human
reproduction

ORIGINAL ARTICLE *Gynaecology*

The antibody-mediated targeted delivery of interleukin-10 inhibits endometriosis in a syngeneic mouse model

Kathrin Schwager[1], Frank Bootz[1], Patrick Imesch[2], Manuela Kaspar[1], Eveline Trachsel[1,*], and Dario Neri[3]

[1]Philochem AG, c/o ETH Zurich, Institute of Pharmaceutical Sciences, Wolfgang-Pauli-Str. 10 HCI E520, CH-8093 Zurich, Switzerland
[2]UniversitätsSpital Zürich, Klinik für Gynäkologie, Frauenklinikstrasse 10, CH-8091 Zürich, Switzerland [3]Institute of Pharmaceutical Sciences, ETH Zürich, Wolfgang-Pauli-Strasse 10, CH-8093 Zurich, Switzerland

*Correspondence address. Tel: +41-43-544-88-03; E-mail: eveline.trachsel@philochem.ch

BACKGROUND: Endometriosis is still a highly underdiagnosed disease, and the current medical and surgical treatment of endometriosis is associated with a high recurrence rate. This study investigates the use of derivatives of the human antibody F8, specific to the alternatively spliced extra-domain A of fibronectin (Fn), for the imaging and treatment of endometriosis.

METHODS: Immunohistochemistry and immunofluorescence was used to evaluate antigen expression in endometriotic tissue of human endometriosis and of a syngeneic mouse model of the disease. The *in vivo* targeting performance of a fluorescent derivative of the F8 antibody was assessed by imaging mice with endometriosis using a near-infrared fluorescence imager, 24 h following i.v. injection of the antibody conjugate. Furthermore, the mouse model was used for therapy experiments using two recombinant F8-based immunocytokines [F8-interleukin-10 (IL10) and F8-IL2] or saline for the treatment groups.

RESULTS: A very strong vascular expression of splice isoforms of Fn and of tenascin-C was observed in human endometriotic lesions by immunohistochemistry and immunofluorescence techniques. After i.v. administration, a selective accumulation of the F8 antibody in endometriotic lesions could be observed in a syngeneic mouse model. These targeting data were used as a basis for therapy experiments with a pro-inflammatory (F8-IL2) and an anti-inflammatory (F8-IL10) cytokine fusion protein of the F8 antibody. The average lesion size in the F8-IL10 treatment group was clearly reduced compared with the saline control group and with the F8-IL2 group, for which no therapeutic effects were observed.

CONCLUSIONS: The F8 antibody targets endometriotic lesions *in vivo* in a mouse model of endometriosis and may be used for the non-invasive imaging of the disease and for the pharmacodelivery of anti-inflammatory cytokines, such as IL10.

Key words: endometriosis / angiogenesis / extra-domain A of fibronectin / targeting / interleukin-10

Introduction

Endometriosis, a common gynecological disorder characterized by extrauterine growth of endometrium-like tissue, affects ∼10% of women. Common symptoms include dysmenorrhea, abdominal pain and infertility. Endometriosis is considered to be an estrogen-dependent disease because and is almost exclusively found in women between menarche and menopause (Sampson, 1927; Giudice and Kao, 2004).

Due to unspecific symptoms of the disease, the only way to confirm and diagnose peritoneal endometriosis is by laparoscopy.

Attempts to develop reliable non-invasive instruments to detect endometriosis have mainly remained elusive. Therefore, the disease is highly underdiagnosed and the time interval between the appearance of symptoms to diagnosis can be very long with an average of 11.7 years in the USA (Hadfield et al., 1996), 8 years in the UK (Hadfield et al., 1996) and 6.7 years in Norway (Husby et al., 2003). Furthermore, the development of new treatments for endometriosis is challenging since monitoring of the disease is difficult. A non-invasive diagnostic method is urgently needed to decrease time to diagnosis and to allow monitoring of the disease.

Treatment of endometriosis has two aims: relief of pain and/or amelioration of infertility. Conservative surgical removal of endometriosis effectively provides pain relief and increases the chances of pregnancy. However, symptoms recur in upto 75% of women within 2 years (Giudice and Kao, 2004). Most of the current medical therapies induce a hypoestrogenic state in patients and are associated with severe side effects (e.g. reduction in bone mineral density) (Rice, 2002). The development of new therapeutic options that do not disturb the normal menstrual cycle and fertility is a current challenge of endometriosis research (Fedele et al., 2008).

Angiogenesis, the formation of new blood vessels, plays an essential role in the growth and survival of endometriotic lesions. Similar to tumor metastases, endometriotic implants require revascularization to guarantee oxygen and nutrient supply. Correspondingly, a typical clinical feature of endometriotic lesions is their dense vascular network. The high vascularization and mitotic activity are observed particularly in red lesions, suggesting that these lesions are very active and probably the first stage of early implantation of endometrial glands and stroma (Nisolle et al., 1993; McLaren et al., 1996). The lower vessel maturation index in red lesion compared with black lesion supports this observation. It has been shown that angiogenic cytokines are elevated in the peritoneal fluid, serum and endometriotic tissue of women suffering from the disease (Taylor et al., 2002; Laschke and Menger, 2007).

Molecules capable of selectively targeting markers of angiogenesis may offer opportunities for the in vivo imaging of endometriosis and for the selective delivery of therapeutic agents to endometriotic lesions.

Our group has extensively studied the use of monoclonal antibodies specific to splice isoforms of fibronectin (Fn) and tenascin-C (TnC) for the targeted delivery of cytokines to the tumor neovasculature. This pharmacodelivery strategy has been investigated for cancer therapy applications, leading to the preclinical (Gillies et al., 1998; Nilsson et al., 2001; Halin et al., 2002; Gafner et al., 2006; Huang et al., 2007, Kaspar et al., 2007; Marlind et al., 2008; Schliemann et al., 2009; Pedretti et al., 2010b), and clinical (King et al., 2004; Schrama et al., 2006; Sauer et al., 2009) investigation of several antibody-cytokine fusion proteins. The best characterized clinical-stage human antibodies developed by our group include F8 (specific to the alternatively spliced extra-domain A (EDA) of Fn (Villa et al., 2008)), L19 (specific to the alternatively spliced extra-domain B (EDB) of Fn (Pini et al., 1998)) and F16 (specific to the alternatively normal adult tissues (Brack et al., 2006), but are strongly expressed (typically around vascular structures) in conditions of tissue remodeling (Zardi et al., 1987; Carnemolla et al., 1989; Chiquet-Ehrismann and Chiquet, 2003; Neri and Bicknell, 2005). At present, the pro-inflammatory antibody−cytokine fusion proteins L19-interleukin-2 (IL2) (Carnemolla et al., 2002), L19-tumor necrosis factor (TNF) (Borsi et al., 2003) and F16-IL2 (Marlind et al., 2008) are currently investigated in multiple Phase II clinical trials in patients with cancer.

