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Enhanced local and systemic inflammatory cytokine mRNA expression in women with endometriosis evokes compensatory adaptive regulatory mRNA response that mediates immune suppression and impairs cytotoxicity

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Abstract

Problem: Endometriosis is a disease characterized by ectopic implantation of endometrium and impaired immune responses. To explore its pathogenic mechanisms, we studied the local and systemic cytokine mRNA profiles and their role in the immunity of patients with endometriosis and healthy controls.

Method of Study: mRNA for eleven cytokines defining cytotoxic Th1, humoral Th2, regulatory Tr1/Th3, and inflammatory cytokine profiles was characterized locally in endometriotic tissue and endometrium, and systemically in PBMCs from women with endometriosis and healthy controls, using real-time qRT-PCR. In addition, immunohistochemical stainings with monoclonal antibodies were performed looking for T regulatory cells in endometriotic lesions.

Results: We found a downregulation of mRNA for cytokines mediating cytotoxicity and antibody response and an upregulation of inflammatory and T-regulatory cytokines in the endometriotic tissues and endometrium from the patients with endometriosis, suggesting enhanced local inflammation and priming of an adaptive regulatory response. Consistent with those findings, there was an abundancy of T regulatory cells in the endometriotic lesions.

Conclusions: The ectopic implantation seen in endometriosis could be possible as a consequence of increased inflammation and priming of adaptive T regulatory cells, resulting in impaired cytotoxicity and enhanced immune suppression.

KEYWORDS

Cytokines, Cytotoxicity, Endometriosis, Immune suppression, Inflammation, Regulatory T-Lymphocytes

1 | INTRODUCTION

Endometriosis, affecting 10% of women worldwide, is an estrogen-dependent disease-causing chronic abdominal pain and infertility in women of reproductive age. It results from implantation and subsequent growth of endometrial tissue outside the uterine cavity.[1,2] The most widely accepted hypothesis for the development of pelvic endometriosis is ectopic dissemination of endometrial tissue through retrograde menstruation, first proposed in the 1920s.[3,4] However, although approximately 90% of women have evidence of retrograde menstruation,[5] only about 10% develop endometriosis. Several factors are likely to influence the susceptibility to the disease. Despite long-time research, endometriosis is still an enigma and its cause(s) are so far unknown.

It is suggested that aberrant immunological mechanisms causing a dysfunction of immune cells and mediators are involved in the pathogenesis of endometriosis. The ectopically disseminated endometrium is allowed to escape immune surveillance. It implants, proliferates, and invades the underlying tissue, forming painful endometriotic lesions that grow under the hormonal influence of the menstrual cycle.[2] Endometriosis is considered a benign disease, but has many features in common with tumours such as clonal proliferation, dissemination, and tissue invasion.[6] Research suggests heritability for endometriosis, estimated to be around 50% in twin studies.[7]

Cytokines may play a significant role in the current understanding of the pathogenesis of endometriosis by modulating the patients' immune system toward acceptance of ectopic implantation of endometrial tissue thus allowing chronic disease progression.[2,8] Cytokines, secreted by a variety of cells, are small proteins/peptides that are key mediators of intercellular communication. The ability of the immune system to regulate immunity and inflammation, promote or prevent cell growth and movement and exert immune surveillance is associated with different cytokine profiles. These are operating locally and mediate a variety of immune responses, denoted T helper (Th) 1, Th2, inflammatory, and T regulatory response. A cytokine profile dominated by Th1 cytokines such as IFNγ and IL15 promotes a Th1 response, that is, cytotoxicity; a Th 2 cytokine profile dominated by IL 4 promotes a Th2 response, that is, a humoral response, while an inflammatory cytokine profile including IL1β, IL6, IL8, tumour necrosis factor (TNF)α, and LTA/TNFβ promotes inflammation, and a Treg cytokine profile dominated by IL10 (Tr1) and/or TGFβ1 (Th3)

