REPRODUCTION AND THE INFLAMMATORY RESPONSE

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REPRODUCTION AND THE INFLAMMATORY RESPONSE

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Table of Contents

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Editorial: Reproduction and the Inflammatory Response

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Keywords: endocrine disrupting compound (EDC), endometriosis, infection, inflammation, inflammasome, polycystic ovary syndrome (PCOS), preeclampsia, repeat implantation failure

Editorial on the Research Topic

Reproduction and the Inflammatory Response

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Reproduction is a fundamental feature of all known life entailing a remarkably complex and tightly regulated series of events that are essential in supporting species' long-term survival. While the development of knowledge of reproductive processes across species is of utmost importance, this Research Topic primarily focuses on those factors that influence the pathophysiology of reproductive complications or gynecological conditions in humans.

Unlike many other species, human reproduction is surprisingly inefficient. Infertility for example is a global public health issue rising in prevalence that affects upwards of 15% of reproductive-aged couples, or a total of 186 million individuals worldwide (1, 2). Even amongst those couples able to initiate pregnancy, challenges are still faced. Embryo loss between fertilization and birth is estimated between 40-60% (3), while pregnancy complications, including preeclampsia (PE, 2-8% of pregnancies) and preterm birth (10.6% of births), are the leading cause of death amongst women of reproductive years (4, 5). Gynecological conditions, such as endometriosis (10%) (6), polycystic ovary syndrome (PCOS, 5-20%) are common (7), in addition to sexually transmitted infections (Chlamydia, 2016 = 127 million estimated infections) (8) and parental lifestyle factors not only influence fertility but also lead to adverse birth outcomes and lay the foundations for offspring susceptibility to disease (9). While numerous contributing factors influence our susceptibility to these reproductive challenges, one area that is intimately involved in all areas of reproductive function and contributes to reproductive pathologies is inflammation and dysregulation of the immune system (10).

The immune system plays an integral role in both the physiology and pathophysiology of reproduction (10, 11). Across both males and females, there is a close functional relationship between the male and female reproductive tracts and the immune system, which is tightly regulated to meet the physiologically challenging demands of successful reproduction (12). In females, events including ovulation, menstruation, implantation, and parturition are all associated with the induction of inflammatory mediators, while in males, the development and function of testes

and epididymis are influenced by their immune microenvironment (12, 13). Correct programming of the maternal immune environment supports implantation and subsequent pregnancy success (14). Thus, altered immune function during, or even prior to gestation can have a significant impact on not only our reproductive health, but also on the long-term health of offspring.

The reasons behind the decline in fertility and increased prevalence in subfertility are complex, but there is extensive evidence that lifestyle and environmental factors are major contributors (15). Endocrine Disrupting Compounds (EDCs) for example, influence reproductive function through their potential to mimic or block the actions of endogenous hormones (9). In this Research Topic, Schjenken et al., highlight the capacity of EDCs to also affect immune system development and function, and propose that in addition to disruptions in hormone signaling that regulate reproductive physiology, that alterations to the immune environment caused by EDC exposure during gestation may disrupt the establishment of maternal immune tolerance that is required to support robust placentation and fetal development (Schjenken et al.). Additionally, infection during pregnancy is also linked with adverse pregnancy outcomes. TORCH infections, which classically comprise toxoplasmosis, Treponema pallidum, rubella, cytomegalovirus, herpesvirus, hepatitis viruses, human immunodeficiency virus, and other infections, such as varicella, parvovirus B19, and enteroviruses are major contributors to prenatal and post-natal morbidity and mortality (16). However, most studies only focus on infections that occurs during pregnancy. In this Research Topic, Liu et al., explored the impact of previous TORCH infections on maternal and neonatal outcomes in an assisted reproduction setting and show that prior infections were not associated with adverse pregnancy or neonatal outcomes, thus highlighting that infections that occur during pregnancy should be the major focus of future study.

Alterations to the maternal immune environment are also implicated as a central predisposing factor in many of the common pregnancy complications, such as repeat implantation failure (RIF) and PE (17–19). Both conditions are commonly characterized by dysregulated activation of both innate and adaptive arms of the immune system (17, 19). In this Research Topic, Ho et al. explored how the proportion of peripheral natural killer cells influenced ART outcomes following intravenous immunoglobulin treatment while di Rivero Vaccari and Shirasuna et al. discussed recent reports that link pregnancy complications such as PE with altered inflammasome activation. In both complications, the authors show the potential benefit of developing a greater understanding of immune cell function in developing diagnostics or therapeutics to treat these pregnancy complications. In the case of RIF, intravenous immunoglobulin (IVIG) treatment is beneficial only in some patients, and Ho et al. demonstrated the prognostic capacity of assessment of peripheral populations of natural killer cells prior to conception. They showed that peripheral CD56+CD16+ NK cells with a population of ≤10.6% in RIF patients were likely to benefit from IVIG therapy with better implantation and pregnancy rate outcomes. In the case of PE, both di Rivero Vaccari and Shirasuna et al. highlighted the potential contribution of altered inflammasome activation to the pathophysiology of PE. Additionally, these reports highlight the therapeutic potential of inflammasome targeted treatments to treat reproductive associated problems, where inflammation and dysregulation of the maternal immune system is a contributing factor (di Rivero Vaccari, Shirasuna et al.)

Inflammation is also influential in the pathophysiology of common gynecological conditions such as endometriosis and PCOS. These conditions are characterized by chronic inflammation and alterations to immune cell phenotype and function. Endometriosis is characterized by the presence of endometrial cells outside of the uterine cavity that leads to symptoms including painful periods, chronic pelvic pain, and infertility (6, 20). A combination of factors, including dysregulation of the immune response and alterations in sex hormone signaling are thought to contribute to an inability to clear ectopic endometrium from the pelvic cavity (6, Garcia–Gómez et al., Hogg et al.). In this Research Topic, Garcia–Gómez et al., Hogg et al., and Borelli et al. all explored different aspects of the role of inflammation in the pathogenesis of endometriosis. Garcia–Gómez et al. highlighted the intimate association between hormones and inflammation in endometriosis. They showed that alterations to the cellular response to steroid hormones lead to dysregulation of the inflammasome pathway, with these changes contributing to disease progression through the prevention of cell death and promotion of adhesion, invasion, and cell proliferation (Borelli et al.). Hogg et al. provided an up–to–date review on the central role that macrophages play in the pathogenesis of endometriosis, focusing on their origins, phenotype, and function. They highlighted that modification of macrophage phenotype and function under disease–modifying conditions promote the growth, development, vascularization, and innervation of lesions in addition to the generation of pain symptoms (Hogg et al.). Finally, Borelli et al. explored the levels of mast cells in peritoneal fluid of women with endometriosis and examined whether dysregulation in the mast cell population may influence sperm function. They showed that mast cells and their main mediator tryptase are more represented in the peritoneal fluid of patients with endometriosis. Finally, using an *in vitro* model of mast cell–sperm interaction, the authors were unable to show a direct effect on sperm motility. However, incubation of the mast cell line, LAD2 with sperm and peritoneal fluid from patients with endometriosis did lead to a significant increase in the mast–cell degranulation response, which may influence sperm function (Borelli et al.).

PCOS is the most common endocrine disease and is a complex and heterogeneous disease characterized by a combination of signs and symptoms of 3 phenotypic characteristics: hyperandrogenism, ovulatory dysfunction, and polycystic ovarian morphology (7, 21). This condition is associated with various cardiometabolic risk factors, chronic inflammation, and an increased risk of infertility (22, 23). In this Research Topic, two manuscripts explored the

association of PCOS with inflammation. In the first study, Ganie et al. explored biomarkers of inflammation in an Indian population of PCOS patients and compared dietary status to their inflammatory load. It is estimated that in India, 22.5% of reproductive–aged women have PCOS (24), and in this study, Ganie et al. demonstrated that in an Indian population, PCOS caused increases in serum pro–inflammatory cytokines (Tumor Necrosis Factor–alpha and Interleukin–6) and decreases in anti–inflammatory molecules (Interleukin–10 and Adiponectin) compared to healthy controls (23). In the other study, Cao et al. performed a retrospective study that explored the seasonal susceptibility of PCOS patients undergoing IVF treatments to ovarian hyperstimulation syndrome (OHSS), a severe complication of controlled ovarian hyperstimulation that is more common in patients with PCOS (25). Their data was used to generate a predictive model that showed that the incidence of patients with PCOS that were at high risk for OHSS was significantly higher during the winter and summer months in Henan province in China (Cao et al.).

Taken together, the papers that form this Research Topic present an overview of research surrounding the roles of inflammation in reproduction. In view of recent advances that define the pivotal role of inflammation in the normal physiology of reproduction and pathophysiology, there is a pressing need to determine how dysregulation of inflammation and immune responses contributes to pathologies that surround reproduction. Ultimately, improved mechanistic understanding of the immune contributions to reproductive success will identify novel targets for diagnostic and therapeutic intervention strategies aimed at alleviating the rising burden of infertility and subfertility.

AUTHOR CONTRIBUTIONS

YY, HC, and JS all contributed to compilation, drafting and writing of this editorial. HC and YY are considered equal first authors. All authors contributed to the article and approved the submitted version.

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Comparative Evaluation of Biomarkers of Inflammation Among Indian Women With Polycystic Ovary Syndrome (PCOS) Consuming Vegetarian vs. Non-vegetarian Diet

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Background: Sub-inflammation and insulin resistance characterize women with PCOS. Data on dietary modulation of inflammation among PCOS women is scant, particularly from Indian subcontinent. The present study aimed to assess the effect of plant based vs. animal origin diets on serum markers of inflammation (primary outcome measure).

Methods: This observational case-control study compared age and BMI matched PCOS and apparently healthy women from two populations following different dietary practices. The vegetarian women from New-Delhi ($n = 82$ PCOS and $n = 179$ healthy) and non-vegetarian women from Srinagar ($n = 62$ PCOS and $n = 141$ healthy) formed the groups. Using a uniform methodology, detailed clinical, biochemical, hormonal, and inflammatory marker assessment was undertaken.

Results: The mean age of the overall cohort was 26.23 ± 4.59 years with a mean BMI of 24.39 ± 3.72 kg/m^2. Overall pro-inflammatory markers (TNF-α, IL-6, IL-1β, hs-CRP and serum resistin) were significantly higher ($p \leq 0.05$) and anti-inflammatory markers (IL-10 and adiponectin) were lower among women with PCOS than healthy subjects. On comparing vegetarian women with non-vegetarians, higher daily calorie intake (1895.46 ± 258.19 vs. 1860.13 ± 323.96 Kcal) with a higher protein and fat and lower carbohydrate intake was recorded in the latter, although the percent energy derived from carbohydrates was higher among vegetarians. Clinical and biochemical parameters were comparable among the groups except mFG score, total serum testosterone and serum lipid levels which were higher among non-vegetarian women as compared to their vegetarian counterparts from both categories (PCOS and healthy). Interestingly, vegetarian women with PCOS and healthy women had higher serum pro-inflammatory and lower anti-inflammatory markers compared to their non-vegetarian counterparts.

Conclusion: Women with PCOS consuming Indian vegetarian diet have higher pro-inflammatory and lower anti-inflammatory marker levels than their age and BMI matched healthy non-vegetarian counterparts. This interesting observation can be attributed to the dietary composition, among other factors and needs confirmation from well-designed randomized studies on a larger cohort.

Clinical Trial Registration: The study was registered with CTRI database under registration number CTRI/2013/09/003996.

Keywords: PCOS, inflammation, insulin resistance, testosterone, hs-CRP, adiponectin

INTRODUCTION

Polycystic ovary syndrome (PCOS), is a multifaceted disorder associated with a host of co-morbidities, including obesity, metabolic syndrome (MS), insulin resistance (IR), abnormal glucose tolerance (AGT), non-alcoholic fatty liver disease (NAFLD), psychiatric disturbances, elevated cardiovascular disease (CVD), and cancer risk etc. (1–6), in addition to several reproductive and cosmetic dysfunctions. The condition is known to affect 5–10% women of reproductive age in the West but is commoner in India with preliminary reports suggesting a prevalence as high as 22.5% (7, 8). Exact etiology being uncertain, the condition is characterized by two dominant pathogenic mechanisms namely hyperandrogenism and IR, both of which may lead to distinct clinical phenotypes and ovarian morphological patterns on ultrasound in women with PCOS (9). Chronic inflammation, a common accompaniment of these metabolic conditions, is characterized by elevation in pro-inflammatory cytokines, chemokines and markers of oxidative stress which in turn is linked to IR (10–13). Published data suggests higher levels of inflammatory markers or their gene polymorphisms among women with PCOS (14–16). Boulman et al. demonstrated elevated hs-CRP levels among insulin resistant women with PCOS similar to that observed by Mazibrada et al. (14, 17) while a few reports documented TNF-α and IL-6 gene polymorphisms among PCOS women in relation to hyperandrogenic phenotypic traits (15, 16). Although the reason of this sub-inflammation is unclear, higher BMI particularly visceral adiposity has been implicated (10, 18).

Dietary patterns have been reported to independently influence inflammatory and endothelial markers among healthy individuals (19, 20). Mediterranean diet, primarily based on sufficient intake of green vegetables, fruits, whole grains, sea food, and low red meat consumption (21) has been reported to have a beneficial effect upon development of type 2 diabetes mellitus (T2DM), inflammatory markers (such as IL-6, hs-CRP, and adiponectin), endothelial function and coagulation (20, 22–25). Similarly, vegetarian diets have been shown to lower lipid parameters (26) and hs-CRP (27). On the contrary, higher carbohydrate intake rather than high fat intake was associated with high total mortality in a recent large multinational epidemiological cohort (28). South Asians, particularly Indians have been demonstrated to have a higher prevalence of T2DM (29), CVD (30), PCOS (8) and sub-inflammation. These higher risks are partly attributed to dietary patterns, typically consisting of high percentage of carbohydrates and saturated fats from vegetables, rice, chapatis, or breads etc. (31). Whether the composition of Indian diet actually has a link to the epidemic of these disorders remains to be seen. In view of the paucity of data evaluating impact of Indian diet patterns on inflammatory markers among women with PCOS, we undertook this study assessing effect of plant based vs. animal origin diets on serum markers of inflammation as a primary outcome measure and clinical and metabolic parameters as secondary outcome measures.

MATERIALS AND METHODS

This cross sectional study recruited subjects from December 2014 to January 2018, from two cities located in North India- New Delhi and Srinagar. The women consuming plant based diets (vegetarian) form New Delhi and those consuming animal derived diets (non-vegetarian) from Srinagar, Jammu and Kashmir were eligible for the study. The study was conducted in accordance with the guidelines enshrined under the Helsinki 1975 declaration and was approved by the Ethics Committees of the respective study institutions. An informed written consent was taken from all the subjects before their enrolment.

Subjects

All consecutive women (18–40 years) attending outpatient clinics of endocrinology and gynecology of All India Institute of Medical Sciences (AIIMS), New Delhi and Sheri-Kashmir Institute of Medical Sciences (SKIMS), Srinagar, Jammu and Kashmir for complaints of unwanted hair growth, irregular menstrual cycles and other symptoms of PCOS were informed about the study. Women who fulfilled Rotterdam 2003 criteria for the diagnosis of PCOS and volunteered to be part of the study were required to sign an informed consent. In order to remove the confounding of age and body weight on inflammatory markers and other metabolic parameters, the women were recruited in blocks of age (18–20, 21–25, 26–30, 31–35, 36–40 years) and BMI categories(<20, 20–25, >25 kg/m^2). Study tools, methods of data capture, SOPs, investigator training, lab evaluation etc. followed a uniform protocol at both the centers.

Clinical Assessment

All women were interviewed for details of their menstrual cyclicity (age of menarche, duration and number of cycles per year), features of hyperandrogenism (duration and extent of unwanted hair growth, acne vulgaris, and androgenic alopecia), weight gain, infertility, history of drug intake etc. as per the the pre-designed uniform questionnaire (**Supplementary Table 2**) at both the participating centers. Oligomenorrhea was defined as a cycle interval of $\geq$35 days or $\leq$8 cycles per year and amenorrhea as cessation of cycles for more than 6 months. A detailed diet review using a food frequency questionnaire (FFQ) and 72 h dietary recall undertaken by qualified and trained dieticians to quantify various dietary components using specially designed diet software (Diet Cal, Profound Tech solutions, New Delhi) at both centers. For purposes of the study, women who consumed meat/chicken/fish/egg at least 5 days a week at least for last 1 year were considered as non-vegetarian and those who strictly adhered to plant based diets were taken as vegetarians. Women refusing consent, consuming medications such as glucocorticoids, insulin sensitizers, anti-epileptics, NSAIDs etc. known to affect glucose tolerance, insulin sensitivity, inflammatory markers, pregnant women, or women with history suggestive of controlled or uncontrolled hyperprolactinemia or androgen-secreting tumors, Cushing's syndrome, thyroid dysfunction, non-classical congenital hyperplasia, diabetes or AGT, at the time of enrolment were excluded from the study. Other exclusions included any prior history (at least 2 weeks) of infection, trauma, surgery or significant stress such as exams, bereavement, psychiatric comorbidity etc. known to generate an inflammatory response.

Body weight, height, waist circumference were measured using standard calibrated instruments (SECA 213, Hamburg Germany) followed by a detailed systemic examination including measurement of blood pressure (Omron HEM7120). A mean of three readings was taken as the final value for these parameters. Quantitation of hirsutism using modified Ferriman-Gallwey (mFG) score (8 or above out of a total of 36 from nine body areas taken as significant), grading of acanthosis nigricans, acne vulgaris, and androgenic alopecia was done by a single observer at each center. The inter-observer variation between the FG scores among the trained observers at two centers was <4%.

Laboratory Evaluation

After an overnight (10–12 h) fast all the participants were subjected to blood sampling arranged in the follicular phase (2nd to 7th day) of a spontaneous or medroxyprogesterone induced menstrual cycle. The samples were immediately separated in a cold centrifuge and aliquoted for biochemistry, hormones, and inflammatory markers. Biochemical parameters were assayed on the spot while the aliquots for hormones and inflammatory markers were stored at -80°C until the assay. The PCO morphology was assessed with trans-abdominal ultrasonography performed in the follicular phase by a single sonologist at each center using 7.5 mHz probe (AlokaSSD-500, Tokyo, Japan) to quantitate ovarian volume, count ovarian follicle number, and assess thecal hyper echogenicity with a common SOP.

Controls

Apparently healthy, age, and BMI matched women were recruited from community clusters as part of health awareness-cum-screening outreach programmes conducted by the respective institutes. These women underwent similar clinical and laboratory evaluation as in cases.

Assays

Biochemical parameters (plasma glucose, lipids, uric acid, calcium, phosphorus, liver and kidney function) were estimated using standard commercially available kits as per manufacturer's instructions on fully automated biochemical analysers (Hitachi 920, Japan). Samples for hormonal parameters (serum total T4, TSH, LH, FSH, PRL, cortisol,17OHP, total testosterone and insulin) from both the centers, were assayed using Electrochemiluminescence immunoassay (ECLIA) using Cobas e411(Roche Diagnostics Limited, USA). The inter- and intra-assay coefficients of variation were <7%. Serum inflammatory marker (TNF-α, IL-1β, IL-6, IL-10, hs-CRP, resistin, and adiponectin) levels were assayed by ELISA, using commercially available kits and according to supplier's protocol (Diaclone, France and Calbiotech, CA, USA). The inter- and intra-assay coefficients of variation were as per the manufacture prescriptions. Both hormonal and inflammatory markers were assayed at departmental laboratory of AIIMS New Delhi.

Sample Size Calculation

Sample size was calculated using G*Power software (version 3.1.9.2). Considering type one error (α) as 0.05, power of study as 90% and effect size 0.3 with reference from a recent study comparing the inflammatory profile of vegetarians and omnivores (32), a minimum of 50 subjects were required per group. Therefore, to account for non-response and incompleteness of data, we planned to recruit a minimum number of 62 cases in each group and 124 controls in each group with case to control ratio of 2:1.

Statistical Analysis

The Statistical Package for Social Sciences-22 software was used for statistical analysis (SPSS Inc., Chicago, IL, USA). Data has been depicted as mean ± standard deviation and was log transformed, wherever necessary. For assessment of normality, Kolmogorov Smirnov test was employed. One way ANOVA was used for comparing more than two groups and Mann-Witney *U*-test were used for comparing two groups. Parameters with $p \leq 0.05$ were considered as statistically significant.

RESULTS

A total of 200 women, qualifying Rotterdam 2003 for the diagnosis of PCOS, were screened ($n = 100$ at each center) out of which 62 non-vegetarian (from Srinagar) and 82 vegetarian (from Delhi) consented and had complete data for analysis. Another 400 apparently healthy age and BMI matched women ($n = 200$ at each center) were screened and invited to participate as controls out of which a total of 320 women (179

vegetarian women from Delhi and 141 non-vegetarian women from Srinagar) consented and had complete data available for analysis (**Figure 1**). Group-wise comparisons of their clinical, biochemical, hormonal and inflammatory marker profiles are shown in **Tables 1–3**.

Comparison Between Women With PCOS and Healthy Controls

The overall respective mean age of PCOS subjects ($n = 144$) and controls ($n = 320$) was 26.06 ± 4.12 vs. 26.55 ± 5.05 years while as their mean BMI was 24.81 ± 3.53 vs. 23.97 ± 3.90 kg/m^2. The mean number of menstrual cycles per year (8.10 ± 2.83 vs. 11.86 ± 2.88) was significantly lower while as mFG scores (11.57 ± 4.36 vs. 5.88 ± 1.77), serum LH (7.58 ± 3.57 vs. 6.42 ± 2.37 IU/ml) and serum total testosterone (0.52 ± 0.27 vs. 0.27 ± 0.13 ng/ml) levels were significantly higher among women with PCOS as compared to healthy women from both the centers. Plasma 25OHD levels were marginally lower (11.45 ± 8.19 vs. 15.78 ± 8.02 ng/ml) among women with PCOS (**Supplementary Table 1**). Fasting plasma insulin (12.57 ± 7.27 vs. 8.59 ± 6.26 mIU/ml), HOMA-IR (2.67 ± 1.58 vs. 1.73 ± 1.15) were higher while as FGIR (9.05 ± 4.76 vs. 15.21 ± 10.57) and QUICKI (0.35 ± 0.06 vs. 0.37 ± 0.04) were significantly lower among PCOS women than healthy controls. Pro-inflammatory markers (TNF-α, IL-6, IL-1β, hs-CRP, and serum resistin) were significantly higher ($p \leq 0.05$) and anti-inflammatory markers (IL-10 and adiponectin) significantly lower among women with PCOS than their healthy counterparts (**Supplementary Table 1**). Other parameters like waist circumference, blood pressure, uric acid and serum phosphorous did not differ significantly among the groups.

Comparison Between Vegetarian and Non-vegetarian Women With PCOS

Age (25.68 ± 3.81 vs. 26.13 ± 4.43 years) and BMI (24.94 ± 3.61 vs. 24.68 ± 3.45 kg/m^2) matched vegetarian and non-vegetarian PCOS women were comparable with regard to most of the clinical (mean number of menstrual cycles per year, mFG score, BP), biochemical (mean plasma glucose, HOMA-IR, urea, uric acid, SGOT) and hormonal (serum LH, FSH, PRL) parameters. Biochemical parameters such as serum creatinine, serum triglycerides and LDL cholesterol levels were higher ($p \leq 0.05$) among the non-vegetarian PCOS women as compared to their vegetarian counterparts. Interestingly, hirsutism scores and serum total testosterone levels were higher among PCOS women from Srinagar (**Tables 1–3**). Another interesting observation was that the pro-inflammatory markers (serum hs-CRP, TNF-α, IL-6, and IL-1β) were elevated and anti-inflammatory (serum adiponectin and IL-10) were lower among vegetarian women with PCOS as compared to non-vegetarian women with PCOS, although statisticalyl significant difference was found only in cases of serum hs-CRP, resistin and adiponectin levels (**Table 3**). A comparison of macronutrient intake between two PCOS groups showed a higher self-reported daily calorie intake among non-vegetarian women (1895.51 ± 308.28 vs. 1862.78 ± 262.33 Kcal, $p \leq 0.05$) with higher daily fat (49.07 ± 12.35 g vs. 43.11 ± 14.72 g) and protein intake (55.16 ± 12.34 g vs. 50.63 ± 11.48 g) and a lower carbohydrate intake (301.86 ± 58.45 vs. 318.24 ± 53.94 g) than their vegetarian counterparts ($p \leq 0.05$).

Comparison Between Healthy Vegetarian and Non-vegetarian Women

Most of the clinical, biochemical, and hormonal parameters were comparable among the subgroups (vegetarian vs. non-vegetarian) of healthy women. Few exceptions were mFG score, serum creatinine, TG, LDL cholesterol levels and total testosterone levels, which were higher among healthy non-vegetarian women from Srinagar, similar to the trend found in women with PCOS subgroups. Healthy vegetarian women from Delhi had higher mean fasting plasma glucose and insulin levels. Similar to the observations in PCOS subgroups, pro-inflammatory markers were higher and anti-inflammatory markers were lower among the healthy vegetarian women from Delhi. However, again this attained statistical significance only in cases of serum hs-CRP, IL-6, and adiponectin. A comparison of macronutrient intake between two healthy groups showed a higher self-reported daily calorie intake among non-vegetarian women (1895.40 ± 208.11 vs. 1857.47 ± 385.59 Kcal, $p \leq 0.05$) with higher daily fat (46.10 ± 9.46 vs. 42.05 ± 16.93 g) and protein intake (54.19 ± 11.03 vs. 52.02 ± 16.62 g) and a lower carbohydrate intake (304.93 ± 44.16 vs. 313.83 ± 72.87 g; $p \leq 0.05$) than their vegetarian counterparts.

On comparing the per cent energy consumption from macronutrients, vegetarian women consumed lower energy from proteins (11.01 ± 2.22% vs. 11.48 ± 2.34%) and fats (20.36 ± 6.34 vs. 22.54 ± 5.05%) ($p \leq 0.05$) and higher energy from carbohydrates (66.04 ± 7.82% vs. 63.13 ± 7.32%) ($p \leq 0.05$) as compared to non-vegetarian women in our cohort.

Figure 2 shows overall trends of serum inflammatory marker profiles among healthy and PCOS women from both vegetarian and non-vegetarian subgroups. Overall the pro-inflammatory markers (TNF-α, IL-6, IL-1β, hs-CRP, and serum resistin) were highest among vegetarian women with PCOS followed by age and BMI matched non-vegetarian women with PCOS which was in turn were higher than healthy vegetarian women. The lowest levels of pro-inflammatory markers were observed in the healthy non-vegetarian women. A similar but reverse trend was observed in anti-inflammatory markers (IL-10 and adiponectin) among these subgroups.

DISCUSSION

In the present study, we aimed to compare the inflammatory biomarker profiles of Indian women with PCOS following vegetarian and non-vegetarian dietary patterns from two different centers in North India. The key results revealed higher pro-inflammatory markers (TNF-α, IL-6, IL-1β, resistin and hs-CRP) among women with PCOS from both the centers as compared to age and BMI matched healthy controls from the respective populations. On comparing inflammatory markers among the PCOS subgroups pursuing different dietary patterns, vegetarian women had higher levels of serum pro-inflammatory

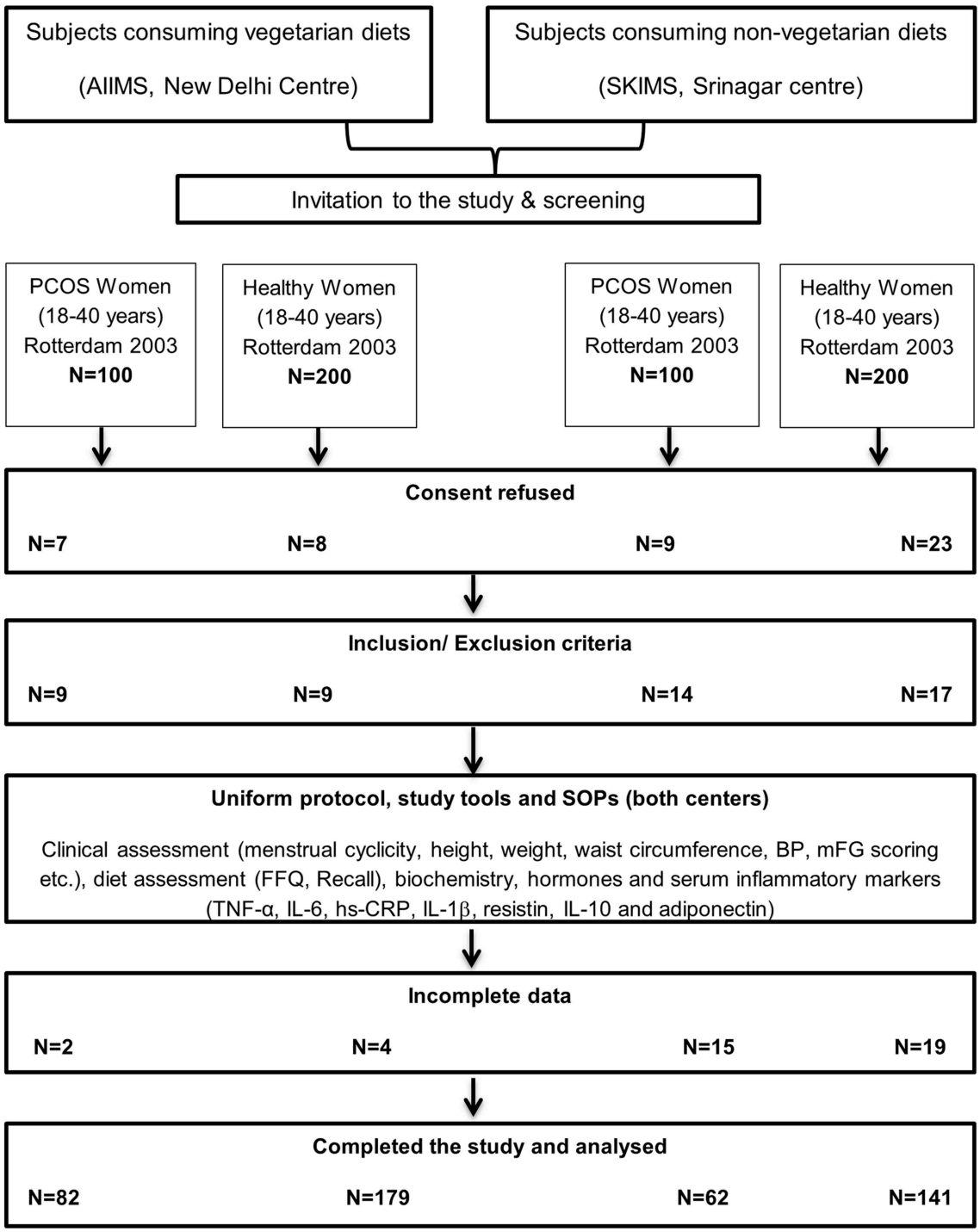

FIGURE 1 | Showing PERT chart describing the flow of subjects throughout this study.

(TNF-α, IL-6, hs-CRP, resistin and IL-1β) and lower levels of anti-inflammatory markers (serum IL-10 and adiponectin) reaching statistical significance in case of serum hs-CRP, resistin and adiponectin. A similar trend was observed when healthy control groups from two populations were compared.

However, the study results can furnish limited inferences owing to some limitations such as lack of data on micronutrients including vitamin B12, omega-3 fatty acids, quantitation of visceral fat mass and weighing of lifestyle differences such as pollution (pollution index of Delhi vs. Srinagar: 91.74 vs. 35.01),

TABLE 1 | Showing comparison of clinical and hormonal parameters among PCOS vs. healthy women from Delhi and Srinagar.