Recently, we were able to demonstrate that splice isoforms of Fn and TnC are present in inflammatory conditions such as atherosclerosis, rheumatoid arthritis or psoriasis (Matter et al., 2004; Trachsel et al., 2007a,b; Schwager et al., 2009; Pedretti et al., 2010a). The pro-inflammatory immunocytokine L19-IL2 was found to promote plaque remodeling and reduce atherosclerotic plaque load in the Apo E−/− mouse model of atherosclerosis (Dietrich et al., 2007),

but the same product worsened inflammation in the collagen-induced arthritis (CIA) mouse model of arthritis. By contrast, both L19-IL10 (Trachsel et al., 2007a) and F8-IL10 (Schwager et al., 2009) were found to be preferentially localized at sites of arthritis and to potently inhibit disease progression in the CIA model. The promising results of these studies encouraged us to investigate antibodies specific to splice isoforms of Fn and TnC for the imaging of endometriosis and as vehicles for the pharmacodelivery of IL2 and IL10 to endometriotic lesions in vivo. 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. The peritoneal environment of women with endometriosis is altered and highly activated. (Giudice and Kao, 2004). However, it remains unclear whether changes in inflammatory mediators are a cause or a consequence of the disease. Anti-inflammatory (e.g. anti-TNF) (D'Antonio et al., 2000) as well as immunostimulatory (e.g. IL-12) (Somigliana et al., 1999) approaches have shown efficacy in mouse models of endometriosis.

Materials and Methods

Immunohistochemical analysis of human and mouse endometriotic tissue

A deep infiltrating inguinal endometriosis sample was obtained according to standard operating procedures for the analysis of tissue specimens at the Department of Gynecology of the University Hospital Zurich, during the early phase of the menstrual cycle from a non-hormonal pretreated woman. For immunohistochemistry, 10 μm cryostat sections were fixed in ice-cold acetone and stained for Fn-EDA, Fn-EDB and TnC-A1. These antibodies do not work on freshly frozen paraffin-embedded specimens. Primary antibodies in small immunoprotein (SIP) format (biotinylated) were added onto the sections in a final concentration of 2 μg/ml and detected with streptavidin-alkaline phosphatase complex (Biospa, Milan, Italy). Fast Red TRSalt (Sigma-Aldrich, St Louis, USA) was used as the phosphatase substrate. Sections were counterstained with hematoxylin, mounted with Glycergel mounting medium (Dako, Glostrup, Denmark) and analyzed with an Axiovert S100 TV microscope (Zeiss, Feldbach, Switzerland).

For immunofluorescence, a double staining for Fn-EDA, Fn-EDB, TnC-A1 and von Willebrand factor was carried out. The following primary antibodies were used: biotinylated SIP(F8), SIP(L19), SIP(F16) and polyclonal rabbit anti-human von Willebrand factor (Dako). For the detection of the biotinylated SIPs Streptavidin Alexa Fluor 488 (Invitrogen, Basel, Switzerland) was used. As secondary detection antibody for the anti-von Willebrand factor antibody, Alexa Fluor 488 goat anti-rabbit (Invitrogen) antibody was used. Slides were mounted and analyzed as described before.

Mouse model of endometriosis

Mouse experiments were performed in agreement with Swiss regulations and under a project license granted by the Veterinäramt des Kantons Zürich, Switzerland (122/2009). C57BL/6 mice (6−8 week old) were subjected to ovariectomy 7 days prior to induction of endometriosis. Mice were anesthesized by isoflurane (Attane™, Minrad Inc., Buffalo/NY, USA) in combination with carprofen (Rimadyl, Pfizer GmbH, Berlin, Germany). Carprofen (0.15 mg/mouse) was injected subcutaneously 2 h before surgery. After ovariectomy, mice were estrogen-treated (Sigma-Aldrich) (3×/week 4 μg estradiol/mouse in a volume of 100 μl

arachis oil (Haenseler AG, Herisau, Switzerland) subcutaneously injected, starting 2 days after ovariectomy). Ovariectomy and estrogen treatment of the mice were done in order to abrogate differences related to the stage of the estrous cycle. One week after ovariectomy, mice were divided into two 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 was detached from the uterine muscle and finely chopped using a scalpel. Endometrial fragments were suspended in saline and injected into the peritoneal cavity using a 19 gauge disposable needle (BBraun, Melsungen, Germany). Mice were killed 4 weeks after transplantation. Endometriotic lesions were embedded in cryoembedding compound (Microm, Walldorf, Germany) and stored at $-80°C$ until processed. Sections (10 μm) were cut and fixed in acetone. Immunohistochemistry and hematoxylin staining on mouse tissue was conducted as described already, using biotinylated F8 antibody in the SIP format.

Near-infrared imaging of endometriosis lesions in mice

Three weeks after transplantation of endometrial tissue, mice were used for imaging experiments. The selective accumulation of SIP(F8) in the syngeneic mouse model of endometriosis was tested by near-infrared imaging analysis, as described by Birchler et al. (1999a). Briefly, SIP(F8) and SIP(F16) (as a negative control) were labeled using amine-reactive Alexa Fluor 750 carboxylic acid (Invitrogen), according to the manufacturer's recommendations. Fluorescently, labeled proteins were separated from unincorporated dye by gel-filtration on disposable PD-10 columns (GE Healthcare, Glattbrugg, Switzerland). Labeled proteins (300 μg) were injected into the tail vein of endometriosis mice. Mice were killed and imaged in a near-infrared mouse imager (Birchler et al., 1999b) 24 h after injection.

Ex vivo detection of SIP(F8)-Alexa750

After near-infrared imaging, endometriotic lesions were embedded in cryoembedding compound (Microm) and stored at $-80°C$ until being processed. Sections (10 μm) were cut and fixed in acetone. SIP(F8)-Alexa750 was detected using a rabbit anti-human immunoglobulin (Ig) E antibody (Dako) followed by Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen). For the detection of blood vessels, a double staining with a rat anti-CD31 antibody followed by Alexa Fluor 594 donkey anti-rat IgG (Invitrogen) was carried out.

