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MACROPHAGES DISPLAY PRO-INFLAMMATORY PHENOTYPES IN EUTOPIC ENDOMETRIUM OF WOMEN WITH ENDOMETRIOSIS WITH RELEVANCE TO AN INFECTIOUS ETIOLOGY OF THE DISEASE

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Abstract

Objective: To phenotype transcriptomically macrophages M1 (Mφ1) and M2 (Mφ2) in the endometrium of women with endometriosis.

Design: Prospective experimental study.

Setting: University research laboratory.

Patient(s): Six women with endometriosis and five controls without disease, in the secretory phase of the menstrual cycle.

Intervention(s): Mφ1, Mφ2, uterine natural killer (uNK) and regulatory-T (Treg) cells were isolated from human endometrium using a uniquely designed cell-specific fluorescence activating cell sorting panel. Transcriptome profiles were assessed by RNA-high sequencing, bioinformatic and biological pathway analyses.

Main Outcomes Measure(s): Differential gene expression between Mφ1 and Mφ2 in women with and without endometriosis, and in Mφ1 versus Mφ2 in each group, were determined and involved different bioloigcal and signaling pathways.

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Result(s): Flow cytometry analysis showed no significant differences in total numbers of leukocytes between control and endometriosis groups, although Mφ1 were higher in endometriosis versus controls. Statistical transcriptomic analysis was performed only in Mφ1 and Mφ2 populations due to larger sample sizes. Bioinformatic analyses revealed that in women with endometriosis endometrial Mφ1 are more pro-inflammatory than controls and Mφ2 paradoxically have a pro-inflammatory phenotype.

Conclusion(s): As Mφ are phenotypically plastic and their polarization state depends on their microenvironment, the altered endometrial environment in women with endometriosis may promote endometrial Mφ2 polarization and an Mφ1 pro-inflammatory phenotype. Moreover, aberrant phenotypes of Mφ may contribute to abnormal gene expression of eutopic endometrium and a pro-inflammatory environment in women with endometriosis relevant to the pathophysiology of the disease and compromised reproductive outcomes.

CAPSULE

RNA-Seq analyses showed that macrophages-M1 are more pro-inflammatory in eutopic endometrium of women with endometriosis than in control endometrium and that macrophages-M2 paradoxically display a pro-inflammatory phenotype in disease.

Keywords

endometriosis; macrophages; inflammation; endometrium; infection

INTRODUCTION

Endometriosis is an estrogen-dependent inflammatory disease that results in pelvic pain and/or infertility. It affects approximately 10% of reproductive age women (1–4) and is characterized by the presence of endometrial-like tissue outside the uterus where it elicits an inflammatory response (1,3,4). The eutopic endometrium of women with endometriosis has been widely studied with regard to dysfunctionality of steroid hormone response, stem cell populations, and recruitment of immune populations for immune tolerance and overall tissue homeostasis and pregnancy success versus women without disease (1–3). However, there are scant data about the function and phenotypes of eutopic endometrial immune cells in women with and without endometriosis. As the endometrial immune niche involves multiple cell types with varying degrees of activation and communications among immune and non-immune cells that dictate functionality of the tissue, characterizing the endometrial immune niche is of great relevance to understanding endometrial function and dysfunction.

Uterine natural killer cells (uNK) secrete angiogenic factors that contribute to the maturation of blood vessels having a role in embryo implantation and successful pregnancy (6,7). In healthy endometrium, their cytotoxic activity diminishes during the secretory phase of the menstrual cycle which allows embryo implantation (8–10). However, in infertile endometriosis patients, uNK have high cytotoxicity in eutopic endometrium that could lead to an inhospitable environment for embryo implantation (11). Other immune cells, such as T regulatory cells (Treg), have been also described to behave differently in the endometrium of women with endometriosis. In healthy endometrium, they increase in the proliferative and

decrease during the secretory phase, with the latter creating an immune-tolerant environment allowing embryo implantation. However, in infertile women with endometriosis, Treg are increased in the peri-implantation endometrium, leading to an implantation failure (12).

Endometriosis has been referred to as “a disease of the macrophage” (13), based mainly on a replete literature on the roles and functionality of this cell type in peritoneal fluid of women with disease and in establishment of endometriosis lesions and associated processes of angiogenesis and fibrosis. Mφ are key effector cells in both innate and humoral immunity as they phagocytose pathogens, act as antigen presenting cells, and have a role in tissue regeneration, angiogenesis and wound healing (14). In eutopic endometrium of women without endometriosis, their numbers vary throughout the menstrual cycle, increasing in the secretory and menstrual phases (15). This increase may be attributed to their phagocytic properties and role in clearing cell debris and apoptotic cells during endometrial shedding (16). Cycle variation among endometrial Mφ does not occur in women with endometriosis (17), suggesting that survival of shed and refluxed endometrial cells may be enhanced, enabling them to migrate to the peritoneal cavity and establish disease. Mφ are classified as either “classically activated” Mφ (Mφ1) or “alternatively activated” Mφ (Mφ2) (14) and, depending on the microenvironment, they can switch from one type to the other (18). Mφ1 have a role in pro-inflammatory responses; whereas, Mφ2 are involved in angiogenesis, anti-inflammatory reactions, and tissue repair (14,19). In healthy endometrium, the predominant population is Mφ2 (19,20), suggesting that the normal environment is anti-inflammatory. Taken together, most of the studies in eutopic endometrium have focused on the number of immune cells in this tissue and how they fluctuate throughout the cycle, but little is known about their functionality in women with endometriosis.