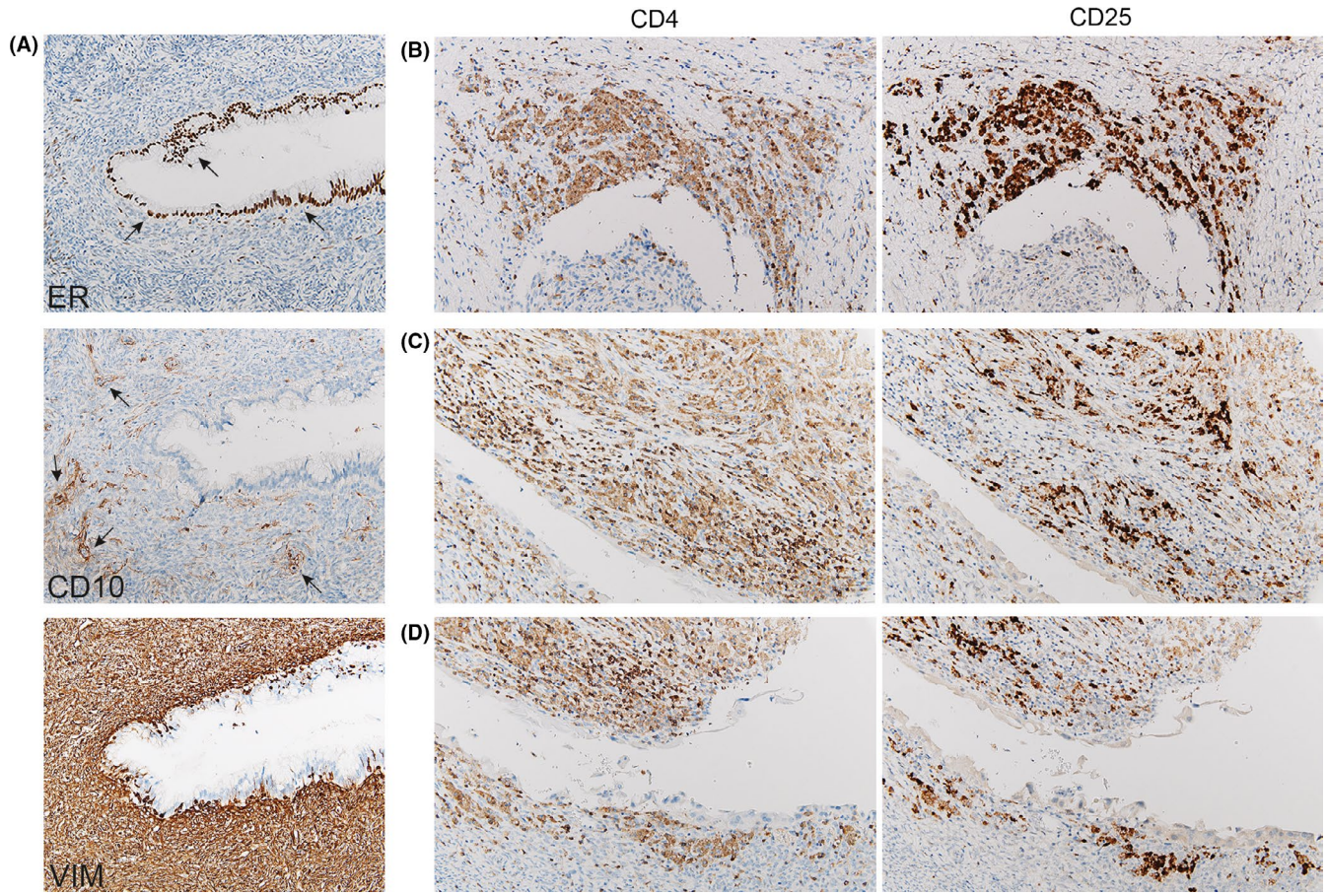

FIGURE 1 Immunohistochemical (IHC) staining of endometriotic lesions with monoclonal antibodies. (A) IHC staining of estrogen receptors (ER), CD10, and Vimentin (VIM), all specific markers used in pathoanatomical diagnosis (PAD) of endometriosis. (B, C, and D) IHC staining of serial sections from three randomly chosen endometriotic tissue samples with monoclonal antibodies against CD4 and CD25, showing that $CD4^{+}CD25^{++}$ T regulatory cells are abundant in endometriotic lesions. Arrows in the sections in (A) point to specific staining of cells/structures, positively identifying endometriosis

promotes an adaptive and/or natural/innate regulatory responses, respectively.[9]

To understand the interplay and contribution of cytokines in the overall immune response in endometriosis, here, using real-time quantitative RT-PCR, we investigated the cytokine profiles locally in the endometriotic and endometrial tissues, and systemically in peripheral blood mononuclear cells (PBMC), and compared them to the cytokine profiles in healthy controls. We studied the simultaneous relative mRNA expression for a broad panel of 11 cytokines, defining the specific immune responses described above. In addition, using immunohistochemistry with monoclonal antibodies, we analyzed the presence of immune cells in endometriotic lesions focusing on T regulatory cells.

2 | MATERIALS AND METHODS

2.1 | Ethical statement

This investigation was approved by the Swedish Ethical Review Authority (dnr 09-108M). All patients and controls donated samples after informed consent.

