be observed with CXCL10-F8(scFv), compared to mice treated with saline [Figure 21g]. Ex vivo detection of tumor infiltrating cells was done on cryostat sections (10 μm) of tumors taken three days after the last injection, revealing an increased infiltration of Natural Killer and CD45R positive cells in CXCL10-F8 treated tumors compared to tumors treated with saline [Figure 25].

![Figure 25. (a) Ex vivo immunofluorescence analysis of tumor infiltrating cells on F9 tumor sections following injections of PBS or CXCL10-F8(scFv), respectively. Red = anti CD31. Scale bars: 100 μm.](image)

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**Figure 25. (a)** Ex vivo immunofluorescence analysis of tumor infiltrating cells on F9 tumor sections following injections of PBS or CXCL10-F8(scFv), respectively. Red = anti CD31. Scale bars: 100 μm.
4.1.2 Discussion

This article presents the first comprehensive characterization of several antibody-chemokine fusions for tumor targeting applications. Ten immunocytokines were cloned and expression was attempted in mammalian cells, such as CHO-S, HEK293 and Sf9 cells. Four immunochemokines could be expressed in CHO-S cells with yields > 1 mg/L and purified to an acceptable degree of homogeneity. Expression of the fusion proteins in HEK293 and Sf21 cells (data not shown) was inferior to expression in CHO-S cells. Yields and downstream processing of immunochemokines were substantially worse, compared to the use of cytokine payloads (which can easily be appended at the N- or C-terminus of antibody fragments in scFv or diabody format).

Importantly, fusion to chemokines abrogated the tumor homing properties of the F8 antibody and no preferential accumulation in the neoplastic mass could be observed following intravenous administration. In the case of CXCL10-F8, the addition of heparin to the culture medium facilitated the isolation of an intact fusion protein. Immunochemokines appear to be proteolytically unstable during production in mammalian cells. Post-translational processing of chemokines by various proteases has been reported in in vivo studies (334, 335). However, Western blot analysis of CXCL10-F8 culture supernatants confirmed that the fusion protein is stable in the culture medium and that addition of heparin improves the biochemical purity of the immunochemokine but also that the fusion protein could not be observed in the flow-through and wash fractions and did not elute from a protein A column (in a low volume test-expression), suggesting that it may aggregate and/or precipitate when reaching high local concentrations in the affinity chromatography procedure or degrade during purification procedure.

Immunochemokines could be analyzed by SDS-PAGE, but their size-exclusion chromatographic characterization proved to be difficult, due to the interaction of the chemokine moiety with standard Superdex 200 size exclusion dextran matrix. We had observed similar problems with recombinant chemokines (e.g. CCL5; data not published). Size exclusion chromatography profiles are typically not shown in publications which describe the production and biological function of
chemokines. However, chemokines can be purified by reversed phase, ion exchange and heparin affinity chromatography methods (336, 337).

For biodistribution studies, radioiodinated immunochemokine preparations were found to be immunoreactive when tested by affinity chromatography on antigen columns, but none was found to display a preferential accumulation at the tumor site following intravenous administration. Unfavorable biophysical properties, such as a high pl-value and glycosylation (338-340), as well as trapping by high expression of the cognate receptor in a tissue (e.g. CXCR3 expression in the liver) or decoy receptors such as D6, DARC or CCX-CKR lacking classical signaling activities after chemokine binding (341, 342), may prevent an efficient tumor targeting process. Chemokine receptor expression in the liver or clearance of the immunochemokines via the hepatobiliary route may explain the high liver uptake 20 min after intravenous administration of CCL19-F8. Moreover, tumor to organ ratios improved by prior application of unlabeled CCL19-F8 in biodistribution experiments suggesting that both a receptor-mediated trapping mechanism and a fast clearance of the fusion protein may hinder an efficient tumor homing process.

The tumor targeting performance of antibodies specific to splice isoforms of fibronectin (e.g., L19 or F8) (313, 314) are dramatically different, when these antibodies are fused to cytokines or chemokines. Most L19- and F8-based immunocytokines exhibit a preferential tumor accumulation in preclinical models of cancer (277), rendering the findings with immunochemokines even more surprising and unexpected. However, certain payloads have previously been reported to completely abrogate the tumor-targeting potential of the parental antibody in mouse models of cancer (343, 344).

It remains to be investigated whether the choice of alternative antibody formats (e.g. IgG) may improve biodistribution results. In one published report, the IgG-based LEC/chTNT-3 immunochemokine was found to accumulate with 2.4 %ID/g at 12 and 24 hours post injection in MAD109 tumors (345), while the parental antibody exhibited a tumor uptake of 13.5 %ID/g on day 3 after injection in MAD109 lung adenocarcinoma bearing BALB/c mice (346). This protein
mediated cancer cures, when used in combination with CD25⁺ depletion (347). However, CD25⁺ depletion itself led to a significant reduction of tumor growth and a recently published non-targeted LEC fusion protein consisting of human LEC and soluble Fc (LEC-Fc) showed similar tumor regression as the targeted LEC fusion protein (LEC/chTNT-3) (348).

At this moment in time, it is not known whether immunochemokines may establish a concentration gradient in vivo, which is permissive and conducing for the migration of leukocytes. It is also not known whether immunochemokines can alter the vasculature at the site of disease, or regulate the function of immune cells, affecting tumor fibrosis, necrosis and immune regulation. The fusion proteins tested in this article exhibited a chemotactic activity in vitro experiments, which was comparable to the one of a commercial recombinant chemokine. However, it will be possible to study the full therapeutic potential of immunochemokines only when in vivo pharmacodelivery problems can be solved. The target antigen for the F8 antibody is located in the sub-endothelial extracellular matrix of tumor blood vessels (86, 314, 349, 350). Thus, in order to achieve preferential tumor localization, F8-based fusion proteins need to cross the endothelial cell layer and bind to the cognate antigen. The pI values of the 10 chemokines which we fused to the F8 antibody ranged between 8.76 (CCL5) and 10.62 (CXCL9). Our group has previously reported that highly positively charged (e.g., murine VEGF-164(351)) or negatively charged (e.g., calmodulin (338)) payloads can abrogate the tumor-targeting properties of antibodies, probably because the many charges inhibit the extravasation process.

Immunochemokines capable of selective in vivo targeting of tumor blood vessels may alter the expression of vascular cell adhesion molecules in the lumen to enable the migration of responsive cells into the tumor mass. Furthermore, chemokines with proven antiangiogenic functions (e.g. CXCL10 (352)) should be able to exert direct effects on endothelial cells potentially leading to fibrosis and tumor necrosis. The characterization of protein production and the results of quantitative biodistribution studies, as reported in this article, should facilitate future research efforts in the field of immunochemokines, while critically
increasing awareness about the impact which payloads may have on the efficiency of antibody-based pharmacodelivery strategies.

4.1.3 Material and methods

4.1.3.1 Cloning of chemokine-F8 fusion proteins

Cloning of the CCL5-F8 immunochemokine is described here. Cloning of all other chemokine-F8 constructs was performed in a similar fashion and all the corresponding primer sequences can be found in the appendix. A murine CCL5 cDNA clone (Sinobiologicals Inc.; Beijing, China) was amplified by polymerase chain reaction (PCR) using primers CCL5-SIP_fw (5’-CCTGTTCCTCGCTGCTGGCTACAGGTGTGCACTCGTCACCATATGGCTCGGACACCACCCTC-3’), that appends a NheI restriction site and part of the signal peptide (SIP), and CCL5-linker_rev (5’-CCGCCACACCTCGCTGAACCGCTCCACCAGCTCATCTCCAATAAGTGTTGATGTG-3’), that appends part of the 14-amino acid GGGGS linker peptide between the chemokine and the antibody moiety. The F8 diabody gene was PCR amplified using the primers Linker-F8_fw (5’-TCAGGCGGAGGTGGCTCTGGCGGTGGCGAGAGGTGCACTGATCCTCCTGTTCCTCGTCGCTGTGGC-3’) that appends a part of the GGGGS-linker peptide (including a 23 base pair overlap) and F8-NOT_I_rev (5’-TTTTCTTTTGGCCGCTCATTATTTGATTTCACCTGTCACCTGTCATCGCTGGC-3’) which appends two stop codons as well as a NotI restriction site. The murine CCL5-linker and linker-diabody(F8) DNA fragments were PCR-assembled using primers Nhel_Sip_fw (5’-CCCAGCTAGCGTGACCGATGGGGCTGGAGTGATCCTCCTCGCTGACCTGTCGCTGGC-3’), containing a Nhel restriction site followed by the N-terminal part of the SIP sequence (21 base pair overlap with CCL5-SIP_fw), and F8-NOT_I_rev. The PCR-assembled full length CCL5-F8 gene was double digested with Nhel/NotI restriction endonucleases and cloned into the mammalian cell expression vector pcDNA3.1 (+) (Invitrogen). CXCL10-F8 was cloned also in scFv(F8) format according to the
same procedure but with a scFv(F8) template instead of the diabody(F8) template (314).

The chimeric chemokine termed ITIP, was assembled using the C-terminal part of the IFN-inducible protein 10 (IP10)/CXCL10 and N-terminal part of the IFN-inducible T cell alpha chemoattractant (ITAC)/CXCL11 sequence (353). Further cloning steps were performed as described above.

4.1.3.2 Cell culture / cell lines and animals

CHO-S cells (ATCC) in suspension were cultured in shaker incubators using PowerCHO-2CD medium (Lonza, Switzerland) supplemented with 8 mM Ultraglutamine, HT supplement (Lonza, Switzerland) and antibiotics. The murine teratocarcinoma F9 cell line was used for biodistribution and therapy studies (CRL-1720, ATCC). Cells were grown in 0.1% gelatin coated tissue flasks in DMEM (Gibco) supplemented with 10% fetal calf serum (FCS). Cells were incubated at 37 °C and 5% CO₂. Tumor cells were implanted subcutaneously in the flank of twelve weeks old female 129/SvEv mice (Charles River, Germany) using 25 x 10⁶ cells. Experiments were performed under a project license granted by the Veterinaeramt des Kantons Zürich, Switzerland (Bew. Nr. 42/2012).

4.1.3.3 Transient gene expression and characterization

All fusion proteins used in this study were expressed using transient gene expression as previously described (343) with the following modifications: Expression time was reduced to three days and in case of CXCL10-F8(scFv) expression, heparin was added to the medium (0.1 mg/ml). Proteins were purified by protein A affinity chromatography followed by ion exchange chromatography and analyzed by SDS-PAGE, ELISA and surface plasmon resonance (BIAcore). For CCL19-F8, CCL20-F8 and CCL21-F8 ELISA experiments, biotinylated EDA antigen or the biotinylated BCD-domain of Tenascin-C was coated (10⁻⁷ M) on
streptavidin stripes (Roche, Switzerland) and different concentrations of the fusion proteins were applied. Detection was done with protein A horseradish peroxidase (HRP) conjugate (Ge Healthcare) and the chromogenic substrate POD (Roche) at 450 nm. The F8 antibody in small immunoprotein format (SIP(F8)) at a concentration of 250 nM served as positive control. In case of CXCL10-F8(scFv), unbiotinylated EDA antigen (10^{-6} M) was coated on a Nunc MaxiSorp flat bottom 96 well plate (Thermo Scientific) and CXCL10-F8(scFv) was added at different concentrations. Detection was done with a biotinylated rabbit anti-mCXCL10 antibody (0.01 mg/mL, Peprotech) followed by streptavidin-HRP (GE Healthcare). BIAcore measurements were performed on an EDA antigen-coated sensor chip.

None of the fusion proteins could be recovered after size exclusion chromatography in phosphate buffered saline (PBS) on a Superdex 200 10/300 GL column (GE Healthcare). All fusion proteins could be purified to homogeneity by ion exchange chromatography on a 1 ml ATOLL MiniChrom column in a 20 mM NaP-buffer at pH 6.8. Elution was done with a salt gradient (one step for CCL19-F8, CCL21-F8 and CXCL10-F8; 2-step gradient for CCL20-F8 purification) of 20 mM NaP buffer with 1 M NaCl. Samples were then dialyzed in PBS over night at 4 °C.

4.1.3.4 Quantitative biodistribution studies

The in vivo targeting performance of chemokine-F8 fusion proteins was evaluated by biodistribution analysis with radioiodinated protein preparations as described before (344). Six to seven days after tumor implantation, mice were grouped and injected into the lateral tail vein with 10 µg of the radio labeled chemokine-F8 fusion proteins. Mice were sacrificed 24 hrs after injection, organs were excised, weighed and radioactivity was measured Packard Cobra γ-counter). Values are given in percentage of injected dose per gram of tissue (%ID/g +/- standard error). With CCL19-F8 another two biodistribution studies were performed. In one experiment mice were sacrificed already 20 min after injection of the
radioiodinated protein and in the 2nd experiment 30 µg of unlabeled (cold) fusion protein was injected prior to 10 µg of the radiolabeled protein and the mice were sacrificed after 24 hrs.

4.1.3.5 Intratumoral therapy studies

When tumors were clearly palpable, mice were randomly grouped and received on five consecutive days intratumoral (i.t.) injections of 10 µg CXCL10-F8(scFv) in PBS buffer, which was also used for injecting the negative control group. Weight and tumor volume (volume = length x width² x 0.5) of all mice was monitored daily.

4.1.3.6 CCL19-F8 and CCL21-F8 transwell migration assay

To test the chemotactic activity of CCL19 and CCL21, a DC chemotaxis assay was performed with lipopolysaccharide-matured murine dendritic cells in transwell plates (3-5µm, Corning®). CCL19-F8 and CCL21-F8 were added at 100 nM concentration to the lower transwell chamber, while cells were added to the upper transwell chamber. The number of migrated cells is expressed as % migrated cells in comparison to DCs present in the 100% control well. Cell numbers could easily be monitored by fluorescence-activated cell sorting (Canto) due to their YFP-expression. Recombinant murine CCL21 served as positive control. All the material for this assay was kindly provided by Dr. Alvaro Teijeira Sanchez (ETH Zurich).

4.1.3.7 Immunofluorescence studies of CXCL10-F8(scFv) treated tumors

Ex vivo detection of tumor infiltrating cells after intratumoral application of CXCL10-F8(scFv) was done with tumors taken three days after the last injection.
Tumors were excised, embedded in cryoembedding medium (Thermo Scientific) and cryostat sections (10 μm) were stained using the following antibodies: CD4 (BioXCell), CD8 (BioXcell), F4/80 (Abcam), CD3e (eBioscience), CD45 (BD Biosciences), CD45R (eBioscience), Asialo GM1 (Wako Pure Chemical Industries), Foxp3 (eBioscience) and CD31 (Santa Cruz Biotechnology). Detection was done with AlexaFluor488 and AlexaFluor594 conjugated secondary antibodies (Invitrogen). Slides were analyzed with an Axioskop2 mot plus microscope (Zeiss). Scale bars: 100 μm.
4.2 Tumor-targeting properties of novel immunocytokines based on murine IL1beta and IL6

This Section has been adapted from the following publication:


4.2.1 Results

The published structures of the receptor-cytokine complexes revealed that both termini of IL1β and of IL6 were available for coupling to the tumor targeting F8 antibody. Therefore, each cytokine was fused with a flexible 14 amino acid linker to either the N-terminal or the C-terminal extremity of the recombinant F8 antibody in noncovalent homodimeric diabody format. The four F8-based immunocytokines were denominated F8-IL1β, IL1β-F8, F8-IL6 and IL6-F8, in order to represent the N- or C-terminal position of the cytokine. In addition, the KSF antibody in diabody format, specific to hen egg lysozyme, was fused to IL1β and IL6, serving as negative control of irrelevant specificity in the mouse (344).

The immunocytokine genes were PCR-assembled, cloned into a mammalian expression vector, expressed in stably transfected CHO-S cells and purified to homogeneity [Figure 26a/b and Figure 27a/b]. All fusion proteins could be expressed with good yields (up to 10 mg/L) and were highly pure (> 95%) after a single protein-A affinity chromatography step. SDS-PAGE gel analysis revealed the existence of a small covalently linked dimer fraction for IL1β-based immunocytokines [in Figure 27 only barely visible and only for KSF-IL1β] due to an unpaired, poorly accessible cysteine in the murine IL1β protein (354). Further, IL1β-F8 shows a slightly different appearance (two bands) on the SDS-PAGE gel, compared to F8-IL1β and control proteins which migrated as a single band. All fusion proteins exhibited excellent biochemical properties in size-exclusion chromatography, ELISA assays and surface plasmon resonance studies [Figure 26c-e and Figure 27c-e]. Cytokine activities were assessed using cell proliferations assays with cytokine-dependent cell lines [Figure 26f and Figure
Furthermore, radioiodinated preparations of the four immunocytokines revealed retention of immunoreactivity, as assessed by affinity chromatography procedures (data not shown).

