Recombinant antibodies for the imaging and treatment of rheumatoid arthritis and endometriosis

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Recombinant antibodies for the imaging and treatment of rheumatoid arthritis and endometriosis

A dissertation submitted to
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for the degree of
Doctor of Sciences

presented by

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Table of contents

1. Summary .............................................................................................................................. 6

2. Introduction ......................................................................................................................... 10

   2.1 Antibodies for pharmaceutical applications ................................................................. 10
      2.1.1 Antibody structure and antibody formats ................................................................. 10
      2.1.2 Mechanisms of action in vivo .................................................................................. 12
      2.1.3 Technologies for antibody production ................................................................. 14

   2.2 Antibody-based vascular targeting ............................................................................... 17
      2.2.1 General concepts ...................................................................................................... 17
      2.2.2 Angiogenesis ........................................................................................................... 18
      2.2.3 Markers of angiogenesis for vascular targeting .................................................... 19

   2.3 Rheumatoid arthritis .................................................................................................... 27
      2.3.1 Disease .................................................................................................................... 27
      2.3.2 Angiogenesis and the expression of EDA in rheumatoid arthritis ......................... 28
      2.3.3 Treatment of rheumatoid arthritis ......................................................................... 29
      2.3.4 Collagen induced arthritis mouse model ............................................................... 31

   2.4 Interleukin 10 ............................................................................................................... 33
      2.4.1 Structure .................................................................................................................. 33
      2.4.2 Receptor signaling .................................................................................................. 33
      2.4.3 Biological activities of IL-10 .................................................................................. 34
      2.4.4 Interleukin-10 in Diseases ..................................................................................... 35
      2.4.5 Tenovil (recombinant human IL-10) ..................................................................... 40

   2.5 Endometriosis .............................................................................................................. 44
      2.5.1 Disease .................................................................................................................... 44
      2.5.2 Diagnosis and treatment ......................................................................................... 46
      2.5.3 Imaging of endometriosis ...................................................................................... 47
      2.5.4 Angiogenesis in endometriosis .............................................................................. 48
      2.5.5 Mouse models of endometriosis ............................................................................ 49
      2.5.6 The role of IL-10 in endometriosis ....................................................................... 49

   2.6 The aim of this thesis .................................................................................................... 50

3. Results .................................................................................................................................. 52
3.1 Immunohistochemical analysis of rheumatoid synovial tissue specimens .......52
3.2 Cloning and in vitro characterization of F8-IL10........................................54
3.3 F8-IL10 selectively targets arthritic lesions and tumors in mice ...............55
3.4 Inhibition of arthritis progression in the collagen-induced model of arthritis 59
3.5 Evaluation of cellular and humoral immunity ...........................................61
3.6 Safety pharmacology profile of F8-IL10....................................................63
3.7 Evaluation of antigen expression in different chronic inflammatory diseases64
3.8 Mouse model of endometriosis...................................................................66
3.9 Targeting of F8 in the syngeneic mouse model of endometriosis..............66
3.10 Preliminary therapy experiment using the two immunocytokines F8-IL10 and F8-IL2 ........................................................................................................... 67

4 Discussion.....................................................................................................69

5 Materials and Methods ..............................................................................72
  5.1 Immunohistochemical analysis of rheumatoid synovial tissue specimens ....72
  5.2 Cloning, expression and characterization of a scFv(F8) fusion proteins .......72
    5.2.1 Cloning and in vitro characterization of F8-IL10.................................72
    5.2.2 Cloning of a TNF receptor fusion protein...........................................74
    5.2.3 Cloning and characterization of F8-IL2...............................................74
  5.3 Targeting experiments in the collagen induced model of arthritis ..........75
    5.3.1 Collagen induced arthritis mouse model..............................................75
    5.3.2 Near infrared imaging of arthritic paws.................................................76
    5.3.3 Phosphorimaging analysis of arthritic paws with radiolabeled F8-IL10....76
    5.3.4 Quantitative biodistribution studies in tumor mice ..............................76
  5.4 Therapy experiments in the collagen induced model of arthritis ............77
    5.4.1 Combination therapy study with Methotrexate....................................77
    5.4.2 Comparison of targeted and untargeted delivery of IL-10....................77
    5.4.3 Ex vivo immunohistochemical detection of F8-IL10 and HyHel10-IL10 in arthritis paws .................................................................78
    5.4.4 Statistical analysis ..................................................................................78
  5.5 Evaluation of cellular and humoral immunity .........................................78
    5.5.1 Immunofluorescence studies of infiltrating cells..................................78
    5.5.2 Anti-bovine collagen II Antibodies ......................................................79
5.5.3 Analysis of mouse plasma cytokine levels ................................................................. 79
5.5.4 Statistical analysis ..................................................................................................... 79
5.6 Safety pharmacology profile of F8-IL10 .................................................................. 80
  5.6.1 Toxicology studies in cynomolgus monkey ............................................................. 80
  5.6.2 Single dose intravenous toxicity study in mice ...................................................... 80
5.7 Evaluation of antigen expression in different chronic inflammatory diseases 81
5.8 Mouse model of Endometriosis .............................................................................. 81
5.9 Targeting of F8 in the syngeneic mouse model of endometriosis ......................... 82
  5.10 Preliminary therapy experiment using the two immunocytokines F8-IL10 and
      F8-IL2 ............................................................................................................................ 82
6 References .................................................................................................................. 83
7 Curriculum Vitae ........................................................................................................ 106
8 Acknowledgments ........................................................................................................ 106
1. Summary

One of the most promising avenues towards the development of better drugs consists in the antibody-mediated delivery of therapeutic agents (e.g. cytokines, drugs or radionuclides) to the site of the disease, while sparing normal tissue. Targeting the neovasculature of tumors or inflammation seems to be a particularly promising avenue, considering the fact that the formation of new blood vessels is a crucial step for disease progression. Furthermore, antigens in the modified neovasculature of diseases are ideal for antibody-based pharmacodelivery applications, in view of their abundance, stability and specificity.

In this thesis we show that spliced isoforms of fibronectin and tenascin C, markers of angiogenesis, are expressed in synovial tissue of rheumatoid arthritis specimens and in endometriosis lesions of patients. The antibody F8, specific to the EDA domain of fibronectin, showed the strongest staining in comparative immunohistochemistry performed on tissue samples of various inflammatory diseases and was thus selected for the development of a fully human fusion protein with IL-10.

IL-10 is a particularly attractive anti-inflammatory cytokine for treatment of chronic inflammation, which has exhibited an excellent tolerability profile in rodents, monkeys, and patients, and which has been investigated in clinical trials for the treatment of patients with inflammatory diseases (e.g. rheumatoid arthritis). The clinical development of IL-10 was discontinued because of insufficient efficacy of the compound in humans. However, in a placebo controlled phase I/II study for the treatment of rheumatoid arthritis ACR20 responses were 63% for the IL-10 groups, compared to 10% for placebo. Similar results were observed with TNF blockers.

The fusion protein F8-IL10 was produced and purified to homogeneity in CHO cells and shown to comprise biological active antibody and cytokine moieties by binding assays on recombinant antigen and by MC/9 cell proliferation assays. Following radioiodination, F8-IL10 was able to selectively target arthritic lesions and tumor neo-vascular structures in mice, as evidenced by autoradiographic analysis and quantitative biodistribution studies. The subcutaneous administration route led to equivalent targeting results when compared with intravenous administration and was thus
selected for the clinical development of the product. F8-IL10 potently inhibited progression of established arthritis in the collagen-induced mouse model when tested alone and in combination with methotrexate. In preparation for clinical trials in patients with rheumatoid arthritis, F8-IL10 was studied in rodents and in cynomolgus monkeys, revealing an excellent safety profile at doses tenfold higher than the planned starting dose for clinical phase I trials.

In a second part of this thesis the targeting potential of F8 was studied in a mouse model of endometriosis. A selective uptake of fluorescently labeled F8 in endometriosis lesions was demonstrated using near infrared imaging.
Zusammenfassung


Wir konnten zeigen, dass gespleisste Isoformen von Fibronektin und Tenascin C, sowohl im Synovium von Patienten mit Rheumatoide Arthritis, als auch in Endometriosegewebe vorkommen. Der Antikörper F8, der spezifisch an die alternativ gespleisste EDA Domäne von Fibronektin bindet, zeigte in diesen Geweben die stärkste Färbung und wurde daher ausgewählt um ein humanes Fusionsprotein mit Interleukin-10 (IL-10) zu entwickeln.


wurde deshalb für die klinische Entwicklung ausgewählt. In einem Arthritis-
Mausmodell resultierte die subkutane Verabreichung von F8-IL10 zu einer deutlichen
Verlangsamung des Fortschreiten der Krankheit. In Kombination mit Methotrexat
konnten die Therapieergebnisse sogar noch weiter verbessert werden.
Als Vorbereitung auf klinische Studien an Patienten mit Rheumatoider Arthritis wurden
Toxikologiestudien an Primaten und Mäusen durchgeführt. Hierbei zeigte sich F8-IL10
ein sehr gutes Toxizitätsprofil, auch bei bis zu 10-fach höherer Dosis als die geplante
Startdosis in den klinischen Studien.
Im zweiten Teil dieser Dissertation wurde das Targeting-Potential von F8 in einem
Endometriose-Mausmodell getestet. Mittels Nahinfrarot-Bildgebung konnte gezeigt
werden, dass sich der Antikörper F8 selektiv im Endometriosegewebe anreichert.
2. Introduction

2.1 Antibodies for pharmaceutical applications

2.1.1 Antibody structure and antibody formats

Antibodies are Y shaped immunoglobulins (Igs) capable of specific recognition of certain target molecules called antigens. The basic structure of an antibody consists of two identical light chains and two identical heavy chains which are linked by disulfide bonds. Each heavy chain contains a variable domain ($V_H$) and 3-4 constant domains ($C_H$), whereas each light chain is composed of a variable domain ($V_L$) and a constant domain ($C_L$). The constant region is identical in all antibodies of the same isotype, but differs in antibodies of different isotypes. In placental mammals there are five antibody isotypes known as IgA, IgD, IgE, IgG and IgM. The antibody isotype which is present in the greatest abundance in the body is IgG. Up to 75% of antibodies in the blood are of this type (Figure 2.1). Highly selective binding of antigen is a common hallmark of antibodies. This is mainly mediated by six loops, which are known as the complementarity-determining regions (CDRs), three of which are present in each of the $V_H$ and $V_L$ domains. The Fc portion of the Ig is glycosylated and serves to bind various effector molecules of the immune system, such as the C1 complex of the complement system and a variety of Fc receptors.

![Figure 2.1: Structure of an IgG antibody](Image)

**Figure 2.1: Structure of an IgG antibody:** Light chains are composed of one constant domain ($C_L$) and one variable domain ($V_L$), whereas heavy chains are composed of three constant domains ($C_{H1}$, $C_{H2}$ and $C_{H3}$) and one variable domain ($V_H$). The heavy chains are covalently linked in the hinge region and the light chains are covalently linked to the heavy chain.
IgG antibodies with a molecular weight of about 150 kDa are limited in their ability to extravasate from the vasculature into the diseased tissue. Furthermore, interactions with the Fc receptor located on normal cells could alter the distribution of the mAb, potentially endangering the patient when toxic payloads are attached. Therefore, engineered mAb-based molecules have been developed which still contain the complementarity determining regions (CDRs) to retain binding affinity towards the antigen, but which are smaller in size compared to the whole immunoglobulin. These different antibody formats vary in blood clearance rate, in vivo stability and performance in targeting (Borsi et al. 2002).

Examples of such antibody fragments are the single chain Fv molecule (scFv), in which the Vₜ and Vᵢ domains are sequentially fused by a polypeptide linker, the Fab fragment, where the association of the Vₜ and Vᵢ domains is stabilized by the first constant domain of the heavy chain and the constant domain of the light chain, and the small immunoprotein (SIP) that consists of a scFv molecule fused to a C₄ domain of an IgE resulting in a homodimeric construct. ScFv antibody fragments were used in this thesis.

Usually the polypeptide linker between Vₜ and Vᵢ domain used in our lab is about 14 amino acids in size. By shortening this linker monovalent scFv can be forced to form multimers thereby leading to an increase of avidity. A scFv molecule with a linker of 3 to 12 residues cannot fold into functional monomeric Fv domain and instead associates with a second scFv molecule to form a noncovalent dimer (diabody) (Holliger et al. 1993). These dimers are capable of bivalent binding to the antigen and show improved tumor targeting (Little et al. 2000; Penichet et al. 2004).

![Figure 2: Different formats of antibodies and antibody fragments.](https://example.com/f2.png)

**Figure 2: Different formats of antibodies and antibody fragments.** Vᵢ = variable light chain domain; Vₜ = variable heavy chain domain; Cᵢ = constant light chain domain; C₄ = constant heavy chain domain. a)
Single chain Fv (scFv) 27 kDa; b) Diabody 54 kDa; c) Fab 50 kDa; d) Small Immunoprotein SIP, 75 kDa; e) Immunoglobulin IgG, 150 kDa.

2.1.2 Mechanisms of action in vivo

Therapeutic antibodies can function by different modes of action: by blocking the action of specific molecules, by antibody dependent cellular cytotoxicity (ADCC) or complement dependent cytotoxicity (CDC) or by functioning as signaling molecules. The blocking activity of therapeutic antibodies is achieved by preventing growth factors, cytokines or other soluble mediators reaching their target receptors, which can be accomplished either by the antibody binding to the factor itself (neutralization) or to its receptor. Examples of blocking antibodies are anti-tumor necrosis factor α (TNF-α) antibodies (e.g. infliximab (Remicade)), for the treatment of chronic inflammatory diseases, or bevacizumab (Avastin), an anti-vascular endothelial growth factor (VEGF) antibody for the treatment of cancer (Ignoffo 2004).

The Fc portion, which varies with the isotype, is responsible for the activation of the classical pathway of complement and for the recruitment and activation of Fc receptor expressing immune cells, resulting in complement dependent cytotoxicity (CDC) and in antibody-dependent cellular cytotoxicity (ADCC), respectively. Antibody-dependent cellular cytotoxicity (ADCC) occurs when antibodies bind to antigens on the target cell and the antibody Fc domains engage Fc receptors (FcR) on the surface of immune effector cells, leading to destruction of the target cell. The strongest evidence for an Fc-mediated contribution to antibody efficacy patients was found for rituximab (an anti-CD20 antibody): responsiveness to rituximab in patients with non-Hodgkin’s lymphoma correlates with polymorphisms in FcyRIIIa and FcyRIIa, which are expressed by immune effector cells (Cartron et al. 2002). The response rates to rituximab in patients with follicular non-Hodgkin’s lymphoma are highest in those who are homozygous for the higher-affinity FcyRIIIa variant. In contrast, no such correlation was found for responsiveness to rituximab in patients with B-cell chronic lymphocytic leukemia, indicating that the antitumor activity of antibodies can vary with the clinical setting (Farag et al. 2004). ADCC can be enhanced by engineering the glycan structure or amino-acid sequence of the Fc region to improve binding to FcγRs. Host cell lines have been selected or engineered to express antibodies
with altered glycosylation, such as increased amounts of bisecting N-acetylglucosamine and/or reduced amounts of fucose (Umana et al. 1999; Shields et al. 2002).

**Figure 2.2: Antibody dependent cellular cytotoxicity:**

a) Monoclonal antibodies bind to antigen on the tumor cell surface, providing the target for Fc receptors on the surface of natural killer cells. The cross-linking of receptors triggers release of perforin and granzymes that lyse the tumor cell. b-d) Cell debris is taken up by antigen-presenting cells, which present the tumor antigens to B cells, triggering the release of antibodies with specificities for numerous epitopes on the target antigens and cytotoxic T lymphocytes (CTLs) that are capable of recognizing and killing cells that express the target antigen. Adapted from (Adams et al. 2005).

CDC is another cell-killing method that can be directed by antibodies. IgM is the most effective isotype for complement activation. However, it is not widely used in clinical oncology because IgM does not readily extravasate from vascular structures. IgG1 and IgG3 are both very effective at directing CDC via the classical complement-activation pathway. CDC is initiated by the complement C1q binding to the Fc region of IgG, which is bound to the surface of a target cell (Figure 2.3). This triggers a cascade of events involving a series of other complement proteins and leads to the proteolytic release of the effector-cell chemotactic/activating agents C3a and C5a. The complement cascade ends in the formation of a membrane attack complex, which creates pores in the cell membrane that facilitate free passage of water and solutes into and out of the cell. It is accepted that CDC plays a significant role in the *in vivo* efficacy of rituximab and
alemtuzumab (Golay et al. 2001; Di Gaetano et al. 2003). The ability of an antibody to elicit CDC may depend on the number of copies of its target antigen on the cell surface, as high antibody density is necessary for C1q to be activated by binding simultaneously to at least two IgG molecules on the surface on a target cell. Although CDC is not believed to dominate the cell killing effects elicited by most mAbs, it generates various factors that can enhance ADCC. The release of the chemotactic/activating agents C3a and C5a results in a gradient that draws effector cells, such as NK cells, into the tumor (Adams et al. 2005).