Therapy experiments with the two immunocytokines F8-IL2 and F8-IL10

F8-IL2 and F8-IL10 were produced as described before (Schwager et al., 2009; Frey et al., 2010). The mouse model was set up as described previously. One day after endometriosis, tissue transplantation mice were grouped (10 mice/group) and treated intravenously with F8-IL2 (20 μg), F8-IL10 (200 μg) or saline. The dose of immunocytokine was chosen in accordance with previous studies in animal models of arthritis (Schwager et al., 2009) or in cancer (Carnemolla et al., 2002; Frey et al., 2010). Mice received three injections on Day 1, 4 and 7. Five days after the last injection mice were killed and analyzed. Lesions were counted and weighed using an analytical balance (Mettler Toledo AT460 Delta Range, Greifensee, Switzerland).

Analysis of mouse plasma cytokine levels

Mouse plasma cytokine level analysis was performed at Cytolab (Muelligen, Switzerland). Plasma of four mice of each group (three for the F8-IL10 group) was analyzed. Furthermore, plasma of four healthy F8-IL10-treated mice was analyzed. 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).

Immunofluorescence studies of infiltrating cells

To evaluate the role of effector cell responses *in vivo,* immunofluorescent staining of endometriotic sections of therapy mice was carried out using antibodies against the following antigens: rat anti-mouse F4/80 (anti-macrophage; Abcam, Cambridge, UK), rat anti-mouse CD45 (anti-leukocyte common antigen; BD Biosciences, San Jose, USA) and rabbit anti-asialo GM1 (anti-natural killer cell; Wako Pure Chemical Industries, Tokyo, Japan). Cryosections were fixed by immersion in cold acetone, and primary antibodies were added and incubated overnight at 4°C. For detection, fluorescent Alexa 488- or 594-coupled antibodies (BD Biosciences) were used. Finally, sections were mounted with Glycergel (Dako). Images were obtained using the individual fluorescent channels using an Axioskop 2 mot plus (Carl Zeiss).

Statistical analysis

Differences in lesion weight and plasma cytokine levels between therapeutic groups were compared using the Mann–Whitney U-test. P-values < 0.05 were considered significant.

Results

Evaluation of antigen expression by immunohistochemistry and immunofluorescence

Immunohistochemical and immunofluorescence staining of a human endometriotic tissue revealed a very strong vascular expression of the EDA domain of Fn and of the A1 domain of TnC, while EDB expression was weaker (Fig. 1). Blood vessels were stained in red using an anti-von Willebrand antibody.

F8 and F16 antibodies exhibited a comparable staining performance in endometriosis. Since only F8 antibody is able to recognize the cognate human and murine antigen with identical affinity (Villa et al., 2008), we continued our preclinical investigations with this antibody.

A syngeneic murine model of endometriosis was developed by intraperitoneally injecting endometrial tissue of donor mice into estrogen-treated recipient mice. Three weeks after tissue implantation, mice were killed and endometriosis fragments were analyzed. Typically, 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, were 2–5 mm in diameter. Hematoxylin staining revealed the typical glandular and cystic structures of endometriosis (Fig. 2A).

Figure 2B represents a three-color immunofluorescence staining of mouse endometriotic lesions. The antibodies F8 and L19 are shown in green, blood vessels are stained in red, using an anti-CD31 antibody and nuclei are stained in blue. The staining pattern of the two antibodies is comparable; however, staining with the antibody F8 appears clearly stronger (Fig. 2B).
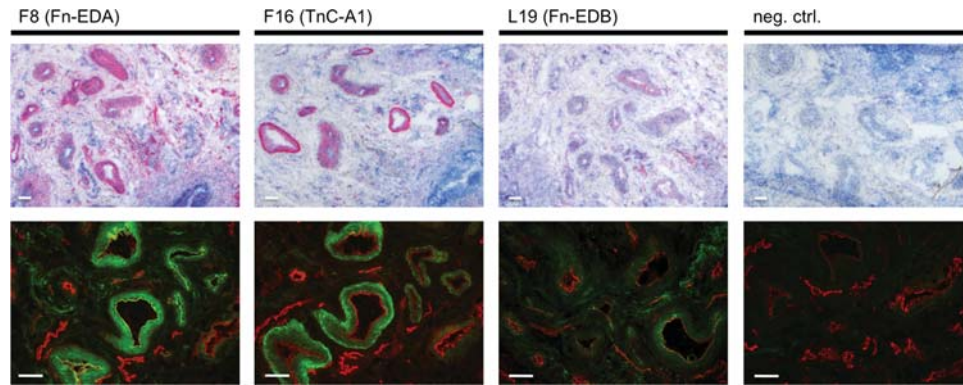
Figure 1 Immunohistochemical and immunofluorescence staining of human endometriotic tissue. Immunohistochemistry with the SIPs L19, F16 and F8 was conducted. Furthermore, immunofluorescence double staining with L19, F16 and F8 (green) and von Willebrand factor (red) was carried out. The anti-von Willebrand factor antibody stains endothelial cells and was used to highlight blood vessels. The antibodies F8 and F16 showed a strong vascular staining pattern. Scale bars = 100 μm.
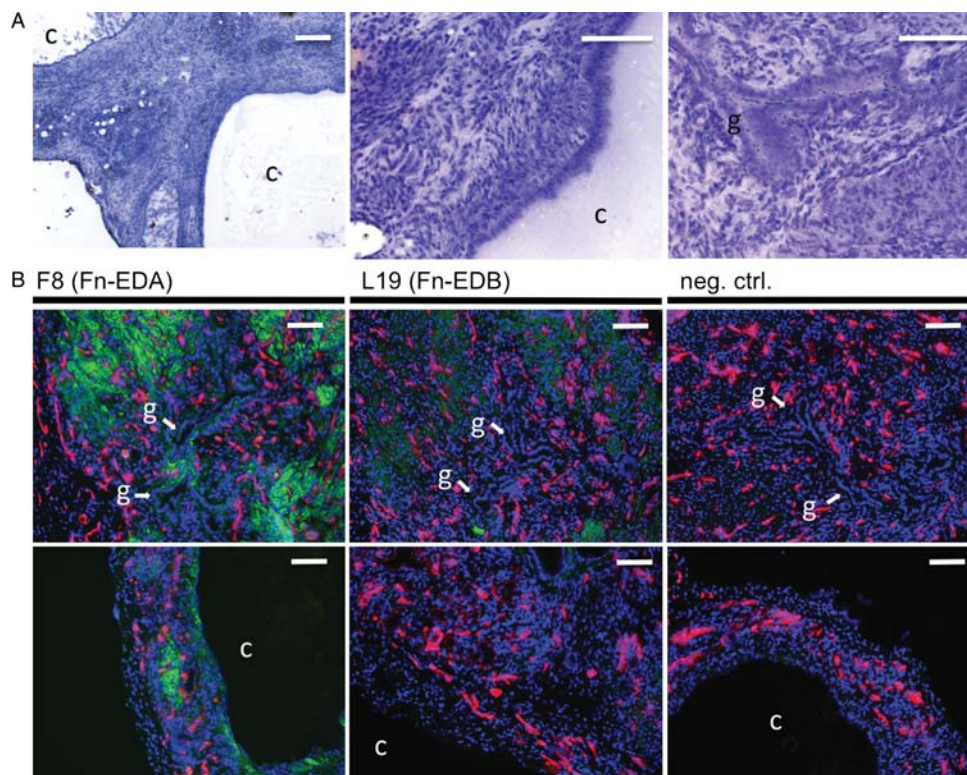

