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## Functional Expression of Aryl Hydrocarbon Receptor on Mast Cells Populating Human Endometriotic Tissues

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### Abstract

Endometriosis is an inflammatory disease characterized by the presence of ectopic endometrial tissue outside the uterus. A diffuse infiltration of mast cells (MCs) is observed throughout endometriotic lesions, but little is known about how these cells contribute to the network of molecules that modulate the growth of ectopic endometrial implants and promote endometriosis-associated inflammation. The Aryl Hydrocarbon Receptor (AhR), a transcription factor known to respond to environmental toxins and endogenous compounds, is present in MCs. In response to AhR activation, MCs produce IL-17 and reactive oxygen species, highlighting the potential impact of AhR ligands on inflammation via MCs. Here, we investigated the possibility that endometrial MCs promote an inflammatory microenvironment by sensing AhR ligands, thus sustaining endometriosis development. Using human endometriotic tissue (ET) samples, we performed the following experiments: i) examined the cytokine expression profile; ii) counted AhR-expressing MCs; iii) verified the phenotype of AhR-expressing MCs to establish whether MCs have a tolerogenic (IL-10-positive) or inflammatory (IL-17-positive) phenotype; iv) measured the presence of AhR ligands (tryptophan-derived kynurenine) and tryptophan-metabolizing enzymes (indoleamine 2,3-dioxygenase 1 (IDO1)); v) treated ET organ cultures with an AhR antagonist *in vitro* to measure changes in the cytokine milieu; and vi) measured the growth of endometrial stromal cells cultured with AhR-activated MC-conditioned medium.

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We found that ET tissue was conducive to cytokine production, orchestrating chronic inflammation and a population of AhR-expressing MCs that are both IL-17 and IL-10-positive. ET was rich in IDO1 and the AhR-ligand kynurenine compared with control tissue, possibly promoting MC activation through AhR. ET was susceptible to treatment with an AhR antagonist, and endometrial stromal cell growth was improved in the presence of soluble factors released by MCs upon AhR activation.

These results suggest a new mechanistic role of MCs in the pathogenesis of endometriosis.

## Keywords

Aryl hydrocarbon receptor; Endometriosis; Interleukin-17; mast cells; kynurenine

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## Introduction

Endometriosis is an inflammatory chronic disease characterized by the presence of ectopic endometrial tissue outside the uterus. Endometriosis primarily affects women of reproductive age, with a general population prevalence between 1.5% and 4% (1,2). However, the prevalence is 71-87% among women with pelvic pain and 5-69% among adolescents with pelvic pain unresponsive to therapy (3). The pathogenesis underlying endometriosis initiation and progression has not been fully clarified. Retrograde menstruation could account for the presence of menstrual tissue in the peritoneal cavity; however, its progressive growth relies on defects of the immune system in addition to genetic and epigenetic predispositions that allow endometrial cells to escape apoptosis, evade immune surveillance, invade the mesothelial surface and acquire vascular supply (4,5).

Several reports have demonstrated elevated levels of proinflammatory cytokines, such as IL-4, IL-6, IL-8, IL-12, TNF- $\alpha$ , MCP-1/CCL2, in the peritoneal fluid and serum of endometriosis patients. These cytokines could contribute to the proliferation and neoangiogenesis of endometriotic implants but also to progesterone-resistance, which in turn impairs the anti-inflammatory action of progesterone (5). Moreover, endometriotic tissues (ETs) reveal abundant inflammatory immune cell infiltrates. However, the role of specific immune cell subsets and their mediators in the *in situ* progression of endometrial lesions has not been sufficiently characterized (6).

Tissue-resident mast cells (MCs) are currently recognized as effector cells in many settings of the immune response, including host defense, immune regulation, allergy, chronic inflammation and autoimmune diseases (7). The pleiotropic functions of MCs reflect their ability to secrete a wide spectrum of preformed or newly synthesized biologically active products in response to multiple signals, with proinflammatory, anti-inflammatory and/or immunosuppressive properties (8). MCs are capable of orchestrating inflammation by modulating the recruitment and function of other immune cells, such as macrophages, granulocytes and lymphocytes, which have been described to be involved in endometriosis pathogenesis (5,9–11). Diffuse infiltration of degranulated tryptase-positive MCs was observed throughout endometriotic stromal lesions, often close to nerve fibers (12–14).

Recently, to investigate MC heterogeneity, Paula and colleagues demonstrated a significant increase only in the number of chymase-positive cells in abdominal wall endometriotic lesions compared with eutopic endometrium controls (15). However, how these cells are activated and to what extent they contribute to the network of locally produced cytokines, chemokines and other mitogenic factors that modulate the growth of the ET and the associated inflammatory profile require further study (16).

Our group recently discovered that the aryl hydrocarbon receptor (AhR) modulates acute and late MC responses (17). AhR, a transcription factor expressed in multiple tissues and in immune cells, is known to respond to environmental toxins, such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and other polyhalogenated aromatic hydrocarbons, as well as to dietary components and endogenous ligands, including heme and tryptophan metabolites (18). AhR influences immune responses and is involved in autoimmune and chronic inflammatory diseases (19–21). AhR is responsible for MC activation in terms of degranulation activity and cytokine responses (17,22,23), while its involvement in controlling MC homeostasis remains unclear (22,24). AhR protein has been found in glandular epithelial cells of the human endometrium, and stromal cells cultured from the endometrium in the proliferative phase express AhR in both the cytoplasm and nucleus (25,26). Several studies have suggested that AhR is involved in the normal function of the endometrium, possibly by modulating cellular proliferation in response to hormones (27). In ETs, AhR mRNA levels are higher than in their healthy eutopic endometrium counterparts. Although dioxin exposure did not affect the expression of AhR in cultured endometrial explants (28,29), the effects of AhR activation on ET components by compounds other than dioxin have not yet been studied.

Here, to establish the immunological phenotype of the ET microenvironment, we compared the cytokine profiles of human endometrial explants cultured from both patients and healthy controls. To test the hypothesis that endometrial MCs can sustain endometriosis development through an AhR-mediated pathway, the levels of endogenous tryptophan-derived AhR ligands and the percentage of AhR-positive MCs producing cytokines known to be modulated by AhR were measured *in situ*. ET explants were exposed to an AhR inhibitor to determine specific changes in the cytokine milieu and last, the growth of endometrial stromal cells cultured with AhR-activated MC-conditioned medium was assessed.

## Material and Methods

### Tissue samples

ETs from patients who had undergone laparoscopic surgery were obtained from the Clinic of Obstetrics and Gynecology, University Hospital of Udine. We included all consecutive women of fertile age at the time of intervention with regular menses and without known endometrial pathology, histological diagnosis of adenomyosis or use of any hormone medication during the year before surgery. This study was conducted according to the Declaration of Helsinki and with Internal Review Board approval (approval number 35/2014). ET representative of stromal and glandular components from ovarian locations was used. As a control, eutopic endometrium from uterine tissues of all women who

underwent hysterectomy for leiomyoma and without known endometrial pathology (polyps, dysfunctional uterine bleeding) or histological diagnosis of adenomyosis was used. In the present study, we included ten women affected by endometriosis and ten controls. Sample extraction and liquid chromatography were performed in nine ET samples and nine eutopic endometria of healthy women, whereas cell culture supernatant analysis was performed in four ET samples and four eutopic endometria of healthy women due to the paucity of available tissue from the remainder of the samples.