Herein, we designed a novel flow cytometry panel to isolate Mφ1, Mφ2, Treg and uNK from eutopic endometrium of women with endometriosis and those with no evidence of disease. RNA High-Sequencing (RNA-Seq) was used to elucidate Mφ1 and Mφ2 phenotypes and possible functions in disease. Overall, the data support a phenotypic switch of the common anti-inflammatory Mφ2 to the pro-inflammatory Mφ1 phenotype and a more exaggerated pro-inflammatory phenotype of the Mφ1 population in women with endometriosis.

MATERIALS AND METHODS

Sample Collection and Processing

Eleven endometrial biopsies in the secretory phase were collected: 6 from women with endometriosis (stage I-IV) and 5 from women with no evidence of endometriosis at the time of the surgery for benign gynecologic disorders. The mean age was 37 and 42 (23-49) years old, respectively. In order to evaluate if the age could confound the results, a non-parametric t-test with a subsequent Mann-Whitney test ($p<0.05$) was performed and no significant differences between groups were found ($p=0.2641$). Patients had not used hormonal therapy for at least three months prior to the study. Endometrial samples were obtained through the University of California San Francisco (UCSF) NIH Human Endometrial Tissue and DNA Bank under approval of the UCSF Committee on Human Research (IRB#10-02786), and written informed consent was obtained from all participants. Endometrial tissue was digested as previously described (21). Briefly, it was minced mechanically and incubated for

one hour at 37ºC in digestion media, which contained collagenase type I and hyaluronidase (21). Subsequently, the single cell suspension was filtered using a 40μm mesh to discard cell clumps, and single cells were cryopreserved in liquid nitrogen until use.

Flow cytometry panel design

A cytometry panel of 10 conjugated antibodies able to separate the immune populations of interest (Mφ1, Mφ2, Treg and uNK) was designed. Specific membrane markers of resident and blood infiltrating immune cells were included to avoid sorting cells derived from the peripheral circulation. The brightest colors were used for the markers with the lowest antigen density. Minimum overlapping of 11 colors (10 antibodies plus the live/dead dye) was achieved. The markers and lasers used for each population are in Table 1 and the gating strategy is in Figure 1.A. First, the cells were gated with CD45 (leukocyte marker), conjugated with brilliant violet 605 (BV605). In the case of Mφ, usually this population is a resident tissue population, thus no specific tissue markers were used. It is challenging to differentiate between the Mφ1 and Mφ2 subpopulations, as they have some common markers and have the ability to polarize from one type to the other. Our strategy was as follows: for both types, CD14 marker conjugated with phycoerythrin (PE) was used. For Mφ2, CD163, a specific marker for this population, was used conjugated with PE-cyanin7 (PE-Cy7). As there are no specific markers for Mφ1 and it was suspected that the concentration of activated Mφ1 would be low in the endometrium (as Mφ2 are higher than Mφ1 in normal endometrium (19)), CD80 (activation marker) conjugated with peridinin chlorophyll protein complex-cyanin 5.5. (PerCP-Cy5.5) antibody, a bright dye, was used. With regard to Treg, they express CD3 and CD4 markers. The most accepted specific marker for Treg is Foxp3. This, is an intracellular marker and thus could not be used for sorting. However, they also express CD25. To be able to discern between tissue Treg and Treg deriving from circulation, CD69, an activation marker for Treg that is expressed in tissue, was included. Thus, $CD3^{+}CD4^{+}CD25^{+}CD69^{+}$ cells (tissue Treg) were isolated. The CD3 antibody was conjugated with ultraviolet B 737 (BUV737), as was the CD4 antibody with ultraviolet B 395 (BUV395). CD25 has a low antigen density, therefore, brilliant blue 515 (BB515) was used, which is one of the brightest dyes. Then, CD69, a tissue activation Treg marker, was conjugated with allophycocyanin-cyanin7 (APC-Cy7), assuring that only resident Treg were isolated. For uNK cells, which are $CD56^{+}$, as it is known that blood NK are $CD16^{+}$; whereas, uNK are $CD16^{Low/-}$ (22–25), $CD56^{+}CD16^{-}$ were collected. For CD56, brilliant violet 421 (BV421), was used and in the case of CD16, allophycocyanin (APC) conjugated antibody was used. Finally, to separate between live and dead cells, Aqua dye was used (sources of all antibodies are listed in Table 1).