TABLE 1 Characteristics of endometriosis patients

Patient (n = 31)	Age (Mean 34, SD 8)	PAD	Dysmenorréa Symptoms	Hormonal treatment at time of sampling
1	39	Yes	Not known	No
2	29	No[a]	Intense	No
3	21	Yes	Intense	No
4	52	Yes	Not known	IUD and post-menopausal
5	39	Yes	Intense	No
6	35	Yes	Insufferable	No
7	43	Yes	No	IUD
8	37	Yes	Insufferable	No
9	21	No[a]	Intense	MPA since 6 weeks
10	23	Yes	Intense	No
11	30	Yes	Insufferable	No
12	39	Yes	Moderate	No
13	28	Yes	Intense	No
14	37	Yes	Moderate	IUD
15	33	Yes	Intense	No
16	28	Yes	No	No
17	42	Yes	Intense	No
18	42	Yes	Intense	IUD and MPA
19	24	Yes	Moderate	IUD
20	42	Yes	Moderate	No
21	31	Yes	Moderate	No
22	34	Yes	Moderate	No
23	25	Yes	No	No
24	46	Yes	Intense	No
25	38	Yes	Moderate	No
26	22	Yes	Insufferable	No
27	40	Yes	Intense	No
28	29	Yes	Not known	No
29	31	Yes	Intense	No
30	47	No[b]	Not known	No
31	30	Yes	Not known	No

Abbreviations: IUD, Intrauterine device Mirena®; MPA, Medroxyprogesterone acetate

[a]Surgical diagnosis, no PAD sample was taken due to fertility reasons

[b]Diagnosis by magnetic resonance and ultrasound.

2.2 | Collection and storage of samples

Thirty-one patients with endometriosis aged 21-52 years, mean age 34 years, and 18 healthy controls aged 19-50 years, mean age 38 years, participated in the study. Tissue from endometriotic lesions, eutopic endometrium and peripheral blood from patients with endometriosis were collected at surgery. Endometrium and peripheral blood were taken from healthy age-matched controls that were not receiving any hormonal treatment. Information about the endometriosis patients and the pathologic anatomic diagnosis (PAD) was extracted from medical records.

After sampling, eutopic endometrium or/and endometriotic tissue was immediately dispersed in RNA later solution or fixed in 4% formalin solution for immunohistochemical (IHC) studies. Within 24 hours (h), the samples for RNA isolation were frozen and kept at −80°C until use. Frozen endometriosis samples, taken from the Västerbotten County's Biobank, were processed with RNA later-ice at −20°C for 24 hours before RNA isolation.

2.3 | Materials

The following antibodies: anti-CD10 (clone 56C6, Novocastra, catalogue nr NCL-L-CD10-270); anti-CD4 (clone SP35, Roche, cataloque nr 790-2596); anti-CD45 (clone LCA, Roche, cataloque nr760-2505); anti-estrogen receptor (ER, clone SP, Roche catalogue nr 790-4324); and anti-FoxP3 (Cell Signalling # 12 653) were all used for IHC staining in Ventana Benchmark with ultraView Universal DAB Detection Kit (Roche, cataloque nr 760-500). The antibodies anti-CD25 (clone 4C9, CellMargue, cataloque nr CMC 12 521 050) and anti-vimentin (VIM, clone V9, Roche, cataloque nr 790-2917) were used for IHC staining in Ventana Benchmark with OptiView DAB IHC Detection Kit (Roche, cataloque nr 760-700). Kits and consumables used in the molecular work: TRIsol Reagent (Invitrogen, Thermo Fisher Scientific), RNeasy Mini kit (QIAGEN, Germany), High-Capacity cDNA Reverse Transcription Kit, TaqMan Gene Expression Master Mix, TaqMan FAM®/MGB probe assays, and primer limited Eukaryotic 18S rRNA Endogenous Control (all from Applied Biosystems, ThermoFisher Scientific).

2.4 | Isolation of peripheral blood mononuclear cells (PBMC) from patients and healthy donors

PBMC were isolated from endometriosis patients and healthy controls within 24 hours from sample collection using Lymphoprep (Nycomed) gradient centrifugation as previously described.[10,11] The interphase containing lymphocytes and macrophages were collected, washed, counted, and kept frozen at −80°C until use.

2.5 | IHC of endometriotic tissue samples

Endometriotic tissue was fixed in 4% formalin solution, imbedded in paraffin, sectioned at 6 μm thickness, and mounted on slides for

TABLE 2 Characteristics of healthy controls

Control (n = 18)	Age (Mean 38, SD 12)	Hormonal treatment at time of sampling	Dysmenorréa symptoms	Day in cycle for sampling from the endometrium
1	22	No	No	21
2	21	No	Moderate	11
3	24	No	No	7
4	19	No	Moderate	19
5	46	No	Unknown	7
6	37	No	No	10
7	48	No	No	-
8	19	No	No	-
9	52	No	Unknown	11
10	37	No	Moderate	-
11	46	No	No	9
12	35	No	No	13
13	34	No	Unknown	19
14	54	No	Moderate	-
15	49	No	Unknown	-
16	47	No	Unknown	-
17	45	No	Unknown	-
18	50	No	No	60

light microscopic studies. Immunohistochemical staining (IHC) on serial sections was performed using Ventana Benchmark immunohistochemistry staining system and the standard protocol. The primary antibodies anti-CD10 were diluted to 1:25 and anti-FoxP3 to 1:600. The rest of the primary antibodies were ready to use and thus applied directly from the manufacturer's bottle. Positive staining was revealed with ultraView Universal DAB Detection Kit and OptiView DAB IHC Detection Kit as explained in Materials. The stained samples were analyzed in Olympus BX53 light microscope. Representative photomicrographs are shown in Figure 1.