### ET culture and multiplex ELISA

Immediately after surgery, ETs and healthy eutopic endometrium samples were brought to the laboratory of Surgical Pathology. After removal of hemorrhagic tissues, the areas macroscopically recognized as endometrial mucosa were gently teased away from the underlying tissue. All samples were cut into 2–3 mm<sup>3</sup> uniform explants with a sterile scalpel blade and cultured in 48-well plates at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> in 0.5 ml of complete RPMI-1640 medium supplemented with 10% FBS (Euroclone, Milano IT). To culture uniform type of explants and to avoid the inclusion of ovarian tissue, frozen sections of zero-time control tissues from each specimen and formalin-fixed and paraffin-embedded of 48 hr-incubated tissues from organ cultures were used to assess tissue architecture and composition (data not shown). Tissue culture medium harvested after 48 hr was centrifuged for 5 min at 2200 g, and supernatants were stored at -80°C. Cytokine, chemokine and growth factor concentrations were measured by quantitative cytokine assays using Bioplex Pro™ human cytokine standard 17-plex panels according to the pre-optimized protocol provided by the manufacturer. The data were collected and analyzed using a Bio-Rad BioPlex 200 instrument equipped with Bio-Plex Manager software version 6.0 (all from Bio-Rad Laboratory, Hercules, CA, USA).

To test the effect of an AhR inhibitor on ET, endometriotic explants were incubated for 48 hr with complete 10% FBS RPMI-1640 medium containing 15 μM –Naphthoflavone (ANF) (Sigma-Aldrich Company Ltd, Gillingham, UK), a specific AhR antagonist, before collecting tissue culture medium and performing a Bioplex Pro™ ELISA as described above. ANF was dissolved in 0.1% dimethyl sulfoxide (DMSO), which was also added to the control samples.

### Antibodies, dilution and incubation

The following panel of commercially available primary anti-human Abs was included in this study: mouse MC tryptase (clone AA1, M7052, Dako, Glostrup DK; dilution for IHC 1:500, incubation 1 h at 37°C); mouse MC tryptase (clone AA1, ab2378, Abcam, Cambridge UK; dilution for IF 1:600, incubation 2 hr at 37°C); mouse MC chymase (clone CC1, MCCXMS, Aczon Biotech, Bologna, IT; dilution for IHC 1:50, incubation 1 h at 37°C); mouse AhR (clone RPT1, GTX22770, GeneTex, Irvine CA, USA; dilution for IHC 1:100, incubation 16 hr at 4°C); rabbit AhR (LS-A3391, LifeSpan BioSciences, Seattle WA USA; dilution for IF 1:30, incubation 16 hr at 4°C); rabbit IDO1 (clone M80, sc-25809, Santa Cruz Biotechnology, Dallas TX, USA; dilution for IHC 1:400, incubation 1 h at 37°C); goat IL-17 (AF-317-NA, R&D System, Minneapolis MN, USA; dilution for IF 1:25, incubation 3 hr at

37°C); rat IL-10 (clone JES3-9D7, 14-7108, eBiosciences, San Diego CA, USA; dilution for IF 1:80, incubation 3 hr at 37°C).

Secondary conjugated Abs for IF were: anti-mouse Alexa Fluor® 488 (Abcam; dilution 1:800), anti-rabbit 649 (dilution 1:400) and anti-rat DyLight 549 (dilution 1:800), both from Jackson ImmunoResearch Europe Ltd. (Suffolk UK), and anti-goat Alexa Fluor® 555 (Invitrogen Thermo Fisher Scientific Inc. Waltham, MA USA; dilution 1:600). Slides were incubated with the fluorescent-labeled secondary Abs for 1 h at 37°C.

### **Immunohistochemistry (IHC) and immunofluorescence (IF)**

IHC was performed on 3.5 µm formalin-fixed paraffin-embedded slides. The slides were deparaffinized and pretreated for epitope retrieval using EnVision™ FLEX citrate buffer, pH 6.0, in PT Link and then placed in an automated Autostainer Link 48 immunostainer (all from Dako, Glostrup, Denmark).

Sections were incubated with primary antibodies (Abs) (see paragraph above) and detected using EnVision™ FLEX system (Dako). Images were obtained using a Leica DMD108 digital microimaging network (Leica Microsystems, Wetzlar, Germany).

Semiquantitative analysis of the IHC staining was performed independently by two pathologists (LM and MO), and glands were considered separately from stroma. Staining was evaluated as follows: percentage of positive cells, intensity of the positivity (evaluated as strong 3, moderate 2 and weak 1) and H-score (product of percentage of positivity and intensity; ranging from 0 to 300 as previously described (30)).

For IF, after incubation with primary Abs (see paragraph above), PBS-washed sections underwent incubation with the fluorescently labeled secondary Abs. Staining specificities were assessed by substituting primary antibodies with isotype controls. The reliability of the triple-staining procedure was evaluated by comparing the three single stains with the triple fluorescent stain in four consecutive sections. Slides were mounted with Vectashield containing DAPI (Vector Laboratories, Burlingame CA, USA) and visualized using a fluorescence-imaging microscope (Leica DMI 6000B) coupled to a CCD camera (Leica DFC350FX). The images were analyzed and composed using Leica LAS AF lite (Leica Microsystems), Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA) and ImageJ software (Open source software, <https://imagej.nih.gov/ij/>). The percentage of tryptase/Ahr double-positive or tryptase/Ahr/IL-17 and tryptase/Ahr/IL-10 triple-positive cells was determined by counting double- or triple-positive cells among tryptase-positive cells with a 40X oil immersion objective lens (numerical aperture: 1.25). All analyses were performed by two independent, blinded observers (GG and RD), and the average counts of the two observers were used.

To test the sensitivity and specificity of the AhR antibody LS-A3391 to AhR protein and not to aryl hydrocarbon receptor nuclear translocator (ARNT), different human cell lines ARNT-positive were stained by IF. U87 glioblastoma and HMC-1 mast cell lines resulted in AhR-positive staining, whereas no positive signal was found in K562 myelogenous leukemia or Jurkat leukemic T-cell lines (Supplementary Figure 1), in accordance with the results

reported by the Human Protein Atlas (<http://www.proteinatlas.org/>) and in ref. (31). Our results indicate that the antibody used herein does not cross react with ARNT.

### Sample extraction, liquid chromatography (LC) and electrospray ionization-mass spectrometry (ESI-MS)

Specimens from healthy endometrium and ETs were collected, immediately frozen in liquid nitrogen and then stored at  $-80^{\circ}\text{C}$  until metabolite extraction and analysis. The frozen samples, ranging between 40 and 200 mg, were placed in 2 ml Eppendorf tubes with 1.5 ml of a methanol/water mixture (1/1) and a metal sphere with a 5 mm diameter. The samples were then homogenized by two 5-min cycles of 50-Hz vibrations (TissueLyser, Qiagen, Redwood City, CA, USA) with an intermediary cooling cycle on dry ice. After centrifugation at 16,000 g for 10 min, each supernatant containing the water-soluble metabolites was dried in a vacuum desiccator at room temperature for 2.5 hr. The obtained residue was re-dissolved in 20  $\mu\text{L}$  water, and 1.6 ml of methanol was added to remove contaminating proteins. Each solution was centrifuged at 16,000 g for 10 min; the supernatant was then divided into 4 aliquots, vacuum-dried and stored at  $-80^{\circ}\text{C}$  until LC/ESI-MS analysis. The tissue pellet was suspended in 1.6 ml of a dichloromethane/methanol mixture (3/1) and homogenized by two 5-min cycles of 50-Hz vibrations with an intermediary cooling cycle on dry ice. After centrifugation at 16,000 g for 10 min, the supernatant was divided into 4 aliquots, dried under vacuum and stored at  $-80^{\circ}\text{C}$ . Each aliquot containing the hydrophilic metabolites was dissolved in milliQ  $\text{H}_2\text{O}$  (200  $\mu\text{L}$ ), and the internal standard (2'-deoxy-adenosine) was added prior to further dilution with milliQ  $\text{H}_2\text{O}$  (0.1%  $\text{HCOOH}$ ). The final internal standard concentration was 12  $\mu\text{M}$ ; 5  $\mu\text{L}$  of each diluted solution (equivalent to the extract from 80  $\mu\text{g}$  of starting tissue) was injected into the LC/ESI-MS system. The analysis of the mixtures was performed with a hybrid QqTOF mass spectrometer AB Sciex QSTAR (Applied Biosystems, Foster City, CA, USA) equipped with an Agilent 1100 Series micro HPLC (Agilent Technologies, Santa Clara, CA, USA). A reversed phase column Jupiter® C4, 150 x 0.5 mm, 5  $\mu\text{m}$ , 300 Å (Phenomenex, Torrance, CA, USA) was used for chromatographic separation; the gradient scheme was: 0-10 min equilibration at 10% B, 10 to 45 min linear gradient from 10% to 90% B and 45-55 min elution at 90% B, where A and B were  $\text{H}_2\text{O}$  MilliQ (0.1% Formic Acid) and  $\text{CH}_3\text{CN}$  (0.1% Formic Acid), respectively, and the flux was 10.0  $\mu\text{L}/\text{min}$ .