Figure 2 (**A**) Hematoxylin staining of mouse endometriotic tissue. Mouse lesions show typical glandular (g) and cystic structures (c). (**B**) Shows three-color immunofluorescence stainings with F8 or L19 (green), an anti-CD31 antibody that stains endothelial cells (red) and the nuclear stain 4(6-diamidino-2-phenylindole) (blue). A similar stromal staining pattern was observed for F8 and L19. However, the staining with F8 is clearly stronger. Scale bars = 100 μm.

Targeting of F8 in the syngeneic mouse model of endometriosis

The *in vivo* targeting performance of F8 was assessed by labeling the antibody with the near-infrared fluorophore Alexa750 (Neri *et al.*, 1997; Birchler *et al.*, 1999a,b). The antibody was used in recombinant SIP format (Borsi *et al.*, 2002), as this format has previously been shown to display favorable pharmacokinetic properties compared with the standard IgG format or the smaller single chain variable fragment (Borsi *et al.*, 2002; Berndorff *et al.*, 2005). Twenty-four
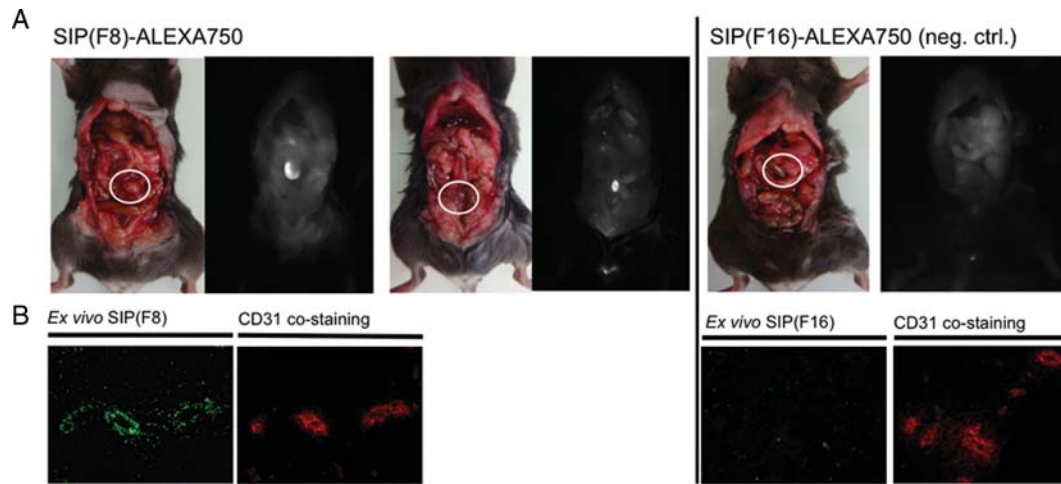
Figure 3 *In vivo* targeting performance of Alexa750 labeled SIP(F8) antibody in the syngeneic mouse model of endometriosis. (**A**) Near-infrared fluorescence imaging. Endometriosis mice were injected with SIP(F8)-Alexa750 or the negative control antibody SIP(F16)-Alexa750. Near-infrared fluorescence imaging analysis was carried out 24 h after injection. (**B**) *Ex vivo* detection of SIP(F8)-Alexa750. To show blood vessels, a double staining with an anti-CD31 antibody was carried out. SIP(F8) is localized around blood vessels in endometriotic lesions. Scale bars = 100 μm.

hours after intravenous injection of the fluorescently labeled SIP(F8) antibody, mice were killed and imaged using an infrared fluorescence imager (Birchler *et al.*, 1999a). A strong and selective antibody accumulation in the endometriotic lesions was observed (Fig. 3) and (Supplementary Fig. 1). By contrast, mice injected with SIP(F16), which is not cross-reactive on mouse tissue and which was used as a negative control, did not show any uptake in endometriotic lesions (Fig. 3A). An *ex vivo* fluorescence detection of SIP(F8) using secondary antibody reagents revealed intense fluorescent staining pattern around vascular structures (Fig. 3B).

Therapy experiment using the two immunocytokines F8-IL10 and F8-IL2

In order to investigate whether the targeted delivery of pro-inflammatory or anti-inflammatory cytokines could provide thera-peutic benefit in a syngeneic mouse model of endometriosis, the immunocytokines F8-IL2 (Frey *et al.*, 2010) and F8-IL10 (Schwager *et al.*, 2009) were used for the treatment of mice with endometriosis. Therapy was started one day after transplantation of endometriotic tissue and mice received three injections (Day 1, 4 and 7) of F8-IL2 (20 μg), F8-IL10 (200 μg) or saline. The dose of immunocytokine used in the study was the one that was found to be effective in animal models of arthritis (Schwager *et al.*, 2009) or in cancer studies (Carnemolla *et al.*, 2002; Frey *et al.*, 2010). Mice were killed 5 days after the last injection and assessed for the number and weight of endometriotic lesions.

In the saline control group, 7 out of 10 mice developed endometriosis, whereas only 3 out of 10 mice developed endometriosis in the F8-IL10 group (Fig. 4A). Furthermore, the average lesion weight was clearly reduced in the F8-IL10 group compared with the saline control group. No therapeutic effect could be observed for F8-IL2 (Fig. 4B).

