Doctoral Thesis

Development of therapeutic proteins for the treatment of rheumatoid arthritis and chronic cardiac rejection

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Development of therapeutic proteins for the treatment of rheumatoid arthritis and chronic cardiac rejection

A dissertation submitted to
ETH ZURICH

for the degree of
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(Dr. sc. ETH Zurich)

presented by

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1 Summary

The antibody-mediated pharmacodelivery of bioactive moieties (e.g., cytokines, cytotoxic drugs and radionuclides) represents a promising avenue to enhance the therapeutic index of pharmaceutical agents, exploiting a selective localization at the site of disease, while sparing normal tissue. In chronic inflammatory disorders and in cancer the formation of new blood vessels (angiogenesis) is important for disease progression. Targeting extracellular matrix antigens specific for the neovasculature by means of monoclonal antibody derivatives is particularly attractive, as these proteins tend to be very stable and abundant. In particular, immunocytokines (i.e., fusion proteins between an antibody or antibody fragment and a cytokine) are especially suited for the implementation of targeting strategies, in which one aims at modulating the activity of the immune system. The F8 antibody, specific to the alternatively-spliced extra-domain A of fibronectin, a marker of angiogenesis, has been fused to various cytokines and has shown promising activity in mouse models of cancer and chronic inflammation.

Our group had previously shown that the immunocytokine F8-huIL10 was able to inhibit disease progression in a mouse model of collagen-induced arthritis, in combination with methotrexate. Stimulated by these promising results, we tested the combination of F8-huIL10 with a murine version of etanercept (muTNFR-Fc). This biopharmaceutical was cloned, expressed and purified by protein A chromatography, yielding a homogenous protein preparation which retained biological activity. In an aggressive model of arthritis, the combination of F8-huIL10 and muTNFR-Fc potently inhibited arthritic score progression.
A chimeric version of the F8-IL10 immunocytokine was produced using murine IL-10 as active payload (F8-muIL10). The protein was pure and retained biological activity as shown in a cell proliferation assay, BIAcore analysis and a biodistribution experiment. In a mouse therapy experiment in rheumatoid arthritis, the chimeric version was found not to be superior to the fully human fusion protein and showed similar generation of mouse anti-fusion protein antibodies.

In a second therapy experiment in mice with collagen-induced arthritis, we could show a selective targeting of arthritic lesions and a substantial therapeutic activity using a new immunocytokine, F8-IL4, which consists of the F8 antibody fused to murine IL-4. Different dosing and combination regimes were tested in the mouse model, complementing the evaluation of arthritic scores with the analysis of serum and tissue cytokine levels. In a combination experiment with F8-IL4 and the anti-inflammatory immunocytokine L19-IL10, a stabilization of established arthritis was observed over a period of 28 days. The combination treatment of F8-IL4 with dexamethasone performed even better, curing 100% of mice with established arthritis.

To our knowledge this is the first report of durable and complete cure in mice with established arthritis. The development and clinical testing of a fully human F8-IL4 fusion protein in patients with rheumatoid arthritis will reveal if the results can be translated to humans.

The antibody F8 binds with identical affinity to EDA of human, mouse or rat origin. In a model of chronic rejection using rats with heterotopic heart transplantation, PET-experiments showed a selective localization of the antibody F8 in small immune protein (SIP) format to the transplanted heart. Stimulated by these encouraging
results, we produced F8-based immunocytokines, containing rat cytokine moieties (F8-ratIL10 and F8-ratIL4). The proteins were cloned, expressed, purified to homogeneity and characterized \textit{in vitro}. A clear targeting activity of F8-ratIL10 was shown in a rat model of chronic cardiac rejection, although treatment with F8-ratIL10 was not capable to inhibit chronic development in a preventive experiment setting.
2 Zusammenfassung


Arthritis inhibierte die Kombination von F8-huIL10 und muTNFR-Fc das Fortschreiten des Arthritisschweregrads.

Eine chimäre Version des F8-IL10 Immunozytokins wurde unter Verwendung des murinen IL-10 (aktives Molekül) hergestellt. Das Protein war rein und behielt seine biologische Aktivität wie mit einem Zell-Proliferations Versuch, einer BIAcore Analyse und einer Biodistributionsstudie bestätigt wurde. In einem Therapieexperiment in Mäusen war die chimäre Version nicht besser als das vollständig humane Fusionsprotein und zeigte ähnliche Bildung von Maus anti-Fusionsprotein Antikörpern.


Nach unserem Wissen ist dies der erste Bericht über eine dauerhafte und komplett Heilung von Mäusen mit etablierter Arthritis. Die Entwicklung und klinische Prüfung eines völlig humanen F8-IL4 Fusionsproteins in Patienten mit rheumatoider Arthritis wird uns zeigen ob die Resultate auf den Menschen übertragbar sind.
3 Introduction

3.1 Antibody-based targeting

The concept of selectively targeting a therapeutic agent to the site of disease was first postulated, a century ago, by Paul Ehrlich. He dreamt of “magic bullets”, which would recognize and kill cognate diseased cells, as antitoxins recognize and block related toxins\(^1\). Antibodies, in a sense can be used as magic bullets. With the knowledge of their structure and function, and being able to generate them against virtually any target molecule, one may use antibodies for pharmacodelivery purposes or for the selective blockade of antigens.

Targeted therapeutic drugs (e.g., monoclonal antibodies; mAb’s) may act directly at the site of disease and therefore spare healthy cells, tissues and organs. Selective therapeutic agents are particularly important for the treatment of cancer and of chronic inflammation. In pharmacodelivery approaches monoclonal antibodies may be used as “vehicles” to selectively bring effector moieties to target proteins at the site of disease\(^2\).

3.1.1 Antibodies

*Structure*

Antibodies are Y-shaped multidomain proteins of the immunoglobulin family (Ig) and are designed to recognize certain target molecules (antigens) with high affinity. They are normally produced by B cells and plasma cells in the frame of the humoral immune response. The Y-shaped structure results from the arrangement of the four polypeptide chains forming an antibody, which is made of the antigen-binding fragment (Fab) linked via the hinge region to the constant region (called crystallizable fragment; Fc) (Figure 1). Two identical light chains consisting of a
variable domain (\(V_L\)) and a constant domain (\(C_L\)) are bound via noncovalent interactions and a covalent disulfide bond to two identical heavy chains with one variable domain (\(V_H\)) and three to four constant domains (\(C_H\)). The heavy chains are stabilized in the flexible hinge region through covalent disulfide bonds giving rise to a full antibody of 150 kDa (IgG).

The bivalent binding of antibodies to their antigens is mediated through the six hypervariable loops (commonly known as complementarity determining regions; CDRs) located on the variable domains of the heavy and light chain and which form the antigen-binding site.

**Figure 1.** Molecular structure of an antibody in the IgG format. Two identical light (\(V_L\) plus \(C_L\)) and two identical heavy (\(V_H\) plus three \(C_H\)) chains form a full antibody. In the hinge region the heavy chains are covalently linked through disulfide bonds. The variable domains of the two chains form the antigen-binding site (Fv). The binding affinity of an antibody is defined by the three complementarity determining regions (1, 2 and 3) within the variable domains. The Fc portion of the antibody is the one binding to the C1q complex of the complement system or to the Fc receptors, exerting some functional effects. Adapted from Brekke & Sandlie.\(^3\)

The constant region is glycosylated at a conserved asparagine residue (N297) and exerts some functional effects through binding to effector molecules of the immune
system (e.g., C1q component of the complement system) and a variety of Fc receptors (e.g., Fcy receptors). The constant region defines the different classes of antibodies (isotypes) and is identical within different molecules belonging to the same class. In mammals the following five isotypes are known: IgA, IgD, IgE, IgG and IgM. IgG for example is the most abundant isotype form in the body and is important for the protection against invading pathogens. IgE, on the other hand, is responsible for allergic reactions and IgA antibodies are important for mucosal immunity.

Function
If an immune response is triggered, B cells and later plasma cells produce antibodies to selectively target and remove pathogens as part of the humoral immune system. Binding of the antibody to the antigen leads to neutralization of the target structure by functional blockade and/or by antigen removal. Additionally, antibodies may mediate the opsonization of pathogens for phagocytosis with the help of the complement system or the destruction of the target cells by antibody-dependent cellular cytotoxicity (ADCC), which involves the action of leukocytes carrying the Fcγ receptors (Figure 2a).

The Fc portion of the antibody molecule triggers various effector functions through binding to cognate Fc receptors (FcR), which can be activating or inhibitory. In addition, the binding of the Fc region to the salvage receptor FcRn is important for the long half-life (approx. 21 days) of human IgG.

In ADCC, antibodies may recognize antigens on the surface of target cells and their Fc region may recruit natural killer cells (NK cells) by means of a binding interaction with the FcγRIII receptor. This cross-linking event leads to the release of perforin
and granzymes from cytoplasmatic granules of NK cells, which induce apoptosis of the target cell.

In the complement-dependent cytotoxicity (CDC) pathway, binding of the complement compound C1 to the Fc region of antibodies in immune complexes (e.g., antibody-coated cells), initiates the classical complement cascade (Figure 2b). This sequence of events involves a series of proteolytic cleavage steps by various complement proteins, ultimately leading to cellular lysis via a membrane attack complex (MAC), which creates pores in the membrane. In addition, certain peptides liberated by proteolysis of complement proteins (e.g., C3a and C5a) may act as anaphylatoxins and stimulate inflammation.

![Figure 2](image-url)

**Figure 2.** Antibody effector functions. (a) In antibody-dependent cellular cytotoxicity (ADCC) the antibody binds to target-associated antigens on the surface of target cells. The Fc domain is free and can bind to FcyRIII receptors on natural killer cells (NK cells). The cross-linking triggers the release of perforin and granzymes from cytolytic granules and induces apoptosis of the selected cell through lysis. (b) In the complement-dependent cytotoxicity pathway (CDC) the C1 complex binds to the Fc region of antibodies bound to target-
associated antigens and initiates the classical complement cascade. The formation of a membrane attack complex (MAC) creating pores in the membrane ultimately leads to cellular lysis. Adapted from Steiner\textsuperscript{6}

\textit{Monoclonal antibodies}

Monoclonal antibodies can be defined as antibodies of high specificity, originating from a single cell. Mouse hybridoma technology was the first technology which allowed the generation of monoclonal antibodies of rodent origin against a wide variety of targets\textsuperscript{7}. The stable fusion of myeloma cells with B cells from immunized mice allows the immortalization of B cell clones, which may express monoclonal antibodies of interest. While mouse-derived monoclonal antibodies have revolutionized biological research, their clinical impact was low due to immunogenicity in humans, weak interaction of mouse antibodies with the human complement system and human Fc receptors as well as their short serum half-life. As rodent antibodies are recognized as “non-self” proteins in patients, leading to a “human anti-mouse antibody” (HAMA) response, efforts have been made to render monoclonal antibodies better tolerated in humans\textsuperscript{5}. For example, the variable domains of a mouse monoclonal antibody can be genetically fused with the constant domains of a human antibody, yielding a “chimeric antibody” product\textsuperscript{8}. In the “antibody humanization process”, the CDR loops of a mouse antibody are grafted onto a human IgG framework\textsuperscript{9,10}. Both chimeric and humanized antibodies, however, still contain rodent sequence portions.

For the generation of fully human antibodies, two main routes (phage-display technology or transgenic mice expressing human IgG genes) can be used. Other technologies for the generation of human antibodies include the use of ribosome-
display and yeast-display libraries, as well as the immortalization of single B lymphocytes isolated from patients\textsuperscript{5}.

Phage-display technology is a methodology for the isolation of binding specificity, which relies on the construction of large combinatorial antibody libraries\textsuperscript{11}. Extensive repertoires of antibody variable domains can be fused to a coat protein on the surface of filamentous phages. Phage particles display an antibody fragment on their surface and, at the same time, contain the gene coding for the antibody in their genome. Thus, phage-display technology provides a link between the binding selectivity of an antibody fragment and the corresponding genetic information. Large antibody libraries with $>10^{10}$ human antibodies can be screened against virtually any antigen of choice, giving rise to fully human antibodies against targets of interest, including proteins which are over-expressed at the site of disease.

Thanks to recent technological advances, the isolation and production of monoclonal antibodies have become efficient processes, thus facilitating the generation of therapeutic antibody products. Indeed, monoclonal antibodies represent the largest and fastest growing sector of pharmaceutical biotechnology, with sales which are larger than for all other types of biological products\textsuperscript{12}. At present, over 40 monoclonal antibody products have gained market approval, especially for oncological and autoimmune conditions.

As previously mentioned, intact antibodies in IgG format may achieve their pharmaceutical activity through various mechanisms, including antigen blockade and activation of ADCC and CDC. The antibody isotype is important, as different Fc regions may trigger different immune responses. This knowledge can be used to modify antibodies in order to exploit the preferred mechanism of action.
**Antibody fragments**

Intact IgG antibodies are large macromolecules (150 kDa), which mediate a bivalent and high-affinity binding by means of the antibody’s two Fab arms (Figure 1). Human monoclonal antibodies display a long serum half-life, due to their interaction with the neonatal Fc receptor (FcRn), but diffuse poorly from the vasculature into tissues. This pharmacokinetic limitation can be overcome by generating antibody fragments, which still contain the variable regions and therefore retain antigen binding, but are smaller than a full IgG. Recombinant antibody fragments vary in stability, clearance from the bloodstream, extravasation, tissue penetration, as well as targeting performance (Figure 3).

**Figure 3.** Antibody formats used for the development of therapeutics. The antibodies differ in size, avidity, Fc receptor binding and clearance from blood. Mw: molecular weight. Adapted from Gutbrodt & Neri.

The smallest fragment which retains the specificity of the parental antibody, is the monovalent single chain Fv (scFv, 27 kDa), which can be obtained by the genetic
fusion of the variable heavy and variable light antibody domains (VH plus VL) through a flexible polypeptide linker. If the linker between the two V domains is shorter than 11 amino-acid residues (e.g. five amino-acids), self-association of VH and VL domains in the scFv molecule is prevented and a noncovalent homodimer (called “diabody”, 54 kDa) is typically formed\textsuperscript{15}. The diabody retains the same avidity as the parental IgG, as it contains two binding sites, while the monomeric scFv fragment contains only one antigen binding site. Fusion of the scFv moiety to an εCH4 domain of a human IgE gives rise to the “small immune protein” (SIP, 75 kDa) format, which forms disulfide-linked homodimers (Figure 3). Similarly, the scFv-Fc format (110 kDa) consists of a fusion between a scFv fragment with the CH2 and CH3 domains of an IgG.

Depending on their size, antibody fragments may diffuse faster than IgG from the vasculature into the tissue. On the other side, small fragments may undergo rapid clearance from the blood circulation.

Until now, only few antibody fragments have been approved for clinical applications. This includes radiolabeled fragments used for imaging (e.g., CEA-scan\textsuperscript{®}, MyoScint\textsuperscript{®}) and neutralizing antibody products (e.g., Lucentis\textsuperscript{®}, Cimzia\textsuperscript{®}, ReoPro\textsuperscript{®}). Antibody fragments can be used for targeted delivery of drugs or cytokines, showing promising results in the treatment of cancer and inflammatory diseases (as discussed in 3.6). Indeed, this thesis mainly focuses on the use of antibody fragments for pharmacodelivery applications.

3.1.2 Antibody-based vascular targeting

General concept
Antibodies are able to selectively recognize their cognate antigen with an excellent specificity. This property can be used in order to deliver bioactive moieties to the site
of disease. In order to perform pharmacodelivery applications, disease-associated markers, which are accessible, abundant and stable, are needed, thus facilitating the discrimination between diseased tissue and healthy organs. Cell-surface markers (e.g., receptors which are overexpressed on tumor cells but not on normal cells) are often used in tumor targeting applications. Alternatively, one may consider targets that are expressed in the tumor stroma.

In principle, also the antibody-based recognition of pathological blood vessels (“vascular targeting”) can be used in order to localize antibodies at the site of disease. Tissue remodeling and the development of new blood vessels are important steps in the progression of many diseases. In solid tumors, for example, angiogenesis, i.e., the generation of new blood vessels from pre-existing ones, is necessary in order to support the growth of the neoplastic mass. Newly formed blood vessels are typically different from normal ones at the molecular level. For this reason, markers of angiogenesis in cancer and in chronic inflammation can be used as targets for antibody-based interventions\(^\text{16}\).

The development of vascular targeting antibodies relies on the identification of accessible antigens, which may facilitate imaging procedures and the antibody-based delivery of payloads (e.g., drugs, cytokines, radionuclides, photosensitizer or pro-coagulant factors) to the site of disease.

**Vascular targets**

Angiogenesis is an essential process during embryogenesis. In adults, angiogenesis is a rare event, mainly confined to wound healing, the female reproductive cycle and certain pathological conditions, in which the process can be induced. Markers of angiogenesis may include proteins expressed on endothelial cells (e.g., vascular endothelial growth factor receptors, integrins) or proteins that
are secreted into the stroma around the vessels (e.g., fibronectin and tenascin-C splice isoforms)\textsuperscript{17} (Figure 4).

**Figure 4.** Illustration of the concept of antibody-based vascular targeting in chronic inflammation. The antibody or antibody fragment is delivered intravenously and homes to the antigen. In rheumatoid arthritis for example, the formation of new blood vessels at sites of inflammation is induced. These newly formed blood vessels express the splice variant EDA of fibronectin and can be targeted by the F8 antibody (here in diabody format). ECM: extracellular matrix. Adapted from Steiner\textsuperscript{6} and Neri & Bicknell\textsuperscript{17}

splice isoforms of fibronectin and tenascin-C are some of the best characterized markers of angiogenesis\textsuperscript{17}. During tissue remodeling, the extra-domain A and B (EDA and EDB) of fibronectin and the extra-domain A1 of tenascin-C are inserted by a mechanism of alternative splicing. These splice variants are almost undetectable in normal adult tissues\textsuperscript{18}. EDB, for example, is not present in the fibronectin molecule in plasma and in most tissues, but EDB isoforms can be found in synovial cells, some vessels and areas of the interstitium of the ovary and in the myometrium\textsuperscript{19,20}.
The monoclonal antibodies F8 and L19\textsuperscript{21,22}, specific to EDA and EDB, respectively, have been developed in our group and have been shown to selectively target tumors\textsuperscript{23-25}, sites of inflammation in rheumatoid arthritis\textsuperscript{26,27} and chronic skin inflammation\textsuperscript{28}, endometriosis\textsuperscript{29} and chronic cardiac rejection\textsuperscript{30} (Figure 5).

Figure 5. Domain structure of fibronectin and targeting antibodies. During angiogenesis the extra-domain A and B (EDA and EDB) of fibronectin are formed by alternative splicing. The monoclonal antibodies F8 and L19 are specific to EDA and EDB, respectively. Adapted from Steiner\textsuperscript{6} and Neri & Bicknell\textsuperscript{17}
**3.2 Rheumatoid Arthritis**

**3.2.1 Disease**

Rheumatoid arthritis (RA) is a chronic inflammatory disease of the joints. The disease is characterized by swelling and destructive inflammation of peripheral joints, which leads to severe disabilities, pain and reduction in quality of life. Because almost all peripheral joints can be affected, the disease is classified as inflammatory polyarthritis. At the beginning of the disease only one or few joints may be affected, most often the hand, feet and knee joints. The disease affects around 0.5-1% of adults in developed countries, with a disease onset between the age of 40 and 60 years and is three times more frequent in women than men.

Rheumatoid arthritis is classified as autoimmune disease due to the formation of autoantibodies e.g. rheumatoid factor, directed against the Fc fragment of IgG’s and autoantibodies against citrullinated epitopes on post-translationally modified proteins. Genetics may play a role in development of RA. There is evidence that genetic variations in molecules of the major histocompatibility complex HLA class II genes (e.g., HLA-DRB1) or protein tyrosine phosphatase (PTPN22), a phosphatase that regulates T-cell activation, may be risk factors leading to disease development. The only environmental factor associated to RA development is smoking.

The etiology and pathogenesis of rheumatoid arthritis is still not completely understood, but many immunological and inflammatory processes play an important role (Figure 6). Immune cells, such as T cells, B cells, monocytes/macrophages, dendritic cells and mast cells, as well as fibroblasts, invade the synovial membrane. They produce many cytokines, e.g., TNFα, IL-1β, IL-6, IL-15 and IL-17, which may shift the balance to the pro-inflammatory side, sustaining the development and
progression of the disease. T cells infiltrating the synovium are mainly CD4+ memory cells and secrete the pro-inflammatory cytokines IL-2 and IFNγ. This leads to a pro-inflammatory T+H1 bias, further activating monocytes, macrophages and synovial fibroblasts, which typically produce TNFα, IL-1β and IL-633,34.

The synoviocytes in the synovial lining layer become hyperplastic and together with activated osteoclasts and tissue-degrading enzymes (e.g., matrix-metalloproteinases; MMPs) form an invasive pannus, which finally leads to severe destruction of the articular cartilage and underlying bone. Pannus formation is accompanied by the extensive growth of new blood vessels.