Mass spectra were recorded in positive ion mode. The pseudo-MRM (Multiple Reaction Monitoring) method was developed in-house; each acquisition cycle included one full-scan MS spectrum and three specific product ion MS/MS spectra for the detection of tryptophan, kynurenine and the internal standard. ESI-MS acquisition parameters for both MS and product ion scans were as follows: nebulizer gas (GS1) and curtain gas (CUR) set at 20.00 and 15.00 psi, respectively; DP (declustering potential), FP (focusing potential) and DP2 (secondary declustering potential) set at 40, 230 and 15 V, respectively, to minimize in-source fragmentation; ion spray voltage (IS) set at 5.50 kV. After optimization of MS/MS product ion scan for the target metabolites, the collision energy (CE) was set at 15 V. Selective narrow windows, with a width of 2 m/z units, were fixed for TOF detection to include the most intense fragment ions obtained by MS/MS scan (187-189 amu, 191-193 amu and 135-137 amu for tryptophan, kynurenine and internal standard, respectively).

The absolute concentration of tryptophan and kynurenine in the analyzed samples was based on standard compound calibration curves (Sigma-Aldrich). The final results, presented in Figure 2, were the ratio of metabolite content/biopsic tissue mass (ng/g or µg/g), which allows a comparison among samples.

### Culture of stromal cells from human endometrium

To isolate and culture stromal cells from normal endometrium, taken at day 4-6 of the cycle (late menstrual) in the early proliferative phase, we used a protocol optimized for culturing stromal cells from human tissues with minor modifications (32–34). Briefly, tissue fragments were enzymatically dissociated using 0.025% collagenase II for 10 min at 37°C (Worthington, Lakewood, NJ). After neutralization of the enzyme, the solution was centrifuged at 600 g for 10 min and filtered to obtain cells less than 70 µm in diameter (Millipore). Freshly isolated cells ( $1.5 \times 10^6$ ) were plated onto human fibronectin (Sigma-Aldrich)-coated 100 mm dishes (Corning BV life sciences, Corning, NY) in proliferation medium (60% low glucose DMEM (Invitrogen), 40% MCDB-201, 1 mg/ml linoleic acid-BSA,  $10^{-9}$  M dexamethasone,  $10^{-4}$  M ascorbic acid-2 phosphate, 1X insulin-transferrin-selenium selenite (all from Sigma-Aldrich), 2% fetal bovine serum (StemCell Technologies), 10 ng/ml human PDGF-BB, 10 ng/ml human EGF (both from Peprotech EC), as in (32–34). The medium was replaced every 4 days. Once cells reached 70-80% confluence, they were detached by TrypLE Express (Invitrogen) and re-plated at a density of  $2 \times 10^3/\text{cm}^2$ . To validate the stromal cultures from human endometrium we have performed flow cytometric and immunofluorescence analysis. At passage 3, all cells expressed vimentin, but not cytokeratins by immunostaining. By flow cytometric analysis, stromal cells were found to express several stromal cells biomarkers, CD29, CD90, CD44, CD49, and CD105. Cells were negative for epithelial markers EPCAM and E-CAD and did not show the expression of CD34, CD38 and CD45, which are specific markers for hematopoietic stem cells and leukocytes, respectively (Supplementary Figure 2).

### Growth kinetics and preparation of MC-conditioned medium

The cell population doubling time (PDT) was calculated by plating cells at the third passage at a density of  $2 \times 10^3$  cells/cm<sup>2</sup> into 96-well plates (BD Falcon™) in MC-conditioned medium (CM), obtained as follows:  $1 \times 10^6$  cells/ml of the human MC line HMC-1 were cultured for 48 hr in the absence or presence of FICZ (300 nM) or, as a positive control, phorbol 12-myristate 13-acetate (PMA, 1 ng/ml) and ionomycin (200 ng/ml) (35), all from Sigma-Aldrich. HMC-1-derived medium was diluted (1:1) in stromal cell culture medium prior to incubation with stromal cells. CM was replaced every 4 days, and images of Hoechst 33342-labeled cells were acquired at different time points (1-2-3-5 days) using a Leica DMI 6000B microscope connected to a Leica DFC350FX camera (Leica Microsystems) and equipped with a 5X objective lens (numerical aperture: 0.12). Cell counts were performed using ImageJ. Experiments were performed in triplicate. PDT was calculated during the log-phase of growth.

### Statistical analysis

The data were analyzed using R (version 3.1.1), and  $p < 0.05$  was considered significant. All p-values presented are for a two-tailed test. The normality of variables was tested with the

Kolmogorov-Smirnov test. Parametric data were described with the mean value and its standard deviation or 95% confidence interval, whereas non-parametric data were presented with the median value and interquartile range (IQR). For univariate analysis, the following statistical tests were applied: the t-test or Wilcoxon test for continuous variables and the Fisher exact or Chi-square test for categorical variables. For the comparison of more than two groups (growth kinetics in the presence of CM), a Friedman test followed by Dunn's test was applied.

## Results

### Population characteristics

The women who underwent surgery for endometriosis had a median age of 45 years (44–47), whereas controls had a median age of 47 years (45–49) ( $p = 0.476$ ). All the ETs analyzed were from ovarian endometriomas, and no significant differences were registered in the cycle phase at the time of surgery between cases and controls (Table 1).

### Inflammatory profiles in explant cultures of human endometrium

The serum and peritoneal fluid of women with endometriosis have been reported to contain high levels of several proinflammatory cytokines (36,37). Thus, to characterize the cytokine milieu present at the ET site, we analyzed a broad range of cytokines produced by healthy eutopic endometrium samples and ETs (Table 2). ETs were characterized by a significant increase in IL-4, IL-10, IL-12, IL-17, IFN- $\gamma$ , MCP-1/CCL2 and MIP-1 $\beta$ /CCL4 production compared with eutopic endometrium samples and a significant reduction of GM-CSF and TNF- $\alpha$  (Table 2). This result indicates that ETs are enriched in numerous cytokines and chemokines that could facilitate chronic disease progression.

### Expression of AhR in MCs within ETs

Altered immune cell function might explain the observed variation in the ET production of cytokines and MCs could contribute to this pattern. As MCs can be activated by AhR, ETs were stained for the presence of this receptor. As reported in the literature (26,38), AhR positivity was distributed in cells of the stroma and in the inflammatory infiltrate (Figure 1A). Positive cells resembling the characteristic granule-rich MC profile were observed (Figure 1B); indeed, immunofluorescence assays confirmed the co-localization of AhR and tryptase in 67% (IQR 57-76%) of MCs present within ovarian ETs and in 89% of MCs in healthy controls (IQR 86-95%) ( $p < 0.05$ ) (Figure 1C-D). Not all MCs populating ETs were AhR-positive, in accordance with previous works showing that exposure to AhR agonists causes AhR-expressing cells to downregulate the receptor (17,39).