Fluorescence Activated Cell Sorting (FACS)

Endometrial samples were thawed at 37ºC. After centrifugation at 1,300rpm for five minutes, the supernatant was discarded and the pellet was washed with 1X PBS. After another centrifugation, cells were resuspended with 1X PBS + 5% bovine serum albumin (BSA) and incubated at room temperature for 30 minutes. A minimum of 100,000 unlabeled cells were separated as a negative control. Ten conjugated antibodies were used to label the samples (Table 1). 1μl of antibody per million cells was used in all cases except for CD45 and CD4, where 2μl per million cells were needed for an optimal cell labelling. A solution

of all Fluorochromes Minus One (FMO) was prepared for each antibody to assess any overlap among the channels in the FACS instrument. After one hour of incubation at 4ºC in 1X PBS + 3% BSA and in the dark, cells were washed with 1X PBS and centrifuged for 5 minutes at 1,300 rpm. The pellet was resuspended with 500µl of 1X PBS and labelled with 1µl of LIVE/DEAD™ Fixable Aqua Dead cell labelling dye (ThermoFisher, Waltham, MA). On the other hand, UltraComp eBeads compensation magnetic beads (ThermoFisher, Waltham, MA, USA) were labelled with each of the 10 antibodies following the manufacturer's instructions, to allow the correction of the spectral overlap between fluorochromes. Using the gating strategy (Figure 1.A), each population was sorted in the FACS Aria Jabba the Hutt (BD Biosciences, East Rutherford, NJ, USA) instrument and collected in 1X PBS. Flow cytometry analysis of the sorted cells was performed using FlowJo.v10 software (FlowJo LLC, Ashland, OR, USA), and statistical analyses (Mann-Whitney test, p-value<0.05) were conducted using GraphPad software (GraphPad Software Inc, San Diego, CA, USA).

RNA extraction

As low yields of cells were obtained after FACS (Figure 1.B), RNeasy micro kit (Qiagen, Hilden, Germany) was used to isolate RNA from Mφ populations (note yields from uNK and Treg cells were too low, and were not used for RNA-Seq, see Methods). From the 22-sorted Mφ samples (Mφ1 and Mφ2 populations of each of the 11 endometrial samples), RNA was extracted following the manufacturer's instructions to perform the total RNA-seq library prep from samples containing >900 cells (9 samples). The library preparation from the remaining samples, which contained at least 20 cells, was performed directly from cells in 1X PBS. RNA was eluted in 10µl of RNase free-water, and the quality (RNA integrity numbers (RIN)) and concentration were measured using a Tapestation4200 System (Agilent, Santa Clara, CA, USA).

RNA-Seq and statistical and bioinformatic analyses

SMART-Seq™ v4 Ultra™ low input RNA kit for sequencing (Clontech, Mountain View, CA, USA) was used to perform the RNA-seq library preparation. It allows RNA-Seq to be performed with very low concentrations of RNA or to use whole cells to preserve sample integrity. In total, library preparations for 22 samples (10 from control and 12 from endometriosis) were performed. The quality of fastq files was tested using the FastQC (v0.11.5) (26) and the Qualimap (rnaseq module – v2.2.1) software (27). Reads were aligned with the STAR mapper (v2.5.2a) (28) to release 88 of the Homo sapiens ENSEMBL version of the genome (GRCh38/hg38 assembly) (29). A raw count of reads per gene was also obtained with STAR (28) . In order to overcome the heterogeneity between samples, first, samples were removed from the analysis if they had <5 million uniquely mapped reads, and the remaining samples were downsampled to 30 million mapped reads when needed. The data have been deposited in NCBI GEO database (accession number GSE130435). The R/Bioconductor package DESeq2 (v1.20.0) (30–32) was used to assess differential expression between experimental groups (Wald statistical test + false discovery rate (FDR) correction). Statistically significant differentially expressed genes (DEG) were considered when FDR<0.05 and log fold change>2 (LogFC>2). Different comparisons performed using Mφ populations are shown in Table 2. Biological significance analyses were conducted using

Ingenuity Pathway Analyses (IPA) software (Ingenuity® Systems, Redwood City, CA, USA), and significant molecular functions were established with an activation Z-score> | 2.00|.

RESULTS

FACS and flow cytometry analyses

After FACS, low cell numbers were obtained (Figure 1.B) that subsequently guided further analyses. Cytometry analyses from all the immune populations showed that $CD45^+$ cells corresponded to an average of 6.8% of the total sample, in agreement with other studies wherein leukocytes comprise 10-20% of total endometrial cells (33–38). No significant differences in $CD45^+$ cells were observed between control and endometriosis groups (Figure 1.C). Statistical analyses comparing percentage of each sub-population between controls and endometriosis were performed, and no significant differences were observed except for Mφ1, which was significantly higher in the endometriosis group ($p=0.0087$) (Figure 1.D). Because resident tissue markers were included in the cytometry panel, contamination of immune populations from the peripheral circulation could also be calculated. In both the control and endometriosis groups, uNK ($CD16^-$) were significantly higher compared to blood NK ($CD16^+$) (Figure 1.E), demonstrating that there was almost no contamination with blood NK cells. The percentage of Treg coming from blood (CD69) was higher than tissue Treg ($CD69^+$), although it was not significant (Figure 1.E). Due to the low number of uNK and Treg cells obtained, RNA-Seq was only performed in the Mφ populations, that had significantly greater numbers of cells. Thus, from the 44 original FACS-sorted immune populations, 22 samples (Mφ populations) from endometriosis and control were used for the transcriptome study.