Parameters	Vegetarian group		Non-vegetarian group					
	Women with PCOS (n = 82) Mean ± SD	Healthy women (n = 179) Mean ± SD	Women with PCOS (n = 62) Mean ± SD	Healthy women (n = 141) Mean ± SD	p-value (a =)	p-value (b =)	p-value (c =)	p-value (d =)
Age (years)	25.68 ± 3.81	26.53 ± 5.99	26.13 ± 4.43	26.57 ± 4.11	0.06	0.59	0.53	0.55
No. of menstrual cycles/year	8.02 ± 2.38	11.72 ± 0.65[a]	8.13 ± 3.40	11.99 ± 0.15[d]	< 0.01	0.705	0.14	< 0.01
Ferriman–Gallwey score (mFG)	11.32 ± 4.52	5.51 ± 2.77[a]	11.82 ± 4.23	6.25 ± 0.76[c,d]	< 0.01	0.43	0.03	< 0.01
BMI (Kg/m^2)	24.94 ± 3.61	23.96 ± 4.17	24.68 ± 3.45	23.97 ± 3.63	0.12	0.467	0.10	0.07
Waist circumference (cm)	83.39 ± 11.49	82.29 ± 12.53	83.42 ± 8.81	83.62 ± 10.94	0.20	0.98	0.09	0.91
Systolic blood pressure (mmHg)	115.49 ± 13.19	115.58 ± 14.51	116.77 ± 8.64	113.43 ± 10.01	0.96	0.53	0.14	0.08
Diastolic blood pressure (mmHg)	77.38 ± 10.08	77.38 ± 10.48	78.15 ± 6.79	76.33 ± 8.78	0.99	0.63	0.34	0.21
Serum total T4 (μg/dl)	8.78 ± 2.23	8.41 ± 1.98	8.27 ± 1.59	8.12 ± 2.12	0.18	0.15	0.22	0.63
Serum TSH (μIU/ml)	3.25 ± 1.81	3.22 ± 1.69	3.45 ± 1.68	3.35 ± 1.39	0.88	0.46	0.48	0.70
Serum prolactin (ng/ml)	16.47 ± 5.38	16.06 ± 4.97	16.17 ± 5.92	15.15 ± 4.25	0.54	0.72	0.11	0.18
Serum LH (IU/ml)	7.75 ± 3.58	6.26 ± 2.36[a]	7.42 ± 3.56	6.59 ± 2.39[d]	< 0.01	0.48	0.31	0.06
Serum FSH (IU/ml)	6.08 ± 2.02	6.99 ± 2.43[a]	6.30 ± 2.13	7.16 ± 1.94[d]	< 0.01	0.51	0.48	< 0.01
Serum total testosterone (ng/ml)	0.48 ± 0.29	0.22 ± 0.18[a]	0.56 ± 0.25[b]	0.31 ± 0.08[c,d]	< 0.01	< 0.01	0.01	< 0.01
Serum 25OHD (ng/ml)	11.66 ± 9.81	15.83 ± 8.80[a]	11.24 ± 6.58	15.74 ± 7.23[d]	0.03	0.72	0.63	< 0.01
Protein (g/day)	50.63 ± 11.48	52.02 ± 16.62	55.16 ± 12.34[b]	54.19 ± 11.03[c]	0.87	0.05	0.05	0.23
%Energy from proteins	10.97 ± 2.04	11.06 ± 2.91	11.47 ± 2.41	11.49 ± 2.26				
Fat (g/day)	43.11 ± 14.72	42.05 ± 16.93	49.07 ± 12.35[b]	46.10 ± 9.46[c]	0.09	< 0.01	< 0.01	0.15
%Energy from fat	20.81 ± 6.03	19.91 ± 6.65	23.11 ± 5.66[b]	21.97 ± 4.37[c]				
Carbohydrate (g/day)	318.24 ± 53.94	313.83 ± 72.87	301.86 ± 58.45[b]	304.93 ± 44.16[c]	0.07	< 0.01	< 0.01	0.54
%Energy from carbohydrates	65.13 ± 7.78	66.94 ± 7.84	62.02 ± 9.12[b]	64.24 ± 5.54[c]				
Energy (Kcal/day)	1862.78 ± 262.33	1857.47 ± 385.59[a]	1895.51 ± 308.28[b]	1895.40 ± 208.11[c]	0.04	< 0.01	< 0.01	0.68

[a]$p < 0.05$ vegetarian PCOS vs. healthy vegetarian women, [b]$p < 0.05$ vegetarian PCOS vs. non-vegetarian PCOS, [c]$p < 0.05$ healthy vegetarian women vs. healthy non-vegetarian, [d]$p < 0.05$ non-vegetarian PCOS vs. non-vegetarian healthy women. For assessment of normality, Kolmogorov Smirnov test was employed.

Values are presented as mean ± standard deviation. P-values were calculated using one-way ANOVA followed by multiple comparisons [post hoc test (Least Significance Difference (LSD))].

stress levels etc. among the two populations. Although, it would have been advantageous to enroll both vegetarian and non-vegetarian women from each center, it was not feasible to enroll pure vegetarian women from the Srinagar centre, since the population is habitually non-vegetarian. Nevertheless, this is the first study reporting impact of diet on inflammatory markers among women with PCOS with a reasonable sample size and age and BMI matched control groups.

Low grade chronic inflammation also referred to as inflammaging is incriminated in the aetiopathogenesis of many chronic illnesses, notably metabolic syndrome (10, 13), obesity (10), T2DM (33), CAD (34), neurodegenerative diseases (35), reproductive dysfunctions including PCOS (13, 14, 17, 36). There is paucity of data on diet-induced inflammation among Asians in general and Indian women with PCOS in particular. Therefore, we undertook this study to evaluate the impact of vegetarian vs. non-vegetarian diet on cytokines among North Indian women with PCOS.

As expected PCOS women (from both centers) had less number of menstrual cycles per year, had more severe hirsutism, elevated serum total testosterone, fasting plasma glucose, and insulin levels as compared to healthy controls, which is in accordance with previously published literature (3, 5, 18). Serum 25OHD levels were lower, while serum alkaline phosphatase was higher among women with PCOS. This could be attributed to their increased body fat content especially the visceral adiposity as reported earlier (37, 38).

We observed that most of the clinical, biochemical, and hormonal parameters were comparable between PCOS women from both centers (vegetarian vs. non-vegetarian) except serum LDL cholesterol, triglycerides and creatinine which were higher among non-vegetarians. This can be attributed to

TABLE 2 | Showing comparison of biochemical parameters among healthy women and women with PCOS from Delhi and Srinagar.

Parameters	Vegetarian group		Non-vegetarian group					
	Women with PCOS (n = 82) Mean ± SD	Healthy women (n = 179) Mean ± SD	Women with PCOS (n = 62) Mean ± SD	Healthy women (n = 141) Mean ± SD	p-value (a =)	p-value (b =)	p-value (c =)	p-value (d =)
Blood glucose- fasting (mg/dl)	87.71 ± 10.53	86.55 ± 10.34	86.57 ± 10.70	83.37 ± 9.13[c,d]	0.91	0.51	< 0.01	0.03
Fasting plasma insulin-(mIU/ml)	13.07 ± 8.62	9.45 ± 8.99[a]	12.06 ± 5.91	7.73 ± 3.52[c,d]	< 0.01	0.41	0.03	< 0.01
HOMA-IR	2.77 ± 1.93	1.72 ± 1.23[a]	2.57 ± 1.24	1.74 ± 1.27[d]	< 0.01	0.41	0.93	< 0.01
QUICKI	0.36 ± 0.09	0.37 ± 0.03[a]	0.34 ± 0.03[b]	0.37 ± 0.04[d]	0.03	0.01	0.52	< 0.01
FGIR	9.12 ± 4.71	14.56 ± 9.21[a]	8.98 ± 4.82	15.87 ± 11.93[d]	0.02	0.94	0.22	< 0.01
Serum urea (mg/dl)	22.12 ± 5.62	23.55 ± 6.84	23.72 ± 4.57	23.77 ± 5.12	0.16	0.14	0.51	0.78
Serum creatinine (mg/dl)	0.65 ± 0.12	0.65 ± 0.17	0.72 ± 0.17[b]	0.78 ± 0.21[c]	0.85	0.03	< 0.01	0.23
Serum total calcium (mg/dl)	9.02 ± 0.73	8.43 ± 1.16	9.24 ± 0.63[b]	8.93 ± 0.75[d]	0.41	0.13	0.26	< 0.01
Serum phosphate (mg/dl)	3.59 ± 0.92	3.84 ± 1.27	4.02 ± 0.89	4.05 ± 0.95	0.26	0.41	0.16	0.58
Serum uric acid (mg/dl)	4.3 ± 1.03	4.13 ± 0.96	4.53 ± 0.82	4.18 ± 0.98	0.07	0.19	0.18	0.06
Serum bilirubin (mg/dl)	0.73 ± 0.23	0.72 ± 0.44	0.79 ± 0.34	0.76 ± 0.34	0.31	0.13	0.41	0.53
Serum SGOT (IU/L)	24.78 ± 8.61	23.72 ± 11.33[a]	23.72 ± 7.21	22.9 ± 8.59[d]	0.02	0.07	0.11	0.05
Serum alkaline phosphatase (IU/L)	107.04 ± 32.16	101.31 ± 33.72[a]	105.71 ± 28.52	94.84 ± 24.3[c,d]	0.03	0.11	< 0.01	< 0.01
Serum total protein (g/dl)	7.34 ± 0.39	7.62 ± 0.59[a]	8.18 ± 0.54b	7.98 ± 1.04[c,d]	< 0.01	0.01	0.05	< 0.01
Serum albumin (g/dl)	4.45 ± 0.43	4.39 ± 0.60	4.55 ± 0.27	4.66 ± 0.51	0.08	0.20	0.12	0.19
Serum total cholesterol (mg/dl)	163.95 ± 32.69	156.08 ± 33.43	168.84 ± 36.62	165.56 ± 34.39[d]	0.08	0.31	0.14	0.02
Serum HDL cholesterol (mg/dl)	46.90 ± 15.59	46.65 ± 12.22	46.62 ± 16.28	48.89 ± 17.75	0.90	0.91	0.18	0.33
Serum LDL cholesterol (mg/dl)	94.4 ± 31.08	85.27 ± 25.48[a]	104.32 ± 24.14[b]	92.48 ± 25.27[c,d]	0.04	0.03	0.02	< 0.01
Serum Triglyceride (mg/dl)	105.18 ± 43.81	101.95 ± 37.15[a]	126.80 ± 39.04[b]	121.63 ± 49.67[c]	0.05	< 0.01	< 0.01	0.07

Values are presented as mean ± standard deviation. p-values were calculated using one-way ANOVA followed by multiple comparisons [post hoc test (Least Significance Difference (LSD))]. [a]$p < 0.05$ vegetarian PCOS vs. healthy vegetarian women, [b]$p < 0.05$ vegetarian PCOS vs. non-vegetarian PCOS, [c]$p < 0.05$ healthy vegetarian women vs. healthy non-vegetarian, [d]$p < 0.05$ non-vegetarian PCOS vs. non-vegetarian healthy women.

TABLE 3 | Showing comparison of serum biomarkers of inflammation among women with PCOS and healthy controls from Delhi and Srinagar.

Parameter	Vegetarian group		Non-vegetarian group					
	Women with PCOS (n = 82) Mean ± SD	Healthy women (n = 179) Mean ± SD	Women with PCOS (n = 62) Mean ± SD	Healthy women (n = 141) Mean ± SD	p-value (a =)	p-value (b =)	p-value (c =)	p-value (d =)
Serum TNF-α (pg/ml)	45.13 ± 35.74	24.18 ± 20.18[a]	35.47 ± 23.68	22.98 ± 16.42[d]	0.03	0.91	0.21	< 0.01
Serum IL-6 (pg/ml)	22.95 ± 14.40	8.44 ± 5.64[a]	19.86 ± 7.85	4.39 ± 3.94[c,d]	< 0.01	0.91	< 0.01	< 0.01
Serum IL-1β (pg/ml)	11.85 ± 7.43	8.55 ± 5.9[a]	9.79 ± 5.12	7.52 ± 4.35[d]	< 0.01	0.43	0.37	0.02
Serum hs-CRP (ng/ml)	3.83 ± 1.68	2.19 ± 1.48[a]	2.38 ± 0.88[b]	1.68 ± 1.52[c,d]	< 0.01	0.01	< 0.01	< 0.01
Serum resistin (ng/ml)	10.84 ± 4.54	6.18 ± 3.69[a]	6.27 ± 2.28[b]	5.48 ± 3.10	< 0.01	< 0.01	0.16	0.03
Serum adiponectin (ng/ml)	3.15 ± 2.01	6.75 ± 4.45[a]	6.33 ± 2.81[b]	7.20 ± 5.38[c,d]	< 0.01	< 0.01	0.05	0.02
Serum IL-10(pg/ml)	6.47 ± 1.93	9.46 ± 3.60[a]	6.71 ± 3.16	10.53 ± 7.47[d]	< 0.01	0.68	0.24	< 0.01

Values are presented as mean ± standard deviation. P-values were calculated using Mann-Whitney U test.
[a]$p < 0.05$ vegetarian PCOS vs. healthy vegetarian women, [b]$p < 0.05$ vegetarian PCOS vs. non-vegetarian PCOS, [c]$p < 0.05$ healthy vegetarian women vs. healthy non-vegetarian, [d]$p < 0.05$ non-vegetarian PCOS vs. non-vegetarian healthy women. For assessment of normality, Kolmogorov Smirnov test was employed.

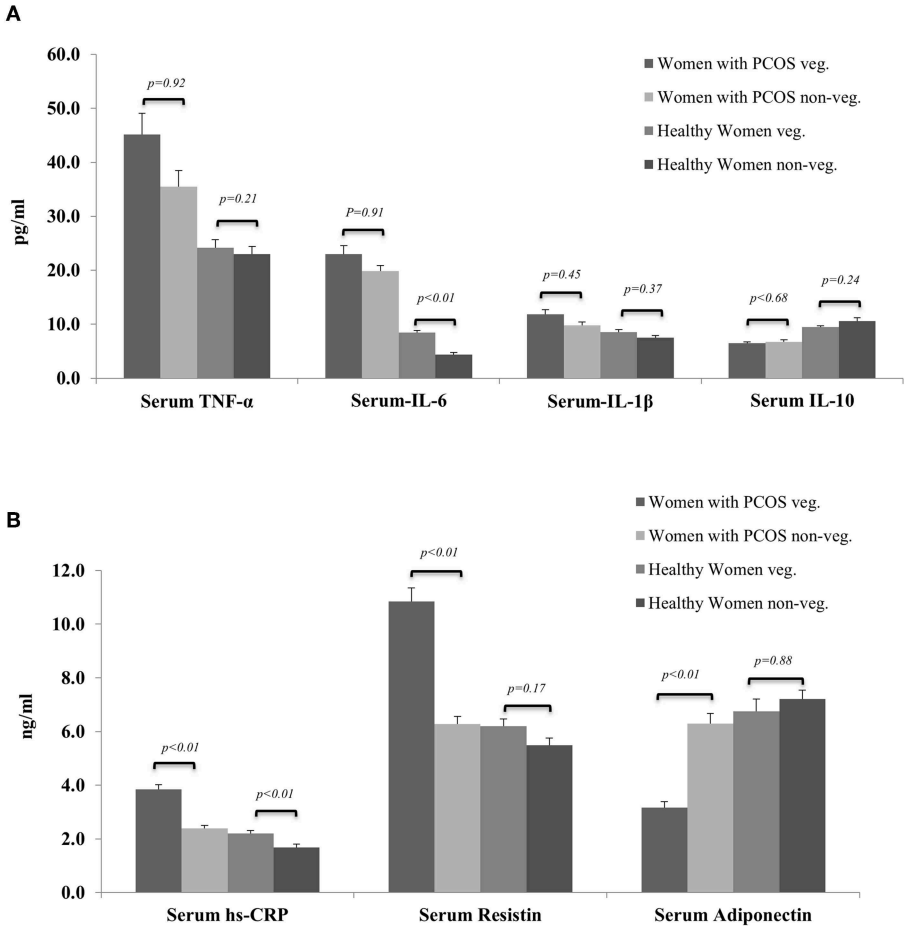

FIGURE 2 | Showing comparison of serum levels of biomarkers of inflammation among women with PCOS and healthy controls from vegetarian and non-vegetarian backgrounds. **(A)** Serum TNF-α, IL-6, IL-1b, and IL-10. **(B)** Serum hs-CRP, resistin, and adiponectin.

higher daily fat and protein intake among the non-vegetarian subgroups and the observation is supported by previous studies suggesting that plant based diets have beneficial effects on serum lipid levels (26, 39). The intriguing finding of higher mFG scores and serum total testosterone among healthy as well as women with PCOS from Srinagar as compared to their age and BMI-matched women from Delhi is not readily explainable. However, this finding is consistent with our previously published data (40) and can be attributed to ethnic differences among the two populations. This finding of higher androgen levels among women from Srinagar centre may be one of the reasons of better inflammatory marker profile although the earlier data analyzing the impact of androgens on inflammation is contradictory and needs further evaluation (41).

The observation of a higher pro-inflammatory state among women with PCOS compared to healthy controls is similar to earlier studies showing higher hs-CRP levels among women with PCOS than healthy controls (14, 17). Although, a systematic review and meta-analysis by Escobar-Morreale et al. (42) negated any difference in the levels of serum IL-6 and TNF-α between women with PCOS and controls, this meta-analysis had some limitations. They included 10 studies enrolling small number of PCOS subjects ($n = 523$) and controls ($n = 330$) with heterogeneous backgrounds from different ethnicities while as we had relatively homogenous population with fair number of subjects in the present study.

Interestingly, among the PCOS subgroups, vegetarian women had significantly higher levels of serum hs-CRP and serum resistin and lower levels of anti-inflammatory adiponectin. Although, there is no data evaluating the impact of different diets among women with PCOS globally, these findings seem to be in contradiction to most previous studies showing lower inflammatory marker levels among non-PCOS subjects following plant-based diets (19, 32, 42). Nettleton et al. in a multi-ethnic study showed that vegetarian diet comprising of whole grains, fruits and green leafy vegetables was inversely related to serum hs-CRP and IL-6 concentrations (19). Randomized studies have shown that a traditional Mediterranean diet is highly beneficial in lowering inflammation (43–45). In a recent meta-analysis of 136,846 participants, Koloverou et al. observed

that strict adherence to Mediterranean diet reduces the risk of developing type 2 DM by 23% (25). Although, a minority of reports among non-PCOS subjects support our observations (46, 47), to the best of our knowledge this is the first study evaluating the impact of diet on inflammatory markers in PCOS and healthy women. There is also suggestion that strict adherence to vegetarian diets without necessary supplementation adversely affects female fertility outcomes in women (48, 49). This higher inflammaging among women (both healthy and PCOS) pursuing vegetarian diet in the present study is unlikely to be due to higher fat mass or higher age as our women were age and BMI matched. This could be attributed to differences in dietary composition (micronutrient or macronutrient) or the methods of preparation of Indian vegetarian diet as opposed to Mediterranean diet. Unlike Mediterranean diet, Indian vegetarian diet is generally rich in carbohydrates and low in omega-3 fatty acids (50) which could also be an explanation for a higher pro-inflammatory marker elevation. A recent cohort study conducted across eighteen countries with participants from different ethnicities by Dehghan et al. found that a higher total fat intake was associated with lower risk of total mortality, cardiovascular diseases and stroke (28). In line with this study, we observed that the vegetarian subjects, despite having lower overall daily calorie intake, recorded a higher per cent energy intake from carbohydrates and lower intake from fats as compared to non-vegetarian subjects and may partly explain the pro-inflammatory state in them. Our results are also supported by recent evidence correlating vegetarian diet with poor health status among Asian Indians in which vegetarianism was found to be associated with higher incidence of metabolic syndrome and obesity (51).

A surge in prevalence of metabolic syndrome, obesity, T2DM, CAD etc. among Asian Indians, despite having lower BMIs has been reported (8, 30, 52) in recent past. The exact etiology being unknown, diet has been incriminated as one of the prime factors in the rising prevalence of these conditions. There is paucity of data on diet-induced inflammation among Asians in general and Indian women with PCOS in particular. Our data is in contravention to earlier studies and traditional advice given to patients, although the implications of this study are not immediately utilizable in clinical practice and may be only generalizable after well-designed, larger long term studies are conducted to reproduce the findings.

In conclusion, this study reports higher inflammatory markers among women with PCOS as compared to healthy controls and the Indian vegetarian diet has adverse impact on this profile.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional ethics committee-All India Institute of Medical Sciences, New Delhi and Institutional ethical committee-Sher-i-Kashmir Institute of Medical Sciences, Srinagar. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

MG, LR, SV, and TP designed the research. MG, TSah, and SN analyzed the data and wrote the manuscript. AR and IW conducted the research. TSat and IG also contributed in writing the paper. All authors approved the final content of the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2019.00699/full#supplementary-material

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Mast Cells in Peritoneal Fluid From Women With Endometriosis and Their Possible Role in Modulating Sperm Function

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Endometriosis is a local pelvic inflammatory process, frequently associated with infertility, with altered function of immune-related cells in the peritoneal environment. Mast cells are known to be key players of the immune system and have been recently involved in endometriosis and in infertility, with their mediators directly suppressing sperm motility. In this study, we evaluated the mast cell population and their mediators in the peritoneal fluid of infertile patients with endometriosis and their impact on human sperm motility. Peritoneal fluids, collected by laparoscopy from 11 infertile patients with endometriosis and 9 fertile controls were evaluated for the presence of mast cells, tryptase levels and their effect on sperm motility. Furthermore, an *in vitro* model of mast cells-sperm interaction in peritoneal fluid was set up, using LAD2 cell line as a mast cell model, and analyzed from a functional as well as a morphological point of view. Mast cell peritoneal fluid population and its main mediator, tryptase, is more represented in endometriosis confirming an involvement of these cells in this disease. Anyway it appears unlikely that tryptase enriched peritoneal fluid, which fails to inhibit sperm motility, could contribute to endometriosis associated infertility. Despite of this, sperm interaction with the mast cell surface (LAD2) induced a significantly mast cell-degranulation response in the peritoneal fluid from endometriosis which could directly modulate sperm function other than motility. This evidence lead us to suppose that there is, between these elements, an interrelationship which deserves further studies.

Keywords: endometriosis, infertility, mast cells, tryptase, sperm

INTRODUCTION

Endometriosis (EMS) is characterized by the presence and growth of endometrial tissue outside the uterus. It is a common disease among women of reproductive age. Population-based studies estimate a prevalence ranging between 1.8 and 3.3% in women aged 15–50 years (Morassutto et al., 2016). Endometriosis is frequently associated with infertility, even if affected women have

an ovulatory activity or a mechanical patency of the fallopian tubes (Gupta et al., 2008; Máté et al., 2018).

The current consensus is that endometriosis is a local pelvic inflammatory process with altered function of immune-related cells in the peritoneal environment. There are recent studies which support this theory, suggesting that the peritoneal fluid of women with endometriosis contains an increased number of activated macrophages that secrete various local products, including growth factors, cytokines and possibly oxidation products (Capobianco and Rovere-Querini, 2013), implicating these factors in the development and progression of endometriosis and endometriosis-associated infertility. Although the contribution of specific immune cell subsets and their mediators to the onset and the course of the inflammatory process in endometrial lesions is still poorly understood, some evidence suggests that mast cells (MC) are crucially involved. Mast cells are known to be key players of the immune system, especially during allergic reactions. However, increasing evidence supports the involvement of MC also in the inflammatory process of EMS. High numbers of degranulated MC have been found in endometriotic lesions (Konno et al., 2003; Kempuraj et al., 2004; Sugamata et al., 2005; Anaf et al., 2006; Kirchhoff et al., 2012; Paula et al., 2015). Of note, this was not the case at unaffected sites of the peritoneum or eutopic endometrial tissue from EMS patients or healthy controls. Additionally, it has been shown that stem cell factor (SCF), the major growth differentiation and chemoattractant factor for MC, is found in higher concentrations in the peritoneal fluid (PF) of EMS patients (Osuga et al., 2000). Despite the possible MC role in the onset and/or the course of the pelvic inflammatory process of endometriosis, neither the amount of MC nor the level of their mediators has been evaluated in EMS-peritoneal fluid to the best of our knowledge.

Endometriosis has been also associated with infertility even in its early stages (Zondervan et al., 2018) before adhesion or anatomic distortion take place. The exact mechanism of endometriosis-associated infertility is not fully understood, although many possible causes have been suggested, among them the potential negative effects of cytokine-rich peritoneal fluid (flushing the tubal and endometrial environment) on sperm function (impairment of acrosome reaction and impairment of sperm motility) (Gupta et al., 2008; Broi et al., 2019). Since in humans there are no selective barriers separating the Fallopian tubes and the peritoneal cavity, PF can be present in the fertilization milieu (Harper, 1988). Although changes in PF volume, cell concentration, hormones, growth factors, cytokines and possibly free oxygen radicals production have been well characterized in endometriosis (Oral et al., 1996; Bedaiwy and Falcone, 2003; Bedaiwy et al., 2007; Nishida et al., 2011; Rižner, 2015), data about the effect of this fluid on sperm function are still controversial (Broi et al., 2019). Most of these studies suggest that substances found in the peritoneal fluid of patients with endometriosis could contribute to infertility through impairment of both sperm function and motion kinetics (Drudy et al., 1994; Zullo et al., 1994; Aeby et al., 1996; Oral et al., 1996; Liu et al., 2000; Luo et al., 2008; Xu et al., 2008; Mansour et al., 2009). However, other studies reported no adverse effect of PF in patients with endometriosis on sperm motility parameters, suggesting that peritoneal fluid in these patients cannot contribute to infertility (Chen et al., 1997; Sharma et al., 1999). Interestingly human recombinant tryptase, the main MC product in all human MC, including those found in the female genital tract (Yamanaka et al., 2000; Sivridis et al., 2001), has been shown to reduce human sperm motility *in vitro* (Weidinger et al., 2003), thus being a good candidate factor for influencing sperm fertilizing ability.

In this study, we evaluated for the first time the amount of MC and their main mediator (tryptase) in the peritoneal fluid of infertile patients with endometriosis and their impact on human sperm motility. Furthermore we evaluated mast cell-sperm interaction in an *in vitro* model by using a human mast cell line (LAD2).

MATERIALS AND METHODS

Patients for PF Collection

Twenty women undergoing either diagnostic or operative laparoscopy at the Institute for Maternal and Child Health, IRCCS Burlo Garofolo, Trieste, Italy were enrolled in a case-control study. The study was reviewed and approved by the Ethical Committee of the Institute for Maternal and Child Health IRCCS Burlo Garofolo, Trieste, Italy (Prot. 1197/2015). Informed consent for participation in the study was obtained from all women.

The study group (EMS group) consisted in total of 11 infertile women, diagnosed with moderate/severe endometriosis (stage III-IV, $n = 9$) and minimal/mild endometriosis (stage I-II, $n = 2$) according to the revised criteria of the American Society for Reproductive Medicine ASRM (Canis et al., 1997). They had normal ovulation and no other identifiable female causes of infertility.

The control group (C group) consisted of a total of 9 fertile women, without endometriosis, subjected to laparoscopy to remove leiomyoma. Medical history and white blood cell count (WBC) were recorded for all the patients.

PF Collection and Cytological Evaluation

Laparoscopy was performed, and all obtainable peritoneal fluid in the pouch of Douglas was aspirated (by a suction unit through a Teflon catheter) immediately after entering the abdominal cavity and collected in sterile plastic tubes. Blood-free samples were immediately transported to the laboratory where total cell numbers were counted (Coulter, Miami, FL, United States) and differential cell counts carried out using an optical microscope on Diff-Quik System (Medion Diagnostics, Gmbh, Düdingen, CH) and Toluidine Blue stained cytospin specimens (Cytospin 2, Shandon Inc., Pittsburgh, PA, United States) on by cytospin preparations after staining with Diff-Quick (Medion Diagnostics, Dudingen, Switzerland). Subsequently, 1 mL of PF was centrifuged (10 min, 600 *g*), filtered through a 0.22-μL membrane (Millipore, Bedford, MA, United States) and stored at −80°C until used (<12 months). The remaining volume of PF (at least 5 mL) was processed for cell blocks preparation by using 10% alcohol–formalin as a fixative agent as described by

Shivakumarswamy et al. (2012). After paraffin embedding 3 μm thickness serial sections were prepared from this cell block and stained immunohistochemically for tryptase (see below).

One of the main advantages of the cell block technique is to obtain multiple sections for conventional stains and immunohistochemistry (Shivakumarswamy et al., 2012). Diagnostic sensitivity may be improved when cytology and cell block preparation are used in tandem.

Immunocytochemical Staining for Tryptase

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded cell block sections 3 μm thin using Ultravision Quanto Large Volume Detection System HRP Polymer (Bio Optica, Milan, Italy) pre-treated with Dewax and HIER Buffer H, pH 8. The sections were incubated for 30 min, at RT Temperature with anti Mast Cell Tryptase monoclonal antibody (15-MOB347 – Bio Optica Milan, Italy) diluted 1:600 in Thermo Scientific Antibody Diluent OP Quanto (Cat. # TA-125-ADQ). The primary antibody was omitted in negative controls (**Figure 2**).

Finally, the sections were incubated for 10 min with 3,3′ Diaminobenzidine chromogen (DAB – Dako Milan, Italy) and Mayer Hematoxylin to nuclear counterstain. Slides were scanned by D-Sight Microscope and Scanner (A. Menarini Diagnostic S.r.l. – Firenze, Italy), then analyzed by VISIA Imaging S.r.l. software.

Protein Content

The PF protein content was quantitated with the Bradford method (Bradford, 1976) using bovine-serum-albumin (BSA) as standard and expressed in mg/mL.

Tryptase Concentration and Enzymatic Activity

Tryptase, a tetrameric serine proteinase, is the major component of mast cell granules, and comprises up to 20% of the total protein of mast cells derived from lung, colon and skin tissue (Schwartz and Bradford, 1986; He and Xie, 2004). A colorimetric assay was used to determine the enzymatic activity of tryptase in the PF samples both from patients and controls (and LAD2 supernatants, see below) as previously described (Borelli et al., 2018). The assay is based on spectrophotometric detection of the chromophore p-nitroaniline (pNA) (Sigma-Aldrich, United States) after cleavage from the labeled substrate tosyl-gly-pro-lys-pNA. The free pNA was quantified using a microtiter plate reader at 405 nm (Biotek Instruments Inc). Samples were assayed in triplicate, and tryptase activity was expressed in (arbitrary unit of absorbance/mL) AU/mL.

Tryptase (Enzyme-Linked Immunosorbent Assay (ELISA) (USCN, Life Sciences Inc) immunoassay kit was used to determine the concentration of tryptase in PF samples and pools both from patients and controls. The assays were performed according to the manufacturer's instructions and the results referred to a calibration curve expressed in ng/mL. Samples were assayed in triplicate.

β-Hexosaminidase Enzyme Activity

β -hexosaminidase (β -hexo), another typical marker of mast cell granules, was assayed spectrophotometrically by the hydrolysis of 4-nitrophenyl N-acetyl-β-D-glucosaminide (Sigma-Aldrich, United States) as previously described (Gri et al., 2008; Medic et al., 2008). Samples were assayed in triplicate and β -hexo activity was expressed in (arbitrary unit of absorbance/mL) AU/mL.

Semen Samples: Isolation of Motile Sperms

Fresh semen was collected (at the Assisted Reproduction Unit of the Institute for Maternal and Child Health IRCCS Burlo Garofolo and University of Trieste) from healthy subjects ($n = 19$, mean age + SD: 39.6 ± 7.3 years) who had given informed consent and had no history of diseases related to infertility. After complete liquefaction, the ejaculates were analyzed according to the standard semen parameters of the World Health Organization (WHO laboratory manual for the examination and processing of human semen, fifth edition. World Health Organization [WHO], 2010). Motility was determined by manual counting of at least 200 sperms observed under 400× phase contrast optics.

A leukocyte count was carried out by using standard peroxidase test, as described in the WHO laboratory manual. All subjects were asymptomatic for genitourinary infections. Only ejaculates with $<1 \times 10^6$ white blood cells/mL, total motility (World Health Organization [WHO], 2010: >40%) and progressive motility (World Health Organization [WHO], 2010: >32%) were used for the experiments.

For isolation of motile sperms, samples were processed using the swim-up technique to eliminate dead spermatozoa and other cells, including bacteria and leukocytes (Ricci et al., 2009). This methodology is based on the active movement of spermatozoa from the prewashed cell pellet into an overlaying medium. Ejaculates were washed (centrifuged at 500 *g* for 10 min) and resuspended in Human Tubular Fluid medium (HTFM, Irvine Scientific, United States), containing 0.05% BSA. The supernatant was discarded, the pellet was suspended in pre-warmed 0,5 mL of HTFM. The pellet was gently over-layered with HTFM in the tube, inclined at 45° and kept at 37°C for 45–60 min. A sterile Pasteur pipette was used to remove the supernatant containing > 95% actively motile sperms whose concentration ranged from 20 to 30 $\times 10^6$/mL.