### Enhanced expression of the AhR-ligand kynurenine and IDO1 in ETs compared with eutopic endometrium

AhR can be triggered by the tryptophan metabolite kynurenine, which can activate MCs (23). Thus, we measured the tissue concentrations of this AhR ligand in eutopic endometrium and ETs. Kynurenine was highly expressed in ETs compared with controls (Figure 2A) ( $p < 0.05$ ). Accordingly, tryptophan was impaired in the same endometriotic specimens (Figure 2B) ( $p < 0.05$ ); thus, the ratio between kynurenine and tryptophan was



significantly higher in ETs than in the eutopic endometrium of controls (median of 86.45 ng/ $\mu$ g, IQR 50.56-105.93 vs 1.76 ng/ $\mu$ g, IQR 1.52-1.88) ( $p < 0.05$ ) (Figure 2C).

It was recently shown that IDO1, the enzyme that catalyzes the degradation of tryptophan to kynurenine, is highly expressed in endometriosis-derived eutopic and ectopic endometrial stromal cells compared with endometriosis-free normal stromal cells (40). Indeed, immunohistochemistry analysis of our samples demonstrated that IDO1 positivity was confined to vascular endothelial cells in the control samples (Figure 2D, panels i and ii), whereas the staining was extended to stromal cells in ETs (Figure 2D, panel iii). The staining intensity of IDO1, represented by the H-score, was significantly higher in the stromal component of ETs (110, IQR 100-140) than in the eutopic endometrial stroma of controls (0, IQR 0-5) ( $p < 0.05$ ) (Figure 2D, panel iv).

Altogether, these results indicate that signalling through the AhR pathway in ETs is likely to occur.

### AhR-positive MCs in ETs express IL-17 and IL-10 cytokines

We have shown that AhR-activated MCs produce IL-17, a critical player in chronic inflammation and autoimmunity, the proinflammatory cytokines IL-6 and IL-13 and the immunomodulatory cytokine IL-10 (17). To establish the phenotype of MCs within ETs, the presence of AhR-positive MCs, as well as the number of AhR-positive cells expressing IL-17, was assessed in ET and control specimens by triple immunofluorescence *in situ* (Figure 3A-B). The results showed co-localization of tryptase, AhR and IL-17 in 54% of MCs in the ET samples (IQR 44-64%), whereas this value was decreased to 2.5% (IQR 2-4%) in healthy controls due to the limited expression of IL-17 (Figure 3C). To establish whether MCs represent an important source of IL-17 in ETs, IL-17 cells expressing the MC-specific marker tryptase were counted among the total IL-17-positive cells (Figure 3D). We observed that 63.0% (IQR 49.0-74.5%) of IL-17 cells were also tryptase-positive.

To determine whether chymase-positive MCs express AhR in a similar manner to tryptase-positive MCs, we first stained serial ET sections with Abs recognizing both enzymes. Then, AhR, chymase and IL-17 were concurrently evaluated in immunofluorescent staining. In our hands, the chymase staining was less diffuse than tryptase (Figure 4-B). Tryptase- and chymase-positive cells showed similar expression patterns and were equally present in ETs (Figure 4C). Thus, we cannot exclude the possibility that MCs in ETs are MC<sub>TC</sub>. Notably, we showed that chymase-positive cells do express AhR and that they are also IL-17-positive (Figure 4D).

As AhR signalling promotes MC-derived IL-10, ETs and controls were stained to highlight the possible presence of IL-10-producing AhR-positive MCs (Figure 5A-B). Indeed, we observed a significant co-localization of MC-tryptase, AhR and IL-10 in 55% of MCs (IQR 53-73%) within ETs but also in 33% (IQR 31-37%) of MCs in control endometrium tissues. Nevertheless, the increase was significant ( $p < 0.05$ ) (Figure 5C). In ETs, 50.2% (IQR 41.4-62.1%) of IL-10-positive cells co-expressed the MC tryptase (Figure 5D), suggesting that MCs contain at least half of the total amount of the two analyzed cytokines. Moreover, by immunofluorescence, we examined whether IL-10 and IL-17 are both present in the same

MCs or in different MC populations. We found that 99% (IQR 98.0-99.0%) of MCs in ET tissues (n=10) co-expressed these two cytokines (Supplementary Figure 3).

### ET microenvironment response to exposure to an AhR antagonist

To prove that the AhR pathway plays a role in modulating the cytokine and chemokine milieu of ETs and to counteract the observed increase in the AhR activating agent kynurenine, four explant cultures of patients were exposed to a single dose of 15  $\mu$ M ANF, an AhR antagonist (41). After 48 hr, the cytokines released in the supernatants were measured and compared with untreated control specimens. A broad downregulation of IL-4, IL-10, IL-17, IFN- $\gamma$ , MCP-1/CCL2 and TNF- $\alpha$  resulted upon treatment with ANF, although only IL-12 and MIP-1/CCL4 levels were significantly reduced in our limited sample size (Figure 6A).

To confirm that AhR-activated MCs are able to release molecules that could affect the growth of endometrium components, we cultured stromal cells from three tissue samples with a CM obtained upon exposure of the HMC-1 MC line to the AhR-activating ligand FICZ (Figure 6B). As a control, PMA and ionomycin, a stimulus known to induce the expression of several cytokines, was used (35). Culturing endometrial stromal cells with CM from FICZ-treated MCs led to a significant decrease in the PDT from 37.7 to 31 hr ( $p < 0.05$ ), similar to what we observed with CM from PMA/ionomycin-treated MCs (from 37.7 to 30.6 hr,  $p < 0.05$ ) (Figure 6B).

Altogether, our results suggest a likely involvement of the AhR pathway, and particularly of AhR-expressing MCs, in the chronic inflammation associated with endometriosis. These results open a new perspective in the treatment of this disease.

### Discussion

The present study is the first to analyze the phenotypic profile of MCs populating ETs in terms of cytokine production instead of granule content or tissue distribution. Furthermore, we envisaged that endometriotic MCs could be activated upon AhR signalling to secrete regulatory cytokines. Indeed, analysis of the inflammatory pattern of ETs showed increased cytokine production compared with the healthy eutopic endometrium obtained from controls. MCs appear to significantly contribute to the observed differences in inflammation. In fact, the majority of MC-tryptase or MC-chymase co-localized with AhR and IL-17 or IL-10 cytokines, and they accounted for half of the population expressing these cytokines within ET lesions. Moreover, there was a significantly increased H-score of the tryptophan-metabolizing enzyme IDO1 and a higher tissue concentration of the AhR-ligand kynurenine in ETs compared with the eutopic endometrium of controls. By contrast, tryptophan was significantly decreased in ETs compared with controls, supporting the interplay between AhR and kynurenine as a positive activator signal for MCs populating ETs. Finally, the involvement of AhR in the pathogenesis of endometriosis is supported by the observed down-modulation of several proinflammatory cytokines and chemokines, including IL-17 and IL-10, upon ET treatment with the AhR antagonist ANF, and the promotion of stromal cell growth by soluble factors released by MCs upon AhR activation. However, due to the relatively small sample size, the results of this observational study must be interpreted with

caution. Accordingly, validation of these findings in a larger independent cohort of patients is needed.