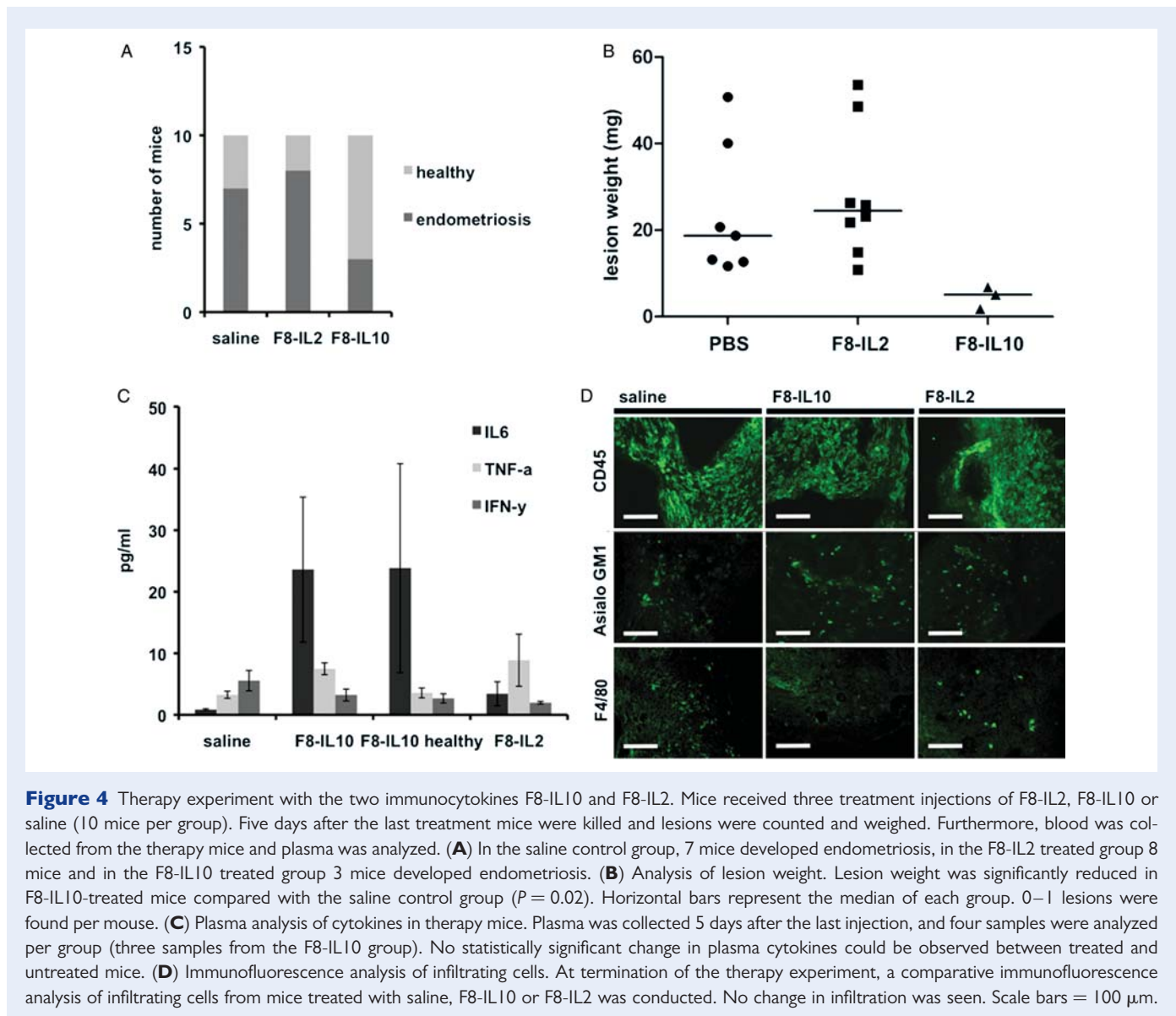At termination of the therapy experiment a comparative immuno-fluorescence analysis of infiltrating cells from mice treated with saline or F8-IL10 was conducted (Fig. 4D). However, no significant change in infiltration could be observed between the treatment groups. Furthermore, analysis of plasma cytokines of killed mice did not show any significant differences of IL6, IL2, TNFα levels between treatment and control groups (Fig. 4C).

Discussion

In this article, we have shown that the EDA domain of Fn is strongly and selectively expressed around vascular structures in human and murine endometriotic lesions. Furthermore, we have observed that the high-affinity human monoclonal antibody F8, specific to EDA, is capable of targeting the neovasculature of endometriosis *in vivo*. Treat-ment with the anti-inflammatory immunocytokine F8-IL10, but not with the pro-inflammatory immunocytokine F8-IL2, resulted in the establishment of significantly smaller endometriotic lesions in a syn-geneic mouse model of the disease after three intravenous injections.

At present, endometriosis is diagnosed at laparoscopy and there would be an urgent need for non-invasive reliable detection techniques. The two most common imaging tests available for diagnosis are the ultra-sound and the magnetic resonance imaging. However, normal results on these tests do not eliminate the possibility of endometriosis. Areas of endometriosis are often too small to be detected by these tests.

Fludeoxyglucose-poistron emission tomography (PET) method-ologies have been reported to successfully image endometriomas in some cases (Jeffry *et al.*, 2004; Derman *et al.*, 2007), but are otherwise not widely used in the clinical practice. Antibody-labeling with the clinical-grade PET radionuclide [124]I has recently become available as an easy route for the use of monoclonal antibodies in immuno-PET pro-cedures (Tijink *et al.*, 2009). [124]I has a half-life of 100 h and is ideally suited to match the pharmacokinetic properties of disease-targeting antibodies. Indeed, clinical trials for the imaging of cancer patients with [124]I-labeled SIP(L19) and SIP(F16) antibodies are currently

Figure 4 Therapy experiment with the two immunocytokines F8-IL10 and F8-IL2. Mice received three treatment injections of F8-IL2, F8-IL10 or saline (10 mice per group). Five days after the last treatment mice were killed and lesions were counted and weighed. Furthermore, blood was collected from the therapy mice and plasma was analyzed. (**A**) In the saline control group, 7 mice developed endometriosis, in the F8-IL2 treated group 8 mice and in the F8-IL10 treated group 3 mice developed endometriosis. (**B**) Analysis of lesion weight. Lesion weight was significantly reduced in F8-IL10-treated mice compared with the saline control group ($P = 0.02$). Horizontal bars represent the median of each group. 0–1 lesions were found per mouse. (**C**) Plasma analysis of cytokines in therapy mice. Plasma was collected 5 days after the last injection, and four samples were analyzed per group (three samples from the F8-IL10 group). No statistically significant change in plasma cytokines could be observed between treated and untreated mice. (**D**) Immunofluorescence analysis of infiltrating cells. At termination of the therapy experiment, a comparative immunofluorescence analysis of infiltrating cells from mice treated with saline, F8-IL10 or F8-IL2 was conducted. No change in infiltration was seen. Scale bars $= 100\ \mu$m.

ongoing, while clinical-grade SIP(F8) has recently become available and will be investigated in immuno-PET studies in the near future.

