J Mol Med (2010) 88:267–278
DOI 10.1007/s00109-009-0559-8

ORIGINAL ARTICLE

Neuroendocrine circuitry and endometriosis: progesterone derivative dampens corticotropin-releasing hormone-induced inflammation by peritoneal cells in vitro

Nadja Tariverdian · Mirjam Rücke · Julia Szekeres-Bartho · Sandra M. Blois · Eva F. Karpf · Peter Sedlmayr · Burghard F. Klapp · Heribert Kentenich · Friederike Siedentopf · Petra C. Arck

Received: 12 May 2009 / Revised: 21 October 2009 / Accepted: 22 October 2009 / Published online: 8 November 2009
© Springer-Verlag 2009

Abstract Clinical symptoms of endometriosis, such as pain and infertility, can be described as persistent stressors. Such continuous exposure to stress may severely affect the equilibrium and bidirectional communication of the endocrine and immune system, hereby further aggravating the progression of endometriosis. In the present study, we aimed to tease apart mediators that are involved in the stress response as well as in the progression of endometriosis. Women undergoing diagnostic laparoscopy due to infertility were recruited ($n=69$). Within this cohort, early stage of endometriosis were diagnosed in $n=30$ and advanced stage of endometriosis in $n=8$. Levels of progesterone in serum were determined. Frequency of progesterone receptor (PR) expression on $CD56^+$ and $CD8^+$ peritoneal lymphocytes was analysed by flow cytometry. The production of tumour necrosis factor (TNF) and interleukin (IL)-10 by peritoneal leukocytes upon stimulation with the potent stress mediator corticotropin-releasing hormone (CRH) and the progesterone derivative dydrogesterone, or both, were evaluated. Furthermore, the production of progesterone-induced blocking factor (PIBF) by peritoneal leukocytes and the expression of PR in endometriotic tissue were investigated. Levels of progesterone in serum were decreased in women with endometriosis and inversely correlated to pain scores. Furthermore, an increased frequency of $CD56^+PR^+$ and $CD8^+PR^+$ peritoneal lymphocytes was present in advanced endometriosis. The TNF/IL-10 ratio, reflecting cytokine secretion by peritoneal cells, was higher in cells derived from endometriosis patients and could be further heightened by CRH stimulation, whereas stimulation with dydrogesterone abrogated the CRH-mediated inflammation. Finally, the expression of PIBF by peritoneal leukocytes was increased in endometriosis. Low levels of progesterone in the follicular phase could be responsible for the progression of endometriosis and related pain. Peripheral CRH, increasing upon high psychological stress, might contribute to the peritoneal inflammation present in endometriosis. The therapeutic

Friederike Siedentopf and Petra C. Arck jointly supervised this work.

N. Tariverdian · M. Rücke · S. M. Blois · B. F. Klapp ·
P. C. Arck (✉)
Center of Internal Medicine and Dermatology,
Division of PsychoNeuroImmunology, Charité,
Universitätsmedizin Berlin,
Campus Virchow, Forum 4, Raum 2.0549,
Augustenburger Platz 1,
13353 Berlin, Germany
e-mail: petra.arck@charite.de

J. Szekeres-Bartho
Department of Medical Microbiology and Immunology,
Pecs University Medical School,
Pecs, Hungary

E. F. Karpf
Institute of Pathology, Medical University of Graz,
Graz, Austria

P. Sedlmayr
Institute of Cell Biology, Histology and Embryology,
Center of Molecular Medicine, Medical University of Graz,
Graz, Austria

H. Kentenich · F. Siedentopf
Department of Obstetrics and Gynaecology,
DRK-Kliniken Westend,
Berlin, Germany

P. C. Arck
Department of Medicine, Brain Body Institute,
McMaster University,
Hamilton, Canada

application of progesterone derivatives, CRH blocking agents as well as improvement of stress coping may disrupt the vicious circle between the chronic peritoneal inflammation and high perception of psychological stress in endometriosis.

Keywords Stress · Sickness behaviour · Progesterone · PIBF · Pain · Peritoneal inflammation · Steroids · Endometriosis

Introduction

Endometriosis is a chronic gynaecological disease. It affects up to 22% of women in their reproductive age [1]. Morphologically, it is characterised by endometrial glands and stroma outside the uterine cavity. Clinical symptoms include severe chronic pain and infertility which interfere with social life, sexuality, psychological well-being and, hence, quality of life. Women with endometriosis report an enhanced stress perception and are more likely to suffer from depression, introversion and anxiety [2–4]. Therefore, the clinical symptoms of endometriosis are viewed as persistent stressors.

Historically, endometriosis has been considered as an oestrogen-dependant disease since estradiol seems to stimulate endometrial proliferation. Clinical studies revealed an improvement of endometriosis-related symptoms during pregnancy, which gave rise to treatments exerting anti-proliferative effects by inducing a pseudo-pregnancy regime, i.e. the application of synthetically produced progestins [5]. A relative or absolute progesterone deficiency or failure of progesterone to appropriately regulate gene expression during endometrial differentiation has also been proposed to be relevant in endometriosis [6]. Intriguingly, progesterone and progestins also alleviate endometriosis-associated pain [7, 8].

A large body of data suggests a pivotal involvement of the peritoneal immune surveillance in the pathogenesis of endometriosis, such as an impaired activation or function of peritoneal cells such as natural killer (NK) cells, macrophages and T lymphocytes [9–12]. These cells of the innate and adaptive immune system are possible targets of progesterone. For example, $CD8^+$ lymphocytes have been proven to be necessary for actions of the progesterone derivative dydrogesterone [13], and peripheral $CD8^+$ blood lymphocytes positive for the progesterone receptor (PR) were proposed to be involved in successful pregnancy [14]. Intriguingly, progesterone has been shown to increase NK cell activity in experimental endometriosis [15].

It is well established that high perception of psychological stress may trigger the incidence or exacerbation of inflammatory diseases ranging from inflammatory bowel disease [16], immune dermatosis [17] and pregnancy complications such as spontaneous abortion and preeclampsia [18]. High levels of perceived stress activate the hypothalamus–pituitary–adrenal (HPA) axis and the release of neurohormones such as corticotropin-releasing hormone (CRH) [19]. Peripheral CRH, which increases upon psychological stress [20], also exerts potent pro-inflammatory actions, as demonstrated, e.g. in the context of intestinal inflammation [21].

Additional to defective cytotoxicity of peritoneal immune cells, inflammation plays a key role in endometriosis. Pro-inflammatory cytokines such as the tumour necrosis factor (TNF), interleukin (IL)-1, but also IL-6—produced in great quantities by peritoneal macrophages in endometriosis [22, 23]—perpetuate pain perception, angiogenesis and exert embryotoxic effects [24, 25]. Intriguingly, CRH is highly expressed in endometriotic lesions [26] and the CRH binding protein is increased in peritoneal fluid in endometriosis [27]. Urocortin, a member of the CRH family of peptides, was also found increased in plasma and cyst contents in ovarian endometriosis [28].

Although high perception of stress and stress-related anxiety are known to be strongly associated with inflammatory cytokines in healthy humans [29], it remains to be elucidated whether psychological stress and up-regulation of CRH can be made attributable for the peritoneal inflammation present in endometriosis.