Figure 26. Cloning, expression and in vitro characterization of non-covalent dimers of IL6-F8 and F8-IL6 fusion proteins. (a) Schematic representation of the expression vectors and the corresponding domain assembly schemes. (b) SDS-PAGE analysis of F8-IL6, IL6-F8 and KSF-IL6; M = molecular marker; NR = non-reducing conditions; R = reducing conditions. (c) Size exclusion analysis of F8-IL6, IL6-F8 and KSF-IL6. (d) ELISA binding assay performed on EDA-antigen coated wells; proteins were applied at 1 μM concentration. (e) Surface plasmon resonance analysis on an EDA-coated sensor chip. Left: F8-IL6 (1 μM, 125 nM and 62.5 nM); right: IL6-F8 (500 nM, 62.5 nM and 31.25 nM). (f) IL6 bioactivity assay performed with F8-IL6, IL6-F8, KSF-IL6 and recombinant human IL6 as positive control on IL6-responsive B9 cells (30 000 cells/well). EC50: F8IL6 (0.48 ng/μl), IL6-F8 (0.16 ng/μl), KSF-IL6 (0.02 ng/μl), hIL6 (3.1 ng/μl).
Figure 27. Cloning, expression and in vitro characterization of non-covalent dimers of IL1β-F8 and F8-IL1β fusion proteins. (a) Schematic representation of the expression vectors and the corresponding domain assembly schemes. (b) SDS-PAGE analysis of F8-IL1β, IL1β-F8 and KSF-IL1β; M = molecular marker; NR = non-reducing conditions; R = reducing conditions. (c) Size exclusion analysis of F8-IL1β, IL1β-F8 and KSF-IL1β. (d) ELISA binding assay performed on EDA-antigen coated wells; proteins were applied at 1 μM concentration. (e) Surface plasmon resonance analysis on an EDA-coated sensor chip. F8-IL1β (left) and IL1β-F8 (right) were measured at 3 different concentrations (1 μM, 125 nM and 62.5 nM). (f) IL1β activity assay performed with F8-IL1β, IL1β-F8, KSF-IL1β and TIB-224 cells (30000 cells/well). As negative control culture medium without any IL1β was used. EC50: F8IL1β (55.9 pg/μl), IL1β-F8 (317 pg/μl), KSF-IL1β (89 pg/μl).

Radioiodinated preparations of F8-IL1β, IL1β-F8, F8-IL6 and IL6-F8 were injected into the tail vein of immunocompetent 129/SvEv mice bearing subcutaneously-grafted murine F9 tumors (IL6-based products: 10 μg per mouse; IL1β-based immunocytokines: 2.5 μg per mouse). A biodistribution analysis performed 24h after injection, revealed differences in the tumor targeting
performance not only between the two different payloads, but also between the orientations of the two cytokines [Figure 28a/b]. IL1β-based immunocytokines exhibited a slow clearance and a relatively high accumulation in most of the measured organs. Tumor uptakes of 3.39 % (N-terminal fusion protein) and 5.8 % (C-terminal fusion protein) injected dose per gram (%ID/g) and tumor to blood ratios of 1 and 7.1 respectively were measured. IL6-based immunocytokines showed generally better profiles with low organ values, tumor uptakes of 2.28 % (N-terminal fusion) and 4.13 %ID/g (C-terminal fusion) and tumor to blood ratios of 13.4 and 21.7. Due to better biodistribution performance of the C-terminal fusion proteins, subsequent therapy studies in the same model of cancer were performed with F8-IL1β and F8-IL6.

![Graphs showing biodistribution study](image)

**Figure 28.** Quantitative biodistribution study of $^{125}$I-labeled preparations of (a) F8-IL6 (black) and IL6-F8 (grey) and (b) F8-IL1β (black) and IL1β-F8 (grey) fusion proteins in subcutaneous F9 teratocarcinoma bearing 129/SvEv mice. Animals were sacrificed after
24 h, organs were excised and radioactivity counted, expressing results as percent of injected dose per gram of tissue (%ID/g ± SE).

As literature reports that IL1β can lead to severe toxicity already when used at sub-microgram doses, a dose-finding study was performed in Balb/c mice [Figure 29a/b]. Different concentrations of IL1β and IL6 were administered and the body weight was monitored as a measure for toxicity, leading to the determination a maximum tolerated dose of 5 µg of F8-IL1β per intravenous injection. F8-IL6 could be given repeatedly up to 100 µg (maximum dose administered) per injection, without any detectable sign of toxicity.

**Figure 29.** (a) F8-IL1β dose-finding study in Balb/c mice, concentrations between 2.5 µg and 30 µg were injected and the body weight was monitored. (d) F8-IL6 dose-finding study in Balb/c mice, concentrations between 10 µg and 100 µg were injected and the body weight was monitored.
Therapy studies in F9 tumor-bearing 129/SvEV mice were performed with three injections of 5 µg F8-IL1β or 100 µg F8-IL6 (every 72 h), starting at an average tumor size of ~100 mm³. Both, F8-IL1β and F8-IL6, exhibited only a modest (<50%) reduction of tumor growth rate [Figure 30a] and only modest increase in survival. In case of IL1β-based immunocytokines there is a significant (**, p = 0.0047) shift between the specifically targeted (F8-IL1β) and the control (KSF-IL1β) fusion protein from day 13 on. However, the weight loss accompanying therapy with 5 µg F8-IL1β per injection did not allow escalation to higher doses of this tumor targeted cytokine [Figure 30b]. However, since there were no noticeable signs of toxicity at a dose of 100 µg of F8-IL6, a second therapy study with four injections (every 48 h) of 225 µg was performed in the same model of cancer. A slight (~5%) weight loss was observed at this dose regimen, but the tumor inhibitory effects remained modest [Figure 30c/d].

**Figure 30.** (a) Evaluation of therapeutic activity of F8-IL1β and F8-IL6 against F9 teratocarcinoma in comparison to the negative control fusion proteins KSF-IL1β and KSF-IL6, specific for egg lysozyme. When F9 tumors were clearly palpable, mice were randomly grouped and injected 3 times (every 72 h; indicated by arrows) with PBS, F8-IL1β (5 µg) or F8-IL6 (100 µg). Data represent mean tumor volumes (+/- SEM), n = 5.
The difference between F8-IL1β and KSF-IL1β is significant (**, p = 0.0047) from day 13 on while there is no significance between F8-IL6 and KSF-IL6 (b) Analysis of toxicity by monitoring changes in weight of treated mice. (c) Evaluation of therapeutic activity of F8-IL6 (high dose) against F9 teratocarcinoma in comparison to the neg. control fusion protein KSF-IL6, specific for egg lysozyme. When F9 tumors were clearly palpable, mice were randomly grouped and injected 4 times (every 48 h) with PBS, KSF-IL6 (225 µg) or F8-IL6 (225 µg). Data represent mean tumor volumes (+/- SEM), n = 5. (d) Analysis of toxicity by monitoring changes in weight of treated mice.

4.2.2 Discussion

In this article, we have described, for the first time, the production and characterization (in vitro and in vivo) of tumor-targeting immunocytokines, based on the potent pro-inflammatory cytokines IL1β and IL6. These payloads are particularly attractive for the development of therapeutic immunocytokines, since they are functionally related to TNF, a pro-inflammatory agent which has successfully been used in clinical-stage products (355). Like TNF, the clinical use of IL1β as payload should be facilitated by the availability of an approved blocking antibody (Ilaris®), which could serve as antidote in case of over-dosing or in case of severe unexpected adverse events. In our experiments, however, the anticancer activity of the new immunocytokines in the F9 teratocarcinoma model of cancer was not as potent as the one of other products based on the F8 antibody (e.g., F8-IL2 (268), F8-TNF (356) F8-IL4 and IL12-F8F8 (109)).

Prior to this study, conflicting reports had indicated either a beneficial or a detrimental action of IL1β and IL6 on tumor growth. We expected that high cytokine concentrations at the tumor site, which can be reached using antibody-mediated targeted delivery strategies, could contribute to a strong pro-inflammatory environment at the neoplastic site. We have recently observed that certain cytokines (e.g. IL4) can exert a potent anti-tumor effect, with a mechanism which could not be predicted based on available literature data. We believe that antibody-based pharmacodelivery strategies are ideally suited to investigate whether a payload displays tumor-promoting or tumor-inhibitory activities.

The biodistribution profile of F8-IL6 and IL6-F8 in F9 tumor-bearing mice was comparable to the one previously reported for other F8-based immunocytokines.
The higher tumor uptake (~4 %ID/g vs. ~2 %ID/g) and better tumor to blood ratio (21.7 vs. 13.4) observed with the C-terminal fusion protein compared to the N-terminal fusion protein convinced us to use F8-IL6 for therapy experiments, in spite of a somewhat higher organ uptake in the intestinal tract, an organ for which variable uptake has previously been observed with F8 derivatives. In case of IL1β-based fusion proteins, the difference in biodistribution results for the two domain orientations was pronounced, with a tumor-to-blood ratio of 7.2 for the C-terminal fusion, compared to a tumor-to-blood ratio of 1 for the N-terminal fusion protein, 24 h after intravenous injection. With high levels of fusion protein observed in the blood and normal organs the profiles of both IL1β-based immunocytokines were unusual, compared to other F8 derivatives previously studied in our laboratory. This observation could not have been anticipated on the basis of biochemical data and on the results obtained incubating a radiolabeled protein preparation with murine blood. Most of the immunocytokine remained in the supernatant, suggesting that the fusion protein had not been trapped by leukocytes carrying the IL1-receptor [Figure 31a/b]. For certain payloads (e.g., IFNγ), we have previously observed that immunocytokine trapping by the cognate receptor may prevent an efficient accumulation at the tumor site in vivo (17). Furthermore, protein extravasation can be inhibited for fusion proteins with extreme pI values (358). Finally, protein glycosylation can have a serious impact on the clearance, extravasation, tumor-targeting and therapy performance (359).
The ultimate goal of our laboratory is to fuse and characterize (*in vitro* and *in vivo*) all cytokines to antibodies specific to fibronectin splice isoforms, which have proven to selectively localize on the tumor sub-endothelial extracellular matrix. The comparative evaluation of different immunocytokines remains of fundamental importance, in order to derive general principles for antibody-based pharmacodelivery applications and to select the most promising candidates for clinical development activities. The therapeutic results obtained with F8-IL6 (and,
to a lesser extent, with F8-IL1β) were unexpected, particularly in view of the excellent biodistribution results obtained in the syngeneic immunocompetent F9 mouse model of cancer. It is possible that this fusion protein, of proven targeting performance in vivo, may find an application for therapeutic intervention in a non-oncological indication. Although it might be that F8-IL1β and F8-IL6 immunocytokines are more effective in other mouse models of cancer (e.g. fibrosarcoma for F8-IL6 and ovarian carcinoma for F8-IL1β), at this moment in time, we do not believe that IL1β and IL6 may represent promising payloads for the development of anticancer immunocytokines, despite their functional similarity to TNF.

4.2.3 Material and methods

4.2.3.1 Cloning of F8-IL1β and F8-IL6 fusion proteins

The cloning of IL1β-F8 and IL6-F8 immunocytokines is described here. Murine Interleukin 1β and Interleukin 6 cDNA clones (Sinobiologicals Inc.; Beijing, China) were PCR amplified using SIP-IL1β-fw (5’-CCTGTTCTCGTCGGCTACAGGTGTGCACTCGGTTCCCATTAAGCTGCAC-3’) and SIP-IL6-fw (5’-CCTGTTCTCGTCGGCTACAGGTGTGCACTCGGTTCCCATTAAGCTGCAC-3’) forward primers, that append a NheI restriction site and part of the SIP secretion signal sequence, and IL1β-linker_rev (5’-CCGCCAGAGCCACCTCCGCCTGAACCGCCTCCACCGGAAGACACGGATTCCACCTTGGCCAGTAGATCTCAAAGTG-3’) reverse primers, that append part of the 14-amino acid GGGS linker peptide between the cytokine and the antibody moiety. The F8 diabody gene was PCR amplified using the primers Linke_F8_fw (5’-TCAGGCGGAGCCACCTCCGCCTGAACCGCCTCCACCGGAGTAGATCTCAAAGTG-3’) and F8-NOT_I_rev (5’-TTTTCCTTTTTTTCGCGGAGTCATTCCATTTTTGACTCCACCTTTCTTGGCC
GAA-3’) which appends two stop codons as well as a NotI restriction site. The murine IL1β-linker/IL6-linker and linker-diabody(F8) DNA fragments were PCR-assembled using primers Nhel_Sip-fw (5’-CCCGCTAGCGTCGACCATGGGCTGGAGCCTGATCCTCCTGTTCCTCGTGCGTGTCG-3’), containing a Nhel restriction site followed by the N-terminal part of the SIP secretion sequence (21bp overlap IL1β/IL6-SIP-fw), and F8-NOT_I_rev containing a stop codon. The PCR-assembled full length cytokine-F8 gene was double digested with Nhel/NotI and cloned into the mammalian cell expression vector pcDNA3.1 (+) (Invitrogen). The C-terminal fusion proteins were cloned analogously with a reverse domain assembly.

4.2.3.2 Cell culture / cell lines

CHO-S (Invitrogen, Zug, Switzerland) cells in suspension were cultured in shaker incubators using PowerCHO-2CD medium (Lonza, Switzerland) supplemented with 8 mM Ultraglutamine, HT supplement (Lonza, Switzerland) and antibiotics. Cells were incubated at 37 °C. For biodistribution and syngeneic tumor mouse therapy studies the murine teratocarcinoma F9 cell line was used (CRL-1720, ATCC, Molsheim-Cedex, France). Cells were grown in tissue flasks coated with 0.1% gelatin in DMEM (GIBCO®) supplemented with 10 % FCS. Murine B9 cells (ACC-211, DSMZ, Germany), TIB-224 cells (ATCC, Molsheim-Cedex, France) were cultured according to supplier’s protocol.

4.2.3.3 Transient gene expression and characterization

All fusion proteins used in this study were initially expressed in CHO-S cells using transient gene expression as previously described (343). From the transient culture, stable cell lines were obtained upon selection of G418 resistant clones. Positive clones were screened for high fusion protein expression levels by ELISA. For ELISA experiments biotinylated EDA antigen was coated (10^{-5} M) on streptavidin stripes (Roche, Switzerland) and protein A-HRP (GE healthcare) was
used for detection. Fusion proteins were purified from the cell culture medium to homogeneity by protein-A chromatography and analyzed by SDS–PAGE (reducing, non-reducing conditions), size exclusion chromatography (Superdex 200 10/300GL, GE Healthcare) and surface plasmon resonance analysis (BIAcore) on an EDA antigen-coated sensor chip.

4.2.3.4 Bioactivity assay

The biological activity of murine IL1β and IL6 was determined by its ability to stimulate the proliferation of TIB-224 and murine B9 cells, respectively. Cells (30000 cells/well) were seeded in 96-well plates in culture medium supplemented with varying concentrations of recombinant fusion proteins (protein range IL1β: 2 ng/µl to 1 pg/µl; protein range IL6: 100 ng/µl to 1 fg/µl). After incubation at 37°C for 48 h, cell proliferation was determined with Cell Titer Aqueous One Solution (Promega). Recombinant human IL6 was used as positive control in the IL6 proliferation assay, while medium without IL1β served as negative control in the IL1β assay. EC50: F8IL6 (0.48 ng/µl), IL6-F8 (0.16 ng/µl), KSF-IL6 (0.02 ng/µl), hIL6 (3.1 ng/µl). EC50: F8IL1β (56 pg/µl), IL1β-F8 (317 pg/µl), KSF-IL1β (89 pg/µl).

4.2.3.5 Quantitative biodistribution studies

The in vivo targeting performance of N- and C-terminal F8 interleukin fusion proteins was evaluated by biodistribution analysis with radioiodinated protein preparations as described before (344). Six to seven days after tumor implantation, mice (n = 5 per group) were grouped and injected into the lateral tail vein with 2.5 µg (~ 3 μCi) of radioiodinated IL1-based fusion proteins or 10 µg (~ 12 μCi) of the radio-labeled IL6 fusion proteins. Mice were sacrificed 24 h after injection, organs were excised, weighed and radioactivity was measured with a Packard Cobra γ-counter. Values are given in percentage of injected dose per gram of tissue (%ID/g +/- SE).
4.2.3.6 Syngeneic tumor mouse models in immunocompetent 129/SvEv mice

Eleven to twelve weeks old female 129/SvEv mice (Charles River, Germany) were subcutaneously injected in the flank with $25 \times 10^6$ cells F9 teratocarcinoma cells. Therapeutic doses of recombinant fusion proteins (5 μg of IL1β-based fusion proteins or 100 μg - 225 μg of IL6-based fusion proteins were given every 72 or 48 h, 3 to four injections, $n = 5$ per group). When weight loss was > 15 % or tumor volumes exceeded 2000 mm$^3$ animals were sacrificed. Experiments were performed under a project license granted by the Verterinaeramt des Kantons Zürich, Switzerland (Bew. Nr. 42/2012).
4.3 The antibody-mediated targeted delivery of interleukin-13 to syngeneic murine tumors mediates a potent anticancer activity

This section has been adapted from the following publication:


4.3.1 Results

Interleukin 13 was fused at the C-terminal extremity of recombinant antibodies in diabody format (360), using a flexible 14 amino acid Gly-Ser linker [Figure 32a/b]. The recombinant F8 antibody was chosen as tumor-homing vehicle, while the KSF antibody (specific to hen egg lysozyme) served as negative control of irrelevant specificity in the mouse (344). The genes coding for the fusion proteins were assembled using a PCR-based procedure, cloned into a mammalian expression vector and transiently expressed in CHO-S cells. Both fusion proteins could be expressed at satisfactory yields (up to 14 mg/L in non-optimized conditions) and purified to homogeneity (> 95%, as judged by SDS-PAGE and gel-filtration analysis) after a single protein-A affinity chromatography step [Figure 32c]. SDS-PAGE gel analysis revealed the presence of both N-glycosylation and O-glycosylation, since not all glycans could be removed by PNGase treatment [Figure39a]. In vitro immunofluorescence staining of F9 tumor sections showed specific binding of the fusion protein to the target antigen EDA located in the subendothelial matrix of blood vessels [Figure 32d]. Both fusion proteins were analyzed by size-exclusion chromatography and SPR studies on an EDA-coated surface [Figure 33a/b]. Cytokine activity was assessed by a proliferation assay with the IL13 cytokine-dependent murine B9 cell line [Figure 33c]. Collectively, these data indicate that both the antibody moiety and the cytokine retained full activity in the F8-IL13 fusion protein.
Figure 32. Cloning, expression and \textit{in vitro} characterization of non-covalent dimers of F8-IL13 fusion protein. (a) Schematic representation of the expression vector for F8-IL13 expression in mammalian cells. (b) Schematic representation of F8-IL13 fusion protein. (c) SDS-PAGE analysis of F8-IL13 and KSF-IL13; M = molecular marker; NR = non-reducing conditions; R = reducing conditions. (d) \textit{Ex vivo} immunofluorescence staining of F9 tumor sections incubated with either F8-IL13 (left) or PBS (right); scale bars: 100 μm.
Figure 33. (a) Size exclusion analysis of F8-IL13 and KSF-IL13. (f) ELISA binding assay performed on EDA-antigen coated wells; proteins were applied at 1 μM concentration. (b) Surface plasmon resonance analysis of 3 different concentrations (1 μM, 500 nM and 250 nM) of F8-IL13 (black) and one concentration (1 μM) of KSF-IL13 (grey) on an EDA-coated sensor chip. (c) IL13 bioactivity assay performed with F8-IL13, KSF-IL13 and rmIL13 on IL13-responsive murine B9 cells. EC50: F8IL13 (58 pg/μl), KSF-IL13 (50 pg/μl), hIL13 (88 pg/μl).