AhR was originally discovered as the mediator of the toxicity of environmental molecules. The effect of dioxin exposure on the risk of developing endometriosis remains controversial. In a non-human primate model, exposure to dioxin was associated with an increased prevalence and severity of endometriosis (42); similarly, several murine models support the possible role of environmental contaminants in the pathophysiology of endometriosis (6,43). However, a detailed review of the original epidemiological studies revealed no significant evidence supporting this relationship (44,45). Nevertheless, a proper understanding of the role of AhR in physiopathological conditions must differentiate between effects triggered by xenobiotic ligands and those triggered by endogenous ligands. A number of chemicals qualify as endogenous or physiological AhR ligands, including tryptophan products (46). Approximately 95% of ingested tryptophan enters the kynurenine pathway, and the first enzyme of the pathway is IDO1, which is stimulated by inflammatory molecules (47). The striking increase in kynurenine content in ETs compared with controls is consistent with the abundance of inflammatory cytokines observed here and in other diseases in which the immune response is activated and IFN- $\gamma$  is produced (48). Significant differences in tryptophan and its metabolites have been observed in the serum and plasma of patients with neurological disorders, affective disorders, autoimmune diseases and malignancy [reviewed in ref (48)]; however, to our knowledge, this study is the first to report a high kynurenine/tryptophan ratio, which is also an index for IDO1 activity, in ETs compared with control tissues.

AhR plays critical modulatory roles in various immune cells during innate and adaptive immune responses, including MCs. Kynurenine promotes MC degranulation, lipid metabolite generation and proinflammatory cytokine production (23) similar to 6-formylindolo[3,2-b]carbazole (FICZ), which is produced upon exposure of tryptophan to visible light or to UVA and UVB irradiation (49). Here, we were unable to measure FICZ compound levels in ETs or control eutopic tissues as it was below the detection limit of the ESI-MS assay (data not shown). Indeed, FICZ has only been identified in human skin, although FICZ-derived sulfate conjugates have been detected in human urine (18).

Tissue-resident MCs can exert their effector functions through direct or indirect actions of a wide variety of preformed or newly synthesized and selectively released mediators, including histamine, proteases, leukotrienes, prostaglandins as well as numerous cytokines, neurotransmitters and growth factors. This unique mediator profile might enable MCs to initiate an inflammatory cascade, leading to the observed symptoms of endometriosis. For example, it could modulate the recruitment, survival, development, phenotype or function of other immune cells described to be involved in endometriosis pathology, including monocytes/macrophages (10,16). Macrophages are recruited to the peritoneal cavity, where they are likely to remove the retrograded endometrial debris in patients with endometriosis (5). They can be stimulated by soluble factors derived from endometriosis-derived stromal cells, such as IDO1-induced IL-33, to become polarized into M2 cells with decreased phagocytic ability (40,50). Endometrium-resident AhR-activated MCs, representing an early source of IL-10, could also promote peritoneal macrophage M2 polarization into a

phenotype possessing tolerogenic activity (51). As a result, M2 cells dampen the immune response and support the survival of the ectopic endometrium in the initial stages of endometriosis. Moreover, concomitant MC production of IL-17, IL-6 and IL-10, together with peritoneal mesothelial cell-derived TGF- $\beta$  (52), could drive regulatory T cells (Treg) to switch to chronic inflammatory Th17 lymphocytes (53). Furthermore, IDO1 activity could directly inhibit the proliferation and activation of effector T cells, thus inducing immune tolerance (54).

In response to AhR activation, MCs also produce reactive oxygen species (ROS) (17,22) that could contribute to the previously described enhanced ROS production in endometriotic cells, which promotes proliferative capabilities of the cells and favors the spreading of the disease (55).

Anaf et al. found increased numbers of activated MCs near endometriotic lesions, often close to nerve fibers (12). It remains to be characterized whether MCs are able to sensitize primary nociceptive neurons by the release of nerve growth factor (NGF) or proinflammatory cytokines in response to AhR activation, thus contributing directly to the development of pain in endometriosis.

To characterize the cytokine milieu present at ET sites and to determine any similarity with results at the periphery, we analyzed a broad range of cytokines produced by explant cultures of human endometrium, as performed by Osteen et al. (56). Interestingly, as opposed to peritoneal fluid, we observed less TNF- $\alpha$  secretion from ETs than their healthy counterparts, a result that could explain why there is still insufficient evidence to support the use of anti-TNF- $\alpha$  drugs in the management of pelvic pain for women with endometriosis (57). Selective AhR modulators have been developed with a view to clinical applications, especially in the context of cancer (18). Considering the complexity of AhR signalling outcomes, the effectiveness of these drugs will depend on multiple factors, including dose and persistence. For instance, we have demonstrated that different MC responses can be achieved depending on the duration of FICZ stimulation. A single dose (that is, singular AhR activation) boosted proinflammatory features of MC activation (histamine and IL-6 production), whereas prolonged exposure shifted MCs toward IL-17 production and impaired degranulation (17). Thus, the short-term organ culture used in this study, which preserved epithelial-stromal and immune cell interactions, could represent a useful tool to assess the therapeutic potential of AhR ligands in modulating the proinflammatory environment of endometriosis, thus mediating disease progression.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

<b>AhR</b>	Aryl Hydrocarbon Receptor
<b>ANF</b>	-Naphthoflavone
<b>ESI-MS</b>	Electrospray Ionization-Mass Spectrometry
<b>ET</b>	Endometriotic Tissue
<b>FICZ</b>	6-formylindolo[3,2-b]carbazole
<b>IDO1</b>	Indoleamine 2,3-dioxygenase-1
<b>IF</b>	Immunofluorescence
<b>IHC</b>	Immunohistochemistry
<b>IL</b>	Interleukin
<b>LC</b>	Liquid Chromatography
<b>MCs</b>	Mast cells
<b>PDT</b>	Population doubling time
<b>PMA</b>	Phorbol 12-myristate 13-acetate