Mechanisms of dampening inflammation have been demonstrated, e.g. for the progesterone derivative dydrogesterone [13, 30, 31]. The progesterone-induced blocking factor (PIBF) has been identified as one factor mediating the immunosuppressive effects of progesterone. Originally demonstrated in $CD8^+$ T cells [32], PIBF is produced by a variety of tumour cell lines, e.g. SRIK-NKL, a $CD8^+$ NK/T cell line; its production is increased by progesterone receptor-dependent pathways [33], and it blocks NK cells [34].

The specific aims of the present study were to compare serum and peritoneal fluid from infertile patients without or with endometriosis (stages I+II and III+IV, respectively) as well as endometriotic tissue in order to evaluate:

(a) if levels of progesterone in serum vary between groups,
(b) if frequencies of $CD56^+PR^+$ or $CD8^+PR^+$ peritoneal lymphocytes differ between the respective groups,
(c) if cytokine profiles of peritoneal leukocytes change upon stimulation with CRH, representative of the stress-related neuroendocrine system,
(d) if the progesterone derivative dydrogesterone is able to counteract the effects of CRH on cytokine production,
(e) if levels of PIBF produced by peritoneal leukocytes fluctuate between the groups,

(f) if endometriotic cells express the PR and if this expression differs between early and advanced endometriosis.

Materials and methods

Study population, collection of peritoneal fluids, endometriotic tissue and peripheral blood

Women undergoing diagnostic laparoscopy due to infertility, defined as the absence of pregnancy despite the wish of having a child and regular sexual intercourse for a duration of 1 year, were included in the present study (Fig. 1). In most cases, pelvic pain was an additional indication for laparoscopy. Women during the luteal phase of the menstrual cycle, taking hormonal or immunosuppressive medication or undergoing in vitro fertilisation treatments were excluded. In total, 69 infertile women during the follicular phase of the menstrual cycle at the time of laparoscopy could be included (Fig. 1). All women had regular cycles of 28–30 days and did not receive medical treatment related to endometriosis since—based on the study design—endometriosis was first diagnosed or excluded macroscopically during laparoscopy; this diagnosis was further substantiated by histological analysis of tissue taken during laparoscopy. All women were invited to provide information with regard to their demographic, biometric data and the intensity of pain during the last 3 days or 4 weeks, respectively, using two numerical rating scales (NRS) from 0 (no pain) to 10 (the worst pain imaginable).

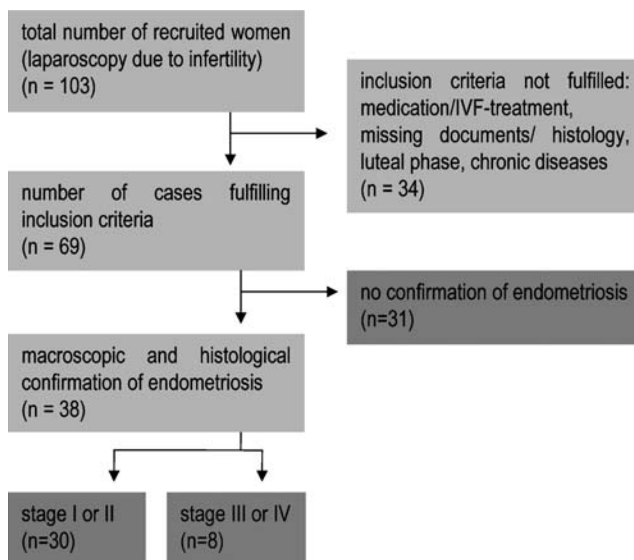

Fig. 1 Flowchart of study population. Diagnosis of endometriosis was made by macroscopic and histological confirmation. Stages were defined according to the revised ASRM classification (ASRM, 1997). *IVF* in vitro fertilisation, *ASRM* American Society for Reproductive Medicine

Laparoscopy was conducted before hysteroscopy to avoid contamination with uterine cells. Peritoneal fluid was collected after the placement of the trocars before any surgical intervention such as biopsy sampling and saved in sterile falcon tubes. If only a very small volume was present, peritoneal flushing with 10 ml saline was necessary to obtain the fluid. Biopsies of macroscopically suspect tissue were analysed microscopically by a pathologist unrelated to the research team. Additionally, one part of the tissue was preserved in sterile phosphate-buffered saline (Dulbecco's PBS, PAA Laboratories GmbH, Pasching, Austria), transferred to the laboratory together with peritoneal fluids within 2–4 h after collection and processed as described below.

According to the laparoscopic macroscopic evaluation and microscopic analysis of biopsies, patients were divided into the following subgroups: with endometriosis (n=38) and without endometriosis (n=31; Fig. 1). Endometriosis was staged according to the revised American Society of Reproductive Medicine classification [35], and endometriosis patients were further divided into the group of patients with early endometriosis at stage I or II (n=30) and the group of patients with advanced endometriosis at stage III or IV (n=8). The median of the day of menstrual cycle was 8 (5–13) in women without endometriosis, 8.5 (4–14) in women with endometriosis stage I or II and 8 (5–10) for stage III or IV, which largely excludes that the insights arising from progesterone analysis are camouflaged by the rise of progesterone near ovulation.

This study, including all analyses performed on tissues, has been approved by the local ethics committee, and written informed consent was obtained from all the patients prior to any intervention.

Determination of serum progesterone concentration

Peripheral blood was taken prior to anaesthesia then centrifuged at 400×g for 10 min at 4°C, and serum was stored at −80°C until analysis. The serum concentrations of progesterone were determined using a commercially available ELISA kit (DRG Instruments GmbH, Marburg, Germany). The assay range of the progesterone ELISA kit we used was 0–40 ng/ml, and the coefficients of variation were as follows: intra-assay, 8.3/4.6/5.2%; inter-assay, 9.9/4.8/6.5%. Samples were then read on a microtitre plate reader (at 450 nm; FLUOstar OPTIMA; BMG LABTECH, Offenburg, Germany), and progesterone concentration was calculated from the standard curve.

Clearly, it would have been desirable to obtain data on progesterone levels not only during the follicular phase but also during the luteal phase. However, women scheduled

for laparoscopy for abdominal pain and infertility routinely undergo surgery in the follicular phase of the menstrual cycle. Considering that progesterone during the luteal phase also shows a large variation, we considered the insights gained from analysing progesterone during the follicular phase as valuable.

Frequency and phenotype of PR^+ peritoneal lymphocytes

Peritoneal fluid was centrifuged at 400×*g* for 10 min at 4°C; supernatants were removed and cells were resuspended in flow cytometer buffer (Dulbecco's PBS with 1% bovine serum albumin (BSA), 5 mM EDTA and 0.1% sodium azide). Three-colour flow cytometry was performed using the following antibodies (Ab): anti-CD8APC, anti-CD56PE (BD Biosciences, San José, CA, USA), anti-PR (DAKO Denmark A/S, Glostrup, Denmark), biotinylated goat-anti-rabbit (Jackson ImmunoResearch Europe, Newmarket, Suffolk, UK) and streptavidinPerCP (BD Biosciences, San José, CA, USA) at concentrations of 1:10 for anti-PR and biotinylated Abs and 1:20 for APC-, PE- and PerCP-conjugated Abs. As a negative control, cells were stained with the corresponding isotype-matched Ab.