![Figure 2.3: Complement-directed cytotoxicity. a) Binding of monoclonal antibodies to antigen on the cell surface. b) Exposure of binding site on monoclonal antibodies for proteins that initiate the complement cascade. c) Release of chemotactic factors. d) Formation of the membrane attack complex, which promotes target-cell lysis. Adapted from (Adams et al. 2005).](image)

The signaling effect of antibodies is predicated on either inducing cross-linking of receptors that are, in turn, connected to mediators of cell division or programmed cell death, or directing them towards specific receptors to act as agonists for the activation of specific cell populations (Brekke et al. 2003; Adams et al. 2005).

2.1.3 Technologies for antibody production

The very first pharmaceutically useful antibodies were obtained from large animals (e.g. horses) that had been immunized with a certain antigen thereby developing adaptive immunity, including antigen-specific polyclonal antibodies which could be extracted from the serum. With the discovery of hybridoma technology by Kohler and Milstein (Kohler et al. 1975), antibodies could be produced in reasonable amounts of
monoclonal quality for diagnostic and clinical use. To produce monoclonal antibodies, B cells are removed from the spleen of an animal that has previously been immunized with a relevant antigen. These B cells are then fused with myeloma tumor cells that can grow indefinitely in culture. Antibodies derived from hybridoma technology are usually of murine origin, which limits their use for human therapy, because of induction of a human anti-mouse antibody (HAMA) response. This not only inactivates and eliminates murine antibodies after repeated administration; the formation of antibody-HAMA complexes also can lead to allergic reactions up to anaphylactic shock, largely limiting the possibility of repeated administration. Furthermore, murine antibodies have only short circulating half-life and due to differences between the murine and the human immune system, the mouse Fc portion also impaired the possibility of inducing complement-mediated and antibody-dependent cellular cytotoxicity, restraining therapeutic possibilities (Stern et al. 2005).

To circumvent the immunogenicity problems and to improve immunologic efficacy, genetic engineering was used to generate so called chimeric antibodies. Constant domains of the murine molecule were replaced by their respective human counterparts. However, although chimeric antibodies were perceived as less foreign, and therefore less immunogenic, than mouse monoclonal antibodies, human anti-chimeric antibody responses (HACAs) have nonetheless been observed (Morrison et al. 1984; Bell et al. 2000). Further minimization of the mouse component of antibodies was achieved through CDR grafting, resulting in a humanized antibody of roughly 95% human origin (Jones et al. 1986).

With the isolation of genes encoding human variable regions, their successful expression in E. Coli, and the introduction of phage-display technology, the task of selecting fully human variable domains has been greatly simplified. Selection from phage-display libraries of human antibody fragments is today the most used and well-established technology for the development of new human antibodies. The display of antibody fragments on the surface of filamentous phage allows the construction of large (> 10⁹ clones) libraries of human antibodies, from which monoclonal antibodies can be isolated by panning the phage library onto an immobilized antigen (Winter et al. 1994; Viti et al. 2000). When required, antibody affinity can be “matured” using combinatorial mutagenesis of the antibody gene and stringent selection strategies (Schier et al. 1995; Pini et al. 1998). Another approach to generate completely human
antibodies is to use mice that are transgenic for the human Ig locus. Immunization of such a transgenic mouse results in a human antibody response, from which hybridomas that produce human antibodies can be generated (Green 1999).
2.2 Antibody-based vascular targeting

2.2.1 General concepts

A substantial proportion of the currently available treatments for disease are limited by a lack of specificity. Therefore, one avenue for the development of better therapeutics relies on the construction of drugs which selectively accumulate at the site of disease while sparing healthy tissues and thereby leading to less unwanted side effects. Antibody-based targeting relates to the targeted delivery of bioactive effector molecules such as drugs, cytokines, radionuclides, photosensitizers or procoagulant factors to the site of disease by means of antibodies specific for disease-associated markers. With this strategy, the therapeutic index of bioactive agent can be improved. An advantage of antibody-based targeting approaches is the synergy between imaging opportunities and therapeutic applications. In principle, the same binding molecule can be used for the delivery not only of a therapeutically active molecule, but also of an imaging agent which allows the detection of the disease (e.g. radionuclides, fluorophores, microbubbles).

Since tumors as well as inflammatory processes are dependent on their capacity to acquire a blood supply, the formation of new blood vessels (angiogenesis) is an important step in disease progression. It has long been known, that the endothelium and surrounding stroma of newly formed blood vessels differ from the ones in normal tissues. Molecules capable of selectively targeting markers of angiogenesis may offer opportunities for the in vivo imaging of and for the selective delivery of therapeutic agents to the site of disease.
Figure 2.4: The concept of antibody-based vascular targeting using the example of a cancer patient. The targeted compound, consisting of an antibody as a carrier molecule and an effector moiety, is applied intravenously and homes to the tumor-specific vascular antigen, resulting in the accumulation of the pharmaceutical at the tumor site. The vascular antigen can be either expressed on the luminal surface of endothelial cells (EC) or in the perivascular extracellular matrix (ECM).

2.2.2 Angiogenesis

Angiogenesis, i.e. the sprouting of new blood capillaries from existing vessels, is an essential process for embryonic development. Once the vascular network is in place, endothelial cells lining the blood vessels are quiescent and angiogenesis is triggered only locally and transiently during a number of physiological conditions like the female reproductive cycle and wound healing (Bischoff 1995). However, angiogenesis is an important feature of a range of different pathological conditions (Folkman 1995). The growth of new capillaries is often triggered in conditions of pathological cellular proliferation, ischemia or chronic inflammation, where an increase in blood supply may compensate for hypoxia and insufficient delivery of nutrients to the tissue (Carmeliet 2003; Pugh et al. 2003). Examples are cancer, rheumatoid arthritis, diabetic retinopathy, psoriasis and juvenile hemangiomas. Angiogenesis is a tightly regulated process, in which pro-angiogenic and anti-angiogenic factors are in equilibrium to neutralize one another. Upregulation of proangiogenic or downregulation of anti-angiogenic mediators induces angiogenesis. To initiate the formation of new capillaries, endothelial cells of existing blood vessels must degrade the underlying basement membrane and invade into the stroma of the
neighboring tissue. A number of growth factors, proteases, adhesion molecules and other angiogenic mediators which enable endothelial cell migration or proliferation regulate this process. Vascular endothelial growth factor (VEGF) is considered to be one of the most important growth factors in angiogenesis (Ferrara et al. 2003). It increases the permeability of existing blood vessels and acts as endothelial cell survival factor, as well as being a potent endothelial cell mitogen. 

During angiogenesis, the associated tissue remodeling leads to the expression and/or exposure of marker molecules around the new blood vessels, which are inaccessible, much lower in abundance, or even undetectable in healthy adult tissues. Such markers of angiogenesis therefore include candidate targets for ligand-based targeting approaches.

2.2.3 Markers of angiogenesis for vascular targeting

Vascular targeting strategies fundamentally depend on structural and molecular differences in the endothelium or the surrounding stroma of disease and normal vessels. Vascular targets include proteins expressed on the endothelial cell, e.g. integrins, as well as those that are secreted into the stroma around the vessels, such as the differentially spliced isoforms of fibronectin and tenascin.

Endothelial membrane proteins and tumor cell markers

Vascular endothelial growth factors and their receptors: Vascular endothelial growth factors (VEGFs) are key mediators in the regulation of tumor-associated angiogenesis (Ferrara et al. 2003). The elevated expression of VEGFs and their receptors in tumors makes them attractive antigens for antibody-based targeting approaches (Ke et al. 1996; Brekken et al. 2000; Cooke et al. 2001; Backer et al. 2007; Korpanty et al. 2007). In a recent study, Backer et al. described the generation of single-chain VEGF-based probes labeled with different contrast agents for the molecular imaging of VEGF receptors in angiogenic vasculature (Backer et al. 2007). However, the absolute quantities of antibodies against these antigens accumulating at the tumor site were in general modest, which possibly reflects kinetic limitations in the targeting of low or medium abundance antigens, even when they are easily accessible from the blood (Halin et al. 2002).
The contribution of VEGF-A to cancer progression has been highlighted by the approval of the humanized anti-VEGF monoclonal antibody bevacizumab (Avastin, Genentech/Roche) for the first-line treatment of advanced colorectal cancer in combination with chemotherapy (Hurwitz et al. 2004) and, recently, for the treatment of non-small-cell lung cancer (Sandler et al. 2006), breast cancer (Miller et al. 2005), glioblastoma and metastatic renal cell carcinoma.

**Integrins:** Integrins are heterodimeric cell-surface receptors that mediate cell adhesion to extracellular matrix proteins or cellular receptor proteins. During vascular remodeling and angiogenesis, endothelial cells exhibit increased expression of several cell-surface molecules that potentiate cell invasion and proliferation (Hood et al. 2002; Ruoslahti 2002).

The integrins $\alpha_3\beta_3$ and $\alpha_5\beta_3$ are upregulated in endothelial cells undergoing angiogenesis and have been shown to be critically involved in angiogenesis events (Brooks et al. 1994; Friedlander et al. 1995; Hynes 2002; Li et al. 2004). Despite the fact that $\alpha_3\beta_3$ integrin and other integrins have also been detected in several non-diseased cells such as smooth muscle cells, osteoclasts and hematopoietic cells, integrins have been proposed as targets for ligand-directed delivery of therapeutic agents to the tumor vasculature (Max et al. 1997; Byzova et al. 1998).

A high-affinity humanized anti-$\alpha_3\beta_3$ antibody (Vitaxin) is in clinical development as an anti-angiogenic therapeutic. However, so far its tumor-targeting performance for cancer imaging has been unsatisfactory (Posey et al. 2001). A further affinity matured version, named Abegrin (MEDI-522), has completed phase II clinical trials as an anti-angiogenic agent (McNeel et al. 2005; Mulgrew et al. 2006).

**Annexin A1:** Annexins are cytosolic proteins, that can associate with cell membranes in a calcium-dependent manner. Some annexins may translocate the lipid bilayer to the external cell surface. Annexin A1 was discovered as a tumor endothelial target using terminal perfusion of tumor-bearing rats with silica beads (Oh et al. 2004). A monoclonal antibody to this antigen has been successfully used for the radioimmunoscintigraphic detection of solid tumor lesions in a rat model. Furthermore, relatively low radioactive doses of the same antibody labeled with $^{125}$I (single injection of 50 $\mu$Ci radiolabeled antibody per animal) showed therapeutic efficacy in tumor-bearing rats (Oh et al. 2004).
**Nucleolin:** Nucleolin was originally described as a nuclear protein involved in the regulation of cell proliferation, replication and nucleogenesis (Srivastava et al. 1999). More recently it was discovered that nucleolin is also expressed on the cell surface of angiogenic endothelial cells, whereas it is restricted to the nucleus in quiescent endothelium (Christian et al. 2003). During the angiogenic process it is shuttled from the nucleus to the cell-surface of endothelial cells where it is involved in the regulation of cell migration and capillary-tubule formation (Huang et al. 2006). A 31-amino acid synthetic peptide (F3) is internalized into tumor endothelial cells and tumor cells upon binding to Nucleolin. However, despite its restricted expression and its ability to internalize binding agents, tumor vascular targeting applications based on antibodies specific to nucleolin have not been reported yet.

**Prostate-specific membrane antigen (PSMA):** PSMA is a membrane glycoprotein, present on the surface of almost all prostate cancer cells and is used as target for antibody-based imaging of prostate adenocarcinoma. The interest for vascular targeting applications of PSMA has been stimulated by the observation that PSMA is over-expressed in the neo-vasculature of several solid tumor types (Liu et al. 1997; Chang et al. 1999), while expression around blood vessels in normal tissues is limited to breast, kidney, duodenum and prostate (Chang et al. 1999). The monoclonal anti-PSMA antibody J591 in fusion with various radionuclides has shown promising targeting efficacy in patients not only with prostate cancer but also with other solid tumors (Bander et al. 2003; Milowsky et al. 2004; Bander et al. 2005; Milowsky et al. 2007).

**Endoglin (CD105):** Endoglin (CD105) is a homodimeric transmembrane glycoprotein and acts as a transforming growth factor-β (TGF-β) co-receptor. It is overexpressed in neovascular endothelial cells of various human cancers (Wang et al. 1993; Burrows et al. 1995; Minhajat et al. 2006). However, extensive immunohistochemical studies have demonstrated a significant expression of endoglin also in normal tissues (Matsubara et al. 2000; Balza et al. 2001; Minhajat et al. 2006) and the tumor accumulation (%ID/g) in quantitative biodistribution studies in rodent models was not impressive (Bredow et al. 2000).

**Phosphatidyl serine phospholipids (PS):** Phosphatidyl serine phospholipids (PS) are major components of the cell membrane which are preferentially found in the inner leaflet of the cell membrane lipid bilayer. During certain biological processes such as cellular stress, apoptosis and endothelial-cell proliferation in tumors, the exposure of
phosphatidyl serine to the outer leaflet of endothelial cell membranes has been reported. Annexin V, an intracellular phospholipids binding protein, and monoclonal antibodies have been used to confirm the surface accessibility of the phosphatidyl serine moiety on endothelial cells in vitro (for example, after treatment with hydrogen peroxide) and in vivo (Ran et al. 2002). A monoclonal antibody to anionic phospholipids inhibited the growth of human breast tumors in mice by stimulating antibody-dependent cellular cytotoxicity toward tumor vessels (Huang et al. 2005). The chimeric antibody bavituximab is currently being tested in clinical trials for the treatment of cancer and hepatitis C.

**CD44**: CD44 is a type I transmembrane cell surface glycoprotein consisting of a single polypeptide chain. It is encoded by a single gene, with alternative splicing providing the potential for multiple isoforms. CD44 is expressed throughout development on many different cell types including hematopoietic cells, fibroblasts, some epithelial and endothelial cells, and cells in the central nervous system. Changes in CD44 expression, including up-regulation, down-regulation and changes in the isoforms expressed have been associated with a variety of malignancies and inflammation (Lesley et al. 1997). The monoclonal antibody TES-23, specific to an isoform of CD44, has been associated with some of the most impressive tumor targeting performances in rodent models of cancer. Tumor values as high as 50-150% injected dose per gram (%ID/g) have been reported as early as one hour after intravenous injection (Wakai et al. 2000). TES-23 recognizes a widely distributed form of CD44 lacking variant exons, termed CD44H. However, the epitope was shown to include a post-translational modification which is found in an activated, tumor-associated form of CD44H (Taniguchi et al. 2000). The rapid accumulation indicates that the antigen is readily accessible from the tumor vasculature.

CD44 splice variants which contain exon 6, CD44v6, are involved in tumor progression (Wielenga et al. 1993; Heider et al. 2004). The antibody-drug conjugate bivatuzumab mertansine, specific for CD44v6, has been tested in phase I clinical trials for squamous cell carcinoma of the head and neck. However, binding of the antibody to CD44v6 on skin keratinocytes mediated serious skin toxicity and the clinical development of bivatuzumab mertansine was discontinued (Tijink et al. 2006).

**Magic roundabout or Robo4**: The Robo family consists of Robo1, Robo2, Robo3, and Robo4, the latter also known as magic roundabout. Robos 1, 2, and 3 are highly
expressed in the nervous system and undetectable in the vascular system. All known Robo family members have a large extracellular domain composed of five immunoglobulin and three fibronectin motifs except Robo4, which has only two immunoglobulin and two fibronectin motifs, and diverges significantly from the other Robos. Robo4 is absent from adult tissues except at sites of active angiogenesis, including tumors. It is highly expressed in the embryo and one presumes it is intimately involved in the development of the vasculature. The pattern of Robo4 expression makes it ideally suited to enable vascular targeting. Biodistribution studies with radiolabeled ligands (e.g., monoclonal antibodies) are required in order to assess the real potential of Robo4 as a target for imaging and/or biomolecular therapeutic intervention (Huminiecki et al. 2002; Seth et al. 2005).

**Extracellular Matrix Proteins**

Markers on the luminal surface of vascular endothelial cells are the most readily accessible targets for an intravenously administered ligand, but may be low in abundance, thus allowing only a small quantity of ligand to accumulate. Antigens expressed in the stroma around the neovasculature can also be reached by the ligand after extravasation and offer the advantage of a usually more abundant and stable expression.

**Splice isoforms of fibronectin:** Fibronectins (FNs) are high molecular weight adhesive glycoproteins present in soluble form in plasma and other body fluids and in insoluble form in the extracellular matrix. FNs are involved in different biological processes including the establishment and maintenance of normal cell morphology, cell adhesion, migration, hemostasis, thrombosis, wound healing and oncogenic transformation.

Although FNs are the product of a single gene, the resulting protein can exist in multiple forms. Their polymorphism is due to alternative splicing in three regions, IIICS, EDA and EDB and to post-translational modifications. In transformed cells and in malignancies, the splicing pattern of FN-pre-mRNA is altered leading to an increased expression of FN isoforms containing the IIICS, EDA and EDB sequences (Ebbinghaus et al. 2004).
A fibronectin subunit consists of a series of repeating units of three different types: type I, type II, type III. The protein sequences undergoing alternative splicing are indicated in green (EDB), orange (EDA) and violet (IIICS).