2.6 | Total RNA extraction and real-time quantitative reverse transcription-polymerase chain reaction (RT-qPCR)

The gene expression analysis of cytokines was performed by real-time RT-qPCR following the MIQE requirements.[12]

2.6.1 | RNA extraction

For RNA isolation, about 30 mg of each tissue sample was minced by lancet into small pieces and further processed according to a standard protocol described below.

Total RNA from tissue biopsies and PBMC was extracted using TRIsol and RNeasy Mini kit, respectively. PBMC samples were immediately disintegrated in 350 μl of lysis buffer at room temperature according to RNeasy Mini manual without DNase treatment.

2.6.2 | Overall quality evaluation of isolated total RNA

RNA yield (on average 245 ng/μl) and purity (on average A_{260}/A_{280} = 1.6) were assessed in 2 μl of RNA sample by using NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc, USA).

2.6.3 | Reverse transcription

For each tissue/cell sample, 400 ng of total RNA in reaction volume 20 μL was transcribed into cDNA using High-Capacity cDNA Reverse Transcription Kit according to the manufacture manual. 60 μL of sterile Milli-Q water was added to every sample to adjust cDNA concentration equal to 5 ng/μL total RNA.

2.6.4 | Real-time quantitative PCR amplification

One μL of cDNA per well in 20 μL reaction volume was used in all tests, on QuantStudio 5 Real-Time PCR System instrument with factory default settings for 10x TaqMan Gene Expression Master

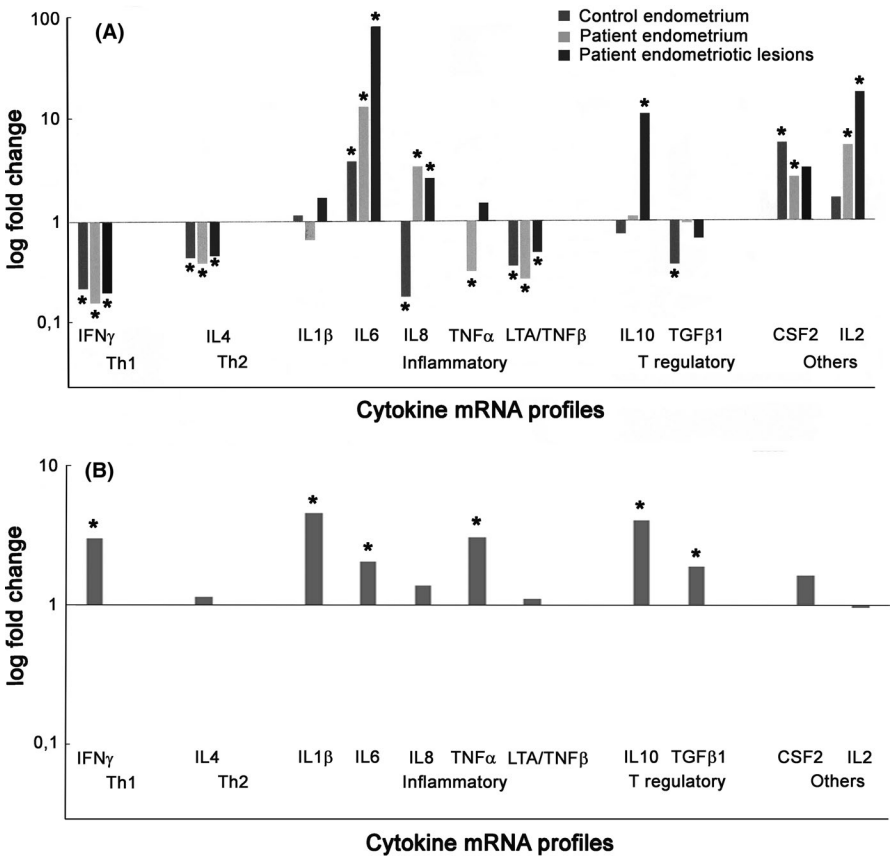

FIGURE 2 The local cytokine mRNA expression in endometrium and endometriotic lesions dominates over the systemic cytokine mRNA expression in isolated PBMC. (A) Relative cytokine mRNA expression in endometrium from healthy controls (blue), endometrium from endometriosis patients (orange) and from endometriosis lesions (gray). Results are presented as a fold change of the relative gene expression in tissue compared to the gene expression (defined as 1) in PBMC from healthy controls (blue) and from endometriosis patients (orange and gray). (B) Relative cytokine mRNA expression in PBMC from endometriosis patients compared to PBMC from healthy controls (defined as 1) illustrating the statistically enhanced systemic inflammatory and regulatory mRNA response in endometriosis patients. * designates statistical significance for p-values see Table S1