Cells were incubated with the respective Abs for 30 min at room temperature in the dark, thereafter incubated with 10% lysis buffer (A. dest. with 1.56 M NH_4Cl, 100 mM $KHCO_3$, 0.78 mM EDTA) to lyse red blood cells for 10 min in the dark, followed by a wash with flow cytometer buffer. Cells were fixed using CellFIX solution (BD Biosciences, Erembodegem, Belgium) and left overnight at 4°C in the dark. The next morning, cells were washed with PBS azide (Dulbecco's PBS with 0.1% sodium azide), resuspended and measured using a FACSCalibur (BD Biosciences, San José, CA, USA). Flow cytometer instrument compensation was set in each sample using single-colour stained samples. The threshold was set to exclude dead cells in the analyses. Data were analysed using CellQuest Pro software (BD Biosciences, San José, CA, USA). Examples of lymphocyte gating and histograms of the respective staining are shown in Fig. 3a–c.

In vitro stimulation of peritoneal leukocytes

In 17 samples, we performed in vitro stimulation studies to investigate the cytokine profile and PIBF production of peritoneal leukocytes. Samples were derived from women without (n=8), with early (n=8) or advanced (n=1) endometriosis. After pretesting different concentrations of CRH and dydrogesterone, finally, 10^4 cells per well were incubated on a 96-well plate (SARSTEDT, Nümbrecht, Germany) in AIMV medium (Invitrogen, Paisley, UK) containing L-glutamine, 50 µg/ml streptomycin sulphate and 10 µg/ml gentamicin sulphate, supplemented with 10% foetal calf serum (Biochrom, Berlin, Germany) and either 10^{-9} M CRH (BACHEM, Bubendorf, Switzerland), 10^{-5} M dydrogesterone (SOLVAY, Hannover, Germany), both or none of the substances. A medium control was performed for every substance to identify spontaneously released cytokine levels. Supernatants were taken after an incubation of 24 h at 37°C in a humidified atmosphere of 5% CO_2 and stored at −80°C until further analysis. Cellular death was excluded by the Trypan blue method.

Quantitative determination of cytokines in cell supernatants

Test measurements of several inflammatory and regulatory cytokines including IL-2, IL4, IL-6 and Interferon-γ revealed that TNF and IL-10 were the optimal cytokines in the samples, as their levels were consistently in the sensitivity range of cytometric bead arrays (CBA). Subsequently, levels of TNF and IL-10 were analysed using the CBA Flex Set system (BD Biosciences, San José, CA, USA). Briefly, 50 µl of each sample and standard dilution were mixed with 50 µl of mixed capture beads and incubated for 1 h at room temperature in the dark before adding 50 µl of the mixed PE detection reagent to each assay tube. After 2 h of incubation at room temperature in the dark, samples were washed once and resuspended in 300 µl of washing buffer before acquisition on a FACSCalibur cytometer (BD Biosciences, San José, CA, USA; Fig. 4a, b). Data were analysed using CBA software (BD Biosciences, San José, CA, USA). Standard curves were generated for each cytokine. The concentration of each cytokine in cell supernatants was determined from the corresponding standard curve. We further calculated the TNF/IL-10 ratio with high values representing a Th1-dominant cytokine profile. In addition, we calculated the relative changes of the TNF/IL-10 ratio in CRH-, dydrogesterone- or CRH and dydrogesterone-stimulated samples (ratio b) over the native (=unstimulated) sample (ratio a). Furthermore, the relative change of the TNF/IL-10 ratio in CRH-stimulated (ratio a) over CRH and dydrogesterone-stimulated samples (ratio b) was calculated. The following formula was used: $[(\text{ratio b} - -\text{ratio a})/\text{ratio a}] \times 100$.

Determination of PIBF concentration in cell supernatants

To determine the native amount of PIBF in the supernatants of cells incubated only with medium, we used a non-commercial ELISA. In brief, a 96-well microtiter plate was coated with 100 µl of recombinant human PIBF (rhuPIBF) 0.125 µg/ml in 0.5 M Tris buffer, pH6.5, incubated overnight at 4°C and washed three times with PBS-Tween. The rhuPIBF standard (1.24, 25, 50, 100, 1,000 ng/ml) was diluted in 0.5 M phosphate buffer (pH

7.3–7.4) and then mixed with the same volume of biotinylated anti-rhuPIBF IgG at a dilution of 1:2,500 in 0.5 M PBS buffer. Samples were diluted 1:2.5 and mixed 1:1 with 1:2,500 biotinylated anti-rhuPIBF IgG in 0.5 M PBS to achieve a final dilution of 1:5 (samples) and 1:5,000 (anti-rhuPIBF). Standards and diluted samples were incubated for 1 h at 37°C. The coated ELISA plate was washed three times with PBS-Tween and blocked with 0.1% BSA, 0.5% gelatine in PBS-Tween during 1 h at 37°C. One hundred microlitres of diluted standards and samples were added to the plate and incubated for 1 h at 37°C. After triple washing, horseradish peroxidase-conjugated streptavidin (Amersham-Pharmacia, Hungary) diluted 1:1,000 in 0.1% BSA PBS-Tween was added and incubated for 30 min at 37°C. After repeated washing, TMB solution (BD OptEIA; BD Biosciences, San José, CA, USA) was added and the reaction was terminated by 1 M H_2SO_4 30 min later. Absorbance was determined at 450 nm, and the concentrations of PIBF were calculated from the corresponding standard curve.

Immunohistochemistry of PR on endometriotic tissue

Endometriotic tissue of women with early or advanced endometriosis was stored in 10% formalin at 4°C until paraffin embedment. Paraffin sections (5 µm) of endometriotic lesions were stained for PR using automated tissue staining at the Institute for Pathology, Charité, University Medicine Berlin. Briefly, sections were incubated with anti-PR Ab (DAKO Denmark A/S, Glostrup, Denmark), a rabbit anti-human Ab which recognises both PR-A and PR-B. The Ab was diluted 1:50 before application. The signal was detected by incubating sections with Fast Red substrate. Slides were examined using a Zeiss Axioscope light microscope (Jena, Germany). Photodocumentation was performed using digital image analysis system (Zeiss KS400, Jena, Germany). Biopsies from malignant breast tumours were labelled in the same setting and served as a positive control for the staining.

Statistical analysis

SPSS 15.0 for windows was used for statistical analysis. The significance of differences between the groups was determined using the chi-square test for nominal variables or the Mann–Whitney *U* test for continuous variables, respectively. Correlations were calculated using the non-parametric Spearman test. Intra-group variation was determined with the Wilcoxon test. Significance was set at $P < 0.05$. Results are shown as medians for continuous variables or as absolute numbers (*n*) and percentages (%) for nominal variables or Spearman's correlation coefficient (*r*), respectively.

Results

Demographic and biometric evaluation

Among our study cohort of infertile women, 38 women were newly diagnosed with endometriosis (Fig. 1). The analysis of the questionnaires and clinical documentation is provided in Table 1. Women without endometriosis, with endometriosis at stages I and II and at stages III and IV did not show significant differences with regard to age, body mass index (BMI) and socioeconomic or medical factors. Pelvic pain perception was reported as higher by women suffering from endometriosis compared to women without endometriosis. However, these differences did not reach levels of significance.

Low levels of progesterone in endometriosis

Levels of progesterone in serum were decreased in women with early and advanced endometriosis (Fig. 2); the latter stage reached levels of significance compared to women without endometriosis.