Effect of Peritoneal Fluid on Human Sperm Viability and Motility

Motility-enriched sperm samples ($N = 19$) were incubated in HTFM containing 0.05% BSA (as negative control) or peritoneal fluid in the ratio 1:4 (125 μL sperm samples and 500 μL PF). For the seek of simplicity we preferred to evaluate many fold peritoneal fluid pools than the single PF, accordingly, a pool of peritoneal fluids was prepared from either groups by mixing equal volumes of fluid samples taken from patients with endometriosis.

Sperm viability [Eosin-Nigrosin staining technique (Björndahl et al., 2003)], total and progressive motility variables were evaluated at 0, 3 (Weidinger et al., 2003) and 24 h (Mansour et al., 2009) by manual counting of sperm observed under phase contrast microscopy.

After 3 h no significant difference in viability was observed in comparison with starting conditions (after swim up, T0), while after 24 h viability was significantly decreased with respect to T0, in the presence of HTFM, of peritoneal fluid from both women with endometriosis and control patients ($p < 0.05$), but no difference was observed when comparing viability of spermatozoa incubated for 3 h or 24 h in either PF-C or PF-EMS, to that of sperm incubated for the same period in HTF medium alone (**Figure 3**).

LAD2 Human Mast Cell Line

The human MC line LAD2 was kindly provided by Prof. Carlo Pucillo (Department of Medicine Medical Area, University of Udine, Udine, Italy). The cell line was established from bone marrow aspirates of a patient with MC sarcoma leukemia and is closely related to hMC (Kirshenbaum et al., 2003). This cell line has been widely used as a suitable model for analyzing mast cell functions particularly degranulation and cytokine secretion (Gage et al., 2009; Chan et al., 2014; Ramis et al., 2015) suggesting that they are endowed with a well-defined secretory apparatus and can be used to investigate the mechanism of activation of their secretory pathways. Furthermore LAD2 have increased expression of tryptase, which represent the mast cell mediator of interest in our study, with respect to other human mast cell line HMC-1 (Kanerva et al., 2009; Guhl et al., 2010; Rådinger et al., 2010).

LAD2 cells were grown in serum-free medium StemPro-34 (Gibco, Grand Island, NY) supplemented with 2 mM L-glutamine (Gibco), 1% penicillin-streptomycin (Gibco), and 100 ng/mL human stem cell factor (PeproTech Inc., Rocky Hill, NJ, United States) in a humidified atmosphere of 5% CO2 in air at 37°C. LAD2 were periodically tested for c-Kit expression on the cell surface by flow cytometry (FACScan, Becton Dickinson, San Diego, CA, United States). LAD2 cells were suspended at 1–2 × 10^6/mL in Tyrode's buffer containing 0.02% BSA (TyB).

In vitro Experimental Model of MC-Sperm Interaction in the Peritoneal Environment

LAD2 cells were incubated (30 min at 37°C) with or without highly motile sperms (1:3–5 ratio of cells to sperms), in Tyrode's buffer containing 0.02% BSA (TyB) or in the presence of 10% pool PF-EMS or PF-C, then spun down at 250 × *g* for 7 min. With the aim of evaluating if sperm represents a secretory stimulus for MC, supernatants (SN) and pellets obtained after centrifugation were evaluated for tryptase and β-hexo activity (degranulation assay, see below).

Degranulation Assay

Tryptase and β-hexo activity (degranulation assay) were evaluated in the supernatant (SN) and pellets using as enzyme substrates, tosyl-gly-pro-lys-p-nitroanilide (0.25 mM final concentration) for tryptase (see above) and 4-nitrophenyl N-acetyl-β-D-glucosaminide (0.5 mM final concentration) for β-hexo [as previously described (Gri et al., 2008; Medic et al., 2008)]. The enzymatic activity was measured in an ELISA reader (Bio-tek instruments INC) at 450 nm. The extent of degranulation was calculated as the percentage of free pNA (for tryptase) or 4-p-nitrophenol (for β-hexo) absorbance in the supernatants taking the sum of the activities found in the supernatants and in cell pellets solubilized in hexadecyl trimethyl ammonium bromide (CTAB) (Sigma-Aldrich, United States) 0.05%, as 100%. Of note, we choose mainly β-hexo activity as a marker of LAD2 degranulation since tryptase activity in PF was too high and covered up the secretory response of LAD2. 4-p-nitrophenol absorbance values were adjusted subtracting the β-hexo activity of sperms, PF-EMS and PF-C alone. A positive control was obtained by cell stimulation with compound 48/80 (10 μg/mL final concentration), a well-known cationic mast cell stimulating agent, for 30–60 min.

SEM Analysis of MC-Sperm Interaction

At the end of the incubation carried out for the degranulation assay, two hundred microliters of the cell suspension (see paragraph *In vitro experimental model of MC-sperm interaction in the peritoneal environment*) were diluted with PBS (Dulbecco's modified phosphate buffered saline (PBS) (Sigma-Aldrich, United States) and placed on a poly-L-lysine-coated glass coverslip (18 mm diameter) (Menzler Glasser). The immobilized cells were allowed to adhere to the coverslip over a 30-min incubation period on ice. The supernatant was decanted, and adherent cells were fixed in 2% glutaraldehyde PBS pH 7.4 at room temperature for 30 min. Fixation was followed by rinsing in PBS and then dehydration through graded ethanols. Samples were transferred to a critical point dryer (Bal-Tec; EM Technology and Application, Furstentum, Liechtenstein) in 100% ethanol and dried through CO2. Coverslips were mounted on aluminum sample stubs and gold coated by sputtering (Edwards S150A apparatus, Edwards High Vacuum, Crawley, West Sussex, United Kingdom).

Cells were observed under both low (X 2,000–4,000) and high (X 35,000–40,000) magnification and representative areas were photographed. SEM micrographs are acquired by a Leica Stereoscan 430i scanning electron microscope (Leica Cambridge Ltd., Cambridge, United Kingdom). In each specimen prepared from two different experiments more than 100 SEM fields, including more than 600 LAD2s, were scored for the presence of adherent sperms. The site of sperm-LAD2 interaction, head/midportion (HM) or tail, was also specified.

Statistical Analysis

Statistical analysis was performed using Graph Pad Prism (Graph Pad Software Inc., La Jolla, CA, United States). The data (levels of blood parameters, PF cellular, and soluble parameters, number of interacting LAD2 and number of LAD2-interacting sperms and the percentage of degranulation) are presented as mean ± Standard Error (SE) or Standard Deviation (SD) (accordingly to the number of the sample

scored). The Kolmogorov–Smirnov test was applied to assess the normality of the studied data. Independent samples Student *t*-test (unpaired) was used to compare data between the two groups. Kruskal–Wallis test was used to evaluate differences in spermatozoa parameters and, when statistically significant differences were present, Dunn's *post hoc* was used to individuate group differences. In all instances, the level of significance for statistical analysis was set at 5% ($p < 0.05$). Power analysis was made using G*Power software (Faul et al., 2007).

Power analysis was performed to assess feasibility of the study: considering effect size = 1.15 and $a = 0.05$, power is equal to 0.79, indicating that highly probable to find statistical significant differences.

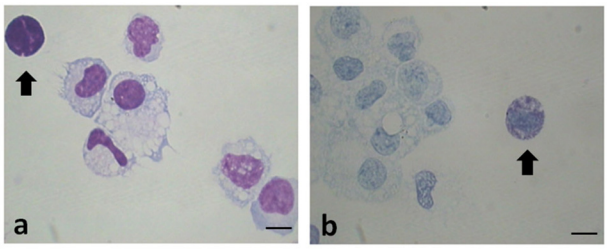

FIGURE 1 | Representative images of cytocentrifuged smears of PF (EMS) stained with Diff-Quick **(a)** and with Toluidin blue (TB) **(b)**. Mast cells/basophils are indicated by the arrows. Original magnification 1,000×. Scale bars = 10 μm.

RESULTS

Blood Cell Counts and Peritoneal Fluid Characterization of the Cellular Components

Table 1 shows white blood counts performed before surgery. The analysis of blood counts showed no statistically significant difference in the number of total circulating leukocytes and different leukocyte subpopulations between the two groups, except that for basophilic granulocytes, whose relative percent (compared to total leukocytes) was significantly lower ($p = 0.021$) in the study group (mean ± SD: 0.54 ± 0.23) as compared to the control group (mean ± SD: 0.87 ± 0.35).

Table 2 shows the data related to the characteristics of peritoneal fluid of the study group and the control group: volume (mL); cell concentration (expressed in 10^6cells/mL); total cells (10^6cells) and percentages for different leukocyte populations evaluated on cytocentrifuged samples of peritoneal fluid stained using the Diff-Quick (DQ) (**Figure 1a**). To evaluate specifically the population of MC the Toluidin Blue (TB) staining (**Figure 1b**) was also performed and the results expressed as % of TB positive cells (**Table 2**). There were no significant differences ($p = 0.158$) neither between the volumes of the PF (mL) in the study group (mean ± SD: 10.1 5.7; $n = 11$) and control groups (mean ± SD: 8.7 ± 3.5; $n = 9$), nor between the total cell count (expressed in 10^6 cells/mL) of the PF in the study group (1.6 ± 1.3; $n = 11$) and the control group (1.7 ± 1.3; $n = 9$). Furthermore, the PF counts (performed on Diff-Quick stained PF cytocentrifugates) show no statistically significant difference in the percentage of different subpopulations between the two groups with except of neutrophils. As expected (Wang et al., 2018) the percentage of the latter cells resulted significantly increased on the average.

Table 2 also shows the data related to the MC counts evaluated on PF cytocentrifugates stained using TB, which

TABLE 1 | Demographic and clinical characteristics of patients in the study group: infertile women with endometriosis (EMS, *N* = 11) and control group: fertile women without EMS (C, *N* = 9).

		Blood (CBC, complete blood count)					
	Age (years)	Leukocytes (10^6/mL)	Neut %	Lymph %	Mono %	Eos %	Bas %
Control (C) group (*n* = 9)	38.4 ± 6.1	7.9 ± 2.3	58.1 ± 8.9	30.3 ± 6.3	4,8 ± 0.9	3.9 ± 2.5	0.87 ± 0.35
Study (EMS) group (*n* = 11)	32.3 ± 4.9	6.6 ± 0.9	54.0 ± 6.6	34.2 ± 5.9	6.5 ± 2.4	3.0 ± 1.3	0.54 ± 0.23
Statistical analysis	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*p* = 0.021

Neut, neutrophils; Lymph, lymphocytes; Mono, monocytes; Eos, eosinophils and Bas, basophils. Values are the mean ± SD. Statistical analysis: Student t-test.

TABLE 2 | Cytological peritoneal fluid characterization in the study and control group.

			Diff Quick stain TB					
	Vol PF (mL)	10^6 cells/mL	MΦ/mono %	Lymph %	Neut %	Eos %	Meso %	MC/baso
Control (C) Group (*n* = 9)	8.7 ± 3.5	1.7 ± 1.3	73.0 ± 10.4	16.0 ± 8.5	8.6 ± 4.5	1.1 ± 0.5	1.3 ± 0.8	0.5 ± 0.5
Study (EMS) Group (*n* = 11)	10.1 ± 5.7	1.6 ± 1.3	56.3 ± 13.4	14.1 ± 10.0	24.6 ± 7.0	2.5 ± 3.8	2.5 ± 5.6	2.8 ± 3.2
Statistical Analysis	*ns*	*ns*	*ns*	*ns*	$p < 0.05$	*ns*	*ns*	*ns*

Neut, neutrophils; Lymph, lymphocytes; MΦ/Mono, macrophages/monocytes; Eos, eosinophils; Bas, basophils; MC, mast cells and Meso, mesothelial like cells; TB, toluidine blue; n, patient number. Values, reported as percentage of total cell counted (always more than 200), are the mean ± SD. Statistical analysis: Student t-test.

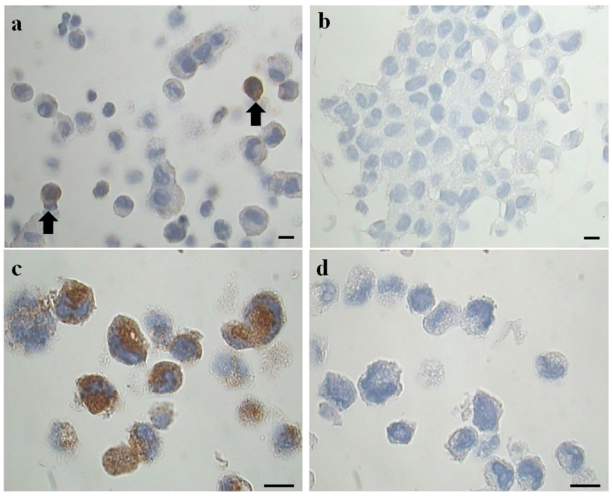

FIGURE 2 | Representative images of immunohistochemical analysis for tryptase on cell blocks' sections. **(a)** Two tryptase positive cells (from the EMS study group) (arrows). **(b)** Negative control (primary antibody omitted). LAD2 cells in **(c)** represent a positive maker control for tryptase, in **(d)** the negative control (primary antibody omitted) is shown. Original magnification 1,000×. Scale bars = 10 μm.

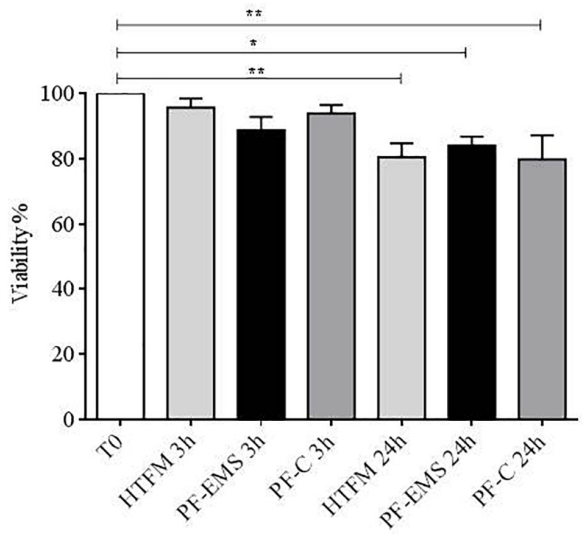

FIGURE 3 | Sperm viability (mean % + SE) after swim-up (T0), after 3 and 24 h incubation in HTF medium (HTFM), peritoneal fluid from women with endometriosis (PF-EMS) and from controls (PF-C). T0 vs. HTFM 3 h: $p = 0.9271$; T0 vs. PF-EMS 3 h: $p = 0.2077$; T0 vs. PF-C 3 h $p = 0.7543$; T0 vs. HFTM 24 $p = 0.0083$; T0 vs. PF-EMS 24 h $p = 0.0386$; T0 vs. PF-C 24 h $p = 0.0061$. $^{*}p < 0.05$; $^{**}p < 0.01$.

makes recognizing MC more easy (EMS vs. C mean % ± SD: 2.8 ± 3.2 vs. 0.5 ± 0.5 $p = 0.616$). MC population is reduced on the average in the control group, but these values were not significantly different.

To clarify the results obtained with the cytocentrifugates the remaining volume of PF was fixed in formalin (4%) and cell blocks were prepared. Serial sections (3 μm) obtained from these cell-blocks were processed for immunohistochemical analysis for tryptase, a specific MC marker (**Figures 2a,b**), using LAD2 cells as a positive marker control (**Figures 2c,d**). The results, expressed as percentage of tryptase positive cells on total cells counted showed that: the study group is characterized by a percentage of tryptase-positive cells (MC) significantly ($p = 0.044$) higher (mean % ± SD: 1.2 ± 0.6; $n = 11$) compared to the control group (mean % ± SD: 0.6 ± 0.3; $n = 9$). These findings agree with the trend revealed by the preliminary analysis of cytocentrifuged samples stained with TB reported in **Table 2**. Of note the density of tryptase-positive cells in the endometriotic lesions (**Supplementary Figure S1** and **Supplementary Table S2**) is comparabe with those reported in the literature (Konno et al., 2003; Kempuraj et al., 2004; Sugamata et al., 2005; Anaf et al., 2006; Kirchhoff et al., 2012; Paula et al., 2015).

Peritoneal Fluid Characterization of Soluble Mediators

There were no significant differences ($p = 0.572$) between the protein concentration (as measured by the Bradford method) of the PF between cases (mean ± SD: 1.19 ± 0.04 mg/mL) and controls (mean ± SD: 1.21 ± 0.06 mg/mL) (**Table 3**).

The PF study group (EMS) is characterized by a statistically significant ($p = 0.031$) increased tryptase enzymatic activity (mean ± SD: 4.2 ± 1.2) compared to the control group (mean ± SD: 3.0 ± 0.8) (**Table 3**). The concentration of tryptase expressed as ng/mL of PF is also shown in **Table 3**. Similarly to what was found for the enzymatic activity of the tryptase in the PF, the study group was characterized by a concentration of tryptase (mean ± SD: 16.4 ± 3.9) significantly higher ($p = 0.023$) than that of the control group (mean ± SD: 12.3 ± 2.3).

Results reported in **Table 3** show that there were no statistically significant differences between the peritoneal levels of β-hexosaminidase enzymatic activity in the two groups ($p = 0.408$). This result is not surprising, since this enzyme is not MC-specific as tryptase is.

TABLE 3 | Biochemical characterization of the peritoneal fluid in the study and control group.

	Control (C) Group ($n = 9$)	Study (EMS) Group ($n = 11$)	Statistical analysis
Protein concentration (mg/ml) *Mean ± SD*	1.21 ± 0.04	1.19 ± 0.06	*ns*
β-hexosaminidase enzyme activity (AU/ml) *Mean ± SD*	5.9 ± 1.3	6.5 ± 1.3	*ns*
Tryptase enzyme activity (AU/ml) *Mean ± SD*	3.0 ± 0.8	4.2 ± 1.2	$p = 0.031$
Tryptase concentration (ng/ml) *Mean ± SD*	12.3 ± 2.3	16.4 ± 3.9	$p = 0.023$

Arbitrary units (AU) are expressed as: absorbance of 4-nitrophenol/ml for β-hexosaminidase; absorbance of p-nitroaniline/ml, for Tryptase. For more details see text. Statistical analysis: Student t-test, two tailed unpaired.

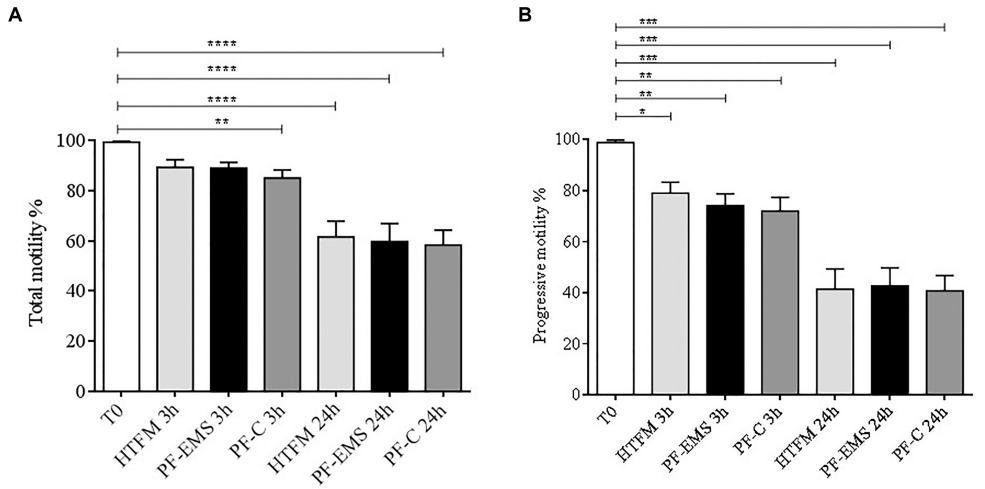

FIGURE 4 | Total **(A)** and progressive **(B)** sperm motility (*mean % ± SE*) after swim-up (T0 starting condition), after 3 and 24 h incubation in HTF medium alone (HTFM), peritoneal fluid from women with endometriosis (PF-EMS) and from controls (PF-C). In (A) T0 vs. HTFM 3 h: $p = 0.4617$; T0 vs. PF-EMS 3 h: $p = 0.0642$; T0 vs. PF-C 3 h $p = 0.0058$; T0 vs. HFTM 24 h $p < 0.0001$; T0 vs. PF-EMS 24 h $p < 0.0001$; T0 vs. PF-C 24 h < 0.0001. In **(B)** T0 vs. HTFM 3 h: $p = 0.0133$; T0 vs. PF-EMS 3 h: $p = 0.0043$; T0 vs. PF-C 3 h $p = 0.0027$; T0 vs. HFTM 24 $p < 0.0001$; T0 vs. PF-EMS 24 h $p < 0.0001$; T0 vs. PF-C 24 h $p < 0.0001$. In both figures $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ and $****p < 0.0001$.

Modulation of Human Sperm Function by Peritoneal Fluid

Motility-enriched sperm samples (n = 19) were incubated in HTFM containing 0.05% BSA or peritoneal fluid pools, respectively, obtained by mixing equal volumes of fluid samples taken from patients with endometriosis [PF-EMS (#11): final tryptase concentration = 14.7 ng/mL] and those collected from women endometriosis-free [PF-C (# 9): final tryptase concentration = 11.1 ng/mL].

Total and progressive sperm motility (**Figures 4A,B**) was significantly decreased in comparison with starting conditions (T0) both after 3 and 24 h incubation period in all condition tested ($p < 0.05$). Anyway, no significant differences were observed when comparing total and progressive motility of spermatozoa incubated for 3 or 24 h in either PF-C or PF-EMS, to that of sperm incubated for the same period in HTF medium alone.

SEM Analysis of LAD2-Sperm Interaction

The mast cell line LAD2 was incubated for 1 h at 37°C alone or with sperms (cells ratio: sperms = 1:3–5) in TyB alone or supplemented with either 10% of peritoneal fluid of study (PF-EMS) or control (PF-C) group. LAD2 cells-sperm interaction was evaluated by scanning electron microscopy (SEM) in samples prepared from two separate experiments. When incubated for 1 h LAD2 cells and sperms frequently show cell to cell adhesive interaction. This interaction is realized either through the sperm head/midportion (HM) or the sperm tail as previously described for macrophages (Blanco et al., 1992). **Figure 5** summarizes these types of interaction as monitored by SEM analysis. **Figure 5a** represents the LAD2-sperm interaction in the presence of PF-C. At least four tail-LAD2 interactions are apparent with three LAD2, while only one head-LAD2 interaction can be seen (arrow). Conversely, in **Figure 5b**, which is representative of LAD2-sperm interaction in the presence of PF-EMS, two sperms in contact with one LAD2 are visible (arrowhead).

With the purpose of evaluating in detail the extent of LAD2-sperm interaction we analyzed more than 100 SEM fields and scored the number of sperm-head/midportion (HM) and sperm-tail interaction with LAD2. **Table 4** shows the result of this analysis. On average, the total sperm-interacting LAD2 (either by HM or by tail) in the presence of PF-EMS accounted for 2.3 cells/field while the interacting cells in the presence of PF-C were 1.3 (ns). The HM sperm-LAD2 interaction in PF-EMS were significantly higher, accounting for 1.2 interactions/field with respect to that observed in the presence of PF-C (0.7 interactions/field). The difference was statistically significant with $p < 0.05$. Conversely the tail sperm-LAD interaction was almost the same in either PF-EMS or PF-C and was not further considered. **Figure 5c** shows two intimate adhesions between sperm and LAD2 surface in the presence of PF-EM. In both cases, extracellular secretion/degranulation can be seen, as either massive extrusion (arrow in c) or single granule secretion (inset in c).

Since degranulation is a reliable marker of MC activation, we assayed cell culture supernatants for the presence of β-hexo (β-hexo), an enzyme located in MC granules.

LAD2 Degranulation Induced by Sperm Interaction

β-hexo is used as a typical marker of mast cell degranulation *in vitro*. **Table 5** shows that the amounts of β -hexo secreted by LAD2 cells is significantly higher after the addition of motile

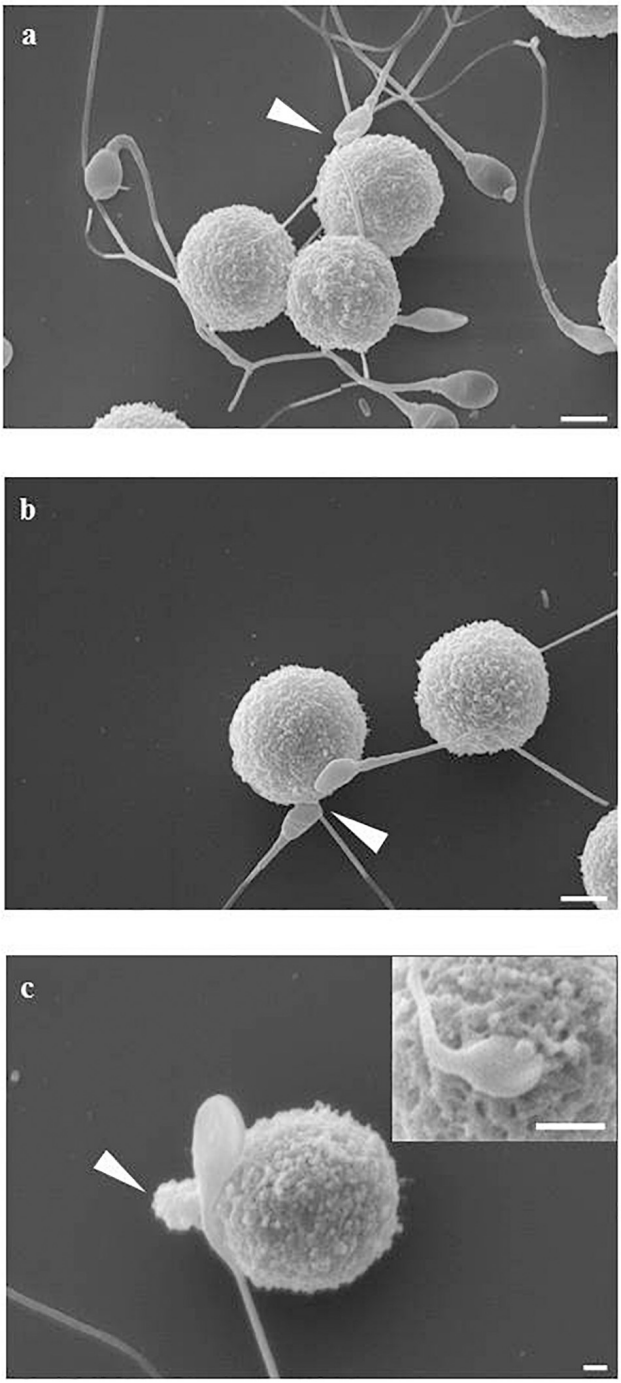

FIGURE 5 | LAD2-sperm interaction evaluated by SEM analysis. **(a)** represents the LAD2-sperm interaction in the presence of PF-C. At least four tail-LAD interactions are apparent with three LAD, while only one head-LAD interaction can be seen (arrow). **(b)** is representative of LAD2-sperm interaction in the presence of PF-EMS, two sperms in contact with one LAD2 are visible (arrow). **(c)** and inset shows two intimate HM interactions between sperm and LAD surface in the presence of PF-EMS. In both cases, extracellular secretion can be seen, as either massive extrusion [arrow in **(c)**] or single granule secretion [inset in **(c)**]. Magnification bars: in **(a,b)** = 3 μm, in **(c)** = 1 μm and in the inset of **(c)** = 3 μm.

sperm to resting cells (mean % ± SD: 6.3 ± 0.7 $n = 3$ vs. 4.2 ± 0.9 $n = 5$ $p = 0.0119$) (**Table 5**).

LAD2 can release also tryptase in the extracellular medium and, likewise to β-hexo, the amount of tryptase secreted by LAD2 cells is higher after the addition of motile sperms to resting cells (% tryptase release, mean of two experiments: 26.3 vs. 15.2, $n = 2$). Anyway we choose β -hexo activity as LAD2-degranulation marker in the following experiments, since tryptase activity in PF was too high and could mask and underestimate the secretory response of LAD2. LAD2 co-incubated (30 min) with human motile sperms in the presence of PF (10%) pool from controls or PF pool from EMS patients were evaluated for β -hexo release (**Table 5**). While the extent of secretion didn't change significantly in the presence of PF-C with respect to the presence of sperm only (mean % ± SD: 7.0 ± 2.0 $n = 3$ and 6.3 ± 0.7 $n = 3$ – $p = 0.5954$), it is significantly increased in the presence of PF-EMS (mean % ± SD: 16.67 ± 2.50 $n = 3$ vs. 6.30 ± 0.66 $n = 3$– $p < 0.0001$) and, interestingly, resulted comparable to the secretion induced by 48/80, a potent mast cell-secretagogue, reported for comparison as a positive control (**Table 5**). However, considering that the ratio between the percent of LAD2 degranulation (% degranulation) and the percent of LAD2 interacting with head-MD of sperms (% HM interacting LAD2) (**Supplementary Table S1**) is almost the same either in the presence of PF-EMS or PF-CTRL), it appears that the higher sperm-induced LAD2 degranulation in PF- is almost completely dependent on the HM event number.

DISCUSSION

Endometriosis (EMS) is a common disease among women of reproductive age and is frequently associated with infertility (Gupta et al., 2008). However, the pathogenesis of this disease is still unknown, so that in several cases the available treatments for symptoms are ineffective (Zito et al., 2014). Increasing evidence supports an involvement of MC either in the inflammatory process of EMS (Kirchhoff et al., 2012) and infertility (Meineke et al., 2000; Weidinger et al., 2003, 2005; Roaiah et al., 2007; Haidl et al., 2011; Menzies et al., 2011). MC are normal constituents of the human myometrium and endometrium and are present in the female genital tract (Sivridis et al., 2001; Hunt and Lynn, 2002). In particular, MC was demonstrated in the oviduct wall (Hunt and Lynn, 2002) and under the lining epithelium of human Fallopian tubes (Weidinger et al., 2003). Endometriotic lesions are characterized by high numbers of degranulated MC (Konno et al., 2003; Kempuraj et al., 2004; Sugamata et al., 2005; Anaf et al., 2006; Kirchhoff et al., 2012; Paula et al., 2015). Mast cells leave evidence, a "fingerprint," of their participation in acute clinical events, that is an elevation in levels of their secreted mediators/metabolites. Of these, tryptase is currently one of the diagnostic criteria for mast cell activation (Butterfield et al., 2018). While clearly showing MC in the female genital tract, it remains unclear whether or not tryptase from the tubal or uterine MC can reach the lumina of these organs. Up to now the concentration of tryptase in the uterus or Fallopian tubal fluid has not been

TABLE 4 | Quantitative and qualitative analysis of LAD2-sperm interaction.

	Field scored (n)	LAD2 scored (n)	LAD2/Field (*mean ± SE*)	InteractingLAD2/ field (*mean ± SE*)	HM interactions/ field (*mean ± SE*)	Tail interactions/ field (*mean ± SE*)
PF-C	100	719	8.8 ± 0.50	1.3 ± 0.3	0.7 ± 0.2	1.2 ± 0.3
PF-EMS	120	646	7.3 ± 0.5	2.3 ± 0.3	1.2 ± 0.1	1.3 ± 0.2
Statistical analysis				*ns*	$p < 0.05$	*ns*

HM, head/midportion. Values are the mean ± SE, obtained from the evaluation of two different experiments. Statistical Analysis: Student t-test, two tails unpaired.

reported. The presence of tryptase in the female genital tract have been only supposed from its presence in the follicular fluid. In this study, we characterized the peritoneal environment associated with endometriosis and infertility (comparing it to control, fertile and endometriosis-free conditions), as regards the MC cellular component and its main product: tryptase.

Our data quantify, for the first time, the presence of an increased number of tryptase-positive cells/MC in the peritoneal fluid of infertile EMS affected women. We also found a significantly reduced number of blood basophils in EMS-group. These cells share with MC the hematopoietic precursor (Yu et al., 2018) and many other features (Marone et al., 2005) and may in principle be involved in EMS pathogenesis, as well. We could speculate a mobilization of basophils from the circulation to the peritoneal cavity. However, this contribution is difficult to evaluate since standard staining techniques are not able to distinguish MC from basophilic granulocytes. Anyway, since normal blood basophils express only trace amounts of tryptase (Li et al., 1998; Samorapoompichit et al., 2001), which has been the main tool and target of our research, and the main diagnostic criteria for identifying mast cell (Butterfield et al., 2018), their contribution was not further considered and should deserves a dedicated research. The increase of MC in the peritoneal environment we found does not prove by itself that MC are involved in the EMS pathogenesis, but is in agreement with the increment of tissue MC population in EMS condition described by other authors (Konno et al., 2003; Kempuraj et al., 2004; Sugamata et al., 2005; Anaf et al., 2006; Kirchhoff et al., 2012; Paula et al., 2015).