A radioiodinated preparation of F8-IL13 retained antigen-binding activity, as assessed by affinity chromatography on EDA resin. Ten micrograms of the product were injected into the tail vein of immunocompetent 129/SvEv mice bearing subcutaneously-grafted murine F9 tumors, revealing a preferential accumulation in the neoplastic mass, with over 16 %ID/g in F9 tumors 24 h after injection and with a tumor : blood ratio of 7.35 [Figure 34a]. An ex vivo analysis of tumor sections confirmed a preferential accumulation of F8-IL13 (but not of KSF-IL13) around tumor neo-vascular structures [Figure 34d].
Figure 34. (a) Quantitative biodistribution study of $^{125}$I-labeled preparation of F8-IL13 fusion protein in subcutaneous F9 teratocarcinoma bearing 129/SvEv mice. Animals were sacrificed 24 h after injection, organs were excised and radioactivity counted, expressing results as percent of injected dose per gram of tissue (%ID/g ± SE). (b) Combination treatment of F8-IL13 with IL12-F8F8 in F9 tumor model. When F9 tumors were clearly palpable, mice were randomly grouped (n = 5) and injected four times (every 48 h) with PBS, 200 µg F8-IL13, 8.75 µg IL12-F8F8 or the combination of both agents (150µg F8-IL13 and 8.75 µg IL12-F8F8). (c) Monitoring of toxicity by the measurement of weight of treated mice. (d) In vivo immunofluorescence analysis of F9 tumor sections of mice treated with F8-IL13, KSF-IL13 or PBS; scale bars: 200 µm.

A pilot dose escalation study, performed in F9 tumor-bearing mice, revealed that doses of F8-IL13 could be administered up to 250 µg by i.v. injection, without any detectable sign of toxicity (e.g., weight loss) and with evidence of potent antitumor activity. As a result, therapy studies in the same model were performed administering four injections of 200 µg F8-IL13 or KSF-IL13, used as single agents, starting treatment when lesions had reached a volume of ~100 mm³. Additionally, stimulated by recent reports of therapy potentiation by targeted delivery of murine IL12 (361-363), 150 µg F8-IL13 were administered to a group of mice in combination with 8.75 µg IL12-F8F8. The use of F8-IL13 led to strong tumor growth retardation, with 2/5 cured mice when the product was given in combination with IL12-F8F8 [Figure 34b]. However, upon rechallenge with F9 cells, both animals developed tumors (data not shown). Some mice receiving F8-
IL13 (at a dose of 200 μg) showed mild diarrhea, but body weight loss remained < 10% [Figure 34c]. Therapy experiments were also performed with the combination of F8-IL13 plus IL12-F8F8 in mice with larger tumors (average tumor volume = 990 mm³, n = 4). Some of the treated mice responded well to treatment, in one case with disappearance of the neoplastic mass, but eventually the tumor grew back [Figure39b]. A microscopic analysis of tumor sections following treatment revealed a rich infiltrate of a variety of leukocytes in the immunocytokine treated groups, without obvious changes in vascular density. Moreover, the tumor density of FoxP3-positive cells was not increased in any of the treatment groups [Figure 38a].

Biodistribution studies with F8-IL13 were also performed in immunocompetent Balb/c mice bearing subcutaneously-grafted murine Wehi-164 sarcoma tumors. In this model, a substantial reduction of antibody uptake in the tumor was observed, compared to the F9 teratocarcinoma model. The tumor : organ ratios for F8-IL13, however, were similar to the ones observed for the parental F8 antibody in small-immune protein format (215), but an example of potent anti-tumor activity has previously been reported for another F8-based immunocytokine in Wehi-164 tumor-bearing mice (278) and an ex vivo analysis of tumor sections confirmed a preferential accumulation of F8-IL13 (but not of KSF-IL13) around tumor neo-vascular structures [Figure 36a]. Three injections were given and F8-IL13 was tested as single agent at two different doses (100 μg and 200 μg). All other experimental conditions were identical as for the study in F9-tumor-bearing mice. F8-IL13 exhibited a strong reduction of tumor growth rate [Figure 36b] and led to cures in 4/5 mice, when administered in combination with IL12-F8F8. As in the F9 model, F8-IL13 was generally well tolerated, but few mice developed diarrhea but without considerable body weight loss [Figure 36c]. Unlike what previously observed in teratocarcinoma-bearing mice, the induction of protective immunity was observed in the Wehi-164 model of sarcoma, as none of the cured mice developed a tumor when rechallenged with tumor cells 75 days after treatment [Figure 36d]. Also in this model therapy experiments were performed in mice with larger tumors (i.e., above 500 mm³). A marked reduction of tumor size was observed for F8-IL13 (when used alone or in combination with
IL12-F8-F8 or F8-IL4 (363)), but not for the IL12-F8-F8 treatment group [**Figure 37a**]. The selective *in vivo* depletion of immune cells with antibodies revealed that the anti-tumor effect in mice with Wehi-164 sarcomas was predominantly mediated by CD4+ T cells [**Figure 37b**]. A flow cytometric analysis of depletion efficacy was performed. Spleens of CD4+, CD8+ and NK cell depleted mice were homogenized and analyzed by a FITC-labeled secondary antibody [**Figure 39c**]. Cytokine levels in Wehi-164 sarcoma tissue samples were measured using a multiplex bead-based assay in tumors one day after the 2nd injection, but no obvious changes in cytokine concentrations could be observed in any of the treatment groups [**Figure 35**]. A microscopic analysis of tumor sections following treatment revealed the presence of an infiltrate of various types of leukocytes [**Figure 38b**].
Figure 35. One day after the 2nd injection, mice bearing Wehi-164 tumors were sacrificed and the tumors were homogenized and concentrations of 13 different cytokines were measured using multiplex bead-based flow cytometry assay kit. IFN, interferon; IL, interleukin; TNF, tumor necrosis factor.
Figure 36. (a) Quantitative biodistribution study of 125I-labeled preparation of F8-IL13 (black, n = 5) and SIP(F8) control (grey, n = 5) in subcutaneous Wehi-164 sarcoma bearing Balb/c mice. Animals were sacrificed 24 h after injection, organs were excised and radioactivity counted, expressing results as percent of injected dose per gram of tissue (%ID/g ± SE) and below an in vivo immunofluorescence analysis of Wehi-164 tumor sections of mice treated with F8-IL13, KSF-IL13 or PBS is shown; scale bars: 100 μm. (b) Therapy study in Wehi-164 sarcoma bearing mice performed with F8-IL13 (two different doses of 100 μg and 200 μg) IL12-F8F8 (8.75 μg) and the combination of F8-IL13 with IL12-F8F8 (150 μg and 8.75 μg) in comparison to the negative control fusion protein KSF-IL13 (two different doses of 100 μg and 200 μg) and the saline group. When F9 tumors were clearly palpable, mice were randomly grouped and injected 3 times (every 48 h; indicated by black arrows). Data represent mean tumor volumes (+/- SEM), n = 5. (c) Analysis of toxicity by monitoring changes in weight of treated mice. (d) Mice cured by the combination treatment of F8-IL13 and IL12-F8F8 (grey, n = 4) as well as naïve mice (black, n = 4) were injected with Wehi-164 sarcoma cells and the tumor growth was measured over time.
Figure 37. (a) Wehi-164 bearing mice were injected (i.v.) three times (every 48 hr) with F8-IL13 (150 μg), IL12-F8F8 (8.75 μg), or the combination of F8-IL13 (150 μg) with IL12-F8F8 (8.75 μg) or IL4 (70 μg) starting when the average tumor size exceeded 500 mm3. (b) In vivo depletion of CD4+, CD8+ and NK cells in Wehi-164 bearing Balb/c mice. Depletion antibodies were injected i.p. on days 4, 7, 10 and 13 (grey arrows) while therapeutic antibody combination (120 μg F8-IL13 and 7 μg IL12-F8F8) therapy as well as the PBS control group was injected i.v. on days 5, 7 and 9 (black arrows). Data represent mean tumor volumes (+/- SEM).
Figure 38. (a) Immunofluorescence analysis of tumor infiltrating cells on 10 μm F9 teratocarcinoma tumor sections following treatment with PBS, KSF-IL13, F8-IL13, IL12-F8F8 and F8-IL13 in combination with IL12-F8F8 (red: anti-CD31). Scale bars: 100 μm. (b) Immunofluorescence analysis of tumor infiltrating cells on 10 μm Wehi-164 sarcoma tumor sections following treatment with PBS, KSF-IL13, F8-IL13, IL12-F8F8 and F8-IL13 in combination with IL12-F8F8 (red: anti-CD31). Scale bars: 100 μm.
Figure 39. (a) SDS-PAGE analysis of PNGase treated of F8-IL13 (left) and untreated F8-IL13 (right). (b) Treatment of mice bearing F9 teratocarcinoma tumors with the combination of F8-IL13 (150 μg) and IL12-F8F8 (8.75 μg) starting at an average tumor size of 990 mm³, injections are indicated with colored arrows (corresponding to individual mice), one mouse (orange markers) had to be sacrificed due to body weight loss. (c) Flow cytometric analysis of depletion efficacy. Spleens of CD4+, CD8+ and NK cell depleted mice were homogenized and analyzed by a FITC-labeled secondary antibody.

4.3.2 Discussion

In this article, we describe for the first time the production and characterization (in vitro and in vivo) of an immunocytokine based on IL13. We used the F8 antibody, which is specific to the alternatively-spliced EDA domain of fibronectin (a marker of angiogenesis) and which has previously been shown to selectively localize on tumor blood vessels in vivo, as tumor targeting moiety. Interleukin-13 was considered to be an attractive payload for the study, since it is functionally related to IL4, a cytokine which has successfully been used for the preparation of
immunocytokines with potent activity in mouse models of cancer (109) and of rheumatoid arthritis (364).

F8-IL13 was able to selectively home to tumor blood vessels in the two immunocompetent mouse models of cancer tested (F9 and Wehi-164) and displayed a potent single-agent activity, which however did not result in cancer cures. When the product was combined with an immunocytokine based on F8 and murine IL12 (IL12-F8F8), long-lasting tumor eradications could be observed in both models in a high proportion of treated mice. Balb/c mice cured from Wehi-164 tumors were able to reject subsequent challenges with tumor cells, indicating the induction of a protective immunity. By contrast, a similar effect could not be observed in 129/SvEv mice bearing F9 teratocarcinomas, indicating that the mouse strain or the intrinsic tumor characteristics may contribute to the induction of anticancer immunity.

We have previously reported the induction of cancer cures and of protective immunity for F8-based immunocytokines containing IL2, IL4, IL12 or TNF as payloads, whenever these products were used in combination with other cytokines (109, 365), cytotoxic agents (114, 268, 278, 362, 365, 366) or IgG-based therapeutic antibodies (279). In all tested cases, the anti-tumor effect depended on the action of both NK cells and CD8+ T cells. Surprisingly, F8-IL13 conferred a potent anti-tumor activity mainly through the action of CD4+ T cells, as revealed by in vivo depletion experiments [Figure 37b]. The significance of these findings still remains to be investigated, but examples of potent anti-tumor mediated by CD4+ T cells have previously been described. Transferred naïve tumor/self-specific CD4+ T cells expanded, differentiated and eradicated established melanoma tumors. Surprisingly, CD4+ T cells developed cytotoxic activity leading to a class II- restricted recognition of tumors cells (367, 368).

Interleukin-13 is one of the last, in order of time, among many cytokines which have been fused to tumor-targeting antibodies, including the L19 and F8 antibodies, specific to splice isoforms of fibronectin. The targeted delivery of a potent immunomodulatory payload to the sub-endothelial extracellular matrix results in a high local concentration of cytokine at the site of disease, where this
agent can interact with *in transit* leukocytes. The study of many different cytokines as partners for antibody fusion is justified by the potent antitumor activity, which has been observed for some payloads (e.g., IL2, IL4, IL12, IL13, TNF), and by striking differences in tumor-homing properties (17, 274, 369). Similar to other types of armed antibody products (e.g., antibody-drug conjugates), also immunocytokines can mediate cancer eradication in immunocompetent mouse models of cancer. However, cytokine-based products typically do not exhibit a direct toxicity to clearance-related organs and to the bone marrow, thus making them ideally suited for combination with conventional anticancer cytotoxic agents.

The therapeutic activity of F8-IL13 was encouraging, particularly when this product was combined with an IL12-based immunocytokine. Similar to previous reports on F8-IL4, the strong synergy observed with IL12-F8F8 was unexpected, as IL13 and IL12 are thought to control mutually exclusive fates of T-cell development and activation. The F8 antibody recognizes the majority of human cancer types (215) and its cognate antigen is conserved in mouse and man, making F8-IL13 an attractive candidate for industrial development activities in oncology. As for other immunocytokine products, it is difficult to predict clinical activity on the basis of preclinical data, as cytokine function can be different in mouse and man.

### 4.3.3 Material and methods

#### 4.3.3.1 Cloning of F8-IL13 and KSF-IL13 fusion proteins

Murine Interleukin 13 cDNA clone (Sinobiologicals Inc.; Beijing, China) was PCR amplified using linker-IL13 fw (5’-TCAGGGAGGTGGCTCTGGCGG TGGCGGA CCGGTGCCAAGATCTGTGTCTCTCC-3’) forward primer, that appends N-terminally part of the glycine-serine-linker sequence, and IL13-NotI rev (5’-TTTTCTTTTTGCGCGCCGCTCATTAGAAGGGGCCGTTGCCGAAA CAGTTGC-3’) reverse primer containing a NotI restriction site. The F8 diabody gene was PCR amplified using the primers SIP-F8 fw (5’-
CCTGTTCCCTCGTCTGCTGCTGACTACAGGTGTGCACTCGGAGGTGCAGCTGT
TGGAGTCTGGGG-3') that N-terminally appends part of the signal peptide DNA sequence and F8-linker_rev (5'-CCGCCAGAGCCACCTCCGCCT
GAACCGCCTCACCCTTGGATTCCACCTTGCTCCCTTGG-3') which appends part of the linker peptide (including a 23 amino acid overlap) at the C-terminus. The murine IL13 and diabody(F8) DNA fragments were PCR-assembled using primers NheI_Sip_fw (5'-CCCGCTAGCGACCATGGCTGGAGCCTGA
TCCTCCTGTCTCCTCCTGCTGTGCGC-3'), containing a NheI restriction site followed by the N-terminal part of the SIP sequence (21bp overlap with SIP-F8_fw), and IL13_NotI_rev. The PCR-assembled full length immunocytokine gene was double digested with NheI/NotI restriction endonucleases and cloned into the mammalian cell expression vector pcDNA3.1 (+) (Invitrogen). The KSF-IL13 fusion protein was cloned analogously with the KSF antibody template instead of the F8 antibody template (annealing parts are the same, hence the same primers could be used). The cloning and expression of IL12-F8F8 is described elsewhere (370).