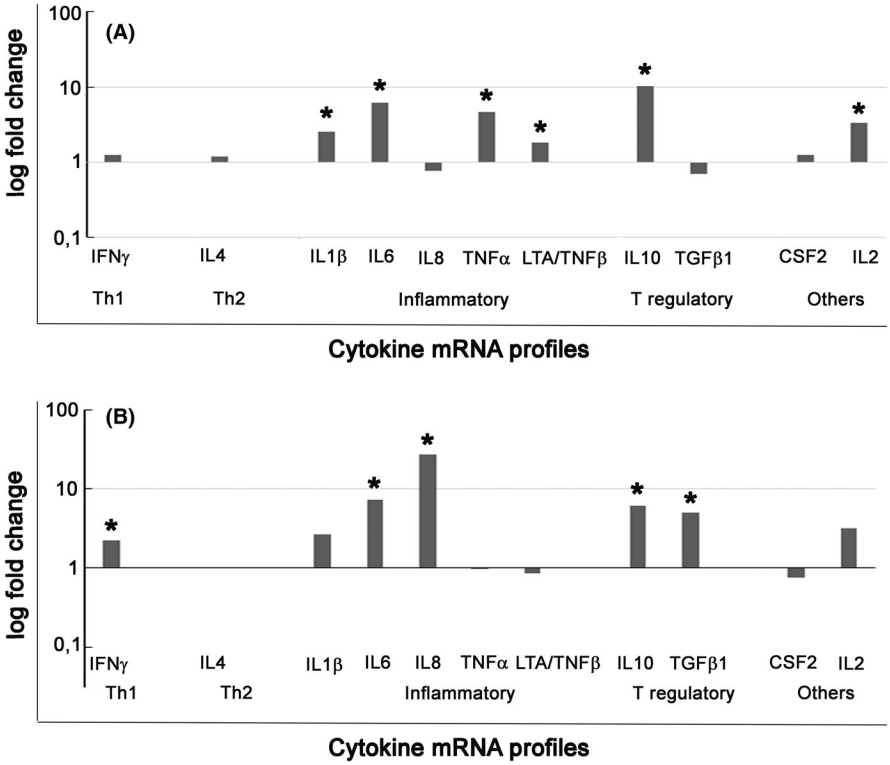

FIGURE 3 Local cytokine mRNA response in endometriosis patients compared to healthy controls. (A) Cytokine mRNA profile in endometriotic lesions compared to endometrium of endometriosis patients, defined as 1 and (B) in endometrium from endometriosis patients and endometrium from healthy controls, defined as 1. Results are presented as a log fold change of the relative gene expression for the individual cytokines representing cytotoxic, humoral, inflammatory, and T-regulatory response. * designates statistical significance for *P*-values see Table S1

Mix, 20x TaqMan FAM®/MGB probe Assays and 50x primer limited Eukaryotic 18S rRNA Endogenous Control (all from Thermo Fisher Scientific). The multiplexed PCR tests were run simultaneously detecting the target gene and the 18S rRNA in each well. Each plate/test was furnished with positive and negative controls for which we used normal PBMC stimulated by PMA/ionomycin and a negative control omitting the template, respectively.

2.7 | Processing of data and statistical analyses

To analyze the data, a comparative or ΔΔCt method was used with an array of relative quantities (RQ) for each study group. The ratios were calculated by dividing the group average of each study group by a reference group average. A non-parametric bootstrap test was used to evaluate the statistical significance of the ratios compared to the null hypothesis that the ratios were less or equal to one. *P*-values ≤ .05 were considered significant.

3 | RESULTS

3.1 | Patients and controls

A brief summary describing the patients and healthy controls is presented in Tables 1 and 2, respectively. Twenty-eight of the patients were diagnosed with PAD, one with MR and ultrasound. Two of the patients were diagnosed by observation of obvious and large endometriomas at surgery where only small amounts of tissue were taken for this study to keep as good ovarian reserve as possible. Only 6 patients were receiving treatment with hormones at the time of sampling. None of the age-matched controls were receiving any hormonal treatment.

3.2 | $CD4^+$ $CD25^{++}$ T regulatory cells are abundant in endometriotic lesions

To study the presence/absence of T regulatory (Treg) cells in endometriotic lesions, IHC stainings with monoclonal antibodies were performed on six randomly chosen samples. Stainings of CD10, estrogen receptor (ER), and vimentin (Figure 1A) were used to confirm the diagnosis of endometriotic lesions. The leukocyte common antigen CD45 was used as a positive control to evaluate the overall presence of leukocytes in the tissue. To reveal adaptive Treg cells, we stained serial tissue sections for CD4 and CD25 markers, looking for the human Treg cell phenotype $CD4^+CD25^{++}$ cells[13] (Figure 1). We found $CD45^+$ leukocytes present in substantial amounts in all samples and among them a great abundancy of $CD4^+CD25^{++}$ cells (Figure 1B,C,D). The finding of abundant $CD4^+CD25^{++}$ Treg cells corroborates with our real-time RT-qPCR data showing that enhanced expression of IL-2 was revealed locally in the endometriotic lesions together with a dominant IL-10 expression. In addition, we tried to stain for FoxP3, a marker mainly found in innate Treg cells, but did not obtain positive results despite trying 3 different monoclonal antibodies (not shown). There are 2 possible explanations for this: (a) According to our mRNA analyses, the Treg cells at the endometriotic lesions are primed by IL-10 and IL-2 and thus comprise adaptive Treg

cells of tr1 type that are known to scarcely express FoxP3, or (b) The anti-FoxP3 antibodies did not perform in IHC staining. IHC stainings of $CD4^{+}CD25^{++}$ T regs in serial sections of 3 representative samples of endometriotic tissue are shown in Figure 1.