**Figure 6.** Illustration of a normal and a rheumatoid arthritis joint. In rheumatoid arthritis the synovial membrane becomes hyperplastic and infiltrated by inflammatory cells, like T cells, B cells, macrophages and dendritic cells. The synoviocytes together with activated osteoclasts and tissue-degrading enzymes form an invasive pannus, which destroys the articular cartilage and underlying bone. Adapted from Strand *et al.*35
3.2.2 Diagnosis and Treatment

Rheumatoid arthritis is diagnosed clinically using the classification system of the American College of Rheumatology (ACR). In 2010, the classification criteria’s from 1987 have been revised together with the European League Against Rheumatism (EULAR). The new classification system focuses on features of early stages of the disease to emphasize the importance of an early diagnosis and to prompt initiation of disease-suppressing therapy to ideally prevent or minimize progression of the disease\textsuperscript{36}.

The strategies for the treatment of rheumatoid arthritis have changed over the last decades. Traditionally, therapy was built on a pyramidal model, with a broad basis of physical therapy and non-pharmacological interventions. At a later time point, first pharmacological interventions would include treatment with non-steroidal anti-inflammatory drugs (NSAIDs). If these therapeutic options are not sufficient, patients would receive disease-modifying anti-rheumatic drugs (DMARDs). Nowadays, the treatment strategy has changed and the pyramid has been inverted. Early diagnosis and aggressive treatment from the beginning represent key factors for a better long-term prognosis. Patients are treated early with DMARDs to control the disease and NSAIDs and corticosteroids are only added to reduce pain and inflammation in acute flares of the disease. The first-line DMARD commonly used for therapy of patients with moderate to severe RA is the folate antagonist methotrexate. Leflunomide, azathioprine and combinations of methotrexate and other agents are used if the patients do not respond sufficiently to methotrexate.

The new generation of DMARDs, i.e. biological products, are becoming more and more important for long-time therapy and suppression of arthritis. Biologicals may display various mechanisms of action, such as the blockade of pro-inflammatory
cytokines (e.g., TNFα, IL-1β and IL-6) or the removal of cells involved in inflammation and in the joint destruction process. Alternatively, the administration of anti-inflammatory cytokines (e.g., IL-4, IL-10 or IL-13), that may shift the pro-inflammatory environment into a more normal condition, can be considered. The first biologicals to enter the market were the tumor necrosis factor (TNF) inhibitors infliximab (Remicade®, a blocking chimeric monoclonal antibody) and the fusion protein etanercept (Enbrel®, consisting of the ligand-binding soluble portion of the TNF receptor (p75) and the Fc fragment of a human IgG1). Adalimumab (Humira®), the first fully human mAb, was approved in 2002. Other approved TNF blockers include certolizumab pegol (Cimzia®), a pegylated humanized antibody Fab fragment and golimumab (Simponi®), a human blocking antibody.

Other biologicals with different mechanisms of action have been approved for the treatment of RA. Anakinra (Kineret®) is a recombinant interleukin 1 receptor antagonist and is approved as single agent or in combination with DMARDs (other than TNF blockers). Tocilizumab (RoActemra®), a humanized mAb binding to the interleukin 6 receptor, abatacept (Orencia®), a CTLA-4-Fc fusion protein inhibiting co-stimulation of T cells through binding against CD28, as well as rituximab (MabThera®), a product which depletes B cells by CD20 binding, represent drugs currently used for the treatment of RA if first line treatment is not adequate (Table 1).
Table 1. Approved biological agents for the treatment of rheumatoid arthritis. Adapted from van Vollenhoven\textsuperscript{37}, Hansel \textit{et al.}\textsuperscript{39} and EMA website (04/2014)\textsuperscript{40}

<table>
<thead>
<tr>
<th>Target</th>
<th>Drug</th>
<th>Trade Name</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF</td>
<td>infliximab</td>
<td>Remicade\textsuperscript{®}</td>
<td>chimeric monoclonal antibody</td>
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Even though many new therapeutic agents with different mechanisms of action have been approved in the last 20 years for the treatment of rheumatoid arthritis, the need for new and more effective drugs is still high. Not all patients respond adequately to existing drugs. Remissions are rare and side-effects are not uncommon. New drugs may include inhibitors of other pro-inflammatory cytokines (e.g., IL-2, IL-12, IL-15, IL-17, IL-18), the use of anti-inflammatory cytokines (IL-4, IL-10, IL-13), as well as immunocytokines (i.e., antibodies fused to anti-inflammatory cytokines; discussed in 3.6.1). In addition, inhibitors of proteases (e.g., MMPs), inhibitors of signal-transduction cascades, chemokine blockers, inhibitors of angiogenesis, agents targeting cell surface antigens of T cells, B cells, adhesion
molecules and Toll-like receptors (TLRs) as well as drugs interfering with the activation of osteoclasts have been considered as possible drugs\textsuperscript{33,35}.

3.2.3 Angiogenesis in rheumatoid arthritis

Inflammation, infiltration and growth of the synovial membrane in rheumatoid arthritis is accompanied by neovascularization. Due to synovial hyperplasia, cells may grow at a distance from blood vessels and may therefore be in demand of oxygen and nutrients. Hypoxia triggers angiogenesis, followed by an increase in number of synovial vessels, which facilitates leukocyte extravasation into the synovium\textsuperscript{41}. In addition, angiogenic factors (e.g., vascular endothelial growth factor; VEGF and fibroblast growth factor; FGF) are overexpressed and many of the induced immunomodulatory factors have direct or indirect angiogenic activity, e.g., TNF\(\alpha\), IL-1, IL-6 and the prostaglandins E1 and E2\textsuperscript{42,43}.

As previously discussed, the expression of alternatively spliced isoforms of fibronectin (EDA and EDB) may occur in angiogenesis and is associated with tissue remodeling processes. Using immunohistochemical techniques on rheumatoid arthritis tissues samples, EDA fibronectin deposition in the synovial lining layer was detected and the expression of the splice isoform was correlated with hyperplasia. Additionally, expression of EDB fibronectin was found in the invasive pannus\textsuperscript{44}. Measurements of synovial fluid EDA fibronectin concentrations in RA patients showed a positive correlation with the progression of joint destruction. Indeed, EDA overexpression in synovial fluid can be used as a prognostic factor for joint destruction\textsuperscript{45}.
3.2.4 Mouse model of rheumatoid arthritis

The collagen-induced arthritis (CIA) model in mice is one of the most important animal models of rheumatoid arthritis. After immunization with type II collagen, a major protein in cartilage, emulsified in Complete Freund’s Adjuvant (CFA), mice show signs of arthritis. The development of arthritis is mediated by an anti-type II collagen autoimmunity and by binding of antibodies to the surface of intact articular cartilage\(^\text{46}\).

This model has been used to test several biological agents that are now on the market, including TNF blockers\(^\text{47}\). The model shows similarities in pathogenesis compared to rheumatoid arthritis in humans, such as synovial hyperplasia, mononuclear cell infiltration, pannus formation, cartilage and bone destruction. Sensitivity to collagen immunization and arthritis development are linked to polymorphisms in the major histocompatibility locus\(^\text{46,47}\).
3.3 Chronic cardiac allograft rejection

3.3.1 Disease
For patients with advanced heart failure refractory to medical therapy, cardiac transplantation is the gold standard. The number of heart transplantations is estimated to be more than 5'000 per year as reported by the International Society for Heart and Lung Transplantation (ISHLT). Mean survival after transplantation is 13 years and survival rates of 85% after 1 year and 75% after 5 years can be observed.

Acute allograft rejection, presenting as cardiac graft failure, can be sufficiently treated with immunosuppressive therapy (e.g., cyclosporine). The incidence of rejection has diminished due to improvements in donor matching, early onset of immunosuppressive treatment and the development of efficient drugs like mTOR inhibitors. The main limiting factor for long-term survival after heart transplantation is cardiac allograft vasculopathy (CAV). The incidence of CAV is 30% at five years and 50% at 10 years. The treatment of this condition is much more difficult than for the acute allograft rejection and therefore contributes to morbidity and mortality after heart transplantation.

Chronic rejection is mainly represented by cardiac allograft rejection (CAV) and cardiac interstitial fibrosis (CIF) leading to severe complications like myocardial infarction or diastolic dysfunction. Both processes are accompanied by distinct structural and functional alterations of the cardiac extracellular matrix (ECM) including a re-occurrence of fetal splicing variants of cell-adhesion modulating proteins such as fibronectin and tenascin-C. These molecules are of functional importance during heart development and are virtually absent in healthy adult
organs, qualifying them as excellent tissue biomarkers of rejection-associated cardiac tissue remodeling.

Cardiac allograft vasculopathy is an accelerated and special form of atherosclerotic coronary disease. CAV is characterized by progressive concentric thickening of the arterial intima and pathological remodeling. An injury of the arterial vasculature caused by immune and non-immune factors may be responsible for the onset of CAV development. The cause of CAV is mostly immunologic, with several cytokines (e.g., IFNγ), chemokines, adhesion molecules and co-stimulatory signals playing an important role in the generation of CAV. A non-immune risk factor can be ischemia. CIF is characterized by activated fibroblasts/myofibroblasts (MyoFbs), which are important for the cardiac extracellular matrix (cECM) re-organization and pathological tissue remodeling.

### 3.3.2 Diagnosis and Treatment

Early diagnosis of CAV is important as it enables physicians to start treatment as soon as possible to prevent progression and further harmful changes as well as to improve prognosis. However, early diagnosis of CAV remains a challenge, because the disease is clinically silent over a long time period. One method to diagnose CAV is coronary angiography, which is routinely performed in patients. The most sensitive method for early detection would be intravascular ultrasound (IVUS), which is a minimally invasive procedure. Other possibilities to detect CAV include the assessment of endothelial and microvascular function, non-invasive methods like myocardial perfusion scintigraphy, dobutamine stress echocardiography and CT or MRI imaging, endomyocardial biopsy or analysis of biomarkers (e.g., immunological factors, genetics or proteins).
Because it is difficult to treat fully established CAV, it is important to start preventive measures early. This includes prevention before and after transplantation (with optimal immunosuppressive therapy), control of cardiovascular risk factors (e.g., hypertension, dyslipidemia and diabetes) and prophylaxis for cytomegalovirus infection. Immunosuppressive therapy often starts with a triple therapy consisting of calcineurin inhibitors (e.g., cyclosporine or tacrolimus) in combination with azathioprine or mycophenolate mofetil plus corticosteroids\(^{49,58}\).

New targets for the treatment of CAV include cytokines, chemokines, co-stimulatory signal molecules, cell proliferation and modulation of apoptosis. The use of DNA-based products, such as antisense oligodeoxynucleotides (ODNs) to regulate transcription of disease-related genes, may represent another strategy for the therapy of CAV\(^{55}\).

Risking restenosis, treatment of established CAV can be performed using coronary angioplasty. Often the best and final therapy may involve re-transplantation\(^{59}\).

### 3.3.3 EDA expression in chronic allograft rejection

Re-expression of the fetal fibronectin splice variant EDA has been shown to be associated with changes in the extracellular matrix (ECM) occurring during chronic cardiac allograft rejection\(^{53,60}\). CAV and CIF are both important factors during this rejection process and are accompanied with structural and functional changes of the cardiac ECM\(^{51}\). In a way, chronic rejection resembles exaggerated wound healing, where overexpression of EDA is seen\(^{61}\).

In a model of chronic rejection using rats with heterotopic heart transplantation, EDA positive tissue staining was observed especially in vessel structures showing CAV and fibrotic areas. In addition, increased EDA protein deposition was associated with a higher rejection grade\(^{62}\). There might also be functional importance of EDA
for CIF development, as shown in an experiment with EDA deficient (EDA−/−) mice\textsuperscript{61}. In a heterotopic rat heart transplantation model targeting of the antibody F8, specifically recognizing EDA, showed selective imaging of chronic cardiac rejection by near infrared fluorescence imaging (NIRF) and microscopic analysis. The F8 antibody stained diseased blood vessels and fibrotic regions\textsuperscript{30}. EDA might be a useful marker for novel molecular imaging strategies to detect CAV and CIF for diagnosis. On the other hand, targeted therapeutic strategies with antibody-based fusion proteins (e.g., coupled to drugs or cytokines) may prevent CAV and CIF, conditions for which at the moment no optimal therapy is known\textsuperscript{51}. Eventually, functional blocking of EDA may prevent CIF and can be used as a target for therapeutic interventions\textsuperscript{61}.

\subsection{3.3.4 Heterotopic rat heart transplantation model}

To understand and analyze chronic cardiac rejection, a rat model of heterotopic transplantation can be used. The model was first described by Ono and Lindsey\textsuperscript{63} in 1969 and has been used as an animal model to assess heart transplantation and related issues. The method was set in a way that the heart is “non-working” and is therefore different from the orthotopic heart. Yokoyama and colleagues refined the method to get a “working” left heart model in rats using a heterotopic abdominal transplantation technique\textsuperscript{64}. This syngeneic immune-competent model of heart transplantation represents the new standard for various studies, allowing to investigate the effect of therapeutic interventions and can be used to induce chronic cardiac allograft rejection. The model has the advantage that both hearts (i.e., the donor heart and the orthotopic heart) can be evaluated at the end of the experiment. A study analyzing cardiac ECM gene expression showed lower gene expression levels in the corresponding orthotopic heart compared to the allograft, but the levels
were elevated compared to healthy control hearts, indicating a transplantation associated systemic effect\textsuperscript{65}.
3.4 Interleukin 10

3.4.1 Structure and mechanism of action

**Structure**

Interleukin 10 (IL-10) is a homodimeric cytokine with a molecular weight of 37 kDa. The homodimer consists of two 160 amino-acid monomers (18.5 kDa), each monomer having four cysteine residues, which form two intramolecular disulfide bonds. The structure of IL-10 was studied by X-ray crystal structure analysis and showed a noncovalent dimer built through two identical interpenetrating polypeptide chains, rotated by 180° to each other. The structure has topological similarity to IFNγ, a cytokine with antagonistic biological properties\(^ {66-68}\) (Figure 7). Human and murine IL-10 exhibit a 80% amino-acid homology\(^ {67}\). Human IL-10, in contrast to murine IL-10, is active on both mouse and human cells.

![Structure of IL-10 with its receptor](image)

**Figure 7.** Structure of IL-10 with its receptor. Ribbon diagram of the 1:2 IL-10/sIL-10R1 complex, where the IL-10 dimer binds two sIL-10R1 molecules. IL-10: blue/yellow; sIL-10R1: green. Adapted from Josephson *et al.*\(^ {69}\)

**IL-10 receptor signaling**

The action of IL-10 is mediated through the cell surface IL-10 receptor signaling complex. The complex consists of two subunits, the IL-10 receptor 1 and 2 (IL-10R1
and IL-10R2, respectively), which are members of the class-2 cytokine receptor family that also include the IFN receptors. Initially, IL-10 is binding with high affinity to IL-10R1, which is the main signaling component. Subsequently this intermediate complex binds with lower affinity to the IL-10R2, resulting in an active signaling complex\textsuperscript{70}. The solved crystal structure of the IL-10/IL-10R1 complex shows the molecular details of how IL-10 binds to its receptor (Figure 8)\textsuperscript{69}.

\begin{figure}[h]
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\caption{Model of the biologically active IL-10 receptor complex. IL-10 requires the two receptors to form an active complex for cell signaling. Initially IL-10 binds with high affinity to IL-10R1 (orange), which is the main signaling component and subsequently this intermediate complex binds with lower affinity to the IL-10R2 (yellow), resulting in an active signaling complex. Black line: cell membrane. Adapted from Josephson \textit{et al.}\textsuperscript{69}}
\end{figure}

The active signaling complex IL-10/IL-10R induces intracellular tyrosine phosphorylation and activation of latent transcription factors through the JAK/STAT system. In detail, the intracellular signal transduction pathway starts after the IL-10/IL-10R complex formation with the activation of the tyrosine kinases JAK1 and TYK2, which are associated with the IL-10R1 and IL-10R2, respectively. The tyrosine phosphorylation activates the latent transcription factors STAT1, 3 and 5, which start gene activation\textsuperscript{67,68}. IL-10 signaling may inhibit the transcription factor NF\textsuperscript{κ}B, which is important for the transcriptional control of many inflammatory
proteins involved in immune functions, thus exerting an anti-inflammatory activity\textsuperscript{67,71}.

\textit{Biological activities}

IL-10 plays a central role in suppressing inflammatory responses and is important for the regulation of immune responses. Activated monocytes, T cells, B cells and other cell populations can produce IL-10, although macrophages are the major source of this cytokine.

IL-10 was first known as cytokine synthesis inhibiting factor (CSIF), because it inhibits the synthesis of several pro-inflammatory cytokines, such as IFN\textgamma.\textsuperscript{72} It also suppresses the expression of pro-inflammatory chemokines, adhesion molecules and antigen-presenting and co-stimulatory molecules on macrophages, neutrophils and T cells. The main targets of IL-10 are antigen-presenting cells (APC’s) and lymphocytes. The effect of IL-10 on these cells regulates the T\textsubscript{H}1/T\textsubscript{H}2 balance and reverses the T\textsubscript{H}1 bias back to normal through induction of a type 2 cytokine pattern.

IL-10 has some direct effects on T cells, inhibiting the proliferation and cytokine synthesis of CD4\textsuperscript{+} T cells. The effect on natural killer (NK) cells is mainly stimulatory. Taken together, IL-10 is a pluripotent cytokine with many effects on different immune cells. The classification of IL-10 as an immunosuppressive cytokine has changed and IL-10 is now considered an immunoregulatory cytokine, because of its distinct properties, which are not only inhibitory but also stimulatory\textsuperscript{67,68}.

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3.4.2 IL-10 as therapeutic agent

*Rheumatoid arthritis*

In rheumatoid arthritis, an autoimmune disease where T<sub>H</sub>1 cytokines play a major role in the pathogenesis, IL-10 has a protective role. IL-10 is expressed in the arthritic joints by synovial macrophages and T cells and is an important immunoregulatory component that inhibits the production of inflammatory cytokines<sup>73</sup>. The protective role of IL-10 in rheumatoid arthritis has been shown in several animal models. Kasama and colleagues evaluated the contribution of IL-10 during the evolution of type II collagen-induced arthritis (CIA). Mice receiving neutralizing IL-10 antibodies developed arthritis faster and with a more severe phenotype, suggesting that IL-10 plays an important regulatory role during the development of collagen-induced arthritis in mice<sup>74</sup>. IL-10 treatment significantly suppressed the severity of arthritis when given during development of CIA, especially when starting early<sup>75</sup>. Another study could show a potential therapeutic role of IL-10 in the CIA model, where pharmacological treatment with IL-10 inhibited paw swelling, disease progression and cartilage destruction<sup>76</sup>. Combining IL-10 with IL-4 in the treatment of CIA showed a synergistic effect. The combination treatment reduced cellular infiltrates in the synovial tissue, caused protection against cartilage destruction and reduced TNFα and IL-1 mRNA levels<sup>77</sup>. In general, IL-10 administration to animals with collagen-induced arthritis reduced joint swelling, cellular infiltration, inflammation, cytokine production, cartilage degradation and joint destruction<sup>67,68</sup>.

Due to these encouraging results from animal experiments, IL-10 has been considered a promising candidate for therapeutic treatment in patients with
rheumatoid arthritis. Human recombinant IL-10 (Tenovil®, ilodecakin) has been investigated in controlled clinical trials in healthy volunteers and patients with different autoimmune diseases, e.g., rheumatoid arthritis, Crohn’s disease and psoriasis, or diseases like hepatitis C infection and HIV infection as well as for the inhibition of therapy associated cytokine release in organ transplantation.

In phase I clinical trials in healthy volunteers, safety, tolerance, pharmacokinetic and pharmacodynamics properties, as well as immunological and hematological effects of IL-10 were tested at different doses. Overall, IL-10 treatment was well tolerated and induced a number of desired immunological changes, such as the inhibition of the production of the pro-inflammatory cytokines TNFα and IL-1β. However, some side effects (like mild to moderate flu-like symptoms, changes in white blood cell populations, transient neutrophilia, lymphocytopenia and delayed decrease in platelet counts) were observed.

The therapeutic effects of IL-10 have been evaluated in several large multicenter studies in patients with different forms of Crohn’s disease. Additionally, a number of clinical trials tested the activity of IL-10 in patients with psoriasis. For the evaluation of IL-10 in rheumatoid arthritis only limited data is available. Rheumatoid arthritis patients receiving IL-10 (Tenovil®) for 28 days showed initial evidence of efficacy and a good safety profile. The combination of IL-10 and methotrexate was tested in a multicenter, placebo-controlled, dose-escalating clinical study. Patients with rheumatoid arthritis receiving Tenovil® at a dose of 8 µg/kg four times daily or 8 µg/kg three times per week showed an ACR20 response in 50% compared with 63% when treated with 20 µg/kg three times per week and only 10% in patients receiving placebo as control. However, ACR50 response was less impressive and these rather discouraging results probably led to the discontinuation of the clinical development. The role of IL-10 in the treatment of rheumatoid arthritis remains
unclear. The anti-inflammatory properties and the therapeutic effects in animal experiments suggested good efficacy also in patients, which unfortunately could not be reproduced until now.