4.3.3.2 Cell culture / cell lines

Chinese hamster ovary (CHO-S) (Invitrogen, Switzerland) cells in suspension were cultured in shaker incubators using PowerCHO-2CD medium (Lonza, Switzerland) supplemented with HT supplement (GIBCO®), 8 mM Ultraglutamine (Lonza, Switzerland), and 1% antibiotics (GIBCO®). Cells were incubated at 37 °C. For biodistribution and syngeneic tumor mouse therapy studies the murine teratocarcinoma F9 (CRL-1720, ATCC, Molsheim-Cedex, France) and Wehi-164 (CLS cell line service) cell lines were used. F9 cells were grown in tissue flasks coated with 0.1% gelatin in DMEM (GIBCO®) supplemented with 10 % FCS. Wehi-164 cells were cultured according to supplier’s protocol in RPMI (GIBCO®) medium. Murine B9 hybridoma cells (DSMZ, ACC-211, Germany) were cultured according to supplier’s protocol.
4.3.3.3 Transient gene expression and characterization

F8-IL13 and KSF-IL13 fusion proteins were expressed in CHO-S cells using transient gene expression as previously described (343). Fusion proteins were purified from the supernatant to homogeneity by protein-A affinity chromatography and analyzed by SDS–PAGE (reducing, non-reducing conditions, Invitrogen), size exclusion chromatography (Superdex 200 10/300GL, GE Healthcare) ELISA, and surface plasmon resonance analysis (BIAcore) on an EDA antigen-coated sensor chip. ELISA experiments were performed on streptavidin stripes (Roche, Switzerland) coated with biotinylated EDA antigen (10^{-7} M). Detection was done with protein A-HRP (GE healthcare).

4.3.3.4 Bioactivity assay

The biological activity of murine IL13 was determined by its ability to stimulate the proliferation of murine B9 cells. Ten thousand cells per well were seeded in 96-well plates in culture medium supplemented with varying concentrations of recombinant fusion proteins (protein range 7.5 pg/μl to 1000 pg/μl). After incubation at 37°C for 48 h, cell proliferation was determined with Cell Titer Aqueous One Solution (Promega).

4.3.3.5 Quantitative biodistribution studies

The in vivo targeting performance of F8-IL13 fusion protein was evaluated by biodistribution analysis with ^{125}I-labeled protein preparations as described before (344). Six days after tumor cell injection, mice (n = 5 per group) were grouped and injected into the lateral tail vein with 10 μg of radioiodinated F8-IL13 fusion protein. Mice were sacrificed 24 h after injection, organs were excised, weighed and radioactivity was measured with a Packard Cobra γ-counter. Values are given in percentage of injected dose per gram of tissue (%ID/g +/- SE).
4.3.3.6 Syngenic tumor mouse models in immunocompetent 129/SvEv and Balb/c mice

Twelve week old female 129/SvEv mice (Charles River, Germany) were subcutaneously (s.c.) injected in the flank with $25 \times 10^6$ F9 teratocarcinoma cells. Balb/c mice (Charles River, Germany) were s.c. injected in the flank with $3 \times 10^6$ cells Wehi-164 sarcoma cells.

Four intravenous (i.v.) injections (every 48 hours) of recombinant fusion proteins (200 μg of F8-IL13 and KSF-IL13 as single agents, 150 μg F8-IL13 and 8.75 μg of IL12-F8F8 in combination therapy modality) were given to F9 bearing mice, while three injections were given in the Wehi-164 model (n = 5). Animals were sacrificed when weight loss was > 15 % or one day before tumor volumes were exceeding 2000 mm$^3$. Experiments were performed under a project license granted by the Verterinaeramt des Kantons Zürich, Switzerland (Bew. Nr. 42/2012).

4.3.3.7 In vivo depletion of CD4+, CD8+ and NK cells in Wehi-164 bearing Balb/c mice

In vivo depletion of CD4$^+$, CD8$^+$ and NK cells was performed in Balb/c mice bearing Wehi-164 tumors. Mice were injected with rat anti-CD4 (BioXCell), anti-CD8 (BioXCell) and rabbit anti-Asialo GM1 (Wako Chemicals) antibodies on day 4, 7, 10 and 13 after s.c. injection of tumor cells. On day 5, 7 and 9 after tumor implantation, mice were injected with 120 μg F8-IL13 in combination with 7 μg IL12F8F8.
4.3.3.8 Immunofluorescence studies of treated tumors

*Ex vivo* detection of tumor infiltrating cells after i.v application of therapeutic proteins was done with tumors taken one day after the 2nd injection. Tumors were excised, embedded in cryoembedding medium (Thermo Scientific) and cryostat sections (10 μm) were stained using the following antibodies: CD4 (BioXCell), CD8 (BioXcell), F4/80 (Abcam), CD3e (eBioscience), CD45 (BD Biosciences), CD45R (eBioscience), Asialo GM1 (Wako Pure Chemical Industries), Foxp3 (eBioscience) and CD31 (Santa Cruz Biotechnology). Detection was done with AlexaFluor488 and AlexaFluor594 coupled secondary antibodies (Invitrogen) and the slides were then analyzed with an Axioskop2 mot plus microscope (Zeiss).

4.3.3.9 Tumor rechallenge studies

More than 50 days after therapy studies, cured mice were injected (s.c.) again with 25 x 10⁶ F9 cells/mouse and 3 x 10⁶ Wehi-164 cells/mouse respectively to see if mice acquired a protective immunity against cancer cells upon treatment with immunocytokines.

4.3.3.10 Cytokine determination assay

Cytokine levels in tumor tissue samples one day after the 2nd injection of all therapy groups were analyzed by the Th1/Th2/Th17/Th22 13plex FlowCytomix Multiplex (eBioscience) kit, following the supplier’s protocol. Fluorescence-activated cell sorting analysis was performed on a BD FACS Canto (BD Bioscience, Allschwil, Switzerland). Data evaluation was done with FlowCytomix Pro 3.0 software (eBioscience). Standard curves generated with standard protein samples allowed the subsequent quantification of 13 different cytokines in tumor specimens.
4.3.3.11 Statistical analysis

Data are expressed as mean ± standard deviation (biodistribution studies) or standard error of the mean (therapy studies). Differences between therapeutic groups were compared using GraphPad Prism’s (GraphPad Software Inc., La Jolla, CA, USA) two-way ANOVA multiple-comparison analysis (P < 0.05 significance level). Differences in cytokine levels were compared using a Mann–Whitney-U-test (P <0.05 significance level).
4.4 Tumor targeting properties of antibody fusion proteins based on different members of the murine tumor necrosis superfamily

This section has been adapted from the following publication:


4.4.1 Results and discussion

The F8 antibody was fused in scFv format to seven murine members of the TNF superfamily: CD40L, TRAIL, FasL, LiGHT, VEGI, lymphotixin alpha, lymphotixin beta and lymphotixin alpha1/beta2. The fusion proteins could be expressed by mammalian cells and were characterized by SDS-Page analysis, size exclusion chromatography and surface plasmon resonance (BIAcore) analysis on an EDA coated sensor chip [Figure 40].
Figure 40. Expression and characterization of antibody-fusion proteins based on members of the TNF superfamily. Schematic representation of the domain structures, SDS-Page (M: molecular marker; N: non-reducing conditions; R: reducing conditions), size exclusion chromatography (SEC200) and surface plasmon analysis of purified protein preparations on an EDA coated sensor chip.

In order to test the antigen binding activity of the F8 moiety in vitro, the fusion proteins were studied by surface plasmon resonance on a BIAcore microsensor chip coated with recombinant EDA. All immunocytokine bound to the cognate antigen with comparable BIAcore profiles [Figure 40]. However, when tested in vivo by quantitative biodistribution experiments in immunocompetent mice carrying subcutaneously grafted F9 teratocarcinoma tumors (a highly vascularized tumor expressing EDA around its blood vessels (314)), radioiodinated preparations of F8-CD40L, F8-FasL, F8-TRAIL, F8-TRAILtrunc, F8-VEGI, F8-VEGItrunc, F8-LIGHT, F8-LTA, F8-ltb and F8-LTα1β2 displayed different and distinctive distribution patterns when compared to F8-TNF [Figure 41]. While F8 fusions with TRAIL, truncated TRAIL and LiGHT preferentially localized at the tumor site 24 h after intravenous injection with only low uptake in healthy organs, F8-LTA was unable reach its cognate antigen in vivo. F8-CD40L, F8-FasL, F8-VEGI, F8-VEGItrunc and F8-ltb exhibited tumor uptake, but those immunocytokines were also found in certain normal tissues, such as spleen and liver. Thus, our data reveal that not all members of the tumor-necrosis factor family are suitable payloads for the antibody-based pharmacodelivery to solid tumors because not all can be efficiently and selectively delivered to the tumor site as fusions proteins.

Bifunctional proteins, such as immunocytokines, do not always efficiently extravasate and reach their cognate tumor-associated antigen in vivo, even if the parental antibody displays good tumor targeting properties as an unmodified protein (as is the case for the F8 antibody). For example, the preferential tumor uptake of antibodies specific to splice isoforms of fibronectin can be completely abrogated by fusion with highly charged payloads (e.g., Calmodulin (338), VEGF-164 (351) and HIV-1 TAT peptides (339)), bulky payloads (370), highly glycosylated payloads (340) and payloads prone to receptor trapping when administered at low doses (e.g., IFNγ (371)). Other payloads can lead to a high uptake in unspecific healthy organs due to the binding activity of the payload itself.
(e.g. receptor binding) [Figure 41].

![Figure 41](image)

The results are expressed as percent of antibody injected dose per gram of tissue (%ID/g ± SEM)

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<th>spleen (µCi)</th>
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<td>F8-TRAILtrunc</td>
<td>5.42 ± 2.70</td>
<td>1.23 ± 0.70</td>
<td>0.61 ± 0.10</td>
<td>2.45 ± 0.39</td>
<td>0.21 ± 0.06</td>
<td>1.63 ± 0.22</td>
<td>0.84 ± 0.12</td>
<td>0.32 ± 0.10</td>
<td>0.40 ± 0.28</td>
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<tr>
<td>F8-VEG1</td>
<td>2.62 ± 0.38</td>
<td>2.03 ± 0.13</td>
<td>0.88 ± 0.28</td>
<td>3.68 ± 0.45</td>
<td>0.40 ± 0.03</td>
<td>2.32 ± 0.32</td>
<td>2.46 ± 0.70</td>
<td>0.54 ± 0.30</td>
<td>0.35 ± 0.06</td>
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<tr>
<td>F8-VEGItrunc</td>
<td>3.47 ± 0.22</td>
<td>4.76 ± 0.21</td>
<td>0.74 ± 0.06</td>
<td>7.08 ± 0.29</td>
<td>0.44 ± 0.04</td>
<td>3.64 ± 0.11</td>
<td>2.10 ± 0.24</td>
<td>0.66 ± 0.29</td>
<td>0.36 ± 0.06</td>
</tr>
<tr>
<td>LIGHT-F8</td>
<td>0.92 ± 0.33</td>
<td>0.35 ± 0.14</td>
<td>0.49 ± 0.21</td>
<td>0.34 ± 0.06</td>
<td>0.06 ± 0.04</td>
<td>0.31 ± 0.12</td>
<td>0.17 ± 0.07</td>
<td>0.09 ± 0.02</td>
<td>0.31 ± 0.10</td>
</tr>
<tr>
<td>LTA-F8</td>
<td>0.09 ± 0.02</td>
<td>1.70 ± 0.32</td>
<td>0.13 ± 0.05</td>
<td>3.12 ± 1.36</td>
<td>0.09 ± 0.03</td>
<td>0.55 ± 0.05</td>
<td>0.08 ± 0.02</td>
<td>0.11 ± 0.04</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td>F8-Ltb</td>
<td>1.26 ± 0.22</td>
<td>0.99 ± 0.18</td>
<td>0.83 ± 0.17</td>
<td>1.92 ± 0.51</td>
<td>0.19 ± 0.01</td>
<td>0.87 ± 0.06</td>
<td>0.85 ± 0.37</td>
<td>0.15 ± 0.02</td>
<td>0.32 ± 0.11</td>
</tr>
<tr>
<td>F8-LTa1b2</td>
<td>2.32 ± 0.21</td>
<td>0.79 ± 0.01</td>
<td>0.86 ± 0.12</td>
<td>1.06 ± 0.51</td>
<td>0.23 ± 0.03</td>
<td>1.12 ± 0.16</td>
<td>1.04 ± 0.04</td>
<td>0.19 ± 0.07</td>
<td>0.46 ± 0.08</td>
</tr>
</tbody>
</table>

**Figure 41.** Comparative biodistribution analysis. Radioiodinated protein preparations (10µg) were intravenously administrated to fully immunocompetent F9 tumor-bearing mice. After 24h, mice were sacrificed and organs were excised, weighted and counted for radioactivity, expressing results as percent of injected dose per gram of tissue (%ID/g) ± SEM.

This is the first complete report of a comparative analysis of the properties of closely-related antibody-cytokine fusion proteins *in vitro* and *in vivo*. The seven novel immunocytokines belonging to the TNF superfamily could be expressed and purified in functional form, which was similar to the one of F8-TNF used as reference product. However, the *in vivo* analysis of biodistribution data revealed distinctive differences in the tumor targeting properties of these closely-related
biopharmaceuticals. Armed antibodies represent a fast-growing class of pharmaceutical biotechnology products, which will continue to benefit in the future from "precision medicine" approaches, involving nuclear medicine imaging procedures and quantitative biodistribution studies.

4.4.2 Material and methods

4.4.2.1 Cell lines and animals

CHO cells (Invitrogen, Zug, Switzerland), LM-Fibroblasts (ATCC-LCG, Molsheim-Cedex, France) and the murine tumor cell lines F9 teratocarcinoma (ATCC-LCG) and WEHI-164 sarcoma (ATCC-LCG) were cultured according to supplier’s protocol.

4.4.2.2 Cloning, expression and purification of fusion proteins

The fusion proteins were designed as the F8 antibody (specific to the alternatively-spliced EDA domain of fibronectin (314)) sequentially fused to a murine member of the tumor-necrosis factor super family (cDNA from Sino Biologicals). The genes of the F8 antibody and the respective cytokine were PCR amplified, PCR assembled and cloned into the mammalian cell expression vector pcDNA3.1 (+) (Invitrogen) by HindIII/NotI or Nhel/NotI (F8-VEGI and F8-VEGItrunc) restriction sites. The fusion proteins were expressed by transient gene expression and purified from supernatant by protein A chromatography as previously described (372). Purified protein was analyzed by SDS-PAGE and size exclusion chromatography (Superdex200 10/300GL, GE Healthcare, Freiburg, Germany). For all further experiments, fusion proteins were purified to homogeneity by preparative gel filtration (Superdex200 10/300GL). Binding affinity of the antibody moiety was analyzed by surface plasmon resonance (BIAcore) on an EDA antigen-coated sensor chip (314, 373).
4.4.2.3 Biodistribution experiments

The *in vivo* targeting performance was evaluated by quantitative biodistribution analysis as previously described. For the tumor model, female 129/SvEv mice were obtained from Charles River (Germany) and implanted subcutaneously with 25 x 10⁶ F9 teratocarcinoma cells in the flank. Tumor-bearing mice were injected with 10 ug radioiodinated protein into the lateral tail vein. Mice were sacrificed 24 hours after injection, organs were excised and radioactivity was measured in Cobra gamma counter (Packard, Meriden, CT, US). Radioactivity is expressed as percentage of the injected dose per gram of tissue (%ID/g). Biodistribution experiments were performed in accordance with the Swiss regulations and under a project license granted by the Veterinäramt des Kantons Zürich (42/2012).
4.5 Glycosylation patterns determine extravasation and disease-targeting properties of armed antibodies

This section has been adapted from the following publication:


4.5.1 Results

We focused our attention on interleukin-9 (IL9) as a therapeutic payload, based on reports on its potent T cell-mediated anti-tumor activities (319, 320). Interleukin-9 is a special cytokine as it contains four distinct N-glycosylation sites while being devoid of O-linked glycans. In this study, we genetically fused IL9 to the C-terminus of a non-glycosylated F8-based diabody and expressed the recombinant immunocytokine either by transient gene expression (TGE) or by stable expression (SE) in stably transfected CHO cells [Figure 42a and 42b] (374). Stably transfected polyclonal CHO cells were cultured at a higher cells density and modified media composition. SDS-PAGE analysis showed similar patterns for protein preparations obtained in various experimental conditions. The mass difference of around 10 kDa observed for glycosylated F8-IL9 samples in SDS-PAGE suggested similar glycosylation site occupancies irrespective of the production method. F8-IL9 preparations tested in vivo eluted as a single peak in gel-filtration and displayed comparable EDA-binding kinetics in SPR analysis [Figure 42d and 42e]. The products could be converted into a fully deglycosylated form upon PNGase F treatment [Figure42c]. Ex vivo, all F8-IL9 preparations selectively stained the sub-endothelial extracellular matrix of blood vessels in murine F9 teratocarcinoma regardless of their production method and enzymatic modification.
Figure 42. Production methods and protein characterization data of F8-IL9 preparations investigated in vivo. Data from TGE (blue) and SE (red) F8-IL9 batches (1 to 3) are displayed. Desialylated SE (DS, gray) and deglycosylated TGE (DG, black) derived F8-IL9 were compared. A) Expression vector and overview of production methods; B) Schematic of homobivalent diabody format; C) Analytical SDS-PAGE analysis; D) Gel-filtration chromatograms; E) EDA-binding sensograms (SPR analysis).
When various batches of F8-IL9 were studied by quantitative biodistribution analysis in immunocompetent 129/Sv mice bearing subcutaneous F9 tumors, a strikingly different tissue distribution profile was observed for proteins produced using either TGE or SE methodologies [Figure 43a]. F8-IL9 derived from TGE cultures was able to efficiently and selectively localize to tumors 24 hours after intravenous injection with 11.53 ± 0.71 percent injected dose per gram (%ID/g) in the neoplastic lesions while exhibiting favorable tumor-to-organ ratios [Figure 43a]. By contrast, batches of the same protein derived from stable expression cultures failed to target tumors in vivo, with only 0.36 ± 0.05%ID/g in the tumor and less than 0.16 ± 0.01%ID/g in blood. In line with the biodistribution data, immunofluorescence microscopy revealed that only TGE-derived F8-IL9 could successfully localize to the perivascular space of tumor blood vessels in vivo [Figure 43b]. This biodistribution data was surprising, as the stable polyclonal expression cultures were directly derived from preceding TGE cultures via antibiotic selection. Our finding proved to be highly reproducible since the data originate from three independent experiments for each type of sample. Additional biodistribution experiments, performed after treating F8-IL9 with PNGase F, revealed that deglycosylated F8-IL9 from stable expression cultures had regained its tumor targeting ability [Figure 43a]. Importantly, TGE- and SE-produced F8-IL9 displayed close to identical biodistribution profiles after enzymatic deglycosylation with PNGase F [Figure 43a], indicating that the protein components were of equivalent quality. F8-IL9 produced by TGE also failed to target the tumor neovasculature of F9 tumors upon enzymatic removal of terminal sialic acids by α2-3,6,8,9 neuraminidase, pointing out the special role of this carbohydrate residue.