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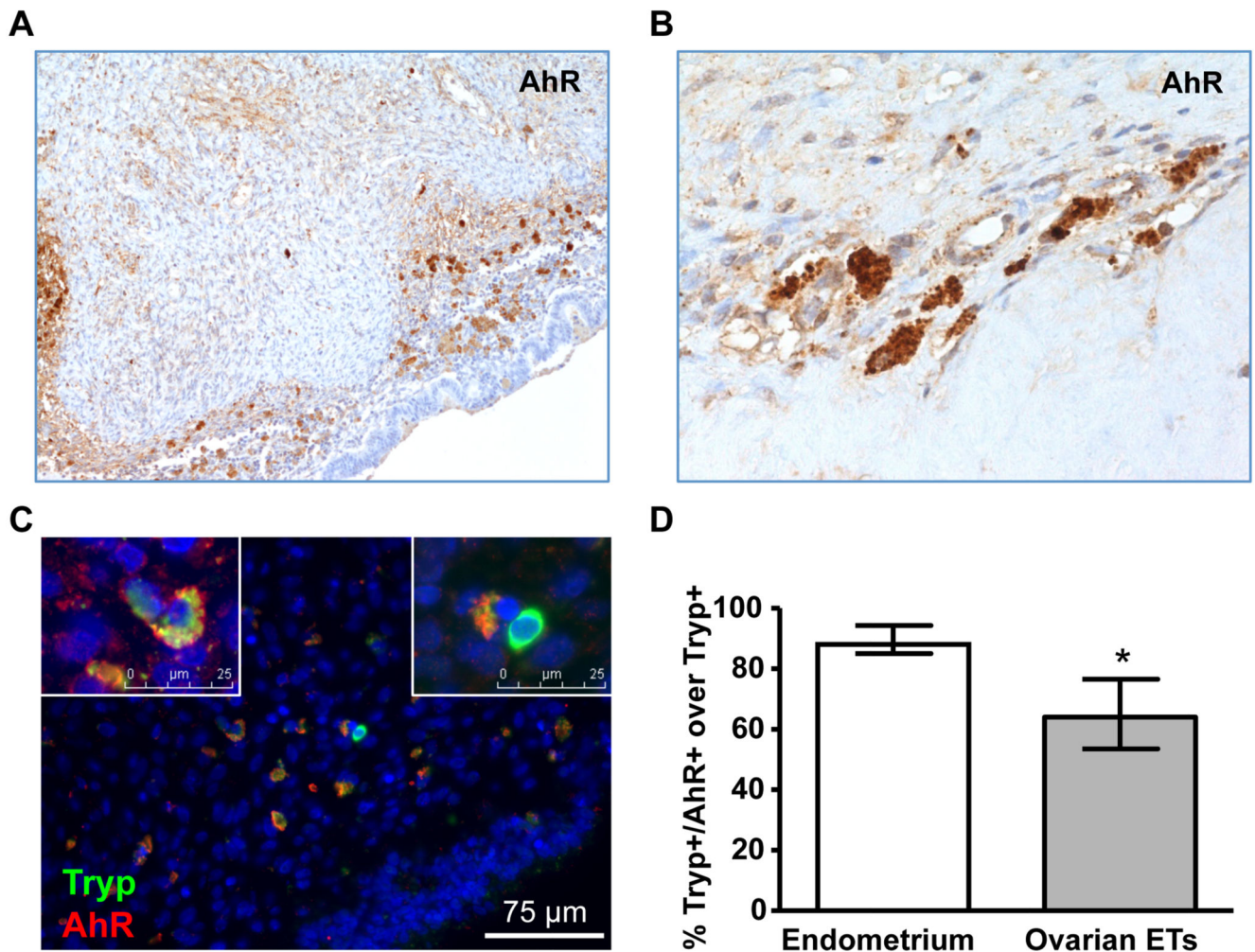
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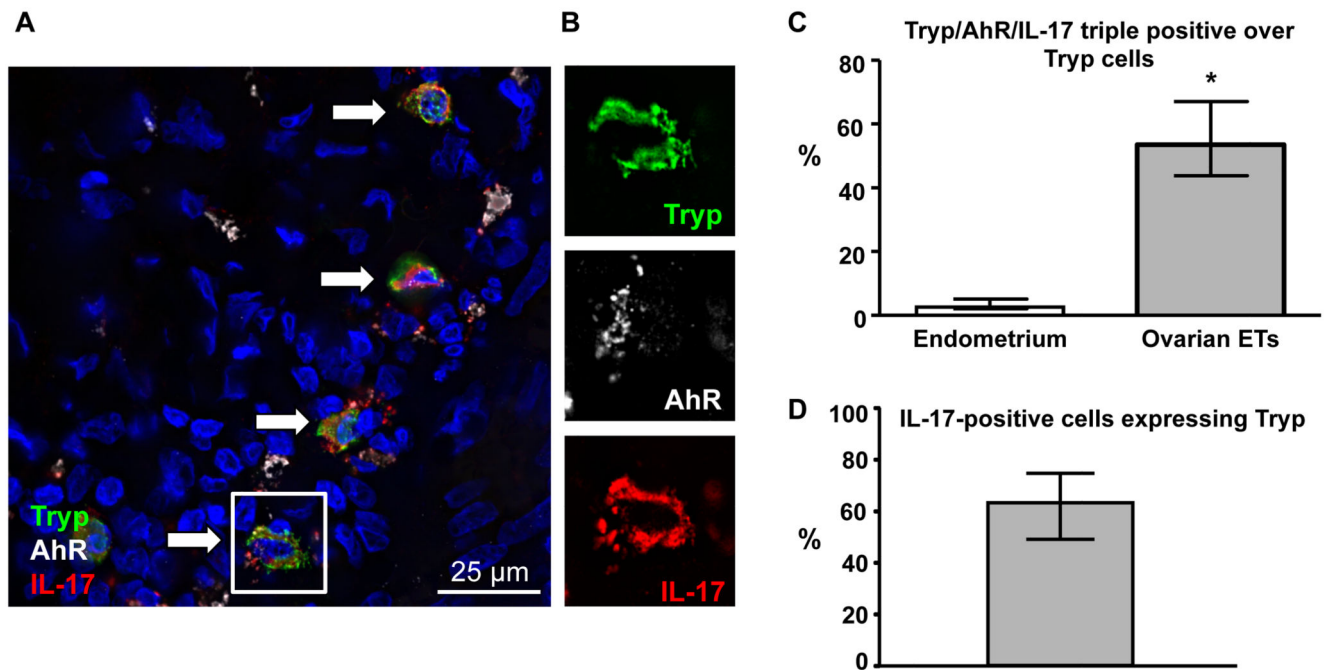


**Figure 1. MCs populating ETs express AhR.**

(A-B) Immunohistochemical localization of AhR protein in human ETs. Magnification, x200 and x400. (C) Double-marker immunofluorescence analysis of AhR and tryptase (Tryp) in sections from ETs (double staining: Tryp green, AhR red). Bars 75  $\mu$ m and 25  $\mu$ m (inset). (D) Percentage of Tryp/AhR (Tryp<sup>+</sup>/AhR<sup>+</sup>) double-positive cells over total Tryp single-positive cells (n=10/group). Cells were detected and counted from ten x400 high-power microscopic fields in each individual sample. Endometrium median 89% (IQR 86-95%); ovarian ET median 67% (IQR 57-76%). \* p<0.05