The extra-domain B (EDB) of fibronectin is a 91-amino-acid type III homology domain which is not present in the fibronectin molecule under physiological conditions but becomes inserted during active tissue remodeling by alternative splicing (Zardi et al. 1987). It displays either predominantly vascular or diffuse stromal patterns of expression, depending on the tumor type (Carnemolla et al. 1989). Despite its distinct expression pattern and its high rate of conservation, a specific role of the EDB domain of fibronectin in angiogenesis has not yet been identified. Fukuda et al. showed that EDB-deficient mice developed normally and that they were fertile (Fukuda et al. 2002). Furthermore, double knock-out mice, lacking the EDB exon and p53 did not show any difference in the length of survival compared to animals expressing EDB.

As the EDB sequence is identical in mouse, rat, rabbit, dog, monkey and man, the generation of anti-EDB antibodies by hybridoma technology has not been possible so far. However, synthetic human antibody phage libraries have allowed the isolation of specific EDB binders (Carnemolla et al. 1996; Neri et al. 1997; Pini et al. 1998). These include the high-affinity human antibody L19, which has been shown to efficiently localize to the tumor vasculature in animal models (Tarli et al. 1999; Demartis et al. 2001; Borsi et al. 2002; Borsi et al. 2003) and in patients with different cancer entities (Santimaria et al. 2003; Birchler et al. 2007) following intravenous administration. Furthermore, our group has demonstrated, that L19 is able to selectively localize at sites of psoriasis in a VEGF-transgenic mouse model, in atherosclerotic plaques of apolipoprotein E-null mice and in arthritic lesions in the collagen induced arthritis mouse model (Matter et al. 2004; Trachsel et al. 2007; Trachsel et al. 2007).

Fibronectin contains a second alternatively spliced domain, extradomain A (EDA). EDA shows a restricted pattern of expression in plasma and normal human tissues, while being over-expressed during active tissue remodeling, such as angiogenesis in tumors, wound healing, and during embryogenesis (Borsi et al. 1998; Muro et al. 1999). A recent
A proteomic study based on the *in vivo* biotinylation of vascular structures has revealed that EDA is a promising vascular marker of metastatic disease (Rybak et al. 2007). As EDB, EDA is a type III homology domain with a sequence of 90 amino acids which is highly conserved between human and mouse (96% identity). To study the *in vivo* function of EDA mice lacking the EDA splicing regulation were generated. Homozygous EDA+/+ and EDA−/− mice were viable and phenotypically similar to the wild-type mice. However, EDA−/− mice showed an abnormal cutaneous skin wound healing. Furthermore, both mouse strains had a significantly shorter lifespan than wild-type animals, suggesting that the presence of both isoforms was necessary for efficient long-term maintenance of biological function (Muro et al. 2003). To understand the role(s) of EDA and EDB splice variants, Astrof and coworkers deleted both EDA and EDB exons from the FN gene. They observed embryonic lethality and embryos displayed multiple embryonic cardiovascular defects (Astrof et al. 2007).

Although the function of EDA is still not clear, it has proven to be an attractive target for antibody-based delivery of bioactive agents to the neo-vasculature of tumors and other angiogenesis related diseases. Our group has reported the generation of 3 high-affinity human monoclonal antibodies to EDA, which efficiently stain neo-vascular structures in animal models of cancer and in human tumor sections. One of these antibodies (termed F8) displayed a dissociation constant for the antigen of 3.1 nM and exhibited an impressive tumor targeting selectivity in biodistribution experiments performed in the diabody format and in the homodimeric SIP format (Villa et al. 2008).

**Large isoforms of tenasin-C:** Tenascins are a family of four extracellular matrix glycoproteins that are found in vertebrates (tenasin-C, -R, -X and -W). They are typically present in many different connective tissues, where they contribute to matrix structure and influence the behavior of cells that are in contact with the ECM (Neri et al. 2005).

Tenasin-C (TnC) is an oligomeric glycoprotein composed of six individual polypeptides with molecular weights ranging from 180 to 300 kDa that are joined at their N-terminus by disulfide bonds. It has a multimeric, six-armed structure called a hexabrachion. Each arm consists of an amino-terminal TN assembly (TA) domain that allows individual TnC polypeptides to interact at their amino termini through a number of cysteine residues and heptad repeats. The molecule further contains a group of assemblies.
epidermal growth factor-like (EGFL) repeats, a series of fibronectin type III domains and a globular fibrinogen-homology domain in the distal region (Carnemolla et al. 1999; Jones et al. 2000). TnC exists in several polymorphic isoforms due to alternative splicing between domains A1 and D in the fibronectin-like type III repeats.

A variety of functions including immunosupression, cell migration, inhibition of focal adhesion, inhibition of apoptosis, and promotion of cell survival leading to tumor metastasis and progression has been attributed to TnC.

The molecule is synthesized dynamically by a variety of cell types during embryogenesis, whereas in normal adult tissues, large TnC expression is less abundant, but it is induced during tissue involution, neovascularization and wound healing. In addition, certain pathologies are characterized by a marked up-regulation of TnC synthesis, for example during infection, inflammation, or tumorgenesis. One of the major site of TnC expression are tumor tissues. Tenascin-C was originally discovered as a glioma mesenchymal extracellular matrix antigen. In the meantime many studies have investigated the presence of TN-C in different types of brain tumors, breast cancers, melanoma, and lung tumors. (Kim et al. 2000; Tokes et al. 2000; Chiquet-Ehrismann et al. 2003).

The tumor-targeting properties of the high-affinity human antibody G11, directed against the domain C of tenascin-C, have been recently demonstrated, using immunohistochemical analyses and quantitative biodistribution studies in an orthotopic rat glioma model (Silacci et al. 2006). Similarly, using the high-affinity human antibody F16 reactive with the domain A1 of tenascin-C, our group has recently shown a strong expression of the antigen in a variety of primary and metastatic tumors and an excellent tumor-targeting performance (Carnemolla et al. 1999; Brack et al. 2006).

Figure 2.6: Structure of Tenascin-C: Schematic representation of an individual TnC polypeptide.
2.3 Rheumatoid arthritis

2.3.1 Disease

Rheumatoid arthritis (RA) is a chronic inflammatory and destructive joint disease that affects ~0.8% of the adult population in the industrialized world. Disease onset typically occurs between 30 and 50 years of age, and it is two to three times more frequent in women than in men. RA leads to significant disability and a consequent reduction in quality of life. RA is classified as an inflammatory polyarthritis because it involves many joints (6 or more), although in the early stages of the disease, only one or a few joints might be afflicted. Virtually all peripheral joints can be affected by the diseases. However, the most commonly involved joints are those of the hands, feet and knees.

The etiology and pathogenesis of RA remain unsolved. It has long been speculated that RA could be triggered by infectious agents, but proof of this is still lacking. The major risk factors that have so far been reproducibly identified are genetic variations in class II major histocompatibility complex, DR beta 1 (HLA-DRβ1) and protein tyrosine phosphatase (PTPN22) genes. The sole environmental factor consistently associated with RA is cigarette smoking (Begovich et al. 2004; Strand et al. 2007). Rheumatoid arthritis is regarded as an autoimmune disease. There is a strong association between RA and several types of autoantibodies, such as rheumatoid factor (against the Fc region of other antibodies) and anti-CCP (against citrullinated epitopes on post-translationally modified proteins), whose presence is associated with a worse prognosis (Klareskog et al. 2006).

Many cell populations are involved in disease pathogenesis: T cells, B cells, monocytes/macrophages, mast cells, dendritic cells and fibroblasts are found in highly increased numbers in the synovial membrane in RA. T cells infiltrating the synovial membrane are primarily CD4+ memory cells, which produce IL-2 and IFN-γ and so clearly have a T_{h1} bias. These T cells activate monocytes, macrophages and synovial fibroblasts, which then overproduce proinflammatory cytokines, mainly TNF-α, IL-1β and IL-6 (Smolen et al. 2003). The synovial lining layer consisting of type A (macrophage like) and type B (fibroblast like) synoviocytes becomes hyperplastic and, at the cartilage-
bone junction, transforms into an aggressive tissue, the ‘pannus’, which contains osteoclasts. The growth of synovial membrane is accompanied by neovascularization.

![Diagram of joint and pannus](image)

**Figure 2.7: Comparison of a normal joint and rheumatoid arthritis joint.** a) In the healthy joint the thin synovial membrane lines the non-weight-bearing aspects of the joint. b) In rheumatoid arthritis the synovial membrane becomes hyperplastic and infiltrated by chronic inflammatory cells. Ultimately it develops into ‘pannus’, which migrates onto and into the articular cartilage and underlying bone. Adapted from (Strand et al. 2007).

### 2.3.2 Angiogenesis and the expression of EDA in rheumatoid arthritis

Angiogenesis is involved in homeostatic processes, such as reproduction, development, and tissue repair, as well as in pathological states including, rheumatoid arthritis, other inflammatory diseases and tumors. In rheumatoid arthritis, leukocytes migrate into the synovium through the vascular endothelium resulting in synovial inflammation and, eventually, joint destruction. The rheumatoid arthritis synovial tissue is rich in newly formed vessels. Angiogenesis enhances leukocyte extravasation into the synovium and thus the progression of rheumatoid arthritis. Potent angiogenic factors, such as VEGF and fibroblast growth factor (FGF), are abundantly expressed in rheumatoid arthritis synovium (Koch 2003). Furthermore, many inflammatory mediators have direct or
indirect angiogenic activities, e.g. prostaglandins E1 and E2, TNF-α, IL-1, IL-6, IL-8 or IL-18 (Taylor 2002; Koch 2003).

Kriegsmann and colleagues have shown EDA fibronectin deposition in the synovial lining layer of osteoarthritis and rheumatoid arthritis tissues, using immunohistochemistry and in situ hybridisation. In specimens of patients with rheumatoid arthritis EDA is detectable in stromal areas, vascular structures, and the synovial lining layer, whereas expression of EDB fibronectin expression is weaker and only some vessels show positive reaction (Kriegsmann et al. 2004). Furthermore, Shiozawa et al. reported a positive correlation with the progression of joint destruction in rheumatoid arthritis and the concentration of EDA fibronectin in the synovial fluid (Shiozawa et al. 2001).

2.3.3 Treatment of rheumatoid arthritis

Traditional therapy of rheumatoid arthritis has been dominated by the therapeutic pyramid that was slowly and gradually built up from a base of physical therapy and non-pharmacological interventions, followed by the use of non-steroidal anti-inflammatory drugs (NSAIDs) and the subsequent initiation of a single disease-modifying anti-rheumatic drug (DMARD). With time, however, attitudes started changing. It has been recognized that successful treatment strategies for RA involve early and aggressive pharmacotherapy with one or more DMARDs, typically supported by NSAIDs and/or corticosteroids to provide pain relief, as well as to control inflammation to some degree. Early use of DMARDs slows disease progression and improves overall long-term prognosis.

DMARDS:

Intramuscular gold has been the standard of care for more than half a century, before the emergence of methotrexate as the first-line DMARD for most patients. To date methotrexate has remained the initial treatment of choice in moderate to severe disease. When response is inadequate, leflunomide, azathioprine, or combination (methotrexate plus another agent) may be considered. Biologic DMARDS, the latest
generation of anti-rheumatic drugs, have novel molecular mechanisms that target cytokines and cells involved in inflammation and joint destruction (Figure 2.9).

![Pathophysiological pathways in rheumatoid arthritis.](image)

**Figure 2.9: Pathophysiological pathways in rheumatoid arthritis.** A schematic showing the simplified pathophysiological pathways in rheumatoid arthritis, and where these processes can be interfered with by currently available biologic agents. Adapted from (van Vollenhoven 2009).

The most successful of the novel RA therapies are the tumor necrosis factor-α (TNF-α) inhibitors. Several TNF inhibitors are well established in the RA market, as well as for several other inflammatory disorders (e.g. Psoriasis). Infliximab (Remicade), a chimeric monoclonal antibody (mAb) that is specific for TNF, and the fusion protein etanercept (Enbrel), which contains the ligand-binding portion of the soluble TNF receptor, have been approved for RA for about a decade. The first fully human TNF-specific mAb adalimumab (Humira) was approved for the treatment of RA in 2002. Certolizumab pegol (Cimzia) is a PEGylated Fab fragment of a humanized TNF inhibitor monoclonal antibody and was approved for the treatment of RA in 2009. In the same year the first once-monthly subcutaneous anti-TNF-α therapy, Golimumab (Simponi) was approved.

Other biologics for RA that have alternative mechanisms of action have been approved: anakinra (Kineret), rituximab (Rituxan), abatacept (Orencia) and tocilizumab (RoActemra). Anakinra is an interleukin-1 receptor antagonist that is approved for the treatment of RA for use alone or in combination with DMARDs other than TNF.
inhibitors. Rituximab, a mAb that binds to CD20 on B cells, is approved for patients who have not responded to TNF inhibitors. Abatacept, a fusion protein that binds to CD28 on T cells, has been approved for patients who have not responded adequately either to oral DMARDs or to TNF inhibitors. Finally, tocilizumab, a humanized mAb that binds to the interleukin-6 receptor, has recently been approved for the treatment of RA in Europe and Japan, for patients who either have responded inadequately to, or were intolerant to, previous therapy with one or more DMARDS (including TNF inhibitors) (Simon 2004; Mount et al. 2005; Stoll et al. 2009; van Vollenhoven 2009).

<table>
<thead>
<tr>
<th>Agent</th>
<th>Structure</th>
<th>Pharmacology</th>
<th>Dosage</th>
<th>Regulatory status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abatacept</td>
<td>Recombinant CTLA4 molecule dimerized on Ig fragment</td>
<td>T-cell co-stimulation blocker</td>
<td>500-1000 mg monthly (i.v.)</td>
<td>Approved in Europe and US</td>
</tr>
<tr>
<td>Adalimumab</td>
<td>Human monoclonal Ab</td>
<td>TNF blockade</td>
<td>40 mg biweekly (s.c.)</td>
<td>Approved in Europe and US</td>
</tr>
<tr>
<td>Anakinra</td>
<td>Recombinant IL-1 receptor antagonist</td>
<td>IL-1 receptor blockade</td>
<td>100 mg daily (s.c.)</td>
<td>Approved in Europe and US</td>
</tr>
<tr>
<td>Certolizumab</td>
<td>Pegylated Fab fragment from humanized mAb</td>
<td>TNF blockade</td>
<td>200 mg biweekly or 400 mg monthly (s.c.)</td>
<td>Approved in US</td>
</tr>
<tr>
<td>Etanercept</td>
<td>Recombinant TNF receptor (p75) dimerized on Ig frame</td>
<td>TNF blockade</td>
<td>50 mg weekly (s.c.)</td>
<td>Approved in Europe and US</td>
</tr>
<tr>
<td>Golimumab</td>
<td>Human mAb</td>
<td>TNF blockade</td>
<td>100 mg every 4 weeks (s.c.)</td>
<td>Approved in US</td>
</tr>
<tr>
<td>Infliximab</td>
<td>Chimeric mAb</td>
<td>TNF blockade</td>
<td>3-10 mg/kg every 4-8 weeks (i.v.)</td>
<td>Approved in Europe and US</td>
</tr>
<tr>
<td>Rituximab</td>
<td>Chimeric mAb</td>
<td>B-cell depletion</td>
<td>1000 mg 2 i.v. infusions 2 weeks apart</td>
<td>Approved in Europe and US</td>
</tr>
<tr>
<td>Tocilizumab</td>
<td>Humanized mAb</td>
<td>IL-6 receptor blockade</td>
<td>8 mg/kg every 4 weeks (i.v.)</td>
<td>Approved in Europe</td>
</tr>
</tbody>
</table>

Table 2.1: The current armamentarium of biologic agents. Adapted from (van Vollenhoven 2009).