The quantitative biodistribution profiles shown in Figure 43a provide a global view of potential glycan-mediated F8-IL9 interactions in vivo. A potential lectin-trapping mechanism (e.g., by immune cells) would be detectable by elevated radioactivity levels in blood and in the spleen. However, only low levels of radiolabeled F8-IL9 were found in normal organs, with the exception of intestinal uptake (often observed using anti-EDA antibody products). We therefore assumed that changes in glycostructures could have an impact both on drug clearance and on extravasation. To support this conclusion, a formal pharmacokinetic analysis
comparing radiolabeled F8-IL9 sample from either TGE or SE cultures was performed [Figure 43c]. However, F8-IL9 produced by the SE method displayed only slightly faster blood clearance, suggesting that the two products could also differ in their ability to cross the tumor endothelium.

![Figure 43](image)

**Figure 43.** In vivo biodistribution profiles, microscopic analysis and pharmacokinetic data. A) Quantitative biodistribution profiles 24h after intravenous administration to F9-tumor bearing mice. TGE (blue), SE (red), desialylated TGE (DS, gray) and deglycosylated SE (DG, black) derived F8-IL9 products are shown; B) Juxtaposition of immunofluorescence detection images of F8-IL9, 24h after intravenous administration or following ex vivo application onto F9 tumor sections (scale bars: 100µm); D) Pharmacokinetic data of SE (red) and TGE (blue) during the first 6h after injection.

The used model system is appropriate to extravasation and vascular targeting because of the specific site of expression of the target antigen EDA in the subendothelial matrix of the tumor-associated vasculature [Figure 43b]. High expression levels of EDA at this site prevent saturation effects over a wide dose range (375). Additionally, the turnover rate for this type of antigens is considered to be very low because bound antibody can be detected up to 5 days after injection (329). Targeting of solid tumors and metastases is often limited by the build-up of an antigen barrier in proximity to the neovasculature (11). Hence, our
findings may also be relevant for other antibody-based pharmacodelivery approaches.

F8-IL9 glycoforms produced by either SE or TGE were extensively characterized using several complementary methods. Hydrophilic interaction chromatography (HILIC)-HPLC-based glycoprofiling of fluorescently labeled glycan pools after PNGase F treatment showed substantial differences between TGE and SE samples [Figure 44a]. We also quantified the amount of terminal N-acetylneuraminic acid (Neu5Ac) moieties in various protein preparations, using two orthogonal assays based either on chemical or enzymatic sialic acid release followed by different fluorescent labeling and detection methods as described in the Methods section. The TGE-produced batches of F8-IL9 exhibited significantly higher Neu5Ac-to-protein ratios, compared to the same protein derived from stably transfected cells in both assays [Figure 44b and 44c]. By comparison, the glycan profiles before and after neuraminidase treatment revealed characteristic peak-shifts for the TGE samples, again indicating terminally sialylated N-glycans [Figure 44a]. MALDI-TOF-TOF MS analysis of permethylated glycans further confirmed this finding [Figure 44d]. The major N-glycan structures from TGE-derived F8-IL9 were sialylated N-glycans with core-fucosylation. On the contrary, MS profiling showed that incomplete galactosylated and neutral N-glycans were the main species present on SE-derived F8-IL9. Further, we have predominantly found α2,3-linked sialic acids on TGE product as confirmed by enzymatic treatment with α2,3 neuraminidase. Surprisingly, we have also observed antennary N-acetyllactosamine (LacNAc) repeats on the glycans of SE F8-IL9. Such poly-LacNAc structures represent ligands for galnectins, which are known to be involved in cell adhesion and tumor progression (376, 377).
Figure 44. Characterization data of N-linked glycan pools. F8-IL9 samples from TGE (blue), SE (red), TGE after desialylation (DS, gray) and SE after deglycosylation (DG, black) were analyzed. A) Representative HILIC-HPLC profiles of 2-AB-labeld N-glycan pools after PNGase F release and neuraminidase treatment (GU: glucose units); B) Quantification of Neu5Ac by reversed-phase HPLC upon mild acid hydrolysis; c) Fluorimetric quantification of terminal Neu5Ac after enzymatic release with α2-3,6,8,9 neuraminidase; D) MALD-TOF-TOF spectra of permethylated glycan pools. In this study, all color schemes were followed by Consortium for Functional Glycomics (378).
Interleukin-9 features four putative glycosylation sites. In order to obtain a detailed view of the glycan microheterogeneity, site-specific glycosylation analysis was performed. After proteolytic cleavage, the four sets of F8-IL9 derived glycopeptides were analyzed by nanoHPLC-HCD-MS/MS. Sialylated glycans were again identified with the help of α2-3,6,8,9 neuraminidase. Site-specific N-linked glycan structures for representative SE and TGE preparations are summarized in Figure 45. Consistent with the data mentioned above, sialylated N-glycans were again predominantly found in the TGE preparations but were also present in the SE product. Notably, sialylation differences were site-specific, i.e., terminal sialic acids as well as galactose residues at the penultimate position were largely absent at glycosylation site 3 in both types of products [Figure 45].

**Figure 45.** Site-specific glycosylation data. Representative TGE- and SE-derived F8-IL9 samples were subjected to nanoHPLC-HCD-MS/MS glycopeptide analysis. Peptide sequences upon cleavage by Trypsin, Glu-C endopeptidase and AspN endopeptidase are shown in the center. Glycosylation sites are numbered from left to right (site 1 to 4).
4.5.2 Discussion

Taken together, the absence of terminal sialic acids and the exposure of terminal galactose or N-acetylgalactosamine residues control both the elimination of the fusion protein from blood and its extravasation properties. Low levels of terminal sialic acids can lead to unacceptably fast clearance as previously described (324). A contribution to protein clearance of either the asialoglycoprotein receptor and of the mannose receptor would be compatible with our analytical data.

Changes in bioavailability and extravasation properties of biopharmaceuticals, including therapeutic antibodies, can have a profound impact on safety and therapeutic action (1). Indeed, substantial differences in pharmacokinetics and biological activities have previously been reported for products undergoing changes in manufacturing processes (379). However, variations in extravasation rates and disease-homing properties may often go unnoticed, unless quantitative biodistribution studies are performed. The findings of this study suggest that the engineering and development of biopharmaceuticals may favor either a complete absence of glycostructures or, alternatively, the engineering of well-defined sialylated carbohydrates (380-382). Pharmacokinetic and biodistribution studies combined with thorough glycan characterization appear mandatory for therapeutic glycoproteins, especially when the production process is modified or when biosimilar products are developed.

4.5.3 Material and methods

4.5.3.1 Cloning of F8-IL9 fusion proteins

Mouse Interleukin-9 cDNA clone (Sino Biologicals Inc.) was amplified by PCR using the forward primer link15-mIl9_for (5'-TCAGCCGGCGGTGGCTCTGCCTGGAGGTGCTCTGGCGGTGGCAGATGAAGATGCAGCACCACATGGGGC-3') that appends the C-terminal part of the flexible (Gly4Ser)3-linker sequence and the
reverse primer mIL9-NotI_rev (5’-TTTTCCTTTTGCACGCTACTATGG TGGCTTTTCTGCCTTTGCTACTCTC -3’) containing two stop codons and a NotI restriction site. The F8 diabody gene was PCR amplified using the primers SIP-F8_for (5’-CCTGTCCTCGTGGCTACAGGTGTGCAGCGCAGCTGTGGC -3’) that appends part of the signal peptide sequence and F8-link15_rev (5’-CCGCCAGAGCCACCTCC GCCTGAACCCTCCACCTTTGATTCCACCTGGGTCCTGGG -3’) which encodes the N-terminal part of the (Gly4Ser)3-linker. The mouse IL9 and F8-diabody DNA fragments were PCR-assembled and amplified using primers Nhel_Sip_for (5’-CCCCTATCGAGCTGGCCGTGGAACATGGGTCCTGGGATCTCC TGTTCCCTGCTGCTGTGGC -3’), containing a Nhel restriction site followed by the N-terminal part of the SIP sequence, and mIL9-NotI rev. The PCR-assembled full length F8-IL9 immunocytokine gene was double-digested with Nhel and NotI restriction endonucleases (New England Biolabs) and ligated into the mammalian cell expression vector pcDNA3.1(+) (Life Technologies) with T4 ligase (New England Biolabs).

### 4.5.3.2 Cell lines and protein expression

Transiently expressed F8-IL9 fusion proteins were produced in suspension cultures of CHO-S cells (Life Technologies) using transient gene expression methodology as previously described. The medium was composed of a 1:1 mixture of ProCHO4 (Lonza) and PowerCHO-2CD (Lonza), both supplemented with HT supplement, i.e. 100µM hypoxanthine and 16µM thymidine (Life Technologies), 2mM Ultraglutamine (Lonza), and 1% antibiotic-antimycotic solution (Life Technologies). Protein expression occurred in a shaking incubator at 31°C for 6 days, starting at an initial cell density of 1 x 106 cells/ml.

To generate stably transfected cells, 10ml of the transient gene expression culture were taken 24h after transfection, centrifuged (at 1000 x g for 4min) and resuspended in RPMI medium (Life Technologies) supplemented with 10% fetal calf serum (FCS), 1% antibiotic-antimycotic solution (Life Technologies) and
0.5mg/ml Geneticin® (Life Technologies). Stable integration into the CHO-S genome was achieved after incubation for >30 days at 37°C and 5% CO2 under antibiotic selection. Polyclonal stably transfected cells were then grown in suspension at 37°C in PowerCHO-2CD medium (Lonza) supplemented as described above with HT supplement, Ultra glutamine, and antibiotic-antimycotic solution. As soon as the cells reached a cell density of 4.5 to 5 x 10^6 cells/ml, cultures were transferred to a 31°C shaking incubator for protein expression until day 5.

F9 teratocarcinoma cells (ATCC No.: CRL-1720) were grown according to supplier’s protocol in 0.1% gelatin-coated tissue culture flasks in DMEM (Life Technologies) supplemented with 10% FCS and 1% antibiotic-antimycotic solution.

### 4.5.3.3 Protein purification and characterization

Both, stably (SE) and transiently (TGE) expressed fusion protein preparations were purified from the supernatant to homogeneity by protein-A affinity chromatography and further analyzed by SDS-PAGE (NuPAGE, Invitrogen), size exclusion chromatography on a Superdex 200 10/300GL column (GE Healthcare) and surface plasmon resonance analysis with a Biacore 3000 (GE Healthcare) on a CM5 sensor chip coated with ~1500RU EDA antigen as described before (215).

### 4.5.3.4 Immunofluorescence analysis

Ex vivo immunofluorescence analysis was performed with F9 teratocarcinoma tumors excised from 129/Sv mice (Charles River), which served as control mice in previous therapy experiments. In vivo immunofluorescence analysis was done with F9 tumors, which were excised 24h after intravenous injection and embedded in NEG 50 cryo-embedding medium (Thermo Scientific). Tumor
sections of 10µm were stained using the following primary antibodies: Goat anti-mCD31 (Santa Cruz Biotechnology) and rat anti-mIL9 antibody RM4A9 (BioLegend). Detection was accomplished with anti-goat AlexaFluor®-488 and anti-rat AlexaFluor®-594-coupled secondary antibodies (Life Technologies). The slides were analyzed with an Axioskop 2 MOT Plus microscope (Zeiss) and processed using Adobe Photoshop.

4.5.3.5 Biodistribution studies and pharmacokinetic analysis

Twelve weeks old female 129/Sv mice were subcutaneously injected in the flank with 2.5 x 10⁷ F9 teratocarcinoma cells. As soon as tumor sizes exceeded >50mm³, mice were grouped (n = 4) and ~10µg of 125I-labeled protein was injected into the lateral tail vein as described before (215). Mice were sacrificed 24h after injection, organs were excised, weighed and radioactivity was quantified with a Packard Cobra γ-counter. Values are given in percentage of injected dose per gram of tissue (%ID/g ± standard deviation). The pharmacokinetic analysis was performed as follows: The radiolabeling of F8-IL9 samples and intravenous administration into healthy 129/Sv mice (n = 4) was performed as described for the quantitative biodistribution analysis. Blood samples at suitable time-points were taken by puncturing the lateral tail vein and withdrawal of 1µL to 5µL of blood, which were analyzed with scintillation counting as described above. One mouse per group was sacrificed after 20min in order to obtain short-time biodistribution profiles. Experiments were performed under the project license granted by the Verterinäramt des Kantons Zürich, Switzerland (Bew. Nr. 42/2012). Experiments were performed under a project license granted by the Verterinäramt des Kantons Zürich, Switzerland (Bew. Nr. 42/2012).

4.5.3.6 Sialic acid-release and quantification

Sialic acids were released enzymatically via Neuraminidase (New England Biolabs), i.e. 3U enzyme per µg F8-IL9 were incubated at 37°C for 16h. The
samples for subsequent in vivo experiments were then purified via protein A affinity chromatography. Alternatively, sialic acids could also be released by mild hydrolysis in 0.5M NaHSO4 (Sigma Aldrich) requiring an incubation period of 40min at 80°C. Enzymatically released N-acetylyneuraminic acid (NeuNAc) was then quantified using a fluorimetric NeuNAc assay kit (BioVision). NeuNAc (Sigma Aldrich) represents the most common sialic acid species and was used as a standard for calibration for both read-outs. Fluorescence was measured in a SpectraMax Paradigm plate reader (Molecular Devices) at excitation/emission (Ex/Em) wavelengths of 535/587nm. Hydrolyzed sialic acids were fluorescently labeled with o-phenylenediamine (OPD, Sigma Aldrich) in 0.5M NaHSO4 for 2h at 80°C. RP-HPLC was performed with a Hitachi Lachrom D-7000 HPLC-system (Merck) equipped with an Xterra® 5µm, 4.6 x150mm C18 column (Waters). Sialic acid derivatives were eluted using an isocratic buffer system and detected Ex/Em 280/425nm as previously described (383). To verify glycosidic α2,3-linkage of terminal sialic acids, samples were incubated with 20U α2,3 neuraminidase (New England Biolabs) per µg F8-IL9 for 16h at 37°C.

4.5.3.7 Enzymatic release of N-glycan pools

N-linked oligosaccharides were released by glycerol-free PNGase F (New England Biolabs), i.e. 10U enzyme per µg protein were incubated for 24h at 37°C. Samples for biodistribution experiments were deglycosylated in PBS buffer followed by an additional protein A purification step to remove the enzyme and contaminants. All other samples were dialysed against 50mM ammonium biocarbonate (Sigma Aldrich) pH8.0 buffer before deglycosylation reactions were performed for a prolonged incubation time of up to 24 hours. Released glycans were then separated form proteins by VivaSpin 500 centrifugal filter units (Sartorius Stedim) with a 10kDa cut-off and washed with 4 x 400µL deionized water. The flow through was collected in an Eppendorf tube and vacuum dried.
4.5.3.8 HILIC-HPLC-based glycoprofiling

Vacuum-dried N-glycan samples were fluorescently labeled with 2-aminobenzamide (2-AB) (384), using a GlycoProfile labeling kit (Sigma Aldrich) and purified via GlycoClean S cartridges (Prozyme) according to the providers' instructions. Samples were again reduced to dryness in a vacuum centrifuge before they were dissolved in 200µL 50% acetonitrile/water. HPLC-HILIC was performed with the Hitachi Lachrom D-7000 HPLC-system equipped with a TSKgel Amide-80 column (TOSOH Bioscience). The injected sample volume was 12µL. Gradient elution was achieved using a buffer system consisting of acetonitrile (Sigma Aldrich) and 0.1M ammonium formate (ARCOS Chemicals) pH4.5 solution. A gradient of 0.3%/min starting with 24% 0.1M ammonium formate provided the highest-resolution. A dextran calibration ladder (Waters) was run after each sample as external standard to calibrate the system for subsequent conversion of retention times [min] into glucose units [GU].