Inverse correlation between progesterone levels and pain scores in advanced endometriosis

Correlations between serum progesterone and pain scores are shown in Table 2. In women without and with early endometriosis, there were no significant correlations between levels of progesterone and pain during the last 3 days or 4 weeks. However, in women with advanced stages of endometriosis, levels of progesterone were inversely correlating with pain perception.

High frequency of PR^+ cells among peritoneal lymphocytes in advanced endometriosis

Analysis of peritoneal lymphocytes by flow cytometry, for which exemplary plots and histograms are given in Fig. 3a–e, revealed a higher frequency of PR^+ cells in women with advanced endometriosis compared to early stages or women without endometriosis (Fig. 3f). A co-expression of $CD56^+$ (Fig. 3g) and $CD8^+$ (Fig. 3h) on PR^+ peritoneal lymphocytes could further be identified.

Inflammatory cytokine profile is more prominent in endometriosis

The TNF/IL-10 ratio—analysed by CBA, as exemplarily shown in Fig. 4a, b—was higher in endometriosis than in women without endometriosis under in vitro conditions, mirroring a dominance of TNF over IL-10 production in peritoneal cells derived from patients with endometriosis (Fig. 4c). It should be noted that endometriosis patients of stages

Table 1 Comparison of demographic, anamnestic and biological parameters at time of recruitment between eligible study participants without endometriosis, with endometriosis at stage I or II and endometriosis at stage III or IV

Parameter		No Signs of Endometriosis	Endometriosis stage I+II	Endometriosis stage III+IV
Age (years)	Median	35.5	36.5	35.0
	Available data (*n*)	30	30	8
BMI (kg/m^2)	Median	22.95	21.59	22.10
	Available data (*n*)	29	30	8
Education[a]	*N* (%)	25 (89.3)	29 (96.7)	7 (87.5)
	Available data (*n*)	28	30	8
Work	*N* (%)	25 (83.3)	26 (89.7)	8 (100.0)
	Available data (*n*)	30	29	8
Living with partner	*N* (%)	29 (96.7)	27 (90.0)	6 (75.0)
	Available data (*n*)	30	30	8
Chronic disease[b]	*N* (%)	4 (12.9)	6 (20.0)	2 (25.0)
	Available data (*n*)	31	30	8
Smoking habits[c]	*N* (%)	12 (40.0)	5 (16.7)	1 (12.5)
	Available data (*n*)	30	30	8
Pelvic pain[d]	*N* (%)	4 (12.9)	7 (23.3)	4 (50.0)
	Available data (*n*)	31	30	8
Pain score (3 days)[e]	Median	0.5	0	0
	Available data (*n*)	30	30	8
Pain score (4 weeks)[e]	Median	3	3	5
	Available data (*n*)	29	30	8

Results are shown as medians for continuous variables or as absolute numbers (*n*) and percentages (%) for nominal variables, respectively. Inter-group differences of all parameters were not statistically significant

BMI body mass index, *NRS* numerical rating scale

[a] As defined >10 years at school

[b] Such as hypothyroidism, insulin-dependent diabetes mellitus, atopic diseases, irritable bowel syndrome (note: a significant effect on the parameters investigated was excluded)

[c] As defined more than two cigarettes per day

[d] Diagnosis as indication for laparoscopy

[e] As reported by NRS scores

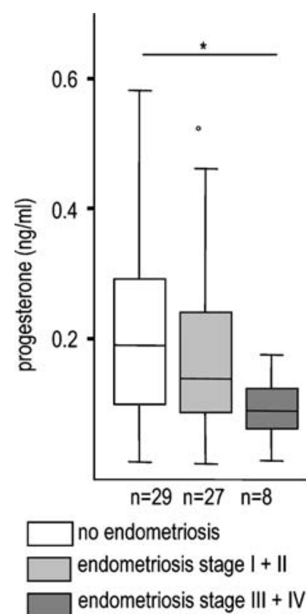

Fig. 2 Levels of progesterone in serum. Levels of serum progesterone in women without endometriosis and at stages I and II or stages III and IV of endometriosis. Results are shown as *box plots* with outliers (*circle*). Medians are 0.19/0.14/0.09 ng/ml for women without, with stage I and II or stage III and IV of endometriosis, respectively. *$P<0.05$

I–IV, respectively, were merged in one group due to the small sample size of stages III and IV. Single cytokine production was not significantly different between the two groups after stimulation with CRH, dydrogesterone or both (Table 3).

Dydrogesterone dampens the CRH-induced TNF>IL-10 skew in endometriosis samples

In both patients with and without endometriosis, we observed an increase of the TNF/IL-10 ratio upon CRH stimulation, indicating a TNF>IL-10 skew, which was dampened when dydrogesterone was added to the culture (Fig. 4c).

Relative changes of the TNF/IL-10 ratio upon stimulation with CHR and dydrogesterone

In order to further test the biological significance of the variation from basal (native, unstimulated) levels seen

Table 2 Correlation between serum progesterone and pain scores (pain during the last 3 days or 4 weeks, respectively) in eligible study participants without endometriosis, with endometriosis at stage I or II and endometriosis at stage III or IV

Parameters		No signs of endometriosis	Endometriosis stage I+II	Endometriosis stage III+IV
Progesterone/Pain (3 days)[a]	*r*	−0.248	−0.064	−0.518
	Available data (*n*)	28	27	8
Progesterone/Pain (4 weeks)[a]	*r*	−0.148	0.101	−0.766[b]
	Available data (*n*)	27	27	8

Spearman's correlation coefficient (*r*) of each correlation is shown

NRS numerical rating scale

[a] As reported by NRS scores

[b] Equals $P<0.05$

with regard to the TNF/IL-10 ratio in the various groups and in vitro conditions , we calculated the relative changes of the TNF/IL-10 ratios upon stimulation with CRH, dydrogesterone or both vs. native (=unstimulated) samples. Furthermore, the relative changes induced by CRH stimulation alone compared to the effect of CRH in the presence of dydrogesterone were tested (Fig. 4d). This approach confirmed the findings displayed in Fig. 4c, as stimulation in the CRH-induced inflammatory response (relative change of TNF/IL-10 ratio >1) was abrogated in samples co-stimulated with dydrogesterone (relative change of TNF/IL-10 ratio <1). This effect was particularly profound in samples from patients with endometriosis.