TABLE 5 | Secretory response of LAD2 induced by sperm interaction.

	Degranulation: % β-hexo release (*mean ± SD*)	*n*	Statistical analysis
Resting LAD2	4.2 ± 0.9	5	
LAD2+sperm	6.3 ± 0.7	3	[1] vs. [2]: $p = 0{,}0119$
LAD2+sperm in PF-C	7.0 ± 2.0	3	[2] vs. [3]: *ns* [4] vs. [3]: $p = 0{,}0007$
LAD2+sperm in PF-EMS	16.7 ± 2.5	3	[2] vs. [4]: $p < 0.0001$
LAD2+48/80	17.4 ± 2.1	3	[1] vs. [5]: $p < 0.0001$

Percent of release of β-hexo. form LAD2 cells alone (Resting LAD2) and challenged (30 min) with sperm in the absence (in TyB) and the presence of pools of PF from EMS (PF-EMS) and control subjects (PF-C). The positive control was obtained by cell stimulation with compound 48/80. β-hexo, β-hexosaminidase. Values are expressed as percentage of enzymatical activity released, taken the total activity of LAD2- βhexo as 100%. For more details see text. Statistical analysis: Dunn's post hoc was used to individuate group difference.

Accordingly, the peritoneal fluid in infertile-EMS conditions was characterized by higher levels of enzymatically active tryptase, the main MC product. Higher EMS peritoneal fluid levels of tryptase could be ascribed not only to the increment of peritoneal MC population but also to that of tissue population. These cells are responsive to many different receptors and can be activated by various kind of stimuli (Redegeld et al., 2018). Now, the physical interaction with sperms should be added to the list. The released mediators could reach in the peritoneum a high level which in principle could affect sperm motility and fertility as previously suggested. Human recombinant tryptase has been shown to inhibit sperm motility *in vitro* (Weidinger et al., 2003, 2005) and accordingly tryptase has been supposed as a yet unrecognized factor capable of influencing sperm fertilizing ability. Anyway, up to now tryptase levels have been evaluated only in human seminal plasma of andrological patients (4.18 ± 1.95 ng/mL) and in human follicular fluid (ranging from 1.60 to 3.73 ng/mL), where they results far below the lower level capable of inhibit sperm motility *in vitro* (10 ng/mL). Accordingly, seminal fluid's levels of tryptase were not correlated to sperm motility (Weidinger et al., 2003).

Infertility is a condition associated with EMS and the effect of peritoneal fluid associated with the condition of endometriosis on sperm motility has been a long controversial topic: some experimental evidence support a decrease in sperm motility in this environment (Aeby et al., 1996; Oral et al., 1996; Liu et al., 2000; Luo et al., 2008; Xu et al., 2008), while other authors report no effect (Chen et al., 1997; Sharma et al., 1999; Munuce et al., 2003).

Interestingly, the concentration of enzimatically active tryptase that we found in the peritoneal fluid of both study (EMS: 16.4 ± 3.9 ng/mL) and control group (C: 12.3 ± 2.3 ng/mL) was higher than those reported in human seminal plasma of andrological patients and human follicular fluid, and higher than the minimum concentration capable of inhibiting *in vitro* sperm motility (Weidinger et al., 2003). In spite of this we didn't find any differences when analyzing sperm viability and motility (total and progressive) in the presence of tryptase enriched peritoneal fluid associated with endometriosis and infertility with respect to control, fertile and endometriosis-free conditions (nor to control medium alone), both in short and long term incubation. Our data are in agreement with previous reports showing the absence of any effect of EMS peritoneal fluid on sperm motility (Chen et al., 1997; Sharma et al., 1999; Munuce et al., 2003) and suggest that tryptase levels, able of inhibiting sperm motility *in vitro*, are ineffective in the peritoneal environment. Anyway we cannot exclude that tryptase (or other still unrecognized MC

mediators) could modulate other sperm function *in vivo*, eg. acrosome reaction (Arumugam, 1994; Munuce et al., 2003) or sperm-oocyte interaction (Aeby et al., 1996; Wong et al., 2001; Caille et al., 2012). While the present report was focused on sperm motility in the presence of EMS-PF, the possible effects of tryptase on sperm functions, will be the subject of future research.

Furthermore, Cincik and Sezen previously reported that the presence of MC in semen, by itself negatively affects sperm motility (Cincik and Sezen, 2003). Accordingly, since the number of tryptase-positive cells/MC increases in the peritoneal fluid of infertile EMS affected women we decided to investigate the possible interaction between these cells and sperm in the peritoneal environment. We co-incubated mast cells (cell line LAD2) and sperms in a pool of PF (10% v/v) obtained from infertile women with EMS or from fertile endometriosis-free controls. Sperm was shown to interact more with LAD2 in the presence of PF-EMS. Furthermore LAD2-sperm interaction was shown to induce a secretory response from LAD2 cells (by morphological and secretory evaluations), which was significantly higher in EMS conditions. We suggest that this secretory response could contribute to increase the level of tryptase present in PF-EMS.

CONCLUSION

In conclusion, on the basis of our findings it appears unlikely that tryptase enriched peritoneal fluid could affect sperm motility.

Anyway the present study presents some reasons for caution, as follows:

- The sample size was limited. A broader study concerning different stages of endometriosis would increase the value of our results.
- Human resident peritoneal mast cells are not readily purified. To overcome this limitation in studying in human beings the MC-sperm interaction, we used the most differentiated human mast cells line available, endowed with a high content of tryptase.
- The sperm parameters analyzed were limited to viability and motility, so we cannot exclude that tryptase (or other still unrecognized MC mediators) could modulate other sperm functions *in vivo*, contributing to the infertility associated with endometriosis.

The novelty reported in this paper is that the presence of a tryptase rich PF can stimulate the sperm-mast cell interaction and induce degranulation from these cells. However, even if tryptase-positive cells are present in a higher percentage in the PF-EMS, they remain quantitatively underrepresented, and therefore their strong interaction with the sperm in the EMS conditions couldn't be enough to affect sperm function. We think that this strong physical and functional mast cell-sperm interaction could be more effective in the male genital tract where MC mediated negative effects on sperm functions has been reported but not clarified so far and require further investigation (Agarwal et al., 1987; Hashimoto et al., 1988; Nagai et al., 1992; Hussein et al., 2005; El-Karaksy et al., 2007; Haidl et al., 2011; Menzies et al., 2011; Windschüttl et al., 2014) One potential benefit to assisted reproductive clinics could derive from targeting mast cells - sperm interaction to treat male infertility due to testicular pathologies associated with inflammation and germ cell loss, as recently suggested (Mayerhofer et al., 2018).

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Comitato Indipendente di Bioetica (CIB)-Burlo Garofolo. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

VB developed the idea for the manuscript, formulated the study design, and performed the peritoneal fluid analyses, interpretation of the data, and manuscript drafting. FR, FF, and GR performed the surgical procedures. FC contributed to the statistical analyses. GR conducted the clinical evaluations and contributed to the statistical analyses, data interpretation, and manuscript drafting. MM and SL recruited patients and performed the sperm analysis. FZ and CB performed the immunocytochemical/immunohistochemical staining and data interpretation. FV performed the scanning electron microscopy analysis. ET performed the evaluation with LAD2 cell line. GZ participated in a critical revision and manuscript drafting. All authors have approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2019.01543/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Peripheral CD56^{+}CD16^{+} NK Cell Populations in the Early Follicular Phase Are Associated With Successful Clinical Outcomes of Intravenous Immunoglobulin Treatment in Women With Repeated Implantation Failure

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The percentage of peripheral CD56^{+}CD16^{+} NK cells in the early follicular phase on days 2–3 of the menstrual cycle in repeated implantation failure (RIF) patients was used to evaluate the impact of intravenous immunoglobulin (IVIG) on ART cycles. A total 283 patients with RIF consisting of at least 3 ART failures and at least 2 high quality embryo transfers were recruited. A logistic regression analysis for the peripheral immunological profile was completed to predict implantation success and compare the implantation and pregnancy rates between groups with ≤10.6 and >10.6% of CD56^{+}CD16^{+} NK cells in the early follicular phase. The logistic regression and receiving operating curve analyses showed that patients with ≤10.6% of peripheral CD56^{+}CD16^{+} NK cells in the early follicular phase showed a lower pregnancy rate within the RIF group without IVIG. Patients with peripheral CD56^{+}CD16^{+} NK cells ≤10.6% and without IVIG treatment showed significantly lower implantation and pregnancy rates (12.3 and 30.3%, respectively) when compared with the CD56^{+}CD16^{+} NK cells >10.6% group (24.9 and 48.0%, respectively, $p < 0.05$). Furthermore, the patients with CD56^{+}CD16^{+} NK cells ≤10.6% given IVIG starting before ET had significantly higher implantation, pregnancy, and live birth rates (27.5, 57.4, and 45.6%, respectively) when compared with the non-IVIG group (12.3, 30.3, and 22.7%, respectively, $p < 0.05$). Our results showed that a low percentage of peripheral CD56^{+}CD16^{+} NK cells (≤10.6%) in the early follicular phase is a potential indicator of reduced pregnancy and implantation success rates in RIF patients, and IVIG treatment will likely benefit this patient subgroup.

Keywords: natural killer cells, intravenous immunoglobulin, repeated implantation failure, infertility, lymphocytes

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INTRODUCTION

With advances in assisted reproduction techniques (ART), high quality embryos can be imbedded into the uterus for pregnancy. However, a substantial number of women suffer from the repeated implantation failure (RIF) of several embryos, regardless of quality (1). For many years, defective crosstalk between the embryo and endometrium in unexplained RIF patients was attributed to circulating peripheral blood mononuclear cells (PBMC) and immunological responses, with the exception of inherent genetic, anatomical, chromosomal, or endocrine abnormalities (2). Fujiwara et al. suggest that circulating blood cells positively contribute to maternal tissue remodeling (2).

Pregnancy evolves through different immunological stages with a pro-inflammatory or anti-inflammatory predominant profile, depending on the stage of gestation analyzed (3, 4). Increasing evidence indicates that immune cell or immunologic factors play an important role in the failure of both natural and ART-induced pregnancies (3–5). Monocyte/macrophage lineage cell markers increase in the decidua/myometrium during pregnancy and may control trophoblast cell invasion into the myometrium while preventing a rejection of the semi-allogenic conceptus to provide an important barrier against invading pathogens (5, 6).

For RIF patients, there are beneficial effects of intravenous immunoglobulin (IVIG) purified from the pooled blood plasma of healthy donors (7, 8). The proposed mechanisms of action of IVIG are categorized into direct antibody effects (9) and immune-modulation (10). Furthermore, preconception immune testing from peripheral blood may be a critical tool for determining which patients will benefit from IVIG therapy (7). Measurements of peripheral blood immune cells by flow cytometry are easier and less invasive because this technique does not require obtaining an endometrial biopsy sample.

Jurisic et al. in 2007 reported that increase in the concentration of IgG immunoglobulins significantly correlated with increase of NK cell activity (11). NK cells constitute 5–10% of peripheral blood lymphocytes (PBL) and have a $CD3^{-}CD16^{+}$ $CD56^{+}$ phenotype (12). NK cells play an important role in cancer (11, 12), viral infections and gynecology (13, 14), transplantation immunology (15), especially because they are cells of innate immunity. Furthermore, natural killer cells play an essential role in defense of the rise and spread of malignancy (11). The multiple myeloma patients with higher NK cell activity at presentation have better cumulative survival in comparison with those with low NK cell activity.

Previous studies indicated that the percentage of peripheral blood NK cells in the luteal phase were significantly increased in women with recurrent pregnancy losses or implantation failures (16, 17). However, a systematic review by Tang et al. reported that the prognostic value of measuring pNK or uNK cell parameters remains unclear (18), and more studies are needed to confirm or refute the role of NK cell assessments as a predictive test for screening in recurrent miscarriage (RM) or RIF patients. Therefore, the relevance between peripheral mononuclear profiles and RIF deserves further investigation. In the present study, peripheral blood monocytes (PBMC) samples were collected from RIF women in the early follicular phase instead of the luteal phase. We compared the PBMC profile in the early follicular phase with controlled ovarian stimulation and IVF outcomes after IVIG in RIF patients to identify candidates for IVIG treatment.

MATERIALS AND METHODS

Patient Selection

The entire study population was comprised of 283 women with RIF who were referred to the Lee Women's Hospital and treated with *in vitro* fertilization (IVF) protocols between Jan. 2007 and Oct. 2011. This study consisted of Human Subject Research. The study protocol was approved by the Institutional Review Board of the Chung Shan Medical University Hospital (CSMUN No. CS:12033). All participants provided their written informed consent to participate in this study; in addition, all participants signed standard IVF consent forms. The written consents of IVIG treatment were obtained from journal meeting records or patient treatment charts in the administration department at Lee's Women Hospital. The journal meetings or consultations in the IVF laboratory at Lee's Women Hospital were held every week, and all participants signed a consent form after the meeting. At least one signature of each participant was recorded during study. Written consent was not obtained from patients in these meetings who were not associated this study or participated in other unpublished studies. The ethics committees/IRBs approved this consent procedure, and the invasion of patient privacy was avoided in this study. All patients were recruited based upon a history of repeat implantation failure with unknown reasons. After delicate counseling, we provided IVIG treatment as an alternative strategy for the possible immune reasons. The choice of IVIG treatment was dependent on the couples. Patients who decided to receive IVIG therapy signed an IVIG consent form that explained the possible risks, the nature of the medication, and the lack of sufficient evidence-proof for treatment efficacy. Inclusion criteria of RIF patients in this study included patients who experienced >2 failures of IVF–embryo transfer therapy with at least two good embryos transferred each session. The following exclusion criteria were used for this study: (i) abnormal uterine anatomy evaluated by hysterosalpingography and /or hysteroscopy; (ii) abnormal blood karyotype in the female or male partner; (iii) positive titer for the lupus anticoagulant; (iv) endometriosis; (v) recurrent miscarriage; (vi) endometrium $\leq$ 7 mm on the day of hCG injection; or (vii) BMI$\geq$30.

IVF Protocol

All women underwent a program consisting of a long protocol for GnRH agonist administration (19). Participating women were administered leuprolide acetate (Lupron, Takeda Chemical Industries, Ltd., Osaka, Japan) starting at the midluteal phase to produce down-regulation. All patients subsequently received recombinant follicular stimulation hormone (rFSH; Gonal-F, Serono, Bari, Italy) for ovarian stimulation from cycle day 3 until the dominant follicle reached a diameter of >18 mm. Next, patients received an injection of 250 micrograms of human

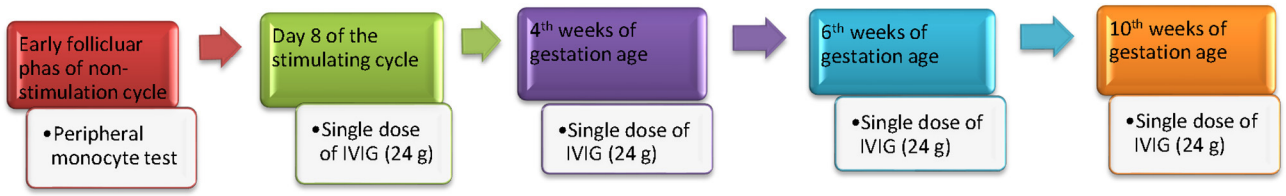

FIGURE 1 | The timing and protocol of IVIG treatment. Peripheral monocyte test was performed on the 2–3rd day of the menstrual cycle prior to ovarian hyperstimulation. Women received the first dose of IVIG (24 g TBSF human immunoglobulin; CSL Limited, Broadmeadous, Australia) on day 8 of the stimulating cycle. If a viable pregnancy was confirmed by serum hCG concentrations and ultrasound, IVIG was continued in 4, 6, and 10th weeks of gestation age (a total dose of 96 g) according to the published protocol.

chorionic gonadotropin (hCG; Ovidriell, Serono) 36 h prior to oocyte retrieval.

IVIG Treatment Protocol

The IVF and IVIG treatment protocols are shown in **Figure 1**. Patients received the first dose of IVIG (24 g TBSF human immunoglobulin; CSL Limited, Broadmeadous, Australia) on day 8 of the stimulating cycle. If a viable pregnancy was confirmed by serum hCG concentrations and ultrasound, IVIG was continued in the 4, 6, and 10th weeks of gestation age (a total dose of 96 g) according to the published protocol (20). Patients in the non-IVIG treatment group did not receive a placebo treatment during stimulation and pregnancy.

Embryo Culture

After retrieval, oocytes were cultured in Quinn's Advantage Fertilization Medium (Sage BioPharma, Inc., Trumbull, CT, USA) with a 10% serum protein substitute (SPS, Sage BioPharma, Inc) in a triple gas phase of 5% CO_2, 5% O_2, and 90% N_2. Following conventional insemination or ICSI, all embryos were furthered cultured in microdrops of cleavage medium (Sage BioPharma, Inc., Trumbull, CT, USA) with a 10% serum protein substitute. Fertilization was verified by the presence of two pronuclei 17–19 h after insemination or injection. The embryo transfer was performed on day 3. Embryos with the most favorable cell number, fragmentation, and asymmetry scores were selected for transfer. Each patient's age, history, and number and morphology of available embryos were utilized to determine the number of embryos to transfer. Clinical pregnancies were diagnosed by the presence of a gestational sac on transvaginal ultrasound 5 weeks after oocyte retrieval.

Peripheral Blood Test and Flow Cytometry

The peripheral blood of RIF women was sampled simultaneously on days 2–3 of the menstrual cycle prior to ovarian hyperstimulation. All of the blood samples from RIF patients were tested for the presence of autoantibodies, such as lupus anticoagulant (LA), anti-cardiolipin antibodies and antinuclear antibodies (ANA). No patients had any type of infection during the last month before blood collection. In the same blood sample, the PBMC profile was determined using flow cytometry. Three-color flow cytometry (FACS Calibur, BD Biosciences) was used to evaluate different mononuclear cell subpopulations. The examination was completed using fresh blood prior to the IVF procedure. The differently labeled monoclonal antibodies were used in following combinations: $CD3^+$, $CD4^+$, $CD8^+$, $CD19^+$, CD-AT (activated T cell; $CD3^+$/HLA-DR^+) and NK ($CD16^+CD56^+$). All of the reagents were produced by Becton Dickinson (BD Biosciences, Franklin Lakes, NJ, USA). The results are presented as a percentage of total lymphocytes (**Figure 2**).

Statistical Analysis

Differences between the IVIG and non-IVIG (control) groups with regard to age, numbers of embryo transfer attempts, numbers of transferred embryos, and numbers of good quality embryos were analyzed by Student's *t*-test. A Chi-square test was used for comparisons of the clinical pregnancy rate, implantation rate and live birth rate between groups. A $P < 0.05$ was considered significant. All calculations were performed using SPSS 17.0 (StatSoft Inc., Tulsa, USA) and MedCalc Statistical Software version 12.7.7 (MedCalc Software bvba, Ostend, Belgium; 2013).

RESULTS

Table 1 shows the demographic data and treatment outcomes for the control and IVIG groups. There was a total of 283 completed treatment cycles comprised of 115 cycles in the IVIG group and 168 cycles in the control (non-IVIG) group. The mean of age, etiology of infertility, previous IVF times, and embryo transferred numbers between IVIG and non-IVIG groups were not significantly different. The implantation [(26.5%, 108/408) vs. (20.1%, 111/553)] and pregnancy [(59.1%, 68/115) vs. (41.1%, 69/168)] rates in the IVIG group were significantly higher when compared with the non-IVIG group. The live birth rate [(30.4%, 51/168) vs. (43.5%, 50/1,150; $p = 0.079$)] in the IVIG group showed a non-significant trend toward an increase when compared with the non-IVIG group. The abortion rates, fetal body weight and gestational age of birth were not significantly different between the two groups.

No adverse effects related to the infusion occurred in the IVIG group. None of the patients discontinued IVIG therapy because of side effects, and the IVIG treatment did not produce significant toxicity in the mother or fetus.

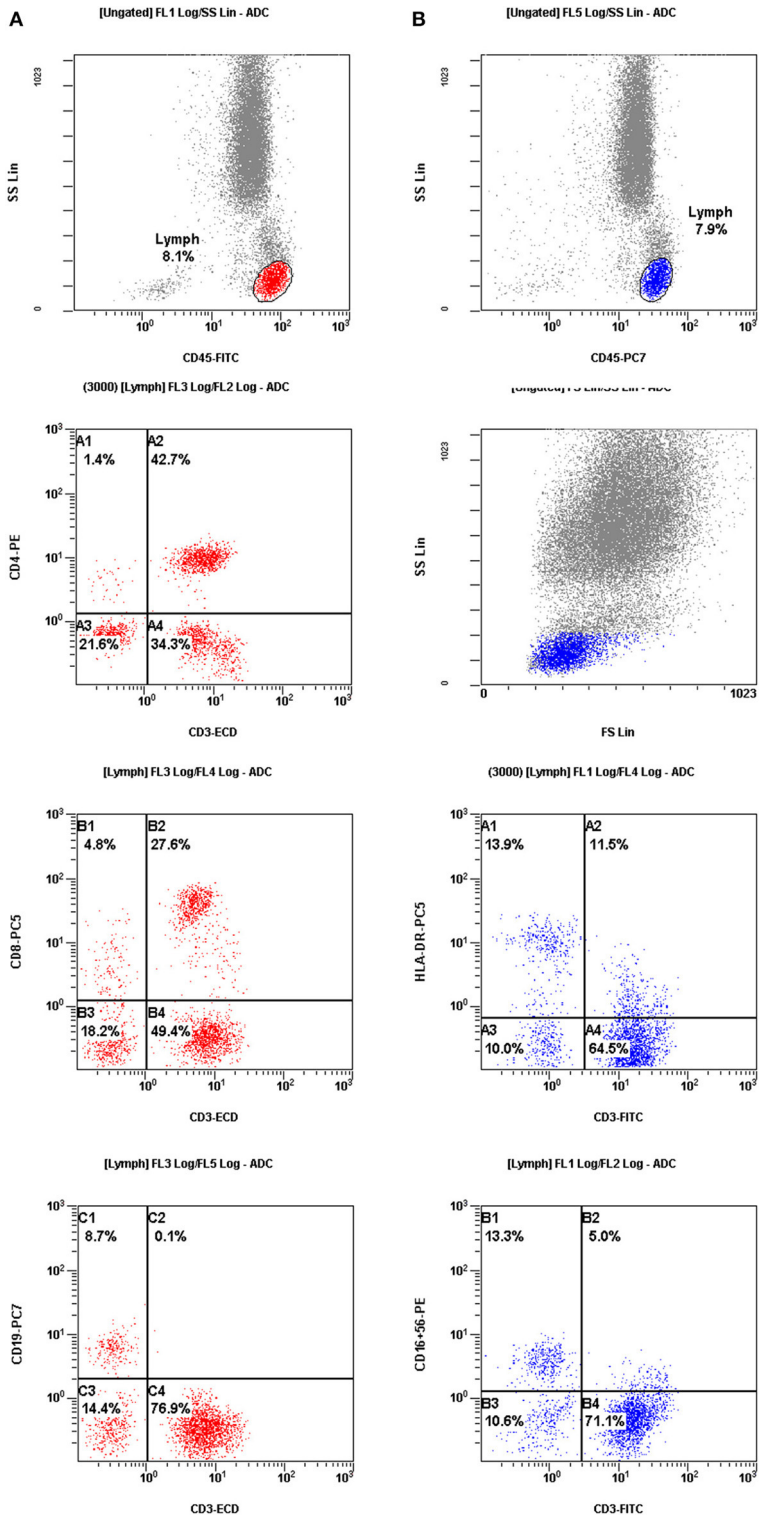

FIGURE 2 | The results were presented as a percentage of total lymphocytes. Cell sorting for assessment of the mononuclear cell profiles in peripheral blood from patients with repeated implantation failure (RIF). **(A)** (red color) Mononuclear cells were gated by fluorescence intensity of CD45 vs. side light scatter (SSL). The T cells ($CD3^+$, C4) and B cells ($CD19^+$, C1) were gated by fluorescence intensity of CD3 vs. CD19. The helper T cells ($CD3^+CD4^+$, A2) were analyzed for their expression of CD3 and CD4. The suppressor T cells ($CD3^+CD8^+$, B2) were gated by fluorescence intensity of CD3 vs. CD8. **(B)** (blue color) Activated T cells ($CD3^+$ $HLA\text{-}DR^+$, A2) were analyzed by the expression of CD3 and HLA-DR. CD56+CD16+ NK cells ($CD3^-CD56^+CD16^+$, B1) were gated by fluorescence intensity of CD3 versus $CD16^+CD56^+$.

TABLE 1 | Demographic data of the control (Non-IVIG) and IVIG treatment groups.

	Control ($n = 168$)	IVIG ($n = 115$)	P*
Age (years)	36.5 ± 4.4	35.4 ± 4.7	0.082
BMI (Kg/m^2)	21.4 ± 3.1	21.3 ± 2.7	0.254
Infertility			
Male factor (%)	31.5 (53/168)	32.3 (44/115)	0.887
Female factor (%)	39.3 (66/168)	31.3 (36/115)	0.169
Combined factor (%)	8.9 (15/168)	10.4 (12/115)	0.673
Unexplained (%)	20.2 (34/168)	20.0 (23/115)	0.967
Previous IVF times	5.1 ± 2.4	5.4 ± 2.9	0.244
Oocyte number	14.7 ± 9.5	15.3 ± 10.6	0.579
MII number	11.8 ± 7.8	12.3 ± 8.5	0.606
Fertilized embryo number	9.5 ± 6.6	9.9 ± 7.0	0.589
High qualified embryo rate	72.1 ± 14.0	70.0 ± 12.6	0.201
Transferred embryos	3.3 ± 0.8	3.5 ± 0.6	0.078
Implantation rate (%)	20.1 (111/553)	26.5 (108/408)	0.019
Pregnancy rate (%)	41.1 (69/168)	59.1 (68/115)	0.003
Live birth rate (%)	30.4 (51/168)	43.5 (50/115)	0.079
Abortion rate (%)	24.6 (17/69)	25.0 (17/68)	0.957
Fetal body weight (g)	2,710 ± 622	2,489 ± 619	0.083
Gestational age of delivery (weeks)	36.8 ± 2.8	36.2 ± 2.5	0.254

**Comparison by Mann Whitney U test or X^2 test as the condition determined.*
The data are presented with mean± standard deviation (SD) or percentage (%).

TABLE 2 | Logistic regression analysis for the peripheral immunological profile to predict pregnancy (implantation success) by control group (Non-IVIG treatment, $n = 168$).

	Mono-variable	
	Regression coefficient (95% CI)	***p***
CD3	0.976 (0.940–1.014)	0.208
CD4	0.986 (0.947–1.026)	0.474
CD8	0.997 (0.956–1.039)	0.874
CDAT	1.078 (0.995–1.168)	0.065
CD19	0.922 (0.859–0.990)	0.026
NK	1.071 (1.021–1.124)	0.005

The Percentage of Peripheral CD56$^+$CD16$^+$ NK Cell in the Early Follicle Phase May Predict the Art Outcome

A logistic regression analysis of the control group was completed to define the correlation between the peripheral mononuclear cell profile and pregnancy (at least one embryo implantation) outcome of IVF treatment. When a single variable in the peripheral monocyte profile was used in the analysis model, both CD19 and CD56$^+$CD16$^+$ NK cell percentages demonstrated a significant correlation with ART pregnancy outcome (**Table 2**).

A receiver operating characteristic (ROC) curve analysis revealed the cut-off value for pregnancy outcome was 10.6% of CD56$^+$CD16$^+$ NK cells and 9.3% of CD19$^+$ B cells (**Figures 3A,B**). This selected value of NK cells had a sensitivity of 71.0% (95% CI: 58.8–81.3) and a specificity of 46.5% (95% CI: 36.4–56.8). The selected values of B cells had a sensitivity of 40.6% (95% CI: 28.9–53.1) and a specificity of 80.8% (95% CI: 71.7–88.0).

None of the parameters in the peripheral mononuclear profile were found to be independent. Therefore, we compared each parameter of peripheral mononuclear cells based on the NK percentage. The patients with lower NK percentages (≤10.6%) had higher levels of CD19 cells, CDAT cells, and CD4 cells (**Figure 4**). In contrast, the levels of cytotoxic CD8 cells were similar between low and high NK groups (**Figure 4**). To determine the independence of the parameters in the peripheral mononuclear profile, we used multivariable logistic regression model for further analysis. The results indicated that the CD56$^+$CD16$^+$ NK cell percentage was the sole factor relevant to the ART outcome. The adjusted odds ratio for CD56$^+$CD16$^+$ NK cell percentage to correlated with pregnancy outcome is 1.061 (95% CI: 1.011–1.115), $P = 0.017$.

A Percentage of ≤10.6% for Peripheral NK Cells in the Early Follicle Phase Is an Indicator for IVIG Treatment

All patients were divided into four subgroups according to the percentage of peripheral CD56$^+$CD16$^+$ NK cells in the early follicular phase: (1) IVIG group with >10.6% of CD56$^+$CD16$^+$ NK cells, (2) IVIG group with ≤10.6% of CD56$^+$CD16$^+$ NK cells, (3) non-IVIG group with >10.6% of CD56$^+$CD16$^+$ NK cells, and (4) non-IVIG group with ≤10.6% of CD56$^+$CD16$^+$ NK cells. In the non-IVIG groups, patients with ≤10.6% of CD56$^+$CD16$^+$ NK cells showed significantly lower implantation and pregnancy rates (12.3 and 30.3%, respectively) than patients with >10.6% of CD56$^+$CD16$^+$ NK cells (24.9 and 48.0%, respectively). The implantation, pregnancy and live birth rates in the IVIG group with a ≤10.6% NK percentage (27.5, 57.4, and 45.6%, respectively) were significantly higher when compared with the non-IVIG group with a ≤10.6% NK percentage (12.3, 30.3, and 22.7%, respectively). The benefit of IVIG treatment on implantation (25.0 vs. 24.9%) and pregnancy (61.7 vs. 48.0%) rates in the group with a >10.6% NK percentage was not significantly different from the non-IVIG group with a >10.6 NK percentage (**Table 3**). IVIG improved all outcome measures in those with NK ≤ 10.6%.

After combining the patients and then subdividing into low NK and high NK groups, the patients in the high NK group showed a trend toward a higher implantation rate [20.4% (93/456) vs. 25.0% (126/505); $P = 0.052$] and abortion rate [20.3% (12/59) vs. 28.2% (22/78); $P = 0.073$] when compared with the low NK group.

DISCUSSION

In the present study, we found that a decreased percentage of peripheral CD56$^+$CD16$^+$ NK cells in the early follicle phase was significantly associated with low pregnancy rates using a logistic regression analysis. Furthermore, we demonstrated that an IVIG infusion was beneficial for implantation rates, pregnancy rates, and live-birth rates in RIF patients with a CD56$^+$CD16$^+$ NK cell population ≤ 0.6%. These data suggest that the peripheral blood

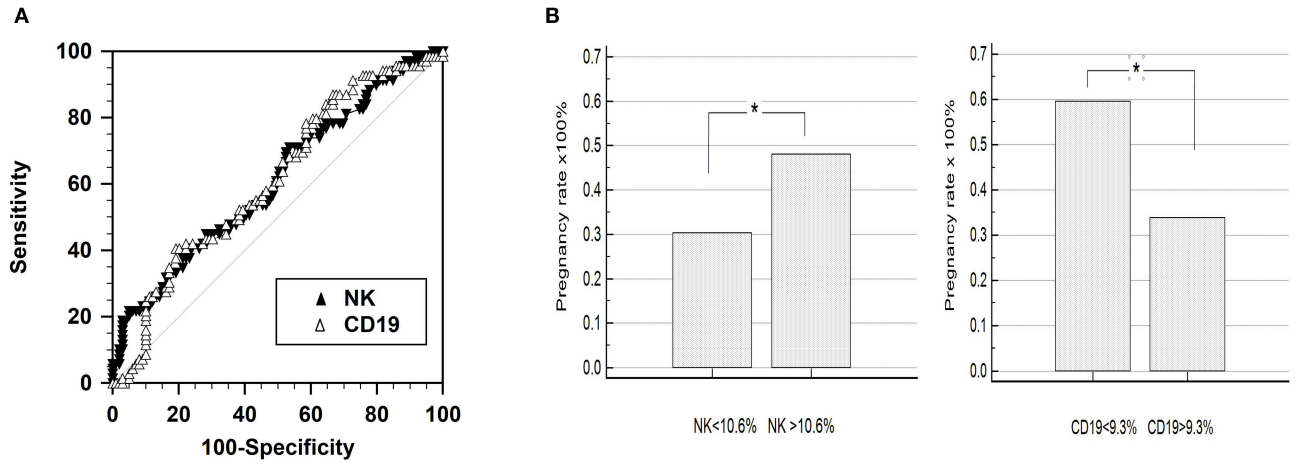

FIGURE 3 | Receiver operating characteristic (ROC) curve analysis of CD56+CD16+ NK cell percentage. **(A)** Comparison of the predictive value for pregnancy outcome by means of area under the curve of receiver operating characteristic (ROC_{AUC}) curve analysis. The percentage of peripheral natural killer (NK; CD56+CD16+) and B (CD19) cells featured similar ROC_{AUC} for ART outcome. **(B)** Pregnancy outcome of the groups divided by the criteria selected by the ROC curve analysis: 10.6% for the NK cells (CD3-CD56+CD16+) and 9.3% for the B cells (CD19+), respectively.