4.5.3.9 MALDI-TOF-TOF analysis for permethylated glycans

Glycans of F8-IL9 were released by PNGase F (Promega), isolated by C18 Sep-Pak® (Waters) and permethylated as described by Dell et al. (385). Permethylated glycans samples were mixed 1:1 with dihydroxybenzonic acid matrix (15mg/ml in 75% acetonitrile in water with 0.1% FA), and then spotted onto a matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-TOF MS) target plate. Data acquisition was performed manually on Model 4800 Proteomics Analyzer (Applied Biosystems) with an Nd:YAG laser and 1,000 shots were accumulated in the reflectron positive ion mode. MALDI-TOF-TOF mass spectrometer was calibrated externally by permethylated N-glycans from RNase B (Sigma). N-glycan structures of m/z 2081.1 and 2362.2 from SE F8-IL9 were further confirmed by MALDI-TOF-TOF MS/MS. All other annotations are based on the current knowledge for N-glycosylation synthesis pathway and the m/z value of each peak was labeled as monoisotopic mass for spectra.
acquired in reflectron mode. Data interpretation was processed manually or by the help of GlycoWorkbench 2.0 (386).

4.5.3.10 Sample preparation for glycopeptides

50μg of purified F8-IL9 proteins from TGE and SE methods were digested by filter-assisted sample preparation (FASP) procedure (387) before MS measurement. Briefly, proteins were reduced by 50mM dithiothreitol in 50mM ammonium bicarbonate buffer (pH 8.5) at 37°C for 1h, following by alkylation by 65mM iodoacetamide at 37°C in the dark for 1h. After four times washing of filter device by ammonium bicarbonate buffer, proteins were digested by sequencing-grade modified trypsin (Promega) at a ratio of 50:1 at 37°C overnight. All digested peptides and glycopeptides were collected by centrifugation and dried by vacuum centrifuge. Additionally, two-thirds of the samples were further treated with Glu-C endopeptidase (Promega), AspN endopeptidase (Promega), neuraminidase (Calbiochem) and PNGase F (Roche) individually. All samples were desalted by Zip-Tip® C18 (Millipore) prior to nanoLC-MS/MS analysis.

4.5.3.11 Glycopeptide analysis by nanoHPLC-HCD-MS/MS

Samples were analyzed on a calibrated LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific) coupled to an Eksigent-Nano-HPLC system (Eksigent Technologies). Peptides were resuspended in 2.5% acetonitrile and 0.1% formic acid and loaded on a self-made fritted column (75μm × 150mm) packed with reverse phase C18 material (AQ, 1.9μm 200Å, Bischoff GmbH) and eluted with a flow rate of 300nl per min by a gradient from 3 to 30% of B in 22min, 50% B in 25min, 97% B in 27min. One scan cycle comprised of a full scan MS survey spectrum, followed by up to 10 sequential HCD MS/MS on the most intense signals above a threshold of 2000. Full-scan MS spectra (700–2000 m/z) were acquired in the FT-Orbitrap at a resolution of 60,000 at 400 m/z, while HCD MS/MS spectra were recorded in the FT-Orbitrap at a resolution of 15,000 at 400
m/z. HCD was performed with a target value of 1e5 and stepped collision energy rolling from 35, 40 and 45 V was applied. AGC target values were 5e5 for full FTMS. For all experiments, dynamic exclusion was used with 1 repeat count, 15s repeat duration, and 60s exclusion duration.

4.5.3.12 Database analysis and site-specific glycosylation analysis

MS and MS/MS data were processed into the Mascot generic format (mgfs) files and searched against Swissprot database (version 201408) through Mascot engine (version 2.4) with the consideration of carbamidomethylation at cysteine and oxidation at methionine. For PNGase F digestions, deamination was considered as a variable modification. The monoisotopic masses of 2+ or more charged peptides were searched with a peptide tolerance of 10 ppm and a MS/MS tolerance of 0.25Da for fragment ions. Only peptides with a maximum of two missing cleavage sites were allowed in database searches. Positive identification of deaminated peptides was performed using a variety of strict criteria including manual inspection of spectra. For site-specific glycosylation analysis, it was proceeded according to our unpublished study (388) and all data were interpreted manually. Here, XCalibur 2.2 sp1.48 was used for data analysis.
5 Conclusions and outlook

There is an increasing interest in pharmaceutical research activities aimed at the development of targeted therapies. The arming of antibodies with bioactive payloads, in order to achieve a selective localization at the site of disease while sparing normal tissue, is a promising avenue to overcome pharmacokinetic limitations (e.g., systemic toxicity), which may hinder the therapeutic performance of untargeted bioactive agents. Targeted drugs are able to specifically localize at sites of disease, thus increasing the therapeutic index of the corresponding untargeted drug.

All projects presented in the thesis started with the design, cloning and expression of novel fusion proteins for cancer therapy. Even though all moieties used to generate fusion proteins were already well characterized, we did not know a priori whether the corresponding fusion protein could be expressed, would be well behaved from a biochemical perspective and would display the desired therapeutic activity in vivo. This thesis aimed at exploring the tumor targeting and, when possible, also the therapeutic performance of novel fusion proteins based on the F8 antibody and a variety of murine chemokines and cytokines in mouse models of cancer. The development of immunocytokines and immunochemokines is facilitated by the ease of expression of these products as fusion proteins, without the need for subsequent chemical modification. We have performed the first comprehensive characterization of antibody-chemokine fusion proteins for tumor targeting applications. Only four out of ten immunochemokines could be reproducibly expressed in mammalian cells. Yields and downstream processing were substantially worse, compared to the closely related and intensively studied class of immunocytokines. When injected into tumor bearing mice, all chemokine payloads led to abrogation of the tumor homing properties of the parental antibody. Unfavorable biophysical properties may prevent an efficient tumor targeting process. The observation that prior application of unlabeled CCL19-F8 led to an improved tumor to organ ratio, suggests that a receptor-mediated trapping mechanism in the bloodstream or an organ may hinder an efficient tumor homing process of immunochemokines. Expression of chemokine receptors in the liver or fast clearance of the immunochemokines via
the hepatobiliary route, may explain the observed high liver uptake after 20 min. Further, for many chemokines the formation of dimers or higher order oligomers has been described (389) suggesting that also oligomerization or aggregation processes may hinder extravasation due to the high molecular weight of the resulting complexes. **Figure 46** shows CCL5 oligomerization and how this oligomers may interact with GAGs.

![Figure 46](image-url)

**Figure 46.** CCL5 oligomerizes to even order structures in a pH-dependent manner (left). The model exposes GAG- and CCR5-binding residues, for simultaneous interaction (right). Adapted from (390).

As described in the literature (391) and reflected by the (initial) degradation problems when expressed as fusion proteins, chemokines are the substrate of various proteases, making them susceptible to degradation. Since a preferential tumor targeting could not be observed in biodistribution studies, antibody chemokine fusion proteins seem to be stable *in vivo*, as their proteolytic degradation would lead to parental antibody fragments, which are known to selectively locate to tumors. We could show that addition of heparin to the expression medium prevented CXCL10 from *in vitro* degradation. The expression of other chemokines did not benefit to the same extent from the addition of this GAG. For future experiments, it may be interesting to explore the addition of other GAGs or GAG mixtures for their stabilizing effect during immunochemokine expression. However, the addition of GAGs could potentially promote the formation of high molecular weight complexes, thus hindering pharmacodelivery experiments. CXCL10 has been shown to form three different tetramers (M, H and L forms). The M tetramer shows basic residues in each monomeric unit being
arranged on the surface to form a continuous binding strip across the entire oligomeric structure, thus facilitating interaction with GAGs (389). If indeed also four CXCL10 molecules fused to antibodies were able to arrange in this conformation, the formation of a high molecular weight complex would be therefore preventing efficient tumor homing. In principle the engineering of non-oligomerizing or non-GAG binding chemokine mutants could be considered for the antibody mediated delivery to the tumor site. However, both oligomerization and GAG binding seem to be important for chemokine function (389) and may result in mutants with impaired functionality. Whether the choice of alternative antibody formats (e.g. IgG) may improve biodistribution results remains to be investigated. There is a single report in the literature describing an IgG based immunochemokine capable of selective accumulation at the tumor site (345). Antibody engineering plays an important role in the development process, since the choice of antibody format affects the pharmaceutical quality of fusion proteins and has a significant impact on the disease-targeting performance. The IgG format has often been used for the development of immunocytokines, despite its long circulatory half-life. The difficulty in producing immunochemokines capable of selective tumor targeting in vivo, prevented the possibility to study whether in vivo chemokine gradients could be generated ‘on demand’. It is also not known whether immunochemokines can alter the vasculature at the site of disease, or regulate the function of immune cells. The characterization of protein production and the results of quantitative biodistribution studies, as performed in this thesis, should facilitate future research efforts in the field of immunochemokines, while critically increasing awareness about the impact which payloads may have on the efficiency of antibody-based pharmacodelivery strategies.

The tumor targeting performance of the same F8 antibody is dramatically different, when fused to cytokines compared to chemokines (313, 314). Multiple immunocytokines have already been cloned and expressed and a wealth of biodistribution data has been collected in animals, thus allowing a comparative evaluation of different fusion proteins. Many F8-based immunocytokines exhibit a preferential tumor accumulation in preclinical models of cancer. Preclinical studies with some of our immunocytokine products have exhibited promising
results with eradication of cancer in a number of murine models. However, there is no certainty that a cytokine will exhibit a comparable therapeutic activity in species as different as mouse and man. Similarly, using the same immunocytokine, different therapeutic outcomes can sometimes be observed in different mouse models of cancer. As single agents, immunocytokines rarely exhibit complete cures of cancer in animal models and in patients, while combination therapy modalities have resulted in complete and long-lasting tumor eradications.

The anti-cancer activity of the novel immunocytokines based on IL1β and IL6 investigated in this thesis was (in the F9 teratocarcinoma model of cancer) not as potent as the one of other products based on the F8 antibody (e.g., F8-TNF) (109, 268, 356). Conflicting reports about their influence on tumor growth had indicated either a beneficial or a detrimental effect, depending on the administered dose of IL1β and IL6. We expected that high cytokine concentrations at the tumor site could contribute to a strong pro-inflammatory environment in the tumor mass and a beneficial outcome, but this hypothesis was not experimentally confirmed. It is conceivable however, that F8-IL1β and F8-IL6, two fusion proteins of proven in vivo targeting performance, find an application for therapeutic intervention in a non-oncological indication. However, the results of this study suggest that neither IL1β nor IL6 represent promising payloads for the development of anti-cancer immunocytokine products.

The F8-IL13 immunocytokine was able to selectively home to tumor blood vessels. In subsequent therapy studies, this fusion protein displayed a potent single-agent activity, which however did not result in cancer cures. In combination with IL12-F8F8, long-lasting tumor eradications could be observed and Balb/c mice cured from Wehi-164 tumors were even able to reject subsequent challenges with tumor cells, indicating the induction of a protective immunity. The fact that a similar effect could not be observed in the F9 teratocarcinoma tumor model indicated that the mouse strain and intrinsic tumor characteristics may contribute to the induction of anticancer immunity. Depletion studies revealed that F8-IL13 conferred a potent anti-tumor activity mainly through the action of CD4+ T cells. This observation is in contrast to all previous reports, in which CD8+ and
NK cells were found to be the main contributors to antitumor immunity. F8-IL13 seems to act through a different mechanism of action and may therefore display also synergistic effects with those previously studied immunocytokines acting mainly through the action of CD8+ cells. It is possible that the anti-tumor effect of IL13 is mediated by suppression of IL17A production at the tumor site, since there is evidence that polarized murine CD4+ Th17 cells express a functional IL13-receptor and that IL13 signaling attenuates Th17 cytokine (IL17A) production (392). Moreover, it has been shown that blockade of IL-17A at tumor sites may help suppress tumor growth by inhibiting angiogenesis and activating cytotoxic T lymphocytes at tumor sites (393). IL17A expression has also been shown to promote chemoresistance and proliferation of tumor cells (394).

As intrinsic tumor characteristics may contribute to the induction or absence of anticancer immunity, it is largely unknown why some patients respond well to therapy and some do not. Similarly, the same immunocytokine can lead to variable therapeutic efficacies in different mouse models of cancer. For the future, it remains to be studied if F8-IL13 is as effective in humans as it is in mice and whether the tumor rejecting immunological mechanisms observed in mice also apply to the human situation. In addition, it will be important to learn whether a synergy between tumor targeted IL12 and IL13 can be observed also in humans. There are several examples where synergistic effects have been observed when immunocytokines were combined with other immunocytokines or chemotherapeutic drugs. However the underlying mechanisms are at present not known and should be further investigated in order to allow the rational design of future combination studies. The dose, application method, duration, schedule and even the sequence of administration can profoundly impact on the success of therapeutic intervention. Because the EDA of fibronectin is conserved in mouse and man and the fact that the F8 antibody recognizes the majority of human cancer types (215), F8-IL13 represents an attractive candidate for industrial development activities in oncology. The therapeutic effect of F8-IL13 in murine models of cancer seems to be at least as strong as the one of F8-IL4, an immunocytokine that has recently begun industrial development. As for other immunocytokine products, due to potentially different cytokine functions in mouse
and man, it is difficult to predict clinical activity on the basis of preclinical data. The strong anti-cancer activity observed in murine models and the rather high concentrations that can be administered may be positive in terms of dosing and product safety in humans. However, it is not known if the same side effects, such as diarrhea would occur also in humans. Furthermore, IL13 is a highly glycosylated cytokine what might lead to additional complications in industrial development as the experiments with the four fold N-glycosylated F8-IL9 immunocytokine have shown.

The impact of glycosylation on the targeting performance of armed antibodies has not been intensely studied in the past, in part due to the inherent heterogeneity of glycan structures and to the lack of suitable model systems. It has been already known that low levels of terminal sialic acids can lead to unacceptably fast clearance of glycoproteins via hepatic glycan receptors (324), but as we discovered for F8-IL9, different manufacturing processes can lead to varying amounts of terminal sialic acids, with a dramatic impact on the antibody’s tissue localization and clearance from the body. Indeed, even small changes in manufacturing processes can lead to dramatic changes in bioavailability of biopharmaceuticals and can have a profound impact on safety and therapeutic efficacy (1, 379). In order to avoid that changes in extravasation rates and disease-homing properties of biopharmaceuticals may compromise pharmaceutical performance, quantitative biodistribution studies together with pharmacokinetic studies should be routinely performed. Development of biopharmaceuticals may therefore favor either complete absence or engineering of defined (highly sialylated) carbohydrates (380-382). These considerations are not only relevant for protein design purposes, but also for changes in the manufacturing process and for the development of biosimilar products. Protein preparations of F8-IL9, which have been obtained by transient gene expression, show selective tumor targeting performance in vivo. The therapeutic activity of these proteins would now deserve to be investigated in mice.

The fact that not all payloads could be efficiently delivered to the site of disease in vivo underlines the importance of performing quantitative biodistribution studies. Such investigations provide information about the in vivo tissue
distribution of a certain biopharmaceutical product at an early development stage, thus allowing to stop projects which exhibit PK liabilities. Surprisingly, the tumor targeting properties even of members of the same family of immunomodulatory proteins may be strikingly different, as we have reported for, members of the TNF superfamily (e.g. F8-VEGI vs F8-TNF). The different PK and tumor targeting profiles were observed, in spite of the fact that these proteins are very similar in terms of their molecular weight, protein fold, and valence. Due to the unfavorable tissue distribution of F8-VEGI and F8-VEGItrunc no further experiments are planned with this immunocytokine.