High production of PIBF by peritoneal lymphocytes in endometriosis

Levels of PIBF secreted by peritoneal cells in vitro were higher in endometriosis as compared to women without

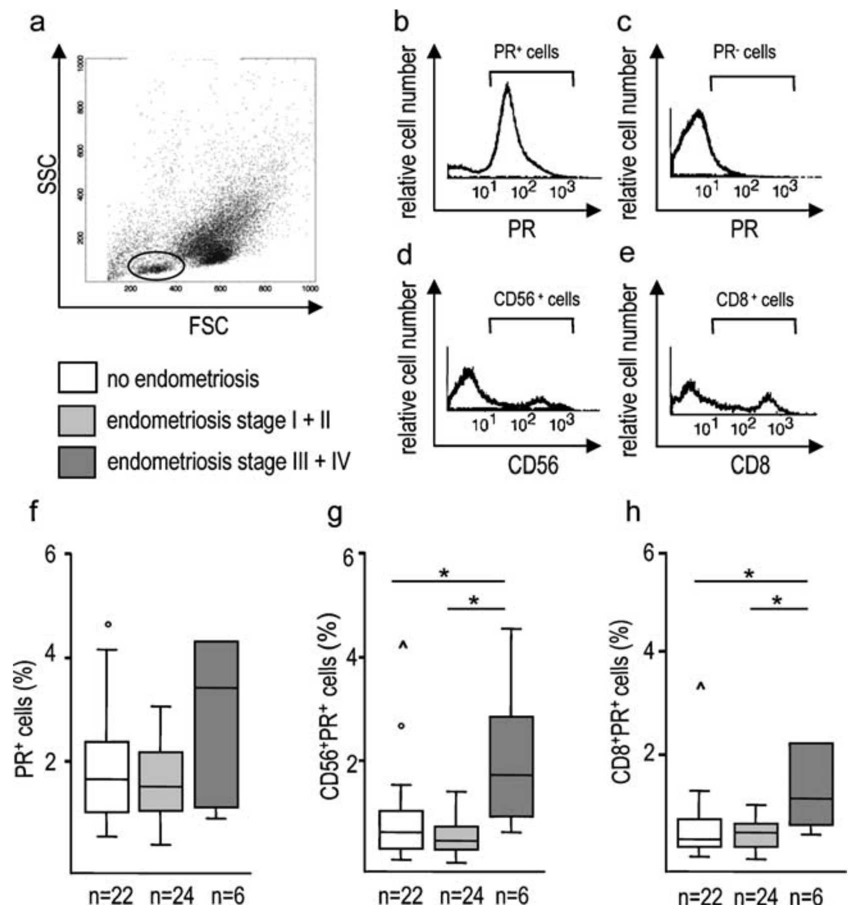

Fig. 3 Progesterone receptor on peritoneal lymphocytes. Representative examples of flow cytometry on peritoneal lymphocytes. Lymphocyte scatter gate was set as shown in **a**; histograms showing results from PR^+ cell labelling (**b**), the respective isotype control (**c**). Cell surface staining for $CD56^+$ is shown in **d**, for $CD8^+$ cells in **e**, staining for PR^+ is depicted in **f**. The frequency of $CD56^+PR^+$ cells is displayed in **g** and of $CD8^+PR^+$ cells in **h**. Frequency is expressed as percentages (%) of all lymphocytes. Results are shown as *box plots* with outliers (*circle*) and extremes (*caret*). Medians are 1.6/1.5/3.4% (**f**), 0.6/0.5/1.7% (**g**) and 0.4/0.5/1.2% (**h**) for women without, with stage I and II or stage III and IV of endometriosis, respectively. *$P<0.05$. *SSC* side scatter, *FSC* forward scatter, *PR* progesterone receptor, *CD* cluster of differentiation

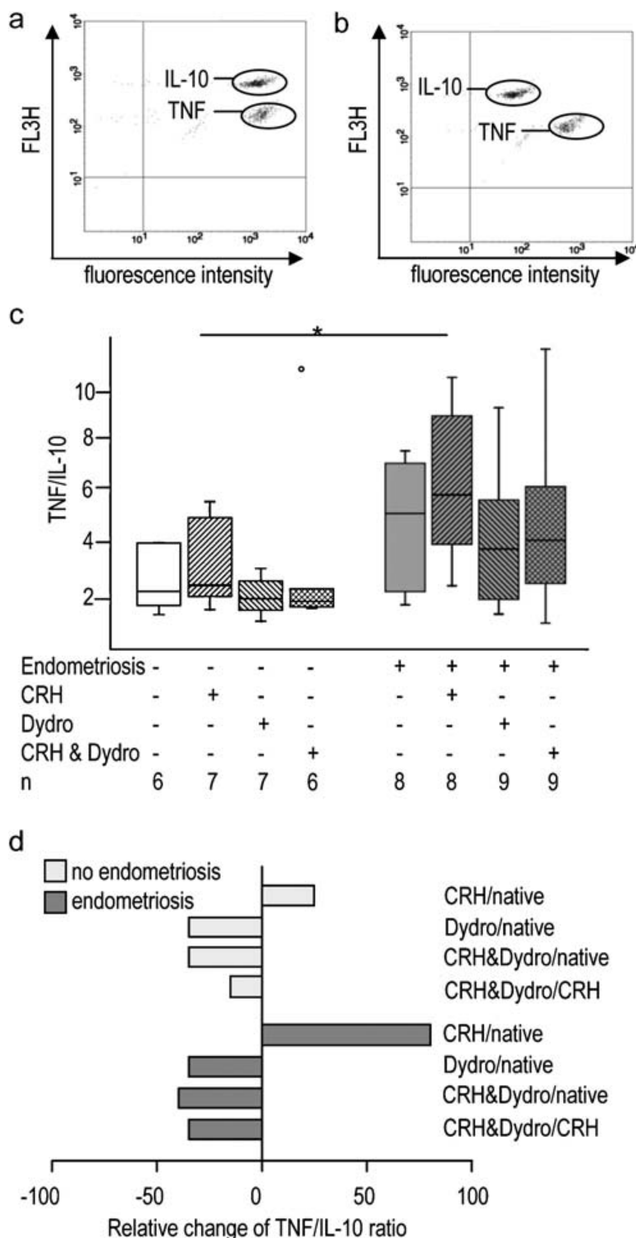

Fig. 4 Effect of CRH and dydrogesterone in vitro stimulation on cytokine secretion of peritoneal leukocytes. Cytokine secretion of peritoneal leukocytes was analysed by CBA flex; representative examples of the acquisition of TNF and IL-10 are shown in **a**, **b**. Peritoneal leukocytes were either unstimulated, stimulated with CRH, dydrogesterone or both. **c** The ratio of TNF to IL-10 cytokine levels are shown in *box plots* with outliers (*circle*). Medians are 2.1/5.2 (native), 2.3/5.9 (CRH), 1.8/3.8 (dydrogesterone) and 1.7/4.2 (CRH and dydrogesterone) for women without or with endometriosis, respectively. *$P<0.05$. It should be noted that one sample of advanced endometriosis (III+IV) was integrated in these analyses; the rest of the tissue was obtained from patients with endometriosis at stages I+II. Exclusion of the sample from advanced endometriosis did not change the overall outcome of the experiment. **d** Percent changes (%) of the native=unstimulated TNF/IL-10 ratio in samples deriving from women without or with endometriosis upon stimulation with CRH was 21.6/78.6, upon stimulation with dydrogesterone was 32.6/33.0 and CRH plus dydrogesterone was 32.8/37.1. Percent changes of the TNF/IL-10 ratio under CRH upon stimulation with CRH plus dydrogesterone was 14.5/32.7. *TNF* tumour necrosis factor, *IL* interleukin, *CBA* cytometric bead array, *CRH* corticotropin releasing hormone

endometriosis; however, levels of significance were not reached (Fig. 5a).

Expression of PR on endometriotic tissue

Next, we wished to analyse the expression of the PR on endometriotic lesions taken during laparoscopy. However, quantification of the overall PR expression was camouflaged by the tissue heterogeneity and, at least in some cases, very small amounts of material; therefore, we refrained from an in-depth analysis of the expression of PR subtypes. We could observe an intense expression of PR throughout the samples of which representative examples are shown in Fig. 5b, c showing H&E staining used for orientation.