$CD56^{+}CD16^{+}$ NK cell levels in the early follicle phase can be used to select patients who will benefit from IVIG.

A recent meta-analysis reported that the use of IVIG was beneficial for ART cycles in women with abnormal or elevated natural killer (NK) cells in the luteal phase; however, the strength of this evidence is poor (21). In this study, we examined peripheral monocytes in the early follicle phase rather than the luteal phase in order to merge the blood tests for ovarian reserves and peripheral mononuclear profiles for patients' convenience. Furthermore, this enabled us to merge the IVIG and IVF stimulation protocols. In addition, an early blood collection extends the available time for the patient to decide to undergo the rather costly IVIG treatment. Therefore, clinicians should balance efficiency vs. cost when deciding to treat certain conditions with IVIG. Appropriate patient selection and criteria for peripheral NK cell populations are crucial factors that determine the success of IVIG treatment.

The NK cells in the follicle and luteal phases may play different roles in implantation. During menstrual cycle, the percentage of peripheral blood NK cells (22) and the total number of peripheral NK-lymphocytes (23) significantly increased from the early follicular to luteal phase. However, Souza et al. (23) reported that NK cytotoxicity in the luteal phase was significantly reduced when compared with the follicular phase ($P < 0.0001$) in healthy women (24). Indeed, *in vitro* NK lymphocytes can differentiate into cells with NK1 (Th1) or NK2 (Th2) phenotypes similar to those of helper T lymphocytes (25). A shift in NK-lymphocytes toward a "Th2-type"-like response is only present during pregnancy and not in the luteal phase of the ovarian cycle. Thus, the NK cell and lymphocyte response shifts away from a type 1 immune response during pregnancy (26). Bouman et al. reported that monocytes may not be activated and are more sensitive in the luteal phase, whereas these cells are suppressed and less sensitive in the follicular phase (27). It has been suggested that a follicular ovarian factor exists that is capable of suppressing the non-specific immune system during the follicular phase (28, 29). Once this factor disappears (in the luteal phase or during pregnancy), the non-specific immune system is no longer suppressed and appears to be "activated and more sensitive"; thus, the specific immune system shifts toward a Th2 response (23, 30).

We suggest that NK cells in the early follicle may be an immune suppressing factor. Therefore, a lower NK population may reflect an unbalanced immune system, which leads to a specific immune system shift in the luteal phase after hormone stimulation. Although NK cells in the luteal phase were not examined in our study, we predict that the NK cells in the luteal phase of RIF patients were elevated, which further impaired embryo implantation. The physiological meaning of this phenomenon may be the preparation of the maternal immune system for potential implantation of the semi-allogenic blastocyst. However, further investigation is needed to confirm the occurrence of this phenomenon.

The uterine NK (uNK) cells are dominant and increase in absolute numbers in the decidua and to remodel the uterine arteries during pregnancy (14, 31, 32). The uNK cells are increase in the invading trophoblast and probably contribute to implantation (14). The uNK cells have been also described that their number increasing in the proliferative phase and reaching the maximal level in the late secretory phase during the menstrual cycle. These uNK cell numeric variations have been correlated to hormone-induced decasualization (30). The origin of uNK cells are presently unknown, and it is still debated whether they arise from NK cell progenitors present in the uterus prior to pregnancy, or are recruited from peripheral NK cell populations (30, 33). Carlino et al. suggest that peripheral NK cell recruitment

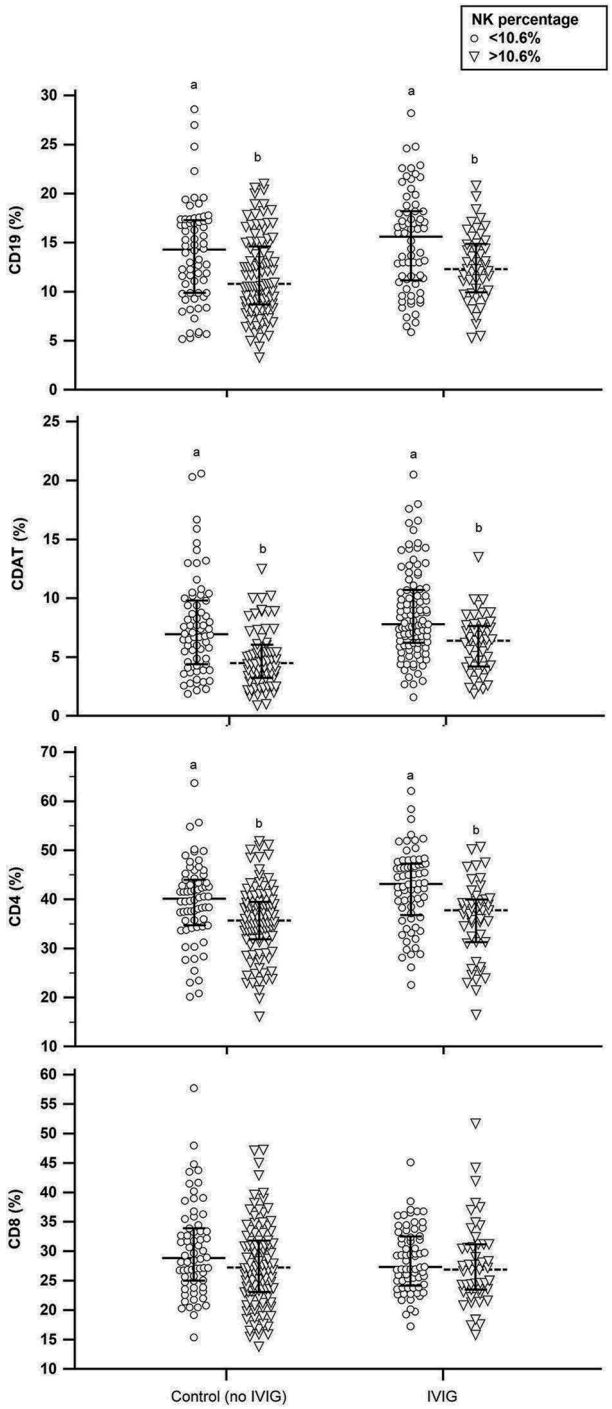

FIGURE 4 | The distribution of peripheral mononuclear cell profiles between high and low percentage of CD56+CD16+ NK cells in intravenous immunoglobulin (IVIG) and non-IVIG groups. Different letters in the same subset figure indicate a significant difference, $P < 0.01$, using Mann-Whitney *U*-test.

to the uterus contributes to the accumulation of NK cells during early pregnancy and that progesterone plays a crucial role in this event (34).

The low NK population in the early follicle phase may reflect a lower recruitment of NK to the endometrium. The mechanisms controlling the accumulation of NK cells in the endometrium remain largely unknown. The propensity of NK cells to move into the decidua has been observed in all species investigated to date, suggesting a significant role for NK cells in normal pregnancy (35). Santillán et al. reported a positive correlation between blood and endometrial $CD56^+$ NK cells (36), and Park et al. showed a correlation between the numbers of peripheral blood NK cells and endometrial NK cells from decidual tissue (37). Hanna et al. indicated that $CD16^-$ NK cells are attracted from the peripheral blood to the decidua via CXCR4 and CXCL12 interactions; thus, the composition of the peripheral lymphocyte population is likely the key to a proper fetomaternal immune tolerance (38). In mouse models, decidual NK cells are recruited from peripheral sites rather than created from self-renewal processes in the uterine mucosa (39). Lee et al. reported that the increase of peripheral blood NK cells in the luteal phase may contribute to the recruitment of uterine NK cells from peripheral blood (22). Furthermore, Okitsu et al. used an autologous PBMC intrauterine administration to effectively improve embryo implantation in RIF patients (40). These data suggest that peripheral $CD56^+CD16^+$ NK cells are recruited into endometrium prior to embryo implantation. The results of our current study suggest that RIF women with low peripheral blood $CD56^+CD16^+$ NK cells in the early follicular phase may reflect an insufficiency of endometrial $CD56^+CD16^+$ NK cell recruitment and a defective microenvironment for embryo implantation.

However, previously published reports showed that a significantly elevated peripheral blood NK cell percentage in the luteal phase impaired female reproductive function (18, 41, 42). An appropriate NK population or recruitment in the luteal phase is very important. Our observations suggest that the percentage of peripheral NK cells in the early follicular phase also plays an important role in the regulation and recruitment of endometrial NK cells.

In our study, we found that the immune cell population in the early follicle phase between different menstrual cycles was similar. We collected samples from two separate no-stimulation cycles in a portion of RIF patients, and the peripheral mononuclear cell profiles (NK, CD19, or CD-AT) were not significantly different (data not shown). Therefore, the peripheral monocyte profile, especially the $CD56^+CD16^+$ NK cell population in the early follicle of RIF patients, may accurately reflect an immune unbalance associated with endometrial receptivity or fetomaternal immune tolerance. The chance of successful implantation in these patients could be strengthened by IVIG treatment.

In the present study, the reduced level of $CD56^+CD16^+$ NK cells was accompanied by an elevated percentage of CD4, CD-AT, and CD19 cells. These data suggest that in addition to $CD56^+CD16^+$ NK cells, T cells or B cells may represent an imbalance of innate and adaptive immune system function. The total number of monocytes was significantly

TABLE 3 | Comparison of pregnancy outcomes of IVIG treatment between low and high NK cell percentages.

(Reference range)	Low NK percentage (≤10.6%)		High NK percentage (>10.6%)	
Groups	**Non-IVIG**	**IVIG**	**Non-IVIG**	**IVIG**
Cycles	66	68	102	47
Age (years)	36.1 ± 3.7	34.8 ± 4.4	36.7 ± 4.8	36.3 ± 5.1
Oocyte number	13.7 ± 9.4	14.0 ± 8.6	15.3 ± 9.6	17.2 ± 12.8
MII number	10.6 ± 7.7	11.3 ± 7.2	12.5 ± 8.0	13.8 ± 10.0
Fertilized embryo number	8.6 ± 9.2	9.2 ± 6.0	10.0 ± 6.9	11.0 ± 8.1
High qualified embryo rate	73.3 ± 14.9	70.3 ± 12.6	71.3 ± 13.5	69.7 ± 12.7
Transferred embryos	3.2 ± 0.8	3.6 ± 0.7	3.3 ± 0.8	3.5 ± 0.8
Implantation rate (%)	12.3 (26/212)[a]	27.5 (67/244)[c]	24.9 (85/341)	25.0 (41/164)
Pregnancy rate (%)	30.3 (20/66)[b]	57.4 (39/68)[d]	48.0 (49/102)	61.7 (29/47)
Live birth rate (%)	22.7 (15/66)	45.6 (31/68)[e]	35.3 (36/102)	40.4 (19/47)
Abortion rate (%)	25.0 (5/20)	17.9 (7/39)	24.5 (12/49)	34.5 (10/29)
Fetal body weight (gm)	2,939 ± 611	2,480 ± 678	2,625 ± 613	2,504 ± 527
Gestational age of delivery (weeks)	37.6 ± 2.8	36.9 ± 2.7	36.5 ± 2.8	35.6 ± 2.0

The cutoff value of NK cell percentage (10.6 %) is selected by receiver operating characteristics curve analysis.
The data are presented with mean± standard deviation (SD) or percentage (%).
[a]$P = 0.0003$, [b]$P = 0.023$ compared with Non-IVIG group combined with high NK percentage (>10.6%) by X^2 test.
[c]$P < 0.001$, [d]$P = 0.002$, [e]$P = 0.005$ compared with Non-IVIG group with low NK percentage (≤10.6%) by X^2 test.

lower in the follicular phase when compared with luteal phase (22, 43) and the changes may be connected to the immunobiology of implantation (22). Furthermore, the effect of different T regulatory cells (subsets of $CD4^+$ T cells) (44) or CD-AT cells (45) correlated with ART outcome and implantation failure.

The strength of this study is the recruitment of a population of RIF patients receiving IVIG treatment to confirm the logistic regression analysis results from non-IVIG group and the benefit of IVIG treatment. The effect of IVIG infusion is prominent in patients with a low percentage of $CD56^+CD16^+$ NK cells when compared with patients who have a high NK percentage. NK cells are immune cells that can be distinguished by CD56 and CD16 surface antigen expression. In this study, only the $CD56^+CD16^+$ NK cells in the PBMC profile were detected because they represent the majority of all NK cells in the blood (46). NK cells are capable of binding to immune-complexed IgG via CD16-Fc gamma RIIIA molecules on the surface (47). NK cells produce cytokines that have pro-inflammatory and immunosuppressive effects [e.g., IFN-γ, tumor necrosis factor-α (TNF-α), and interleukin (IL)-10] and growth factors, such as granulocyte macrophage colony-stimulating factor (GM-CSF) and granulocyte colony-stimulating factor (G-CSF) (48, 49). Stimulation of CD16 on NK cells results in the production of cytokines, such as IFN-γ, TNF-α, and GM-CSF (47). IVIG induces antibody-dependent cellular cytotoxicity (ADCC) of mature dendritic cells (DCs) by NK cells, which downsizes the antigen-presenting pool and inhibits T-cell priming. By influencing the interaction between DCs and NK cells, IVIG modulates the ability of the innate immune system to trigger T-cell activation. This represents a mechanism that can "cool down" the immune system during times of activation (50). Several recent observations have emphasized the effects of IVIG therapy on a variety of cells from the innate and adaptive aspects of the immune system, including $CD56^+CD16^+$ NK cells, and various subsets of T cells and B cells (51). The results of the present study are in agreement with previous reports that show an association between unexplained RIF and immune imbalance in peripheral blood mononuclear cell profiles and the ability of IVIG to act as an immune modulator to enhance embryo implantation.

The limitation of this study was the lack of PBMC profiles from a fertile control group to compare with RIF groups. Further studies are needed to determine the NK profile after IVIG treatment. To confirm the importance of peripheral NK cells and monocytes in implantation, we have designed future studies to assess the PBMC population and cytokine expression in the early follicular phase without stimulation and the luteal phase after IVIG treatment in RIF patients.

In conclusion, we are the first to report that the peripheral $CD56^+CD16^+$ NK cell population in the early follicular phase is associated with IVIG outcomes in RIF patients. For RIF patients with a $CD56^+CD16^+$ NK cell population ≤10.6%, the implantation potential from IVF cycles is significantly lower than patients with NK cells >10.6%. In addition, IVIG treatment may be beneficial for these patients.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institute of Review Board of the Chung Shan Medical University Hospital. The patients/participants

provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Y-KH and H-HC were involved in conception and design of the study, performing experiments, acquisition of data, analysis and interpretation of data, and drafting of the article. T-HL and M-SL were involved in the analysis and interpretation of data, critical revision of the article, and final approval of the article. C-CH, C-IL, and P-YL was involved in the analysis and interpretation of data.

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REVIEW
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Endometriosis-Associated Macrophages: Origin, Phenotype, and Function

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Endometriosis is a complex, heterogeneous, chronic inflammatory condition impacting ∼176 million women worldwide. It is associated with chronic pelvic pain, infertility, and fatigue, and has a substantial impact on health-related quality of life. Endometriosis is defined by the growth of endometrial-like tissue outside the uterus, typically on the lining of the pelvic cavity and ovaries (known as "lesions"). Macrophages are complex cells at the center of this enigmatic condition; they are critical for the growth, development, vascularization, and innervation of lesions as well as generation of pain symptoms. In health, tissue-resident macrophages are seeded during early embryonic life are vital for development and homeostasis of tissues. In the adult, under inflammatory challenge, monocytes are recruited from the blood and differentiate into macrophages in tissues where they fulfill functions, such as fighting infection and repairing wounds. The interplay between tissue-resident and recruited macrophages is now at the forefront of macrophage research due to their differential roles in inflammatory disorders. In some cancers, tumor-associated macrophages (TAMs) are comprised of tissue-resident macrophages and recruited inflammatory monocytes that differentiate into macrophages within the tumor. These macrophages of different origins play differential roles in disease progression. Herein, we review the complexities of macrophage dynamics in health and disease and explore the paradigm that under disease-modified conditions, macrophages that normally maintain homeostasis become modified such that they promote disease. We also interrogate the evidence to support the existence of multiple phenotypic populations and origins of macrophages in endometriosis and how this could be exploited for therapy.

Keywords: endometriosis, macrophage, monocyte, origin, phenotype

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BACKGROUND

Endometriosis is defined by the presence of endometrial-like tissue outside the uterus ("lesions"), typically on the lining of the pelvic cavity (peritoneum) or on the ovaries. Endometriosis is a heterogeneous disease, and lesions can be categorized into three sub-types: superficial peritoneal, deep (infiltrating), and ovarian ("endometriomas"), where more than one sub-type can exist in the same patient and superficial peritoneal endometriosis is the most common form of disease (1, 2). It is associated with debilitating chronic pelvic pain, infertility, and fatigue. It is estimated to affect 6–10% of women of reproductive age (3), up to 50% of infertile women (4) and is prevalent in

71–97% of women with chronic pelvic pain (5). Endometriosis-associated symptoms can negatively impact mental, physical and social well-being and quality of life (6). Poor pregnancy outcomes are also associated with the disease, including preterm labor, pre-eclampsia, ectopic pregnancy, miscarriage, and intrauterine growth restriction (7). Endometriosis has a significant socioeconomic impact, costing the UK an estimated £8.5 billion pounds each year, with societal cost being mostly attributed to loss of productivity (8, 9). Diagnosis from onset of symptoms can take an average of 7–8 years. Generally, a diagnosis of endometriosis is achieved by laparoscopic evaluation of the pelvis, however imaging techniques such as transvaginal sonography and magnetic resonance imaging may be utilized to diagnose deep lesions and endometriomas (10–12).

Endometriosis lesions are characterized by the presence of ectopic endometrial-like tissue containing glands and stroma, however recent re-evaluation of disease definition suggests that fibrosis and smooth muscle cells are more consistent features of lesions (13). Endometriosis is classified as an estrogen-dependent *chronic inflammatory condition*: symptoms are modulated by ovarian hormones and lesions generate intense inflammation within the pelvic cavity. Lesions also become vascularized and are infiltrated by sensory nerve fibers (**Figure 1**). The ectopic endometrial cells and local inflammatory environment activate nerve fibers in lesions, establishing a dialogue with the central nervous system and generating pain in the condition. Lesions behave like the eutopic endometrium and exhibit cyclical bleeding into the pelvic cavity in response to ovarian hormones, and this acts to potentiate inflammation (14). Disease classification (rAFS/rASRM) is currently based on lesion size, location, extent of lesion infiltration into tissue and the presence of adhesions. Classification ranges from stage I ("minimal") to stage IV ("severe") (15).

Current treatments for endometriosis aim to alleviate endometriosis-associated pain and/or to treat infertility associated with the disease and include surgical and medical management (2, 3). Ovarian suppression limits activity and growth of lesions, leading to reduced pain symptoms. Common methods of ovarian suppression include oral contraceptives and gonadotrophin-releasing hormone (GnRH) agonists (16) with add-back HRT. Whilst ovarian suppression may alleviate pain symptoms, treatment is also contraceptive and therefore inappropriate for women aiming to conceive. Additionally, GnRH agonists are associated with side effects such as memory loss, insomnia, and hot flushes in a recent study of endometriosis patients with long term use (17). Treatments can also include non-steroidal anti-inflammatory drugs such as ibuprofen, however long-term pain management for women with endometriosis often encompasses a combination of treatments. As well as medical therapy, laparoscopic surgery to remove lesions can provide symptom relief in some patients, however up to 50% of women experience a relapse of symptoms within 2 years after surgery (11). Current treatment options lack significant clinically proven benefit and aim at alleviating symptoms, rather than treating disease (18). Consequently, there is a compelling clinical need for new non-hormonal treatments that have fewer side effects and effectively treat endometriosis over a life course, without the need for repeated surgeries or suppression of fertility.

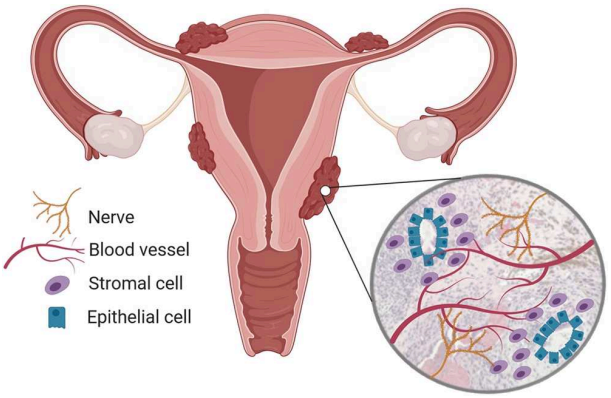

FIGURE 1 | Endometriosis is a chronic inflammatory condition. Endometriosis is characterized by the presence of endometrial-like tissue found outside the uterus, most commonly in the peritoneal cavity. Endometriosis lesions are heterogenous but usually contain endometrial stromal cells and epithelial glands, immune cell infiltrates and are vascularized and innervated by nerves. Created using Biorender.com.

ETIOLOGY AND NATURAL HISTORY

It is widely accepted that endometriosis is a multifactorial disease and the pathophysiology of endometriosis can certainly be associated with a number of elements that clearly contribute to disease. Evidence suggests that endometriosis has a heritable component due to high familial incidence of the disease (19–22). A meta-analysis of eight genome-wide association studies (GWAS) elucidated six loci associated with endometriosis (23). Genes implicated in disease included those involved in the regulation of epithelial cells and hormone metabolism, specifically genes involved in regulating hormone responses in tissues (24, 25). These GWAS results are not surprising since the symptoms of endometriosis are modulated by ovarian sex steroids; early age at menarche is a risk factor for development of endometriosis, suggesting increased exposure to estrogen may incur increased risk of disease (26). Endometriosis lesions aberrantly express a number of steroidogenic enzymes including aromatase and 17β-hydroxysteriod dehydrogenase (17β-HSD), this results in increased synthesis and decreased metabolism of estrogen (27–29) such that local levels remain high. Estrogen signaling modulates a large number of down-stream disease processes within endometriosis lesions, which are reviewed in Yilmaz and Bulun (30), Liang et al. (31), and Rizner (32). Immune cell dysfunction is also intrinsically linked to the pathophysiology of endometriosis. Alterations in immune cell populations have been observed in the peritoneal fluid of women with endometriosis; specifically, women with endometriosis have more peritoneal macrophages (33), neutrophils and dendritic cells (34). Function is also perturbed: NK cells have reduced cytotoxicity (35, 36), and disease severity is positively correlated

with NK cell killing capacity (37). Peritoneal macrophages also exhibit impaired phagocytosis (38). Macrophages are the most abundant immune cells present within endometriosis lesions and are evidently central to the pathophysiology of endometriosis. Whilst studies have highlighted clear functional roles for macrophages in the disorder, little is known regarding the origins and phenotypic heterogeneity of macrophages in endometriosis.

Our understanding of endometriosis etiology remains limited. It is being increasingly recognized that different sub-types of endometriosis may arise from different origins, however evidence for this is still limited (39, 40). A number of theories are discussed below and we speculate on how the origin and role of macrophages may differ in each scenario:

The most widely accepted theory was postulated in 1927 by John Sampson, who observed that during menstruation, endometrial tissue can reflux back up the fallopian tubes and into the pelvic cavity, a physiological process known as *"retrograde menstruation*." Although this process occurs in ∼90% of women, only in some does refluxed endometrial tissue form endometriosis lesions (41) and the mechanisms underpinning the attachment of endometrial tissue and lesion development remain elusive. It could be predicted, and mouse studies have demonstrated that macrophages originating from the endometrium contribute to peritoneal endometriosis lesions (42). These endometrial macrophages could be pivotal in the establishment of lesions since it has previously been demonstrated that macrophages trafficking to the endometrium are most abundant during repair following endometrial breakdown and shedding with a presumed role in repairing the denuded functional layer of the endometrium (43). However, evidence supporting this hypothesis is still absent. Another theory based on the dissemination of cells from the uterus into the peritoneal cavity suggests that neonatal retrograde reflux of endometrial stem/progenitor cells could be responsible for development of lesions. Visible vaginal bleeding is observed in 3–5% of female neonates, whereas occult bleeding may occur at a frequency of between 25 and 60% (44). Bleeding in the immediate postnatal period is similar to menstrual bleeding as it occurs in response to hormone withdrawal from *in utero* progesterone exposure. This theory suggests that stem/progenitor cells could implant into the peritoneal wall where they may remain dormant until adolescence, when elevated estrogen levels may then promote the proliferation and growth of seeded endometrial cells. Whilst, this theory represents a plausible mechanism of lesion formation, current evidence is lacking and proof that endometrial stem/progenitor cells are present in the peritoneal tissue of pre-pubescent girls is absent. The *coelomic metaplasia* theory suggests that endometriosis lesions arise as the result of metaplastic differentiation of the coelomic epithelium into endometrial cells and is supported by evidence suggesting endometriosis lesions can be found in women without a uterus (45). The formation of endometriosis lesions at sites distant from the peritoneal cavity (46, 47), as well as identification in men on rare occasions (48) supports the theory. Upon development of lesions at the onset on adolescence (neonatal stem cell theory) or following metaplasia it would be expected that monocytes are recruited to the site of the lesion and/or that peritoneal macrophages may traffic into the developing lesion and activate repair processes that facilitate establishment of new endometrial-like explants. Notably, stem cells and macrophages are known to have a reciprocal relationship whereby stem cells can contribute to macrophage activation and phenotype during regenerative processes and macrophages can dictate accumulation of progenitor/stem cell-like cells (49). In endometriosis, mesenchymal stem-like cells promote macrophages to adopt a pro-repair phenotype (50) but further studies regarding the relationship between stem cells and macrophages in endometriosis are currently limited. *Müllerianosis* (müllerian rests; normal endometrial, endosalpingeal, and endocervical tissue) predicts that developmentally displaced tissue are incorporated into normal organs during organogenesis (51). Occurrence of deep infiltrating endometriosis particularly lends itself to this theory, where endometrial tissue is found deep within the organ structure. Speculation may infer a role for tissue-resident macrophages in lesions resulting from developmentally displaced endometrial-like tissue. Upon activation of a "dormant" lesion laid down during organogenesis the tissue-resident macrophages may change phenotype and proliferate such that they promote inflammation, growth, and invasion of the lesion. Inflammation arising upon activation of a dormant lesion may also lead to the recruitment of monocytes that differentiate into macrophages such that endometriosis lesion-resident macrophages are constituted by tissue-resident and monocyte-derived macrophages similar to what occurs in tumors (52). Any differences existing in macrophage origin, phenotype and function across the different subtypes of endometriosis lesions remain unknown.

THE MACROPHAGE: A COMPLEX CELL AT THE CENTER OF AN ENIGMATIC CONDITION

Inflammation and immune cell dysfunction are central to the pathophysiology of endometriosis. Whilst, a number of leukocytes exhibit altered numbers and function in endometriosis, it is evident that macrophages play an unrivaled role in disease pathogenesis. We and others have demonstrated that macrophages are critical for licensing lesion growth, promoting vascularization and innervation as well as contributing to pain in the disorder (53–55). Lessons from diverse tissues also place macrophages at the center of disease states such as liver injury (56), multiple sclerosis (57), and cancer (52). Tissue context ultimately dictates the role that macrophages play in disease but a recurring theme indicates that the ontogeny of the macrophages in diseased tissues determines how they respond and contribute to pathogenesis. Below, we review the available literature on macrophage ontogeny, phenotype and function in health and then focus on their role during inflammation and disease states. Ultimately, we discuss the role that macrophages play in endometriosis in light of what can be learnt from other disease states.

Macrophages Have Different Origins and Diverse Phenotypes

Macrophage Ontogeny

Macrophages are mononuclear phagocytes that play critical roles in immunity (phagocytizing pathogens, apoptotic cells and debris, antigen presentation, and modulation of other leukocyte populations). They are present in all tissues of the body (58, 59) and play diverse tissue specific roles in maintaining homeostasis. Much of our knowledge regarding macrophage ontogeny is derived from studies conducted in mice. Macrophages are derived from three key populations; the yolk sac of the embryo, the fetal liver and postnatally, hematopoiesis in the bone marrow (**Figure 2**). The earliest macrophages arise from erythro-myeloid progenitors (EMPs) produced during primitive hematopoiesis in the extra-embryonic yolk sac at embryonic day (E) 7.5 and 8.25. After blood circulation is established, EMP derived macrophages seed fetal tissue. Excluding microglia, these macrophages are partially or fully replaced by monocytes originating from the fetal liver, which differentiate into macrophages in tissues. Fetal liver monocyte-derived macrophages can persist into adulthood and form the tissue resident macrophage population, undergoing self-renewal, for example in the peritoneum, spleen, lung, skin, and liver. In other organs, tissue macrophages derived from fetal liver monocytes are gradually replaced by recruited monocytes from the bone marrow. This process occurs in tissues such as the gut and dermis (60–64).

In humans, peripheral blood monocytes form two main populations; $CD14^{hi}$ $CD16^{lo}$ and $CD14^{lo}$ $CD16^{hi}$, although an intermediate population can be identified. The $CD14^{hi}$ $CD16^{lo}$ (classical) subset is the most abundant in the blood. Under inflammatory conditions, classical monocytes extravasate into tissues, differentiate into macrophages or dendritic cells (65) and fulfill functions such as clearance of apoptotic bodies, stimulating angiogenesis and restoring integrity of tissues (66). $CD14^{lo}$ $CD16^{hi}$ ("non-classical") monocytes also exhibit extravasation into tissues during inflammation, but they infiltrate tissues later in the inflammatory process and exhibit a bias toward differentiating into "wound-healing" macrophages (67). A key role of the non-classical monocyte population is to patrol the blood vessels along the endothelial cell layer, providing immunosurveillance of vasculature and the surrounding tissues (68). Classical monocytes also patrol tissues and play a homeostatic role in steady state conditions, without differentiating into macrophages (69). Classical and non-classical monocytes in humans are analogous to $Ly6C^{hi}$ classical and $Ly6C^{lo}$ non-classical monocytes in mice and exhibit significant homology at transcriptional analysis (70, 71). In mice, classical monocytes can be classified as $Ly6C^{hi}$ $CX3CR1^{lo}$ $CD43^{lo}CCR2^{hi}$, and non-classical monocytes as $Ly6C^{lo}$ $CX3CR1^{hi}$ $CD43^{hi}CCR2^{lo}$, with all monocyte populations being $CD11b^{hi}$ $F4/80^{int}$ (65).

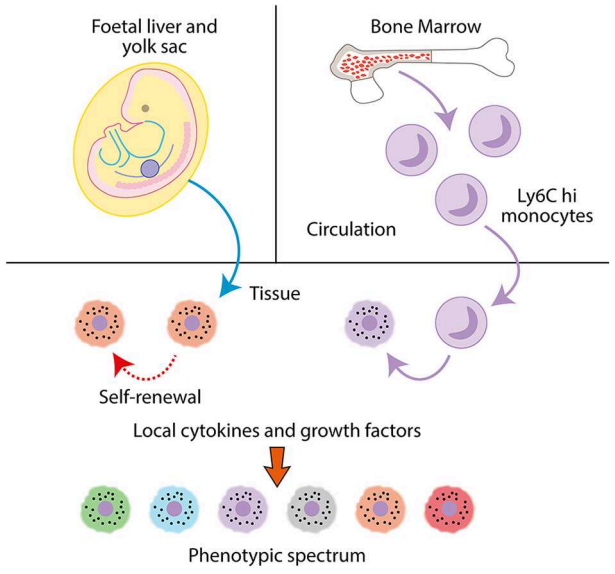

FIGURE 2 | Macrophages are mononuclear phagocytes. Tissue macrophages are seeded during fetal life from the fetal liver and yolk sack and undergo self-renewal. In adults, monocyte precursors extravasate from the bone marrow into the circulation, where they can then infiltrate into tissues and differentiate into macrophages. In tissues, macrophages modulate their phenotype dependent on local cytokines and growth factors to specific tissue or disease-associated phenotypes.