In summary, our work and the work of other groups indicates that immunocytokines can be efficient biopharmaceutical agents, with potent activity in mice and in humans, but a careful characterization of the disease-homing properties and of biochemical features is mandatory, in order to judiciously select the best candidates for product development.
6 Abbreviations

ALL  acute lymphoblastic leukemia
AA   amino acid
ALCL anaplastic large-cell lymphoma
Immunocytokines antibody chemokine fusion proteins
ADCC antibody dependent cellular cytotoxicity
ADCs antibody drug conjugates
AG   antigens
BCR  B cell receptor
BiTEs bispecific T cell engagers
CEA  carcinoembryonic antigen
CHO  Chinese hamster ovary
CLL  chronic lymphocytic leukemia
CSF  colony-stimulating factors
CDC  complement dependent cytotoxicity
CTL  cytotoxic T lymphocytes
DG   deglycosylated
DCs  dendritic cells
DS   desialylated
DTPA diethylenetriaminepentaacetic acid
DNL  Dock-and-Lock®
DART dual-affinity retargeting
ELR  Glu-Leu-Arg
EpCAM endothelial cell adhesion molecule
EGF  epidermal growth factor
EGFRRvIII epidermal growth factor receptor variant III
<table>
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<th>Abbreviation</th>
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<tbody>
<tr>
<td>EDA</td>
<td>extra domain A</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>FcγRs</td>
<td>Fc receptors</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>Fn</td>
<td>Fibronectin</td>
</tr>
<tr>
<td>GAGs</td>
<td>glycosaminoglycans</td>
</tr>
<tr>
<td>GPCRs</td>
<td>G-protein coupled receptors</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>HILIC</td>
<td>hydrophilic interaction chromatography</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>hypoxia inducible factor</td>
</tr>
<tr>
<td>IL1RA</td>
<td>IL1-receptor antagonist</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulins</td>
</tr>
<tr>
<td>Immune-PET</td>
<td>immuno-positron emission tomography</td>
</tr>
<tr>
<td>ITAM</td>
<td>immunoreceptor tyrosine-based activation motifs</td>
</tr>
<tr>
<td>%ID/g</td>
<td>percent injected dose per gram</td>
</tr>
<tr>
<td>IGF</td>
<td>insulin-like growth factor</td>
</tr>
<tr>
<td>IL12</td>
<td>Interleukin-12</td>
</tr>
<tr>
<td>IL2</td>
<td>Interleukin-2</td>
</tr>
<tr>
<td>IL9</td>
<td>Interleukin-9</td>
</tr>
<tr>
<td>i.t.</td>
<td>intratumoral</td>
</tr>
<tr>
<td>i.v.</td>
<td>intravenous</td>
</tr>
<tr>
<td>LTα</td>
<td>lymphotixin alpha</td>
</tr>
<tr>
<td>DOTA</td>
<td>macrocyclic tetraazacyclododecane-tetraacetic acid</td>
</tr>
<tr>
<td>MTD</td>
<td>maximum tolerated dose</td>
</tr>
<tr>
<td>MAC</td>
<td>membrane attack complex</td>
</tr>
<tr>
<td>mAbs</td>
<td>monoclonal antibodies</td>
</tr>
</tbody>
</table>
mIL9  
murine interleukin-9

AML  
myeloid leukemia

NeuNAc  
N-acetylyeuraminic acid

PNGase F  
peptide N-glycosidase F

PBS  
phosphate buffered saline

PCR  
polymerase chain reaction

PSMA  
prostate-specific membrane antigen

PKA  
protein kinase A

RIT  
Radioimmunotherapy

rhIL1β  
recombinant human IL1β

SIP  
signal peptide

scFv  
single-chain Fv

SIP  
small immune protein

SE  
stable expression

s.c.  
subcutaneous

SPR  
surface plasmon resonance

TandAb  
tandem diabody

TNFR  
TNF receptor

TGF  
transforming growth factors

TGE  
transient gene expression

T-DM1  
trastuzumab-DM1

VEGItrunc  
truncated version of VEGI

TNF  
tumor necrosis factor (formerly known as TNFα)

VEGF  
vascular endothelial growth factor

VEGI  
vascular endothelial growth inhibitor
7 References


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202. Buhmann R, Michael S, Juergen H, Horst L, Peschel C, Kolb HJ. Immunotherapy with FBTA05 (Bi20), a trifunctional bispecific anti-CD3 x anti-CD20 antibody and donor lymphocyte infusion (DLI) in relapsed or refractory B-cell lymphoma after allogeneic stem cell


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I am truly thankful to many friends who have been always supportive over the years and helped me to clear my mind whenever necessary. My deepest thanks go to my family and to Cirille for their love and support over the last couple of years as well as all the years before.
9 Appendix

9.1 F8-IL5 and KSF-IL5 immunocytokines

When F8-IL13 and KSF-IL13 were cloned and expressed, F8-IL5 and KSF-IL5 immunocytokines were cloned and expressed in parallel. These fusion proteins could be expressed with an acceptable yield (< 5 mg/L) but the size exclusion chromatography profile on a Superdex 200 column revealed the presence of F8-IL5 multimers. The fractions of the monomeric peak were collected and re-purified by SEC on the same column, resulting in a single peak and a protein of good pharmaceutical quality. The purified protein was then further analyzed by ELISA and SPR in analogy to F8-IL13 described above. A biodistribution in F9 tumor bearing mice revealed specific accumulation at the tumor site (>16 % ID/g). However, in a preliminary therapy experiment performed with one F9 tumor bearing mouse per group no therapeutic benefit could be observed. One of the two mice injected with F8-IL5 developed a 2nd tumor at the front left extremity at day 14 leading to the decision to stop the further investigation of this immunocytokine.
Figure A1. Cloning, expression, purification of the F8-IL5 immunocytokine. (a) SDS-PAGE analysis of F8-IL5 under non-reducing (NR) and reducing (R) conditions. (b) SEC purification on a S200 column, the peak corresponding to the monomeric fraction was collected and (c) re-purified on the same column. (d) ELISA analysis on EDA coated SA wells. (e) SPR analysis on an EDA coated sensor chip (concentrations between 250 nM and 1 μM). (f) Size exclusion profile of KSF-IL5 on a S200 column. (g) Biodistribution profile of F8-IL5 in F9 bearing 129Sv mice, 10 μg of radioiodinated protein were injected i.v. and the mice were sacrificed after 24 hours, organs were weighted and radioactivity was measured. (h) Preliminary therapy experiment in 129Sv mice with F9 tumors, administered doses are indicated (arrows).
### 9.2 Nucleotide Sequences

- **TCACCA** → Cytokine/Chemokine sequence
- **GGTGG** → GS-linker sequence
- **GCAGC** → F8 V_{H} sequence
- **ATTGT** → F8 V_{L} sequence

**CCL5-F8**

```
TCACCATATGGCTCGGACACCCACCACCTCCCTGCTGCTTGGCTACCTCCCTCGCCGCTGCTGTCCTGCCTGCCTGCCCAGTCAAGGAACTGTGCTATCTGCTGACCTCCCAAGAGAAGAGTTGAGGGGTCTGGGACAGACTTCACTCTCACCATCAGCAGACTGGAGCCTGAAGATTTTGCAGTGTATTA
```

**CCL19-F8**

```
GGTGCTAATGATCGGGAAGACTGTGCTGCTGACCCAGCAGCCCATCCCTCCCTGGGAACATCAGTGGAAAGAAGAATGCTGCTCTCTCGCCAGAGAGAAGGAGGCCTGTATATTACTGTGCGAAAAAATCTCATTTGTATCTTTTTGACTACCTGGGGCCAGGAACCCTGGTCACCGTCTCGAGTGAGTGGGTCTCAGCTATTAGTGGTAGTGGTAGCACATACTACGCAGACTCCGTGAAGGGCCGGTTCACCATCTCCAGAGACAATTCCAAGAACACGCTGTATCTGCAAATGAACAGCCTGAGAGCCGAAGAACGGCCGTATATTACTGTGCGAAAAGTACTCATTTGTATCTTTTTGACT
```

188
CXCL10-F8

ATCCCTCTCGCAAGGACGGTCCGCTGCAACTGCATATCGATGACGGGCCAGTGAGAATGAGGCCATAGGGAAGCTTGAAGGGCCATAGGGAAGCTTGAATACCATCCCTGCGAGCCTAT
CTCTGCCCACGTGTTGAGATCCTG

F8-Il1β

GAGGTGCAGCTTGGAGATCTGGGGAGGAGGCTTGCTGAGCTGAGCTGGTGCTTCGGACGGCTCAGG
GAAGGGGGCTGAATCTGCTTCTATACAGGCTCCGAAGGCTGACAGAATATCAACAAACAT

190
F8-IL6

GAGGTGCAGCTGGGAGTCTGGGAGGCTTGATACAGCTGGGAGGCTTGACAGCTGGGGGGTCCCTGAGACTCTCCTGTGCAGCCTCTGGATTACCTTTAGCCTGTTTACGATGAGCTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTCTCAGCTATTAGTGGTAGTGGTGGTAGCACATACTACGCAGACTCCGTGAAGGGCCGGTTCACCATCTCCAGAGACAATTCCAAGAACACGCTGTATCTGCAAAATGAACAGCCCTGAGAGCCGAGGACACGGCCGTATATTACTGTGCGAAAAGTACTCATTTGTATCTTTTTGACTACTGGGGCCAGGGAACCTGGTCACCGTCTCGAGTCAGGCGGATCTGGCGAAATTGTGTTGACGCAGTCTCCAGGCACCCTGTCTTTGTCTCCAGGGGAAAGAGCCACCCTCTCCTGCAAGGGCCAGTCAGAGTGTTAGCATGCCGTTTTTAGCCTGGTACCAGCAGAAACCTGGCCAGGCTCCCCAGGCTCCTCATCTATGGTGCATCCAGCAGGGCCACTGGCATCCCAGACAGGTTCAGTGGCAGTGGGTCTGGGACAGACTTCACTCTCACCATCAGCAGACTGGAGCCTGAAGATTTTGCAGGTATTACTGTCAGCAGATGCGTGGTCGGCCGCCGACGTTCGGCCAAGGGACCAAGGTGGAAAATCTGAAGAATTTCTAAAAGTCACCACTGACGAGATGCTACCAAACTGGATATAATCAGGAAATTTGCTATTTGGAATTTTCCTCGTGTCTCTCCCTCTGACCCTTAAGGAGCTTATTGAGGAGCTGAGCAACATCACACAAGACCA

F8-IL13

GAGGTGCAGCTGGGAGTCTGGGAGGCTTGATACAGCTGGGAGGCTTGACAGCTGGGGGGTCCCTGAGACTCTCCTGTGCAGCCTCTGGATTACCTTTAGCCTGTTTACGATGAGCTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTCTCAGCTATTAGTGGTAGTGGTGGTAGCACATACTACGCAGACTCCGTGAAGGGCCGGTTCACCATCTCCAGAGACAATTCCAAGAACACGCTGTATCTGCAAAATGAACAGCCCTGAGAGCCGAGGACACGGCCGTATATTACTGTGCGAAAAGTACTCATTTGTATCTTTTTGACTACTGGGGCCAGGGAACCTGGTCACCGTCTCGAGTCAGGCGGATCTGGCGAAATTGTGTTGACGCAGTCTCCAGGCACCCTGTCTTTGTCTCCAGGGGAAAGAGCCACCCTCTCCTGCAAGGGCCAGTCAGAGTGTTAGCATGCCGTTTTTAGCCTGGTACCAGCAGAAACCTGGCCAGGCTCCCCAGGCTCCTCATCTATGGTGCATCCAGCAGGGCCACTGGCATCCCAGACAGGTTCAGTGGCAGTGGGTCTGGGACAGACTTCACTCTCACCATCAGCAGACTGGAGCCTGAAGATTTTGCAGGTATTACTGTCAGCAGATGCGTGGTCGGCCGCCGACGTTCGGCCAAGGGACCAAGGTGGAAAATCTGAAGAATTTCTAAAAGTCACCACTGACGAGATGCTACCAAACTGGATATAATCAGGAAATTTGCTATTTGGAATTTTCCTCGTGTCTCTCCCTCTGACCCTTAAGGAGCTTATTGAGGAGCTGAGCAACATCACACAAGACCA

191
F8-VEGI

GAGGTGCAGCTGTTGGAGTCTGGGAGGTTCAGGCTAGCCTGGGGGTCCCTGAGACTCTCCTGTGCAAGCTGTTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTCCCTGAGACTCTCCTGTGCAGCCTCTGGATTCACCTTTAGCCTGTTTACGATGAGCTGGGTCCGCCAGGCTCCAGGGGAAGGGGCTGGAGTGGGTCTCAGCTATTAGTGGTAGTGGTGGTAGCACATACTACGCAGACTCCGTGAAGGGCCGGTTACCATCTCCAGAGACAATTCCAAGAACACGCTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCCGTATATTACTGTGCGAAAAGTACTCATTTGTATCTTTTGGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCGAGTGTTGGAGGCGGTTCAGGCGGAAGTGGCTCTGGCGGTGGCGGAAGAATTGTGTTGACGCAGTCTCCAGGCACCCTGTCTTTGTCTCCAGGGGAAAGAGCCACCCTCTCCTGCAGGGCCAGTCAGAGTGTTAGCATGCCGTTTTTAGCCGTGGTACCAGCAAACCTGGCCAGGCTCCCAGGCTCCTCATCTATGGTGCATCCAGCAGGGCCACTGGCATCCCAGACAGGTTCAGTGGCAGTGGGTCTGGGACAGACTTCACTCTCACCATCAGCAGACTGGAGCCTGAAGATTTTGCAGTGTATTACTGTCAGCAGATGCGTGGTCGGCCGCCGACTTCGGCCAAGGGACCAAGGTGGAAATCAAAATCTTCCTCATCGGGTAGTAGCTCTTCCGGCTCCTAGTCCAGCGGCCTACCTCCCAGAGGCAAGCCGAGAGACACACCTGACAATTAAGAAACCCCAGCACCACATCTGAAAATACGCTCCTGCTCTACACTGGGAACATGACCTAGGGGATGAAGTACATCAACAAATCCCTGGTGATCCCAGAGTCAGGACTATTTCATCTACTCCCAGATCACATTCCGAGGGACCACAATCTGTGTGTGGTGACATCAGTCGGGGGAGACGACCAAACAGTGAGAGCAGACTCCATCACCATGGTTATACGAGACAGCTACATGAAACGTCAGTGCATCCTTGGGATTACACAACAAAAGAAGATAAACCTTCTTGGAGCCTTCTTCTTGGCTA

F8-VEGItrunc

GAGGTGCAGCTGTTGGAGTCTGGGAGGTTCAGGCTAGCCTGGGGGTCCCTGAGACTCTCCTGTGCAAGCTGTTGGAGTCTGGGGGAGGCTTGGTACAGCCTGGGGGGTCCCTGAGACTCTCCTGTGCAGCCTCTGGATTCACCTTTAGCCTGTTTACGATGAGCTGGGTCCGCCAGGCTCCAGGGGAAGGGGCTGGAGTGGGTCTCAGCTATTAGTGGTAGTGGTGGTAGCACATACTACGCAGACTCCGTGAAGGGCCGGTTACCATCTCCAGAGACAATTCCAAGAACACGCTGTATCTGCAAATGAACAGCCTGAGAGCCGAGGACACGGCCGTATATTACTGTGCGAAAAGTACTCATTTGTATCTTTTGGACTACTGGGGCCAGGGAACCCTGGTCACCGTCTCGAGTGTTGGAGGCGGTTCAGGCGGAAGTGGCTCTGGCGGTGGCGGAAGAATTGTGTTGACGCAGTCTCCAGGCACCCTGTCTTTGTCTCCAGGGGAAAGAGCCACCCTCTCCTGCAGGGCCAGTCAGAGTGTTAGCATGCCGTTTTTAGCCGTGGTACCAGCAAACCTGGCCAGGCTCCCAGGCTCCTCATCTATGGTGCATCCAGCAGGGCCACTGGCATCCCAGACAGGTTCAGTGGCAGTGGGTCTGGGACAGACTTCACTCTCACCATCAGCAGACTGGAGCCTGAAGATTTTGCAGTGTATTACTGTCAGCAGATGCGTGGTCGGCCGCCGACTTCGGCCAAGGGACCAAGGTGGAAATCAAAATCTTCCTCATCGGGTAGTAGCTCTTCCGGCTCCTAGTCCAGCGGCCTACCTCCCAGAGGCAAGCCGAGAGACACACCTGACAATTAAGAAACCCCAGCACCACATCTGAAAATACGCTCCTGCTCTACACTGGGAACATGACCTAGGGGATGAAGTACATCAACAAATCCCTGGTGATCCCAGAGTCAGGACTATTTCATCTACTCCCAGATCACATTCCGAGGGACCACAATCTGTGTGTGGTGACATCAGTCGGGGGAGACGACCAAACAGTGAGAGCAGACTCCATCACCATGGTTATACGAGACAGCTACATGAAACGTCAGTGCATCCTTGGGATTACACAACAAAAGAAGATAAACCTTCTTGGAGCCTTCTTCTTGGCTA
TACCCTGAGCTGCCCCCTTACTAACAGGGTCCAAGTCTGTTGGAATAAGCAACAATTGGGTCCAGTCGTAAT

**F8-IL9**

GAGGTGCAGCTTTGGAGTCTGGGGGAGGCTTGGGTACAGCCTGGGGGGTCCCTGAGACTACCTCTCTGTGCAGCCTCTGGATTCACCTTTAGCCTGTTTACGATGAGCTGGGTCCGCCAGGCTCCAGGGAAGGGGCTGGAGTGGGTCTCAGCTATTAGTGGTAGTGGTGAAGACAGACTACCGAGACTCGTGAAGGGCCGCTTACCACATCTCTCTACCTGCTGTAACGAGCAGACGACTCTGCTCGAGGGGTAGCGGGAGGTGGCTCTGGCGGTGGCGGATCAAGATGCAGCACCACATGGGGCATCAGAGACACCAATTACCTTATTGAAATCTGAAGGATGATCCACCAGGTATTACTGTCAGCAGATGCGTGCTTCTGTACGTTCGGCCAAGGGACCAAGGTGGAAAAAGTCAAAAGGTGGAGGCGGTTACAGCTGACCAATGCCACACAGAAATCAAGACATCTTGCCTGTTTTCCATCGGATGAAAAGGATAGTTGAAGTCCTAAAGAACATCACGTGTCGCTTTTCCTGCGAAAAGCCAAGCAACCAGACCATGGCAGGATGGCAGCACCACATGEGAAGGAAGACTGTACGTACAGCTTCTCTTCCTGCTCCACGTGGTTAATTTCAGAGATGCAAAGGCAAAGACCTGGAAGAAGGAGTCCACGTTTACAGCTGACCAATGCCACACAGAAATCAAGACATCTTGCCTGTTTTCCATCGGATGAAAAGGATAGTTGAAGTCCTAAAGAACATCACGTGTCGCTTTTCCTGCGAAAAGCCAAGCA