Discussion

In our cohort of 69 infertile women, 55.1% were diagnosed with endometriosis. This high prevalence is in line with a previous study revealing that 68% of infertile women additionally suffer from endometriosis [36].

Serum progesterone levels in endometriosis patients were decreased as compared to women without endometriosis, particularly at advanced stages, whereby none of these women was under any treatment with steroid hormones. Although highly speculative and currently not supported by published evidence, it may be conceivable that these low levels of progesterone could be responsible for the progression of endometriosis to advanced stages, e.g. by prolonging the proliferative phase in the ectopic tissue due to a relative predominance of oestrogen. However, to further clarify the role of progesterone and estrogens in endometriosis, more research including patients in the luteal phase of the menstrual cycle as well as experimental studies of the effects of progesterone and estrogens on endometriotic tissue would be required.

Low levels of late luteal progesterone in endometriosis were shown in earlier studies [37], although fertile women served as controls in that investigation. In the present study, we could show that levels of progesterone in all study participants—with and without endometriosis—were overall lower than levels of progesterone in healthy women during the follicular phase of the menstrual cycle, which are 0.2–1.4 ng/ml. This suggests a role for progesterone in infertility independent of endometriosis. However, serial measurements of progesterone are recommended due to intra-individual variations to sustain such suggestion, which could not be performed in the setting of this study.

Table 3 Individual cytokine levels for TNF and IL-10 in supernatants after in vitro culture of peritoneal cells

Cytokine levels (pg/ml)	CRH in culture	Dydro in culture	No signs of endometriosis	Endometriosis
TNF	−	−	5.4	13.8
	+	−	4.9	10.9
	−	+	7.3	10.8
	+	+	5.1	11.3
IL-10	−	−	2.0	2.1
	+	−	1.8	1.7
	−	+	3.7	3.0
	+	+	3.5	2.4

Cytokine levels are presented as medians. Samples from eight patients without and nine patients with endometriosis were analysed, except for the analysis of patients without endometriosis and CRH/Dydro supplementation of the culture; here, one sample was lost. Intergroup differences were not statistically significant. *TNF* tumour necrosis factor, *IL* interleukin, *CBA* cytometric bead arrays, *CRH* corticotropin releasing hormone, *Dydro* dydrogesterone

Due to tissue constraints available for hormonal analyses, we prioritised the analysis of progesterone. However, additional insights on other sexual steroids like estradiol and androgens as well as the anti-mullerian hormone as indicator for the ovarian reserve would also be of great interest. This information could clarify whether low levels of progesterone are specifically linked to endometriosis or to other hormonal changes within the patients' cohort.

In the present study, levels of progesterone were strongly inversely correlated to pain scores in advanced endometriosis, which is in line with studies indicating that progesterone is able to alleviate pain [7, 31], e.g. via down-regulation of a receptor involved in mediating pain, the neurokinin (NK)-1 receptor [31]. In this context, it would be of interest to elucidate whether the NK-1 receptor is involved in the mediation of pain in endometriosis. In the present study, high pain perception reported for the period of the last 4 weeks inversely correlated with progesterone levels. It would be desirable to gain more insights on the effects of progesterone on pelvic pain at various times of the cycle since levels of progesterone undergo considerable variations during the menstrual cycle.

We further detected an increased expression of surface PR on peritoneal lymphocytes in advanced endometriosis. It can be speculated that this is a compensatory up-regulation due to low levels of progesterone detected in serum. Unfortunately, due to the necessary peritoneal flushing in several cases, we could not assess the quantity of progesterone in peritoneal fluid, whilst all the above presented assays were not influenced by this dilution. The expression of PR on lymphocytes, such as $CD56^+$ NK cells and $CD8^+$ T cells, suggests that these cells are possible targets for progesterone. Our in vitro analyses revealed that the production of PIBF by unstimulated peritoneal leukocytes was increased in endometriosis. PIBF is known to functionally block NK cells, which may be undesirable in the context of endometriosis where activated NK cells could strengthen the immunosurveillance within the peritoneal cavity. However, this interpretation requires more studies on the production of PIBF in endometriosis and may be challenged by one study which indicates that dienogest, a synthetic steroid with progestational activity, increases NK cell activity [15].

In vitro, the TNF/IL-10 ratio was increased in endometriosis, supporting the well-established model of sterile inflammation in endometriosis which is likely to drive angiogenesis, pain and infertility. Our study could confirm an inflammatory effect of CRH on peritoneal cells, as we observed that the TNF/IL-10 ratio was increased by CRH, which in turn could be dampened when the progesterone derivative dydrogesterone was added to the culture. Dydrogestrone has previously been shown to suppress inflammation due to a predominance of anti-inflammatory cytokines [30, 38]. Not surprisingly, these effects were also present in cultures of cells from non-endometriosis patients, although at a lower scale. As previously mentioned, mast cell-derived CRH and urocortin were found at high levels

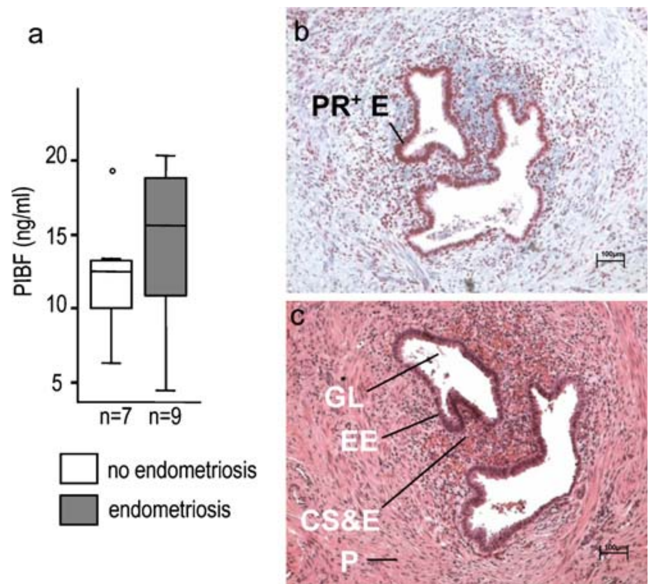

Fig. 5 PIBF levels in cell supernatant and endometrial tissue expression of progesterone receptor. Levels of PIBF in cell supernatants of unstimulated peritoneal leukocytes are shown in **a**. Results are shown as *box plots* with outliers (*circle*). Medians are 12.5/15.8 ng/ml for women without or with endometriosis, respectively. Representative example of immunohistochemical staining for PR (**b**) in endometriotic tissue (**b**). H&E staining (**c**) was used for orientation (**b**, **c**). *PIBF* progesterone-induced blocking factor, *H&E* hematoxylin–eosin, *PR*$^+$ *E* PR+ epithelial cell, *GL* glandular lumen, *EE* endometrial epithelium, *CS&E* cytogenic stroma, erythrocytes, *P* peritoneum

in endometriotic tissue [26, 28], and the increase of CRH binding protein in peritoneal fluid in endometriosis [27] may be interpreted as an attempt of the body to down-regulate high levels of peripheral CRH, derived from immunocytes and/or descending nerve fibres [39–41]. However, the results of the present study should be confirmed in a larger sample of endometriosis and non-endometriosis patients. Additionally, it should be considered that in endometriosis, inflammation is often followed by adhesions in advanced stages which are responsible for clinical symptoms like chronic pain. Thus, it would be pivotal to therapeutically dampen inflammation before clinical symptoms can occur.