3.3 | Cytokine mRNA profiles in endometriosis compared to healthy age-matched controls

Using real-time quantitative RT-PCR, we analyzed and compared the cytokine mRNA profiles in patients and controls in (a) PBMC, reflecting systemic cytokine responses; (b) endometrium, reflecting local mucosal responses in the uterine cavity; and (c) endometriotic lesions extracted from intraperitoneal locations at surgery of endometriosis patients. The chosen primers and probes distinguish between the main cytokine mRNA patterns for Th1, Th2, Th3, Tr1, and inflammatory response and the analyzed cytokines are known to be the index cytokines defining cytotoxicity, antibody response, inflammation, and immune regulation.[14] For obvious reasons, the endometriotic tissue could not be compared to an analogue tissue in the controls. Instead, we compared endometriotic tissue to the PBMC and the endometrium of the patients, thus making comparisons between local and systemic responses in paired samples of the endometriosis patients. Results are presented as relative mRNA expression visualized as logarithmic fold change differences in Figures 2 and 3. The presented results in Figures 2 and 3 are based on the whole patient group including six hormonally treated patients. We chose to present the whole endometriosis group as the statistical significance of the cytokine mRNA expression was very similar between the total group of patients (n = 31) and when the six patients with hormonal treatment were excluded (n = 25) as shown in Tables S1 and S2, respectively, where the bootstrap p-values for the analyses of the individual cytokines are presented.

The local cytokine mRNA response in endometriotic lesions and endometrium of endometriosis patients compared to the respective systemic response reveals downregulation of cytotoxicity and antibody response combined with enhanced upregulation of inflammation and adaptive T-regulatory responses.

In Figure 2A, the local relative cytokine mRNA expression in endometrium and endometriotic tissue in patients and endometrium in controls is compared to the mRNA profiles in PBMC from peripheral blood of patients and controls, respectively. The results are presented as quotes of the mRNA expression in patient's endometrium or endometriotic lesion and the mRNA expression in the same patient's PBMC, designated as 1. The same analytic procedure was used for the estimation of mRNA expression in the endometrium of healthy controls. As can be seen, there is a significantly enhanced inflammatory and regulatory cytokine mRNA expression in the endometriosis patients compared to healthy controls. The mRNA expression of the inflammatory cytokines IL-6 and IL-8 is highly upregulated, particularly in the endometriotic tissue, where mRNA for IL-8 and IL-6 reaches 4 to 80 times higher fold change, respectively, compared to the expression in the controls. In contrast, TNFα and LTA/TNFβ were locally downregulated compared to the systemic response (=1, Figure 2A). Another prominent finding was the high expression of IL-10 (fold change about 10). At the same time, the mRNA expression of TGFβ1, priming Th3 innate regulatory cells, was not upregulated but somewhat suppressed. Notable is also that there was a simultaneous statistically significant fivefold to 20-fold higher expression of IL-2 locally in the patients' endometrium and in endometriotic lesions. Together, the IL-10 mRNA response, combined with the IL-2 response, suggests a local upregulation and priming of Tr1 adaptive regulatory cells. Despite the rigorous inflammatory mRNA response, concomitantly, there was a notable significant downregulation of the Th1 and Th2 responses represented here by the relative expression of the index cytokines IFNγ and IL-4, respectively. In Figure 2B, the systemic cytokine mRNA response in PBMCs of patients and healthy controls (relative expression = 1) is compared. The systemic cytokine response in endometriosis patients was generally enhanced compared to healthy controls. Notable was that the systemic IFNγ response in PBMC from endometriosis patients was upregulated compared to PBMC from healthy controls. Furthermore, in contrast to the local response (Figure 2A), there was an mRNA upregulation of both IL-10 and TGFβ but no expression of IL-2 systemically. The systemic inflammatory response was dominated by IL-1β and IL-6, and in contrast to the local inflammatory response (Figure 2A), there was an upregulation of TNFα as well.

The local cytokine mRNA response in patients' endometrium and endometriotic lesions suggests an enhanced local inflammation and priming of the adaptive T regulatory response in the endometriotic lesions.