RNA extraction and gene expression analyses (RNA-Seq)

RNA concentrations extracted from Mφ ranged between 5-45ng/μl. After RNA-Seq and quality controls, we excluded any FastQ sequences for which the number of reads did not reach our threshold of five million reads/sequence. Thus, the populations analyzed were: 5 Mφ1 endometriosis, 3 Mφ1 control, 6 Mφ2 endometriosis and 4 Mφ2 control. After statistical analysis, DEG (FDR<0.05 and LogFC≥2) were found in all comparisons (Table 2; Supplemental Table 1).

Biological significance of the DEG analyses revealed significant molecular functions, relevant molecules secreted by Mφ1 and Mφ2, activation/inhibition of upstream regulators and de-regulated networks in each comparison (activation Z-score≥2.00) (Table 3). The 25 top de-regulated networks are in Supplemental Table 2. Increase in cell-cell contact was observed along with repression of RNA molecular functions, when comparing Mφ1 endometriosis versus Mφ1 control (**Comparison 4,** Table 2). Increased cell-cell contact is consistent with, e.g., increased adhesion to bacteria to accomplish bacterial engulfment. Top de-regulated networks showed overexpression of cellular development, growth and proliferation, and overexpression of immune response-related networks, such as infectious disease and antimicrobial and inflammatory responses (Table 3). These data indicate that

Mφ1 in endometriosis have a more extensive pro-inflammatory phenotype than Mφ1 in the control group.

In contrast, molecular functions upregulated in Mφ2 in endometriosis (**Comparison 5,** Table 2) included an accumulation of Ca^{2+}, increase in carbohydrate transport, and internalization of bacteria (Table 3). When comparing Mφ1 of women with versus without endometriosis (**comparison 4,** Table 2), the upstream regulator TNFα was predicted to be increased (Table 3). Increased internalization of bacteria is consistent with phagocytic properties of the pro-inflammatory Mφ1 phenotype. The top networks in endometrial Mφ2 from women with endometriosis included deregulation of connective tissue disorders, endocrine system development and function, lipid metabolism, inflammatory disease/response, and drug metabolism (Table 3). These data overall demonstrate that Mφ2 in eutopic endometrium of women with endometriosis have a pro-inflammatory phenotype, compared to Mφ2 in control women.

DISCUSSION

In the current study, we developed a cytometry panel that allowed for separating circulating immune cells and tissue resident cells and different immune cell types within human endometrium. Thus, the analyzed immune populations were purely tissue-activated resident cells devoid of contamination by circulating immune cells. One goal was to develop and optimize this panel for the current study. However, it will also have value for other researchers aiming to separate these tissue-specific populations, since it is a challenging panel to design due to the multiple colors used and the possible overlap between channels.

After cytometry analyses, where Mφ1 were found to be significantly higher in endometriosis, Mφ were studied in more detail by transcriptomic analyses. To our knowledge this is the first RNA-Seq dataset of Mφ in eutopic endometrium of women with endometriosis. Abnormal distribution of Mφ within eutopic endometrium of women with disease could contribute to the aberrant distribution of immune cells in the pelvic cavity and the abnormal development and gene expression of this tissue. While Mφ maintain organ homeostasis and facilitate host defense and wound healing, they also underlie the pathogenesis of many chronic inflammatory diseases (39).

The increased de-regulated molecular functions and networks in Mφ1 in endometrium of women with endometriosis indicate these cells have a more pro-inflammatory phenotype than Mφ1 in the control group. In addition, a significantly higher number of sorted Mφ1 was observed in endometriosis patients (Figure 1.D), confirming a previous report (40). Moreover, these results suggesting that eutopic endometrium of women with endometriosis is more pro-inflammatory than control endometrium, is consistent with findings from other groups (17,41).

An unexpected finding herein was the pro-inflammatory phenotype exhibited by endometrial Mφ2 from women with endometriosis. Mφ2 in other tissues generally display an anti-inflammatory phenotype (39), and, importantly, Mφ are phenotypically plastic with regard to their polarization state depending on their microenvironment (42). Moreover, Mφ1 and Mφ2

gene expression signatures often overlap, and the resultant phenotype depends on the tissue microenvironment (40). Thus, endometrial Mφ2 of women with endometriosis could undergo polarization *in situ* to Mφ1, adopting a pro-inflammatory phenotype, due to an altered environment. The paradigm of different subpopulations of Mφ is controversial in the immunology literature. Specifically, it is unclear whether there are unique Mφ populations (as Mφ1, Mφ2) or if Mφ comprise a unique population that alters its phenotype depending on environmental cues. Herein, we have referred to Mφ as two different subpopulations (Mφ1 and Mφ2), although the dynamics and mechanisms driving pro-inflammatory and anti-inflammatory Mφ functional phenotypes remain to be determined.