*Chronic cardiac allograft rejection*

IL-10 administration was tested in a model of rat cardiac allograft rejection and was able to prolong cardiac allograft survival and reduce parameters of rejection. In this study, the viral IL-10 gene was transferred into rat hearts by intracoronary administration using adenoviruses. Combination with low dose cyclosporine A demonstrated a synergistic effect on prolongation of allograft survival\(^8^4\). Another study using adeno-associated viral vector-mediated stable expression of IL-10 in a rat heart transplantation model could show significant prolonged allograft survival, reduced T cell and macrophage infiltration, suppressed development of transplant arteriosclerosis and prevention of chronic allograft deterioration\(^8^5\). In a mouse model of chronic heart transplantation rejection in IL-10 transgenic mice, IL-10 expression inhibited the development of cardiac allograft vasculopathy (CAV) through reduced mononuclear cell recruitment and altered cytokine profile\(^8^6\).

In animal models of liver transplantation a potential role for IL-10 therapy, with adenovirus mediated gene transfer of IL-10 or IL-10-engineered mesenchymal stem cells (MSCs), has been shown\(^8^7,^{8^8}\).

The therapeutic potential of IL-10 in chronic cardiac allograft rejection needs to be further analyzed because not much is known until now, but the mechanism of action of IL-10 makes it an interesting therapeutic product for this disease. The anti-inflammatory and anti-proliferative actions of IL-10 could, in principle, mediate beneficial effects in cardiac transplant rejection.
3.5 Interleukin 4

3.5.1 Structure and mechanism of action

Structure
Interleukin 4 (IL-4) is a 14 kDa glycoprotein, which consists of 153 amino-acids and is stabilized by 3 intramolecular disulfide bonds. IL-4 belongs to the family of IL-4/IL-13 cytokines, that are T cell-derived. IL-4 shares 20% homology with IL-13 on the amino-acid level and numerous biological properties. The crystal structure of IL-4 shows a highly compact and globular protein with a hydrophobic core and four α-helices, which are arranged in a left handed antiparallel bundle. The human and murine IL-4 protein share a 41% sequence identity and show a strict species specificity in terms of their biological activity.

IL-4 receptor signaling
The cellular responses of IL-4 are mediated through a high affinity binding to the α-chain of the cognate receptor (IL-4Rα) and the formation of a IL-4 receptor complex (Figure 9). This complex subsequently binds to the low affinity common γ chain (γc or IL-2Rγy) to form a signaling heterodimer, but can also bind to the IL-13Rα1 receptor chain. After formation of the full complex, IL-4 signaling involves the intracellular activation of the tyrosine kinases JAK1 and JAK3, activation of STAT6 and IRS1/2 (insulin receptor substrate 1/2) and promotes proliferation and differentiation. The STAT6 signaling induces changes in gene expression and differentiation, whereas IRS2 signaling gives rise to mitogenic and anti-apoptotic signals. The IL-4 receptor can be found on hematopoietic, endothelial, epithelial, muscle, fibroblast, hepatocyte and brain cells.
**Figure 9.** Structure of IL-4 with its receptor. IL-4 (red) binds with high affinity to the ectodomain of the IL-4 receptor α-chain (IL4-BP, blue/yellow). The five IL4-BP loops interacting with IL-4 are highlighted in yellow. Adapted from Hage et al.91

**Biological activities**

Interleukin 4 is a pleiotropic type I cytokine, originally identified as B cell growth factor. IL-4 exhibits stimulatory and suppressive effects on the growth, differentiation and activity of B and T lymphocytes, myeloid cells and non-hematopoietic cells. The IL-4 cell surface receptor is found on different cell types, and, as a consequence, IL-4 displays multiple effects, e.g., proliferation of activated B and T cells, increase of the expression of class II MHC molecules in B cells, production of IgE through specific immunoglobulin class switching, increase of the cytotoxic activity of lymphocytes and monocytes and enhancement of the functional activity of myeloid cells.90 IL-4 itself is produced by a subgroup of CD4⁺ T cells (often referred to as Th2 cells), basophils and mast cells. IL-4 suppresses the activity of IFNγ-producing CD4⁺ T cells, playing an important role in the decision between a Th1 or Th2 T cell commitment. IL-4 is important for regulating the differentiation of antigen-stimulated naive T cells into Th2-like cells capable of producing IL-4 and related cytokines, such as IL-5, IL-10 and IL-13.95
Taken together, IL-4 is a key regulator of humoral and adaptive immunity. Over time, it has become clear that IL-4 can act as an anti-inflammatory cytokine. IL-4 can inhibit the synthesis of pro-inflammatory cytokines like IL-1, TNFα, IL-6, IL-8 and IL-12 and on the other side can stimulate the synthesis of cytokine inhibitors, e.g., interleukin 1 receptor antagonist (IL-1Ra).

3.5.2 IL-4 as therapeutic agent

Rheumatoid arthritis
In arthritis, IL-4 may have protective effects on extracellular matrix degeneration, because it suppresses the production of metalloproteinases and inhibits osteoclast activity and survival. The role of IL-4 was investigated in different animal models of rheumatoid arthritis. In a collagen-induced mouse model of arthritis, IL-4 alone did not mediate any detectable therapeutic effect, but together with IL-10 it suppressed the macroscopic signs of inflammation after early and delayed treatment. The combined treatment also reduced cellular synovial tissue infiltration and protected the cartilage from destruction. In addition, the mRNA levels of TNFα and IL-1 were suppressed, and the levels of IL-1 receptor antagonist elevated. In a second study, Joosten and colleagues investigated the tissue protective effect of IL-4 treatment in the CIA model. The treatment with IL-4 in mice with established collagen-induced arthritis suppressed disease activity and protected against cartilage and bone destruction. In addition, the combination of IL-4 with low-dose prednisolone suppressed joint swelling and inflammatory parameters. A study, in which IL-4 was administered by electroporation of IL-4-DNA, could show a significant reduction of CIA onset and the degree of synovitis, cartilage erosion and decreased expression of IL-1β.
The use of IL-4 in clinical trials in patients with rheumatoid arthritis reported a lack of efficacy, which may have resulted from suboptimal dosing of IL-4 in the trials, with low concentrations at the site of disease. Higher systemic doses of IL-4 were not tolerated as they lead to severe side effects\textsuperscript{98}. In principle, this problem could be overcome by the selective delivery of IL-4 to the site of disease using monoclonal antibodies (discussed in chapter 3.6.1 Immunocytokines).

Interleukin-4 has also been investigated in several cancer types, but only minimal anti-tumor activity was observed. Substantial toxicity occurred when the drug was used in daily injections of 25 μg/kg\textsuperscript{99}.
3.6 Armed antibodies

Antibodies are important vehicles for the selective pharmacodelivery of bioactive molecules and are gaining importance in oncology and inflammatory disorders. Cytotoxic drugs, cytokines, radionuclides, photosensitizer or procoagulant factors can be directly fused to the antibody, either by chemical conjugation or, in the case of cytokines, by genetic fusion. Additional strategies for the “arming” of antibodies for therapeutic purposes include immunoliposome-mediated drug delivery, T-cell recruitment with bispecific antibodies or the engineering of T-cells expressing chimeric antigen receptors (CARs)$^{100}$. Bispecific antibodies are recombinant antibody products with dual antigen binding specificities$^{101}$. In this thesis, we focused on antibody-cytokine fusion proteins (immunocytokines), for the treatment of chronic inflammatory conditions.

3.6.1 Immunocytokines

As discussed in the previous chapters, cytokines are highly active molecules with immune-modulating activities. Some cytokines may display beneficial effects against inflammatory disorders and cancer, but can also show systemic toxicities even at low doses, preventing the escalation to therapeutically active regimens. Only few cytokines have been approved for clinical use, including IL-2 (Proleukin®), TNF (Beromun®) and IFNα (Roferon A® and Intron A®) for the treatment of cancer. Other cytokines have received marketing authorization for the treatment of viral and bacterial infections and chronic inflammatory conditions, like IFN-α for the therapy of hepatitis, IFN-1β for the treatment of multiple sclerosis and IFN-γ for the treatment of chronic granulomatous disease$^{14,102}$.

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The selective delivery of cytokines using monoclonal antibodies may help circumvent the toxicity problems (Figure 10). Immunocytokines may improve the therapeutic index of the corresponding cytokine moiety by a selective localization at the site of disease. Cytokines may be fused to full antibodies or antibody fragments as described in chapter 3.1.1. Depending on the antibody and cytokine moieties, these immunocytokines show different targeting, localization and diffusion properties. Through interaction with its natural receptor, the cytokine may profoundly alter the activity of the immune system\textsuperscript{14}. In tumor therapy, pro-inflammatory cytokines may facilitate the infiltration of leukocytes and the induction of an antitumor immune response\textsuperscript{103-105}.

The preclinical testing of several immunocytokines has been reported and is summarized in Pasche et al.\textsuperscript{106}, whereas Gutbrodt et al.\textsuperscript{14} has recently reviewed the immunocytokine products which are currently in clinical development.

**Figure 10.** List of cytokine payloads used for the generation of immunocytokines. The cytokines are grouped according to their main biological activity. For IL-4, the classification is not so clear, although the perception of IL-4 has changed towards an anti-inflammatory cytokine. Some cytokines may have different activities, depending on the dose and context. Adapted from Hemmerle\textsuperscript{2} and Kontermann\textsuperscript{107}
The development of immunocytokines has been facilitated by advances in antibody engineering and by the identification of accessible markers of disease. There is a growing interest in the exploration of immunocytokines in combination with other drugs\textsuperscript{14}.

**IL-10 based immunocytokines**

Recombinant interleukin 10 (Tenovil\textsuperscript{®}) has been tested in clinical trials in patients with rheumatoid arthritis, but clinical results were not as positive as those observed with TNF blockers, which led to the discontinuation of clinical development\textsuperscript{67}. To overcome these problems, the immunocytokines L19-IL10 and F8-IL10 have been developed in our group, fusing IL-10 to the L19 and F8 antibodies, specific to EDB and EDA, respectively. Preclinical testing of L19-IL10 in a collagen-induced model of arthritis showed therapeutic activity, which was superior to the activity of IL-10 fused to an antibody of irrelevant specificity in the mouse\textsuperscript{26}. Also the F8-IL10 immunocytokine was able to selectively target arthritic lesions in mice and inhibited progression of established arthritis when tested alone and in combination with methotrexate. In addition F8-IL10 was studied in rodents and in cynomolgus monkeys, showing an excellent safety profile\textsuperscript{27}. Based on these promising results the immunocytokine F8-IL10 is currently being investigated in clinical trials in patients with rheumatoid arthritis, showing encouraging interim results\textsuperscript{108}.

In a mouse model of psoriasis, EDB expression in psoriatic lesions could be detected using the L19 antibody, but L19-based delivery of IL-10 did not improve inflammation in a transgenic mouse model of chronic skin inflammation\textsuperscript{28}. It remains to be seen, whether IL-10 is the best cytokine to use in psoriasis, even though clinical results with Tenovil\textsuperscript{®} were encouraging\textsuperscript{67}.
The immunocytokine F8-IL10 was tested in a syngeneic mouse model of endometriosis and showed a preferential accumulation in endometriotic lesions and a reduction in average lesion size. The F8 antibody may be used for the non-invasive imaging of the disease and for the pharmacodelivery of anti-inflammatory cytokines, such as IL10\textsuperscript{29,107}.

*IL-4 based immunocytokines*

Interleukin 4 has many immune-modulatory effects as previously discussed. This cytokine has been fused to the antibody F8, forming the immunocytokine F8-IL4. This product has been shown to inhibit tumor growth as single agent in three different immunocompetent murine cancer models. Together with immunocytokines based on IL-2 and IL-12, F8-IL4 showed synergistic effects. The combined use of F8-IL4 and F8-IL12 resulted in complete tumor eradication\textsuperscript{99}. F8-IL4 has also been tested in three immunocompetent mouse models of skin inflammation and showed a superior therapeutic benefit to the non-targeted version of IL-4 and led to increased levels of key regulatory cytokines in the inflamed skin\textsuperscript{109}.
3.7 Aim of the thesis

The search for potent and selective therapeutic agents characterizes modern pharmaceutical research. The use of recombinant cytokines is often limited due to systemic toxicities even at low concentrations, preventing dose escalation to therapeutically active regimens. The antibody-based pharmacodelivery of cytokines may help mitigate the toxicity problem. Such targeting strategies are gaining importance in oncology and inflammatory disorders. In chronic inflammatory conditions, tissue remodeling and the formation of new blood vessels (angiogenesis) are important for disease progression. Targeting accessible, abundant and stable antigens in the neovasculature with immunocytokines represents a promising avenue for the treatment of these diseases.

Patients affected by rheumatoid arthritis suffer from pain, severe disabilities and reduction in quality of life. Even though in the last 20 years many new therapeutic agents have been approved, the need for new and more effective drugs is still high, as not all patients respond adequately to existing drugs. In arthritis remissions are rare, while drugs can have severe side effects.

The main aim of this thesis was the development of new therapeutics for the treatment of rheumatoid arthritis. We first investigated the therapeutic potential of F8-IL10$^{27}$ in a combination treatment with a murine version of Enbrel® (muTNFR-Fc) in a mouse model of arthritis. In addition, we studied the effect of immunogenicity on therapeutic outcome and formation of mouse anti-fusion protein antibodies.
In the search for immunocytokines with more potent activity compared to F8-IL10, we tested the F8-IL4 fusion protein, alone and in combination with either other immunocytokines (e.g., L19-IL10) or with the glucocorticoid dexamethasone, in mice with collagen-induced arthritis.

In a second part of this thesis, we produced novel chimeric immunocytokines and tested their activity in a rat model of chronic cardiac rejection in collaboration with the group of PD Marcus Franz at the University of Jena (Germany). I cloned and expressed rat versions of the IL-10 and IL-4 based immunocytokines (F8-ratIL10 and F8-ratIL4), while our collaborators performed the therapy experiments. In addition, PET imaging experiments were performed in Zürich, in collaboration with the group of Prof. R. Schibli.
4 Results

4.1 Murine analogues of etanercept and of F8-IL10 inhibit the progression of collagen-induced arthritis in the mouse

4.1.1 Cloning, expression and characterization of fusion proteins

The murine fusion protein muTNFR-Fc was cloned and expressed by stable transfection in CHO cells, appending the extracellular part (amino-acids 23 to 258) of the murine p75-TNF receptor at the N-terminus of a murine IgG1 Fc portion, containing the hinge region (Figure 11a,b). A complete sequence of muTNFR-Fc is reported in 12.1.1.

The fusion protein was purified from the culture supernatant by protein A chromatography, yielding a preparation that was pure in SDS-PAGE analysis and size exclusion chromatography (Figure 11c,d). The biological activity of muTNFR-Fc was tested by inhibition of TNF-mediated killing of lung murine fibroblasts\(^1\), exhibiting a half-maximal inhibitory concentration value of 0.1 nM (Figure 11e).

\(^1\) Adapted from F. Doll \textit{et al.} Arthritis Research & Therapy 2013
Figure 11. Cloning, expression and in vitro characterization of muTNFR-Fc. (a) Schematic representation of the cloning strategy of muTNFR-Fc. muTNFR was directly fused to the Fc fragment (hinge, CH2 and CH3 regions) of murine IgG1, containing a signal sequence (SS) for secretion of the protein at the N-terminus. (b) Schematic representation of the formation of a muTNFR-Fc dimer through disulfide bridges in the hinge region. (c) SDS-PAGE analysis of purified muTNFR-Fc. M, molecular weight marker; NR, nonreducing conditions; R, reducing conditions. (d) Size exclusion chromatography (SEC200) of covalent homodimeric muTNFR-Fc. (e) Bioactivity assay of muTNFR-Fc. muTNFR-Fc inhibited tumor necrosis factor (TNF)-induced killing of mouse fibroblasts with a half-maximal inhibitory concentration of 0.1 nM (mean ± standard deviation, n = 3).

The production and characterization of the fully human fusion protein F8-huIL10 have previously been described by our group. In addition, the fusion protein F8-muIL10 was cloned and expressed by stable transfection in CHO cells. Murine IL-10 was appended at the C-terminus of the F8 antibody in noncovalent homodimeric scFv (diabody) format, using a five-amino-acid linker between variable heavy chain (VH) and variable light chain (VL), and a 15-amino-acid linker between the
antibody and muIL10 (Figure 12a,b). A complete sequence of F8-muIL10 is reported in 12.1.2. Also in this case, the fusion protein was purified by protein A chromatography from the cell culture supernatant, yielding a well-behaved protein preparation (Figure 12c,d).

**Figure 12.** Cloning, expression and *in vitro* characterization of F8-muIL10. (a) Schematic representation of the cloning strategy of F8-muIL10. The murine IL-10 moiety was fused by a 15-amino-acid linker (SSSSG)₃ to the C-terminus of the F8 scFv antibody fragment in
diabody format (five-amino-acid linker between variable heavy chain ($V_H$) and variable light chain ($V_L$)). SS, signal sequence. (b) Schematic representation of protein domain assembly of the noncovalent F8-muIL10 dimer. (c) SDS-PAGE analysis of purified F8-muIL10. M, molecular weight marker; NR, nonreducing conditions; R, reducing conditions. (d) Size exclusion chromatography (SEC200) of noncovalent homodimeric F8-muIL10. (e) MC/9 cell proliferation assay. F8-muIL10 and recombinant murine IL-10 (rmuIL10) induced proliferation of MC/9 cells (mean ± standard deviation (SD), n = 3). (f) BIAcore analysis of F8-muIL10 on extra-domain A of fibronectin (EDA)-coated chip. (g) Quantitative biodistribution study of radioiodinated F8-muIL10. Mice bearing subcutaneous F9 tumors were injected intravenously with 15 µg radiolabeled protein (n = 3). Mice were sacrificed after 24 hours and organs were excised and radioactivity counted, expressing results as percent of injected dose per gram of tissue (%ID/g ± SD).

The biological activity of F8-muIL10 was tested by a proliferation assay on MC/9 cells, as previously described (Figure 12e)\textsuperscript{27}. Furthermore, the formation of a high-affinity and kinetically stable complex between F8-muIL10 and its cognate antigen (the alternatively spliced EDA domain of fibronectin) was confirmed by BIAcore analysis (Figure 12f). \textit{In vivo} targeting was assessed by quantitative biodistribution using radioiodinated F8-muIL10 injected i.v. into mice bearing subcutaneous F9 tumors. Selective tumor targeting was seen, with a tumor to blood ratio of 12, but spleen and liver uptake were also high (Figure 12g).