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### Primer sequences for chemokine amplification:

<table>
<thead>
<tr>
<th>cDNA clone</th>
<th>Gene bank ref. ID</th>
<th>Primer Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Murine CCL5</td>
<td>NM_013653.3</td>
<td>CCL5-SIP_FW: CCTGTTCCTCGTCGCTGTGGCTACAGGTTGCGACTCGTCACCATAATGGCTCGGACACCACCTC &lt;br&gt; CCL5-LINKER_REV: CGGCCAGGACCCACCTCGCCGCTGAACCGCCTCCACCGCTCATCTCAAAATAGTTGATG</td>
</tr>
<tr>
<td>Murine CCL17</td>
<td>NM_011332.2</td>
<td>CCL17-SIP_FW: CCTGTTCCTCGTCGCTGTGGCTACAGGTTGCGACTCGGACAGCTCAGGCCGACCAATG &lt;br&gt; CCL17-LINKER_REV: CGGCCAGGACCCACCTCGCCGCTGAACCGCCTCCACCGCTCATCTCAAAATAGTTGATG</td>
</tr>
<tr>
<td>Murine CCL19</td>
<td>NM_011888.2</td>
<td>CCL19-SIP_FW: CCTGTTCCTCGTCGCTGTGGCTACAGGTTGCGACTCGGACAGCTCAGGCCGACCAATG &lt;br&gt; CCL19-LINKER_REV: CGGCCAGGACCCACCTCGCCGCTGAACCGCCTCCACCGCTCATCTCAAAATAGTTGATG</td>
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<tr>
<td>Murine CCL20</td>
<td>NM_016960.1</td>
<td>CCL20-SIP_FW: CCTGTTCCTCGTCGCTGTGGCTACAGGTTGCGACTCGGACAGCTCAGGCCGACCAATG &lt;br&gt; CCL20-LINKER_REV: CGGCCAGGACCCACCTCGCCGCTGAACCGCCTCCACCGCTCATCTCAAAATAGTTGATG</td>
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<tr>
<td>Murine CCL21</td>
<td>NM_023052.2</td>
<td>CCL21-SIP_FW: CCTGTTCCTCGTCGCTGTGGCTACAGGTTGCGACTCGGACAGCTCAGGCCGACCAATG &lt;br&gt; CCL21-LINKER_REV: CGGCCAGGACCCACCTCGCCGCTGAACCGCCTCCACCGCTCATCTCAAAATAGTTGATG</td>
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<tr>
<td>Murine CXCL4</td>
<td>NM_019932.4</td>
<td>CXCL4-SIP_FW: CCTGTTCCTCGTCGCTGTGGCTACAGGTTGCGACTCGGACAGCTCAGGCCGACCAATG &lt;br&gt; CXCL4-LINKER_REV: CGGCCAGGACCCACCTCGCCGCTGAACCGCCTCCACCGCTCATCTCAAAATAGTTGATG</td>
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<tr>
<td>Murine CXCL9</td>
<td>NM_008599.4</td>
<td>CXCL9-SIP_FW: CCTGTTCCTCGTCGCTGTGGCTACAGGTTGCGACTCGGACAGCTCAGGCCGACCAATG &lt;br&gt; CXCL9-LINKER_REV: CGGCCAGGACCCACCTCGCCGCTGAACCGCCTCCACCGCTCATCTCAAAATAGTTGATG</td>
</tr>
<tr>
<td>Murine CXCL10</td>
<td>NM_021274.1</td>
<td>CXCL10-SIP_FW: CCTGTTCCTCGTCGCTGTGGCTACAGGTTGCGACTCGGACAGCTCAGGCCGACCAATG &lt;br&gt; CXCL10-LINKER_REV: CGGCCAGGACCCACCTCGCCGCTGAACCGCCTCCACCGCTCATCTCAAAATAGTTGATG</td>
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<tr>
<td>Murine CXCL11</td>
<td>NM_019494.1</td>
<td>CXCL11-SIP_FW: CCTGTTCCTCGTCGCTGTGGCTACAGGTTGCGACTCGGACAGCTCAGGCCGACCAATG &lt;br&gt; CXCL11-LINKER_REV: CGGCCAGGACCCACCTCGCCGCTGAACCGCCTCCACCGCTCATCTCAAAATAGTTGATG</td>
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</table>
9.3 Statistical analysis

Statistical analysis of i.t. CXCL10-F8(scFv) therapy:

<table>
<thead>
<tr>
<th>Column B</th>
<th>CXCL10-F8(scFv) vs.</th>
<th>Column A</th>
<th>PBS</th>
</tr>
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<tbody>
<tr>
<td>Paired t test</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P value</td>
<td>0.0404</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P value summary</td>
<td>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Significantly different? (P &lt; 0.05)</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>One- or two-tailed P value?</td>
<td>Two-tailed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>t, df</td>
<td>t=2.509 df=7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of pairs</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean of differences</td>
<td>-154.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SD of differences</td>
<td>174.1</td>
<td></td>
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<tr>
<td>SEM of differences</td>
<td>61.56</td>
<td></td>
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<tr>
<td>95% confidence interval</td>
<td>-300.0 to -8.901</td>
<td></td>
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<tr>
<td>R squared</td>
<td>0.4735</td>
<td></td>
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<tr>
<td>Correlation coefficient (r)</td>
<td>0.9909</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P value (one tailed)</td>
<td>&lt; 0.0001</td>
<td></td>
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<tr>
<td>P value summary</td>
<td>****</td>
<td></td>
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<tr>
<td>Was the pairing significantly effective?</td>
<td>Yes</td>
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F8-IL13: Wehi-164 therapy

Statistical analysis (Day 12):

<table>
<thead>
<tr>
<th>Bonferroni's multiple comparisons test</th>
<th>Mean Diff.</th>
<th>99% CI of diff.</th>
<th>Significant?</th>
<th>Summary</th>
<th>Adjusted P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS vs. F8-IL13 100ug</td>
<td>466.2</td>
<td>194.5 to 737.9</td>
<td>Yes</td>
<td>****</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>PBS vs. KSF-IL13 100ug</td>
<td>-27</td>
<td>-298.7 to 244.7</td>
<td>No</td>
<td>ns</td>
<td>&gt; 0.9999</td>
</tr>
<tr>
<td>PBS vs. F8-IL13 200ug</td>
<td>641.8</td>
<td>370.1 to 913.5</td>
<td>Yes</td>
<td>****</td>
<td>&lt; 0.0001</td>
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<tr>
<td>PBS vs. KSF-IL13 200ug</td>
<td>32.2</td>
<td>-239.5 to 303.9</td>
<td>No</td>
<td>ns</td>
<td>&gt; 0.9999</td>
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<tr>
<td>PBS vs. IL12-F8F8</td>
<td>519.8</td>
<td>248.1 to 791.5</td>
<td>Yes</td>
<td>****</td>
<td>&lt; 0.0001</td>
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<td>Group Comparison</td>
<td>Mean Difference</td>
<td>95% CI</td>
<td>Significance</td>
<td>p-value</td>
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<td>-----------------</td>
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<tr>
<td>PBS vs. Combo</td>
<td>708.6</td>
<td>436.9 to 980.3</td>
<td>Yes ****</td>
<td>&lt; 0.0001</td>
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<tr>
<td>F8-IL13 100ug vs. KSF-IL13 100ug</td>
<td>-493.2</td>
<td>-764.9 to -221.5</td>
<td>Yes ****</td>
<td>&lt; 0.0001</td>
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<tr>
<td>F8-IL13 100ug vs. F8-IL13 200ug</td>
<td>175.6</td>
<td>-96.14 to 447.3</td>
<td>No ns</td>
<td>0.4827</td>
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<tr>
<td>F8-IL13 100ug vs. KSF-IL13 200ug</td>
<td>-434</td>
<td>-705.7 to -162.3</td>
<td>Yes ****</td>
<td>&lt; 0.0001</td>
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<tr>
<td>F8-IL13 100ug vs. IL12-F8F8</td>
<td>53.6</td>
<td>-218.1 to 325.3</td>
<td>No ns</td>
<td>&gt; 0.9999</td>
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</tr>
<tr>
<td>F8-IL13 100ug vs. Combo</td>
<td>242.4</td>
<td>-29.34 to 514.1</td>
<td>No *</td>
<td>0.0374</td>
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<tr>
<td>KSF-IL13 100ug vs. F8-IL13 200ug</td>
<td>668.8</td>
<td>397.1 to 940.5</td>
<td>Yes ****</td>
<td>&lt; 0.0001</td>
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</tr>
<tr>
<td>KSF-IL13 100ug vs. KSF-IL13 200ug</td>
<td>59.2</td>
<td>-212.5 to 330.9</td>
<td>No ns</td>
<td>&gt; 0.9999</td>
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<tr>
<td>KSF-IL13 100ug vs. IL12-F8F8</td>
<td>546.8</td>
<td>275.1 to 818.5</td>
<td>Yes ****</td>
<td>&lt; 0.0001</td>
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<tr>
<td>KSF-IL13 100ug vs. Combo</td>
<td>735.6</td>
<td>463.9 to 1007</td>
<td>Yes ****</td>
<td>&lt; 0.0001</td>
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<tr>
<td>F8-IL13 200ug vs. KSF-IL13 200ug</td>
<td>-609.6</td>
<td>-881.3 to -337.9</td>
<td>Yes ****</td>
<td>&lt; 0.0001</td>
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<tr>
<td>F8-IL13 200ug vs. IL12-F8F8</td>
<td>-122</td>
<td>-393.7 to 149.7</td>
<td>No ns</td>
<td>&gt; 0.9999</td>
<td></td>
</tr>
<tr>
<td>F8-IL13 200ug vs. Combo</td>
<td>66.8</td>
<td>-204.9 to 338.5</td>
<td>No ns</td>
<td>&gt; 0.9999</td>
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</tr>
<tr>
<td>KSF-IL13 200ug vs. IL12-F8F8</td>
<td>487.6</td>
<td>215.9 to 759.3</td>
<td>Yes ****</td>
<td>&lt; 0.0001</td>
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<tr>
<td>KSF-IL13 200ug vs. Combo</td>
<td>676.4</td>
<td>404.7 to 948.1</td>
<td>Yes ****</td>
<td>&lt; 0.0001</td>
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<tr>
<td>IL12-F8F8 vs. Combo</td>
<td>188.8</td>
<td>-82.94 to 460.5</td>
<td>No ns</td>
<td>0.3062</td>
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### F8-IL13: F9 therapy

**Statistical analysis (Day 12):**

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<th>Bonferroni's multiple comparisons test</th>
<th>Mean Diff.</th>
<th>99% CI of diff.</th>
<th>Significant?</th>
<th>Summary</th>
<th>Adjusted P Value</th>
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</thead>
<tbody>
<tr>
<td>PBS vs. F8-IL13</td>
<td>466.2</td>
<td>61.90 to 870.5</td>
<td>Yes</td>
<td>**</td>
<td>0.0016</td>
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<tr>
<td>PBS vs. KSF-IL13</td>
<td>-27.00</td>
<td>-431.3 to 377.3</td>
<td>No</td>
<td>ns</td>
<td>&gt; 0.9999</td>
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<tr>
<td>PBS vs. IL12-F8F8</td>
<td>14.80</td>
<td>-389.5 to 419.1</td>
<td>No</td>
<td>ns</td>
<td>&gt; 0.9999</td>
</tr>
<tr>
<td>PBS vs. Combo</td>
<td>518.2</td>
<td>113.9 to 922.5</td>
<td>Yes</td>
<td>***</td>
<td>0.0003</td>
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<tr>
<td>F8-IL13 vs. KSF-IL13</td>
<td>-493.2</td>
<td>-897.5 to -88.90</td>
<td>Yes</td>
<td>***</td>
<td>0.0007</td>
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<tr>
<td>F8-IL13 vs. IL12-F8F8</td>
<td>-451.4</td>
<td>-855.7 to -47.10</td>
<td>Yes</td>
<td>**</td>
<td>0.0025</td>
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<tr>
<td>F8-IL13 vs. Combo</td>
<td>52.00</td>
<td>-352.3 to 456.3</td>
<td>No</td>
<td>ns</td>
<td>&gt; 0.9999</td>
</tr>
<tr>
<td>KSF-IL13 vs. IL12-F8F8</td>
<td>41.80</td>
<td>-362.5 to 446.1</td>
<td>No</td>
<td>ns</td>
<td>&gt; 0.9999</td>
</tr>
<tr>
<td>KSF-IL13 vs. Combo</td>
<td>545.2</td>
<td>140.9 to 949.5</td>
<td>Yes</td>
<td>***</td>
<td>0.0001</td>
</tr>
<tr>
<td>IL12-F8F8 vs. Combo</td>
<td>503.4</td>
<td>99.10 to 907.7</td>
<td>Yes</td>
<td>***</td>
<td>0.0005</td>
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### F8-IL13: Depletion Therapy (Wehi-164)

**Statistical analysis (Day 14):**

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<tr>
<th>Bonferroni's multiple comparisons test</th>
<th>Mean Diff.</th>
<th>99% CI of diff.</th>
<th>Significant?</th>
<th>Summary</th>
<th>Adjusted P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS vs. CD4+ depleted</td>
<td>530.2</td>
<td>340.0 to 720.4</td>
<td>Yes</td>
<td>****</td>
<td>&lt; 0.0001</td>
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<tr>
<td>PBS vs. CD8+ depleted</td>
<td>930.0</td>
<td>739.8 to 1120</td>
<td>Yes</td>
<td>****</td>
<td>&lt; 0.0001</td>
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<tr>
<td>PBS vs. NK depleted</td>
<td>915.0</td>
<td>724.8 to 1105</td>
<td>Yes</td>
<td>****</td>
<td>&lt; 0.0001</td>
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<tr>
<td>PBS vs. Combo</td>
<td>954.4</td>
<td>764.2 to 1145</td>
<td>Yes</td>
<td>****</td>
<td>&lt; 0.0001</td>
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<tr>
<td>CD4+ depleted vs. CD8+ depleted</td>
<td>399.8</td>
<td>209.6 to 590.0</td>
<td>Yes</td>
<td>****</td>
<td>&lt; 0.0001</td>
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<tr>
<td>Comparison</td>
<td>Mean Diff</td>
<td>99% CI of diff</td>
<td>Significant</td>
<td>Summary</td>
<td>Adjusted P Value</td>
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<tr>
<td>------------------------------------</td>
<td>-----------</td>
<td>----------------</td>
<td>-------------</td>
<td>---------</td>
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</tr>
<tr>
<td>CD4+ depleted vs. NK depleted</td>
<td>384.8</td>
<td>194.6 to 575.0</td>
<td>Yes</td>
<td>****</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>CD4+ depleted vs. Combo</td>
<td>424.2</td>
<td>234.0 to 614.4</td>
<td>Yes</td>
<td>****</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>CD8+ depleted vs. NK depleted</td>
<td>-15.00</td>
<td>-205.2 to 175.2</td>
<td>No</td>
<td>ns</td>
<td>&gt; 0.9999</td>
</tr>
<tr>
<td>CD8+ depleted vs. Combo</td>
<td>24.40</td>
<td>-165.8 to 214.6</td>
<td>No</td>
<td>ns</td>
<td>&gt; 0.9999</td>
</tr>
<tr>
<td>NK depleted vs. Combo</td>
<td>39.40</td>
<td>-150.8 to 229.6</td>
<td>No</td>
<td>ns</td>
<td>&gt; 0.9999</td>
</tr>
</tbody>
</table>

**F8-IL13: Big Tumor Therapy**

**Statistical analysis (Day 14):**

<table>
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<tr>
<th>Bonferroni's multiple comparisons test</th>
<th>Mean Diff</th>
<th>99% CI of diff</th>
<th>Significant</th>
<th>Summary</th>
<th>Adjusted P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>F8-IL13 100ug vs. IL12-F8F8</td>
<td>-809.4</td>
<td>-1278 to -340.4</td>
<td>Yes</td>
<td>****</td>
<td>&lt; 0.0001</td>
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<tr>
<td>F8-IL13 100ug vs. Combo (IL13/IL12)</td>
<td>52.80</td>
<td>-416.2 to 521.8</td>
<td>No</td>
<td>ns</td>
<td>&gt; 0.9999</td>
</tr>
<tr>
<td>F8-IL13 100ug vs. Combo (IL13/IL4)</td>
<td>89.20</td>
<td>-379.8 to 558.2</td>
<td>No</td>
<td>ns</td>
<td>&gt; 0.9999</td>
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<tr>
<td>IL12-F8F8 vs. Combo (IL13/IL12)</td>
<td>862.2</td>
<td>393.2 to 1331</td>
<td>Yes</td>
<td>****</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>IL12-F8F8 vs. Combo (IL13/IL4)</td>
<td>898.6</td>
<td>429.6 to 1368</td>
<td>Yes</td>
<td>****</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Combo (IL13/IL12) vs. Combo (IL13/IL4)</td>
<td>36.40</td>
<td>-432.6 to 505.4</td>
<td>No</td>
<td>ns</td>
<td>&gt; 0.9999</td>
</tr>
</tbody>
</table>