Notably, tumors take advantage of macrophage plasticity. For example, in the early phases of cancer, high production of Mφ1 inflammatory mediators activates the adaptive immune response capable of eliminating nascent neoplastic cells, and also support neoplastic transformation (40). In contrast, once the tumor is stablished, the main population of Mφ is Mφ2, producing an anti-inflammatory environment, which allows tumor growth. Endometriosis it is not a malignancy, however, it shares some characteristics with cancers. In endometriotic lesions and peritoneal fluid of women with endometriosis, e.g., Mφ2 are increased (43), indicating that, as in cancer, an anti-inflammatory environment prevails favoring development and growth of the endometriotic lesions. In addition, that Mφ2 have a role in angiogenesis further supports this paradigm. Finally, Mφ2 are also involved in nerve growth, suggesting they may also have a role in endometriosis-related pain (44).

The initial pro-inflammatory phenotype of Mφ in cancer increases NFKB and downstream events and increases transcription of pro-inflammatory cytokines such as TNFα, IL12, IL23, IL1β, IL6, and ROS. In the current study, the NFKB pathway was activated in Mφ1 of endometriosis, which does not occur in Mφ1 of control women (Table 3). Indeed, it has been described that the NFKB pathway is de-regulated in the eutopic endometrium of women with endometriosis (45), which also indicates that the microenvironment in endometrium of women with disease is more pro-inflammatory than heathy tissue.

Notably, an increase of transport of carbohydrates was observed in Mφ2 of women with endometriosis. It is known that glycolysis is high in Mφ1 and is decreased in Mφ2 and Mφ polarization may derive from a reprogramming of glucose metabolism (46). Several studies have suggested that altering nutrient availability or blocking specific metabolic pathways skews the Mφ phenotype and alters their effector functions in chronic inflammatory diseases (47). In this regard, Mφ metabolism modulation could open a new therapeutic window for treating inflammatory diseases including endometriosis.

Finally, the upstream regulator TNFα was increased in IPA analysis when comparing Mφ1 of women with versus without endometriosis (**comparison 4,** Table 2), as well as increased Ca^{2+} accumulation was activated in Mφ2 (Table 3). It has been noted that a transient increase of Ca^{2+} plays a role in the expression of TNFα by Mφ1 (48). Intracellular Ca^{2+} oscillations are likely to induce permanent changes in Mφ physiology, and a supra-physiologic elevation of Ca^{2+} in mitochondria can be cytotoxic and induce apoptosis in the long term (48). Whether TNFα-mediated events play a role in Mφ function awaits further studies.

Over the past decade, high-throughput sequencing techniques have challenged the dogma of the sterility of the uterine endometrium (49–54), and in particular an altered endometrial microbiome in women with endometriosis has been proposed (55). In addition, the endometrial microbiome also correlates with IVF outcomes (52), although whether this occurs in women with endometriosis awaits further study. However, treatment with antibiotics resulted in reduced numbers of endometriosis lesions in a mouse model, with concomitant alteration of the gut microbiome (56), although the endometrial microbiome was not reported in this study (54). Interestingly, a recent systematic review supports the use of antibiotics prior to oocyte retrieval in patients with endometriosis, among other gynecologic disorders (57). The presence of pathogenic, non-commensal bacteria in the endometrium may induce an altered immune cell profile and activation (increased numbers and activation of Mφ1 and activation of Mφ2) that could impact the production of cytokines by immune resident cells that adversely affect embryo implantation (58). In addition to effects on reproductive outcomes, the observed greater pro-inflammatory endometrial environment herein could be related to the pathophysiology of the disease. While attractive, we are aware that the sample size of the study is small. Therefore, these results should be taken with caution. Finally, whether the pro-inflammatory phenotype of the Mφ2 population reported herein is in response to commensal bacteria or pathogens, or if Mφ populations are implicated in reproductive outcomes, is not clear. However, it is anticipated that this important area of research could have profound implications clinically and diagnostically.