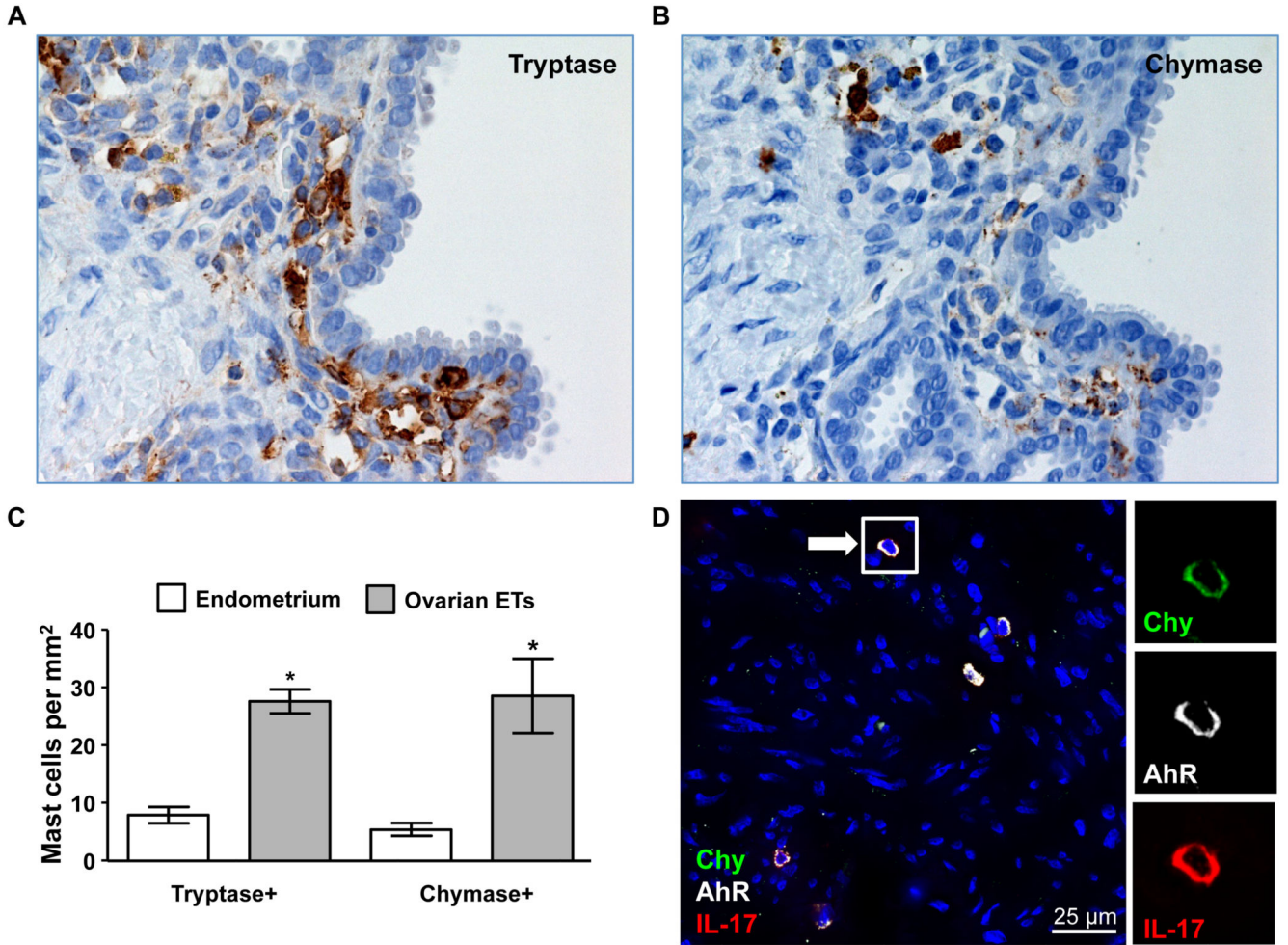


mass (ng/ $\mu$ g). **(D)** Representative IDO1 immunostaining in healthy proliferative (i) and secretory (ii) endometrium and in ET (iii) are shown. Magnification, x200; (iv) show the significant differences in stromal H-score values of IDO1 expression between ET samples and healthy endometrium (n = 10/group). The p-values (\*) reported in the plot area refer to Wilcoxon test.

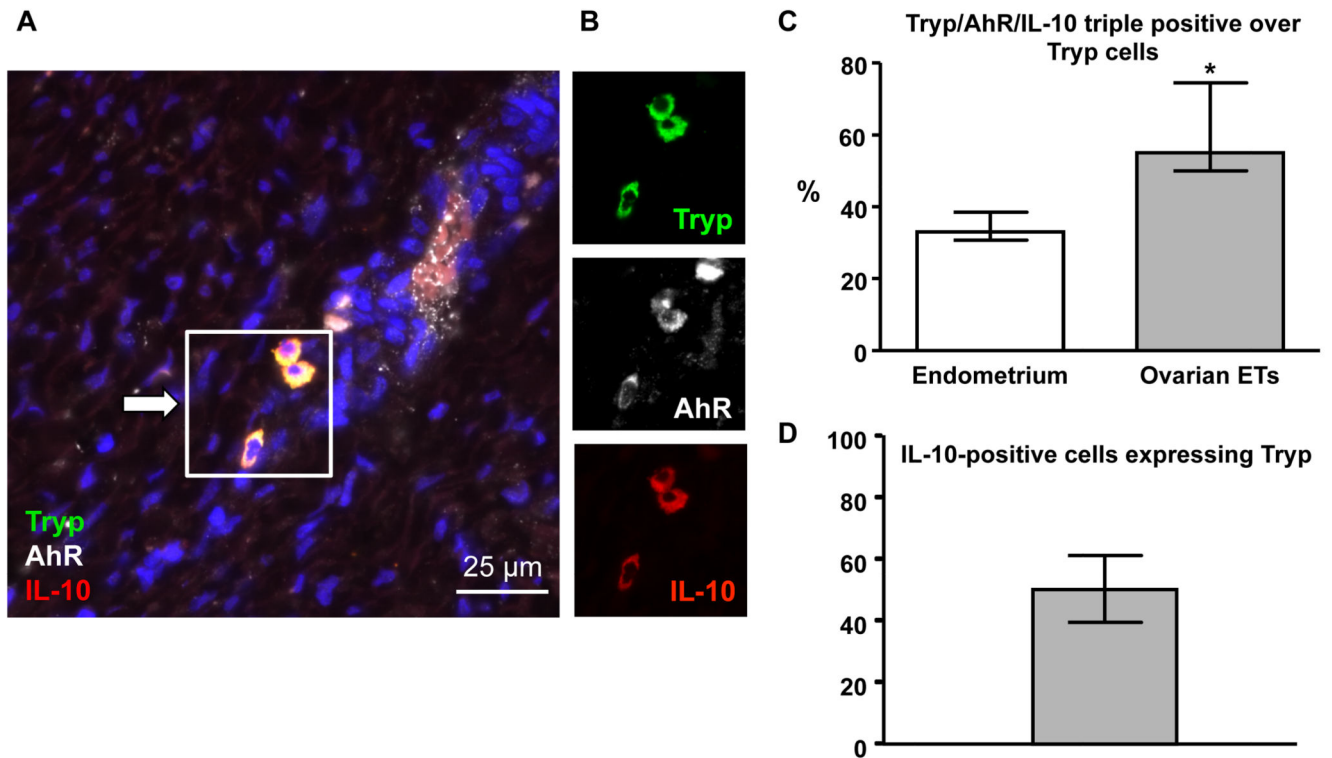


**Figure 3. AhR-positive MCs express the inflammatory cytokine IL-17 in ETs.**

(A) Immunofluorescence triple staining showing that several tryptase-expressing MCs (Tryp, green) co-express both AhR (white) and IL-17 (red) (*arrows*). Scale bar, 25  $\mu$ m. (B) A higher magnification view of the boxed area in (A) comparing uncoupled fluorescence micrographs of a MC. The DAPI staining shows that this field of cells is primarily a monolayer, allowing assessment of co-positivity for tryptase, AhR and IL-17. (C) The percentage of triple-positive cells among tryptase-expressing MCs in 10 individual ET samples is shown. Cells were detected and counted from ten x400 high-power microscopic fields in each sample. Endometrium median 2.5% (IQR 2-4%); ovarian ET median 54% (IQR 44-64%). \*  $p < 0.05$  (Wilcoxon test). (D) IL-17 single-positive and IL-17 and tryptase double-positive cells were counted in ovarian ET samples as in C. The results are expressed as the median of IL-17-positive cells expressing tryptase 63.0% (IQR 49.0-74.5%).