2.3.4 Collagen induced arthritis mouse model

The collagen-induced arthritis (CIA) mouse model is the most commonly studied animal model of rheumatoid arthritis. It shares several pathological features with RA, and Collagen II is a major protein in cartilage, the target tissue of RA. In the last years, the CIA model has been instrumental in the testing and development of the new biologically based therapeutics, such as those that target TNF-α (Williams et al. 1992). CIA is elicited in genetically susceptible strains of mice by immunization with CII emulsified in complete Freund’s adjuvant. The ensuing pathogenesis shares several
pathological features with RA, including synovial hyperplasia, mononuclear cell infiltration, cartilage degradation, and, like RA, susceptibility is linked to the expression of specific MHC class I genes (Courtenay et al. 1980; Bliven et al. 1986; Brand 2005; Brand et al. 2007).
2.4 Interleukin 10

2.4.1 Structure

Interleukin-10 (IL-10) is a homodimeric cytokine, produced by activated monocytes and T cells, that plays an important role in the regulation of inflammatory responses and immune reactions. IL-10 is located on chromosome 1q, the human IL-10 gene spans 4.7kb and consists of five exons separated by four introns. The mature human IL-10 (hIL-10) protein consists of 160 amino acids and has a predicted molecular weight of 18'647 Da (Vieira et al. 1991). It runs as a single species with an apparent molecular weight of 17 kDa in SDS-PAGE. Human IL-10 contains four cysteine residues which form two intramolecular disulfide bonds. Human and murine IL-10 exhibit a 73% amino acid homology. Human IL-10 is active on both mouse and human cells, whereas mIL-10 is effective only on mouse cells. Viral homologues of IL-10 have been found in Epstein Barr Virus (EBV), equine herpes virus type 2 (EHV2), and poxvirus Orf genomes. The EBV-encoded IL-10 (viral IL-10) has maintained some, but not all the biological activities of its cellular counterpart. Viral IL-10 (vIL-10) has 90% homology with hIL-10 (Vieira et al. 1991). Recombinant hIL-10 and vIL-10 are not N-glycosylated. Murine IL-10 (mIL-10) appears to be heterogeneously N-glycosylated. However, glycosylation of mIL-10 has no known influence on biological activity (Vieira et al. 1991; Windsor et al. 1993).

Early studies suggested that IL-10 is dimeric. Biochemical and X-ray crystallographic analyses of hIL-10 and vIL-10 demonstrated that IL-10 is an acid-sensitive, noncovalent homodimer of two interpenetrating polypeptide chains, similar to interferon-γ (IFN-γ) (Syto et al. 1998; Moore et al. 2001). An engineered hIL-10 altered to favor monomer formation bound to the IL-10 receptor and retained biological activity, although with reduced affinity and lower specific activity in a biological assay compared to the wild-type cytokine (Josephson et al. 2000).

2.4.2 Receptor signaling

The IL-10 receptor complex is composed of two subunits, of the IL-10 receptor 1 (IL-10R1) and IL-10 receptor 2 (IL-10R2), that are members of the interferon receptor family. IL-10 is thought to bind IL-10R1 first with high affinity and then IL-10R2 receptor 2 with a
lower affinity. IL-10R1 appears to be the major signaling component and IL-10R2 perhaps acts as a common chain in different cytokine receptors (Fickenscher et al. 2002). The best characterized IL-10 signaling pathway is the Jak/Stat system. The IL-10/IL-10R interaction engages the Jak family tyrosine kinases Jak1 and Tyk2, which are constitutively associated with IL-10R1 and IL-10R2 respectively. IL-10 induces tyrosine phosphorylation and activation of the latent transcription factors Stat3, Stat1, and in non-macrophage cells, Stat5 (Finbloom et al. 1995; Wehinger et al. 1996). Macrophages from Jak1-/- mice do not respond to IL-10, which indicates that Jak1 plays an obligatory early role in IL-10 signaling (Rodig et al. 1998). Stat3 is also implicated strongly as a key mediator of IL-10 response (Takeda et al. 1999). Mice with a cell type-specific disruption of the Stat3 gene in macrophages and neutrophils develop a chronic enterocholitis, and their macrophages are completely refractory to the effects of IL-10 (Takeda et al. 1999). In addition, IL-10 signaling in immune cells mediates some inhibitory effects via inhibition of the nuclear factor κB (NFκB), a transcription factor that controls the expression of many inflammatory proteins (Wang et al. 1995).

2.4.3 Biological activities of IL-10

IL-10 affects many aspects of inflammatory and immune responses. It has dominant suppressive effects on the production of proinflammatory cytokines by monocytes and neutrophils, and downregulates the expression of activating and costimulatory molecules on monocytes and dendritic cells. IL-10 potently inhibits not only the production of cytokines (e.g. IL-1β, IL-6, IL-12, IL-18, GM-CSF and TNFα) by activated monocytes, but also of CC chemokines and CXC chemokines. The inhibitory effects of IL-10 on IL-1β and TNF-α production are crucial to its anti-inflammatory activities, because these cytokines often have synergistic activites on inflammatory pathways and processes, and amplify these responses by inducing secondary mediators such as chemokines, prostaglandins, and platelet-activating factor (PAF).

IL-10 not only inhibits production of these effectors, but in addition enhances expression of their natural antagonists (e.g. IL-1RA and soluble p55 and p75 TNFR). Downregulation of these stimulatory or co-stimulatory molecules significantly affected the T cell-activating capacity of antigen presenting cells (APCs). Furthermore, IL-10
strongly inhibits cytokine production and proliferation of T cells activated in the presence of APCs (Fiorentino et al. 1991). However, T cells do express IL-10 receptor and IL-10 also directly modulates T cell function. In particular, inhibitory effects have been described on CD4+ T cells. IL-10 inhibits the proliferation as well as the cytokine synthesis of these cells. It affects their IL-2 and IFN-γ as well as their IL-4 and IL-5 production. Therefore, IL-10 seems to inhibit both the Th1-type and the Th2-type responses, although the effect on Th1 cells appears to be stronger (Del Prete et al. 1993; Romagnani 1995; Groux et al. 1996). In contrast, IL-10 does not exert potent direct inhibitory effects on CD8+ T cells. It can even activate CD8+ T cells under certain conditions (Groux et al. 1998).

IL-10 inhibits production of prostaglandin E2 (PGE2), another proinflammatory mediator, through downregulation of cyclooxygenase 2 (COX-2) expression (Mertz et al. 1994). This also affected the expression of matrix metalloproteinases, which are regulated by a PGE-cAMP pathway.

IL-10 has various but weak stimulatory effects on B cells. It prevents apoptosis and enhances the proliferation and differentiation toward plasma cells as well as the IgM synthesis (Levy et al. 1994).

2.4.4 Interleukin-10 in Diseases

**Malignant Diseases**

The profound immunosuppressive effects of IL-10 have prompted numerous studies of its expression and function in association with cancer.

Elevated IL-10 levels have been observed in certain cancers, for example ovarian cancer, various carcinomas, melanoma, and lymphoma (Moore et al. 2001; Asadullah et al. 2003). IL-10 overexpression can occur for multiple reasons with very different implications. IL-10 can be expressed by tumor cells themselves, possibly suppressing antitumor responses, or IL-10 could be produced by activated cells involved in a host antitumor reaction. Thus it is difficult to interpret the significance of elevated IL-10 expression in many studies.

IL-10 production was found in a high percentage of melanoma and melanoma metastases (Dummer et al. 1996) and it has been shown that IL-10 functions as a
The ability of IL-10 to inhibit induction and effector function of T cell mediated and inflammatory immune responses led to numerous studies of its expression function, and potential utility in bone marrow and organ transplantation. However, the role of IL-10 in transplantation is poorly understood. IL-10 pretreatment is associated with improved graft acceptance. Different studies showed that IL-10 treatment of recipient animals prior to grafting enhanced graft survival, whereas providing IL-10 at or after the time of grafting had little beneficial effect or even enhanced rejection (Qian et al. 1996; Li et al. 1997; Li et al. 1999). Studies of bone marrow transplantation (BMT) and

Transplantation

The ability of IL-10 to inhibit induction and effector function of T cell mediated and inflammatory immune responses led to numerous studies of its expression function, and potential utility in bone marrow and organ transplantation. However, the role of IL-10 in transplantation is poorly understood. IL-10 pretreatment is associated with improved graft acceptance. Different studies showed that IL-10 treatment of recipient animals prior to grafting enhanced graft survival, whereas providing IL-10 at or after the time of grafting had little beneficial effect or even enhanced rejection (Qian et al. 1996; Li et al. 1997; Li et al. 1999). Studies of bone marrow transplantation (BMT) and
graft-versus-host disease (GVHD) also support this concept. Patients exhibiting elevated levels of IL-10 production prior to bone marrow transplantation have lower incidence of GVHD and improved survival. In contrast high IL-10 levels in post BMT GVHD patients indicate a poor prognosis for survival (Bacchetta et al. 1994; Hempel et al. 1997; Baker et al. 1999; Holler et al. 2000). Furthermore, posttransplant administration of IL-10 to mice in models of BMT/GVHD was generally deleterious, resulting in unimproved or increased mortality (Blazar et al. 1995).

Experimental Autoimmune Encephalomyelitis (EAE)

IL-10 has been tested in mouse and rat models of experimental autoimmune encephalomyelitis (EAE) which are in part representative of the pathology observed in multiple sclerosis in humans. Systemic administration of IL-10 during the initiation phase of the disease suppressed disease induction, and reduced T cell proliferation to myelin basic protein and CNS infiltration (Rott et al. 1994). In addition, IL-10 prevented TNF-α-induced relapses in SJL mice that had partially or completely recovered from acute EAE. Furthermore, neutralization of endogenous IL-10 increased both the incidence and severity of staphylococcal enterotoxin B or TNF-α induced relapses, suggesting a protective role for IL-10 in the development of disease (Crisi et al. 1995). Elevations of IL-10 mRNA levels were observed in the recovery phase of acute EAE in mice and rats (Kennedy et al. 1992; Issazadeh et al. 1996), and a lack of IL-10 production was observed in chronic relapsing EAE (Issazadeh et al. 1996). In vitro a combination of the immunosuppressive drugs, vitamin D3 and Dexamethasone, induced human and mouse naive CD4-T cells to differentiate into regulatory CD4+CD25+ T cells. These cells produced IL-10 and retained strong proliferative capacity. The regulatory function of these cells that in vitro inhibit both Th1-and Th2-inducting cytokines was demonstrated in vivo by their ability to prevent EAE, when targeted to the site of inflammation (Barrat et al. 2002).

In most models IL-10 was active in preventing EAE whereas its efficiency in ongoing or established EAE was less clear. Interestingly, IFN-β application, the best established therapeutic approach in multiple sclerosis in patients, modulates the IL-10/IL-12 cytokine circuit resulting in dominance of IL-10 (Tuohy et al. 2000).

Chronic Inflammatory Bowel Disease
Crohn’s disease and ulcerative colitis are complex chronic diseases of the gut, the etiology and pathogenesis of which are poorly understood. The gastrointestinal tract serves as a barrier between the host and several foreign antigens and pathogens that are contained within its lumen. The mucosa-associated immune system must balance two opposing functions: the development of an immune response to pathogens while maintaining tolerance to antigens derived from food and ‘natural’ microbial flora. The regulation of this balance is very complex but cytokines seem to play a key role. Intestinal epithelial cells from inflamed and healthy colonic tissue express IL-10 mRNA and protein to the same extent. However, compared with non-inflamed gut, significantly increased numbers of mononuclear cells producing IL-10 are present in the submucosa of inflamed gastrointestinal tissue.

Further evidence for the pivotal role of IL-10 in mucosal immunoregulation comes from gene-targeted IL-10 knock-out (IL-10−/−) mice, which develop a spontaneous ileocolitis by 2-3 months of age with many similarities to Crohn’s disease (Kuhn et al. 1993). The development of inflammation is entirely dependent on exposure to luminal bacteria. IL-10−/− mice kept in germ-free conditions remain disease-free, while inoculation with specific bacterial strains induces colitis via an antigen-driven Th1 response. This suggests that IL-10 is important in the maintenance of tolerance to components of the intestinal flora. In its absence, luminal antigen stimulates a Th1-dependent response that leads to prolonged intestinal inflammation, in part sustained by the uncontrolled release of IL-12 and IFN-γ (Kuhn et al. 1993; Kullberg et al. 1998; Sellon et al. 1998; Lindsay et al. 2001). IL-10 has proven efficacy in several mouse models. Data from patients suggest that overexpression of IFN-γ and TNF-α and low levels of IL-10 might lead to development of severe Crohn’s disease. Furthermore low ileal IL-10 concentrations are believed to be predictive of endoscopic recurrence after surgery in Crohn’s disease. IL-10 therefore seems to be an important regulator of intestinal immune response (Schreiber et al. 1995; Meresse et al. 2002).

Rheumatoid Arthritis
A number of studies have shown a correlative relationship between IL-10 and rheumatoid arthritis. Endogenous IL-10 produced in the joint by synovial macrophages and T cells, inhibited production of inflammatory cytokines by synovial cells, suggesting
that IL-10 may have a protective role in vivo (Katsikis et al. 1994). Ex vivo studies showed that IL-10 can effectively block the production of the proinflammatory cytokines TNF-α, IL-1, and IL-8 by synovial macrophages and synoviocytes. However, IL-10 has also been correlated with an increased autoantibody production, serum factor, and B cell activation in RA patients (Chomarat et al. 1995; Hart et al. 1995; Hart et al. 1996). The therapeutic potential of IL-10 in a collagen induced model of arthritis (CIA) in mice has been demonstrated by several groups. Elevated levels of IL-10 have been observed at the onset of CIA and a regulatory role for IL-10 was shown by increased disease activity after neutralization of IL-10 (Kasama et al. 1995). When administered to animals before and/or after induction of disease, IL-10 reduced joint swelling, infiltration, cytokine production, and cartilage degradation in animal models of arthritis. Feldmann and coworkers showed therapeutic effect of hIL-10 in the collagen induced mouse model which had a highly synergistic effect when applied together with anti-TNF-α antibody (Walmsley et al. 1996). In a different in vivo study IL-4 or low dose prednisolone in combination with IL-10 synergistically reduced joint inflammation in acute and chronic arthritis models (Joosten et al. 1997; Joosten et al. 1999).

Psoriasis
Psoriasis is a common cutaneous disorder characterized by inflammation and abnormal epidermal proliferation. T cells and cytokines are of major importance in the pathogenesis of the disease. According to the predominant expression of IL-2 and IFN-γ and the lack of IL-4 in skin lesions, psoriasis is believed to be characterized by a type 1 cytokine pattern (Uyemura et al. 1993; Schlaak et al. 1994). The level of IL-10 mRNA expression does not differ from healthy skin, even though numerous proinflammatory cytokines are overexpressed. This indicates a relative IL-10 deficiency in psoriasis (Asadullah et al. 1998). These results are supported by immunohistochemical findings of low cutaneous IL-10 protein expression and by quantification of IL-10 protein in blister fluids. Accordingly it has also been shown that T cells involved in psoriasis vulgaris belong to the Th1 subset (Schlaak et al. 1994).
Several antipsoriatic treatments, such as UV radiation (Enk et al. 1995), fumaric acid esters (Asadullah et al. 1997), and calcitriol, have been shown to increase IL-10 or IL-10 receptor expression (Asadullah et al. 1999).
Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is a chronic autoimmune connective tissue disease with no predilection to involve specific organs, characterized by polyclonal B cell activation, high levels of serum autoantibodies (anti-nuclear antibodies) and glomerular immune complex deposition. Both B cells and macrophages from SLE patients spontaneously produce high levels of IL-10 (Llorente et al. 1993), and several studies have shown a correlation between serum levels of IL-10 and disease activity (Houssiau et al. 1995; Llorente et al. 1997). Studies in mouse models of SLE showed that autoantibody production and immune complex pathology could be substantially inhibited by treatment with anti-IL-10 antibodies (Ishida et al. 1994; Llorente et al. 1995). A murine IgG1 anti-IL-10 mAb was given for 21 days at a dose of 20 mg/d to 6 SLE patients with active, steroid-dependent disease in an open label pilot study. Cutaneous lesions and joint symptoms improved and prednisone dose was decreased. Maximal benefit was evident at 2 months, with continued responses over 3-6 months (Llorente et al. 2000). Phase I trials are expected with a human anti-IL-10 mAb.

2.4.5 Tenovil (recombinant human IL-10)

Phase I Trials in healthy volunteers

IL-10 has been considered an attractive candidate for therapeutic use based on its potent in vitro immunomodulating activities and proven effects in several animal models of chronic inflammation. In phase I clinical trials, safety, tolerance, pharmacokinetics, pharmacodynamics, immunological, and hematological effects of single or multiple doses of IL-10 administered by intravenous (i.v.) or subcutaneous (s.c.) route have been investigated in various settings on healthy volunteers (Chernoff et al. 1995; Huhn et al. 1996; Huhn et al. 1997). Overall, these studies showed that IL-10 is well tolerated without serious side effects at doses up to 25 μg/kg; mild to moderate flu-like symptoms were observed in a fraction of recipients at doses up to 100 μg/kg. Production of neutralizing antibodies was not observed in any of the studies. Following a single i.v. or s.c. doses of IL-10 a transient neutrophilia, lymphocytopenia, monocytosis, and a delayed decrease in platelet counts was observed. There was also marked inhibition in the production of the pro-inflammatory cytokines IL-1b and TNF-α,
whereas levels of their respective antagonists, IL-1Ra and soluble TNFR p55, were unaffected (Chernoff et al. 1995).

Pharmacokinetic parameters of IL-10 were determined following i.v. or s.c. administration of doses ranging from 0.1 to 100 µg/kg. IL-10 has a terminal half life of two to three hours. Because hIL-10 is nonglycosylated, it is cleared mainly through the kidneys. Subcutaneous administration of IL-10 resulted in slow absorption and led to a prolonged but lower AUC with a terminal half life of 2.7 to 4.5 hours. This resulted in a extended immunosuppressive effect (Radwanski et al. 1998).