In principle, near-infrared fluorescence imaging modalities could also be considered for the molecular imaging of endometriosis, using fluorescently labeled antibodies (Folli *et al.*, 1994; Neri *et al.*, 1997; Birchler *et al.*, 1999a,b; Rudin and Weissleder, 2003). Light transmission through tissue is maximal at around 800 nm wavelength, with a 10% transmittance through 1 cm of most human tissues (Wan *et al.*, 1981). For this reason, near-infrared fluorophores such as Alexa750 derivatives used in this study should be considered. Immunophotodetection procedures have been used for the imaging of colorectal cancer in mice and in patients (Gutowski *et al.*, 2001). Furthermore, the clinically approved near-infrared fluorophore indocyanine green has been successfully used for the non-invasive detection of 10-cm deep breast cancer lesions, with the help of diffuse optical tomography imaging methodologies (Ntziachristos *et al.*, 2000). As fluorescence imaging methodologies are often confined to lesions that are few centimeters deep in abdominal locations, immuno-

PET imaging procedures are likely to be more useful for the detection of endometriosis in patients. On the other hand, real-time fluorescence imaging methodologies could help for disease detection during surgical procedures.

The therapy outcome in this study indicates a promising biological activity for the antibody-mediated pharmacodelivery of the anti-inflammatory cytokine IL-10 to sites of endometriosis. In the past, there have been contrasting views as to whether anti-inflammatory or pro-inflammatory treatment regimens would be preferable for the treatment of patients with endometriosis. On the one hand, it has been claimed that, the capacity of refluxed endometrial cells to evade immune-surveillance might be critical for the development of the disease and a variety of immuno-stimulatory drugs have shown results in animal models (Somigliana *et al.*, 1999). On the other hand, endometriosis represents an inflammatory disease, which is believed to progress with the support of an inflammatory milieu, and anti-inflammatory drugs, such as anti-TNF, have shown encouraging results in animal models of endometriosis (D'Antonio *et al.*, 2000; D'Hooghe *et al.*, 2006).

We used 10 mice per treatment group, which led to statistically significant conclusions. This number of mice per study group could not be extended because of the practical complexity in setting up the mouse model and because of the ethical principle of minimization of study mice. Scoring of lesions in mice was not blinded, but lesions were submitted to histological analysis.

In contrast to previous experience of our group in the plasma analysis as a response to immunocytokine treatment (Schwager *et al.*, 2009), a clear effect of F8-IL10 treatment on plasma levels of mouse cytokines could not be seen. This may reflect an intrinsic difference in the action of F8-IL10 in mice with endometriosis. Subtle variations in cytokine levels may require the study of more than four mice per group, as described in this paper.

Future studies will be needed to investigate whether prolonged treatment of mice with F8-IL10 or similar agents results in a complete disappearance of endometriotic lesions. These studies will probably require the use of fully murine phage-derived antibodies (Sommavilla *et al.*, 2010) fused to murine IL10, since the repeated administration of the fully human F8-IL10 immunocytokine is immunogenic in the mouse. Additionally, combination studies could be performed, to investigate whether F8-IL10 synergizes with established treatment modalities, such as GnRH analog, nuclear factor-κB (NF-κB)-inhibitors and progestins. During treatment with GnRH-a, several inflammatory molecules are down-regulated in the peritoneal fluid of women with endometriosis (Ferrero *et al.*, 2009) and a decrease in inflammatory and angiogenic responses can be observed (Khan *et al.*, 2010). Recent studies show that NF-κB-mediated gene transcription promotes inflammation, invasion, angiogenesis and cell proliferation of endometriotic cells. Inhibitors of this pathway could have positive effects in the treatment of endometriosis and a synergistic effect with F8-IL10 is possible. Dienogest, a selective progesterone receptor agonist and a promising therapeutic option, inhibits prostaglandin E_2 production (Shimizu *et al.*, 2011). A combination with F8-IL10 may be beneficial.

Recombinant human IL10 (Tenovil™) has been extensively tested in patients with inflammatory disorders and has exhibited an excellent tolerability profile at doses up to 25 μg/kg (Huhn *et al.*, 1996; Rosenblum *et al.*, 2002). It 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. Therapeutic efficacy has been observed in Phase I/II clinical trials in rheumatoid arthritis and psoriasis patients. The clinical development of Tenovil™ was discontinued because of insufficient efficacy of the compound in humans. Up to now, IL10 has not been investigated in endometriosis.

F8-IL10 has exhibited an excellent safety profile in rodents and cynomolgus monkeys and is currently in Phase I clinical development in combination with methotrexate for the treatment of patients with rheumatoid arthritis who have failed at least two lines of biological treatment (Schwager *et al.*, 2009).

In summary, we have shown that the F8 antibody appears to be a suitable tool for the pharmacodelivery of bioactive moieties to sites of endometriosis *in vivo*, which should facilitate imaging and therapy for the disease. Furthermore, the F8 antibody can now be considered as a 'building block' for the development of additional antibody derivatives and for the implementation of pharmacodelivery strategies in endometriosis.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

Authors' roles

Conflict of interest

Funding

References

Berndorff D, Borkowski S, Sieger S, Rother A, Friebe M, Viti F, Hilger CS, Cyr JE, Dinkelborg LM. Radioimmunotherapy of solid tumors by targeting extra domain B fibronectin: identification of the best-suited radioimmunoconjugate. *Clin Cancer Res* 2005;**11**:7053s–7063s.

Birchler M, Neri G, Tarli L, Halin C, Viti F, Neri D. Infrared photodetection for the *in vivo* localisation of phage-derived antibodies directed against angiogenic markers. *J Immunol Methods* 1999a;**231**:239–248.

Birchler M, Viti F, Zardi L, Spiess B, Neri D. Selective targeting and photocoagulation of ocular angiogenesis mediated by a phage-derived human antibody fragment. *Nat Biotechnol* 1999b;**17**:984–988.

Borsi L, Balza E, Bestagno M, Castellani P, Carnemolla B, Biro A, Leprini A, Sepulveda J, Burrone O, Neri D et al. Selective targeting of tumoral vasculature: comparison of different formats of an antibody (L19) to the ED-B domain of fibronectin. *Int J Cancer* 2002;**102**:75–85.

Borsi L, Balza E, Carnemolla B, Sassi F, Castellani P, Berndt A, Kosmehl H, Biro A, Siri A, Orecchia P et al. Selective targeted delivery of TNFalpha to tumor blood vessels. *Blood* 2003;**102**:4384–4392.

Brack SS, Silacci M, Birchler M, Neri D. Tumor-targeting properties of novel antibodies specific to the large isoform of tenascin-C. *Clin Cancer Res* 2006;**12**:3200–3208.

Carnemolla B, Balza E, Siri A, Zardi L, Nicotra MR, Bigotti A, Natali PG. A tumor-associated fibronectin isoform generated by alternative splicing of messenger RNA precursors. *J Cell Biol* 1989;**108**:1139–1148.

Carnemolla B, Borsi L, Balza E, Castellani P, Meazza R, Berndt A, Ferrini S, Kosmehl H, Neri D, Zardi L. Enhancement of the antitumor properties of interleukin-2 by its targeted delivery to the tumor blood vessel extracellular matrix. *Blood* 2002;**99**:1659–1665.