In addition, the \textit{in vivo} targeting properties of F8-muIL10 and F8-huIL10 were investigated in mice with collagen-induced arthritis, using near-infrared fluorescence imaging. The fusion proteins, studied at 100 µg and 200 µg doses after labeling with IRDye 750, were injected i.v. and mice were imaged 1, 4, 24, and 48 hours after injection, always using the same acquisition parameters. The experiments showed a preferential accumulation of the F8-IL10 fusion proteins in inflamed paws and toes, which slowly declined over time (Figure 13).
Figure 13. *In vivo* near-infrared fluorescence imaging of F8-IL10 fusion proteins in mice with collagen-induced arthritis. Arthritic mice (n = 1) were injected intravenously with 200 or 100 µg F8-muIL10 or F8-huIL10 labeled with IRDye 750. (a) Mice injected with 200 µg of fusion protein were imaged 1, 4, 24 and 48 hours after injection. (b) After 48 hours mice were sacrificed and individual paws were imaged. In addition, photographs of paws are shown to illustrate the paw swelling. (c) Mice injected with 100 µg fusion protein were imaged 1, 4, and 24 hours after injection. (d) After 24 hours mice were sacrificed and individual paws were imaged. Indicated numbers represent the score of the according paw: 1, one toe inflamed and swollen; 2, more than one toe, but not entire paw, inflamed and swollen or mild swelling of entire paw; 3, entire paw inflamed and swollen. FL, front left; FR, front right; BL, back left; BR, back right; plus the according score. *Site of immunization.
4.1.2 Therapy experiments in the collagen-induced model of arthritis

The therapeutic activity of muTNFR-Fc and of F8-IL10 proteins (F8-huIL10 and F8-muIL10) was tested in the collagen-induced model of rheumatoid arthritis in male DBA/1J mice. In a first experiment, we used a full collagen induction (0.05 ml emulsion of bovine type II collagen emulsified in Completes Freund’s Adjuvant and a booster injection of 0.05 ml of the same emulsion), which led to a rapid development of the disease. Arthritis severity was monitored using a conventional arthritic score (1 point for each limb with at least one affected toe, 2 points if the whole paw is swollen) and a modified score (1 point if one toe per limb is affected, 2 points if more than one toe per limb is affected or a moderate paw swelling is observed, 3 points if the entire paw is swollen, 4 points if the paw is severely swollen). Treatment was started when mice displayed a total arthritic score of 1, 2 or 3 (Figure 14). However, out of 50 mice scheduled for treatment, 15 (30%) developed an explosive disease (moved within 1 day from score 0 to score >3) and could not be included in the therapy experiment. The remaining mice were treated with three injections of saline (s.c.), muTNFR-Fc (10 µg, i.v.), F8-huIL10 (200 µg, s.c.) or the combination of muTNFR-Fc and F8-huIL10. The strongest inhibition of arthritis progression (both in terms of arthritic score and of paw swelling) was observed for the combination treatment (p <0.01 vs. saline), whereas the single agents did not display a significant activity in this aggressive model of arthritis with full collagen induction (Figure 14a,b,c).
**Figure 14.** Therapy studies in mice with collagen-induced arthritis. (a) to (d) Mice were immunized according to the full collagen induction protocol, included in the therapy when showing symptoms and injected on days 1, 4 and 7 (arrows). *Fifteen of 50 mice (30%) developed an explosive disease and could not be included in the therapy. Mice received saline subcutaneously (s.c.; squares), 200 µg F8-huIL10 s.c. (circles), 10 µg muTNFR-Fc intravenously (i.v.; triangles) or a combination of F8-huIL10 and muTNFR-Fc (crosses). The clinical score was evaluated daily and expressed as (a) arthritic score or (b) modified arthritic score with mean and standard error of the mean (SEM; n = 7). (c) Paw swelling was measured daily and paw thickness expressed as the mean of all four paws (mean and SEM). (d) Weight was monitored daily and expressed as percent of weight loss (mean and SEM). (e) to (h) Mice were immunized according to the reduced collagen induction protocol.
Six of 60 mice (10%) developed disease too rapidly and could not be included in the therapy. Mice received saline s.c. (squares), 200 µg F8-huIL10 s.c. (circles), 30 µg muTNFR-Fc i.v. (triangles), a combination of F8-huIL10 and muTNFR-Fc (crosses) or 200 µg F8-muLL10 s.c. (open circles). (e) Arthritic score or (f) modified arthritic score with mean and SEM (n = 10). (g) Paw swelling was measured daily and paw thickness expressed as the mean of all four paws (mean and SEM). (h) Weight was monitored daily and expressed as percent of weight loss (mean and SEM). *p <0.05, **p <0.01 combo versus saline; *p <0.05, **p <0.01 muTNFR-Fc versus saline; *p <0.05 combo versus muTNFR-Fc.

We repeated the experiment using a reduced collagen induction schedule, featuring a reduced booster injection (reduction to 80%, 0.04 ml emulsion instead of 0.05 ml). In this case, only 6/60 mice (10%) could not be included into the therapy experiment, because they progressed too rapidly from score 0 to score >3. F8-muLL10 (200 µg s.c.), F8-huLL10 (200 µg, s.c.) and muTNFR-Fc (30 µg, i.v.; alone or in combination with F8-huLL10) displayed an inhibition of disease progression compared with saline treatment (Figure 14e,f,g), being statistically significant for muTNFR-Fc alone or in combination with F8-huLL10 compared with saline from day 6 onwards (p <0.05). Over time, mice lost weight because of arthritis progression, an effect that was reduced by the combination therapy (Figure 14d,h). The administration of IL-10 fused to an antibody of irrelevant specificity in the mouse (HyHel10-IL10) has previously been described, showing inhibition activities that were lower than the corresponding fusion proteins based on the F8 or L19 antibodies.

4.1.3 Characterization of mouse plasma
Plasma was collected from mice before the first therapeutic injection and when they were sacrificed. We used BIAcore technology in order to assess whether mice developed a mouse anti-fusion protein antibody (MAFA) response as a result of
treatment with the fully human F8-huIL10 or the chimeric F8-muIL10 fusion protein (Figure 15a,b). Plasma samples were studied at a dilution of 1:500, whereas monoclonal antibodies specific to human and murine IL-10 were used as positive controls in this assay at concentrations of 1 and 4 µg/ml. Plasma samples before immunocytokine treatment (day 1) did not display a detectable BIAcore response, whereas all samples at the end of treatment revealed the presence of a MAFA reaction.

Figure 15. Analysis of mouse anti-fusion protein antibody response. (a) Schematic representation of the BIAcore experiment. F8-huIL10 or F8-muIL10 was immobilized on a microsensor chip. Plasma of mice treated with either the fully human F8-huIL10 or the chimeric F8-muIL10 fusion protein or positive control antibodies (anti-huIL10 or anti-muIL10) were passed over the different flow cells. (b) The flow rate over the sensor surface was 30 µl/minute for 3 minutes and the response was recorded 30 seconds after the end of the injection. (c) Relative response (RU) of plasma samples and positive controls. Samples were passed over the two different flow cells coated with F8-huIL10 (black bars) or F8-muIL10 (grey bars) and binding of mouse anti-fusion protein antibody (MAFA) expressed as relative response.
In this assay, there was no significant difference between the use of the fully human F8-huIL10 or of the chimeric F8-muIL10 fusion protein, suggesting that the main immunogenic contribution in the mouse was due to the human F8 antibody moiety (Figure 15c). An assessment of immunogenicity based on binding velocity on BIAcore chips, rather than on BIAcore response units at saturation, did not reveal a substantial difference for the quantification of mouse anti-fusion protein antibodies (see chapter 12.2).

Mouse plasma was also used to measure cytokine levels using a multiplex bead-based assay. The results of these measurements are reported in Figure 16 and Figure 17, corresponding to the therapy experiments with full collagen induction and reduced collagen induction. For each sample and cytokine, measurements were repeated on a different day in order to have an independent replicate of the assay (see chapter 12.3).

![Figure 16](image_url)

**Figure 16.** Analysis of cytokine levels in mice with full collagen-induced arthritis. At the end of the therapy 13 different cytokine concentrations were measured in plasma using multiplex bead-based flow cytometry. Data points of cytokine concentrations above detection level are represented in a scatter plot with the mean ± standard error of the mean.
(n = 7). Standard curves defined with positive control samples (see 12.3) were used to generate a level of quantification (LOQ; dotted red line). Most of the measured concentrations were below the LOQ, but a significant increase in the level of IL-27 was observed for F8-huIL10 and the combination versus saline (* p = 0.0017 and p = 0.038, respectively).

Using standard curves defined with positive control samples (see chapter 12.3) a level of quantification was assigned to every cytokine. Most of the measured concentrations were below the level of quantification, but a significant increase in the level of IL-27 was observed for F8-huIL10 and the combination versus saline (p = 0.0017 and p = 0.038, respectively) in the full collagen induction setting.

**Figure 17.** Analysis of cytokine levels in mice with reduced collagen-induced arthritis. At the end of the therapy 13 different cytokine concentrations were measured in plasma using multiplex bead-based flow cytometry. Data points of cytokine concentrations above detection level are represented in a scatter plot with the mean ± standard error of the mean (n = 10). Standard curves defined with positive control samples (see chapter 12.3) were used to generate a level of quantification (LOQ; dotted red line).
4.1.4 Incubation of immunocytokines with whole blood

Immunocytokines are expected to exert their therapeutic activity by extravasation from blood vessels at sites of disease, followed by antibody binding to its cognate antigen and by a prolonged interaction of the cytokine moiety with leukocytes. Since the binding of IL-10 to its receptor on blood cells could potentially inhibit extravasation and disease-targeting activity, we measured the ability of radioiodinated preparations of F8-huIL10 and F8-muL10 to interact with blood cells by a centrifugation-based assay. The two fusion proteins were incubated with human and murine blood, respectively, for 10 minutes at various concentrations. A centrifugation step followed by radioactivity counting in plasma and in the cellular pellet allowed one to determine the fraction of unbound immunocytokine. Figure 18 shows that ~90% of F8-huIL10 is not bound to blood cells at concentrations as low as 0.2 µg/ml, while F8-muL10 revealed ~40% binding to blood at a concentration of 1 µg/ml.

![Figure 18](image1.png)

**Figure 18.** Incubation experiment of radiolabeled immunocytokines with blood, analyzed by a centrifugation-based assay. (a) Different concentrations of F8-huIL10 were incubated with fresh human blood. (b) Different concentrations of F8-muL10 were incubated with fresh mouse blood. In both cases, inhibitors of coagulation were used. After a centrifugation step and separation of plasma from the cell pellet, radioactivity was counted and expressed as percent of binding.
4.2 The antibody-based delivery of IL-4 to the neo-vasculature cures mice with arthritis

4.2.1 Cloning, expression and characterization of F8-IL4

F8-IL4 is a fusion protein consisting of the F8 antibody in noncovalent homodimeric scFv format ("diabody")\(^1\), fused to murine IL-4. The diabody format has previously been shown to allow an efficient accumulation at sites of disease, while being rapidly cleared from circulation\(^2\). We expressed F8-IL4 and KSF-IL4 (an immunocytokine of irrelevant specificity in the mouse, serving as negative control as it recognizes hen egg lysozyme) in CHO cells, yielding homogenous protein preparations after affinity chromatography (Figure 19a-c). A radioiodinated preparation of F8-IL4, incubated with mouse blood, remained in the plasma after a centrifugation step, indicating that the protein is not efficiently trapped by leukocytes (Figure 19d). An intravenous administration of radioiodinated F8-IL4 and KSF-IL4 into mice with collagen-induced arthritis revealed that F8-IL4 (but not KSF-IL4) was able to selectively localize at sites of arthritis (e.g., inflamed toes and paws) in the mouse, as revealed by an autoradiographic analysis 24 h after injection (Figure 19e). A microscopic analysis of antigen expression and of the immunocytokine localization in arthritic lesions confirmed that the F8-IL4 fusion protein was able to selectively target the sub-endothelial extracellular matrix of newly formed blood vessels (Figure 19f).

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\(^1\) Adapted from Hemmerle, T.*, Doll, F.* & Neri, D. Proc Natl Acad Sci U S A. 2014. * contributed equally to the study
Figure 19. Cloning, expression and characterization of F8-IL4. (a) Schematic representation of an antibody-cytokine fusion protein based on the F8 antibody in diabody format and IL-4 (F8-IL4 respectively KSF-IL4). (b) SDS-PAGE analysis of purified fusion protein (M, molecular-weight marker; R, F8-IL4 under reducing conditions; NR, F8-IL4 under non-reducing conditions). Monomeric fusion proteins are expected to have a molecular weight of 40 kDa. (c) Size exclusion chromatography profile of purified F8-IL4 (SEC 200). The peak eluting at a retention volume of 13.2 ml corresponds to the noncovalent homodimeric form of F8-IL4. (d) Incubation experiment of radiolabeled protein preparation with murine blood. Radioiodinated F8-IL4 (50 μg/ml) was incubated with fresh mouse blood containing inhibitors of coagulation. After centrifugation and separation of plasma from the cell pellet, radioactivity was counted and expressed as percent of initial dose. (e) Investigation of selective accumulation of F8-IL4 in inflamed paws. Arthritic mice were injected with ¹²⁵I-labelled F8-IL4 or KSF-IL4 (untargeted IL-4; specific to hen egg lysozyme; negative control). Uptake of radioiodinated antibodies was analyzed by phosphorimaging 24 hours after injection (score 0, no inflammation or swelling; score 1, one inflamed and swollen toe; score 2, two or more inflamed and swollen toes). (f) Immunofluorescence analysis of targeting. Healthy and inflamed paw tissues were stained ex vivo with F8-SIP or KSF-SIP (green). Additionally, mice with established arthritis were injected with F8-IL4 or KSF-IL4 and antibody accumulation was analyzed by staining for IL-4 (green). (Vascular CD31 staining in magenta; Scale bar = 100 μm)
4.2.2 Therapy experiments in the collagen-induced model of arthritis

The therapeutic activity of F8-IL4 was assessed in mice with collagen-induced arthritis. F8-IL4 showed a dose-dependent disease-modulating effect with a superior performance to a murine analogue of etanercept (TNFR-Fc) in a model of severe arthritis\(^1\)\(^\text{12}\). The therapeutic benefit also resulted in a reduction of body weight loss and correlated with decreased IL-6 and increased IL-13 serum levels (Figure 20a, Appendix 12.3). When comparing F8-IL4 to untargeted IL-4 (KSF-IL4), the F8-based immunocytokine exhibited a clear superiority over KSF-IL4 (p <0.01) (Figure 20b, Appendix 12.3). A comparison between intravenous and subcutaneous administration of F8-IL4 was also performed, as the subcutaneous route is more convenient for patients. Both treatments were similarly efficacious in terms of disease score, but the subcutaneous administration of F8-IL4 resulted in a decreased body weight loss (Figure 20c).
Figure 20. Therapeutic activity of F8-IL4 in the collagen-induced arthritis model. The therapeutic proteins were administered 3 times every 72 h (n = 7-9; SEM; *, p <0.05, IL-6 and IL-13 levels were determined in serum). (a) Dose finding and proof of principle study with low dose F8-IL4 (5 μg) and high dose F8-IL4 (100 μg) compared to vehicle (PBS; negative control) and 30 μg murine TNFR-Fc (positive control). (b) Comparison of targeted to untargeted delivery of IL-4. F8-IL4 displayed superior therapeutic activity to KSF-IL4. (c) Effect of the route of administration on efficacy of F8-IL4. No significant difference of subcutaneous to intravenous administration could be observed. (d) Combination therapy with murine TNFR-Fc. No additive effect of F8-IL4 with TNF-blockade was observed.

While the combination with murine TNFR-Fc exhibited no superior disease-reduction than F8-IL4 as monotherapy (Figure 20d, Appendix 12.3), the treatment with a combination of F8-IL4 and a previously described IL-10-based immunocytokine (L19-IL10)26 resulted in disease stabilization, which lasted for 28 days (Figure 21a-c). Surprisingly, the combination of F8-IL4 with dexamethasone resulted in a highly potent disease-modulating activity, with a complete disappearance of any sign of arthritis in 100% (9/9) of the study animals (Figure 21a-c). A cytokine analysis in paws of animals at the end of the experiment confirmed that the F8-IL4 plus dexamethasone combination treatment resulted in a complete normalization of cytokine concentrations. The most striking reduction of cytokine levels was observed for IL-10, IL-13, IL-17, IL-21, IL-22 and TNF levels (Figure 21d, Appendix 12.3). By contrast, only a trend to normalization in anti-collagen antibody levels was observed (Figure 22). F8-IL4 treatment did not lead to increased IgE levels, in keeping with the observation that IL-4 blockade had no effect on circulating eosinophils and IgE levels in allergy patients (Figure 23)113.
Figure 21. The combination of F8-IL4 and dexamethasone cures mice with collagen-induced arthritis. Therapeutic activity was investigated by arthritic score, measurement of paw thickness, observation of changes in weight and analysis of cytokine levels. Therapeutic proteins were administered 3 times every 72 h and dexamethasone every 24 h for 9 days (n = 8-10; SEM). (a) F8-IL4 in combination with L19-IL10 stabilized arthritis progression over a 4 weeks period. Combination of F8-IL4 with dexamethasone lead to complete regression of arthritis in all mice. (b) Therapeutic regimes were well tolerated. Weight was monitored daily and expressed as percent of weight loss. (c) Combination treatment with F8-IL4 and dexamethasone decreased paw swelling to baseline value (dotted line at 1.8 mm, determined with healthy mice). (d) Cytokine levels were determined in paw tissue lysates.
Figure 22. Determination of bovine type II collagen-specific total IgG, IgG1 and IgG2a antibody titers in serum by ELISA. (a) Comparison of PBS (vehicle control) treated mice to F8-IL4 treated mice. (b) Investigation of the different anti-collagen antibody levels in the cured F8-IL4 combination group with dexamethasone and the stabilized F8-IL4 combination group with L19-IL10 to the PBS control group and healthy mice.

Figure 23. Detection of circulating IgE in serum by ELISA. No difference in IgE concentration in serum of PBS treated mice to mice treated with intravenous F8-IL4 could be observed.
4.3 Cloning, production and characterization of rat immunocytokines for the treatment of chronic cardiac allograft rejection

4.3.1 In vivo PET imaging of F8-SIP

The targeting properties of F8-SIP\textsuperscript{21} were studied in a heterotopic rat heart transplantation model with chronic cardiac rejection using in vivo PET detection. Animals received injections of \textsuperscript{124}I-labeled F8-SIP and the negative control antibody KSF-SIP. One hour after injection both antibodies (\textsuperscript{124}I-F8-SIP and \textsuperscript{124}I-KSF-SIP) were visible in the rats, with a prominent localization in the blood pool, the liver, the kidney and the bladder (Figure 24). In both rats, the transplanted heart (allograft) in the abdominal region was clearly visible 1 hour after injection. After 24 hours, targeting of the F8-SIP antibody to the transplanted heart was found to be more intense, compared to the control antibody KSF-SIP (Figure 25).

\textsuperscript{III} Adapted from Franz, M.*, Doll, F.* et al. Manuscript in preparation. * contributed equally to this study
Figure 24. In vivo PET of heart-transplanted rats using $^{124}$I-F8-SIP or $^{124}$I-KSF-SIP antibody. Rats were imaged 1 h and 24 h post injection (p.i.). Representative coronal and sagittal images with cross hairs indicating transplanted heart. He/Lu, heart/lung region; Sp, spleen; Li, liver; Ki, kidney; tHe, transplanted heart; Bl, urinary bladder; L, anatomical left; R: anatomical right; P, posterior (dorsal); A, anterior (ventral). SUV max = 1 for images 1 h p.i., SUV max = 0.5 and 0.05 for images 24 h p.i. of $^{124}$I-F8-SIP and $^{124}$I-KSF-SIP, respectively.
Figure 25. Magnified images of normal heart and transplanted heart regions 1 h and 24 h after injection of $^{124}$I-F8-SIP or $^{124}$I-KSF-SIP. Images are from the same rats as in Figure 24. Cross hairs indicate recipient heart or transplanted heart (allograft). SUV max = 1.5 in images 1 h p.i. and 0.5 and 0.05 for images 24 h p.i. of $^{124}$I-F8-SIP and $^{124}$I-KSF-SIP, respectively.
4.3.2 Cloning, expression and *in vitro* characterization of rat immunocytokines

**F8-ratIL10**

The chimeric fusion protein F8-ratIL10 was cloned into a mammalian cell expression vector, fusing the F8 antibody in diabody format\(^{21}\) to the sequence of rat IL-10 (amino-acid 19-178), and expressed in stably transfected CHO cells (Figure 26a; for the complete sequence of F8-ratIL10 see Appendix 12.1.3). The short five-amino-acid linker between V\(_H\) and V\(_L\) forces the formation of the noncovalent homodimeric scFv in diabody format (Figure 26b). F8-ratIL10 was purified by protein A chromatography from cell culture supernatant, yielding a pure protein preparation as tested by SDS-PAGE and size exclusion chromatography analysis (Figure 26c,d). The protein retained comparable biological activity to recombinant rat IL-10 in a MC/9 cell proliferation assay (Figure 26e). The binding capacity of the antibody F8 to its cognate EDA antigen (the alternatively spliced EDA domain of fibronectin) was intact, as confirmed by BIAcore analysis (Figure 26f).

The cloning, expression and characterization of KSF-huIL10 was performed in analogy to F8-huIL10 as previously described\(^{27}\). Both proteins, F8-huIL10 and the negative control fusion protein KSF-huIL10, were produced at high purity and in sufficient amounts for targeting, together with F8-ratIL10, in a heterotopic rat heart transplantation model.
**Figure 26.** Cloning, expression and characterization of F8-ratIL10. (a) Schematic representation of the chimeric antibody-cytokine fusion protein F8-ratIL10. The rat IL-10 gene was fused via a 15-amino-acid linker (SSSSG)₃ to the C-terminus of the F8 antibody in diabody format (V₇ fused to V₉ by a five-amino-acid linker). The sequence was ligated into the mammalian cell-expression vector pcDNA3.1(+) after NheI/NotI double digest. SS, signal sequence. (b) Schematic representation of the different moieties of the noncovalent F8-ratIL10 dimer. (c) SDS-PAGE analysis of purified F8-ratIL10. M, molecular weight marker; NR, non-reducing conditions; R, reducing conditions; expected molecular weight of the monomer = 45 kDa. (d) Size exclusion chromatography (SEC200) profile of F8-ratIL10 with a single peak showing the noncovalent dimer. (e) Biological activity of the IL-10 moiety of the fusion protein was tested in a proliferation assay with MC/9 cells. Both, the fusion protein F8-ratIL10 and recombinant rat IL-10 at equivalent doses, induced proliferation of the MC/9 cells (mean ± SD, n = 3). (f) Binding analysis of the F8 antibody to its cognate antigen EDA in a BIAcore experiment revealed intact binding capacity of the fusion protein.