Macrophage Phenotype

Macrophages respond to their local microenvironment and change both their transcriptome and phenotype in response to local signals (72). Historically, macrophages have been divided into either "M1" classically or "M2" alternatively activated cells. Classically activated macrophages are associated with inflammation, and express pro-inflammatory markers. Alternatively activated macrophages are associated with homeostasis, wound healing and immunomodulation (73). These extreme polarization states only really exist *in vitro*, where studies commonly use stimulation with granulocyte-macrophage colony-stimulating factor (GM-CSF) and the cytokine IFN-γ (Interferon γ) to generate M1 macrophages, and stimulation with macrophage colony-stimulating factor (M-CSF) and the cytokines interleukin-4 (IL-4) and interleukin-10 (IL-10) to generate M2 activated macrophages (74). Whilst, this classification system is a useful tool for investigating macrophages at extremes of activation, it is now appreciated that *in vivo* macrophages exhibit a broad spectrum of phenotypes that are tissue and disease specific, and the M1/M2 system cannot represent the diverse nature and complexities of macrophage phenotype (75, 76). Transcriptional analysis of mouse macrophage populations from different tissues demonstrates minimal overlap in mRNA expression, reflecting a divergence in gene expression patterns (77). This heterogeneity reflects the ability of macrophages to modulate their gene expression in response to local tissue signals, becoming specialized to their tissue niche, be that in a healthy or diseased state. In disease, macrophages may modulate their phenotype dependent on disease stage or severity, and the mechanisms behind this are crucial for understanding their exact role in pathogenesis. Thus, defining macrophage phenotypes in disease states, with the potential of modulating macrophage phenotype

or specifically targeting disease specific macrophages for clinical benefit is a key focus for research (72, 78–80).

Endometrial Macrophages

The endometrium is a unique and highly dynamic tissue that undergoes cyclic proliferation, differentiation, shedding (menstruation), and repair in response to ovarian-derived estrogen and progesterone during the menstrual cycle. In the normal cycling endometrium, an influx of macrophages occurs during the secretory and menstrual phases, along with a concomitant increase in macrophage-derived cytokines and proteases (81). Evidence from a mouse model of endometrial breakdown and repair identified an influx of classical monocytes which differentiated into macrophages in the endometrium during the repair phase of the menstrual cycle (43). Monocyte extravasation from blood vessels into the endometrium is regulated by CCL2 (82, 83) and CX3C chemokine receptor 1 (CX3CR1) (84). The influx of macrophages into the endometrium is in line with the numerous roles they are presumed to play in modulating endometrial differentiation, breakdown, and repair. During the proliferative phase, macrophages have been postulated to play a role in regeneration and proliferation of the functional layer of the endometrium and express activation and adhesion markers CD54, CD69, and CD71 (85). Macrophages are also implicated in regulating gland remodeling (86) and angiogenesis during the secretory phase via production of vascular endothelial growth factor (VEGF) (87). At menstruation macrophages play a role in initiation of endometrial shedding by secreting matrix metalloproteinases (MMPs) (88). Specifically, secretion of MMP-12, MMP-9, and MMP-14 are required for the breakdown of the functional layer of the endometrium during menstruation (89–91).

In response to estrogen, macrophages increase their proliferative capacity and undergo activation to adopt a phenotype which represents a more "wound healing-like" population (92). Thus, estrogen signaling can accelerate the wound healing process and this is in part regulated by increasing the production of macrophage-derived proteases, MMPs, fibroblast growth factor, VEGF and cytokines such as resistin like alpha (RELMα) (92–94). Endometrial macrophages do not express the progesterone receptor (95), however macrophage gene expression is significantly altered in response to progesterone (96) suggesting an indirect method of regulation. Interestingly, exposure to cortisol was demonstrated to increase expression of angiogenic genes such as CXCL2, CXCL8, and VEGFC in macrophages *in vitro*, suggesting that local cortisol levels could be important for regulating angiogenesis within the remodeling endometrium (97). Taken together this evidence indicates that macrophages are key players in augmenting dynamic remodeling and repair in the endometrium and this is regulated by exposure to local cytokines, growth factors and hormones that modulate their phenotype, function, and recruitment throughout the menstrual cycle. However, compared to other tissue macrophages, the phenotype and function of endometrial macrophages and the mechanisms governing their recruitment and activation are significantly less well-characterized.

Peritoneal Macrophages

Mouse

Peritoneal cavity macrophages are one of the most studied macrophage populations in mice, largely due to their ease of isolation. Two subsets of peritoneal macrophages are recognized in mice based on differential expression of F4/80 and MHC II. The tissue resident, so called "large" (due to their larger size) peritoneal macrophages (LpM) are F4/80^{hi}, MHC II^{lo}, and the monocyte-derived "small" peritoneal macrophages (SpM) are F4/80^{lo} MHC II^{hi} (98). LpM are the most abundant macrophage population in the peritoneal cavity at steady state and form the tissue resident population, they are phagocytic and perform immunosurveillance and homeostatic roles in the peritoneal cavity (98) as well as mediating recruitment and maintenance of B1 B cells. They are also linked to regulation of intestinal immunity (99). LpM self-renew and the proliferative capacity of LpM is regulated by GATA-binding factor 6 (Gata6), a transcription factor uniquely expressed by LpM in the peritoneal cavity, which also regulates macrophage phenotype (100). In mice, the LpM population consists primarily of embryonic-derived cells, however monocyte-derived macrophages do replace embryonic-derived LpM over time, a process that is highly sex and age dependent, and slower in females. Over time, Ly6C^{hi} monocytes enter the peritoneal cavity in a CCR2-dependent manner and differentiate transiently into SpM, prior to transitioning into tissue resident LpM (101). Thus, the LpM constitute both embryonic and monocyte-derived cells and the two populations have been shown to be transcriptionally distinct from each other (101). SpM are implicated in the inflammatory response in the peritoneal cavity, however their role in the steady state peritoneal cavity remains unclear (102).

Human

In humans, macrophages constitute 50% of peritoneal cavity leukocytes (103). Tissue resident peritoneal macrophages have been defined by high expression of complement receptor of the immunoglobulin superfamily (CRIg) and low expression of CCR2. These cells are highly phagocytic and more numerous in steady state, also displaying similar transcriptional profiles to the mouse LpM population (104). Human monocyte-derived macrophages in the peritoneal cavity, analogous to F4/80^{lo} MHC II^{hi} SpM in the mouse, have been defined as CRIg^{lo}, CCR2^{hi}. This CRIg^{lo}, CCR2^{hi} population in humans has a reduced phagocytic capacity and is lower in number compared to CRIg^{hi} CCR2^{lo} tissue macrophages, consistent with characteristics of SpM. It must be noted however that in humans, Gata6 was found to be more highly up-regulated in the pro-inflammatory CRIg^{lo} CCR2^{hi} population (104), highlighting that key differences between human and mouse peritoneal macrophages exist, and further research is critically required to clarify these differences.

Peritoneal Macrophage Dynamics During Inflammation

Under inflammatory conditions, LpM respond to stimuli in a phenomenon known as the macrophage disappearance reaction (MDR) (105): in mice the LpM compartment undergoes a dramatic reduction in numbers largely by migration to the

omentum, mediated by retinoic acid and Gata6 (106). The degree of loss in the LpM population is highly dependent on the dose of inflammatory stimuli and has been studied in a number of inflammatory models, such as lipopolysaccharide (LPS), zymosan or thioglycollate induced peritonitis (107–109). LpM that persist during inflammation have been hypothesized to play a regulatory role in the peritoneal cavity by secretion of IL-10, an anti-inflammatory cytokine which has also been shown to regulate inflammatory SpM number (109). LpM also play a key role in clearance of apoptotic cells during inflammation (108), and exhibit high expression of T-cell immunoglobulin and mucin domain containing 4 (Tim4) which recognizes phosphatidyl-serine on apoptotic cell bodies (110). Upon resolution of inflammation, the depleted LpM population increases its proliferative capacity through a colony stimulating factor 1 receptor (Csf-1r) mediated mechanism to restore LpM number (107). Interestingly, LpM have been shown to infiltrate the liver by a non-vascular route in response to the damage-associated molecular pattern molecule (DAMP) ATP, where they play a key role in regeneration and tissue repair in the liver after sterile injury, modulating their phenotype in response to local tissue microenvironmental cues (111). This migration implies that LpM have the ability to execute wound repair and tissue regeneration in visceral organs. Furthermore, with a reduction of LpM numbers a concurrent increase in SpM and inflammatory Ly6C^{hi} monocytes is observed in a number of mouse models of peritoneal inflammation (105). SpM exhibit a pro-inflammatory response when challenged with LPS *in vitro*, producing high levels of chemokine (C-C motif) ligand 5 (Ccl5), chemokine (C-C motif) ligand 3 (Ccl3), and tumor necrosis factor-α (Tnf-α), as opposed to LpM which produce G-CSF and GM-CSF under LPS stimuli (102). In an *in vivo* model of peritonitis, SpM also produce high amounts of pro-inflammatory cytokines including Tnf-α, interleukin-1β (Il-1β), and Ifn-γ (112), and are critical for clearance of infection in the peritoneal cavity after bacterial challenge in the mouse (113). The ability of SpM to respond to inflammatory stimuli by producing pro-inflammatory cytokines enables rapid response to immunological challenge in the peritoneal cavity. At resolution of inflammation, SpM have been shown to undergo apoptosis (108) but can also migrate to local draining lymph nodes (114). However, SpM have also been shown to persist in the cavity and can eventually differentiate into F4/80^{hi} MHC II^{lo} cells (115), suggesting that inflammation has the potential to alter the complement of peritoneal cavity macrophage populations, even after homeostasis has been restored. The multiple fates of SpM reflect the heterogeneity in this cell compartment, but the roles of SpM sub-populations in inflammation are still largely undefined. In summary, under steady-state/homeostatic conditions LpM exhibit an immune-surveillance and immune-regulatory role and act to remove apoptotic and senescent cells. The roles of SpM are less well-defined but the markers they express suggests roles in antigen presentation and T cell activation. Inflammatory challenge with thioglycolate, zymosan or LPS (lipopolysaccharide) causes loss of LpM and expansion of SpM via monocyte recruitment and differentiation. New SpM are pro-inflammatory, expressing high levels of Tnfα, Il-1β, and Ifnγ and are better able to engulf microbes compared to homeostatic SpM. Of note, type-2 inflammation characterized by elevated levels of IL-4 does not induce MDR and instead F4/80^{hi} LpM accumulate in the peritoneal cavity and exhibit a pro-repair phenotype (116). Thus, it seems that under different inflammatory challenge LpM are biased to exhibit a pro-repair phenotype whilst SpM adopt a pro-inflammatory phenotype. Mechanistic studies on peritoneal macrophages in humans are challenging and therefore knowledge of this physiological process in humans is minimal.

Macrophages Can Promote Disease

The unique and diverse roles that macrophages play in the maintenance of healthy tissues is mirrored by their pivotal roles in development, maintenance, and progression of a number of diseases (72). Peritoneal cavity macrophage perturbations and functional dysregulation are linked to a number of adverse clinical outcomes. For example, an increase in peritoneal macrophages was associated with negative outcomes in patients with peritonitis (109), and dysregulation of peritoneal macrophages has been linked to acute pancreatitis, where peritoneal macrophages produce increased levels of pro-inflammatory cytokines that exacerbate disease (115). Conversely, macrophages have been shown to be protective against the formation of adhesions, a common complication after abdominal surgery (117) and indicating that macrophage dysfunction could contribute to adhesion formation. Thus, macrophages are intrinsically linked to disease in the peritoneal cavity in humans.

Although endometriosis is a benign condition a number of parallels can be drawn between the condition and cancer (118). Macrophages are unambiguously at the center of the pathophysiology of both diseases. Macrophage infiltration in tumors is a predictor of poor clinical outcomes in malignancy (119, 120), attributed to the fact that macrophages promote initiation, progression, and metastasis in most cancers (75). In the last decade, a major focus has been to define the populations that constitute tumor-associated macrophages (TAMs). In a mouse model of breast cancer, Ly6C^{hi} inflammatory monocytes are recruited to metastatic sites via a CCR2/CCL2 mediated mechanism to form TAMs. Inhibition of CCL2/CCR2 signaling with an anti-CCL2 antibody inhibited monocyte recruitment thereby inhibiting metastasis and prolonging survival of the mice (121). Similarly, mouse models of Lewis lung carcinoma demonstrated that TAMs were derived from CCR2 driven recruitment of Ly6C^{hi} monocytes and blockage of CCL2 decreased tumor growth (122, 123). Furthermore, tissue resident macrophages have also been implicated in cancer pathophysiology and can contribute to the TAM population. For example, in a mouse model of pancreatic ductal adenocarcinoma, Zhu et al. demonstrated using a parabiosis model that TAMs were derived from both embryonically derived tissue resident macrophages as well as from circulating Ly6C^{hi} monocytes. During tumor development embryonically derived macrophages expanded via *in situ* proliferation and had a pro-fibrotic role in tumors. Using a Csf-1r neutralizing antibody and clodronate liposome treatment to deplete tissue resident macrophages a

reduction in tumor size and increased survival of mice was observed. Monocyte-derived macrophages however played a key role in antigen presentation. Use of CCR2 knockout mice or a CCR2 inhibitor to prevent recruitment of Ly6C^{hi} monocytes did not affect tumor growth (52). This study highlights the importance of defining the ontogeny of TAMs in order to decipher which populations are fundamentally required for tumor growth, with the aim of improving clinical outcomes.

Whilst TAMs may have multiple origins, it has been demonstrated that the tumor microenvironment can modulate macrophage phenotype to promote malignancy, indicating that origin does not wholly define function when macrophages are exposed to cytokines and growth factors locally in the tumor. A number of different macrophage populations within tumors have been described which play differential roles and have different phenotypes. For example, populations of invasive, perivascular, metastasis associated, angiogenic (Tie2^{+}), and immunosuppressive macrophages which secrete high levels of IL-10 have been described (75). Detailed profiling of hepatocellular carcinoma biopsies demonstrated the presence of various macrophage sub-types in tumors that had both pro and anti-tumoral properties (124).

The Role of Macrophages in Endometriosis

Macrophage Ontogeny in Endometriosis

Whilst, a role for macrophages in endometriosis pathophysiology is established (and discussed below), the ontogeny of endometriosis-associated macrophages is still poorly understood. Greaves et al. demonstrated in a syngeneic mouse model of endometriosis that lesion resident macrophages are derived from both the (donor) endometrium and (recipient) infiltrating macrophage populations (42) (**Figure 3**). These infiltrating macrophage populations are likely to constitute peritoneal or recruited monocyte-derived macrophages, however the exact origins of these populations is currently unknown. Although, peritoneal macrophages contribute to inflammation in endometriosis, it remains unknown whether they infiltrate endometriosis lesions and thus the role these cells play within the ectopic tissue is not known. Using bone marrow chimeras Sekiguchi et al. demonstrated that CD11b^{+} cells from the bone marrow infiltrate and accumulate in endometriosis lesions in a mouse model (125). These cells could represent a monocyte/macrophage population, although CD11b^{+} cells could also constitute neutrophils, eosinophils and or certain subsets of dendritic cells (126). Capobianco et al. demonstrated that bone marrow derived Tie2^{+} cells infiltrated endometriosis lesions in a mouse model, again demonstrating that bone marrow derived cells that ultimately express macrophage markers within lesions could be recruited from blood vessels (127).

Macrophage Phenotype and Function in Endometriosis

Endometrial macrophages exhibit differential properties in endometriosis. Reflecting on the theory of retrograde menstruation and studies in mice identifying endometrial macrophages in lesions, the presence of macrophages in refluxed endometrial tissue in women has the potential to augment disease development in the peritoneal cavity. A number of studies have demonstrated perturbations in macrophage populations in the eutopic endometrium of endometriosis patients. Women with endometriosis have more endometrial macrophages that express lower levels of the "wound-healing" marker CD163 compared to women without disease, however the exact mechanisms behind these alterations are unknown (128, 129). Analogous to this, increased levels of CCL2 can be observed in the endometrium of women with disease which corresponds

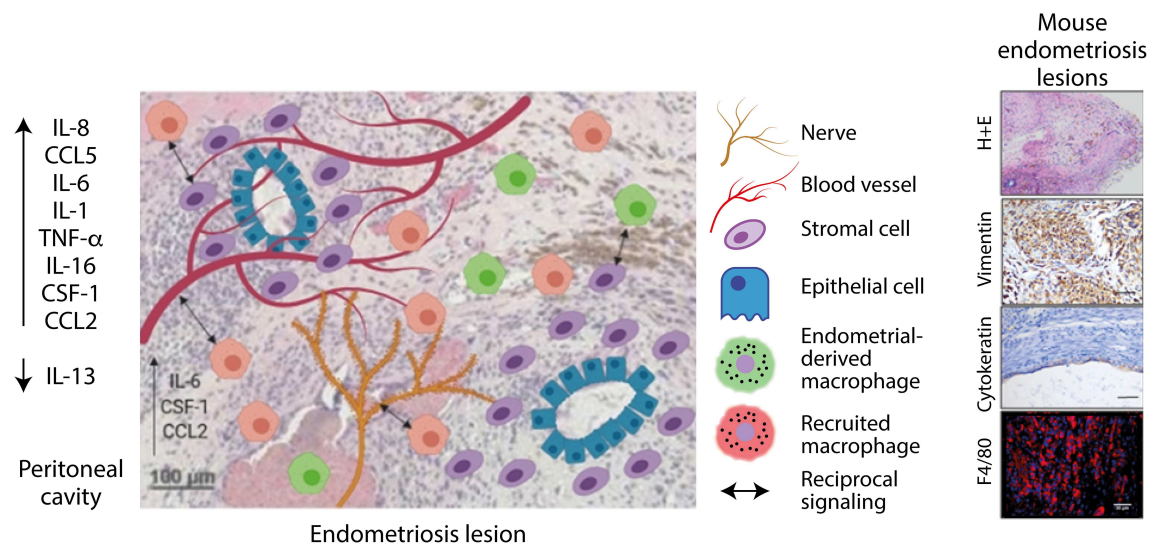

FIGURE 3 | Endometriosis lesions are infiltrated by blood vessels, nerves, and macrophages. Lesion resident macrophages are derived from macrophages originating from the endometrium and recruited macrophages. Macrophages interact with blood vessels and nerves to stimulate their growth. Signaling also occurs between macrophages and stromal cells, which increases their clonal expansion and invasive properties. Created using Biorender.com.

to disease severity, suggesting increased influx of monocytes in disease that can then differentiate into macrophages (130). Increased matrix metalloproteinase-9 (MMP-9) co-localized with $CD68^+$ macrophages in the endometrium of women with endometriosis is indicative of an increase in the number of macrophages implicated in tissue remodeling. This may enhance the ability of ectopic endometrial tissue deposits to implant in the peritoneal cavity (131). Whilst evidence of macrophage perturbations in the eutopic endometrium of women with endometriosis exists, the role of endometrial macrophages in endometriosis has not been defined as functional studies in this area are lacking.

Women with endometriosis evidently have an increased number of peritoneal macrophages that exhibit a dysfunctional phenotype. Peritoneal macrophages collected from women with endometriosis have reduced phagocytic capacity due to low levels and activity of matrix metalloproteinase 9, which is required for extracellular matrix degradation and is regulated by prostaglandin E2 (PGE2) (38). In a co-culture system, in the presence of endometrial stromal cells isolated from ectopic endometrial tissues, monocyte-derived macrophages secreted IL-10 and TGF-β, which in turn suppressed cytotoxicity and viability of NK cells (132), suggesting that macrophages are immunosuppressive in the presence of ectopic endometrial stromal cells and can act to suppress NK cells in the peritoneal cavity. Whilst, a few studies have investigated peritoneal macrophages in women with endometriosis the cells have been evaluated as a global population and there are no studies pertaining to the constitution and function of the individual $CRIg^{hi}$ and $CRIg^{lo}$ populations of these cells: the abundance and behavior of $CRIg^{hi}$ population in women with endometriosis is not known, although inflammation and survival of refluxed endometrial tissue suggests that in endometriosis this tissue resident population could act to create a permissive and mitogenic environment for the formation of lesions. Analogous to this, a study by Beste et al. demonstrated enhanced expression of both pro- and anti-inflammatory cytokines by macrophages collected from the peritoneal fluid of women with endometriosis, this could reflect the mixed population of cells present (133). The increased number of peritoneal macrophages in women with endometriosis suggests that in the condition the "macrophage disappearance reaction" (MDR) does not occur. Indeed, it has been previously demonstrated that in type-2 inflammation characterized by high level of IL-4 the MDR does not happen and peritoneal macrophages accumulate as a result of *in situ* proliferation (116). IL-4 concentrations are elevated in the peritoneal fluid of women with endometriosis (134) suggesting that this could be a mechanism for macrophage accumulation. However, because the abundance of the different populations has not been characterized this hypothesis remains to be proven. Mouse models of endometriosis provide conflicting evidence of peritoneal macrophage dynamics: Yuan et al. demonstrated that in a model that injects syngeneic, estradiol primed, endometrial fragments into intact mice, those with endometriosis exhibited significantly lower numbers of LpM and more abundant SpM compared to control mice, consistent with the MDR. These perturbations in peritoneal macrophage populations were evident from 0.25 to 42 days post tissue injection (135). However, in a model injecting "menses-like" endometrial tissue into ovariectomized recipients supplemented with estradiol valerate, loss of LpM was not observed, and mice with endometriosis had more abundant LpM compared to naïve and sham animals [although the increase was not statistically significant (54)]. The second study seems to more closely recapitulate macrophage dynamics in women with endometriosis, although evidence is very limited. The differences observed in these two studies could be a result of several differences in experimental design including the nature of the donor endometrium injected into the peritoneal cavity as well as manipulations performed on the recipient mice. Yuan et al. also demonstrated that in mice with endometriosis LpM exhibited a "pro-inflammatory" activation state and SpM were more "pro-repair" in nature (135). This interpretation was based on expression of NOS2 (inflammatory) and CD206 (repair) and is contradictory to others studies reporting the pro-inflammatory status of SpM and pro-repair status of LpM in response to different inflammatory stimuli.

Although, it has been demonstrated that monocytes are recruited to lesions from the bone marrow, little evidence exists to characterize their role and dynamics once they infiltrate ectopic tissue. Johan et al. examined infiltrating macrophage phenotype over time in endometriosis lesions in a heterologous mouse model and found that macrophage phenotype was progressively altered over time. Macrophages initially expressed pro-inflammatory markers iNOS and major histocompatibility complex II (MHC II), however at 7 and 14 days post lesion induction a higher proportion of macrophages expressed arginase 1 and CD204 (scavenger receptor A), which are more associated with a tissue remodeling phenotype (136). This study therefore demonstrates that macrophage phenotype in endometriosis lesions is dynamic and progressively changes as lesions develop in the peritoneal cavity. However, as with other studies, the limited number of markers assessed makes it difficult to truly re-capitulate the complex phenotype of macrophages in the tissue.

Endometriosis lesions from women are highly infiltrated by CD68+ macrophages that are present within the stroma of the tissue and can also be found in close proximity to glands (42, 53). Studies in women have strongly implied a role for macrophages in endometriosis, but the mechanistic studies performed in experimental models have significantly improved our understanding of the role of macrophages in the condition. Studies to date have largely focused on defining the role of macrophages in syngeneic mouse models using various cell depletion approaches. A commonly utilized depletion method uses liposomes encapsulating bisphosphonates. These liposomes are taken up by phagocytic cells, which degrade the liposomes, releasing bisphosphonate and causing subsequent cell death. This method therefore selectively depletes phagocytic cells and is non-toxic to non-phagocytic cells, and has been commonly used to deplete phagocytic macrophage populations (137). In a syngeneic mouse model of disease, Bacci et al. used clodronate liposomes and a monoclonal anti-F4/80 antibody to deplete/inhibit peritoneal macrophage function in mice with induced endometriosis and demonstrated that both treatments

caused a reduction in growth and blood vessel formation in lesions (53). Adoptive transfer of *in vitro* generated "pro-inflammatory" (stimulated with IFN-γ), "anti-inflammatory" (stimulated with macrophage-colony-stimulating factor and IL-10), or "non-polarized" (stimulated with macrophage-colony-stimulating factor) macrophages lead to differential effects on lesion development. "Non-polarized" macrophages had no effect on lesion number or weight, however adoptive transfer of pro-inflammatory macrophages reduced lesion weight. Conversely, adoptive transfer of anti-inflammatory macrophages caused an increase in lesion weight. The authors noted that lesion architecture was also disrupted in mice which had received adoptive transfer of pro-inflammatory macrophages (53). Together, this data suggests that anti-inflammatory/pro-repair macrophages may be important for the growth and development of lesions and pro-inflammatory macrophages have an antagonistic effect, clearing ectopic endometrial tissue and disrupting lesion architecture. Whilst this data provides an important insight into the roles of macrophage phenotypes in endometriosis, the use of the M1/M2 paradigm is limited and the exact phenotype and phenotypic heterogeneity of macrophages in endometriosis and their role in disease is currently unknown. Capobianco et al. identified Tie-2 expressing macrophages that infiltrated mouse and human lesions. Depletion of Tie-2^+ macrophages was achieved using a bone marrow chimera from mice expressing a suicide gene (herpes simplex virus type 1 thymidine kinase) expressed under control of the Tie2 promoter into wild-type mice. After treatment with ganciclovir (an anti-viral drug), bone-marrow derived $Tie2^+$ cells were selectively depleted and growth of endometriosis lesions was inhibited, with loss of neovascularization and glandular organization in the resultant lesions (127). Sekiguchi et al. demonstrated that VEGFR1 knockout mice had smaller and less vascularized lesions than WT in a mouse model where sections of uterus were sutured onto the peritoneal wall. Using bone marrow chimeras they demonstrated that $VEGFR1^+$ cells in lesions were bone marrow derived $CD11b^+$ macrophages (125). WT endometriosis mice were also treated with clophosome N which depleted phagocytic cells in the peritoneal cavity at the time of endometriosis induction, and demonstrated that growth and angiogenesis in lesions was reduced (125). A similar study using liposomal bisphosphonate to deplete phagocytic peritoneal populations also demonstrated reduced growth of endometriosis lesions in a rat model (138). Thus, it seems clear that in experimental models of endometriosis, depletion of peritoneal phagocytic macrophage populations inhibits growth and angiogenesis of induced lesions.

Endometriosis lesions exhibit cyclical bleeding in response to ovarian steroids in the same context as the eutopic endometrium, thus lesions can perceived as wounds undergoing recurrent tissue injury and repair (40). The process in lesions has been described to involve epithelial-mesenchymal transition, fibroblast-myofibroblast transdifferentiation, smooth muscle cell metaplasia and fibrosis (139). Macrophages are critical for successful repair and regeneration in tissues; they stimulate local fibroblasts to differentiate into myofibroblasts to facilitate wound contraction (140). During wound repair the proliferation and expansion of local stromal cells is also regulated by macrophages and if the injury is severe, macrophages may activate additional stem cell and local progenitor cell populations that participate in repair (49). In line with these established roles in tissue injury and repair *in vitro* studies have aimed at assessing the interaction between endometrial stromal cells and macrophages in endometriosis. In a co-culture system, culture of endometrial stromal cells with autologous macrophages isolated from women with endometriosis increased the invasive and clonogenic ability of stromal cells (141). Co-culture with ectopic endometrial stromal cells was also shown to decrease the phagocytic capacity of macrophages and increased the survival and proliferation of stromal cells compared to eutopic endometrial stromal cells in a study by Mei et al. (142). A similar effect was also reported by Shao et al. (143). Reciprocal signaling therefore appears to be occurring between ectopic endometrial stromal cells and macrophages, which could contribute to their survival and the formation of endometriosis lesions in the peritoneal cavity, however the precise mechanisms are yet to be elucidated and the specific macrophage populations involved are unknown. Stromal cells derived from ovarian endometrioma were found to express markers of mesenchymal stromal cells (MSCs), formed colony forming units and exhibited multipotency suggesting characteristics of mesenchymal stem-like cells. The MSCs from endometriomas promoted differentiation of monocytes to spindle shaped pro-repair/immunosuppressive macrophages *in vitro* (50). The results suggest that MSC influence macrophages such that they exhibit an immunosuppressive phenotype and support lesion growth. The coordination of monocytes and macrophage activation states during inflammation and repair is tightly and temporally controlled. If disturbances occur at any point in the process this can lead to aberrant repair and the formation of pathological fibrosis (49). For example, persistent activation and sustained recruitment of pro-repair macrophages may contribute to pathological fibrosis (144). Since endometriosis lesions are undergoing consistent and repeated episodes of injury and repair and lesions exhibit fibrotic content, the events required for efficient, scarless repair may be disturbed. Depletion studies have demonstrated that macrophage depletion significantly reduces the fibrosis in lesions. Moreover, adoptive transfer of macrophages polarized *in vitro* to exhibit an M2a phenotype (activated with IL-4) increased the fibrotic content of lesions (139). Thus, it seems that unlike the physiological wound repair process, endometriosis lesions cannot enter the resolution phase of inflammation and repair and the local inflammatory environment causes persistent activation of pro-repair macrophages that contribute to fibrosis.

A role for macrophages in neurogenesis in endometriosis lesions has been established in the literature, suggesting a role in the generation of endometriosis-associated pain. Indeed, nerve infiltration in lesions is positively correlated with higher reported pain scores in women (145). Cholinergic, adrenergic, sensory Aδ and C nerve fibers have been identified in lesions (146, 147), and macrophages are densely populated in areas of high nerve density (55, 148). Greaves et al. reported that in response to estradiol, nerve fibers secreted CCL2 and CSF-1, which attracted macrophages, which in turn secreted neurotrophin 3 and brain-derived neurotrophin factor,

stimulating neurogenesis (55). Recently a role for macrophage-derived insulin-like growth factor-1 (IGF-1) as a key signal for nerve outgrowth and sensitization in endometriosis has also been described (54): depletion of peritoneal macrophages by clodronate liposomes reversed abnormal pain behavior in mice with induced endometriosis and notably reduced the number of lesions in the peritoneal cavity, providing a direct link between macrophages and endometriosis-associated pain/lesion development. Macrophages treated with peritoneal fluid from women with endometriosis exhibit an up regulation of IGF-1 at the mRNA level, and mechanistically macrophage-derived IGF-1 increased the growth of embryonic rat dorsal root ganglion explants and this was reversed by an IGF-1 inhibitor. Similarly, IGF-1 inhibition by the IGF-1 receptor inhibitor linsitinib in a mouse model could reverse abnormal pain behaviors (54). Taken together, macrophages are evidently pivotal to facilitating neurogenesis and the generation endometriosis-associated pain symptoms, and this is at least in part mediated by IGF-1. The reciprocal signaling that occurs between macrophages and nerve fibers therefore appears critical in regulating neurogenesis in lesions and neuroinflammation is a key driver of endometriosis pathophysiology.

Studies mechanistically indicate that macrophages play key roles in growth, vascularization, and neurogenesis in lesions, as well as generating pain in the condition and these experiments have given insight into some of the factors expressed by macrophages. However, the phenotype of macrophages in endometriosis has not been fully characterized. Macrophages in endometriosis lesions have long been described as being wound healing and "M2-like," however few studies have taken into consideration the complexities of macrophage phenotype, where pro-inflammatory and wound-healing like markers often co-exist in response to complex signals from the local tissue microenvironment (76). In humans, lesion resident macrophages express the scavenger receptors CD163 and CD206, associated with hemoglobin scavenging and silent clearance of debris (53). Cominelli et al. also identified $CD163^+$ $CD206^+$ macrophages in superficial lesions from women, which also expressed high levels of matrix metalloproteinase-27, associated with tissue remodeling (149). Duan et al. characterized nitric oxide synthase ($iNOS^+$) pro-inflammatory and $CD163^+$ wound healing-like macrophages in mouse endometriosis lesions (139). In a rhesus macaque model of endometriosis, lesions were highly infiltrated by $CD163^+$ macrophages (150). Whilst macrophages in endometriosis lesions possessing a "wound healing" like phenotype is synergistic with their role in growth and angiogenesis in lesions, a more comprehensive analysis of macrophage phenotype in endometriosis lesions is required. It is also unknown whether different phenotypes exist within endometriosis lesions, which could play differential roles in pathology, and identifying these populations is key for understanding which macrophage populations are driving pathology.