Chiquet-Ehrismann R, Chiquet M. Tenascins: regulation and putative functions during pathological stress. *J Pathol* 2003;**200**:488–499.

D'Antonio M, Martelli F, Peano S, Papoian R, Borrelli F. Ability of recombinant human TNF binding protein-1 (r-hTBP-1) to inhibit the development of experimentally-induced endometriosis in rats. *J Reprod Immunol* 2000;**48**:81–98.

D'Hooghe TM, Nugent NP, Cuneo S, Chai DC, Deer F, Debrock S, Kyama CM, Mihalyi A, Mwenda JM. Recombinant human TNFRSF1A (r-hTBP1) inhibits the development of endometriosis in baboons: a prospective, randomized, placebo- and drug-controlled study. *Biol Reprod* 2006;**74**:131–136.

Derman AY, Sperling D, Merav A, Jain VR, Levin M, Jana S, Haramati LB. Endometrioma presenting as a cavitary lung mass with intense 18F-FDG uptake on PET-CT. *J Thorac Imaging* 2007;**22**:172–175.

Dietrich T, Atrott K, Stawowy P, Menssen H, Willuda J, Menrad A, Fleck E, Graf K. Targeted immunocytokine therapy with interleukin-2 reduces plaque formation in aortic lesions from ApoE-deficient mice(abstr). *Eur Heart J* 2007;**28**(Suppl):746–747.

Fedele L, Somigliana E, Frontino G, Benaglia L, Vigano P. New drugs in development for the treatment of endometriosis. *Expert Opin Investig Drugs* 2008;**17**:1187–1202.

Ferrero S, Gillott DJ, Remorgida V, Anserini P, Ragni N, Grudzinskas JG. GnRH analogue remarkably down-regulates inflammatory proteins in peritoneal fluid proteome of women with endometriosis. *J Reprod Med* 2009;**54**:223–231.

Folli S, Westermann P, Braichotte D, Pelegrin A, Wagnieres G, van den Bergh H, Mach JP. Antibody-indocyanin conjugates for immunophotodetection of human squamous cell carcinoma in nude mice. *Cancer Res* 1994;**54**:2643–2649.

Frey K, Schliemann C, Schwager K, Giavazzi R, Johannsen M, Neri D. The immunocytokine F8-IL2 improves the therapeutic performance of sunitinib in a mouse model of renal cell carcinoma. *J Urol* 2010;**184**:2540–2548.

Gafner V, Trachsel E, Neri D. An engineered antibody-interleukin-12 fusion protein with enhanced tumor vascular targeting properties. *Int J Cancer* 2006;**119**:2205–2212.

Gillies SD, Lan Y, Wesolowski JS, Qian X, Reisfeld RA, Holden S, Super M, Lo KM. Antibody-IL-12 fusion proteins are effective in SCID mouse models of prostate and colon carcinoma metastases. *J Immunol* 1998;**160**:6195–6203.

Giudice LC, Kao LC. Endometriosis. *Lancet* 2004;**364**:1789–1799.

Gutowski M, Carcenac M, Pourquier D, Larroque C, Saint-Aubert B, Rouanet P, Pelegrin A. Intraoperative immunophotodetection for radical resection of cancers: evaluation in an experimental model. *Clin Cancer Res* 2001;**7**:1142–1148.

Hadfield R, Mardon H, Barlow D, Kennedy S. Delay in the diagnosis of endometriosis: a survey of women from the USA and the UK. *Hum Reprod* 1996;**11**:878–880.

Halin C, Rondini S, Nilsson F, Berndt A, Kosmehl H, Zardi L, Neri D. Enhancement of the antitumor activity of interleukin-12 by targeted delivery to neovasculature. *Nat Biotechnol* 2002;**20**:264–269.

Huang TH, Chintalacharuvu KR, Morrison SL. Targeting IFN-alpha to B cell lymphoma by a tumor-specific antibody elicits potent antitumor activities. *J Immunol* 2007;**179**:6881–6888.

Huhn RD, Radwanski E, O'Connell SM, Sturgill MG, Clarke L, Cody RP, Affrime MB, Cutler DL. Pharmacokinetics and immunomodulatory properties of intravenously administered recombinant human interleukin-10 in healthy volunteers. *Blood* 1996;**87**:699–705.

Husby GK, Haugen RS, Moen MH. Diagnostic delay in women with pain and endometriosis. *Acta Obstet Gynecol Scand* 2003;**82**:649–653.

Jeffry L, Kerrou K, Camatte S, Metzger U, Lelievre L, Talbot JN, Lecuru F. Endometriosis with FDG uptake on PET. *Eur J Obstet Gynecol Reprod Biol* 2004;**117**:236–239.

Kaspar M, Trachsel E, Neri D. The antibody-mediated targeted delivery of interleukin-15 and GM-CSF to the tumor neovasculature inhibits tumor growth and metastasis. *Cancer Res* 2007;**67**:4940–4948.

Khan KN, Kitajima M, Hiraki K, Fujishita A, Sekine I, Ishimaru T, Masuzaki H. Changes in tissue inflammation, angiogenesis and apoptosis in endometriosis, adenomyosis and uterine myoma after GnRH agonist therapy. *Hum Reprod* 2010;**25**:642–653.

King DM, Albertini MR, Schalch H, Hank JA, Gan J, Surfus J, Mahvi D, Schiller JH, Warner T, Kim K *et al.* () Phase I clinical trial of the immunocytokine EMD 273063 in melanoma patients. *J Clin Oncol* 2004;**22**:4463–4473.

Laschke MW, Menger MD. *In vitro* and *in vivo* approaches to study angiogenesis in the pathophysiology and therapy of endometriosis. *Hum Reprod Update* 2007;**13**:331–342.

Marlind J, Kaspar M, Trachsel E, Sommavilla R, Hindle S, Bacci C, Giovannoni L, Neri D. Antibody-mediated delivery of interleukin-2 to the stroma of breast cancer strongly enhances the potency of chemotherapy. *Clin Cancer Res* 2008;**14**:6515–6524.

Matter CM, Schuler PK, Alessi P, Meier P, Ricci R, Zhang D, Halin C, Castellani P, Zardi L, Hofer CK *et al.* Molecular imaging of atherosclerotic plaques using a human antibody against the extra-domain B of fibronectin. *Circ Res* 2004;**95**:1225–1233.

McLaren J, Prentice A, Charnock-Jones DS, Smith SK. Vascular endothelial growth factor (VEGF) concentrations are elevated in peritoneal fluid of women with endometriosis. *Hum Reprod* 1996;**11**:220–223.