**Crohn’s disease**

Based on the successful experimental findings in animal models of intestinal inflammation, IL-10 therapy was introduced as a potential new anti-inflammatory therapy in Crohn’s disease (CD). Several large multicenter trials were performed, testing multiple IL-10 dosages in patients with mild/moderate or therapy refractory CD, as well as in patients undergoing curative ileal or ileocolonic resection to prevent endoscopic postoperative occurrence by systemic administration. Overall the data indicates that IL-10 therapy is safe and well tolerated. However, IL-10 treatment did not result in significantly higher remission rates or clinical improvement compared with placebo treatment (Fedorak et al. 2000; Schreiber et al. 2000; Colombel et al. 2001).

There are several explanations for the disappointment with this therapeutic strategy. It has been proposed that with the administered dose of IL-10 in the clinical trials, the ultimate local IL-10 concentration in the intestine would be too low to result in downregulation of inflammatory mediators. Increasing the dose of systemically administered IL-10 is limited due to side effects (for example, anemia, and headache). In patients treated with the highest dose of IL-10 (20µg/kg) a significant increase in neopterin, which is produced by human monocytes/macrophages in response to IFN-γ, as well as an increase in phytohemagglutinin-induced IFN-γ production, was observed. The upregulation of IFN-γ and neopterin could counterbalance the immunosuppressive properties of IL-10 and this could be a reason for the lack of efficacy of high doses of IL-10 in the treatment of Crohn’s disease (Tilg et al. 2002).
Rheumatoid Arthritis

Limited data regarding the effects of IL-10 in rheumatoid arthritis are available. A combination of IL-10 and methotrexate in a multicenter, placebo-controlled, dose escalating study in RA patients was described where 8 µg/kg daily or 8 µg/kg three times per week produced an ACR20 response in 50% of patients compared with 63% at a dose of 20 µg/kg three times per week and 10% with placebo. Similarly, ACR50 was achieved in 13% (8 µg/kg daily) and in 25 and 13% (8 and 20 µg/kg three times per week, respectively). A good safety profile was observed and 70% of the patients were treated for > 12 month. No anti-IL-10 antibodies were found (Weinblatt et al. 1999).

Psoriasis

Therapeutic effects of hIL-10 in psoriatic patients have been studied in seven clinical trials. Results are summarized in Table 2.2.

<table>
<thead>
<tr>
<th>Study</th>
<th>Design, patients, and interventions</th>
<th>Outcome and remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pilot study in exacerbated psoriasis vulgaris</td>
<td>Open label, not placebo controlled; three patients with moderate to severe psoriasis; 8 µg/kg/day, s.c. over a 24-day period</td>
<td>Safe, clinical, and histological confirmed improvement (Asadullah et al. 1998)</td>
</tr>
<tr>
<td>Pilot study in exacerbated psoriasis vulgaris</td>
<td>Open label, not placebo controlled, ten patients with moderate to severe psoriasis; 4 µg/kg/day s.c. over 42 days</td>
<td>Safe, good to moderate response, histological confirmed; significant mean decrease in PASI by 40% after 3 weeks and 68% after 6 weeks. These patients are a subgroup of the study by (Reich et al. 1998)</td>
</tr>
<tr>
<td>Phase II in exacerbated psoriasis vulgaris</td>
<td>Open label, not placebo controlled, ten patients with moderate to severe psoriasis; 4 µg/kg/day s.c. over 49 days</td>
<td>Safe, good to moderate response, histological confirmed, significant mean decrease in PASI by 55% after 7 weeks (Asadullah et al. 1999)</td>
</tr>
<tr>
<td>Phase II in exacerbated psoriasis vulgaris</td>
<td>Open label, not placebo-controlled, 15 patients with moderate to severe disease; 4 µg/kg/day, s.c. over 42 days</td>
<td>Safe, good to moderate response, histological confirmed; 14 patients evaluated, mean decrease in PASI by 50% after 4 weeks and 59% after 6 weeks (Reich et al. 2001)</td>
</tr>
<tr>
<td>Phase II in psoriatic arthritis</td>
<td>Placebo-controlled, double-blind, 29 patients with psoriatic arthritis, dose escalating with 1, 5, or 10 µg/kg, s.c. daily for 28 days</td>
<td>Well tolerated, significant clinical improvement in skin (&gt;30%) PASI reduction: 50% in IL-10 group (10 µg/kg) versus 10% in placebo after 4 weeks. No decrease in articular disease activity (McInnes et al. 2001)</td>
</tr>
<tr>
<td>Phase II in exacerbated psoriasis vulgaris</td>
<td>Randomized, double-blind, placebo-controlled, 28 patients with moderate to severe psoriasis; 20 µg/kg three times per week for 12 weeks</td>
<td>Modest trend towards improvement after 6 and 8 weeks (mean decrease in PASI by 31% and 33% in IL-10 group versus 7% and 13% in placebo group, respectively), but no significant difference at week 12 (17% versus 13% in placebo group) (Kimball et al. 2002)</td>
</tr>
<tr>
<td>Phase II in psoriasis vulgaris in remission</td>
<td>Placebo-controlled, double-blind, 17 patients with moderate to severe psoriasis in remission; 10 µg/kg, IL-10 (n = 7) or placebo (n = 10) three times per week for 4 months</td>
<td>Well tolerated; 90% relapse in the placebo group versus 28% in the IL-10 treated group; significant prolongation of relapse-free interval (101 days versus 66 in placebo) (Friedrich et al. 2002)</td>
</tr>
</tbody>
</table>

Table 2: Clinical trials in psoriasis. Adapted from Asadullah et al. 2003
Overall IL-10 therapy was well tolerated and immunologically effective in all studies in Psoriasis.

Chronic Hepatitis C and Human Immunodeficiency Virus (HIV)
A randomized, double-blind, placebo-controlled, multicenter trial was performed to determine the effect of multiple subcutaneous doses of rhIL-10 on plasma HIV RNA levels and CD4 T-cell counts. RhIL-10 was well tolerated but no significant change in plasma HIV RNA concentrations or CD4 T-cell counts could be observed in any of the groups (Angel et al. 2000).

There are several reports on an association between IL-10 polymorphisms and the course of hepatitis C infection. Moreover, IL-10 is able to express anti-fibrotic properties in experimental models of liver cirrhosis (Boyer et al. 2000). IL-10 normalized serum levels of alanine aminotransferase, a marker for hepatic inflammation, improved liver histology and reduced liver fibrosis in a large proportion of patients. However, IL-10 did not reduce serum HCV RNA levels, indicating that it did not affect viral load (Dharancy et al. 2000; Schuppan et al. 2000).
2.5 Endometriosis

2.5.1 Disease

Endometriosis is a common, benign, oestrogen dependent, chronic gynecological disorder. It is characterized by the presence of endometrial tissue outside the normal location, mainly on the pelvic peritoneum, but also on the ovaries and in the rectovaginal septum, and more rarely in the pericardium, pleura, and even the brain. It is estimated that 10 to 15% of women of reproductive age suffer from this disease. In women with abdominal pain, infertility, or both, the frequency is 35-50%. Common symptoms include dysmenorrhea, dyspareunia, abdominal pain and infertility. Due to variability in symptoms and signs time to diagnosis can be very long. Endometriosis is considered to be an estrogen-dependent disease because women are almost exclusively affected between menarche and menopause.

The most widely accepted theory on the pathogenesis of endometriosis is that the disorder originates from retrograde menstruation of endometrial tissue into the peritoneal cavity, followed by attachment of endometrial fragments to the epithelium of the peritoneum, establishment of a blood supply, and generation of suboptimum immune response that does not adequately clear the implants (Sampson 1927; Giudice et al. 2004). The retrograde menstruation theory is supported by the finding that women with endometriosis have higher volumes of refluxed menstrual blood and endometrial tissue fragments than women without the disorder. The immune system is believed to play an important role in the pathogenesis of endometriosis. A lack of adequate immune surveillance in the peritoneum is thought to be a cause of the disorder (Lebovic et al. 2001). A decreased NK activity and cytotoxicity against autologous endometrial cells in women with endometriosis has been observed (Oosterlynck et al. 1991). Furthermore, there is much evidence of activation of peritoneal macrophages with increased cytokine production in women with endometriosis, although there is decreased phagocytic activity (Koninckx et al. 1998).

High concentrations of cytokines like IL-6 and IL-8, growth factors, and angiogenic factors have been found in the peritoneal fluid (Gazvani et al. 2002). Once endometriotic lesions are formed, they secrete several proinflammatory molecules and
estrogens. Monocyte chemoattractant protein 1 (MCP-1), IL-8, and RANTES attract granulocytes, NK cells, and macrophages and autoregulatory positive feedback loops ensure further accumulation of these cells, cytokines, and chemokines in established lesions (Lebovic et al. 2001).

The role of the immune system in endometriosis pathophysiology has increasingly gained new attention in the last years. Immunological alterations in patients have been noticed for decades, but were regarded as secondary side effects and have not been further evaluated as potential cause of the disease or possible targets for therapeutic approaches.

Figure 2.8: Retrograde menstruation: Schematic diagram of events occurring in the pelvis with retrograde menstruation and the cellular and biochemical principles involved in the pathogenesis of endometriosis. Adapted from (Giudice et al. 2004).

The morphology of endometriosis lesions is categorized as red (red, red-pink and clear lesions), white (white, yellow-brown) and black (black and blue lesions) (ASRM 1997). The classification system of the American Society for Reproductive Medicine is based on a 20-point scale and includes four stages (ASRM 1997). The stage of the endometriosis
is based on the location, number of places the implants are found, the depth the implants penetrate into normal tissue and severity of the scar tissue (adhesions) around the implants.

2.5.2 Diagnosis and treatment

Due to unspecific symptoms of the disease, the only way to confirm and diagnose endometriosis is by laparoscopy or other types of surgery. Unfortunately, the disease is highly underdiagnosed and time to diagnosis often exceeds 6 to 8 years. A non-invasive diagnostic method is urgently needed to decrease time to diagnosis and is a current challenge in endometriosis research.

Treatment of endometriosis has two aims: relief of pain and amelioration of infertility. Surgery via laparoscopy or laparotomy removes lesions via excision; however, this procedure is far from ideal due to implied risks associated with all surgical interventions. Furthermore, 20% of patients which underwent laparoscopic surgery did not report an improvement of pain (Abbott et al. 2004) and the symptoms recur in up to 75% of women within 2 years.

The hormonal dependence of endometrial implants has provided the basis for medical management of endometriosis. Agents to suppress ovarian function and lower circulating oestradiol concentrations include androgens, progestagens, GnRH agonists, and contraceptive steroids. The first drug approved for endometriosis treatment by the FDA was danazol. This agent has androgenic agonist effects and inhibits ovarian steroidogenesis resulting in decreased secretion of estradiol (Lessey 2000). Several studies have demonstrated beneficial effects of danazol on the extent of endometriosis and symptoms of pelvic pain, but few data support its use for the treatment of infertility. Furthermore, the use of danazol is limited by its masculinizing side-effects. Its role as a treatment for endometriosis has been largely replaced by the GnRH agonists (Buserelin, Goserelin, Leuprorelin, Leuprolide Nafarelin, and Triptorelin). GnRH agonists bind to the GnRH receptors and result in stimulation of gonadotropin release. After this initial increase in gonadotropin release, cessation follows due to the effect that GnRH agonists have a remarkably longer half life than natural GnRHs. This results in a downregulation of GnRH receptors, which effectively desensitizes the pituitary and results in decreased gonadotropin secretion (Conn et al. 1987). Consequently, secretion
of estrogen is blocked, resulting in postmenopausal estrogen levels and pain relief is achieved in most cases. Side effects of this treatment are triggered by the hypoestrogenic status and range from dizziness, vaginal dryness and increased risk of thrombosis. Most importantly, prolonged hypoestrogenism results in loss of bone density with increased risk of fractures. Thus, to avoid these long-term effects, treatment duration with GnRH agonists is usually restricted to a time period of <6 months, which has proven to lead to regression of macroscopic lesions and pain relief in the vast majority of patients (Griesinger et al. 2005). However, symptoms usually recur within 6-12 month.

The oral contraceptive pill, leading to a pregnancy-pretending hormonal state, has been used to treat symptoms of endometriosis since the 1960s. The pill inhibits ovulation and decreases mean gonadotropin levels and menstrual flow. The advantage of an oral contraceptive over other hormonal treatments is that it can be taken indefinitely and fewer side effects are observed. However, like other anti-hormonal treatments, it eliminates the possibility for pregnancy in affected women desiring children. Combined oral contraceptives are widely used as initial therapy, often together with analgesic non-steroidal anti-inflammatory drugs (NSAIDs) (Vercellini et al. 1993). Several progestins have been used for the treatment of endometriosis. They induce a pseudopregnancy state and have an anti-oestrogenic effect. Medoxyprogesterone acetate (MPA) has been the most studied. A meta-analysis showed that progestin have a comparable efficacy in relieving pain as danazol and GnRH agonists. Side effects include weight gain, acne, depression and breakthrough bleeding (Vercellini et al. 1997). New agents to treat endometriosis are under investigation, which either target the hormonal metabolism (e.g. SERM, aromatase inhibitors) or try to modulate the immune system (e.g. COX-2 inhibitors, TNF-α blocker) or to influence angiogenic processes (e.g. VEGF inhibitors) (Vercellini et al. 2008).

2.5.3 Imaging of endometriosis

Use of imaging tests may identify endometriotic cysts or larger endometriotic areas. The two most common imaging tests are ultrasound and magnetic resonance imaging MRI. However, normal results on theses tests do not eliminate the possibility of endometriosis. Areas of endometriosis are often too small to be seen by these tests.
There are very few cases known where endometriosis lesions were detected by chance in FDG-PET scans (Figure 2.8) (Jeffry et al. 2004; Derman et al. 2007). However, most endometriotic lesions do not give positive results on FDG-PET.

**Figure 2.8: Imaging of Endometriosis**: Whole body F-FDG PET-CT scan reveals a 5.5 cm cavitory mass in the left lower lobe with a corresponding area of intense FDG radiotracer uptake. The histological diagnosis was endometriosis (Derman et al. 2007).

### 2.5.4 Angiogenesis in endometriosis

Normal tissue growth such as in embryonic development, wound healing, ovarian function, and the menstrual cycle, depends on new vessel formation for the supply of oxygen and nutrients as well as the removal of waste products. The analogy of the establishment of an endometriotic lesion and a metastasizing tumor cell is remarkable. Endometrial tissue adheres to the peritoneal surface, invades the extracellular matrix and initiates angiogenesis in order to generate new blood vessels needed to survive. However, tumor metastases are usually single cells or small clumps of cells, which do not require an immediate angiogenic response as the cell masses have not yet reached the critical diameter of 1-2 mm. The requirement for oxygen and nutrients for the implanting endometrial tissue is more acute. Only tissue fragments large enough (> 1mm) to preserve the original tissue architecture were shown to have invasive
properties (Taylor et al. 2002; Nap et al. 2003; Laschke et al. 2007). It has been shown that angiogenic cytokines (e.g. VEGF-A, IL-6, IL-8) are elevated in the peritoneal fluid, serum and endometriotic tissue of women suffering from the disease.

2.5.5 Mouse models of endometriosis

The only species developing spontaneous endometriosis are several non-human primates, but ethical considerations concerning primate experiments in general as well as high costs make this model impractical for systematic evaluation in endometriosis research. A frequently used animal model for endometriosis is the mouse model, whether as heterologous or syngeneic approach. Unlike non-human primates, rodents do not menstruate and, therefore, do not spontaneously develop endometriosis. Heterologous mouse models all include transplantation of injection of non-mouse material in immunodeficient mice. Human endometrium from women with or without endometriosis or fragments from endometriotic lesions are utilized in these models, whether intraperitoneally or subcutaneously (Zamah et al. 1984; Grummer et al. 2001). The first autologous mouse model was described in 1995 by Cummings and Metcalf (1995). In this model endometrial fragments of donor mice are injected into the peritoneal cavity of recipient, syngeneic mice. These ectopic endometrial fragments show histological characteristics of the human disease, including the formation of multiple highly vascularized lesions containing endometrial glands, stroma and cysts, independent of their peritoneal localization in the abdomen. Corresponding to the situation in humans, the growth of the ectopic endometrial tissue is estrogen dependent. As endometrial fragments from syngeneic mice are implanted, immunocompetent mice can be used and this mouse model is better suited to study the effect of immune-modulating drugs on endometriosis than the heterologous mouse model (Cummings et al. 1995; Somigliana et al. 1999).

2.5.6 The role of IL-10 in endometriosis

The immune system is believed to be involved in the pathogenesis of endometriosis, and a lack of adequate immune surveillance in the peritoneum is thought to be a cause
of the disorder (Lebovic et al. 2001). It has been reported that proinflammatory cytokines, such as IL-6, IL-8 and TNF-α are elevated in the peritoneal fluid of women with endometriosis (Harada et al. 1997; Harada et al. 2001).