Identification of endometrial macrophages and bone-marrow monocyte-derived macrophages in endometriosis lesions demonstrates that endometriosis-associated macrophages have different origins, however differential roles for these populations have not yet been investigated. It is possible that the endometrial macrophages in lesions are monocyte-derived since a rapid influx of classical monocytes into the endometrium is observed during endometrial repair. Evidence of embryonically derived tissue resident macrophages in lesions is yet to be demonstrated. Previous studies have demonstrated that depletion of peritoneal macrophages has pronounced effects on lesion size and vascularization (53), depletion of this population translates to reduced number of macrophages in lesions and attenuates pain in mice (54). It remains unknown how endometrial and recruited monocytes contribute to lesion establishment and maintenance. Depletion of different macrophage populations prior to inducing endometriosis in mice and at different time-points during the life-course of the lesion will yield important insights into the role of these pivotal cells in the disorder. Whilst macrophages from 3 origins have been described the true heterogeneity of macrophage phenotype in lesions and in the peritoneal fluid, in endometriosis, is unknown. Application of single cell discovery techniques and digital molecular pathology could provide vital information on the complexities of endometriosis-associated macrophage phenotype, and coupled with *in vivo* functional studies identification of a disease-promoting population that exhibits unique markers that differ from healthy macrophages may be possible.

THE FUTURE: MACROPHAGE TARGETED THERAPIES

Macrophages offer an attractive therapeutic target due to their instrumental role in a number of pathologies (79). Inhibition of macrophage signaling or recruitment, as well as re-education of disease-associated macrophages to a "healthy" phenotype could be of clinical benefit to patients where macrophages are implicated in disease pathophysiology. Identification of disease promoting macrophage populations and a detailed understanding of their regulation, recruitment and phenotype is a fundamental step before the development of therapeutics which specifically target disease-associated macrophages is possible. Due to the pivotal role that macrophages play in many cancers, macrophage-targeted therapies have received much attention in the literature and a number of *in vivo* studies and clinical trials have demonstrated efficacy in using macrophage-mediated treatments to improve clinical outcomes (79). A subset of studies has targeted proliferation of TAMs in an effort to alleviate tumor burden and improve clinical outcomes. Strachan et al. demonstrated that targeting the Csf-1-receptor with a small molecule inhibitor attenuated the turnover rate of TAMs and decreased tumor growth in mouse models of breast and cervical cancer (151). A phase I trial demonstrated a significant reduction in macrophage number in solid tumors after anti-Csf-1r treatment (152), and Csf-1r inhibition showed an improvement in clinical outcomes including improvement of symptoms in patients with diffuse-type giant cell tumors (153). Inhibiting macrophage proliferation therefore appears to be of clinical benefit in cancer models and subsets of cancer patients. Future treatments should aim to specifically

target disease-associated macrophage populations; Csf-1 is a key regulator of macrophage proliferation and survival in most tissues and neutralization or inhibition would affect healthy macrophage populations and as such is not an ideal therapy (154). The proliferative capacity of endometriosis lesion-resident macrophages is currently unknown, thus further research is required to determine whether this treatment strategy would be of benefit to women with endometriosis.

Another potential mechanism of therapeutic intervention could involve blocking recruitment of disease-promoting macrophage populations. The CCL2/CCR2 recruitment mechanism is implicated in a number of cancers and a CCR2 inhibitor to be administered alongside chemotherapy is currently in phase 1b trials (155). Inhibition of recruitment may be beneficial in blocking infiltration of macrophages into endometriosis lesions, however the mechanisms, which regulate recruitment into lesions, are currently poorly understood.

Whilst progression of research into macrophage-targeted therapies is promising, current therapies do not specifically target disease-promoting macrophages but have the potential to affect macrophage populations throughout the whole body. However, as our understanding of disease-modified macrophages improves, it is evident that establishing macrophage origins and phenotype heterogeneity in disease are crucial areas of research before specific, targeted treatments can be designed (72). Future work describing macrophage sub-populations, active recruitment mechanisms and macrophage phenotype in endometriosis is therefore critically required before macrophage-targeted treatments may be a possibility for women with endometriosis.

AUTHOR CONTRIBUTIONS

CH performed literature search and wrote manuscript. AH provided feedback. EG conceptualized manuscript, provided feedback, and wrote manuscript.

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The Inflammasome in Reproductive Biology: A Promising Target for Novel Therapies

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The inflammasome is a key regulator of innate immunity involved in the inflammatory response to infections as well as disease through the activation of caspase-1 and the processing of the inflammatory cytokines interleukin (IL)-1β and IL-18. Even though the inflammasome was first described in the context of infections, most research in recent years has focused on targeting the inflammasome as a therapeutic option in sterile inflammatory events. Recent evidence indicates a clear involvement of the inflammasome in Reproductive Biology such as infertility and preeclampsia. In this mini-review, I summarize the current findings on the inflammasome that have been described in the field of Reproductive Biology and highlight the potential that the inflammasome has as a novel therapeutic option in this field. The topics covered in this review as it pertains to the inflammasome field cover the literature published on male and female infertility, endometriosis, preeclampsia, placental inflammation, and reproductive senescence.

Keywords: inflammasome, fertility, inflammation, caspase-1, reproduction

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THE INFLAMMASOME

The inflammasome is a multiprotein complex with a dual role, one on inflammation and the other one on cell death. The most studied role of the inflammasome involves the activation of the cysteine aspartase caspase-1, resulting in the processing of the pro-inflammatory cytokines interleukin (IL)-1β and IL-18 (1). The most recently identified role of the inflammasome is the cell death mechanism of pyroptosis, which involves the cleavage of gasdermin-D and the release, but not activation, of IL-1β (2). The inflammasome is comprised of three basic components: a nucleotide oligomerization domain (NOD)-like receptor (NLR) such as NLRP1, NLRP2, or NLRP3 as well as the adaptor protein known as apoptosis-associated speck-like protein containing a caspase activating recruitment domain (ASC) and the inflammatory cysteine protease caspase-1 (**Figure 1**).

INITIAL STEPS IN THE FIELD OF INFLAMMASOME RESEARCH

The inflammasome was initially discovered by the late Tschopp and colleagues in 2002 as a multiprotein complex involved in the activation of caspase-1, which is responsible for activating IL-1β and IL-18 (1). Most of the initial studies on the inflammasome started focusing on bacterial infections (3). Then these studies were further extended to the role of inflammasomes in viral (4) and fungal infections (5, 6) as well as autoimmune diseases (7). In the mid 2000s, the first studies on the inflammasome in a sterile event were carried on vitiligo (8) and central nervous system

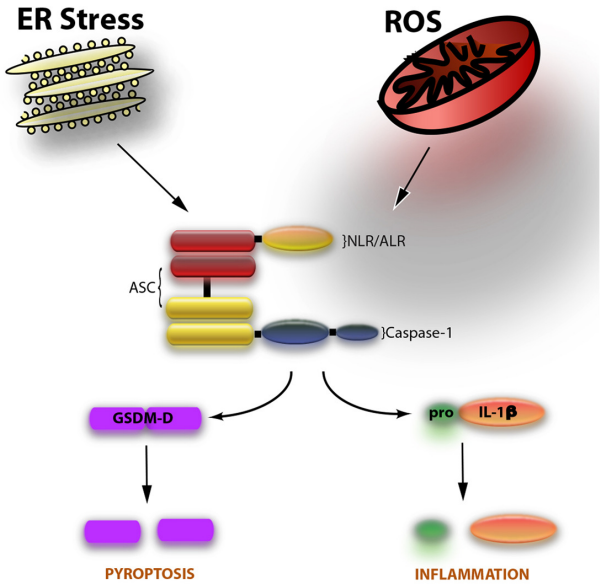

FIGURE 1 | The inflammasome is comprised of caspase-1, ASC, and an NLR such as NLRP1 or NLRP3. Two events involved in the activation of the inflammasome are endoplasmic reticulum (ER) stress and the formation of reactive oxygen species (ROS). Upon activation of the inflammasome, caspase-1 is cleaved. Once cleaved (activated), caspase-1 goes on to cleave the pro-inflammatory cytokine IL-1β to induce inflammation. In addition, the substrate of pyroptosis (inflammasome-mediated cell death) gasdermin-D (GSDM-D) is cleaved. GSDM-D cleavage results in the formation of pores through which IL-1β is then released as well as cell death.

injury (9). Since then, the inflammasome field has started to expand into other indications such as atherosclerosis (10), diabetes (11), nephropathies (12), liver diseases (13), aging (14, 15) as well as in the field of reproductive biology (16, 17), which extent even to the effects of obesity and the inflammatory contribution of the inflammasome to male subfertility (18).

THE INFLAMMASOME IN REPRODUCTIVE BIOLOGY

In the context of reproductive biology, the inflammasome has been studied in areas as diverse as female (19) and male infertility (16, 17), fetal growth (20), endometriosis (21), preeclampsia (22), gestational diabetes (23), perinatal depression (24), placental inflammation (25), preterm births (26), and reproductive senescence (27) (**Table 1**).

Infertility

Effective fertility requires a fine balance between pro- and anti-inflammatory mediators. Thus, an imbalance in the inflammatory response during fertilization and early embryogenesis dooms the process toward pregnancy failure (31). Witkin and colleagues showed that a polymorphism in the gene encoding for NLRP3 (*CIAS1*) is associated with female infertility. Interestingly, this polymorphism increased the likelihood of mycoplasma infection-associated female infertility (19). Moreover, another role for NLRP2 was also described

TABLE 1 | Conditions associated with inflammasome activation in the field of Reproductive Biology.

Condition	Findings	References
Female infertility	NLRP3 gene polymorphism associated with female infertility	(19)
Male infertility	Inflammasome inhibition improves sperm motility in spinal cord injured men	(16, 17)
Endometriosis	Inflammasome signaling proteins are elevated in the endometrium of females with recurrent pregnancy loss	(28)
Preeclampsia	The NLRP3 inflammasome contributes to the inflammatory response seen in preeclampsia	(25, 29, 30)
Preterm births	Caspase-1, ASC, and IL-1β genes are elevated in preterm birth mice	(26)
Reproductive senescence	Inflammasome proteins are carried in EV released by female reproductive organs that reach the brain, contributing to brain inflammation	(27)

for infertility. The NLRP2 inflammasome was first described to be formed in the nervous system (32). In the context of fertility, NLRP2 regulates oocyte quality, which is involved in age-associated fertility loss (33). In addition, a role for NLRP3 in the immune response in the testes has also been described (34). In addition, in the sperm of patients with spinal cord injury, inflammasome proteins are elevated (16), and this increase in inflammasome protein expression is consistent with decrease sperm motility that is improved by inhibition of ASC (16). In a rodent model of spinal cord injury, similar findings have been recently reported (35).

Endometriosis

An abnormal imbalance between pro- and anti-inflammatory proteins in the endometrium results in recurrent miscarriages. Inflammatory proteins like tumor necrosis factor, IL-6, IL-10, and interferon-γ are dysregulated in women with recurrent pregnancy loss (36). Thus, highlighting the importance of an adequate pro- to anti-inflammatory milieu. Similarly, significant research has started to be published in the rea of the inflammasome and the endometrium (37). Accordingly, NLRP3, caspase-1, ASC, IL-1β, and IL-18 are increased in the endometrium of women with recurrent pregnancy loss (28). Thus, future therapeutic alternatives that aim to rebalance the pro- to anti-inflammatory milieu in the endometrium should also consider the inflammasome as part of the equation.

Preeclampsia and Placental Inflammation

A disorder associated with hypertension and proteinuria starting on the 20th week of pregnancy (38), preeclampsia has a significantly heightened inflammatory response in which the inflammasome plays a contributing role (39). In regards to inflammasome regulation in preeclampsia, Weel and colleagues showed that the NLRP3 inflammasome is upregulated, and that it contributes to the damaging effects of inflammation present in preeclampsia (29), a finding that was then corroborated by Stodle et al. who showed that cholesterol and uric acid crystals

activated the NLRP3 inflammasome in preeclampsia (30). A similar role for NLRP3 was suggested in a model of nanosilica-induced placental inflammation in rodents, but not for ASC (25). However, ASC is significantly increased in the amniotic fluid of women who undergo spontaneous labor at term (40). More recently, extracellular vesicles (EV) have been shown to activate the inflammasome in trophoblasts, thus promoting preeclampsia (41). Moreover, in women with anti-phospholipid syndrome, NLRP3 and ASC are responsible for placental dysfunction that increases adverse pregnancy outcomes (42). For instance, ASC specks have been detected in choriodecidual leukocytes isolated from women who underwent spontaneous labor at term (43).

In addition, exacerbated inflammation in the placenta is associated with fetal growth restriction (44), and protein levels of caspase-1 and IL-1β were elevated in cytotrophoblasts exposed to uric acid crystals, suggesting that inflammasome activation may contribute to placental inflammation by exposure to uric acid crystals, which are known to be associated with fetal growth restriction, preeclampsia and inflammasome activation. Taken together, these findings indicate a clear role for the inflammasome in preeclampsia and placental inflammation.

Reproductive Senescence

Reproductive senescence in females is characterized by heightened inflammation, which makes females more prone to the development of certain diseases. Inflammasome proteins have been shown to be present in EV (45). Interestingly, in reproductive senescent females, EV containing a cargo of inflammasome proteins originate in the female reproductive organs such as the ovaries; EV are then transported through the bloodstream to the nervous system by crossing the blood brain barrier, resulting in inflammasome activation in the brain (27). This heightened inflammasome activation in the brain makes females more susceptible to the damaging effects of central nervous system events such as stroke.

THERAPEUTIC POTENTIAL OF THE INFLAMMASOME

As a result of inflammasome involvement in several indications affecting several organ systems, the inflammasome is well-poised for the development of therapeutic interventions that can improve outcomes in a variety of diseases. Recently, as a result of this tremendous therapeutic potential, Big Pharma and the Biotechnology Industry have garnered special interest in licensing and developing therapeutic interventions that are meant to inhibit the inflammasome in a variety of diseases such as neurodegenerative diseases, liver diseases or gout, among others. The therapeutic potential of the inflammasome is so vast that it has been proven difficult to decide what indication to choose for clinical trials targeting the inflammasome.

Testing therapeutic interventions aimed at inhibiting inflammasome activation is of utmost importance since the ultimate role should be to gain a better mechanistic understanding so that efficient and more specific therapies can be eventually tested in patients. In the field of Reproductive Biology, miR-520c-3p has been shown to inhibit the NLRP3 inflammasome in preeclampsia (46). In addition, the NLRP3 inhibitor MCC950 has been shown to reduce preterm birth by 35.7% and neonatal mortality by 26.7% (47). Similarly, the NLRP3 inflammasome inhibitor glibenclamide also decreases inflammasome activation in human trophoblasts, thus highlighting the therapeutic potential of the NLRP3 inflammasome for the treatment of placental disorders (22).

Moreover, other inflammasomes such as the NLRP1 and AIM2 inflammasomes are also promising targets in this field. For instance, omega-3 fatty acids inhibit NLRP1 and AIM2 inflammasome activation and trophoblast cathepsin S release into the cytosol from lysosomes, thus reducing preterm birth associated with infection and inflammation (48).

Taken together, these findings in the area of Reproductive Biology highlight the important role of the inflammasome, and indicate that therapeutic targeting of the inflammasome is a viable option to treat reproduction-related problems. Current evidence points at NLRP1, NRLP2, NLRP3, AIM2, caspase-1, ASC, and IL-1β as potential targets for therapeutic intervention in this field.

FUTURE DIRECTIONS AND CONCLUSIONS

Inflammasome research in the field of Reproductive Biology needs to focus on more mechanistic insights beyond understanding the expression of inflammasome signaling proteins like caspase-1, ASC, and IL-1β (**Figure 1**). Future research should take a deeper look into the potential mechanisms of inflammasome activation such as extracellular potassium levels (22); the role of oxidative stress on inflammasome activation (49, 50); or whether the inflammasome-mediated process of pyroptosis, or the non-canonical inflammasome pathways, involving caspase-11 in rodents (caspase-4/5 in humans), or caspase-8 are involved in conditions associated with reproduction. To this extent, a recent article has been published showing that hypoxia and endoplasmic reticulum stress activate the NLRP3 inflammasome in primary human trophoblasts, resulting in increased expression of Thioredoxin-interacting protein (TXNIP), a key regulator of inflammasome activation (51). Moreover, these findings were consistent with increased cleavage of caspase-1 and GSDM-D, thus indicating that placental pyroptosis contributes to the systemic release of factors involved in preeclampsia (52).

In conclusion, whether it involves female or male reproductive biology, the data published so far indicate that it is critical to maintain an adequate ratio of pro-inflammatory to anti-inflammatory proteins to increase the possibility of successful reproduction. Thus, targeting the inflammasome to decrease the pro-inflammatory environment is a promising approach, but further research in the area of biomarkers will be useful in gaining a better understanding as to what are the right protein concentrations for relevant pro-inflammatory and anti-inflammatory markers that can be used to help

patients with reproductive problems. For instance, one of such studies has been carried looking at increased ASC levels in amniotic fluid obtained from women with clinical chorioamnionitis at term (53). Therefore, further research should focus on mechanistic insights with the goal of developing better therapies and on biomarkers with the goal of diagnosis and monitoring patients once those treatments are tested in clinical trials or delivered to patients in the clinical setting.

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Conflict of Interest: JR is a co-founder and managing member of InflamaCORE, LLC and has licensed patents on inflammasome proteins as biomarkers of injury and disease as well as on targeting inflammasome proteins for therapeutic purposes. JR is a Scientific Advisory Board Member for ZyVersa Therapeutics.

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REVIEW
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Regulation of Inflammation Pathways and Inflammasome by Sex Steroid Hormones in Endometriosis

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Endometriosis is a gynecological disorder characterized by the growth of endometrial tissue (glands and stroma) outside the uterus, mainly in the peritoneal cavity, ovaries, and intestines. This condition shows estrogen dependency and progesterone resistance, and it has been associated with chronic inflammation, severe pain, and infertility, which negatively affect the quality of life in reproductive women. The molecular mechanisms involved in the pathogenesis of endometriosis are not completely understood; however, inflammation plays a key role in the pathophysiology of the disease, mainly by altering the function of immune cells (macrophages, natural killer, and T cells) and increasing levels of pro-inflammatory mediators in the peritoneal cavity, endometrium, and blood. These immune alterations inhibit apoptotic pathways and promote adhesion and proliferation of endometriotic cells, as well as angiogenesis and neurogenesis in endometriotic lesions. It has been demonstrated that hormonal alterations in endometriosis are related to the inflammatory unbalance in this disease. Particularly, steroid hormones (mainly estradiol) promote the expression and release of pro-inflammatory factors. Excessive inflammation in endometriosis contributes to changes of hormonal regulation by modulating sex steroid receptors expression and increasing aromatase activity. In addition, dysregulation of the inflammasome pathway, mediated by an alteration of cellular responses to steroid hormones, participates in disease progression through preventing cell death, promoting adhesion, invasion, and cell proliferation. Furthermore, inflammation is involved in endometriosis-associated infertility, which alters endometrium receptivity by impairing biochemical responses and decidualization. The purpose of this review is to present current research about the role of inflammasome in the pathogenesis of endometriosis as well as the molecular role of sex hormones in the inflammatory responses in endometriosis.

Keywords: endometriosis, inflammation, pro-inflammatory factors, inflammasome, sex steroid hormones, progesterone receptor, estrogen receptor, bacteria

INTRODUCTION

Endometriosis is a multifaceted gynecological condition with an estimated prevalence of ∼10–15% of the general population (1). It is histologically defined as the presence of glands and stroma of endometrial tissue outside the uterus, mainly in the peritoneal cavity and ovaries (2–4). Reports have shown the location of these lesions in sub-peritoneal fat, recto-vaginal or recto-uterine spaces, bowel, bladder, pelvic nerves, uterosacral ligaments, ureters, anterior abdominal wall, as well as abdominal skin, diaphragm, pleura, lungs, pericardium, and brain, although these locations are usually less frequent than in pelvic structures (4–6).

Endometriotic lesions include superficial lesions in peritoneum and serosa, up to ovarian cysts (endometriomas), deep nodules, and severe adhesions (7). Pelvic endometriotic lesions have been systematically classified into superficial implants (peritoneum and ovarian cysts) and deep nodules (parametrium, Douglas pouch, the anterior wall of the rectum, vaginal wall, vesicouterine space, detrusor muscle of the bladder, ureters, and sigmoid colon) (5). The most widely used staging system of endometriosis was defined by the American Society for Reproductive Medicine (ASRM) in 1997, which classifies endometriosis severity into four different stages (I–IV), from minimal to severe, according to the score obtained from the size of the endometrial implants, involved pelvic structures, and spread of the lesions (7).

Endometriosis is the more frequent cause of chronic and cyclic pelvic pain in reproductive-age women, even occurring in adolescents and menopausal women (7); endometriosis encompasses different pain classes, including dysmenorrhea, dyspareunia, dysuria, and dyschezia (8, 9). Especially in cases of deep endometriosis, the pain is due to an invasion of endometrial cells and pro-inflammatory mediators on the nerve fibers, which trigger a disorder of nociceptive modulation of pain increasing the intensity of the neuronal signal toward the somatosensory cerebral cortex (10). Infertility is another consequence of endometriosis, by reducing implantation capacity, increasing risk of pregnancy loss and physical obstruction imposed by endometriotic lesions (11). Moreover, endometriosis symptoms negatively influence women's life quality by affecting their productivity, social life, and emotional health (12).

Treatment for endometriosis usually includes hormonal management and surgical elimination of lesions (10). Hormonal treatment consists of suppression of growth lesions and pain reduction by abolishing ovulation and menstruation through the administration of progestins, oral contraceptives, and gonadotropin-releasing hormone agonists (13). Surgical elimination is frequently made by laparoscopy, the gold standard for diagnosis and treatment, by which peritoneal implants, deep nodules, and ovarian cysts are removed; moreover, this technique is also performed for more radical proceedings as hysterectomy (1, 14). However, there is not an actual cure for the disease, and lesions and pain tend to reappear after treatments (15); therefore, it is important to continue the development of cutting-edge research focused on studying the underlying mechanisms involved in endometriosis pathophysiology.

There are several and not fully confirmed theories that describe endometriosis pathogenesis. The more accepted theory is the origin of lesions from retrograde menstruation, which establishes that during menstruation, residual endometrial tissue reaches the pelvic cavity, by traveling through fallopian tubes, due to uterine contraction disorders (3). This phenomenon is observed in 90% of women in reproductive age; however, it does not explain why only 10% of them develop endometriosis or the presence of lesions in more distal locations (14). Among other proposed theories are the coelomic metaplasia and the theory of Müllerian remnant; the first one involves the transformation of healthy peritoneal tissue into ectopic endometrial tissue; this theory is based on the fact that peritoneal and endometrial cells have a common origin from coelomic epithelium. In contrast, vascular and lymphatic metastasis suggests that reminiscent endometrial tissue travel through the blood and lymphatic vessels to reach ectopic locations; on the other hand, the theory of Müllerian remnant argues that cellular debris from embryonic Müllerian duct transform into endometriotic tissue by the influence of sex hormones rising at the beginning of puberty (3). There are efforts to unify the existent theories (16); however, the precise mechanisms underlying origin and development of endometriosis remain mainly unknown.

At a cellular level, the main alterations in endometriosis are characterized by cell proliferation, inflammation, and angiogenesis, which are closely connected to each other and are caused by an alteration in sex hormonal signaling, that depend on the sustained activation of estradiol (E2)-dependent pathways and the disruption of those dependent on progesterone (P4), through alteration of activity of their cognate receptors. This alteration in the activity of hormone receptors converges in a distinctive phenotype of resistance to progesterone and of estrogen dependence. A recent and detailed revision about the role of P4 and E2 in endometriosis describes the normal molecular hormonal regulation of the physiology of endometrium and its alterations in endometriosis (17) and highlights inflammation as a known key contributor in the pathophysiology of endometriosis, which is strongly associated with chronic pelvic pain and defects in endometrial receptivity and the decidualization process (17, 18). In fact, endometriosis is frequently considered as an inflammatory disease. Therefore, this review presents the current research about the role of inflammation and the inflammasome in the pathogenesis of endometriosis, as well as a discussion about the molecular mechanisms involved in the alteration of immune and inflammatory responses by female steroid hormones in this disease.

PATHOGENESIS OF ENDOMETRIOSIS: ALTERED PATHWAYS

Hormonal imbalance in endometriosis is the main contributor in the alterations of multiple cellular functions such as proliferation, adhesion, and differentiation, as well as evasion of immune clearance, neurogenesis, angiogenesis, pain generation, metabolism, and inflammation (3). This imbalance is due

to an increased expression of aromatase (CYP19A1) (19) and a decreased expression of progesterone-regulated 17β-hydroxysteroid dehydrogenase (17β-HSD) (20), which increases bioavailable levels of E2 in ovaries, peripheral tissue, and endometriotic lesions (21). Besides enzymatic and hormonal changes, the activity of nuclear receptors is also modified, through dysregulation of their expression at both mRNA and protein levels (22, 23). In the case of estrogen receptors (ER), there is an increase of isotype ER-β (*ESR2*) expression associated with hypomethylation of its promoter (24), and a significant reduction of ER-α (*ESR1*) expression by hypermethylation of its promoter and through direct inhibition by ER-β. In contrast, both progesterone receptor isoforms PR-A and PR-B (*PRG*) show a decreased expression; in particular, PR-B undergoes a drastic downregulation (mRNA and protein levels), which is mainly due to hypermethylation of the PR-B promoter (25), therefore affecting downstream hormone target genes (26).

The cellular and molecular processes involved in the development of endometriosis are only partially described. Considering retrograde menstruation as the potential beginning of endometriosis, it has been proposed that viable glandular and stromal endometrial cells contained in the menstrual debris reach the peritoneal cavity and are able to adhere to the peritoneum by interaction of cell surface receptors such as integrins, with membrane adhesion molecules of extracellular matrix, like fibronectin and laminin (27, 28). Besides, this debris contains stem cells that could also be responsible for the progress of endometriosis, by differentiating in endometrial cells that are unable to decidualize and that possess a phenotype of progesterone resistance and immune function alteration (29).

Furthermore, it has been shown that after initial adhesion, endometriotic cells invade peritoneum, possibly through the action of matrix metalloproteinases (MMPs) that degrade the basal lamina containing laminin, fibronectin, and collagen, which allows the remodeling of surrounding tissue (30). Additionally, reports have demonstrated that E2, through the increased activity of ER-β, promotes the cellular survival, by impairing tumor necrosis factor-α (TNF-α)-mediated apoptosis (31), increasing cellular proliferation with the participation of growth factor signaling, and favoring epithelial–mesenchymal transition that contributes to the production of collagen and formation of fibrosis (7). Also, ER-β induces the proliferation and survival of endometriotic cells through the increase of mRNA and protein levels of Ras-like estrogen-regulated growth inhibitor (RERG) that induces ribosomal biogenesis, while significantly stimulating expression of glucocorticoid-regulated kinase (SGK1) that has an anti-apoptotic role (32, 33). Growth factors such as hepatocyte growth factor (HGF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), insulin-like growth factors (IGF), and basic fibroblast growth factor (BFGF) also participate in the progression of endometriotic lesion through their strong mitogenic activity (3).

Additionally, studies have shown that an immunologically permissive environment in the peritoneum (34) is involved in endometriosis pathogenesis. Impaired inflammatory cell function of macrophages with a reduced phagocytic activity (35) and a resistance of endometriotic tissue to be lysed by natural killer cells (NKs) (36) allow the evasion of clearance by the immune system.

Once invasion takes place, angiogenesis and neurogenesis are activated coordinately. Angiogenesis allows the maintenance of lesions, supplying them with functional blood vessels that form a dense vascularization. In this process, diverse growth and pro-angiogenic factors play important roles, such as vascular endothelial growth factor (VEGF) that is regulated by E2 and responds to an inflammatory microenvironment, promoting endothelial cell proliferation (37, 38). Neurogenesis is linked to both inflammatory response and angiogenesis and, along with an imbalance in sensory and sympathetic innervation, contributes to the growth of nerve fibers, subsequent peripheral neuroinflammation, and generation of chronic pain (39). One of the postulated consequences of immune cell activation in the endometriosis microenvironment is the production of cytokines, growth factors, and eicosanoids that simultaneously stimulate lesion innervation and neovascularization through a coordinated mechanism that is known as neuroangiogenesis (40).

Concerning the molecular origin of these alterations, there is an increasing evidence of genetic and epigenetic changes in epithelial and stromal cells, respectively, both in ectopic and eutopic endometrial tissue compared to healthy endometrium. This changes influence of all aspects in the pathophysiology of endometriosis, by modifying the expression of essential components of cellular and biochemical pathways compromised in endometriosis, including the expression of ER and PR genes, and can explain associated inheritance and predisposition to present the disease (41).

Some genetic variants associated with endometriosis risk have been linked to chromosomic regions 7p15.2 and 10q26 by genetic linkage studies. These regions contain *CYP2C19* (cytochrome P450 2C19), *INHBA* (inhibin subunit beta A), *SFRP4* (secreted frizzled-related protein 4), and *HOXA10* (homeobox A10) genes (42). On the other hand, genome-wide association studies have shown 14 genetic loci associated with endometriosis, which are involved in alterations of wingless-related integration site protein (WNT), mitogen-activated protein kinase (MAPK), and signal transducer and activator of transcription 3 (STAT3) signaling (7). Remarkably, cancer driver mutations have been identified in *ARID1A*, *PIK3CA*, *KRAS*, and *PPP2R1A* genes in epithelial cells of endometriotic tissue; however, it has not yet been demonstrated that these changes originate malignant transformation from endometriotic lesions (43).

Different transcriptomic alterations have been detected in endometriosis patients; for example, by using cDNA microarray analysis specific genes that mainly encode components of the immune system and inflammatory pathways, proteins involved in cell adhesion and remodeling of the extracellular matrix as well components of signal transduction pathways were found differentially expressed in ectopic endometrium when compared to eutopic endometrium; some altered genes are those that encode phospholipase A2 group IIA (PLA2 IIA), PLA2 group V (PLA2 V), fatty acid–binding protein 4 (FABP4), prostacyclin synthase (PGIS), complement component 7, claudin 11, heptoglobin, some integrins, and

tissue inhibitors of metalloproteinases 1 and 2 (TIMP-1 and TIMP-2) (44). Furthermore, next-generation sequencing analysis of eutopic endometrium transcriptome has shown abnormalities in comparison with endometrium from healthy women, demonstrating differential expression of genes involved in extracellular matrix remodeling, angiogenesis, cell proliferation and differentiation, such as matrix metallopeptidase 11 (MMP-11), dual specific phosphatase 1 (DUSP1), Fos proto-oncogene (FOS), serpin family E member 1 (SERPINE1), and adenosine deaminase 2 (ADA2) (45).

The regulation of gene expression by epigenetic mechanisms encompasses DNA methylation, post-translational modifications of histones, non-coding RNAs (mainly microRNAs), among others (46). The role of epigenetic mechanisms in the pathogenesis of endometriosis has been recently explored and reviewed (47). Genome-wide DNA methylation studies have shown that endometriotic lesions and eutopic endometrium display an altered epigenetic program compared with endometrial tissue from women without the disease, which in turn has been associated with an altered expression profile in several genes involved in the pathogenesis of endometriosis (29, 47–49). Particularly, an increase in the content of DNA methylation has been reported in the promoter and coding region of *GATA2* gene, and the promoter of *ESR1* and *PGR* genes in endometriotic cells in relation to endometrial cells, whereas *GATA6*, *ESR2*, and *SF1* genes are hypomethylated in endometriotic cells (24, 25, 50, 51). These alterations were associated with the corresponding changes in gene expression, which partly explains the altered progesterone signaling, progesterone resistance, increased inflammation, and the excessive estradiol production observed in this disease (47). Moreover, it has been suggested that histone acetylation and methylation are also involved in the pathogenesis of endometriosis, since alterations in those post-translational modifications have been associated with the presence of the disease (52).