Neri D, Bicknell R. Tumour vascular targeting. *Nat Rev Cancer* 2005;**5**:436–446.

Neri D, Carnemolla B, Nissim A, Leprini A, Querze G, Balza E, Pini A, Tarli L, Halin C, Neri P *et al.* Targeting by affinity-matured recombinant antibody fragments of an angiogenesis associated fibronectin isoform. *Nat Biotechnol* 1997;**15**:1271–1275.

Nilsson F, Kosmehl H, Zardi L, Neri D. Targeted delivery of tissue factor to the ED-B domain of fibronectin, a marker of angiogenesis, mediates the infarction of solid tumors in mice. *Cancer Res* 2001;**61**:711–716.

Nisolle M, Casanas-Roux F, Anaf V, Mine JM, Donnez J. Morphometric study of the stromal vascularization in peritoneal endometriosis. *Fertil Steril* 1993;**59**:681–684.

Ntziachristos V, Yodh AG, Schnall M, Chance B. Concurrent MRI and diffuse optical tomography of breast after indocyanine green enhancement. *Proc Natl Acad Sci USA* 2000;**97**:2767–2772.

Pedretti M, Rancic Z, Soltermann A, Herzog BA, Schliemann C, Lachat M, Neri D, Kaufmann PA. Comparative immunohistochemical staining of atherosclerotic plaques using F16, F8 and L19: Three clinical-grade fully human antibodies. *Atherosclerosis* 2010a;**208**:382–389.

Pedretti M, Verpelli C, Marlind J, Bertani G, Sala C, Neri D, Bello L. Combination of temozolomide with immunocytokine F16-IL2 for the treatment of glioblastoma. *Br J Cancer* 2010b;**103**:827–836.

Pini A, Viti F, Santucci A, Carnemolla B, Zardi L, Neri P, Neri D. Design and use of a phage display library. Human antibodies with subnanomolar affinity against a marker of angiogenesis eluted from a two-dimensional gel. *J Biol Chem* 1998;**273**:21769–21776.

Rice VM. Conventional medical therapies for endometriosis. *Ann NY Acad Sci* 2002;**955**:343–352; discussion 389–393, 396–406.

Rosenblum IY, Johnson RC, Schmahai TJ. Preclinical safety evaluation of recombinant human interleukin-10. *Regul Toxicol Pharmacol* 2002;**35**:56–71.

Rudin M, Weissleder R. Molecular imaging in drug discovery and development. *Nat Rev Drug Discov* 2003;**2**:123–131.

Sampson JA. Peritoneal endometriosis due to the menstrual dissemination of endometrial tissue into the peritoneal cavity. *Am J Obstet Gynecol* 1927;**14**:422–469.

Sauer S, Erba PA, Petrini M, Menrad A, Giovannoni L, Grana C, Hirsch B, Zardi L, Paganelli G, Mariani G *et al.* Expression of the oncofetal ED-B-containing fibronectin isoform in hematologic tumors enables ED-B-targeted 131I-L19SIP radioimmunotherapy in Hodgkin lymphoma patients. *Blood* 2009;**113**:2265–2274.

Schliemann C, Palumbo A, Zuberbuhler K, Villa A, Kaspar M, Trachsel E, Klapper W, Menssen HD, Neri D. Complete eradication of human B-cell lymphoma xenografts using rituximab in combination with the immunocytokine L19-IL2. *Blood* 2009;**113**:2275–2283.

Schrama D, Reisfeld RA, Becker JC. Antibody targeted drugs as cancer therapeutics. *Nat Rev Drug Discov* 2006;**5**:147–159.

Schwager K, Kaspar M, Bootz F, Marcolongo R, Paresce E, Neri D, Trachsel E. Preclinical characterization of DEKAVIL (F8-IL10), a novel clinical-stage immunocytokine which inhibits the progression of collagen-induced arthritis. *Arthritis Res Ther* 2009;**11**:R142.

Shimizu Y, Mita S, Takeuchi T, Notsu T, Mizuguchi K, Kyo S. Dienogest, a synthetic progestin, inhibits prostaglandin E$_2$ production and aromatase expression by human endometrial epithelial cells in a spheroid culture system. *Steroids* 2011;**76**:60–67.

Somigliana E, Vigano P, Rossi G, Carinelli S, Vignali M, Panina-Bordignon P. Endometrial ability to implant in ectopic sites can be prevented by interleukin-12 in a Murine model of endometriosis. *Hum Reprod* 1999;**14**:2944–2950.

Sommavilla R, Lovato V, Villa A, Sgier D, Neri D. Design and construction of a naive mouse antibody phage display library. *J Immunol Methods* 2010;**353**:31–43.

Taylor RN, Lebovic DI, Mueller MD. Angiogenic factors in endometriosis. *Ann NY Acad Sci* 2002;**955**:89–100; discussion 118, 396–406.

Tijink BM, Perk LR, Budde M, Stigter-van Walsum M, Visser GW, Kloet RW, Dinkelborg LM, Leemans CR, Neri D, van Dongen GA. (124)I-L19-SIP for immuno-PET imaging of tumour vasculature and guidance of (131)I-L19-SIP radioimmunotherapy. *Eur J Nucl Med Mol Imaging* 2009;**36**:1235–1244.

Trachsel E, Bootz F, Silacci M, Kaspar M, Kosmehl H, Neri D. Antibody-mediated delivery of IL-10 inhibits the progression of established collagen-induced arthritis. *Arthritis Res Ther* 2007a;**9**:R9.

Trachsel E, Kaspar M, Bootz F, Detmar M, Neri D. A human mAb specific to oncofetal fibronectin selectively targets chronic skin inflammation in vivo. *J Invest Dermatol* 2007b;**127**:881–886.

Vignali DA. Multiplexed particle-based flow cytometric assays. *J Immunol Methods* 2000;**243**:243–255.

Villa A, Trachsel E, Kaspar M, Schliemann C, Sommavilla R, Rybak JN, Rosli C, Borsi L, Neri D. A high-affinity human monoclonal antibody specific to the alternatively spliced EDA domain of fibronectin efficiently targets tumor neo-vasculature in vivo. *Int J Cancer* 2008;**122**:2405–2413.

Wan S, Parrish JA, Anderson RR, Madden M. Transmittance of nonionizing radiation in human tissues. *Photochem Photobiol* 1981;**34**:679–681.

Zardi L, Carnemolla B, Siri A, Petersen TE, Paolella G, Sebastio G, Baralle FE. Transformed human cells produce a new fibronectin isoform by preferential alternative splicing of a previously unobserved exon. *EMBO J* 1987;**6**:2337–2342.