**F8-ratIL4**

The chimeric fusion protein F8-ratIL4 was cloned in analogy to F8-IL4⁹⁹ and F8-ratIL10. The F8 antibody in diabody format²¹ was fused to the sequence of rat IL-4 (amino-acid 25-147) and expressed in stably transfected CHO cells (Figure 27a; for
the complete sequence of F8-ratIL4 see Appendix 12.1.4). Between $V_H$ and $V_L$ a short five-amino-acid linker was introduced and forces the formation of the noncovalent homodimeric scFv in diabody format (Figure 27b). The pure protein preparation of F8-ratIL4 was tested by SDS-PAGE and size exclusion chromatography analysis (Figure 27c,d). With the BIAcore analysis on an EDA coated sensor chip we could demonstrate binding capacity of the F8 antibody to its cognate antigen (Figure 27e).

**Figure 27.** Cloning, expression and characterization of F8-ratIL4. (a) Schematic representation of the antibody-cytokine fusion protein F8-ratIL4. Via a 15-amino-acid linker $(SSSSG)_3$ the rat IL-4 gene was fused to the C-terminus of the F8 antibody. The sequence was ligated into the mammalian cell-expression vector pcDNA3.1(+) after $N_{hel}/NotI$ double
digest. SS, signal sequence. (b) Schematic representation of the noncovalent F8-ratIL4 dimer. (c) SDS-PAGE analysis of purified F8-ratIL4. M, molecular weight marker; NR, non-reducing conditions; R, reducing conditions (d) Size exclusion chromatography (SEC200) profile of F8-ratIL4 with a single peak corresponding to the noncovalent dimer. (e) A BIAcore experiment revealed intact binding capacity of the fusion protein to its cognate antigen EDA.

4.3.3 Targeted delivery of IL-10 in chronic cardiac allograft rejection

In collaboration with the group of PD Marcus Franz in Jena (Germany), the chimeric fusion protein F8-ratIL10 was tested in a rat animal model of chronic cardiac rejection. Some of the results obtained after weekly treatment of the rats with different immunocytokines (F8-ratIL10, F8-huIL10 and KSF-huIL10) over a period of 10 weeks are shown in this chapter.

Clinical and macroscopic findings

Clinical monitoring of heart-transplanted rats was performed at regular intervals. All rats tolerated the immunocytokine treatment well and were in good clinical condition during the entire period of therapy. Irrespective of the transplantation procedure or the kind of immunocytokine that has been injected, there was a comparable increase in body weight in all rats. At day 70, when the experiment was stopped and organs were explanted, the weight of cardiac allografts as well as recipient hearts was estimated. In the groups that received F8-huIL10 or F8-ratIL10, the weight of the allografts was increased compared to the groups that received PBS or KSF-huIL10. Between the PBS group and the F8-ratIL10 group, the differences in allograft weight showed statistical significance (p = 0.025).
**Histological evaluation of allografts and recipient hearts**

Histological evaluation of cardiac allografts, recipients or control hearts was performed using HE-, EvG- as well as Sirius Red-stained tissue sections. In heart-transplanted animals that received PBS or KSF-huIL10, all histological signs of chronic cardiac allograft rejection; i.e. CAV, inflammatory cell infiltration, cardiac myocyte degeneration and CIF, occurred as expected and in an extend that has been demonstrated several times in recent studies using the heterotopic rat model employed in the current experiments. Transplanted rats that received F8-huIL10 or F8-ratIL10 respectively, exhibited a much more severe extent of tissue damage with a clear accentuation of inflammation and CIF compared to the PBS or the KSF-huIL10 group. Control F344 rats did not show any histological signs of tissue damage irrespective of treatment. Representative histological images for the different control and experimental groups are given in Figure 28.
Figure 28. Histological evaluation of cardiac allografts, recipient hearts and controls. (a-d) HE-stained cardiac tissue sections of non-transplanted control rats (F344) did not show any histological signs of tissue damage irrespective of treatment. (e-m) In transplanted animals that received PBS or KSF-huIL10 (e, f, i, k), all histological signs of chronic cardiac allograft rejection (CAV, inflammatory cell infiltration, cardiac myocyte degeneration and CIF) occurred as representatively shown in HE- (e, f) and Sirius Red- (i, k) stained sections. Transplanted animals that received F8-huIL10 or F8-ratIL10 (g, h, l, m), exhibited a much more severe extent of tissue damage with a clear accentuation of inflammatory cell infiltration and CIF as shown in representative HE- (g, h) and Sirius Red- (l, m) stained sections. (Magnification: 20x; scale bars = 100 μm).

Immunohistochemical characterization of immune cell infiltration
All inflammatory cells analyzed by immunohistochemistry (i.e., CD4⁺, CD8⁺, CD68⁺ and CD161⁺ cells), could be specifically detected in all cardiac allografts, although at different levels. Figure 29 presents representative images of immune cell infiltration in transplanted heart sections, from the four different treatment groups (red
fluorescence). A strong increase of immune cell infiltration could be observed in the F8-huIL10 / F8-ratIL10 treatment groups compared to the PBS / KSF-huIL10 groups.

**Figure 29.** Immunohistochemical detection and characterization of immune cell infiltration in rat cardiac allografts of the four different experimental groups by immunofluorescence labeling (red fluorescence): CD4 positive cells (a-d), CD8 positive cells (e-h), CD68 positive cells (i-m), CD 161 positive cells (n-q) and α-SMA positive cells (r-u). For all immune cells as well as for α-SMA positive cells, there was an extensive increase in the F8-huIL10 and / or F8-ratIL10 groups compared to the PBS and / or KSF-huIL10 groups (Magnification: 20x; scale bars = 100 μm).
4.3.4 Analysis of rat anti-fusion protein antibody response

Plasma collected from rats treated with F8-ratIL10, F8-huIL10, KSF-huIL10 and PBS was analyzed using BIAcore technology, in order to assess whether rats had developed a rat anti-fusion protein antibody (RAFA) response as a result of treatment (Figure 30). As positive control we used plasma from arthritic mice treated with F8-huIL10, F8-muIL10 or PBS, which has been analyzed previously using the same experimental protocol (see chapter 4.1.3, Figure 15 and Doll et al.\textsuperscript{112}) and anti-rat and anti-human IL-10 antibodies. Plasma samples from mice had shown generation of mouse anti-fusion protein antibodies (MAFA) as seen previously. Surprisingly, plasma samples from rats treated with the fusion proteins showed no generation of rat anti-fusion protein antibodies (RAFA). Only in one rat treated with F8-ratIL10, a weak RAFA response was seen. Since antibodies were detected on both F8-ratIL10 and F8-huIL10 antigens, we assume that the RAFA response was directed against the human F8 antibody moiety. In this rat, we could observe the generation of RAFA’s at week 5 until week 8, whereas the levels normalized at the end of the therapy experiment (Figure 31).
Figure 30. Analysis of anti-fusion protein antibody response. The relative response (RU) was recorded 30 seconds after the end of injection. Samples were passed over three different flow cells coated with F8-ratIL10 (blue), F8-huIL10 (red) and KSF-huIL10 (green). Positive control antibodies against rat and human IL-10 were used to test the coating at the beginning and the end of the experiment. Plasma samples from mice with rheumatoid arthritis treated with F8-huIL10 (n = 3), F8-muIL10 (n = 3) and PBS (n = 1) were used as positive controls and showed formation of mouse anti-fusion protein antibodies (MAFA). Plasma taken at week 8 from rats treated with F8-ratIL10 (n = 3), F8-huIL10 (n = 3), KSF-huIL10 (n = 3) and PBS (n = 2) was negative in formation of rat anti-fusion protein antibodies (RAFA) except in one rat treated with F8-ratIL10.
Figure 31. Analysis of anti-fusion protein antibody response over time. The relative response (RU) was recorded 30 seconds after the end of injection. Samples were passed over three different flow cells coated with F8-ratIL10 (blue), F8-huIL10 (red) and KSF-huIL10 (green). Plasma taken weekly from rats treated with (a) F8-ratIL10 (n = 3), (b) F8-huIL10 (n = 3), (c) KSF-huIL10 (n = 3) and (d) PBS (n = 2) was analyzed and showed no formation of rat anti-fusion protein antibodies (RAFA). Only one rat treated with F8-ratIL10 produced antibodies against the fusion protein between week 5 and 8. (e) Control animals with no heart transplantation but treatment with F8-ratIL10, F8-huIL10, KSF-huIL10 and PBS (n = 1 for each).
5 Discussion

5.1 Murine analogues of etanercept and of F8-IL10 inhibit the progression of collagen-induced arthritis in the mouse

We have described and characterized a murine version of etanercept and a chimeric version of F8-IL10, which represent useful tools for the preclinical testing of these two therapeutic modalities, that are based on TNF inhibition and on the targeted delivery of an anti-inflammatory immunocytokine, respectively. Murine versions of etanercept have previously been reported\textsuperscript{114-116}, but these articles did not provide full information about the cloning, purification and \textit{in vitro} characterization of the fusion proteins. Barck and colleagues reported that their version of mTNFRII-Fc showed significant treatment effect in a murine model of collagen-induced arthritis at a concentration of 1 mg/kg (approximately 20 µg/mouse), upon analysis of computer-assisted tomography data\textsuperscript{115}. In our study, we see \textit{in vivo} activity in the 10 to 30 µg/mouse dose range.

In a first \textit{in vivo} experiment, we used a full collagen induction protocol (see Materials and Methods). This led to a rapid onset of arthritis, with 30% of the mice developing explosive disease (that is, moved within 1 day from score 0 to score >3; excluded from therapy). The mice included in the therapy experiment showed strong progression in arthritic score, which was only inhibited by the combination therapy (F8-huIL10 plus muTNFR-Fc). In contrast to what has been previously reported by our group in a less aggressive model of arthritis\textsuperscript{27}, F8-huIL10 did not show a significant therapeutic effect, when used as a single agent. In a second \textit{in vivo} experiment the booster injection was reduced to 80%, resulting in a milder development of the disease with only 10% of the mice excluded from therapy. In this

\textsuperscript{IV} Adapted from F. Doll et al. Arthritis Research & Therapy 2013
case, TNF blockade ($p = 0.0035$), F8-huL10 ($p = 0.0561$) and the combination treatment ($p = 0.0023$) inhibited arthritis progression upon day 8 when compared with saline (Figure 14f). We have previously demonstrated that the therapeutic activity of targeted IL-10 (for example, F8-huL10 or L19-huL10) is superior to the one of IL-10 fused to an antibody of irrelevant specificity in the mouse$^{26,27}$.

No correlation could be found between treatment groups and the generation of collagen-specific antibodies (see Appendix 12.4). Furthermore, within the same mouse, healthy and inflamed structures could be observed in different paws, which prevented a homogeneous comparison of leukocyte infiltration in different treatment groups (see Appendix 12.5).

Since one of the goals of our investigation was the assessment of immunogenic reactions using the fully human and the chimeric versions of F8-IL10, MAFAs were measured using BIAcore technology (Figure 15). The results show that chimerization did not reduce protein immunogenicity. Human and murine IL-10 share 73% amino-acid identity, whereas an alignment of corresponding germline V segments of antibody variable genes displays 40 to 80% amino-acid identity between the two species$^{117-119}$. The variability of CDR3 regions further contributes to sequence diversity between human and murine antibodies.

The issue as to whether immunocytokines can be trapped in blood by cellular components has received some attention in the past$^{104,120-122}$. Preferably, the immunocytokine should not bind to leukocytes in blood, as this could prevent its ability to localize on its cognate marker of disease at extravascular sites. Figure 18 shows a surprising difference in cellular trapping between F8-huL10 incubated with human blood and F8-muL10 incubated with mouse blood. This discrepancy may reflect a different abundance of IL-10 receptors between mouse and human
leukocytes. Reassuringly, the clinical-stage product F8-huIL10 was not blocked by blood cells at concentrations as low as 0.2 µg/ml.

Dekavil (F8-huIL10) has shown selective targeting of arthritic lesions and inhibited progression of established disease in mice with collagen-induced arthritis and is now being investigated in a phase 1b clinical trial in patients with arthritis in combination with methotrexate. The results of this preclinical study provide a rationale for the combined use of Dekavil with TNF blockers, in line with previous reports about the combination of TNF blockade and IL-10. Previously, the combined use of TNF blockade with other anti-inflammatory biological agents (for example, combination of etanercept with anakinra) was found to be associated with increased safety risks in humans, including serious infections (0% for etanercept alone, 3.7 to 7.4% for etanercept plus anakinra), injection site reactions and neutropenia.

However, the excellent safety profile of recombinant human IL-10 (Tenovil®) and the fact that no signs of toxicity were observed in mice treated with muTNFR-Fc and F8-huIL10 suggest that a combination of etanercept with Dekavil may indeed be clinically feasible.

5.2 The antibody-based delivery of IL-4 to the neo-vasculature cures mice with arthritis

We have described and characterized the use of the immunocytokine F8-IL4 in a murine model of collagen-induced arthritis. The mechanism of action is not fully understood, but we think that the local accumulation of IL-4 in inflamed joints,

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together with the action of dexamethasone, dramatically alters the cytokine levels at the site of disease, reducing inflammation.

We could show by autoradiography and immunofluorescence experiments that the F8-IL4 immunocytokine was able to selectively localize at sites of inflammation in the mouse model of arthritis. High dose F8-IL4 (100 µg) was even more effective than a murine version of Enbrel\textsuperscript{®} (muTNFR-Fc)\textsuperscript{112} used as positive control. Targeting of IL-4 with the F8 antibody was essential for the therapeutic activity effect, as immunocytokines based on the negative control antibody KSF (specific to hen egg lysozyme) did not have similar therapeutic potency. Our group had previously shown that the therapeutic activity of targeted IL-10 (e.g., F8-huIL10 or L19-huIL10) is superior to the one of IL-10 fused to an antibody of irrelevant specificity in the mouse\textsuperscript{26,27}.

We compared subcutaneous and intravenous administration routes for F8-IL4, as a subcutaneous administration may be better accepted by patients and may lead to better compliance. In our hands, the subcutaneous administration yielded therapeutic results which were comparable to those obtained following the conventional intravenous administration route.

IL-4 has many immunomodulatory effects and it is not completely clear how this product mediates a positive effect in rheumatoid arthritis. In order to study the mechanism of action, we measured the concentrations of cytokines in plasma and lysates from inflamed paws. We could observe an association of pharmacological activity with an elevation of IL-13 levels and decreased IL-6 plasma concentrations. The observed correlation between F8-IL4 treatment and cytokine changes in serum may facilitate the monitoring of patients and/or the implementation of patient stratification procedures.
The promising therapeutic effects of L19-IL10\textsuperscript{26}, F8-IL10\textsuperscript{27} and F8-IL4 as single agents in the collagen-induced mouse model provided the rationale for testing the combination of these two immunocytokines. As the two cytokine moieties have different \textit{in vivo} mechanisms, we hoped that a synergistic effect could be seen in the combination treatment. Indeed, mice treated with F8-IL4 and L19-IL10 showed a stabilization of the arthritic score over a time period of 28 days, with no detectable toxicity.

Dexamethasone is a glucocorticoid used for the treatment of patients with rheumatoid arthritis, especially when they have phases of strong active inflammation. We tested the combination of F8-IL4 and dexamethasone, a well known anti-inflammatory drug, frequently used by arthritis patients. Surprisingly, we were able to cure all study animals (9/9) from established arthritis with this combination treatment. The highly potent disease-modulating activity only started to be completely visible after the end of the treatment and was long lasting until day 28. F8-IL4 plus dexamethasone combination treatment resulted in a complete normalization of cytokine concentrations. This effect on cytokine level is likely to be important for the cures observed in animals.

To our knowledge this is the first report of durable and complete regressions in mice with established RA. The findings are likely to be of clinical significance, since dexamethasone is often used to treat RA patients and since the F8 antibody reacts with identical affinity against murine and human EDA, and since other immunocytokines specific to fibronectin splice isoforms are currently being investigated in clinical trials. While some anti-inflammatory therapeutic proteins have been associated with an elevation of cancer risk in patients, F8-IL4 has been shown
to mediate a strong anti-tumoral activity in immunocompetent mouse models of cancer\textsuperscript{99}.

### 5.3 Cloning, production and characterization of rat immunocytokines for the treatment of chronic cardiac allograft rejection\textsuperscript{VI}

The selective accumulation of the F8 antibody at the site of disease was tested using PET technology in a model of chronic cardiac rejection. Rejection-associated cardiac tissue remodeling leads to the expression of fetal variants of the cell-adhesion modulating protein fibronectin, in particular extra-domain A containing fibronectin (EDA). These variants are not detectable in healthy adult organs but overexpressed in association to tissue remodeling\textsuperscript{125}. The human antibody F8, specific to both human and rat EDA may act as a delivery vehicle for bioactive payloads (drugs or cytokines) or diagnostic agents (radionuclides, magnetic beads)\textsuperscript{106}.

For PET imaging analysis, we injected rats having chronic cardiac rejection with $^{124}\text{I}$-F8-SIP and the negative control antibody $^{124}\text{I}$-KSF-SIP. Selective targeting to the allograft was observed with F8-SIP 24 hours after injection. These results are in line with previously reported data showing selective accumulation of the F8 antibody in cardiac allografts using near infrared fluorescence (NIRF) imaging and microscopic analysis\textsuperscript{30}. The antibody localized to diseased blood vessels and fibrotic regions, sites where expression of EDA is expected. These are the first examples of antibody-mediated imaging of chronic cardiac rejection. In addition to the potential development of novel molecular imaging strategies, these findings may be important

\textsuperscript{VI} Adapted from Franz, M.*, Doll, F.* et al. Manuscript in preparation. * contributed equally to this study
for the development of targeted therapeutic strategies, especially when considering the lack of efficient avenues for the prevention or treatment of CAV and CIF.

As shown in other diseases, e.g., cancer and inflammatory disorders like rheumatoid arthritis\textsuperscript{14,27,106}, the targeted delivery of cytokines using monoclonal antibodies holds promises for the development of more selective therapies.

In the setting of chronic cardiac allograft rejection with CAV and CIF, the F8-IL10 fusion protein was an attractive product for preclinical investigations, since IL-10 is known to mediate anti-inflammatory and anti-proliferative effects.

The current study focused on the antibody-based targeted delivery of IL-10 in an appropriate animal model of chronic cardiac allograft rejection. A new fusion protein (F8-ratIL10) was cloned, expressed, characterized and tested in the rat model, comparing it to F8-huIL10\textsuperscript{27} and KSF-huIL10, an immunocytokine of irrelevant antigen specificity in the rat. All observed treatment effects were transplantation-specific, since the F8 antibody is specific to EDA that is not expressed in healthy hearts but is overexpressed after transplantation\textsuperscript{62,65}. The non-transplanted control animals did not show any alterations in cardiac tissue irrespective of treatment.

In the experimental setting used in this study, treatment with F8-IL10 was not able to inhibit chronic rejection development. At present, it is not clear, why F8-IL10 did not attenuate, but possibly aggravated the extent of rejection or tissue alteration. Although this question cannot be answered comprehensively at present, there are some hypotheses that can be proposed.

The idea that IL-10 might have beneficial effects in cardiac transplant rejection is supported both by its anti-inflammatory and anti-proliferative properties and by a few studies on transplant rejection\textsuperscript{84,85,88}. Our current study revealed contradictory findings in the sense of an acceleration of chronic cardiac allograft rejection in the
F8-IL10 treatment groups. Our observations are supported by an interesting study published by Ebbs and colleagues in 2002. Using a rat model of acute rejection, the authors could show that tacrolimus (which is an established immunosuppressive drug administered in heart transplant recipients) can treat ongoing allograft rejection by inhibiting IL-10 mediated cytotoxic cell infiltration\textsuperscript{126}.

Another aspect that should be critically discussed relates to the use of a chimeric protein, rather than a fully rat fusion protein, for a treatment study in a chronic setting. The F8 antibody is human and a rat equivalent is not available at present. Our group could recently demonstrate in a mouse model of arthritis that anti-fusion protein antibodies are developed when treating murine animals with fully human (F8-huIL10) or chimeric (F8-muIL10) fusion proteins\textsuperscript{112}. Because of this fact, we analyzed plasma from treated rats, with the aim to detect the levels of rat anti-fusion protein antibodies (RAFA’s). The chimeric (F8-ratIL10) but also the fully human (F8-huIL10 and KSF-huIL10) fusion proteins did not show any formation of RAFA’s, except in one rat which had been treated with F8-ratIL10. In this case, the RAFA response disappeared at the end of the treatment. The striking difference in the formation of anti-fusion protein antibodies between the arthritis experiments in the mouse and the graft rejection in the rat may relate to the different inflammatory statuses of the two disease models.
6 Conclusions

The targeted delivery of molecules using antibodies as vehicles has gained importance for the treatment of cancer and inflammatory disorders. The selective targeting of effector moieties (e.g., cytokines, drugs and radionuclides) to sites of disease may increase therapeutic activity, while sparing healthy tissue. Disease-homing antibody-cytokine fusion proteins (“immunocytokines”) are interesting candidates for the therapy of cancer and chronic inflammatory conditions with the potential to modulate the activity of the immune system at the site of disease.

In this thesis, we explored the therapeutic effects of different immunocytokines, alone or in combination with other drugs.