The role of IL-10 in the pathogenesis remains unclear. The main function of IL-10 is to limit and terminate inflammatory responses. In women with endometriosis, Th2 helper cells from peritoneal fluid are reported to aberrantly suppress cell-mediated immunity by up-regulating IL-10 secretions in the peritoneal fluid. On the other side it has been shown that patients with endometriosis that need surgery are more likely to carry a low expressing IL-10 genotype, which results in inadequate IL-10 levels to terminate inflammatory reaction (Juo et al. 2008). In general IL-10 is known as an anti-inflammatory cytokine that inhibits the activation of neutrophils and macrophages, sources of IL-6 and TNF-α synthesis. Recently, it has also been shown that IL-10 suppresses TNF-α induced IL-6 expression levels in endometriotic stromal cells (Tagashira et al. 2009). Certainly IL-10 plays a significant role in regulating the inflammatory environment in patients with endometriosis and may also provide a therapeutic modality for endometriosis.

2.6 The aim of this thesis

The therapeutic potential of recombinant cytokines is often limited by severe toxicities, even at low doses, thus preventing dose escalation and the establishment of a sufficient concentration at target tissues. It is becoming increasingly clear that monoclonal antibodies could be used to deliver cytokines at sites of disease, thus increasing their potency and sparing normal tissues. This pharmacodelivery strategy has been mainly investigated for cancer therapy applications, leading to the preclinical (Gillies et al. 1998; Halin et al. 2002; Gafner et al. 2006; Huang et al. 2007; Schliemann et al. 2008) and clinical (King et al. 2004; Schrama et al. 2006) investigation of several antibody-cytokine fusion proteins. For example, our group has brought to Phase I and Phase II clinical trials immunocytokines based on human IL2 (Carnemolla et al. 2002; Borsi et al. 2003; Menrad et al. 2005; Marlind et al. 2008) and on human TNF (Borsi et al. 2003; Halin et al. 2003; Balza et al. 2006).
Recently, we have observed that antibody-based pharmacodelivery strategies can also be used in the non-oncological setting (Birchler et al. 1999; Trachsel et al. 2007), for example aiming at the targeted delivery of anti-inflammatory cytokines at sites of inflammation. We have reported that the L19 antibody, specific to the alternatively spliced extra-domain B (EDB) of fibronectin (Zardi et al. 1987; Pini et al. 1998), could be fused to human IL10, thus generating an immunocytokine capable of preferential accumulation at neovascular sites of cancer and arthritis and capable of inhibiting the progression of established collagen-induced arthritis (CIA) in the mouse (Trachsel et al. 2007).

The aim of this thesis was to further investigate the expression of alternatively spliced components of the extracellular matrix in chronic inflammatory disorders and to explore the use of immunocytokines in disease models of chronic inflammation.
3. Results

3.1 Immunohistochemical analysis of rheumatoid synovial tissue specimens

Figure 3.1 presents a comparative immunohistochemical and immunofluorescence analysis of the human monoclonal antibodies L19, G11, F16 and F8. In total, pathology specimens of 7 patients were analyzed, four of which are shown in Figure 3.1. Both F16 and F8 displayed a stronger staining pattern compared to L19 and G11. The F8 antibody exhibited sometimes a diffuse stromal staining or a vascular staining pattern, but consistently strongly reacted both with human and with murine specimens of arthritis and was thus selected for pharmacodelivery applications. Furthermore, F8 and F16 exhibited a prominent perivascular staining pattern in tissue specimens from patients suffering from psoriatic arthritis and osteoarthritis. In tumor-bearing mice, the in vivo targeting potential of F8 and L19 was comparable when assessed by quantitative biodistribution studies (Villa et al. 2008).
Figure 3.1: Immunohistochemical analysis of rheumatoid arthritis specimens, psoriatic arthritis specimens and osteoarthritis specimens. Immunohistochemistry with the small immunoproteins L19, G11, F16, and F8 was performed in different pathology specimens obtained from biopsies of patients with rheumatoid arthritis, psoriatic arthritis or osteoarthritis. In total, pathology specimens of 7 patients were analyzed, 4 of them are shown above. Furthermore immunofluorescence double staining with L19, G11, F16 and F8 (red) and von Willebrand factor (green) was performed on rheumatoid synovial tissue specimens of one patient (rheumatoid arthritis (1)). Overall F8 exhibited the strongest staining of all tested antibodies. It showed a diffuse stromal staining in certain areas and a vascular staining pattern in others. For negative controls, the primary antibody was omitted. Scale bars, 100 µm.
3.2 Cloning and *in vitro* characterization of F8-IL10

The immunocytokine F8-IL10 was cloned in a mammalian expression vector by sequentially fusing the F8 in scFv format (Huston et al. 1988; Villa et al. 2008) in frame with the human IL-10 gene, using flexible aminoacid linkers (Figure 3.2). The resulting plasmid pKS1 was used to stably transfect CHO-S cells. A short 5-aminoacid linker was used to bridge \( V_H \) and \( V_L \) domains within the scFv antibody fragment moiety, thus driving the formation of a stable non-covalent homodimer (Figure 3.3 a,b)) (Holliger et al. 1993).

![Figure 3.2: Cloning F8-IL10. Schematic representation of a pcDNA3.1 vector containing the essential elements of the F8-IL10 fusion protein.](image)

F8-IL10 could be purified to homogeneity on protein A (Figure 3.3 a,b)), retained full immunoreactivity when tested by affinity chromatography on an EDA-Sepharose resin (data not shown) and displayed a biological activity comparable to the one of recombinant human IL-10 used in equimolar amounts in a MC/9 cell proliferation assay (Figure 3.3 c)) (Thompson-Snipes et al. 1991)). In a crossreactivity study on tissue microarray none of the healthy tissue sections showed any staining with F8-IL10, except for ovary (1/3), placenta (3/3) and uterus (2/3) (data not shown). This finding is excellent agreement with the known expression of oncofetal antigens in organs of the female reproductive system (Kaspar et al. 2006).
Figure 3.3: Characterization of F8-IL10. a) SDS-PAGE analysis of purified fusion proteins: lane 1, molecular-weight marker; lanes 2 and 3, F8-IL10 under nonreducing and reducing conditions, respectively. b) Gel-filtration analysis of affinity-purified F8-IL10. The peak eluting at a retention volume of 12 ml corresponds to the noncovalent homodimeric form of F8-IL10. c) MC/9 cell proliferation assay. F8-IL10 displayed biological activity comparable with the one of recombinant human IL-10 used as a standard in the assay.

3.3 F8-IL10 selectively targets arthritic lesions and tumors in mice

The in vivo targeting properties of the F8 antibody and of F8-IL10 were tested in CIA mice, using both fluorescently labeled and radioiodinated protein preparations. Figure 3.5 a) shows near-infrared fluorescence images (Neri et al. 1997; Birchler et al. 1999) of arthritic mice 24 hours after intravenous injection of 100 µg SIP(F8) (Neri et al. 1995; Villa et al. 2008) labeled with Alexa750 dye. A preferential accumulation of the F8 antibody could be detected in the inflamed extremities. A more detailed targeting analysis was obtained using 125I-labeled preparations of SIP(F8) and of F8-IL10. Twenty-four hours after intravenous or subcutaneous administration, arthritic limbs were imaged on a PhosphorImager, revealing a preferential protein accumulation at arthritic fingers and paws compared to healthy control paws (Figure 3.5 b, c)). The ranges of
lesion to nonaffected paw ratios measured by phosphorimaging were 7.4–13.9 for SIP(F8) i.v. and 5.0–6.8 for F8-IL10 s.c.. The administration of comparable amounts of antibodies of irrelevant specificity in the mouse in recombinant SIP format did not exhibit any preferential uptake at sites of inflammation (Trachsel et al. 2006).
Figure 3.5: *In vivo* targeting of the small immunoprotein F8 and the fusion protein F8-IL10 in arthritic mice. (a) Near infrared fluorescence imaging. Arthritic mice (*n* = 3) were injected with SIP(F8)-Alexa750 (Molecular Probes, Leiden, The Netherlands). Near infrared fluorescence imaging analysis was performed 24 hours after injection. Arrows indicate grade 2 swelling in the front paws of the mice. (b-c) Phosphorimaging. Arthritic mice (*n* = 2) were injected intravenously with 125I-labelled SIP(F8) (b) or subcutaneously with 125I-labelled F8-IL10 (c). Uptake of radio-iodinated antibodies was analyzed by phosphorimaging 24 hours after injection. The ranges of lesion to nonaffected paw ratios measured by phosphorimaging were 7.4-13.9 for SIP(F8) i.v. and 5.0-6.8 for F8-IL10 s.c..

The subcutaneous administration of therapeutic proteins in patients with arthritis is often preferable compared to the intravenous administration route, which is typically performed at the hospital. In order to investigate whether a selective *in vivo* targeting of lesions could be obtained using F8-IL10 both with s.c. and i.v. administrations, we performed a comparative biodistribution study in tumor-bearing mice. We chose a cancer model rather than an arthritis model for this analysis, since tumor-bearing mice provide a quantitative biodistribution analysis of therapeutic proteins. Figure 3.6 b) illustrates biodistribution results (expressed as % injected dose per gram of tissue) for a radiiodinated preparation of F8-IL10, administered intravenously or subcutaneously. For both administration routes, a preferential tumor uptake could be observed, with excellent tumor:organ ratios at 24h and 48h following injection. An antibody-IL10 fusion protein of irrelevant specificity in the mouse (Smith-Gill et al. 1984; Neri et al. 1995; Halin et al. 2002) exhibited a reduced tumor uptake in the same animal model (Figure 3.6 a)). In order to quantitatively assess the residence time of F8-IL10 on neovascular lesions following subcutaneous administration, a biodistribution study was performed sacrificing tumor-bearing mice at 24, 48, 72 and 96 hours and correcting
for the tumor volume increase during the study period. Figure 3.6 c) shows that the
immunocytokine efficiently and stably localized at the tumor site, while being cleared
from all normal organs. No statistically significant difference could be observed in
terms of tumor uptake between the 48 and 96 hour time points.

**Figure 3.6: Biodistribution study in F9 tumor-bearing mice.** In all biodistribution experiments \( n = 4 \) mice
were analyzed per group. Radioactivity content of tumor or organs is expressed as percentage of the
injected dose per gram of tissue (%ID/g) ± standard error. (a) Comparison of intravenous and
subcutaneous injection. Tumor bearing mice were injected intravenously or subcutaneously with \( ^{125}\text{I} \)-
labelled F8-IL10 and sacrificed 24h or 48h after injection. (b) Comparison of targeted and untargeted IL10.
Mice were injected intravenously with \( ^{125}\text{I} \)-labelled F8-IL10 or \( ^{125}\text{I} \)-labelled HyHel10-IL10 (HyHel10 is an
antibody specific to hen egg lysozyme and is not recognizing any murine antigen). They were sacrificed
24h after injection. (c) Residence time of F8-IL10 following subcutaneous administration. Mice were
injected with \( ^{125}\text{I} \)-labelled F8-IL10 and sacrificed 24h, 48h, 72h, or 96h after injection.
### 3.4 Inhibition of arthritis progression in the collagen-induced model of arthritis

The CIA model was used to assess the therapeutic potential of F8-IL10 when used alone or in combination with methotrexate. Mice were allowed to reach an arthritic score of 1 to 3, before receiving three injections (day 1, 4, 7) of F8-IL10 (200 µg) and/or of methotrexate (100 µg). The F8-IL10 dose for the mouse was calculated from the recommended equivalent dose of 20 µg/kg of recombinant human IL-10 used in clinical trials using a body surface correction algorithm (Reagan-Shaw et al. 2008) and a correction factor for the activity of human IL-10 in mice (Rosenblum et al. 2002).

Figure 3.7 a) shows that mice treated with methotrexate did not exhibit any detectable reduction of arthritis, in line with previously published results where comparable doses of methotrexate in the same mouse model had no significant effect on the onset of CIA (Wunder et al. 2003). Disease progression was substantially inhibited for F8-IL10 with i.v. administration and with s.c. administration. Both s.c. injections of F8-IL10 and the combination treatment of methotrexate + i.v. F8-IL10 allowed to maintain arthritic score below 3 until they were sacrificed (18 days after the beginning of pharmacological treatment). Similar to what previously reported (Trachsel et al. 2007), the therapeutic performance of an antibody-IL10 fusion protein of irrelevant specificity in the mouse exhibited a worse therapeutic benefit, confirming the contribution of selective targeting to therapeutic outcome (Figure 3.7 b)). We were not allowed by the local authorities (Veterinäramt des Kantons Zürich) to extend the duration of the observation period for the mice in order to keep animal discomfort within an acceptable limit, but it would have obviously been of scientific interest to monitor disease stabilization over a longer period of time.

In our mouse model of CIA, the therapeutic activity of F8-IL10 was found to be comparable to the one of a recombinant biopharmaceutical based on the extracellular part of murine TNF receptor 2, administered with the same schedule (Figure 3.7b)).
Figure 3.7: Therapy studies of F8-IL10 in the CIA mouse model. a) Combination with methotrexate. Arthritic mice were given injections with saline (black squares), methotrexate 100 µg i.p. (open circles), F8-IL10 200 µg s.c. (black triangles), F8-IL10 200 µg i.v. (black circles), or a combination of F8-IL10 200 µg i.v. and methotrexate 100 µg i.p (crosses). Injections were started at day 1 after arthritis onset and then repeated every third day for three injections per animal, as indicated by the arrows. The arthritic score was evaluated daily and expressed as the mean ± SEM of 8 mice per group. * = P < 0.05 versus saline; ** = P < 0.05 versus F8-IL10 i.v.. b) Comparison of targeted versus systemic application of IL-10. Arthritic mice were injected subcutaneously with saline (black squares), HyHel10-IL10 200 µg (open circles), F8-TNFRII (crosses), or F8-IL10 200 µg (black circles) every third day for three injections, as indicated by arrows. Arthritic score is expressed as the mean ± SEM of 6-7 mice per group. * = P < 0.05 versus saline.

Paws and blood of mice was analyzed at the end of the therapy and we could demonstrate by immunohistochemistry that F8-IL10 is still detectable in arthritic paws (Figure 3.8a). Analysis of plasma cytokines of sacrificed mice showed significantly (P = 0.004) decreased IL6 levels for F8-IL10 treated mice (Figure 3.8 b)). Furthermore, saline treated mice showed elevated IL-1β levels compared to healthy control and F8-IL10 treated mice. No statistically significant reduction was observed for IL-2 or TNF-α (data not shown).
Figure 3.8: Therapy studies of F8-IL10 in the CIA mouse model. a) *Ex vivo* immunohistochemical detection of F8-IL10 and HyHel10-IL10 in arthritis paws. Analysis of the arthritis paws at the end of therapy (day 12 for F8-IL10 and day 10 for HyHel10-IL10) showed that F8-IL10 is still detectable by immunohistochemistry using an anti-human-IL-10-antibody. b) Analysis of plasma cytokines levels at the end of therapy. F8-IL10 treated mice showed significantly decreased IL6 levels compared with the saline group. Furthermore, IL1b serum levels of F8-IL10 treated mice were below the lower limit of detection (LLD). * = P < 0.05 versus saline.

3.5 Evaluation of cellular and humoral immunity

Figure 3.9 shows a comparative immunofluorescence analysis of infiltrating cells from mice treated with saline or F8-IL10. Staining with an anti-CD45 antibody revealed that F8-IL10 treated mice presented a significantly (P = 0.03) lower level of infiltrating leukocytes in the paw compared with the saline treatment group. In accordance with this finding, staining with an anti-asialo-GM1 antibody, which preferentially stains natural killer cells, with the macrophage-specific antibody F4/80 and with CD4/CD8 antibodies, showed a decreased infiltration of these cells in paws of F8-IL10 treated mice.
Figure 3.9: Immunofluorescence analysis of infiltrating cells: At termination of the therapy experiment a comparative immunofluorescence analysis of infiltrating cells from mice treated with saline or F8-IL10 was performed. a) Representative immunofluorescence images of paw sections. Scale bars, 100 µm. b) Sections were evaluated for area percentage positive staining and a significant decrease of infiltrating leukocytes was observed. * = P < 0.05 versus saline.

Humoral immunity was followed by measurement of serum levels of anti-collagen II immunoglobulin (Ig) isotypes. Serum samples were obtained from both control and F8-IL10 treated animals at the termination of the experiment and total IgG antibody levels, as well as IgG1 and IgG2α isotype levels were determined by ELISA (Figure 3.10). Total IgG levels were significantly lower in F8-IL10 treated animals than in controls (P < 0.05). When analyzing specific isotypes, no significant differences were seen in the anti-collagen II IgG1 levels between the two groups. However, the anti-collagen II IgG2α titers were significantly lower (P < 0.05) in sera from F8-IL10 treated mice, as seen for other anti-arthritis therapeutic interventions in the CIA model (Khoury et al. 2008).
Figure 3.10: Anti type-II collagen antibodies. Titers of bovine type II collagen-specific total IgG, IgG1 and IgG2a antibodies were determined by ELISA. A clear reduction of total IgG and IgG2a, but not IgG1, antibody levels was observed in F8-IL10 treated mice. * = P < 0.05 versus saline.