In the next step, after comparing the local and systemic cytokine mRNA profile in endometriosis patients, we specifically analyzed the local cytokine profile in endometriotic lesions compared to patient endometrium (Figure 3A) and in patient endometrium compared to endometrium from healthy controls (Figure 3B). As can be seen, the dominating mRNA responses in endometriotic lesions (Figure 3A) are as follows: (a) inflammatory, featured by enhanced mRNA for IL-1β, IL-6, TNFα, and LTA/TNFβ and (b) an adaptive Tr1 type, featured by concomitantly expressed IL-10 and IL-2 mRNA suggesting an active local priming of adaptive Tregs in the endometriotic lesions, as shown and supported by the presence of $CD4^{+}CD25^{++}$ cells in serial tissue sections (Figure 1B, C, D). Comparison of cytokine mRNA profile in the patients' endometrium versus healthy controls' endometrium (Figure 3B) shows: (a) about twofold upregulation of IFNγ, (b) statistically significant upregulation of 2 inflammatory markers, IL-6 and IL-8, (c) statistically significant upregulation of both IL-10 and TGFβ1, cytokines characteristic for of adaptive Tr1 and innate Th3 Tregs type, respectively, and (d) conversely, IL-2 mRNA was only slightly but not significantly upregulated.

Combining the results in Figure 3A and B, we show that when implanted outside uterus, the endometrial tissue developing into endometriotic lesions: (a) loses the relative upregulation of INFγ, (b) upregulates the inflammation by enhanced mRNA response of IL-1β, TNFα, and LTA/TNFβ, and (c) promotes Tregs by enhanced upregulation of IL-10 (10-fold in Figure 3A compared to sixfold in Figure 3B), lost/slightly downregulated TGFβ1 mRNA expression

and simultaneously 5-20 times upregulated IL-2 mRNA (shown in Figure 3A).

4 | DISCUSSION

Immune mechanisms are proposed to play a role in the development of endometriosis. In contrast to healthy women, patients suffering from endometriosis appear to have a decreased cell-mediated immunity with suppressed T- and NK-cell cytotoxicity.[15,16] The reason(s) for this is(are) unknown, but altered cytokine response initiating inflammation and suppressing cytotoxicity could permit the ectopic endometrial fragments to survive, implant, and develop into endometriotic lesions.[17] In several previous studies, single cytokines in the peripheral blood and/or peritoneal fluid of endometriosis patient have been investigated,[18,19] looking for the causes of the impaired cytotoxic response. In this study, we have simultaneously evaluated and quantified the relative mRNA expression of 11 cytokines in paired samples of endometriotic tissue, endometrial tissue, and peripheral blood mononuclear cells (PBMC) from endometriosis patients and healthy controls, which, to our knowledge, has not been done before. This approach allows us to study a wide spectrum of cytokine expression at the local and systemic level that gives us a unique view and understanding of the aberrant cytokine responses at the endometriotic site. Using IHC to phenotype lymphocytes in endometriotic biopsies we have further confirmed the notion from our cytokine mRNA analyses indicating local Treg cell priming in endometriotic lesions.

Our results can be summarized as follows: (a) There was an extensive systemic and local inflammatory response in the endometriosis patients compared to healthy controls, (b) there was a lack of expression or impaired upregulation of INFγ, a key cytokine necessary for the Th1 response that mediates NK- and cytotoxic T-cell killing, (c) there was a prominent upregulation of mRNA for IL-10 and IL-2 in the endometriosis patients, most pronounced in endometriotic lesions, consistent with a local Tr1 regulatory response priming naïve T cells into adaptive Treg cells, and (d) in agreement with the previous statement, we found a substantial abundancy of $CD4^{+}CD25^{++}$ cells in the endometriotic lesions as shown by IHC staining of serial tissue sections. Taken together, our findings support the hypothesis of failure to clear disseminated endometriotic tissue from ectopic sites[8,20,21] and propose one possible explanation of the mechanism behind the suppressed cytotoxic potency of NK and cytotoxic T cells.

Consistent with previous studies,[22-25] we found high levels of mRNA expression of inflammatory cytokines, mainly IL-6 and IL-8 but also of IL1β and TNFα thus supporting the view of endometriosis as an inflammatory disease. Simultaneously with the expression of inflammatory cytokines, a strong expression of the anti-inflammatory cytokine IL-10,[26] known to mediate an adaptive Tr1 response, was revealed in the endometriotic lesions, but also to a lesser degree in the eutopic endometrial tissue of endometriosis patients. The prominent local anti-inflammatory response, in tune with a previous study,[19] could be compensatory to the strong local inflammatory response. Interestingly, the enhanced IL-10 mRNA expression at the local level was accompanied by a fivefold to 20-fold higher expression of IL-2, a cytokine essential for stimulation and proliferation of Treg cells. Taken together, these observations indicate that active priming of adaptive Tr1 regulatory cells takes place in endometriosis, probably starting already in the eutopic endometrium of the patients and continuing with enhanced strength in the disseminated endometriotic lesions. Systemically, in the PBMC, mRNA for IL-10 and TGFβ (a cytokine characteristic for innate Treg cells) was upregulated without concomitant upregulation of IL-2, again confirming that Treg priming is a local phenomenon taking place in endometriotic and endometrial tissue. The skewing of the immune response toward Treg as a reaction to inflammation in endometriosis is also seen in other chronic inflammatory diseases such as rheumatoid arthritis, Crohn's disease, systemic lupus erythematosus, and psoriasis.[27]