In a first part of this thesis, we cloned, produced and characterized a murine version of Enbrel® (muTNFR-Fc) and the chimeric immunocytokine F8-muIL10. The murine analogues represent useful research tools for the study of TNF blockade and targeted cytokine delivery in the murine model of RA. The results obtained in this mouse model provide a rationale for the combination of Dekavil (F8-huIL10) with Enbrel® in patients with RA who do not respond sufficiently to either of the monotherapies and other approved drugs.

When using therapeutic proteins, it is important to monitor the emergence of an immunogenic reaction, as this may bias therapy results and safety assessments. In this thesis, we studied the different immunogenicity potential of a fully human (F8-huIL10) and of a chimeric (F8-muIL10) immunocytokine in a mouse model of arthritis. Unexpectedly, chimerization of F8-IL10 did not reduce protein immunogenicity, as demonstrated using BIACore technology.
In a second project, we tested the therapeutic activity of F8-IL4 in the collagen-induced model of arthritis. Previous studies had shown that F8-IL4 could inhibit tumor growth in three different immunocompetent murine cancer models. Therapeutic activity was also observed in different models of skin inflammation. We were able to show that F8-IL4 selectively localizes to neovascular structures at sites of rheumatoid arthritis, leading to high local IL-4 concentrations. The combination with an IL-10 immunocytokine showed synergistic effects and induced a long lasting stabilization of established arthritis. The combination of F8-IL4 with dexamethasone, a clinically used glucocorticoid, cured 100% (9/9) of treated mice with established arthritis. To our knowledge this is the first report of durable and complete regressions in mice with established RA. The treatment of patients with rheumatoid arthritis using a fully human version of F8-IL4 will show whether these promising results can be observed also in the clinic. The need for new therapeutics for the treatment of rheumatoid arthritis is still high, as not all patients benefit from the currently available therapeutics. The disease is still incurable.

In a third project, we studied the performance of rat immunocytokines in a chronic cardiac rejection model. The selective targeting of the F8 antibody to sites of chronic rejection and remodeling of tissue was confirmed in vivo using PET technology. The results are in keeping with previously reported near infrared fluorescence (NIRF) imaging studies. Unfortunately, treatment of rats with F8-IL10 did not inhibit chronic graft rejection. An early treatment with F8-IL10 did not attenuate, but possibly aggravated, the extent of rejection or tissue alteration. Further studies, in which treatment is initiated at later timepoints, are needed in order to reveal whether targeted IL-10 delivery may be effective for the treatment of chronic cardiac rejection. We cloned a F8-based immunocytokine with rat IL-4 and it will be
interesting to measure the therapeutic potential of this chimeric immunocytokine in the rat model.

Ongoing research efforts in the field of rheumatoid arthritis will hopefully help develop better therapeutics for patients suffering from the disease. Dekavil (F8-IL10) is a good candidate, which is currently being investigated in clinical trials. In this thesis we were able to show that combination with Enbrel® is effective and safe. These findings may be of clinical significance, as Enbrel® is frequently administered to rheumatoid arthritis patients.

In addition, the observation of cures in mice with arthritis using F8-IL4 has stimulated clinical development activities with a fully human fusion protein, while also providing mechanistic tools to study how a severe autoimmune disease can be “turned off”.
7 Outlook

In this thesis we have shown several results with translational potential. Here, we discuss advantages and disadvantages, benefits and problems associated with the translation of good preclinical drug candidates to industrial and clinical development programs.

The combination of TNF blockers with targeted cytokines is of interest, because of the synergy between two different mechanisms of action. We investigated the combination of the murine analogues of Enbrel® and F8-IL10 in a mouse model of collagen-induced arthritis. The excellent safety profile of recombinant human IL-10 (Tenovil®)67 in humans and the good tolerability of the combination treatment in the animals suggest a combination of Dekavil (F8-IL10) and Enbrel® in patients with rheumatoid arthritis. However, using a combination of Enbrel® and Kineret®, a recombinant IL-1 receptor antagonist, increased safety risks in humans, including serious infections, injection site reactions and neutropenia, have been reported124. In future clinical trials it will be important to monitor patients with combination treatments well, to reduce severe safety risks.

Similar research efforts are made in the field. For example, a bispecific TNF/IL-17A inhibitor, comprising an interleukin 17A (IL-17A) neutralizing Fynomer fused to a fully human anti-TNF antibody, has recently begun clinical testing127.

The promising therapeutic effects of F8-IL4, alone and in combination with another immunocytokine or dexamethasone, provide a strong rationale for the development of a fully human version of this product for pharmaceutical applications in patients.
The next steps in the pharmaceutical development include GMP manufacturing of a fully human F8-IL4 fusion protein (glycosylated or mutant form), toxicological studies in monkeys and then first dose-finding studies in patients with rheumatoid arthritis. The identification of an optimal dose regimen in humans will pose some challenges. In the mouse model, administration of 100 µg of F8-IL4 (corresponding to 33 µg IL-4) every 72 hours was well tolerated. Previous reports of clinical use of recombinant IL-4 suggest toxicities, including nausea, vomit, headache/pain and cases of grade 4 toxicities in cancer patients treated daily with doses of 5 µg/kg\textsuperscript{128}. Reducing dosing to 5 µg/kg thrice weekly resulted in mild toxicities with no grade 4 non-hematologic toxicity\textsuperscript{129}. In patients with psoriasis, therapy was well tolerated and showed decreased clinical scores using 0.2-0.5 µg/kg recombinant IL-4 subcutaneous three times daily, 5 days a week\textsuperscript{130}.

An asymmetry between the tolerated dose of IL-4 in the mouse and man can be observed, which we will have to consider when starting clinical trials with F8-IL4 in patients. On the other side, we assume that side effects are reduced with the targeted delivery of IL-4 to sites of inflammation and reduction of systemic concentrations.

The durable and complete regression of established arthritis after F8-IL4 and dexamethasone combination treatment is an important finding when translating F8-IL4 therapy to patients. Glucocorticoids are often used to treat patients with rheumatoid arthritis at the beginning of the disease and later as bridge therapy for flare-ups\textsuperscript{131}. Recent findings showed that the combination of the biologic agent tocilizumab with glucocorticoids significantly lowered remission rates\textsuperscript{132} and that the concomitant use of anakinra and steroids at baseline increased the likelihood to develop an infection\textsuperscript{133}. We still see a great potential of combining F8-IL4 with
dexamethasone in patients, but we will have to consider the above mentioned effects with regards to patient stratification. A careful follow-up will also be needed. It would be important to study in more details the mechanism of action for the synergy between F8-IL4 and the glucocorticoid dexamethasone, which remains largely unexplained.

For the therapy of chronic cardiac rejection, it is too early to say something about the therapeutic potential of our immunocytokines in humans. We still have to find the right cytokine and treatment protocol, which may yield a therapeutic benefit in the rat animal model. On the other side, a non-invasive PET imaging may be of substantial clinical interest for early diagnosis of cardiac allograft vasculopathy (CAV). Currently, only invasive methods are used to diagnose CAV, mainly based on biopsies, coronary angiography or the more sensitive intravascular ultrasound. An early diagnosis could enable physicians to start treatment as soon as possible and therefore to prevent progression and further harmful changes. If CAV is detected only at later time points, treatment may be more difficult.
Materials and Methods\textsuperscript{VII}

8.1 Cell lines and animals

CHO-S cells (Invitrogen, Zug, Switzerland) were cultured adherent in RPMI 1640 (Gibco, Zug, Switzerland) supplemented with 10% fetal bovine serum (Gibco), 2 mM ultraglutamine (Lonza, Basel, Switzerland) and antibiotics/antimycotics (Gibco) or in suspension in PowerCHO-2CD (Lonza) with 8 mM ultraglutamine, HT supplement (Gibco) and antibiotics/antimycotics (Gibco) in shaker incubators. Lung murine fibroblasts (CCL-1.3; ATCC, Molsheim Cedex, France) were cultured adherent in Dulbecco’s modified Eagle’s medium (Gibco) with 10% fetal bovine serum and antibiotics/antimycotics. MC/9 cells (murine mast cells, CRL-8306; ATCC) were cultured according to the supplier’s protocol in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM ultraglutamine, 10% rat-T-STIM (BD Becton Dickinson, Allschwil, Switzerland) and 0.05 mM $\beta$-mercaptoethanol (Gibco). Murine F9 teratocarcinoma cells (CRL-1720; ATCC) were cultured on 0.1% gelatin-coated tissue flasks in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics/antimycotics.

Male DBA/1J and female Balb/c mice were obtained from Janvier (Le Genest-St-Isle, France). Female 129/SvEv mice, male Lewis (LEW) rats and male Fisher (F344) rats were obtained from Charles River (Sulzfeld, Germany).

8.2 Cloning of fusion proteins

8.2.1 Cloning of muTNFR-Fc
For cloning of muTNFR-Fc the murine TNFR gene (extracellular domain of TNFR II, amino-acids 23 to 258) was amplified from previously cloned F8(scFv)-TNFR II by polymerase chain reaction (PCR) using the primer pair 5’-CCTGGTTCCTCGTGCCTGTGGCTACAGGTGTGCACTCGGTGCCCGCCCAGGTTGTCTT-3’ and 5’- CAATC CCTGGGCACGCCACCCTTGGTACTTTGTTCC-3’ appending part of a signal sequence at the N-terminus and an overlapping fragment to muFc at the C-terminus. The gene for murine Fc (hinge, CH2, CH3; amino-acids 98 to 324) was amplified from a commercial cDNA (Source BioScience, Berlin, Germany) using the primer pair 5’-CCAAGGGTGCGTGCCCAGGGATTGTGGTTGAAGC-3’ and 5’- TTTTCTTTTGCCGCGCTCATGCTATTACCCAGGAGATGGGAGAGG-3’ appending an overlapping fragment to muTNFR at the N-terminus and a stop codon and NotI restriction site to the C-terminus. muTNFR and muFc sequences were PCR-assembled using the primer pair 5’-CCAAGGGTGCGTGCCCAGGGATTGTGGTTGAAGC-3’ and 5’- TTTTCTTTTGCCGCGCTCATGCTATTACCCAGGAGATGGGAGAGG-3’ appending the second part of the signal sequence and a HindIII restriction site to the N-terminus. The assembled fragment was double digested with HindIII/NotI (New England BioLabs, Allschwil, Switzerland) and ligated into the mammalian cell-expression vector pcDNA3.1(+) (Invitrogen) (for full sequence see Appendix 12.1.1).

8.2.2 Cloning of F8-muIL10
F8-muIL10 was cloned using the sequence for F8 in diabody format with the primers 5’-CCTGGTTCCTCGTGCCTGTGGCTACAGGTGTGCACTCGGAGGTGCGAG CTGTTGGAGTCTGGGG-3’ and 5’-GATGAGCCGGAAGAGCTACTACCCGATGAGG
AAGATTTGATTTCACCTTGGTCCCTTGCCGAA-3’ introducing part of a signal sequence at the N-terminus and part of a C-terminal (SSSG)\textsubscript{3} linker. The sequence for murine IL-10 (amino-acids 19 to 178) was amplified from a commercial cDNA (Sino Biological Inc., Beijing, China) using the primer pair 5’-GGGTAGTAGCTCTTCCGGCTCATCGTCCAGCGGCAGCAGGCGCCAGTACAGC CGGG-3’ and 5’-TTTCCTTTTGCAGGCGCCTAGCTTTTTCATTTTGATCATCATG-3’ appending a complementary part of the (SSSG)\textsubscript{3} linker at the N-terminus and a stop codon and NotI restriction site to the C-terminus. The sequence for murine IL-10 was PCR-assembled using the primers 5’-CCCGCTAGCGTCCGACCATGGGCTGGAGCCTCGATCCTCCTCGTCCGCTCG GTGGC-3’ and 5’-TTTCCTTTTGCAGGCGCCTAGCTTTTTCATTTTGATCATCATG-3’ adding the rest of the signal sequence and a Nhel restriction site to the N-terminus. The assembled fragment was double digested with Nhel/NotI (New England BioLabs) and ligated into the mammalian cell-expression vector pcDNA3.1(+) (for full sequence see Appendix 12.1.2).

### 8.2.3 Cloning of F8-ratIL10

The rat version of F8-IL10 was cloned in analogy to the above described production of F8-muIL10. The sequence for F8 in diabody format\textsuperscript{21} was amplified using the primer pair 5’-CCTGTTCCTCGTCGCTGTGGCTACAGGTGTGCACTCGGAGGTGC AGCTGTGGAGTCTGGG-3’ and 5’-TTGGCCAAGGGACCAGGTGGAATCAA ATCTTCCGATCGGTAGTAGCTCTCCTCCGGCTCATC-3’ introducing part of a signal sequence at the N-terminus and part of a C-terminal (SSSG)\textsubscript{3} linker. Rat IL-10 (aa 19-178) was amplified from a commercial cDNA (Sino Biological Inc., Beijing, China) using the primer pair 5’-GGGTAGTAGCTCTTCCGGCTCATCGTCCGAGCG CAGGAAAGGACATTCCAGG-3’ and 5’-CGTGAACACTCAAAATGAAATTA
GGCGGCCGCGCCGTGGCAC-3’ attaching the complementary part of the (SSSGS)$_3$ linker to the N-terminus and a stop codon and NotI restriction site to the C-terminus. F8 diabody and rat IL-10 sequences were PCR-assembled using the primers 5’-CCGCTAGCCTCGACATGGGCTGGAGCTGATCTCCTCTGTCTAGCTGTGCCTGTGGC-3’ and 5’-CGTGACTCAATAATGAAAGATTAGGGCGCCGCCCCTGGCA C-3’ attaching the rest of the signal sequence and a Nhel restriction site to the N-terminus. The final fragment was double digested using the restriction enzymes Nhel and NotI (New England BioLabs) and ligated into the mammalian cell-expression vector pcDNA3.1(+) (Invitrogen) (for full sequence see Appendix 12.1.3).

8.2.4 Cloning of F8-ratIL4

The rat version of F8-IL4 was cloned in analogy to F8-muIL4 and F8-ratIL10 (see above). The sequence for F8 in diabody format was amplified using the same primer pair as for the cloning of F8-ratIL10, introducing part of a signal sequence at the N-terminus and part of a C-terminal (SSSGS)$_3$ linker. Rat IL-4 (aa 25-147) was amplified from commercial cDNA (Sino Biological Inc.) using the primer pair 5’-GGTCTAGTCCTTCGACCAGGAGCTCTCTCTCAGCGCTTGTAAACGACAGCGCTCTGA GAGAG-3’ and 5’-GCAGTCCTGCACATTCTCATTGTAACGTCGCTGAGAAGGAAAAA-3’ attaching the complementary part of the (SSSGS)$_3$ linker to the N-terminus and a stop codon and NotI restriction site to the C-terminus. F8 diabody and rat IL-4 sequences were PCR-assembled using the primers 5’-CCCAAGCTTGTGTGCTGAGCAGGCTGGAGCTGATCTCCTCTGTCTAGCTGTGCCTGTGGC-3’ and 5’-GCAGTCCTGCACATTCTCATTGTAACGTCGCTGAGAAGGAAAAA-3’ attaching the rest of the signal sequence and a HindIII restriction site to the N-terminus. The final fragment was double digested using the restriction enzymes...
HindIII and NotI (New England BioLabs) and ligated into the mammalian cell-expression vector pcDNA3.1(+) (Invitrogen) (for full sequence see Appendix 12.1.4)

8.2.5 Cloning of other fusion proteins
The cloning and production of F8-IL4 and KSF-IL4 as well as L19-IL10 in diabody format have been previously described. The negative control fusion protein KSF-huIL10 was cloned in analogy to F8-huIL10 and KSF-IL4 (negative control for F8-IL4) as described previously. Briefly, the KSF diabody, specific to hen egg lysozyme, and the human IL-10 gene were amplified separately, PCR assembled and cloned into the pcDNA3.1(+) mammalian cell-expression vector using the procedures described above (for full sequence see Appendix 12.1.5).

8.3 Protein expression and purification
The fusion proteins were expressed in a stable monoclonal cell line as reported before. Briefly, PEI-mediated transient gene expression was used to generate a polyclonal batch of protein. An aliquot of the transient gene expression culture was used to produce a stable cell line using geneticin (G418, 0.5 g/l; Santa Cruz, Heidelberg, Germany) for selection. Monoclonal cells were screened for high expression of protein by ELISA, using L19-TNFα (produced in our laboratory) as coating antigen and a goat anti-mouse IgG (Fc-specific)-peroxidase antibody (Sigma-Aldrich, Buchs, Switzerland) for detection in the case of muTNFR-Fc. For F8-muIL10, F8-ratIL10 and F8-ratIL4, recombinant EDA was used as antigen and protein A–horseradish peroxidase (GE Healthcare, Glattbrugg, Switzerland) for detection. The best producing clone for each construct was grown in PowerCHO-2CD medium in suspension for large-scale production of protein. The proteins were
purified from cell culture supernatant by protein A affinity chromatography and analyzed by SDS-PAGE, size exclusion chromatography (Superdex200 10/300GL; GE Healthcare) and for the F8-based immunocytokines additionally by surface plasmon analysis (BIAcore) on an EDA-coated CM5 sensor chip (GE Healthcare).

8.4 Characterization of fusion proteins

8.4.1 Bioactivity assays

The biological activity of muTNFR-Fc was determined by its ability to inhibit TNFα-induced killing of mouse fibroblasts110. Lung murine fibroblast cells were seeded in a 96-well plate (30'000 cells/well) in 100 µl culture medium and incubated for 24 hours at 37°C, 5% CO2. Medium containing actinomycin D (final concentration 2 µg/ml; Sigma-Aldrich), TNFα (final concentration of trimer 5 pM; eBioscience, Vienna, Austria) and different concentrations of muTNFR-Fc (serially diluted from 50 nM to 0.1 pM) was added to the cells. After incubation at 37°C for 24 hours cell viability was determined by addition of 20 µl Cell Titer Aqueous One Solution (Promega, Dübendorf, Switzerland) and after 2 hours absorption was measured at 490 nm.

For determination of the biological activity of the IL-10 moiety in the F8-muIL10 and F8-ratIL10 fusion proteins, an IL-4 dependent proliferation assay of MC/9 cells was used27. Cells were seeded in a 96-well plate (40'000 cells/well) with 200 µl culture medium (without rat-T-STIM) containing 5 pg (0.05 units)/ml murine IL-4 (eBioscience) and varying concentrations of F8-muIL10 and F8-ratIL10 or recombinant murine IL-10 and recombinant rat IL-10 (eBioscience and AbD Serotec, Puchheim, Germany, respectively). Starting concentrations were 100 ng/ml IL-10 equivalents for the murine IL-10 experiment and 500 ng/ml IL-10 equivalents for the rat IL-10 experiment. After incubation at 37°C for 48 hours 20 µl/well Cell Titer
Aqueous One Solution was added, and after 2 or 4 hours absorption was measured at 490 nm.

8.4.2 Biodistribution
The \textit{in vivo} targeting of F8-muL10 was tested by quantitative biodistribution analysis using radiolabeled protein as described before\textsuperscript{135}. For this analysis 129/SvEv mice were implanted subcutaneously (s.c.) with F9 tumor cells (25 $\times$ 10\textsuperscript{6} cells) in the flank. Purified F8-muL10 (15 $\mu$g/mouse) was radioiodinated with $^{125}$I (Perkin Elmer, Schwerzenbach, Switzerland) and injected intravenously (i.v.) into the lateral tail vein of mice ($n = 3$) grafted with F9 tumors. Mice were sacrificed 24 hours after injection. Organs were excised, weighed and radioactivity was counted using a Cobra $\gamma$ counter (Packard Instrument Company, Meriden, CT, USA). Radioactivity content of representative organs was expressed as percentage of injected dose per gram of tissue (%ID/g) (reference number 42/2012; Veterinäramt des Kantons Zürich, Zürich, Switzerland).

8.4.3 \textit{In vivo} imaging
To test the targeting properties of the murine and human F8-IL10 fusion proteins, a near-infrared fluorescence imaging study was performed. For this purpose, the proteins (11 nmol F8-muL10 and F8-huL10) were incubated for 1 hour with a 20x molar excess of IRDye 750 N-hydroxysuccinimidyl ester (220 nmol; LI-COR, Bad Homburg, Germany) in 10\% dimethylsulfoxide/phosphate-buffered saline (PBS), pH 7.4, at room temperature. Protein was purified from free dye using a PD10 desalting column (GE Healthcare), eluted in 5\% dimethylsulfoxide/PBS and concentrated to 1.3 mg/ml using Amicon Ultra (10 K) centrifugal filter units (Millipore, Zug, Switzerland). Then 200 $\mu$g (or 100 $\mu$g) of each protein were injected i.v. into the
lateral tail vein of mice (n = 1) that had developed arthritis after the second collagen immunization (see section 7.5 Mouse model of collagen-induced arthritis for more details). Mice were imaged at 1, 4, 24 and 48 hours after the injection under isoflurane anesthesia on their ventral side using an IVIS Spectrum machine (Xenogen, Caliper Life Sciences, Oftringen, Switzerland) with the following imaging parameters: $\lambda_{\text{ex}} = 745$ nm, $\lambda_{\text{em}} = 800$ nm, exposure time = 1 second, F/stop = 4, small binning. After 48 hours, mice were sacrificed and paws (arthritic and not affected ones) were photographed and then submitted to fluorescence imaging, using the same parameters.