3.6 Safety pharmacology profile of F8-IL10

In preparation for a dose-finding, pharmacokinetic Phase I study of F8-IL10 in combination with methotrexate in patients with active rheumatoid arthritis we performed a toxicity assessment of F8-IL10 in combination with methotrexate in cynomolgus monkeys. In this study, three groups of monkeys (each group consisting of 3 F and 3 M animals) received administrations of either F8-IL10 alone, F8-IL10 + MTX or saline. During the study F8-IL10 was injected subcutaneously three times a week for 8 weeks at a dosage of 180 µg/kg (60 µg/kg IL-10 equivalents), which reflects 10x the initial human dose intended for administration during the Phase I clinical study. Methotrexate was given on a weekly basis at the standard dosage of 0.65 mg/kg. There were no relevant findings in body weight evolution, food consumption, quantitative electrocardiography parameters or systolic and diastolic blood pressure values. No relevant ophthalmological findings were noted in any groups. A ventricular premature complex was recorded in one female treated with F8-IL10 alone in week 4, after treatment. During the course of the study (week 4), a regenerative anemia was observed, however complete recovery was noted in week 7. No toxicologically relevant findings were observed in the blood biochemical parameters at the end of week 4 and end of the treatment period in any groups. Pharmacokinetic data were obtained during the toxicology study. Blood samples were collected at pre-dose, 5 and 30 min, and 3 and 24 hours after the injection. The serum concentration of F8-IL10 was measured using a validated colorimetric ELISA. Many of the samples analyzed were found to be below the
level of quantification (< 0.25 ng/ml). However, for those samples in which a positive result was obtained, maximum serum levels were generally observed 3 hours after the subcutaneous injection of F8-IL10 with serum levels of about 20 ng/ml. After 24 hours no more detection of F8-IL10 in serum was possible. In conclusion, subcutaneous administration of F8-IL10 alone or in combination with methotrexate was generally well tolerated.

The acute toxicity of F8-IL10 was investigated in mice after intravenous administration of a single dose level of 20 mg/kg, corresponding to 300 times the human starting dose proposed for clinical trials (Reagan-Shaw et al. 2008), followed by a 14 day observation period. Body weights were recorded weekly and necropsy was performed on all animals. No mortality occurred and no clinical signs were noted in both male and female animals. Changes in body weight observed at the end of the study were within the expected range for this strain and age of animals. No changes of toxicological significance were observed in the weight of organs. No abnormalities were detected in all treated animals at the necropsy examination and no abnormalities were observed at the injection site. These results indicate that F8-IL10 had no toxic effect on mice following a single intravenous administration at a dose level of 20 mg/kg body weight. The product was well locally tolerated when injected into the tail vein at the dose level tested.

3.7 Evaluation of antigen expression in different chronic inflammatory diseases

In order to evaluate the expression of EDA, EDB and TnC A1 in other chronic inflammatory disorders, such as psoriasis, multiple sclerosis, ulcerative colitis and endometriosis, an immunohistochemical analysis on human disease specimens was performed (Figure 3.11). Endometriosis tissue samples showed the nicest and strongest staining of all tested samples. A very strong vascular expression of EDA and TnC A1 in immunohistochemistry and immunofluorescence (Figure 3.11 b)) was observed. These promising results suggest that targeting of endometriosis using F8 or F16 should be possible and encouraged us to set up a mouse model of endometriosis.
Figure 3.11: Immunohistochemical analysis of different disease samples. a) Comparison of the expression of EDA, EDB and TnC A1 in various inflammatory diseases. The nicest and strongest staining was observed in endometriosis tissue samples. b) Immunofluorescence double staining with F8, L19 and F16 (red) and von Willebrand Factor (green) on endometriosis specimen.
3.8 Mouse model of endometriosis

To investigate potential targeting applications of the F8 antibody, a syngeneic mouse model of endometriosis was established. Endometrial tissue of donor mice was injected i.p. in estrogen-treated recipient mice. 21 days after implantation of tissue, mice were sacrificed and endometriosis fragments were analyzed. 1-2 lesions were found per animal. Most of the lesions were embedded in the fatty tissue around the uterine horns or attached to the peritoneum. Macroscopically, lesions consisted of white to light yellow nodules that were 2-5 mm in diameter. Hematoxylin staining revealed the typical glandular and cystic structures of endometriosis (Figure 3.12 a)). To investigate the expression of EDA immunohistochemistry was performed (Figure 3.12 b)). F8 showed a nice vascular and stromal staining pattern.

![Figure 3.12: Histological and immunohistochemical examination](image)

Expression of EDA in the syngeneic mouse model of endometriosis was proven by immunohistochemistry.

3.9 Targeting of F8 in the syngeneic mouse model of endometriosis

The in vivo targeting performance of SIP(F8) was assessed by i.v. injection of SIP(F8) labeled with Alexa750 dye. 24h after injection mice were sacrificed and imaged using an infrared fluorescence imager (Birchler et al. 1999). A strong and selective antibody accumulation in the endometriosis lesions was observed (Figure 3.13 a-b)). By contrast, mice injected with SIP(F16), which is not crossreactive on mouse tissue and which was
used as negative control, did not show any uptake in endometriosis lesions (Figure 3.13 c)). An ex vivo fluorescence detection of SIP(F8) using an anti-human IgE antibody showed a nice fluorescent staining pattern around vascular structures (Figure 3.13 d)).

3.10 Preliminary therapy experiment using the two immunocytokines F8-IL10 and F8-IL2

In the last years, the potential role of the immune system in endometriosis pathophysiology has increasingly gained new attention and a pivotal role of the immune system in the pathogenesis of endometriosis has been suggested. However, it is not clear whether changes in inflammatory mediators are a cause or a consequence of the disease and anti-inflammatory (anti-TNF-α) (D'Antonio et al. 2000) as well as immunostimulatory (e.g. IL-12) (Somigliana et al. 1999) drugs have proven efficacy in
mouse models of endometriosis. Therefore we decided to test the anti-inflammatory cytokine F8-IL10 and the immunostimulatory cytokine F8-IL2 in a preliminary therapy experiment. The fusion protein F8-IL2 was cloned in analogy to F8-IL10 (data not shown). Treatment was started one day after transplantation of endometriosis tissue and mice received three injections (day 1, 4 and 7) of F8-IL10 (200 µg), F8-IL2 (20 µg) or saline. Mice were sacrificed 5 days after the last injection. In the saline control group 3 out of 5 mice developed endometriosis, whereas no endometriosis lesions were found in the F8-IL2 group and 1 out of 5 mice developed endometriosis in the F8-IL10 group. Even though these results are not statistically significant, they do strongly suggest that the immunoconjugates F8-IL10 and F8-IL2 have the potential to impact on the development of endometriosis \textit{in vivo}. It will be valuable to obtain statistically more meaningful data in further experiments with more animals.
4 Discussion

In this thesis, we have compared four human monoclonal antibodies specific to alternatively-spliced components of the extracellular matrix and have identified F8 as a suitable candidate for pharmacodelivery applications in rheumatoid arthritis and endometriosis.

In analogy to our previous work in this area (Trachsel et al. 2007), we fused F8 to human IL-10, generating the immunocytokine F8-IL10, which was shown to preferentially localize at sites of arthritis in the collagen induced murine model of the disease. F8-IL10 was able to stabilize clinical features of arthritis in this animal model and was found to be well tolerated in monkeys at human equivalent doses of 20 µg/kg (Reagan-Shaw et al. 2008). Preclinical studies were facilitated by the fact that F8 binds with comparable affinity to EDA of murine, monkey and human origin (Villa et al. 2008).

The rationale behind the development of F8-IL10 as a novel biopharmaceutical relies on the promising, yet not sufficiently satisfactory, preclinical and clinical data reported for recombinant human IL-10 (Tenovil TM). In controlled clinical trials in patients with rheumatoid arthritis, Tenovil exhibited ACR20 values substantially higher than the ones in control groups and comparable to the ACR20 values reported for TNF blockers. However, the ACR50 values observed with Tenovil, while significantly better compared to the ones observed in patients treated only with methotrexate, were not as good as those reported for Humira, Remicade and Enbrel (Maini et al. 1997; Weinblatt et al. 1999; Rau 2002).

In spite of these observations, we and others have extensively demonstrated in animal models that the antibody-based delivery of cytokines to sites of disease can substantially improve the therapeutic index of these biopharmaceuticals (Carnemolla et al. 2002; Borsi et al. 2003; Marling et al. 2008; Schliemann et al. 2008)). To our knowledge, F8-IL10 will be the first anti-inflammatory immunocytokine to be tested in the clinical setting and it will be interesting to learn whether the improved performance and selectivity documented in the mouse model of arthritis holds true for patients with rheumatoid disease. Encouraged by the excellent tolerability profile observed in cynomolgus monkeys, we have submitted a request for clinical trials in
Italy.

When developing F8-IL10 for industrial pharmaceutical programs, care was devoted to identifying a suitable formulation which could be compatible with subcutaneous administration. Using radioiodinated protein preparations, we studied the biodistribution properties of F8-IL10 both in mouse models of arthritis and in tumor-bearing mice, where targeting performance can be expressed as percent injected dose per gram. The conventional intravenous administration route yielded tumor targeting results comparable to the ones obtained following a subcutaneous administration, thus providing a robust rationale for the development of clinical trials featuring subcutaneous injections. Experience gained with TNF blocking antibodies suggests that subcutaneous administration may be better accepted by patients and may lead to a better compliance, reducing the need to visit hospital sites for each administration. The data presented provide a strong rationale for the clinical investigation of F8-IL10 as a novel biopharmaceutical for the therapy of patients with rheumatoid arthritis who have failed at least two lines of biological therapy. Clinical studies will reveal whether the promising preclinical results can be translated to the clinical setting and, potentially, whether F8-IL10 could find a broader clinical applicability as a targeted anti-inflammatory agent for diseases which over-express the EDA domain of fibronectin.

Endometriosis is a highly underdiagnosed disease and time to diagnosis often exceeds 6 to 8 years. A non-invasive diagnostic method is urgently needed to decrease time to diagnosis and is a current challenge in endometriosis research. Furthermore, the current medical and surgical treatment of endometriosis is still associated with a high recurrence rate. In this thesis we could show a very strong vascular expression of EDA and TnC A1 by immunohistochemistry and immunofluorescence in human endometriosis lesions. Furthermore, we have demonstrated that F8 is able to selectively localize at endometriosis lesions in the syngeneic mouse model of endometriosis. The promising results suggest a potential role of F8 for diagnosis or coupled to a bioactive moiety for treatment of endometriosis. The role of the immune system in the pathogenesis of endometriosis remains unclear and anti-inflammatory as well as immunostimulatory drugs have shown efficacy in animal models of endometriosis. We tested the two immunocytokines F8-IL10 and F8-IL2 in a preliminar therapy experiment and the results of this study let assume that F8-IL10 and F8-IL2 have the potential to
impact on the development of endometriosis. However, further experiments with larger groups would be needed to obtain statistically significant results.
5 Materials and Methods

5.1 Immunohistochemical analysis of rheumatoid synovial tissue specimens

For immunohistochemistry on synovial tissue samples, 10 μm cryostat sections were fixed in ice-cold aceton and stained for FN-EDA, FN-EDB, TnC-A1. Primary antibodies in small immunoprotein (SIP) format were added onto the sections in a final concentration of 2 µg/ml and detected with rabbit anti-human IgE antibody (Dako, Glostrup, Denmark) followed by biotinylated goat anti-rabbit IgG antibody (Biospa, Milan, Italy) and streptavidin-alkaline phosphatase (SAP) complex (Biospa, Milan, Italy). Fast Red TRSalt (Sigma-Aldrich, St Louis, MO) was used as the phosphatase substrate. Sections were counterstained with hematoxylin, mounted with Glycergel mounting medium (Dako) and analyzed with an Axiovert S100 TV microscope (Zeiss, Feldbach, Switzerland). In total, freshly frozen pathology specimens of 7 patients were analyzed by immunohistochemistry.

For immunofluorescence, a double staining for FN-EDA, FN-EDB, TnC-A1 resp. TnC-C and von Willebrand factor was performed. The following primary antibodies were used: scFv(F8), scFv(L19), scFv(F16) resp. scFv(G11) and polyclonal rabbit anti-human von Willebrand factor (Dako). As secondary detection antibodies mouse anti-Myc (9E10) monoclonal antibody followed by Alexa Fluor 594 goat anti-mouse IgG (Molecular Probes, Leiden, The Netherlands) was used for scFv and Alexa Fluor 488 goat anti-rabbit (Molecular Probes) for von Willebrand factor. Slides were mounted and analyzed as described before.

5.2 Cloning, expression and characterization of a scFv(F8) fusion proteins

5.2.1 Cloning and in vitro characterization of F8-IL10

Cloning of F8-IL10
The human IL-10 gene was amplified from the previously cloned fusion protein L19-IL10 using the following primer sequences: a backward antisense primer, 5’-TAATGGTGATGGTGATGGTGGTTTCGTATCTTCATTGTCATGTAGGCTTC-3’; and a forward sense primer, 5’-TTTCCCTTTTGCCGCCGCTCATTAGTTTC-GTATCTTCATTGTCATGTA-3’, which appended part of a 15 amino acid linker (SSSSG)3 at its N-terminus and a stop codon and NotI restriction site at its C-terminus. The gene for the single-chain variable fragment (F8) was amplified with a signal peptide using the following primer pair: a backward antisense primer, 5’-CCCAAGCTTGTGACCATGGGCTGGAGCC-3’ and a forward sense primer, 5’-GAGCCGGAAGAGCTACTACCCGATGAGGAAGATTTGATTTCCACCTTG-GTCCCTTG-3’. Using this strategy, a HindIII restriction site was inserted at the N-terminus and a complementary part of the linker sequence was inserted at the C-terminus. The single-chain Fv and IL-10 fragments were then assembled using PCR and cloned into the HindIII and NotI restriction sites of the mammalian cell-expression vector pcDNA3.1(+) (Invitrogen, Basel, Switzerland).

Expression and purification of F8-IL10
CHO-S cells were stably transfected with the previously described plasmid and selection was carried out in the presence of G418 (0.5 g/l). Clones of G418-resistant cells were screened for expression of the fusion protein by ELISA using recombinant EDA of human fibronectin as antigen and Protein A HRP for detection (GE Healthcare, UK). Following generation of monoclonal cell lines, the best expressing clone was adapted to growth in PowerCHO-2 CD protein-free medium (Lonza, Switzerland) for large-scale production of F8-IL10. The fusion protein could be purified from cell culture medium by protein A affinity chromatography, as there is a staphylococcal protein A binding site present on most V\textsubscript{H}3 subclasses (Sasso et al. 1991; Hoogenboom et al. 1992; Silacci et al. 2005). The size of the fusion protein was analyzed in reducing and nonreducing conditions on SDS-PAGE and in native conditions by fast protein liquid chromatography gel filtration on a Superdex S-200 size exclusion column (GE Healthcare).

Bioactivity assay of F8-IL10
Biological activity of human IL10 was determined by its ability to induce the IL-4-dependent proliferation of MC/9 cells (Thompson-Snipes et al. 1991) using a colorimetric thiazole blue (MTT) dye-reduction assay (Sigma). In a 96-well microtitre plate, 10,000 MC/9 (murine mast cell line) (ATCC-LCG, Molesheim Cedex, France) cells/well in 200 µl of medium containing 5 pg (0.05 units)/ml of murine IL-4 (eBiosciences, San Diego, CA, USA) were treated for 48 hours with varying amounts of human IL-10. The human IL-10 standard and fusion protein were used at a maximum concentration of 100 ng/ml IL-10 equivalents and serially diluted. To this, 10 µl of 5 mg/ml MTT was added and the cells were incubated for 3-5 hours. The cells were then centrifuged lysed with dimethylsulfoxide (DMSO) and read for absorbance at 570 nm.

5.2.2 Cloning of a TNF receptor fusion protein

Murine TNFRII extracellular domain was amplified using a backward antisense primer, 5’- TTTTCTTTTGCGGCCGCTCATTA- 3’; and a forward sense primer, 5’-GGGTAGTAGCTTTCCGGCTCATCGTCCAGCGGCGTGCCCAGGTTG-3’, which appended part of a 15 amino acid linker (SSSSG)3 at its N-terminus and a stop codon and NotI restriction site at its C-terminus. The gene for the single-chain variable fragment (F8) was amplified with a signal peptide using the following primer pair: a backward antisense primer, 5’-CCCAAGCTTGTCGACCATGGGCTGGAGCC-3’ and a forward sense primer, 5’-GAGCCGGAAGAGCTACTACCCGATGAGGAAGATTTGATTTCCACCTTG-GTCCCTTG-3’. Using this strategy, a HindIII restriction site was inserted at the N-terminus and a complementary part of the linker sequence was inserted at the C-terminus. The resulting PCR assembly product was cloned into the HindIII and NotI restriction sites of the mammalian cell-expression vector pcDNA3.1(+) expressed in CHO-S cells.