CONCLUSIONS

Overall, the results of the current study lead to the conclusion that both Mφ1 and Mφ2 in eutopic endometrium of women with endometriosis display a higher pro-inflammatory phenotype compared to controls without disease. Endometrial Mφ2 appear to be predisposed to Mφ1 polarization in women with endometriosis, thus increasing their inflammatory phenotype. These findings suggest that eutopic endometrium has different Mφ gene signatures depending on the presence or absence of disease and that the endometrial environment of women with endometriosis is more pro-inflammatory than control endometrium. Whether subtypes of the disease are associated with different subsets of immune and whether the macrophage pro-inflammatory status is related to bacteria in the endometrium of women with disease are yet to be determined. Finally, the results herein may have implications regarding the impact of the macrophage phenotypes on reproductive outcomes and possible novel therapeutics for microbiome-related symptoms and response for fertility and pain in women with endometriosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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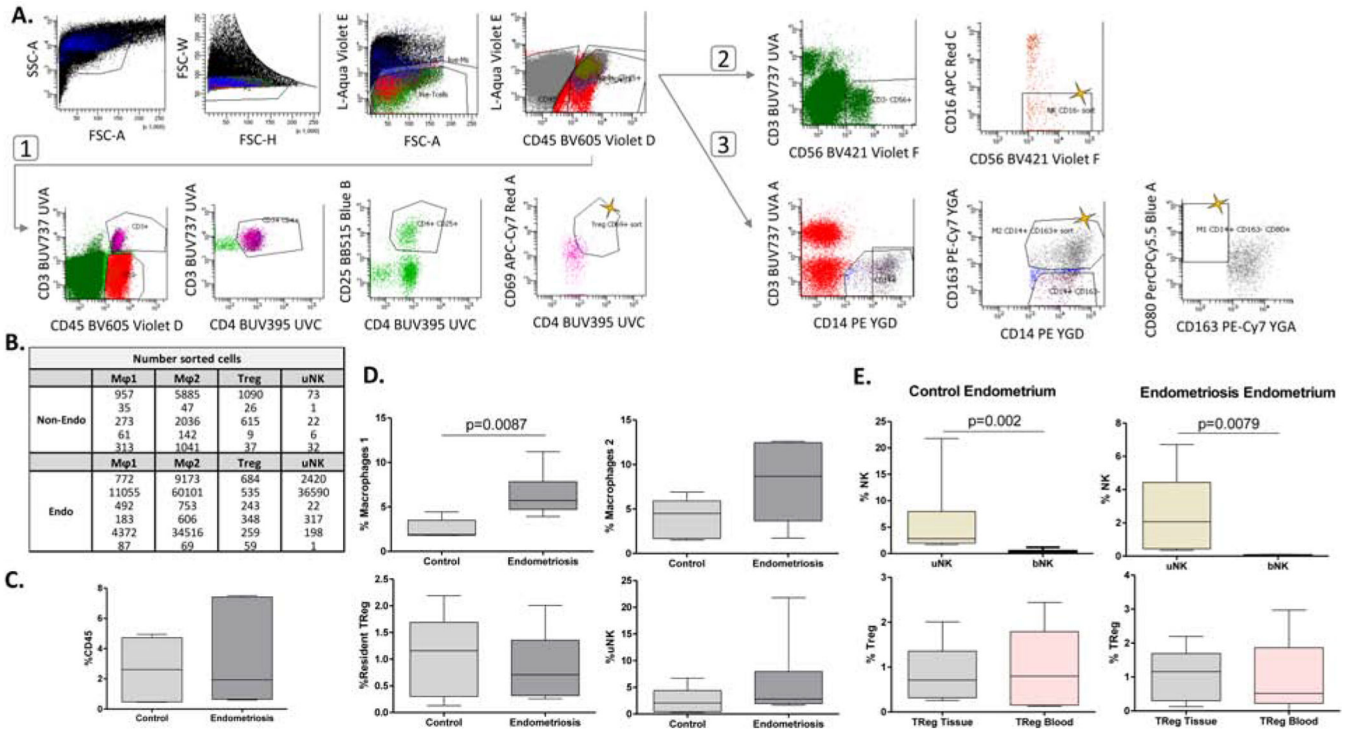

Figure 1. Cytometry panel and flow cytometry analyses.

A. Gating strategy. The different gates used to sort the four desired populations are shown. The X and Y axis show the lasers that were used to gate the cells. In addition, the name of the marker and the fluorochrome used have been inserted on the corresponding axes. First, cells were gated by their complexity (SSC-A/FSC-A). Then, singles cells were gated (FSC-W/FSC-H) as well as live cells (L-Aqua Violet E/FSC-A). In this case, two different gates were made: one for T cells and uNK and a bigger one for Mφ due to the autofluorescence present in the latter. The next step was to gate live $CD45^+$ cells (L-Aqua Violet E/CD45 BV605 Violet D). Thereafter, three strategies were used; 1) gating resident Treg ($CD45^+CD3^+CD4^+CD25^+CD69^+$); 2) gating uNK ($CD45^+CD3^-CD56^+CD16^-$), and 3) gating Mφ2 ($CD3^-CD14^+CD163^+$) and Mφ1 ($CD3^-CD14^+CD163^-CD80^+$). The final sorted populations are marked with a yellow star. **B. Number of sorted cells.** The table shows the number of cells of macrophages M1 (Mφ1), macrophages M2 (Mφ2), resident regulatory T cells (Treg) and uterine natural killer (uNK) cells, obtained after FACS in non-endometriosis and endometriosis patients. **C. Percentage of $CD45^+$ cells.** The figure shows that no significant differences were found in the % $CD45^+$ cells between control (n=5) and endometriosis patients (n=6). **D. Percentage of each subpopulation comparions between control and endometriosis.** Mφ1 increased significantly in numbers in endometriosis endometrium; whereas, no significant differences were found for Mφ2, uNK and Treg. **E. Percentage of uNK and Treg coming from blood circulation.** It can be observed that in both control (left panel) and endometriosis (right panel) endometrium, there is a significant increase of resident uNK ($CD16^-$) compared to circulating NK ($CD16^+$), which indicates that there is almost no NK contamination from the peripheral circulation. In the case of Treg,

it can be observed that there is an increase of Treg coming from blood in both groups, altough it it is not significant.