The HPA axis, when activated by stress, exerts an inhibitory effect on the female reproductive system. CRH is likely to be the key hormone; it inhibits hypothalamic gonadotropin-releasing hormone secretion. Subsequently, glucocorticoids inhibit pituitary luteinising hormone and ovarian oestrogen and progesterone secretion [42]. Interestingly, levels of CRH have been shown to increase not only in the central nervous system but also in the periphery [20]. Thus, in the context of endometriosis, CRH may aggravate the inflammatory response in peritoneal cells via direct effects as well as indirectly via dampening progesterone, a potent suppressor of inflammation. To further strengthen this hypothesis, we aimed at analysing levels of CRH in peritoneal fluid; however, the sensitivity of this approach was not satisfactory.

A rising number of publications force an interdisciplinary approach to complex diseases such as endometriosis by elucidating the links and cross talks between the immune, endocrine and neuronal systems [43]. Peripheral immunological events such as high levels of pro-inflammatory cytokines can signal certain brain regions to induce the so-called sickness behaviour which comprises depressive-like behavioural patterns [44]. These effects are likely to be in parts mediated by vagal afferents [45]. Acetylcholine $(Ach)^+$ nerve fibres were found in endometriotic tissue [46]; thus, in endometriosis, pro-inflammatory cytokines might contribute to behavioural changes and enhanced stress perception by signalling from the peritoneal cavity to the brain via vagal afferents.

Before focussing approaches such as blocking CRH at the periphery, therapeutic possibilities aiming at an improvement of stress coping strategies should be better implemented in the therapy of women who are likely to benefit from such treatment. Endometriosis patients indicated a need for additional therapy strategies for improving their quality of life [47], and there is evidence that relaxation programmes can decrease stress perception and anxious depression and, intriguingly, induce a reduction of TNF, e.g. in the serum of tinnitus patients [48]. Such approaches are getting even more relevant when we keep in mind that many women suffering from endometriosis desire having a child and therefore should not be treated with substances interfering with ovulation or pregnancy maintenance.

Acknowledgements We are indebted to the support of the physicians and operation room staff at the DRK Klinikum Westend; without their enthusiasm and continuous support, it would have been impossible to perform the present study. The authors are grateful for the technical support provided by Evelin Hagen, Petra Moschansky and Petra Busse. We also wish to thank Thomas Keil for assistance with statistical analyses. This work was made possible by research grants from the Charité to PCA. This study was integrated in 'EMBIC', a Network of Excellence co-financed by the European Commission throughout the FP6 framework programme 'Life Science, Genomics and Biotechnology for Health'. NT is supported by the German Merit Foundation and a recipient of a Charité stipend. SMB is supported by the Habilitation Program of the Charité.

Conflict of interest The authors declare that they have no conflicting interests related to this study.

References

1. Moen MH, Muus KM (1991) Endometriosis in pregnant and non-pregnant women at tubal sterilization. Hum Reprod 6:699–702
2. Strauss B, Didzus A, Speidel H (1992) A study of the psychosomatic aspects of endometriosis. Psychother Psychosom Med Psychol 42:242–252
3. Low WY, Edelmann RJ, Sutton C (1993) A psychological profile of endometriosis patients in comparison to patients with pelvic pain of other origins. J Psychosom Res 37:111–116
4. Siedentopf F, Tariverdian N, Rücke M, Kentenich H, Arck PC (2008) Immune status, psychosocial distress and reduced quality of life in infertile patients with endometriosis. Am J Reprod Immunol 60:449–461
5. Prentice A, Deary AJ, Bland E (2000) Progestagens and anti-progestagens for pain associated with endometriosis. Cochrane Database Syst Rev:CD002122
6. Fujishita A, Nakane PK, Koji T, Masuzaki H, Chavez RO, Yamabe T, Ishimaru T (1997) Expression of estrogen and progesterone receptors in endometrium and peritoneal endometriosis: an immunohistochemical and in situ hybridization study. Fertil Steril 67:856–864
7. Vercellini P, Fedele L, Pietropaolo G, Frontino G, Somigliana E, Crosignani PG (2003) Progestogens for endometriosis: forward to the past. Hum Reprod Updat 9:387–396
8. Overton CE, Lindsay PC, Johal B, Collins SA, Siddle NC, Shaw RW, Barlow DH (1994) A randomized, double-blind, placebo-controlled study of luteal phase dydrogesterone (Duphaston) in women with minimal to mild endometriosis. Fertil Steril 62:701–707
9. Oosterlynck DJ, Meuleman C, Waer M, Vandeputte M, Koninckx PR (1992) The natural killer activity of peritoneal fluid lymphocytes is decreased in women with endometriosis. Fertil Steril 58:290–295
10. Braun DP, Gebel H, Rotman C, Rana N, Dmowski WP (1992) The development of cytotoxicity in peritoneal macrophages from women with endometriosis. Fertil Steril 57:1203–1210
11. Ho HN, Chao KH, Chen HF, Wu MY, Yang YS, Lee TY (1995) Peritoneal natural killer cytotoxicity and $CD25^+$ $CD3^+$ lymphocyte subpopulation are decreased in women with stage III–IV endometriosis. Hum Reprod 10:2671–2675
12. Tariverdian N, Siedentopf F, Rucke M, Blois SM, Klapp BF, Kentenich H, Arck PC (2009) Intraperitoneal immune cell status