### 8.4.4 Autoradiography experiments

The *in vivo* targeting performance of F8-IL4 respectively KSF-IL4 was evaluated by autoradiography analysis in mice with collagen-induced arthritis. Radioiodinated protein preparations were injected into the lateral tail vein (100 μg; F8-IL4 7 μCi, KSF-IL4 8 μCi). Mice were sacrificed 24 h after injection and paws were exposed for 16 h to a phosphorimager screen (Fujifilm, Dielsdorf, Switzerland). Accumulated radioactivity was read with a phosphorimager (BAS5000, Fujifilm, Stamfort, CT, USA).

### 8.4.5 Immunofluorescence analysis

Frozen sections of healthy and arthritic paws were fixed in ice-cold acetone and stained for EDA expression using biotinylated F8 and KSF antibody in small immunoprotein (SIP) format. Bound antibody was detected using Streptavidin-Alexa Fluor conjugate (Invitrogen).

To detect the *in vivo* accumulation of immunocytokines, arthritic mice were injected with F8-IL4 or KSF-IL4 (100 μg/mouse, 3 injections, every 72 h). Sections of swollen
paws (score 3) were stained with a rat anti-mouse IL-4 antibody (eBioscience, Allschwil, Switzerland) and an anti-rat IgG Alexa Fluor coupled secondary antibody (Invitrogen). Vascular structures were stained using anti-CD31 antibody (Santa Cruz) and secondary Alexa Fluor coupled antibody (Invitrogen). Slides were mounted with fluorescent mounting medium (Dako, Glostrup, Denmark) and analyzed with an Axioskop2 mot plus microscope (Zeiss, Feldbach, Switzerland).

8.4.6 Incubation experiments of radiolabeled immunocytokines with whole blood
The ability of F8-huIL10, F8-muIL10 and F8-IL4 to interact with blood cells was determined by a centrifugation-based assay with radiolabeled preparations. Purified F8-huIL10, F8-muIL10 and F8-IL4 were radioiodinated with $^{125}$I (Perkin Elmer) as described before and different concentrations of labeled protein were incubated with fresh human and mouse blood. Human blood was collected in S-Monovette tubes (kalium-EDTA (ethylenediamine tetraacetic acid), Sarstedt, Sevelen, Switzerland). Mouse blood was taken from DBA/1J mice for testing of the F8-muIL10 protein and Balb/c mice for F8-IL4, via cardiac puncture after sacrifice using Microtainer LH tubes (lithium heparin, BD Bioscience) to prevent coagulation. After 10 minutes of incubation, tubes were centrifuged for 3 minutes at 2'000 x g. Plasma was separated from the cell pellet and radioactivity of both was counted using a Cobra γ counter (Packard Instrument Company). Radioactivity was expressed as percent of the input.

8.5 Mouse model of collagen-induced arthritis
Male DBA/1J mice (8 weeks old) were immunized by subcutaneous injection at the base of the tail with 0.05 ml (50 μg) emulsion of bovine type II collagen emulsified in
Complete Freund's Adjuvant (Hooke Laboratories, Lawrence, MA, USA). Three weeks later, a booster injection of 0.05 ml bovine collagen/Complete Freund's Adjuvant in the case of the full collagen induction protocol and 0.04 (40 μg) ml for the reduced collagen induction protocol (and for studies with F8-IL4) was given to the mice. After the booster injection, mice were inspected daily and disease was monitored using two different scoring systems. To each limb a clinical score was assigned (0 = normal, 1 = swelling of one or more toes of the same limb and 2 = swelling of the whole paw). A maximum score of eight can be reached in this first scoring system\textsuperscript{27,136}. A more diverted clinical score, the modified score, was also used (0 = normal; 1 = one toe inflamed and swollen; 2 = more than one toe, but not entire paw, inflamed and swollen or mild swelling of entire paw; 3 = entire paw inflamed and swollen; 4 = very inflamed and swollen paw; adapted from Hooke Laboratories). A maximum score of 16 can be reached. In addition, swelling of affected paws was measured daily with a caliper under isoflurane anesthesia. Paw thickness is expressed as the mean of all four paws of each animal. Animals were included into a therapy group when showing signs of joint inflammation with a score of 1 to 3. When the joint inflammation was too strong at day 1 (more than one paw, score >3) mice were not included into the experiment, because according to our project license (208/2010) we are not allowed to keep a mouse alive with a conventional arthritic score ≥4 for more than 4 days. All animal experiments were performed in agreement with swiss ethical regulations. Ethical approval for all experiments was given by the state veterinary office (reference number 208/2010; Veterinäramt des Kantons Zürich, Zürich, Switzerland).
8.6 Therapy experiments in mice with collagen-induced arthritis

8.6.1 Combination therapy of muTNFR-Fc and F8-huIL10
Mice were immunized according to the full collagen induction protocol (50 µg for first immunization and 50 µg for second immunization). Mice with a new clinical score of 1 to 3 were randomly assigned to a treatment or control group and therapy was started (day 1, n = 7). Mice received intravenous injections of muTNFR-Fc (10 µg) into the lateral tail vein or subcutaneous injections of F8-huIL10 (200 µg) or saline or a combination of muTNFR-Fc (10 µg, i.v.) and F8-huIL10 (200 µg, s.c.), three times on days 1, 4 and 7. Seven mice were analyzed per group in a daily, non-blinded fashion and the arthritic clinical score, the thickness of inflamed paws and weight was monitored. Mice were sacrificed at day 5 (PBS), day 8 (F8-huIL10, muTNFR-Fc) or day 13 (combination) due to arthritic score (≥4 for more than 4 days with conventional arthritic score) and weight loss (>15%), in accordance with local regulations.

8.6.2 Comparison of F8-huIL10 and F8-muIL10
Mice were immunized according to the reduced collagen induction protocol (50 µg for first immunization and 40 µg for second immunization). Therapy was performed as described before (n = 10). Mice received either intravenous injections of muTNFR-Fc (30 µg) or subcutaneous injections of F8-huIL10 (200 µg), F8-muIL10 (200 µg) or saline or a combination of muTNFR-Fc (30 µg, i.v.) and F8-huIL10 (200 µg, s.c.). Ten mice were analyzed per group in a daily, non-blinded fashion and the arthritic clinical score, the thickness of inflamed paws and weight was monitored. Mice were sacrificed at day 8 (PBS), day 9 (F8-huIL10, muTNFR-Fc, F8-muIL10) or day 13 (combination) due to arthritic score and weight loss, in accordance with local regulations.
8.6.3 IL-4 dose finding therapy experiment
Mice were immunized with 50 µg bovine collagen/CFA emulsion for the first immunization and 60 µg for the second immunization. When mice developed a new clinical score of 1 to 4, they were randomly assigned to a treatment group and therapy was started (n = 7-8 mice per group). Phosphate-buffered saline (PBS) (vehicle), 30 µg muTNFR-Fc, 5 µg F8-IL4 or 100 µg F8-IL4 were injected into the lateral tail vein on day 1, 4 and 7. Mice were monitored daily for the arthritic clinical score, the thickness of inflamed paws and weight and sacrificed due to the arthritic score and weight loss in accordance with local regulations.

8.6.4 Comparison of targeted to untargeted IL-4 and combination therapy with muTNFR-Fc
For a moderate strength in arthritic inflammation, mice were immunized with 50 µg bovine collagen/CFA emulsion for the first immunization and 40 µg for the second immunization and included in a treatment group with a new clinical score of 1 to 4 (n = 8-9 mice per group). On day 1, 4 and 7, mice were treated intravenously with PBS (vehicle, control), 30 µg muTNFR-Fc, 100 µg F8-IL4, 100 µg KSF-IL4 or the combination of F8-IL4 with muTNFR-Fc (100 µg F8-IL4 with 30 µg muTNFR-Fc).

8.6.5 Comparison of subcutaneous to intravenous administration of F8-IL4 and combination therapy with dexamethasone or the antibody-mediated delivery of IL-10
Mice, immunized for moderate arthritis strength (50 µg and 40 µg), with a new clinical score of 1 to 4 were included in a treatment group and treated with either intravenous PBS (vehicle, control), 100 µg F8-IL4, 200 µg L19-IL10, the combination of F8-IL4 with L19-IL10, subcutaneous 100 µg F8-IL4, intraperitoneal 100 µg dexamethasone or the combination of intravenous F8-IL4 and intraperitoneal...
dexamethasone (n = 8-10 mice per group). Immunocytokine treatments were administered three times (every 72 h) and dexamethasone was administered daily until day 9.

8.7 Rat model of heterotopic heart transplantation for chronic cardiac allograft rejection

The transplantation procedures were performed by our collaborators in Jena. For better understanding the method of transplantation and the therapeutic protocol are described in this chapter.

For heterotopic rat heart transplantation procedures, inbred male Lewis (LEW) and Fisher (F344) rats were used. After delivery, the rats were allowed to acclimatize for at least 7 days before surgery was performed. During this time period the animals had *ad libitum* access to food and water and were exposed to controlled light/dark cycles. In accordance to The Principles of Laboratory Animal Care (NIH publication VI. 25, No. 28, revised 1996) and the current version of the German Law on the Protection of Animals (registration no. 02-005/13), guidelines for animal care were carefully followed during all experiments.

The surgical procedures and techniques of heterotopic rat heart transplantation with LEW rats as donors and F344 rats as cardiac allograft recipients have been described in the past. For the present study, 20 LEW grafts were explanted from the LEW rats (n = 20) as donors and transplanted into F344 rats (n = 20) as recipients after approximately 30-90 minutes of cold ischemia in 4°C Bretschneider cardioplegic solution. As healthy controls not undergoing heart transplantation, 3 F344 rats and 2 LEW rats were used. After transplantation, the heart-transplanted rats received a sub-therapeutic dose of cyclosporine A (2 mg/kg per day) for 14 days post transplant to suppress acute rejection. The animals were allowed to live...
for 10 weeks (70 days). Starting on day 7 after transplantation, the transplanted animals were treated by intravenous injection into the tail vein with PBS (vehicle, control) and the immunocytokines KSF-huIL10, F8-huIL10 and F8-ratIL10. Each treatment group consisted of 5 animals. Injections were performed weekly over 9 weeks (from day 7 until day 63 after transplantation) by injecting 1 mg of the appropriate immunocytokine per rat (injection volume of approximately 300 µl per rat). As non-transplanted controls, the 3 F344 rats mentioned above were treated with immunocytokines (one rat received KSF-huIL10, one F8-huIL10 and one F8-ratIL10). The 2 LEW controls rats did not undergo any treatment. Rats were clinically monitored every day in the first 14 days post transplantation including determination of body weight and estimation of heart function of the cardiac allograft by palpation using a semi-quantitative score from 0 (absence of beating) to 4+ (optimal beating) according to Lange and colleagues\textsuperscript{137}. After week two, body weight estimation and palpation was measured once a week. Additionally, a retro-orbital blood withdrawal was performed weekly in anaesthetized rats. On day 70 after transplantation, rats were sacrificed in deep anesthesia and analgesia and the organs were excised and immediately shock frozen in liquid nitrogen (stored at -80°C) or formalin-fixed and paraffin embedded.

8.8 PET imaging

For PET imaging applications in rats with chronic cardiac rejection, the F8-SIP and KSF-SIP antibodies were labeled with $^{124}$I (Perkin Elmer) using the chloramine T method as described before\textsuperscript{135,138}. Briefly, 1 mg of protein in PBS was incubated with 3.4 mCi of $^{124}$I and an aqueous solution of chloramine T (Sigma-Aldrich, 5 mg/ml; 0.25 µg chloramine T per µg protein) for 1 min 45 s. Using PD-10 (GE Healthcare) gel filtration columns, unincorporated iodine was separated from the
labeled protein. The radiolabeled protein preparation ($^{124}$I-F8-SIP and $^{124}$I-KSF-SIP) were injected i.v. into the lateral tail vein of rats having chronic cardiac rejection (for rat model see above). Normal uptake of iodine by the thyroid gland was blocked by administration of Lugol solution to the drinking water of the rats 2 days before the experiment. One hour before the injection of radiolabeled protein preparations, rats received an oral administration of a sodium perchlorate solution (1 g/ml)$^{139}$.

PET experiments were performed with the dedicated small animal PET camera Vista eXplore (Sedecal, Spain). Rats were anesthetized with 2-3% isoflurane in oxygen-air 10 min before PET acquisition. Depth of anesthesia was monitored by measuring respiratory frequency. Body temperature was controlled by a rectal probe and kept at 37°C by a thermocoupler and a heated air stream. PET acquisitions of the whole body (3 bed position, 4 overlay) were started 1 h or 24 h after injection of 11-23 MBq $^{124}$I-F8-SIP or $^{124}$I-KSF-SIP antibody and lasted for 60 min. Data were reconstructed with the 2D ordered-subsets expectation maximization (2D-OSEM) protocol and analyzed with PMOD v3.5 software (PMOD Technologies Ltd., Zurich, Switzerland). Standardized uptake values (SUV) were calculated as the ratio of regional averaged radioactivity in Becquerel per cubic centimeter and injected radioactivity in Becquerel per gram body weight.

The experiment was performed in agreement with Swiss regulations and under a project license granted by the Veterinäramt des Kantons Zürich, Switzerland (240/2013).

8.9 Evaluation of cardiac allograft rejection

8.9.1 Histological evaluation

In all explanted cardiac allografts (n = 19), 3 representative recipient hearts from the transplanted animals, the hearts of the non-transplanted (but treated) F344 rats as
well as the hearts of the 2 LEW control rats (without any treatment), histological evaluation of tissue damage was performed. Formalin fixed and paraffin embedded tissue sections (4 μm) were subjected to hematoxylin & eosin (HE-), Elastica van Gieson (EvG-) as well as Sirius Red-staining. Histological analysis was done using light microscopy (x10 and x20 objective). The grade of chronic cardiac allograft rejection was assessed according to a recently described scoring system including the parameters of cardiac allograft vasculopathy (CAV), cardiac interstitial fibrosis (CIF), lymphocyte infiltration and cardiac myocyte degeneration.

8.9.2 Immunohistochemical characterization of immune cell infiltration
To analyze infiltrating immune cells and α-smooth muscle actin positive cells, immunofluorescence labeling was performed. For that, 4 μm thick cardiac tissue sections from the allografts or the control organs were fixed in ice-cold acetone for 10 minutes. After drying, primary antibodies (Table 2), diluted in antibody diluent solution (Dako Deutschland GmbH, Hamburg, Germany), were applied to the tissue and allowed to incubate for 1 hour at room temperature.

Table 2. Antibodies used for immunofluorescence labeling.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Subtype</th>
<th>Clone</th>
<th>Dilution</th>
<th>Source</th>
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<tbody>
<tr>
<td>CD 8</td>
<td>IgG1</td>
<td>Ox-8</td>
<td>1:200</td>
<td>AbD Serotec, Oxford, UK</td>
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<tr>
<td>CD 68</td>
<td>IgG1</td>
<td>ED1</td>
<td>1:200</td>
<td>AbD Serotec, Oxford, UK</td>
</tr>
<tr>
<td>CD 161</td>
<td>IgG1</td>
<td></td>
<td>1:500</td>
<td>AbD Serotec, Oxford, UK</td>
</tr>
<tr>
<td>α-SMA</td>
<td>IgG2a</td>
<td>1A4</td>
<td>1:20</td>
<td>Dako Deutschland GmbH, Hamburg, Germany</td>
</tr>
</tbody>
</table>

After thorough rinsing, secondary antibodies were applied for 45 minutes at room temperature. For the detection, the fluorescently labeled donkey-anti-mouse-Cy3
antibody (Jackson ImmunoResearch, West Grove, PA, USA) was used in a dilution of 1:400 in antibody diluent (Dako Deutschland GmbH). After rinsing with TBS-Tween and Aqua dest., the slides were mounted using Vectashield H1200 containing DAPI stain (Linaris biologische Produkte GmbH, Wertheim, Deutschland) and stored at -20°C. To prove staining specificity, the primary antibody was replaced by non-immune serum. Immunofluorescence labeling analysis was performed by confocal laser-scanning microscopy (cLSM) using a Zeiss microscope (LSM 510, Jena, Germany).

8.10 Analysis of anti-fusion protein antibody response in plasma
The formation of anti-fusion protein antibodies was tested using plasma from mice with collagen-induced arthritis and rats with chronic cardiac rejection treated with different immunocytokines.

Blood from mice was obtained at the start of the therapy (day 1, n = 4) from the vena saphena or at the end of therapy from sacrificed mice through cardiac puncture, processed to plasma and stored at -20°C. Blood from rats was obtained during the on-going treatment study. Blood samples were taken weekly from anaesthetized rats via retro-orbital blood withdrawal. The samples were immediately centrifuged at 10 000 rpm for 25 minutes. Supernatants were stored at -20°C until further analysis.

Two different BIAcore experiments were performed. In a first experiment, the immunogenicity of F8-huIL10 and F8-muIL10 in mice with collagen-induced arthritis was assessed by surface plasmon resonance (BIAcore 3000) screening of mouse plasma samples. F8-huIL10 or F8-muIL10 at a concentration of 50 µg/ml were immobilized on a CM5 sensor chip (GE Healthcare) using an amine coupling kit (GE Healthcare). Surface density of 2’600 RU and 2’900 RU was achieved for F8-huIL10
and F8-muIL10, respectively. On a control flow cell, activation by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxy-succinimide (NHS) was performed and immediately blocked by injecting ethanolamine. For binding analysis, positive control samples of anti-human IL-10 and anti-murine IL-10 antibodies (1 and 4 µg/ml; eBioscience) and serum samples diluted 500-fold in HBS-EP buffer (GE Healthcare) were passed over the different flow cells with a flow rate of 30 µl/minute for 3 minutes. The response was recorded 30 seconds after the end of the injection. The positive control was run again at the end of the analysis to confirm binding capacity of the immobilized protein. To regenerate the surface, 10 mM glycine, pH 2.0, was run over the flow cells for 40 seconds at 30 µl/minute.

In a second experiment, rat plasma samples were analyzed. For comparison reasons plasma from mice with rheumatoid arthritis receiving F8-huIL10, F8-muIL10 or PBS was tested in the same experiment setting. On a CM5 sensor chip (GE Healthcare) F8-ratIL10, F8-huIL10 and KSF-huIL10 were immobilized at a concentration of 50 µg/ml on three different flow cells using an amine coupling kit (GE Healthcare). Surface density of 8’400 RU, 6’100 RU and 4’000 RU was achieved for F8-ratIL10, F8-huIL10 and KSF-huIL10, respectively. The control flow cell was activated as described above. For the experiment, plasma samples were diluted 500-fold in HBS-EP buffer (GE Healthcare) and compared to positive control samples of anti-rat IL-10 (Peprotech, London, UK; 10 µg/ml) and anti-human IL-10 (eBioscience; 0.5 µg/ml) antibodies. The samples were passed over the different flow cells at a flow rate of 30 µl/minute for 3 minutes and the response was recorded 30 seconds after the end of the injection. After each run the surface was regenerated with 10 mM glycine, pH 2.0.
8.11 Cytokine levels in plasma, serum and paw lysates

Blood was obtained at the end of therapy from each mouse (see above), processed to plasma or serum and stored at −20°C.

To compare cytokine levels in paws of treated and control mice, hind paws were taken at the end of the therapy experiment. After detaching the skin, paws were cut into small pieces and the tissue fragments were suspended in a 50 mM Tris, 150 mM NaCl buffer containing complete protease inhibitor cocktail (Roche Diagnostics, Rotkreuz, Switzerland). For homogenization, a 5 mm stainless steal bead (Qiagen, Hombrechtikon, Switzerland) was added and the tissue was homogenized in a QIAGEN Tissue Lyzer (4 x 1 minute, 4°C, 30 Hz). The supernatant was harvested after centrifugation (5 min, 4°C, 16'000 x g). The protein concentrations of the extracts were determined by a BCA assay (Thermo Fisher Scientific, Waltham, MA, USA) and samples were normalized according to total protein concentration.

To quantify cytokine levels, a multiplex bead-based flow cytometry analysis was performed using the Mouse Th1/Th2/Th17/Th22 13plex FlowCytomix Multiplex (eBioscience) following the supplier’s protocol. Fluorescence-activated cell sorting (FACS) analysis was performed on a BD FACS Canto (BD Bioscience, Allschwil, Switzerland) and data evaluated with FlowCytomix Pro 3.0 software (eBioscience).