Our notion of a skew toward Treg cell priming was supported by IHC staining revealing a high abundance of $CD4^{+}CD25^{++}$ Tregs in endometriotic tissue. In support of our findings, Takamura et al[27] found an increase of regulatory T cells at the endometriotic site, although not reaching statistical significance. Further, it was shown that regulatory T cells (Treg) are increased in the peritoneal fluid of endometriosis patients.[28] Regulatory T cells were also increased in the eutopic endometrium of endometriosis patients during the secretory phase of the menstrual cycle[29] supporting our notion that this aberration might originate from the endometrium. The endometrial cells entering the peritoneal cavity during retrograde menstruation may then be responsible for the induction of the aberrant cytokine profile observed both in peritoneal fluid and ectopic endometrium.[30-32] Thus, several studies support our results of upregulated Treg cytokines but there are also results that show no difference compared to controls.[19,33] The reasons for these discrepancies are not known but might be due to differences in the disease duration and treatment, the experimental set-up, the methods used, and the timing of the sample collection. To try to accurately reflect the natural course of endometriosis, our samples were collected at the time of the disease diagnosis and the vast majority of the patients (all but 6) were not hormonally treated.

As prominent as the inflammatory and T regulatory responses were, the basic Th1 and Th2 responses, measured by the index cytokines IFNγ and IL-4, were equal to those in normal controls, or were downregulated, as shown in Figures 2 and 3. Inadequate INFγ mRNA response cannot activate the cytotoxic response of NK and cytotoxic T cells as previously described.[8,20,21] The reason(s) for the impairment of T- and NK-cell cytotoxicity is (are) at present not known. A hypothetical explanation could be that the skewed immune response toward immunoregulation by adaptive Treg cells, raised to counteract the vigorous inflammation, could have the unwanted side effect of inhibiting the cytotoxic potency of the immune system in endometriosis causing an impaired immune surveillance. Regulatory T cells could be responsible for suppression of the immune surveillance at the endometriotic site, as these cells have the ability to modify the secretion pattern of

cytokines, and suppress activation and proliferation of immune cells including cytotoxic T and NK cells.[13] The final outcome of the cytokine-mediated Treg immune suppression would then be survival of ectopically disseminated endometrial tissue that, in a vicious circle, will promote more inflammation and thus, more T reg-mediated immune suppression. This could partly provide a mechanistic explanation of the pathogenesis of endometriosis but is by far not enough to explain the etiology of this disease. A second hypothetical explanation could be that other unknown factors or an intrinsic defect(s) downregulate the cytotoxic potential of the T and NK cells and eventually cause the disease and its chronical course. Our results provide convincing evidence supporting the first explanation involving Treg cells. In line with this, a recent review[19] suggested anti-inflammatory cytokines to have an impact on the progression of endometriosis.

In this study, we assessed cytokine mRNA profiles in tissue samples and PBMC from endometriosis patients and healthy controls by measuring cytokine mRNA expression levels in lieu of measuring biologically active molecules at the protein level. There was a considerable time lag between the collection of tissue and serum samples which could jeopardize protein analyses as cytokines are peptides sensitive to degradation if exposed to room temperature, long transportation, and freezing/thawing. Therefore, we used cytokine mRNA profiling by real-time quantitative RT-PCR, a method less affected by post-sampling handling. Recently, we analyzed and compared cytokine mRNA and protein analyses from methodological point of view.

Considering the very high sensitivity and reproducibility of real-time qRT-PCR, we suggest that determination of cytokine mRNA profiles can be used as a proxy for protein-mediated functions in certain circumstances, such as for comparisons between different patient groups and controls, and in defining mechanistic pathways involved in the pathogenesis of various disease conditions. We are aware that the cytokines that contribute to the cytokine milieu in endometriosis are produced by different cell populations in PBMC, endometriotic, and endometrial samples. However, we wanted to analyze the overall cytokine mRNA profile that prevailed locally and systemically in endometriosis, regardless of the individual contributing cells, in order to understand how this cytokine profile molds the micromilieu in endometriosis and contributes to the pathogenesis of this disease.

In conclusion, our results are consistent with the suggested dysfunction of cytotoxic T and NK cells in patients with endometriosis. The dominating cytokine profiles found in this study are consistent with an active local priming of adaptive T regulatory cells, and stand out as a probable mechanism for promotion of immune suppression and impairment of the cytotoxic response, which allows persistency of ectopic endometriotic lesions, chronic pain, and infertility. Future studies with larger cohorts of patients and controls are needed to apprehend in detail the role of cytokines in endometriosis and to prove/disprove the results in this investigation.

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CONFLICTS OF INTEREST

There are no conflicts of interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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