in infertile women with and without endometriosis. J Reprod Immunol 80:80–90
13. Blois SM, Joachim R, Kandil J, Margni R, Tometten M, Klapp BF, Arck PC (2004) Depletion of $CD8^+$ cells abolishes the pregnancy protective effect of progesterone substitution with dydrogesterone in mice by altering the Th1/Th2 cytokine profile. J Immunol 172:5893–5899
14. Chiu L, Nishimura M, Ishii Y, Nieda M, Maeshima M, Takedani Y, Shibata Y, Tadokoro K, Juji T (1996) Enhancement of the expression of progesterone receptor on progesterone-treated lymphocytes after immunotherapy in unexplained recurrent spontaneous abortion. Am J Reprod Immunol 35:552–557
15. Katsuki Y, Takano Y, Futamura Y, Shibutani Y, Aoki D, Udagawa Y, Nozawa S (1998) Effects of dienogest, a synthetic steroid, on experimental endometriosis in rats. Eur J Endocrinol 138:216–226
16. Qiu BS, Vallance BA, Blennerhassett PA, Collins SM (1999) The role of $CD4^+$ lymphocytes in the susceptibility of mice to stress-induced reactivation of experimental colitis. Nat Med 5:1178–1182
17. Paus R, Theoharides TC, Arck PC (2006) Neuroimmunoendocrine circuitry of the 'brain-skin connection'. Trends Immunol 27:32–39
18. Arck P, Knackstedt MK, Blois SM (2006) Current insights and future perspectives on neuro-endocrine-immune circuitry challenging pregnancy maintenance and fetal health. J Repro Med Endo 3:98–102
19. Cacioppo JT, Berntson GG, Malarkey WB, Kiecolt-Glaser JK, Sheridan JF, Poehlmann KM, Burleson MH, Ernst JM, Hawkley LC, Glaser R (1998) Autonomic, neuroendocrine, and immune responses to psychological stress: the reactivity hypothesis. Ann N Y Acad Sci 840:664–673
20. Saunders PR, Santos J, Hanssen NP, Yates D, Groot JA, Perdue MH (2002) Physical and psychological stress in rats enhances colonic epithelial permeability via peripheral CRH. Dig Dis Sci 47:208–215
21. van Tol EA, Petrusz P, Lund PK, Yamauchi M, Sartor RB (1996) Local production of corticotropin releasing hormone is increased in experimental intestinal inflammation in rats. Gut 39:385–392
22. Keenan JA, Chen TT, Chadwell NL, Torry DS, Caudle MR (1994) Interferon-gamma (IFN-gamma) and interleukin-6 (IL-6) in peritoneal fluid and macrophage-conditioned media of women with endometriosis. Am J Reprod Immunol 32:180–183
23. Keenan JA, Chen TT, Chadwell NL, Torry DS, Caudle MR (1995) IL-1 beta, TNF-alpha, and IL-2 in peritoneal fluid and macrophage-conditioned media of women with endometriosis. Am J Reprod Immunol 34:381–385
24. Taketani Y, Kuo TM, Mizuno M (1992) Comparison of cytokine levels and embryo toxicity in peritoneal fluid in infertile women with untreated or treated endometriosis. Am J Obstet Gynecol 167:265–270
25. Arck PC, Merali FS, Manuel J, Chaouat G, Clark DA (1995) Stress-triggered abortion: inhibition of protective suppression and promotion of tumor necrosis factor-alpha (TNF-alpha) release as a mechanism triggering resorptions in mice. Am J Reprod Immunol 33:74–80
26. Kempuraj D, Papadopoulou N, Stanford EJ, Christodoulou S, Madhappan B, Sant GR, Solage K, Adams T, Theoharides TC (2004) Increased numbers of activated mast cells in endometriosis lesions positive for corticotropin-releasing hormone and urocortin. Am J Reprod Immunol 52:267–275
27. Florio P, Busacca M, Vignali M, Vigano P, Woods RJ, Lowry PJ, Genazzani AR, Luisi S, Santuz M, Petraglia F (1998) Peritoneal fluid levels of immunoreactive corticotropin-releasing factor (CRF) and CRF-binding protein (CRF-BP) in healthy and endometriosic women. J Endocrinol Investig 21:37–42
28. Florio P, Reis FM, Torres PB, Calonaci F, Toti P, Bocchi C, Linton EA, Petraglia F (2007) Plasma urocortin levels in the diagnosis of ovarian endometriosis. Obstet Gynecol 110:594–600
29. Maes M, Song C, Lin A, De Jongh R, Van Gastel A, Kenis G, Bosmans E, De Meester I, Benoy I, Neels H, Demedts P, Janca A, Scharpe S, Smith RS (1998) The effects of psychological stress on humans: increased production of pro-inflammatory cytokines and a Th1-like response in stress-induced anxiety. Cytokine 10:313–318
30. Joachim R, Zenclussen AC, Polgar B, Douglas AJ, Fest S, Knackstedt M, Klapp BF, Arck PC (2003) The progesterone derivative dydrogesterone abrogates murine stress-triggered abortion by inducing a Th2 biased local immune response. Steroids 68:931–940
31. Orsal AS, Blois S, Labuz D, Peters EM, Schaefer M, Arck PC (2006) The progesterone derivative dydrogesterone down-regulates neurokinin 1 receptor expression on lymphocytes, induces a Th2 skew and exerts hypoalgesic effects in mice. J Mol Med 84:159–167
32. Szekeres-Bartho J, Autran B, Debre P, Andreu G, Denver L, Chaouat G (1989) Immunoregulatory effects of a suppressor factor from healthy pregnant women's lymphocytes after progesterone induction. Cell Immunol 122:281–294
33. Srivastava MD, Thomas A, Srivastava BI, Check JH (2007) Expression and modulation of progesterone induced blocking factor (PIBF) and innate immune factors in human leukemia cell lines by progesterone and mifepristone. Leuk Lymphoma 48:1610–1617
34. Szekeres-Bartho J, Par G, Dombay G, Smart YC, Volgyi Z (1997) The antiabortive effect of progesterone-induced blocking factor in mice is manifested by modulating NK activity. Cell Immunol 177:194–199
35. ASRM (1997) Revised American Society for Reproductive Medicine classification of endometriosis: 1996. Fertil Steril 67:817–821
36. Koninckx PR, Meuleman C, Demeyere S, Lesaffre E, Cornillie FJ (1991) Suggestive evidence that pelvic endometriosis is a progressive disease, whereas deeply infiltrating endometriosis is associated with pelvic pain. Fertil Steril 55:759–765
37. Cunha-Filho JS, Gross JL, Bastos de Souza CA, Lemos NA, Giugliani C, Freitas F, Passos EP (2003) Physiopathological aspects of corpus luteum defect in infertile patients with mild/minimal endometriosis. J Assist Reprod Genet 20:117–121
38. Raghupathy R, Al Mutawa E, Makhseed M, Azizieh F, Szekeres-Bartho J (2005) Modulation of cytokine production by dydrogesterone in lymphocytes from women with recurrent miscarriage. Bjog 112:1096–1101
39. Kempuraj D, Papadopoulou NG, Lytinas M, Huang M, Kandere-Grzybowska K, Madhappan B, Boucher W, Christodoulou S, Athanassiou A, Theoharides TC (2004) Corticotropin-releasing hormone and its structurally related urocortin are synthesized and secreted by human mast cells. Endocrinology 145:43–48
40. Kravchenco IV, Furalev VA (1994) Secretion of immunoreactive corticotropin releasing factor and adrenocorticotropic hormone by T- and B-lymphocytes in response to cellular stress factors. Biochem Biophys Res Commun 204:828–834
41. Merchenthaler I, Hynes MA, Vigh S, Shally AV, Petrusz P (1983) Immunocytochemical localization of corticotropin releasing factor (CRF) in the rat spinal cord. Brain Res 275:373–377
42. Kalantaridou SN, Makrigiannakis A, Zoumakis E, Chrousos GP (2004) Stress and the female reproductive system. J Reprod Immunol 62:61–68
43. Tariverdian N, Theoharides TC, Siedentopf F, Gutierrez G, Jeschke U, Rabinovich GA, Blois SM, Arck PC (2007) Neuroendocrine-immune disequilibrium and endometriosis: an interdisciplinary approach. Semin Immunopathol 29:193–210
44. Watkins LR, Maier SF (2000) The pain of being sick: implications of immune-to-brain communication for understanding pain. Annu Rev Psychol 51:29–57

45. Luheshi GN, Bluthe RM, Rushforth D, Mulcahy N, Konsman JP, Goldbach M, Dantzer R (2000) Vagotomy attenuates the behavioural but not the pyrogenic effects of interleukin-1 in rats. Auton Neurosci 85:127–132
46. Tokushige N, Markham R, Russell P, Fraser IS (2006) Nerve fibres in peritoneal endometriosis. Hum Reprod 21:3001–3007
47. Cox H, Henderson L, Wood R, Cagliarini G (2003) Learning to take charge: women's experiences of living with endometriosis. Complement Ther Nurs Midwifery 9:62–68
48. Weber C, Arck P, Mazurek B, Klapp BF (2002) Impact of a relaxation training on psychometric and immunologic parameters in tinnitus sufferers. J Psychosom Res 52:29–33