For the analysis of plasma from mice treated with muTNFR-Fc and F8-IL10 the experiment was repeated on a different day in order to have an independent replicate of the assay (see 12.3). Using standard curves generated by the FlowCytomix Pro 3.0 software with positive control samples, a level of quantification was assigned to every cytokine (see 12.3).
8.12 Serum anti-collagen antibodies
For the determination of anti-bovine collagen type II specific antibody levels in mice treated with different immunocytokines, serum was analyzed by ELISA techniques as previously described.\(^\text{27}\).
Bovine collagen II solution (5 µg/ml, Chondrex, Inc., Redmond, WA, USA) was coated and serum samples were tested in triplicates at a 1:800 or 1:1000 dilution. Bound IgG, IgG1 and IgG2a were detected by incubation with horseradish peroxidase conjugated goat anti-mouse Fc (for IgG), IgG1 or IgG2a antibodies (Santa Cruz, Heidelberg, Germany).

8.13 Serum IgE levels
For the determination of IgE levels in mice treated with F8-IL4 compared to levels in mice treated with vehicle control, serum was analyzed using a Mouse IgE Ready-Set-Go ELISA kit (eBioscience) according to the supplier’s protocol. Briefly, wells were coated with anti-mouse IgE monoclonal antibody and bound IgE was detected with biotinylated anti-mouse IgE.

8.14 Statistical analysis
Data are expressed as the mean ± standard deviation or standard error of the mean. Differences in arthritic outcome between therapeutic groups were compared using GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA) grouped two-way ANOVA multiple-comparison (Bonferroni-corrected) analysis, with \( p < 0.05 \) considered significant. Differences in cytokine levels were compared using a Mann-Whitney test, with \( p < 0.05 \) considered significant.
The mean values of all parameters assessed in the rat heart transplantation study were tested for statistical significant differences between the diverse experimental
and control groups using the Kruskal-Wallis test. A p value of ≤0.05 was defined to indicate statistical significance. The analysis was performed using SPSS software (SPSS Inc, Chicago, IL, USA).
### 9 Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>%ID/g</td>
<td>Percentage of injected dose per gram</td>
</tr>
<tr>
<td>aa</td>
<td>Amino-acid</td>
</tr>
<tr>
<td>ACR20</td>
<td>American College of Rheumatology 20% score</td>
</tr>
<tr>
<td>ACR50</td>
<td>American College of Rheumatology 50% score</td>
</tr>
<tr>
<td>ADCC</td>
<td>Antibody-dependent cellular cytotoxicity</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen-presenting cell</td>
</tr>
<tr>
<td>CAV</td>
<td>Cardiac allograft vasculopathy</td>
</tr>
<tr>
<td>CDC</td>
<td>Complement-dependent cytotoxicity</td>
</tr>
<tr>
<td>CDR</td>
<td>Complementarity-determining regions</td>
</tr>
<tr>
<td>C\textsubscript{H}</td>
<td>Constant heavy chain</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary cell line</td>
</tr>
<tr>
<td>CIF</td>
<td>Cardiac interstitial fibrosis</td>
</tr>
<tr>
<td>CIA</td>
<td>Collagen-induced arthritis</td>
</tr>
<tr>
<td>C\textsubscript{L}</td>
<td>Constant light chain</td>
</tr>
<tr>
<td>CSIF</td>
<td>Cytokine synthesis inhibiting factor</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T lymphocyte antigen 4</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EDA</td>
<td>Extra domain A of fibronectin</td>
</tr>
<tr>
<td>EDB</td>
<td>Extra domain B of fibronectin</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbant assay</td>
</tr>
<tr>
<td>EMA</td>
<td>European Medicines Agency</td>
</tr>
<tr>
<td>Fc</td>
<td>Crystallizable fragment of Ig molecule</td>
</tr>
<tr>
<td>FcR</td>
<td>Fc receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>FcγR</td>
<td>Fc gamma receptor</td>
</tr>
<tr>
<td>FcRn</td>
<td>Neonatal Fc receptor</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>Fv</td>
<td>Variable fragment of Ig molecule</td>
</tr>
<tr>
<td>HAMA</td>
<td>Human anti-mouse antibody</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL-4</td>
<td>Interleukin 4</td>
</tr>
<tr>
<td>IL-10</td>
<td>Interleukin 10</td>
</tr>
<tr>
<td>IL-10R1/R2</td>
<td>Interleukin 10 receptor 1/2</td>
</tr>
<tr>
<td>IRS</td>
<td>Insulin receptor substrate</td>
</tr>
<tr>
<td>ISHLT</td>
<td>International Society for Heart and Lung Transplantation</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenously</td>
</tr>
<tr>
<td>IVUS</td>
<td>Intravascular ultrasound</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MAC</td>
<td>Membrane attack complex</td>
</tr>
<tr>
<td>MAFA</td>
<td>Mouse anti-fusion protein antibody</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MSCs</td>
<td>Mesenchymal stem cells</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor kB</td>
</tr>
<tr>
<td>NHS</td>
<td>N-hydroxysuccinimide</td>
</tr>
<tr>
<td>NIRF</td>
<td>Near infrared fluorescence imaging</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>NK cell</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>ODNs</td>
<td>Oligodeoxynucleotides</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PET</td>
<td>Positron emitting tomography</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>RAFA</td>
<td>Rat anti-fusion protein antibody</td>
</tr>
<tr>
<td>rhuIL10</td>
<td>Recombinant human IL10</td>
</tr>
<tr>
<td>rmuIL10</td>
<td>Recombinant murine IL10</td>
</tr>
<tr>
<td>s.c.</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>scFv</td>
<td>Single-chain variable fragment</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecylsulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEC</td>
<td>Size exclusion chromatography</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SIP</td>
<td>Small immonoprotein</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TNFR</td>
<td>Tumor necrosis factor receptor</td>
</tr>
<tr>
<td>Tyk</td>
<td>Tyrosine kinase</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>$V_H$</td>
<td>Variable heavy chain</td>
</tr>
<tr>
<td>$V_L$</td>
<td>Variable light chain</td>
</tr>
<tr>
<td>$\lambda_{em}$</td>
<td>Emmision wavelength</td>
</tr>
<tr>
<td>$\lambda_{ex}$</td>
<td>Excitation wavelength</td>
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</tbody>
</table>
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* The two authors contributed equally to this work
11 References


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12 Appendix

12.1 Nucleotide and amino-acid sequences

12.1.1 muTNFR-Fc

Complete nucleotide sequence of muTNFR-Fc.

HindIII - signal sequence (SS) - muTNFR - muFc (Hinge; CH2; CH3) - Stop - NotI

```
CCCAGCTTGTGACCATATGGGCTGGACTATCATCTCTGTCTCTCGTCTGCTTGCTGCTGCTACAGGTTGTGACAGCTGG
TGCCCCGAGTTTGTCCTTGACACCTACAAACCAGGCAACTGTTGTAGTACAGATCAAGCCTGGCCCTAGCTGCATGGCAGGT
GACAGGAGGCTGCATGACATGCGGTGGCAAGGAGGGCGTGTGTGCGGACTGTGAGGCAAGCATGTATACCAGGTCTGGAACCTC
GCCAATATGTGAAACATTTCGCAACAAGGTGGAGAACCAGGTGGAGACCGTGTGTGCGGACTGTGAGGCAAGCATGTATACCAGG
TTCTTCGTACCATGACATGCGGTGGCAAGGAGGGCGTGTGTGCGGACTGTGAGGCAAGCATGTATACCAGGTCTGGAACCTC
GCCAATATGTGAAACATTTCGCAACAAGGTGGAGA
```

Complete amino-acid sequence of muTNFR-Fc.

```
VPAQVVTLPYKEPYECQISSEQEYDRKAAQMCCAKCPPGQYVKHFCNKTSDTVCADECASMAYQVWNQFRCLS
CSSSCTTDQVEIRACTKQQRVCAACEAGRYCALKTHSGSCRQCMRLSKCGPGFGVASSRAPNGLVCKACAPGT
```
12.1.2 F8-muIL10

Complete nucleotide sequence of F8-muIL10.

<table>
<thead>
<tr>
<th>NheI</th>
<th>- signal sequence (SS) - F8(diabody) - linker - muIL10 - Stop - NcoI</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCC</td>
<td>GCTAGCGTGCACCACATGGGCTGGACCTGATCCTCCTCTGCTCGTGTGCTACAGGTGTGCACTCGG</td>
</tr>
<tr>
<td>AGGTCACGCTTCTGGAGCTGGTGGAGGGAGCTTGGTACAGCCTGGGGGGTCCCTGAGACTCTCCTGTCAGC</td>
<td></td>
</tr>
<tr>
<td>GTATTACATTTAGCTTTTACTATCATACACTACGAGACTCTCGGCTGCCAGGCTCCAGGGAAGGGGCCCTGGTCAGGCTGAGGTGTAAGCATGAGAAGCATTTGAATTCCCTGGGTG</td>
<td>AGAAGCTGAAGACCTCAGGATGCGGCTGAGGCGCTGTCATCGATTTCTCCCCTGTGAAAATAAGAGCAAGGCAGTGGAGCAGGTGAAGAGTGATTTTAATAAGCTCCAAGACCAAGGTGTCTACAAGGCCATGAATGAATTTGACATCTTCATCAACTGCATAGAAGCATACATGATGATCAAAATGAAAAGCTAGCGGCCGCTAAAGGGAAA</td>
</tr>
</tbody>
</table>

Complete amino-acid sequence of F8-muIL10.

| EVQLLESGGGLVQPGGSLRLSCAASGFTSFLFTSWVRQAPGKLEWVAISGSGLSTYADSVKVRFTISRDN |
| SKNTLYLQMNLSRAEDTAVYYCAKSTHLYLFDYWGQGTLVTVSSGGSGGEEIVLTQSPGTLSSPGERATLSCRA |
12.1.3 F8-ratL10

Complete nucleotide sequence of F8-ratL10.

**NheI** - signal sequence (SS) - F8(diabody) - linker - ratL10 - Stop - NotI

```
CCC GCTAGC GTCGACCATGGGCTGGAGCCTGATCCTCCTGTTCCTCGTCGCTGTGGCTACAGGTGTGCACTCG
AGGTGACGCTTTGGAATCTGCGGAGGTGCTAGCATACGTCCTCTGCGTTCTGGGCTGGAGCTGGTGGTAAGCG
```

Complete amino-acid sequence of F8-ratL10.

```
EVQLLESGGGGLVQPGGSLRLSCAASGFTFSLFTMSWVRQAPGKGLEWVSAISGSGSTYYADSVKGRFTISRDN
SKNTLYQMNSLRADMTAVYVKACSTKYLFDYWGQGTLVTVSSGGSGEIVLTQSPGERATLSCRA
SQSVSMPFLAWYQQPGQAPLLYIGASSRATGIPDRFSGSGSTDFTLTISRLLEPEDFAVYRCQMQMRGPTTF
```
12.1.4 F8-ratIL4

Complete nucleotide sequence of F8-ratIL4.

HindIII - signal sequence (SS) - F8(diabody) - linker - ratIL4 - Stop – NotI

Complete amino-acid sequence of F8-ratIL4.

EVQLLESGGGLVQPGGLSRLSCAASGFTSFSLFTMSWVRQAPGKGLEWVSAISGSGGSTYYADSVKGRFTISRDN
SKNTLYLQMNRAEDTAVYVKACSTHTLYFLDYWGQGTLVTVSSGGSGGEIVLQTSPGTLSLSQGERATSLRA
SQSVMFLLAWYKQPGAPRLLIGASRATGIPDRFSGSGSGTDFTLISRLEPEDFAVYYCQQMRGRPPTF
GQGTKVEIKSSSGSSSSGSSSSGSKGHSIRGDNCTHFPVSQTHMLRELRAAFSQVKFFQKDKQDLNILLTD
SLLQDFKRFLGCQALSEMKFYLVEMPFQAGNHPEIKEHLNSLGEKLTLWIQLRCHRFLPCENSKAVEQV
KNDFNKLQDKGVIKAMNEFDIFINCIIEAYVTLMKMN
12.1.5 KSF-huIL10

Complete nucleotide sequence of KSF-huIL10.

HindIII - signal sequence (SS) - KSF(diabody) - linker - huIL10 - Stop - NotI

```
cccAAGCTTGTGACCACATGGGCTGGAGCCCTGTACCTCCTCTGTCTGCTGTGGCTACAGGTGTGCACTCGG
AGGTTGCACTGTGTAGCTGGCTGGGAGGTGGTTCTGAGCTAGCGTGAGCACCCGCTGTAGCTGGCTGGTGTGAG
TGAGCTGACTCAGGACCCTGCTGTGTCTGTGGCCTTGGGACAGACAGTCAGGATCACATGCCAAGGAGACAGTC
TCAGAAGCTATTATGCAAGCTGTTACGACAGCAGAAGCCAGACAGCAGGCCCCCTGTACTTGCTATATGGTAT
AACGCCGCTTCAGGGATCCCAGACCAGCTTTCTCCTCTGTACCTCCCTCTGGAATCGCAGCTGGGCTGTGGTAT
CCGTGGAGGACTTTAAGGGTTACCTGGGTTGCCAAGCCTTGTCTGAGATGATCCAGTTTTACCTGGAGGAG
GTAGATGCCGCACGAGATGAGAACACAGCTGTATCTGCAAATGAACAGCC
```

Complete amino-acid sequence of KSF-huIL10.

```
EVQLLESGGGLVQPGGSLRLSCAASGFTFSSYAMSVWVRQAPGKLEWVSASISGGSTYYADSVKGRFTISRDN
SKNTLYLQMNSLRADTVYYCAKPSKVSLEDFYWQGTTLVTVSSGSGGSELTQDPAVSVALQTVRITCQGDS
LRSYVYISWQQKPGAPVLYIGKNRRPSGIDPRFSGSSGNTASLTTGTGQAEDAYCNSSLPSNLAVVG
GGTKLTVLGSSSSGSIGSGGSSGSSPQGQTQSENCSCTHFPGNLNPMLRDLRRDFSRVKTFQMKDLQDNLLLKE
SLLEDKFGYLGQALSEMIQFYLEEVMPQAEQDPDITKAHVNSLGENKLTRLRLRCHRFLPENCOKAVEQV
KNAFNKLQEGKYKAMSEFDFINYIEAYMTMKRN
```
12.2 Analysis of mouse anti-fusion protein antibody response

Analysis of mouse anti-fusion protein antibody (MAFA) response. (a) The flow rate over the sensor surface was 30 µl/minute for 3 minutes and the initial slope was calculated using the following equation: (response 60 seconds after the start of binding - response at baseline) / 60 seconds. (b) Initial slope of plasma samples and positive controls. Samples were passed over the two different flow cells coated with F8-huIL10 (black bars) or F8-muIL10 (grey bars) and the initial slope was calculated.
12.3 Analysis of cytokine levels
Murine analogues of etanercept and of F8-IL10 inhibit the progression of collagen-induced arthritis in the mouse

Mouse plasma was used to measure cytokine levels using a multiplex bead-based assay. Here we show the independent replication of the assay for each sample and cytokine in the full collagen induction and reduced collagen induction setting. Using standard curves defined with positive control samples a level of quantification was assigned to every cytokine.

Replicate of analysis of cytokine levels in mice with full collagen-induced arthritis.
For each sample and cytokine, measurements were repeated on a different day in order to have an independent replicate of the assay. At the end of the therapy 13 different cytokine concentrations were measured in plasma using multiplex bead-based flow cytometry. Data points of cytokine concentrations above detection level are represented in a scatter plot with the mean ± standard error of the mean (n = 7). Standard curves defined with positive control samples were used to generate a level of quantification (LOQ; dotted red line).
Replicate of analysis of cytokine levels in mice with reduced collagen-induced arthritis. For each sample and cytokine, measurements were repeated on a different day in order to have an independent replicate of the assay. At the end of the therapy 13 different cytokine concentrations were measured in plasma using multiplex bead-based flow cytometry. Data points of cytokine concentrations above detection level are represented in a scatter plot with the mean ± standard error of the mean (n = 10). Standard curves defined with positive control samples were used to generate a level of quantification (LOQ; dotted red line).

Standard curves defined with positive control samples. A mixture of standard cytokines was prepared according to the supplier’s protocol (eBioscience) and serially diluted. To determine cytokine concentration, a fluorescence-activated cell sorting analysis was performed on a BD FACS Canto and data evaluated with FlowCytomix Pro 3.0 software (eBioscience), generating the standard curves. Manually a level of quantification (LOQ) was
superimposed on these standard curves (dotted green line). (For high-resolution figure see online version of Doll et al. Arthritis Research & Therapy 2013[11]).

**Standard curves defined with positive control samples for the repetition experiment.**

A mixture of standard cytokines was prepared according to the supplier’s protocol (eBioscience) and serially diluted. To determine cytokine concentration, a fluorescence-activated cell sorting analysis was performed on a BD FACS Canto and data evaluated with FlowCytomix Pro 3.0 software (eBioscience), generating the standard curves. Manually a level of quantification (LOQ) was superimposed on these standard curves (dotted green line). (For high-resolution figure see online version of Doll et al. Arthritis Research & Therapy 2013[11]).

The antibody-based delivery of IL-4 to the neo-vasculature cures mice with arthritis

To determine the therapeutic benefit of F8-IL4, we measured cytokine levels in plasma of mice with collagen-induced arthritis.

A cytokine analysis in paws of animals at the end of the experiment confirmed that the F8-IL4 plus dexamethasone combination treatment resulted in a complete normalization of cytokine concentrations.
Cytokine analysis in serum of mice treated with PBS, 30 μg muTNFR-Fc, 5 μg F8-IL4 (low F8-IL4) or 100 μg F8-IL4 using multiplex bead-based flow cytometry.
Cytokine levels in serum of mice treated with PBS, 30 µg TNFR-Fc, 100 µg F8-IL4, the combination of F8-IL4 with muTNFR-Fc or 100 µg KSF-IL4.
Cytokine analysis in paw lysates of mice treated with PBS, 100 µg F8-IL4 i.v., 100 µg F8-IL4 s.c., 200 µg L19-IL10, 100 µg dexamethasone, the combination of F8-IL4 with L19-IL10 respectively dexamethasone using multiplex bead-based flow cytometry. The results are shown for the different arthritic scores assigned to the inflamed paws.
Analysis of cytokine levels in serum of mice treated with PBS, 100 µg F8-IL4 i.v., 100 µg F8-IL4 s.c., 200 µg L19-IL10, 100 µg dexamethasone, the combination of F8-IL4 with L19-IL10 respectively dexamethasone using multiplex bead-based flow cytometry.
12.4 Anti type-II collagen antibodies

Titers of bovine type-II collagen-specific total IgG, IgG1 and IgG2a antibodies were determined using standard ELISA techniques. Healthy*, mice were immunized but did not show any signs of inflammation.

12.5 Immunohistochemical and immunofluorescence analysis of paw sections

a) not inflamed

b) inflamed cells

![Image: Comparison of inflamed and non-inflamed cells]

![Image: Immunohistochemical analysis of paw sections]

![Image: Immunofluorescence analysis of paw sections]

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At the end of the therapy, paws from different therapy groups were frozen in cryoembedding medium (Neg50; Thermo Scientific, Wohlen, Switzerland) and stored at −80°C for analysis. (a) Hematoxylin and eosin staining of healthy and inflamed paw. Paw cryosections (10 µm) were fixed in ice-cold acetone and stained with hematoxylin solution Gill No. 2 (Sigma Aldrich) and alcoholic eosin Y solution (Sigma Aldrich). 10x magnification; scale bars = 50 µm. (b) Immunofluorescence analysis of infiltrating cells. Cryosections (10 µm) were fixed in ice-cold acetone and immunofluorescence staining was performed using primary antibodies against the following antigens: rat anti-mouse CD45 (leukocytes, 1:200; BD Bioscience), rabbit anti-asialo GM1 (NK cells, 1:4,000; Wako Pure Chemical Industries, Tokyo, Japan), rat anti-mouse CD4 (CD4+ cells, 1:50; BioXCell, West Lebanon, NH, USA) and rat anti-mouse CD8 (CD8+ cells, 1:50; BioXCell). Donkey anti-rat Alexa Fluor488 (1:200; Invitrogen) and goat anti-rabbit Alexa Fluor488 (1:200; Invitrogen) were used as secondary antibodies for detection. Sections were mounted with fluorescent mounting medium (Dako, Baar, Switzerland) and analyzed with an Axioskop2 mot plus microscope (Zeiss, Feldbach, Switzerland). The following scoring system was used for semiquantitative analysis of infiltrating cells: 0 = negative, 1 = single areas of positive cells with weak to moderate staining intensity, 2 = single areas of positive cells with strong staining intensity or disseminated positivity with weak to moderate staining intensity, 3 = large areas of positive cells within the whole tissue section with moderate to strong staining intensity. 10x magnification; scale bar = 50 µm (for all images). (c) Results of semiquantitative analysis for the different infiltrating cells (n = 2, F8-muIL10 n = 1). Healthy*, mice were immunized but did not show any signs of inflammation.
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First of all, I would like to express my gratitude to Prof. Dario Neri for giving me the opportunity to perform my PhD in his group and for supporting me throughout my PhD. I am grateful for the exciting project, the support and the valuable discussions.

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