5.2.3 Cloning and characterization of F8-IL2

*Cloning of F8-IL2*
The gene for the F8 diabody (Villa et al. 2008) was amplified with a signal peptide using the primers 5’-CCCAAGCTTGGACCATTGGGCTGGAGC-3’ and 5’-CAAGGGACCAAGGTGGAATCAAATCTCCTCATTACGGGTAGCTCTCTCCGGCTCATC-3’, introducing a N-terminal HindIII restriction site and a part of a C-terminal (SSSSG)_3 linker. The human IL-2 gene (aa 21-153) was amplified from the previously cloned immunocytokine F16-IL2 (Marlind et al. 2008) using the primers 5’-TAGCTCTTCCGGCTCATCGTCCAGCGGCACCTACTTCAAGTTTCTACAAAGAAAAACAC-3’ and 5’-GTCAAAGCATCATCTCAACACTGACTTAATGAGCGGCCGCTACAAGGAAAAGAAAGAAA-3’ generating a complementary part of the (SSSSG)_3-linker at its N-terminus and a C-terminal stop codon and NotI restriction site. The F8 diabody and huIL-2 fragments were then assembled by PCR and cloned via HindIII and NotI restriction sites into the mammalian expression vector pcDNA3.1(+) (Invitrogen).

Expression and characterization of F8-IL2
CHO-S cells were stably transfected with the plasmid pcDNA3.1-F8-IL2 and selection was carried out in the presence of G418 (0.5 g/l). Clones of G418-resistant cells were screened for the expression of the fusion protein by ELISA using recombinant EDA of human fibronectin as antigen and proteinA-HRP for detection (GE Healthcare). The best expressing clone was adapted to grow in suspension in PowerCHO-2CD protein free medium for large-scale production of F8-IL2. The fusion protein was purified from the cell culture medium by protein A affinity chromatography. The size of the fusion protein was analyzed under reducing and non-reducing conditions by SDS-PAGE and under native conditions by FPLC gel filtration on a Superdex S-200 size exclusion column (GE Healthcare).

5.3 Targeting experiments in the collagen induced model of arthritis

5.3.1 Collagen induced arthritis mouse model

Male DBA/1 mice (8-10 weeks old) were immunized by intradermal injection at the base of the tail with 150 µg of bovine type II collagen (Chondrex, Inc., US) emulsified with equal volumes of Freund’s complete adjuvant (Chondrex, Inc., US). The procedure was repeated 2 weeks after the first immunization. Mice were inspected daily and each
mouse that exhibited erythema and/or paw swelling in one or more limbs was assigned to an imaging or treatment study.
Arthritis was monitored defining a clinical score. Each limb was graded daily in a nonblinded fashion (0 = normal, 1 = swelling of one or more fingers of the same limb and 2 = swelling of the whole paw), with a maximum score of 8 per animal (Williams et al. 2000).

5.3.2 Near infrared imaging of arthritic paws

The selective accumulation of SIP(F8) in arthritic mice was tested by near-infrared imaging analysis, as described by Birchler and colleagues (Birchler et al. 1999). Briefly, SIP(F8) was labeled using Alexa750 (Molecular Probes), according to the manufacturer’s recommendations, and injected into the tail vein of arthritic mice (n = 3). Mice were anaesthetized using ketamin, 80 mg/kg body weight, and medetomidine, 0.2 mg/kg body weight, and imaged in a near infrared mouse imager 24 hours after injection.

5.3.3 Phosphorimage analysis of arthritic paws with radiolabeled F8-IL10

For a more detailed targeting analysis of SIP(F8) and F8-IL10 the proteins were radioiodinated and injected i.v. or s.c., respectively (150 μg protein, 7 μCi). Mice (n = 2) were sacrificed 24 hours after injection, paws were exposed to a phosphorimager screen (Fujifilm, Dielsdorf, Switzerland) for 1 hour and read in a PhosphorImager (Fujifilm BAS-5000). Data were analyzed using Aida Image Analyzer v.4.15 (Fujifilm).

5.3.4 Quantitative biodistribution studies in tumor mice

To compare the in vivo targeting performance after subcutaneous and intravenous injection quantitative biodistribution analyses using radiolabeled antibody preparations were performed as described before. Briefly, purified F8-IL10 was radioiodinated with 125I and injected i.v. or s.c. into 129Sv mice (n = 4) grafted with a subcutaneous F9 tumor (150 μg, 8 μCi per mouse). Mice were sacrificed 24h, 48h, 72h, or
96h after injection. Organs were weighed and radioactivity was counted using a Cobra γ counter (Packard, Meriden, CT). Radioactivity content of representative organs was expressed as the percentage of the injected dose per gram of tissue (%ID/g ± SE).

In a similar experiment a comparison of targeted and systemic application of IL-10 was performed. HyHel10-IL10 and F8-IL10 were labeled with 125I and intravenously injected into 129Sv mice (n = 4) grafted with a subcutaneous F9 tumor (150 µg, 8 µCi per mouse). Tumor and organ uptake was measured 24h after injection, as described above.

5.4 Therapy experiments in the collagen induced model of arthritis

5.4.1 Combination therapy study with Methotrexate

Each mouse that exhibited erythema and/or swelling of one or more paws was randomly assigned to a treatment or control group and therapy was started. Mice were given an s.c. or i.v. injection of F8-IL10 (3 x 200 µg), saline or an i.p. injection of Methotrexate (3 x 100 µg). For the combination study mice were given an i.v. injection of F8-IL10 (3 x 200 µg) followed by an i.p. injection of Methotrexate (3 x 100 µg). 8 mice were analyzed per group. The arthritic score was evaluated daily in a nonblinded fashion. The results are displayed as the mean ± standard error for each group. Experiments were performed in agreement with Swiss regulations and under a project license granted by the Veterinäramt des Kantons Zürich, Switzerland (171/2007).

5.4.2 Comparison of targeted and untargeted delivery of IL-10

Cloning, expression and purification of an HyHel10-IL10 fusion protein has been described before (Trachsel et al. 2007). Therapy was performed as described above. Briefly, arthritis mice were injected subcutaneously with saline, HyHel10-IL10 (200 µg), TNFRII-fusion (100 µg) or F8-IL10 (200 µg). 6-7 mice were analyzed per group.
5.4.3 Ex vivo immunohistochemical detection of F8-IL10 and HyHel10-IL10 in arthritis paws

At the end of therapy mice were killed and paws were embedded in cryombedding compound (Microm, Walldorf, Germany) and stored at -80 °C. Sections (10 μm were cut and fixed in aceton. F8-IL10 and HyHel10-IL10 were detected using a biotinylated anti-human IL-10 antibody (eBiosciences, San Diego, CA, USA) followed by streptavidin-alkaline phosphatase (SAP) complex (Biospa, Milan, Italy). Fast Red TR Salt (Sigma-Aldrich, St Louis, MO) was used as the phosphatase substrate. Sections were counterstained with hematoxylin, mounted with Glycergel mounting medium (Dako) and analyzed with an Axiovert S100 TV microscope (Zeiss, Feldbach, Switzerland).

5.4.4 Statistical analysis

Data are expressed as the mean ± SEM. Differences in the arthritis score between different groups were compared using Mann-Whitney test.

5.5 Evaluation of cellular and humoral immunity

5.5.1 Immunofluorescence studies of infiltrating cells

To evaluate the role of effector cell responses in vivo immunofluorescent staining of paw sections of therapy mice was performed using antibodies against the following antigens: rat anti-mouse F4/80 (anti-macrophage; Abcam), rat anti mouse CD45 (BD Biosciences), rabbit anti-asialo GM1 (anti-NK; Wako Pure Chemical Industries) and rat anti-mouse CD4 and rat anti-mouse CD8. Cryosections were thawed and fixed by immersion in cold acetone for 10 min. Blocking was done by incubating the sections with 20% donkey/goat serum in PBS for 1h. Following washing with PBS 2 x 5 min at room temperature sections were incubated with the primary antibodies in 12% bovine serum albumin in PBS over night at 4° C. Sections were washed 3 x 5 min with PBS at room temperature and then incubated with fluorescent Alexa 488- or 594-coupled secondary antibodies (BD BiosciencesPharminen) and Hoechst (4,6-diamidino-2-phenylindole) in 12% bovine serum albumin-PBS. Finally, sections were washed 3 x 5
min in PBS and mounted with Glycergel (DAKO) and a coverglass (VWR International). Images were obtained using the individual fluorescent channels using an Axioskop 2 mot plus (Carl Zeiss).

Staining was quantified in representative 10x microscopic images using ImageJ software (http://rsb.info.nih.gov/ij/) and expressed as a percentage of measurement area.

5.5.2 Anti-bovine collagen II Antibodies

Levels of anti-bovine collagen II antibodies at the termination of experiments were determined using standard ELISA techniques as described before (Perez et al. 2002). Microtiter plates were coated with bovine collagen II solution (5 µg/ml) overnight at 4°C. After washing they were blocked for 2h at room temperature with 2% BSA. Samples were tested in triplicates at 1:800 dilution. Bound total IgG, IgG1 and IgG2a were detected by incubation with horse radish peroxidase conjugated goat anti-mouse IgG/IgG1 or IgG2a antibodies (Santa Cruz Biotechnology, US).

5.5.3 Analysis of mouse plasma cytokine levels

Mouse plasma cytokine level analysis was performed at Cytolab (Muelligen, Switzerland). A multiplexed particle-based flow cytometric cytokine assay was used (Vignali 2000). MAP Fluorokine cytokine kits were purchased from R&D (Oxon,UK). The procedures closely followed the manufacturer’s instructions. The analysis was conducted using a conventional flow cytometer (FC500 MPL, BeckmanCoulter, Nyon, Switzerland).

5.5.4 Statistical analysis

Data are expressed as the mean ± SEM. Significances were calculated using Students t test.
5.6 Safety pharmacology profile of F8-IL10

5.6.1 Toxicology studies in cynomolgus monkey

Preclinical toxicology studies were performed at CIT (Centre International de Toxicologie, Evreux) in accordance with GLP guidelines. During the study two groups (group 2 and 3) of three male and three female cynomolgus monkeys received test Dekavil (F8-IL10) by subcutaneous injection in the dorsum at the dose-level of 180 µg/kg/administration, three times a week for 8 weeks. Another group (group1) of three males and three females received the formulation buffer for Dekavil (F8-IL10), under the same experimental conditions, and acted as a control group. Animals in group 3 were also administered Methotrexate starting on day 4, as well as folic acid 24h after each Methotrexate administration. Both these test items were administered by oral gavage with capsules, once a week until the end of the study. Blood samples were taken from all the animals for determination of serum levels of Dekavil (F8-IL10) on day 1 and on the last day of dosing, at designated time-points. Animals were checked daily for reaction to treatment and the following investigations were done: body weight, food consumption, ophthalmoscopy, electrocardiography, blood pressure, hematology and clinical chemistry. On completion of the treatment period, animals were sacrificed and submitted to a complete macroscopic examination.

5.6.2 Single dose intravenous toxicity study in mice

A single group of 5 male and 5 female mice (Hsd:ICR(CD-1)) was intravenously injected with 20mg/kg F8-IL10 followed by a 14 day observation period. A control group of 5 male and 5 female mice (Hsd:ICR(CD-1)) was injected with the vehicle alone (saline). All animals were killed with carbon dioxide at the end of the observation period and subjected to necropsy.
5.7 Evaluation of antigen expression in different chronic inflammatory diseases

For a comparative immunohistochemistry, 10 µm cryostat sections were fixed in ice-cold aceton and stained for FN-EDA, FN-EDB, and TnC-A1. Biotinylated SIPs were added onto the sections in a final concentration of 2 µg/ml and detected with streptavidin-alkaline phosphatase (SAP) complex (Biospa, Milan, Italy). Fast Red TRSalt (Sigma-Aldrich, St Louis, MO) was used as the phosphatase substrate. Sections were counterstained with hematoxylin, mounted with Glycergel mounting medium (Dako) and analyzed with an Axiovert S100 TV microscope (Zeiss, Feldbach, Switzerland). For immunofluorescence, a double staining for FN-EDA/TnC-A1 and von Willebrand factor was performed. Biotinylated SIPs were added onto the sections in a final concentration of 2 µg/ml and detected with streptavidin Alexa488 (Molecular Probes, Leiden, The Netherlands) and Alexa Fluor 594 goat anti-rabbit (Molecular Probes, Leiden, The Netherlands), respectively. Slides were mounted and analyzed as described before.

5.8 Mouse model of Endometriosis

6-8 week old C57BL/6 were subjected to ovariectomy 7 days prior to induction of endometriosis. Mice were anesthesized by isoflurane in combination with carprofen (Rimadyl). After ovariectomy mice were oestrogen-treated (3x/week 4ug estradiol/mouse in a volume of 100 ul arachis oil subcutanously injected, starting at the day of transplantation). Ovariectomy plus oestrogen supplementation was done in order to abrogate differences related to the stage of the oestrous cycle. At day 0 mice were splitted in 2 groups: donor mice (33%) and recipient mice (66%). Donor mice were killed and both uterine horns were removed and subsequently placed in a sterile Petri dish containing sterile saline. Endometrium were detached from the uterine muscle and finley chopped using a scalpel. Endometrial fragments were suspended in saline and injected into the peritoneal cavity. 3 weeks after transplantation mice were sacrificed and lesions were embedded in OCT and frozen at -80 °C. For tissue analysis 10 µm sections were cut. Immunohistochemistry using biotinylated SIP(F8) and SIP(F16) as negative control was performed as described above.
5.9 Targeting of F8 in the syngeneic mouse model of endometriosis

The selective accumulation of SIP(F8) in endometriosis lesions was tested by near-infrared imaging analysis, as described by Birchler and colleagues (Birchler et al. 1999). Briefly, SIP(F8) and SIP(F16) (as a negative control) was labeled using Alexa750 (Molecular Probes), according to the manufacturer’s recommendations, and injected into the tail vein of arthritic mice. Mice were sacrificed and imaged in a near infrared mouse imager 24 hours after injection.

After near infrared imaging endometriosis lesions were embedded in cryoembedding compound (Microm, Walldorf, Germany) and stored at -80 °C. Sections (10 µm) were cut and fixed in aceton. SIP(F8)-ALEXA750 was detected using a rabbit anti-human IgE antibody (Dako, Glostrup, Denmark) followed by Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes, Leiden, The Netherlands). For the detection of blood vessels a double staining with a rat anti-CD31 antibody, followed by Alexa Fluor 594 donkey anti-rat IgG was performed.

5.10 Preliminary therapy experiment using the two immunocytokines F8-IL10 and F8-IL2

One day after endometriosis tissue transplantation mice were grouped (5 mice / group) and treated intravenously with F8-IL10 (200 µg), F8-IL2 (20 µg) or saline. Mice received 3 injections on day 1, 4 and 7. 5 days after the last injection mice were sacrificed and analyzed.
6 References


Del Prete, G., M. De Carli, F. Almerigogna, M. G. Giudizi, R. Biagiotti and S. Romagnani (1993). "Human IL-10 is produced by both type 1 helper (Th1) and type 2 helper (Th2) T cell clones and inhibits their antigen-specific proliferation and cytokine production." J Immunol 150(2): 353-60.


Golay, J., M. Lazzari, V. Facchinetti, S. Bernasconi, G. Borleri, T. Barbui, A. Rambaldi and M. Introna (2001). "CD20 levels determine the in vitro susceptibility to rituximab and


Stat5) and their distinct combinatorial assembly in the promoters of selected genes."

Furst, K. Bulpitt, E. Veys, et al. (1999). rHUuIL-10 (Tenovil) plus methotrexate (MTX) in
active rheumatoid arthritis (RA): a phase I/II study. American College of Rheumatology
63rd Annual Scientific Meeting, Boston, Mass.

Wielenga, V. J., K. H. Heider, G. J. Offerhaus, G. R. Adolf, F. M. van den Berg, H. Ponta, P.

ameliorates joint disease in murine collagen-induced arthritis." Proc Natl Acad Sci U S A
89(20): 9784-8.

"Evaluation of TNF-alpha and IL-1 blockade in collagen-induced arthritis and

Windsor, W. T., R. Syto, A. Tsarbopoulos, R. Zhang, J. Durkin, S. Baldwin, S. Paliwal, P. W.


Wunder, A., U. Muller-Ladner, E. H. Stelzer, J. Funk, E. Neumann, G. Stehle, T. Pap, H. Sinn,

"Interleukin-10 is a growth factor for human melanoma cells and down-regulates HLA
class-I, HLA class-II and ICAM-1 molecules." Int J Cancer 71(4): 630-7.

Zamah, N. M., M. G. Dodson, L. C. Stephens, V. C. Buttram, Jr., P. K. Besch and R. H.

Zardi, L., B. Carnemolla, A. Siri, T. E. Petersen, G. Paolella, G. Sebastio and F. E. Baralle
(1987). "Transformed human cells produce a new fibronectin isoform by preferential
alternative splicing of a previously unobserved exon." Embo J 6(8): 2337-42.
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