Table 1.

Antibodies used for the FACS.

The table shows all the markers used for the sorting of the desired populations, the fluorochrome to which they were conjugated, the antibody, the distributor, and the laser with which the fluorochromes are excited. Excitation and emission wavelenghts for each fluorechrome are also shown.

Marker	Fluorochrome (excitation/emission)	Conjugated Antibody	Manufacturer* and reference	FACS Aria Laser
Live/dead	Aqua (405nm/512nm)	-	ThermoFisher (L34957)	Violet E
CD45	BV605 (405nm/600nm)	CD45-BV605	BD Bioscience (BDB 564047)	Violet
CD14	PE (496nm/578)	CD14-PE	Biolegend (301805)	YGD
CD163	PE-Cy7 (561nm/785nm)	CD163-PE-Cy7	Biolegend (333614)	YGA
CD80	PerCP-Cy5.5 (488nm/695nm)	CD80-PerCP-Cy5.5	Biolegend (305231)	Blue A
CD3	BUV737 (355nm/737nm)	CD3-BUV737	BD Bioscience (BDB 564307)	UVA
CD4	BUV395 (355nm/395nm)	CD4-BUV395	BD Bioscience (BDB 563550)	UVC
CD25	BB515 (488nm/515nm)	CD25-BB515	BD Bioscience (564468)	Blue B
CD69	APC-Cy7 (640nm/785nm)	CD69-APC-Cy7	BD Bioscience (BDB 560737)	RedA
CD56	BV421 (405nm/421nm)	CD56-BV421	BD Bioscience (562752)	Violet F
CD16	APC-Cy7 (640nm/660nm)	CD16-APC	Biolegend (302011)	Red C

*ThermoFisher, Waltham, MA, USA; BD BioScience, East Rutherford, NJ, USA; Biolegend, San Diego, CA, USA.

Table 2.

Comparisons of the Mφ populations analyzed by RNA-seq.

The first column shows each comparison performed after quality control analyses. The second column shows the number of samples used in each population to perform each comparison. The last column shows the number of differentially expressed genes obtained in each comparison by Wald statistical test and FDR. Mφ1: macrophages 1; Mφ2: macrophages 2; DEG: differentially expressed genes; LogFC: log fold change; FDR: false discovery rate.

Comparisons	Sample size	DEG (LogFC≥2/FDR<0.05)
1. Mφ Endo vs. Mφ Control	11 vs. 7	1567
2. Mφ1 Control vs. Mφ2 Control	3 vs. 4	1260
3. Mφ1 Endo vs. Mφ2 Endo	5 vs. 6	705
4. Mφ1 Endo vs. Mφ1 Control	5 vs. 3	1422
5. Mφ2 Endo vs. Mφ2 Control	6 vs. 4	1544

Table 3.

De-regulated molecular functions, networks and upstream regulators in macrophages comparisons.

The table shows the significant molecular de-regulated molecular functions (Z-Score≥2) obtained after IPA analyses of different macrophages comparisons, as well as the diferentially expressed genes of RNA-Seq dataset involved in these functions. The fifth column shows the five top de-regulated networks in each comparison, where the immune-related networks are bolded. Finally, the sixth column shows the upstream regulators predicted to be activated (↑) or inhibited (↓) in the IPA analysis of each comparison. Mφ1: Macrophages 1; Mφ2: Macrophages 2; Endo: Endometriosis; Ctr: Control; ERG: ETS-related gene; TNF: Tumor Necrosis Factor; LH: Lutein hormone; miR-483-3p: micoRNA-483-3p; TGFB1: Transforming Growth Factor Beta 1; SATB: Stabilin 1; INHBA: Inhibin Subunit Beta A; false discovery rate. NFkB: Nuclear Factor kappa Beta; IFNα: Interferon alpha; IL15: Interleukin 15; VCAN: Versican.