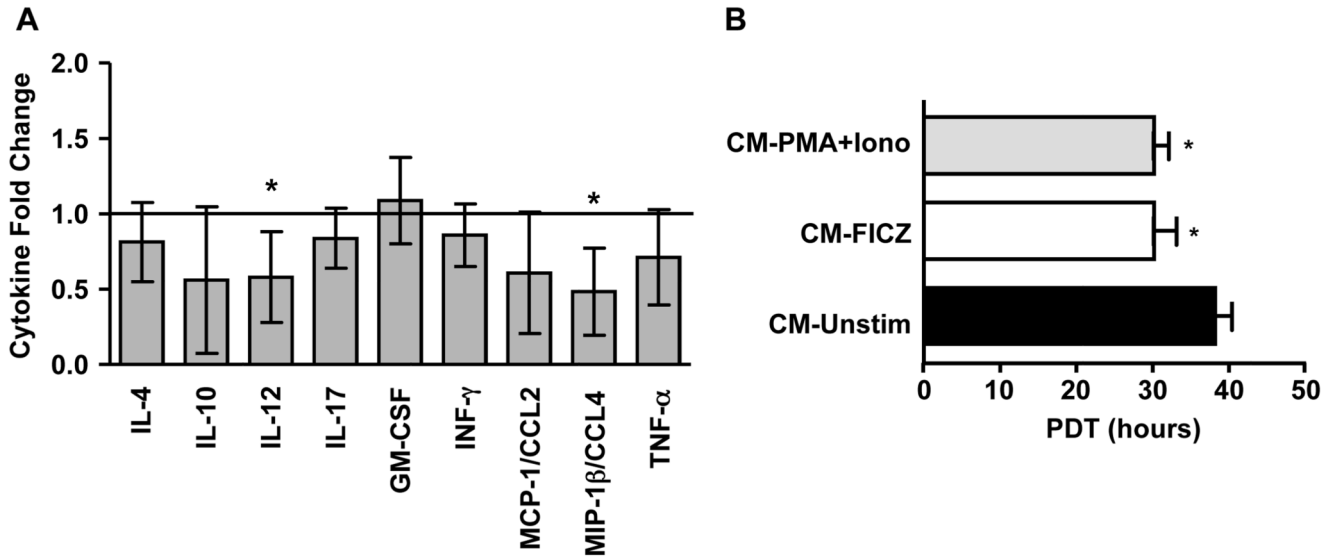


**Figure 4. Chymase-expressing MCs within ETs are AhR- and IL-17-positive.** (A-B) Consecutive ET sections were cut and stained for MC tryptase and chymase, respectively. Magnification, x400. (C) Quantitative analysis of tryptase- and chymase-positive MCs. The data represent the mean  $\pm$  S.D. of the number of cells per mm<sup>2</sup> observed in 10 fields of endometrium biopsies per specimen (n = 8/group). \* p<0.05 (Wilcoxon test). (D) Immunofluorescence triple staining showing that chymase-expressing MCs (Chy, green) co-express both AhR (white) and IL-17 (red) (arrows). Scale bar, 25  $\mu$ m. On the right, a higher-magnification view of the boxed area in (D) comparing uncoupled fluorescence micrographs of the selected MC.



**Figure 5. AhR-positive MCs express the tolerogenic IL-10 cytokine in ETs.**

(A) Immunofluorescence triple staining showing that several tryptase-expressing MCs (Tryp, green) co-express both AhR (white) and IL-10 (red) (arrows). Scale bar, 25  $\mu$ m. (B) A higher magnification view of the boxed area in (A) comparing uncoupled fluorescence micrographs of three MCs. (C) The percentage of triple-positive cells among tryptase-expressing MCs in 10 individual control and ET samples is shown. Cells were detected and counted from ten x400 high-power microscopic fields in each sample. Endometrium median 33% (IQR 31-37%); ovarian median 55% (IQR 53-73%). \*  $p < 0.05$  (Wilcoxon test). (D) IL-10 single-positive and IL-10 and tryptase double-positive cells were counted in ovarian ET samples as in C. IL-10 single-positive and IL-10 and tryptase double-positive cells were counted in ovarian ET samples as in C. The results are expressed as the median of IL-10-positive cells expressing tryptase 50.2% (IQR 41.4-62.1%).



**Figure 6. (A) Ovarian ET cytokine expression upon treatment with an AhR antagonist.** IL-4, IL-10, IL-12, IL-17, GM-CSF, INF- $\gamma$ , MCP-1/CCL2, MIP-1/CCL4 and TNF- $\alpha$  protein expression in supernatants of untreated (control) or ANF-treated ovarian ET cultures measured by ELISA 48 hr after exposure to the AhR-antagonist ANF (15  $\mu$ M). Control protein concentration was assigned a value of 1 and is illustrated as a horizontal line for presentation purposes. Sample size was 4 patients/group. The values are the mean and 95% confidence intervals. \*  $p < 0.05$  (t-test). **(B) Reduced PDT of endometrium stromal cells exposed to FICZ-activated MC-conditioned medium.** Endometrium-derived stromal cells were cultured in conditioned medium (CM) of the HMC-1 MC line treated without (CM-Unstim) or with FICZ (CM-FICZ) or PMA and ionomycin (CM-PMA+Iono) (for details, see Material and Methods section). Results are expressed as the median and IQR ( $n = 3$ ), \*  $p < 0.05$  (Dunn's Test) vs CM-Unstim.

**Table 1****Population description.**

The backgrounds of controls (n = 10) and ovarian endometriotic tissues (ETs) (n = 10) used in this study are shown.

	<b>Controls</b>	<b>ETs</b>	<b>p</b>
Median age of women in years (range)	47 (45-49)	45 (44-47)	0.476
<b>Phases</b>			
Follicular phase	60% (6/10)	60% (6/10)	1.000
Luteal phase	40% (4/10)	40% (4/10)	1.000
<b>Indication for surgery</b>			
Ovarian mass		70% (7/10)	
Chronic pelvic pain		30% (3/10)	
<b>ASRM stage</b>			
I			
II		30% (3/10)	
III		40% (4/10)	
IV		30% (3/10)	



**Table 2**  
**Different cytokine production between the eutopic endometrium of healthy women (controls) and ovarian endometriotic tissues (ETs).**

Cytokine production was evaluated in the supernatants of healthy endometrium or ETs cultured in complete medium for 48 hr. The data are presented as the mean ( $\pm$  standard deviation); p-values refer to t-test or are presented as the median (interquartile range – IQR), and p-value refers to Wilcoxon test (\*). N.D., Not detectable.

	Controls (n=4)	ETs (n=4)	Fold change (ET vs Controls)	p
<b>IL-1b (pg/ml) (*)</b>	181.7 (91.1-275.1)	20.9 (9.7-32.6)		0.266
<b>IL-2 (pg/ml)</b>	71.8 ( $\pm$ 45.3)	112.4 ( $\pm$ 29.5)		0.629
<b>IL-5 (pg/ml)</b>	N.D.	9.4 ( $\pm$ 7.6)		N.D.
<b>IL-6 (ng/ml)</b>	6.7 ( $\pm$ 6.4)	64.0 ( $\pm$ 45.9)		0.162
<b>IL-7 (pg/ml)</b>	91.8 ( $\pm$ 16.2)	80.0 ( $\pm$ 14.1)		0.519
<b>IL-13 (pg/ml)</b>	3.2 ( $\pm$ 4.3)	17.0 ( $\pm$ 12.1)		0.235
<b>G-CSF (ng/ml)</b>	3.4 ( $\pm$ 1.2)	3.9 ( $\pm$ 0.8)		0.482
<b>IL-4 (pg/ml)</b>	17.5 ( $\pm$ 5.5)	40.3 ( $\pm$ 4.2)	+2.3	<0.05
<b>IL-10 (pg/ml)</b>	35.4 ( $\pm$ 18.1)	909 ( $\pm$ 409.8)	+25.7	<0.05
<b>IL-12 (pg/ml)</b>	41.1 ( $\pm$ 20.9)	322.4 ( $\pm$ 139.4)	+7.8	<0.05
<b>IL-17 (pg/ml)</b>	97.7 ( $\pm$ 96.8)	402.0 ( $\pm$ 76.1)	+4.1	<0.05
<b>IFN-<math>\gamma</math> (pg/ml)</b>	785.5 ( $\pm$ 509.8)	1802.3 ( $\pm$ 158.8)	+2.3	<0.05
<b>MCP-1/CCL2 (ng/ml)</b>	1.6 ( $\pm$ 1.4)	4.0 ( $\pm$ 0.9)	+2.5	<0.05
<b>MIP-1<math>\beta</math>/CCL4 (pg/ml)</b>	558.5 ( $\pm$ 354.9)	1347.0 ( $\pm$ 275.3)	+2.4	<0.05
<b>GM-CSF (pg/ml)</b>	826.8 ( $\pm$ 49.5)	184.3 ( $\pm$ 106.1)	-4.5	<0.05
<b>TNF-<math>\alpha</math> (pg/ml) (*)</b>	732.7 (592.9-848.6)	217.5 (197.8-231.8)	-3.4	<0.05