	Molecular Functions	**Z-Score**	**Genes**	**De-regulated Networks**	**Upstream regulators**	**Z-Score**
Mφ-Endo vs Mφ Ctr	Senescence of fibroblast cell line Activation of cells	−2.09 1.95	ARHGAP10, DPY30, EREG, HYAL1, IL1A, LATS1, MAPK1, MATN4, ME2, PBRM1, POT1, PTEN, PTTG1, RBL1 ANGPT1, C3, CADM1, CD1D, CD4, CHRNA7, CSF2, DDIT4, EGF, EIF3A, GP1BA, HLAB1, IL13, IL1A, IL1R1, IL33, ITGA3, LBP, LTA, MYO18A, NOS3, NT5E, PSEN1, PTEN, PTGDR2, PTPN22, RAB5B, SBNO2, SIRPG, SPI1, STIM1, THBS1, TOP2A, VAMP4, VEGFA, WASL	Hematological System Development and Function, **Immune Cell Trafficking, Inflammatory Response** **Infectious Diseases, Antimicrobial Response, Inflammatory Response** Organismal Injury and Abnormalities, Cell Morphology, Cellular Development Cellular Development, Cellular Growth and Proliferation, **Reproductive System Development and Function** Dermatological Diseases and Conditions, **Inflammatory Disease, Inflammatory Response**	↑ERG	2,333
	Molecular Functions	**Z-Score**	**Genes**			**Z-Score**
Mφ1 Endo vs Mφ1 Ctr	Cell-cell contact Repression of RNA	2.25 2.19	ACTN4, ANGPT1, ARF6, ARHGAP19, CBLL1, CLDN2, CTNNB1, DNM2, DSP, ESRP2, F2RL2, GDF15, GSK3B, IL13, ING4, ITGA4, NRDC, NT5E, PLCB1, RAPGEF3, SYK, TJP1 CUL3, DR1, ERCC2, FOXG1, LCOR, MECP2, TAF1	Cellular Development, Cellular Growth and Proliferation, **Lymphoid Tissue Structure and Development** **Infectious Diseases, Antimicrobial Response, Inflammatory Response** Cardiac Arrythmia, Cardiovascular Disease, Hereditary Disorder Cell Morphology, Cellular Movement, Organismal Injury and Abnormalities Cell Signaling, Cellular Assembly and Organization, Cellular Function and Maintenance	↑TNF	2,587
	Molecular Functions	**Z-Score**	**Genes**			**Z-Score**
Mφ2 Endo vs Mφ2 Ctr	Concentration of Ca^{2+} Transport of carbohydrates Internalization of bacteria	2.36 2.21 2.15	DRD1, ITGAL, KLRD1, LPAR4, PLG, TRPV5, VIPR2 ABCB1, AQP2, C3, CSF2RA, NPC1, RALBP1, SLC23A1, SLC2A3, SLC45A1, SLC5A1 C3, CEACAM6, ERBB2, PLG, PRKCA	Connective Tissue Disorders, **Inflammatory Disease, Inflammatory Response** Drug Metabolism, Endocrine System Development and Function, Lipid Metabolism Cell-To-Cell Signaling and Interaction, Hematological System Development and Function, **Immune Cell Trafficking** Cell Death and Survival, Hematological System Development and Function, **Lymphoid Tissue Structure and Development** Behavior, Nervous System Development and Function, Endocrine System Disorders	↓LH	−2,169
	Molecular Functions	**Z-Score**	**Genes**			**Z-Score**
Mφ1 vs Mφ2 Ctr	Cytotoxicity of leukocytes	−2.18	BMPR1A, CBLB, CD244, FCAR, KIR2DL4, KIR3DL1, KLRC1, NOTCH2	Cancer, Cell Death and Survival, Hematological Disease Cellular Function and Maintenance, Cellular Movement, Carbohydrate Metabolism Cell Death and Survival, Cancer, Organismal Injury and Abnormalities Developmental Disorder, Hereditary Disorder, Organismal Injury and Abnormalities Hematological System Development and Function, **Lymphoid Tissue Structure and Development,** Tissue Morphology	↓miR-483-3p ↑TGFB1	−2,000 2,195
	Molecular Functions	*Z-Score*	**Genes**			**Z-Score**

	Molecular Functions	Z-Score	Genes	De-regulated Networks	Upstream regulators	Z-Score
Mφ1 vs Mφ2 Endo	Growth of *S. Cerevisiae* Engulfment of cells	−2.00 2.12	LIG1, PARP1, POLR2K, ZMPSTE24 AP2B1, ATP6V1E1, AXL, CD47, CDC5L, CERK, CSK, DNM2, EGR1, ERBB2, LMBRD1, NR1H3, RHOG, SNX5, STK4, SYK, USPL1	Cell-To-Cell Signaling and Interaction, Hematological System Development and Function, **Immune Cell Trafficking** Cell Death and Survival, **Embryonic Development**, Organismal Injury and Abnormalities Cell Death and Survival, **Inflammatory Response**, Cancer Cellular Assembly and Organization, Cellular Function and Maintenance, Neurological Disease Cancer, Cell Death and Survival, Organismal Injury and Abnormalities	↑TNF ↓SATB1 ↑INHBA ↑NFkB ↑IFNα ↑IL15 ↓VCAN	2,385 −2,400 2,449 2,784 2,186 2,224 −2,000