In spite of being considered a "benign" disease, the complexity of endometriosis is very clear. Its pathogenesis is associated with different molecular and cellular alterations in endometriotic tissue and the surrounding microenvironment; these modifications are closely related to each other and form a complex positive feedback loop, which indicates that probably there is not only one mechanism that originates and influences their pathogenesis. Components of the molecular mechanisms involved in endometriosis pathophysiology show high heterogeneity between patients, notwithstanding that they are analyzed in populations as homogeneous as possible. Indeed, great recent advances in the knowledge about the disease have been made; however, there is still a gap in the information that allows the identification of the key pathway or pathways that start the appearance of endometriotic lesions. According to recent findings, we consider that the phenomenon where all mechanisms converged is the chronic inflammation, which is present in all the clinical manifestations of this gynecological disorder, without forgetting that it is subject to a fine hormonal regulation. For that reason, in the next sections, we described main cellular and molecular alterations of the immune response in endometriosis.

ALTERATION OF INFLAMMATORY FUNCTIONS IN ENDOMETRIOSIS

Inflammation is the main molecular and cellular process involved in the pathophysiology of endometriosis, causing pain, tissue remodeling, lesion formation, fibrosis, and infertility (6). Aberrant production and secretion of immune mediators like cytokines, prostaglandins, and metalloproteinases, as well as alteration of activity and infiltration of immune cells in sites of lesion and peritoneal cavity are some changes involved in this disrupted response (3, 53).

The interaction between immune and hormonal systems considerably impacts endometriosis pathogenesis and development (14). Hormonal signaling differentially regulates immune response; P4 is known by its anti-inflammatory capacity, mediated mainly through PR-B, which overcomes nuclear factor kappa-light-chain-enhancer of active B cells (NF-κB) signaling (54). On the other hand, E2 has a noteworthy role in the promotion of inflammation, by inducing the secretion of cytokines and prostaglandins from peritoneal macrophages (55) by action of ER-β (23), while ER-α has a dual role, with both anti- and pro-inflammatory effects (56). However, in endometriosis, there is an imbalance in the functions of sex steroid hormones, with an important role of estrogens in the exacerbated pro-inflammatory state of endometriosis. Changes induced by sex steroid hormones through their cognate receptors at the immune and inflammatory levels are described below. **Figure 1** shows a schematic model that resumes the mechanisms involved in regulation of inflammation in endometriosis.

Alteration of Immune Cell Function

Inflammation in endometriosis is generally attributed to the recruitment of macrophages and other activated leukocytes from bone marrow to the developing endometriotic lesions and eutopic endometrium, these cells are attracted by chemokines synthesized and released *in situ*. In the endometriotic lesions, immune cells secrete elevated levels of pro-inflammatory cytokines, which in turn stimulate the production of diverse molecules such as chemokines, and growth factors that sustain an inflammatory microenvironment and the remodeling of the ectopic tissue (40). This tissue also has the capacity of releasing pro-inflammatory factors, creating a positive feedback loop that maintains the chronic inflammation state (57).

Macrophages are the first defense line of the immune system, by removing pathogens through phagocytosis and cell debris through cytokine secretion (58). An elevated number of macrophages are found in endometriosis patients during all phases of the menstrual cycle (59). Interleukin-8 (IL-8), C-C chemokine regulated on activation normal T cell expressed and secreted (RANTES, or CCL5), as well as monocyte chemotactic protein-1 (MCP-1, or CCL2) have been found in peritoneal fluid mainly functioning as chemoattractants of recruited macrophages (60–63). As a result, peritoneal macrophages from

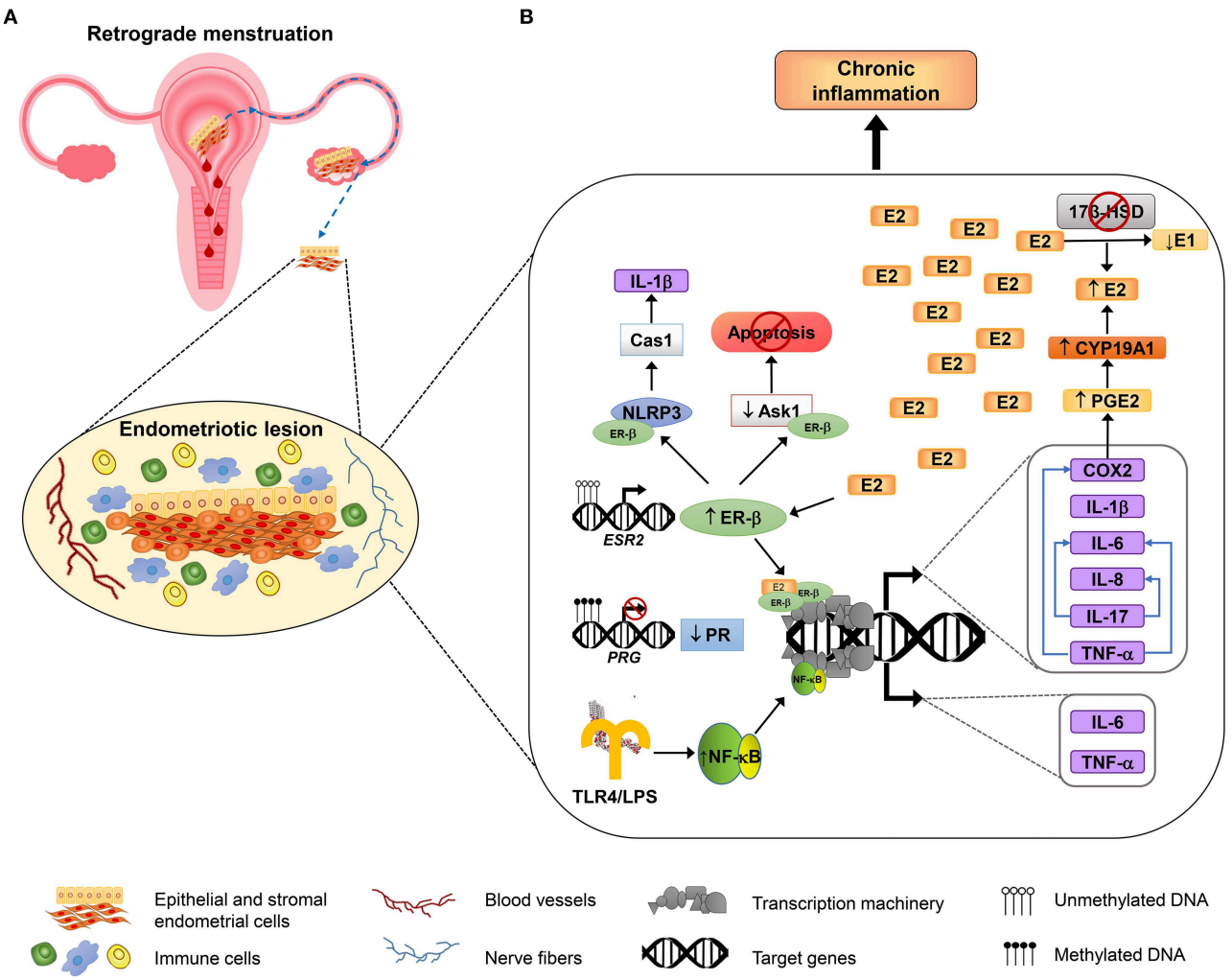

FIGURE 1 | Inflammatory mechanisms involved in endometriosis. An overview about the molecular mechanisms involved in the adhesion, survival, proliferation, inflammation, and neuroangiogenesis of endometriotic lesions. **(A)** According to the most accepted hypothesis of endometriosis origin, stromal and epithelial cells from shed endometrial tissue reach ovaries or peritoneal cavity by retrograde efflux during menstruation. The developing endometriotic lesion stimulates angiogenesis and nerve development and secretes chemoattractant molecules recruiting high numbers of macrophages (showed in blue) and natural killer cells (indicated in green) with reduced phagocytic and cytolytic activity, respectively. **(B)** Alterations in DNA methylation lead to the growth and maintenance of endometriotic lesions by increasing the expression of estrogen receptor β (ER-β) and reducing progesterone receptor (PR) expression. Overexpressed ER-β induces the expression of genes that encode several pro-inflammatory molecules, such as IL-1β, IL-6, IL-8, IL-17, TNF-α, and COX2; some of them, in turn, can stimulate the production of other immune molecules (depicted as blue arrows). COX2 promotes an increase of the synthesis of prostaglandin E2 (PGE_2), and PGE_2 induces aromatase activity (CYP19A1), leading to E2 increased levels. E2 elevated levels are sustained by reduced expression of 17β-HSD, an enzyme that catalyzes the conversion of E2 to estrone (E1), and that is induced by PR (not shown). In addition, ER-β interacts with some inflammasome components such as NLRP3 sensor and caspase 1 (Cas1) to activate IL-1β, and also interacts with apoptosis signal-regulating kinase-1 (ASK-1) to reduce its activity and hence inhibits TNF-α-mediated apoptosis. On the other hand, the induction of genes involved in the inflammatory response in endometriosis is also mediated by NF-κB activation, through the TLR4 interaction with *E. coli* LPS, or possibly through other microbial metabolites associated with infections or dysbiosis, as is proposed by "bacterial contamination" theory.

endometriosis patients overexpress cyclo-oxygenase 2 (COX-2) and secrete higher levels of prostaglandins than those from women without disease (64).

MMPs normally regulate macrophage activity by degrading the extracellular matrix of cells that will be phagocyted, but these molecules show a reduced expression in macrophages of patients with endometriosis, which is subsequently associated with impaired phagocytic activity. Particularly, MMP-9 has a reduction in both expression (mRNA and protein) and enzymatic activity, and this reduction is essentially due to the presence of prostaglandin E_2 (PGE_2) in the peritoneal fluid. Moreover, PGE_2 activity takes place through the EP2/EP4 (receptors for MMPs)-dependent PKA pathway (65). Other studies report that phagocytosis inactivation by PGE2 trough CD36 inhibition is mediated by the EP-2. On the other hand, cytokines secretion activity by these cells is increased; for example, IL-8, IL-10, and

tumor necrosis factor (TNF)-α are found in peritoneal fluid of endometriosis patients at greater levels than fertile women (66); thus, macrophages could promote and sustain an inflammatory environment required for endometriosis progress (67).

Lymphocytes are essential to determining survival, implantation, and proliferation of endometriotic cells (68); among affected lymphocytes in endometriosis are T-cells. Usually, there is a balance between populations of regulatory T-cells (T_{regs}), a subgroup of helper T cells (CD4+) that act as anti-inflammatory cells, and effector or cytotoxic T-cells (CD8+); this equilibrium is required to maintain immune tolerance and to eliminate endometriotic cells. However, the high levels of E2 and the reduced P4 response influence in the elevated concentration of T_{regs} in peritoneal fluid and in the endometriotic lesions, which decrease immune surveillance (58) and suppress the immune response, promote in this way the establishment of lesions (69). In contrast, effector T cells show a decreased activity, while helper T-cells in general are increased and participate in secreting high levels of cytokines (58, 70). Growth of endometriotic lesions is allowed by reduced ratios of Th1 to Th2 cells, with the consequent potentiation of Th1 response, mainly in the first stages of disease (71, 72). The Th1/Th2 ratio depends on the stage of the disease once it has been established, since Th1 cytokines prevalence occurs during minimal to mild endometriosis whereas Th2 cytokines are manifested in severe stages (58). Besides, the Th17 cell subset has a role in endometriosis, mediated by secretion of IL-17 resulting in the secretion of chemokine (C-C motif) ligand 20 (CCL20; also known as macrophage inflammatory protein-3, MIP3A) by endometriotic cells. CCL20 acts as a chemoattractant for Th17 cells and neutrophils to the endometriotic lesion (68). IL-17A also can stimulate IL-8 secretion and COX2 expression that promotes the proliferation of human endometriotic stromal cells (73).

NK cells represent around 15% of leukocytes in peripheral blood and of 30% of peritoneal leukocytes; the main function of NK cells consists in protecting against infections and tumor development through their cytolytic and immunomodulatory capabilities. NK cells destroy other cells by secreting lytic granules that contain granzymes, perforin, cytotoxins, or cytokines (74). NK cells in peritoneal fluid and peripheral blood from patients with diagnosed endometriosis have a decreased cytotoxicity against endometrial cells (75). Hence, they fail in eliminating endometrial fragments from menstrual debris in ectopic sites due to both their diminished activity and resistance from ectopic endometrium to be eliminated, which could be due to the presence of soluble non-specific factors released by human endometrial cells able to interfere with NK cell function, as suggested by the reduced activity of NKs in the presence of conditioned media obtained from human endometrial cell culture, without compromised cell viability (36). Alteration of NK cytotoxic activity in endometriosis patients is related to the reduced levels of granzyme B and perforin. In addition to defects in their activity, NK cells showed reduced numbers both in peritoneal fluid and in peripheral blood of endometriosis patients vs. control women (76, 77). Analysis of populations of peritoneal NK cells has shown that mature NK cells (CD32CD56+) are significantly decreased, in contrast with the proportions of immature NK cells (CD272CD11b2) that are increased, which suggest that the pathogenesis of endometriosis is associated with anomalous differentiation of NK cells (36). On the other hand, the reduction in NK activity correlates with the severity of disease (78), which makes them a potential marker and diagnosis tool of endometriosis and its severity. On the other hand, the reduction of NK function is associated with elevated levels of E2 in endometriosis. The association between NK cell activity and serum E2 levels suggests that immunoendocrine interaction is essential for the progression of endometriosis (77).

Mast cells are another group of leukocytes that are involved in endometriosis by modulating the recruitment, survival, development, phenotype, or function of other immune cells involved in endometriosis pathology, including macrophages, granulocytes, dendritic cells, and T and B cells (79). High numbers of degranulated mast cells have been found in endometriotic lesions, mainly of deep infiltrating type, but not in non-affected areas from peritoneum or eutopic endometrium of patients, indicating that a reaction of hypersensitivity is strongly associated to endometriosis (80); released mediators include TNF-α, IL-8, MCP-1, RANTES, and stem cell factor (SCF) (81). Additionally, the infiltration of mast cells is mainly observed at the periphery of nerve endings, which correlated with chronic pelvic pain, suggesting a relation with nociception in this disease that could be produced through the transient receptor potential vanilloid subfamily 1 (TRPV1) channel (82).

The alteration of the mechanism of maturation, infiltration, and functions of immune cells at the systemic or local level in endometriosis is mediated through modification of molecular and the biochemical environment that converges in the progression of the disease. This makes them potential targets to drug design or looking for alternative treatments that inhibit specific molecules of altered pathways involved in exacerbated inflammation and associated symptoms such as pain or infertility.

Alterations of Immune Mediators

The predominant molecular characteristic of endometriosis is the elevated concentration of immune mediators, mainly pro-inflammatory molecules, both locally and at the systemic level. NF-κB is a key transcriptional factor that is overexpressed and overactivated in endometriotic cells of the lesions and peritoneal macrophages, an effect that is potentiated by the pro-inflammatory environment through action of cytokines. In turn, NF-κB is involved in the positive regulation of these pro-inflammatory factors as well as of chemoattractants, adhesion molecules, and angiogenic factors (83). Immune cells and endometriotic stromal cells are an important source of cytokines, prostaglandins, and chemokines, which are released to the surrounding environment, showing consistency in elevated levels between different fluids and tissues. For example, levels of pro-inflammatory cytokines IL-1β, IL-6, IL-8, IFN-γ, and TNF-α are elevated in peritoneal fluid and serum of patients with endometriosis and could be used as non-invasive markers of the disease (84).

IL-1β is the more studied inflammatory cytokine involved in the pathophysiology of endometriosis, with multiple roles in the disease development. For example, it is involved in activation of cyclooxygenase 2 (COX2), which in turn elevates prostaglandin E_2 (PGE_2) levels that contribute to activation of aromatase and other members of steroidogenic pathway, through an increased binding of steroidogenic factor 1 (SF-1) to promoter of aromatase gene (85). IL-1β also contributes to recruitment of macrophages and neuroangiogenesis by regulating chemokine RANTES and promoting the production of brain-derived neurotrophic factor (BDNF), which co-localizes with macrophages and nerve fibers in endometriotic lesions and cultures of eutopic stromal cells; both events are mediated through c-Jun N-terminal kinase (JNK) and NF-κB signaling pathways. These findings support the notion of interaction between pro-inflammatory factors that favor the communication between different cell types in endometriotic lesions, which as a result promote the recruitment of vessels and nerves to stimulate angiogenesis, growth of the lesion, and generation of pain (40).

IL-6 for its part is found at elevated concentrations in peritoneal fluid and circulation; it is secreted by both macrophages and endometriotic cells, with the macrophages being its main source (86, 87). Serum concentration of IL-6 was analyzed along with the concentration of surface antigen CA125, used frequently as a marker of disease, and both showed an association to moderate-severe endometriosis (87). IL-6 also participates in the decrease of differentiation and cytolytic activity of NK cells, by inducing the tyrosine phosphorylation of phospholipase Cg and tyrosine phosphatase SHP-2, whose expression inhibits cytotoxic activities of NKs, promoting survival of endometriotic cells (86). Multiple functions of IL-6 are mediated by its receptor IL-6R, located on macrophages surface, which simultaneously makes them the source and the target of this cytokine (88). Its elevated expression and secretion by peritoneal macrophages from women with endometriosis is promoted by E2, which in turn is associated with induction of growth of endometriotic lesions (55). Additionally, this pro-inflammatory cytokine participates in the stimulation of aromatase expression in stromal cells from lesions (89), which shows again an interaction between the endocrine system and inflammation processes.

In addition to its function as a chemokine, IL-8 has other important roles in endometriosis development. Its elevated concentration has been shown in endometriotic stromal cells, as an effect of the action of E2 and TNF-α through NF-κB; remarkably, this increase is reversed by P4 treatment, indicating that P4 downregulation in endometriosis contributes to alteration of IL-8 expression during the pathogenesis of the disease (90). Moreover, IL-8 considerably increases the proliferation of stromal cells from ovarian endometriomas (91) and shows a positive correlation with the severity of endometriosis.

On the other hand, TNF-α is considered an essential factor in the pathogenesis of endometriosis. It is secreted in high levels by pelvic macrophages in response to E2 treatment (55). TNF-α promotes the production of prostaglandins PGF_2 and PGE_2 by epithelial and stromal endometrial cells in endometriotic lesions and stromal cells from eutopic endometrium of women with endometriosis by inducing COX2 overexpression through NF-κB activation (92, 93). TNF-α induces endometriosis-associated inflammation by overregulated secretion of other molecules as IL-6, granulocyte-macrophage–colony-stimulating factor (GM-CSF), and MCP-1 (94).

Another pro-inflammatory cytokine involved in endometriosis is IL-17A that stimulates the production of pro-angiogenic cytokines, such as IL-8 or IL-1β, and was found in significantly higher concentrations in the peritoneal fluid of patients with minimal-to-mild endometriosis than those with moderate-to-severe endometriosis and without the disease. Therefore, IL-17 could play an important role in the initial phases of endometriosis by favoring the angiogenesis in the peritoneal surface, which facilitates the survival, implantation, and proliferation of ectopic endometrial tissue (95). In addition to the role in promoting angiogenesis, IL-17 contributes to maintaining a pro-inflammatory environment in the peritoneal cavity needed for the establishment and preservation of endometriosis lesions, where it is produced at elevated levels. Moreover, studies have shown that IL-17 plasma levels are also increased and are dependent on the stage of the disease. This cytokine also maintains the inflammatory and angiogenic milieu by inducing the production of cytokines VEGF, platelet-derived growth factor (PDGF)-AA, C-X-C motif chemokine ligand 12 (CXCL12), and granulocyte colony-stimulating factor (G-CSF) (96).

IFN-γ is also expressed at elevated levels in ectopic endometrium in contrast to eutopic endometrium of patients with endometriosis; a similar situation is observed in serum as well as in peritoneal fluid of patients (97–99), which is related with an unbalanced immune activity in endometriosis. IFN-γ is involved in increasing the resistance of endometrial cells to apoptosis and to stimulate the expression of cell adhesion molecules to allow the establishment of endometriotic lesions (99).

Prostaglandins are locally produced hormones involved in inflammation and pain. In women with endometriosis, PGE_2 and $PGF_{2\alpha}$ are produced in elevated concentrations both in the eutopic and ectopic endometrium. $PGF_{2\alpha}$ contributes to dysmenorrhea through its vasoconstrictive properties and the capacity to induce uterine contractions. In contrast, PGE_2 may induce pain directly. In endometriosis, there is a positive feedback between inflammation mediators and E2. This hormone stimulates COX2 activation that increases PGE_2 production using arachidonic acid as precursor; PGE_2 production in turn influences steroidogenic genes, mainly overexpressing aromatase (100), therefore increasing the E2 levels that stimulate signaling through ER-β. As mentioned before, the inflammatory microenvironment in endometriotic lesions also stimulates PGE_2 production (65). Interestingly, COX2 is differentially regulated in ectopic and eutopic endometrium from endometriosis patients in response to IL-1β, since ectopic tissue is more sensible to this induction that is important to sustain inflammatory microenvironment that in turn sustain lesion development. This increased sensibility is due to IL-1β capacity

to increase COX-2 transcript stability (65, 100). Angiogenic factor VEGF also participates in PGE_2 production by induction of COX2 expression (101), which shows the interplay between inflammation and cell pathways that sustain the maintenance of endometriotic milieu.

Chemoattractants facilitate the recruitment of macrophages at the lesion site, especially chemokines IL-8, MCP-1, and RANTES, whose participation was described in section alteration of immune cells function, which can serve as biomarkers in identifying patients with endometriosis, but the precision of such tests can be enriched by including other inflammatory markers, or in the case of infertility-associated endometriosis, these molecules could be included as endometrial receptivity markers. Interestingly, MCP-1 is overexpressed (mRNA and protein) by action of P4 and E2 only in endometrial endothelial cells from patients with endometriosis, in contrast with cells from healthy women that did not show MCP-1 expression in response to this stimulus. Therefore, hormonal induction of chemokine expression in the endometriotic cells plays an important role in recruiting mononuclear cells, which contribute to inflammation in endometriosis (102).

The pro-inflammatory environment also has the capacity of regulating expression of steroid hormone receptors; Grandi et al. observed in endometrial stromal cells (eutopic tissue) isolated from endometriosis patients that exposition to TNF-α and IL-1β significantly reduces the expression of both PR-A and PR-B (mRNA and protein). This contributes to P4 resistance, by altering the response to hormonal treatments using progestins, which explains the low local response to this therapy and indicates that the origin of inflammation in endometriosis lies not only in the ectopic tissue but also in the cells of eutopic tissue, whose multiple molecular alterations could allow them to survive out the uterus (57).

On the other hand, elevated expression of immunosuppressive cytokines in endometriosis has also been reported, like IL-4 and IL-10, which are involved in the development of disease through stimulating survival, growth, invasion, angiogenesis, and immune escape of endometriotic lesions (103). The elevated concentrations of these cytokines could be due to their insufficient control over pro-inflammatory activities, overcompensating and inhibiting the immune response. IL-4 is found at high levels in endometriotic tissues, where it stimulates the proliferation of endometriotic stromal cells by stimulating the activation of p38MAPK, SAPK/JNK, and p42/44 MAPK signaling (104). IL-10 in serum and peritoneal fluid from patients with endometriosis has shown significantly increased levels in contrast to those from healthy women (105). The role of this cytokine was addressed using a mouse model of surgically induced endometriosis. The blocking activity of IL-10 in mouse decreases the size of lesions, while the administration of this cytokine promoted their growth; additionally, infiltrated dendritic cells were the main secreting source of IL-10. The foregoing suggests that IL-10 contributes to immune suppression necessary for the development of endometriosis (105, 106).

For its part, retinol, which is the precursor of retinoic acid (RA) necessary for endometrial cell differentiation and function, and whose nuclear receptors have also been detected in endometrium, also has a participation in endometriosis, since genes that regulate its synthesis and signaling pathways have an altered expression, which therefore causes reduction of RA. Cell pathways altered in endometriosis and regulated by local retinoic acid production involve MMP secretion, gap junctional intracellular communication, and the expression of a variety of cytokines involved in cell differentiation and immune regulation. Some altered RA-regulated molecules are IL-6, MCP-1, TNF-α, VEGF, connexin 43, various integrins, and *fas* ligand; this dysregulation is related to the progesterone-resistance effect (107, 108). This observation indicates a relationship between diet and nutrition in the disease, supporting the hypothesis of its multifactorial origin.

The use of biochemical markers of inflammation has been proposed to evaluate disease in complement instead of detection of endometriotic lesions through laparoscopic surgery. In this respect, a nested case–control study revealed that plasma IL-1β levels were positively associated with an increased risk to develop endometriosis. However, an association between plasmatic IL-6 and TNF-α levels with disease risk was not found (109), but an association of TNF-α with this risk was found only between women younger than 40, suggesting that young age is a threat factor to develop endometriosis, and the authors concluded that IL-1β and TNF-β could be early markers of disease. Recently, significantly higher concentrations of SCGF-β, IL-8, HGF, and MCP-1 have been reported, as well as lower levels of anti-inflammatory cytokine IL-13 in endometriosis patients such as those found in women without endometriosis (110). Additionally, in the same study, through regression analysis, the combination of SCGF-β, IL-13, and G-CSF concentrations predicted with accuracy the presence of endometriosis (86% sensitivity and 67% specificity) (110). Other studies focused to associate concentrations of pro-inflammatory cytokines and markers of receptivity in peritoneal fluid, such as IL-8, RANTES, osteoprotegerin (OPG), pregnancy-associated plasma protein-A (PAPP-A), TNF-α, midkine, and glycodelin, with the severity of pain according to pain scores. Concentrations of these molecules were found to be correlated with pain level and stage of endometriosis; in particular, this correlation was positive in the case of increased levels of TNF-α and glycodelin, indicating the potential role of this molecule in the severity of pain in endometriosis (111).

In summary, dysregulation of immune molecule expression is manifested both in immune system cells and in endometriosic cells, in response to an altered hormonal environment, which in turn responds to changes in the immune milieu. In fact, the action of these molecules is not individual; they act in a coordinated fashion to regulate the production and function of each other, thus forming a complex network that allows the communication between different cell types in order to maintain the viability and development of endometriotic lesions. Due to the importance of these inflammatory factors, it is necessary to perform more studies about altered immune factors associated with the severity of endometriosis. Since most molecules found in ectopic tissue can also be found at systemic level, that is, they could reflect the local environment that surrounds endometriotic lesion, it is fundamental to propose a complete panel of non-invasive

biomarkers to detect early stages of the disease in patients who are exposed to commonly associated risk factors or have a family history of endometriosis, which could be useful to complement or substitute the surgical diagnosis. In the same way, this knowledge will be advantageous to determine the appropriate therapeutic target and treatment to each patient, in the process of designing a personalized therapy.

EFFECTS OF SEX HORMONES ON INFLAMMASOME REGULATION IN ENDOMETRIOSIS

Inflammasomes are complex molecular structures from the innate immune system that control pyroptosis (inflammatory form of cell death) and the activation of caspase 1, which is an enzyme involved in the proteolytic maturation of the pro-inflammatory cytokines IL-1β, IL-18, and IL-33, which, by caspase-1 influence, are released in a non-classical secretion pathway (112). Production of IL-1β and IL-18 occurs in response against components of pathogen microorganisms (pathogen-associated molecular patterns, PAMPs) or danger signals from damaged and necrotic cells (danger-associated molecular patterns, DAMPs) that activate pattern recognition receptors (PRRs) expressed on the surfaces of macrophages, dendritic, and epithelial cells, among which are Toll-like receptors (TLRs) (113). After recognition of PAMPs or DAMPs by PRRs, adapter molecules are recruited, leading to NF-κB activation and, in consequence, the overexpression of genes that encode pro-inflammatory cytokines. For activation of caspase 1, the assembly of a complex integrated by a cytosolic sensor is necessary [a PRR that consists in a nucleotide-binding domain and leucine-rich-repeat-containing (NLR, also known as NOD-like receptor) or an AIM2-like receptor (ALR) protein], an adapter protein known as apoptosis-associated speck-like protein containing a CARD (ASC), which interacts with procaspase 1, connecting it with sensor that initiates self-cleavage of caspase 1 and the formation of its active form that is necessary for processing of immature cytokines (114).

Nevertheless, alteration of inflammasome is involved in the development of distinct diseases related to inflammatory disorders, for example, inflammatory bowel disease, Crohn's disease, vitiligo, periodontitis neurodegenerative diseases such as multiple sclerosis, Alzheimer's disease, and Parkinson's disease, as well as metabolic disorders that include atherosclerosis, insulin resistance, type 2 diabetes, and obesity (115). Besides the former illnesses, inflammasome also plays an important role in reproduction pathologies.

Recently, it has been demonstrated that human endometrium expresses inflammasome components, including the NLR family member NLRP3 (also identified as NALP-3) and ASC protein, which are significantly overexpressed in the endometrium of patients with recurrent pregnancy loss (without any evidence of infection) in contrast to healthy fertile women. In consequence, caspase 1 showed an increased activity and augmented levels of both IL-1β and IL-18 were detected, which probably led to an abnormal activation of uterine innate immunity that is involved in the establishment of a pro-inflammatory endometrial milieu, which would impede successful implantation, and provoke disturbance of placental development and pregnancy loss (116).

Inflammasome pathways could also be involved in the pathophysiology of endometriosis. Recently, to investigate the role of non-genomic activation of ER-β in this disease, a mouse model of surgically induced endometriosis was used through autotransplantation of fragments of endometrial tissue in the peritoneal cavity, both in mice with overexpression of ER-β (ERβ-OE) and in ERβ-null mice. Lesions in ERβ-OE mice had greater dimensions than in ERβ-null and control mice, confirmed by using a selective antagonist, suggesting an important participation of receptor in the progression of the disease (31).

In the same study, the authors determined interactions of ER-β with proteins involved in apoptosis, such as apoptosis signal-regulating kinase-1 (ASK-1), which participates in apoptosis induced by TNF-α. Nonetheless, ASK-1 has shown reduced activity in ERβ-OE mice; additionally, an ASK-1 interaction with serine/threonine kinase receptor-associated protein (STRAP) and 14-3-3 protein was observed, a complex that also interacts with ER-β; in turn, the STRAP/14-3-3 complex obstructs ASK-1/TNF receptor-associated factor 2 interaction, hence inhibiting TNF-α-mediated apoptosis.

In the same way, the interaction of ER-β with inflammasome components was determined, such as NLRP3 and caspase 1. The processed form (and therefore its activated form) of the latter along with IL-1β was found in high levels in endometriosis lesions of ERβ-OE mice. This is in accordance with previous reports of endometriosis in humans, in which it has been observed that endometriotic implants produce elevated concentrations of IL-1β, in contrast to eutopic endometrium or endometrium of healthy women; likewise, this increase is also observed in the peritoneal fluid of endometriosis patients (117, 118). In conclusion, the mechanism observed in this model of endometriosis progression indicates an interaction between the immune system and estrogen signaling through non-genomic ER-β activity. This interaction, in which ER-β cooperates with components of inflammasome machinery to increase IL-1β levels and to modulate apoptosis induced by TNF-α, activates a mechanism to ensure endometriotic cell survival, adhesion, and proliferation through evasion of immune surveillance and the epithelial–mesenchymal transition pathway (31). However, the interplay between non-genomic and genomic activities of ER-β, as well the participation of ER-α and other steroid receptors in inflammatory processes of endometriosis, remains to be determined. In addition, key components of inflammasome and their interactions with these receptors could be useful as drug targets, designed to interrupt downstream signaling pathways, thus affecting progress of the disease and maintaining it in stages easily controlled by using non-invasive therapies.

CONTRIBUTION OF INFLAMMATION OF BACTERIAL ORIGIN TO ENDOMETRIOSIS

Endometriosis is a multifactorial disease in whose development alteration of sex hormone pathways has a key role and bacterial infections of the female reproductive tract or alterations in its

microbiota composition seem to have significant participation. Inflammation in endometriosis has been related to the presence of bacteria and their products (metabolites or virulence factors) in the peritoneal cavity, which led researchers to propose the "bacterial contamination" hypothesis (119). Some observations indicate the presence of *Escherichia coli* in menstrual blood of patients diagnosed with endometriosis in contrast with control women; bacterial load correlates with elevated concentrations of LPS in menstrual blood and peritoneal fluid. In addition, the concentrations of HGF, VEGF, IL-6, and TNF-α were significantly higher in the culture media of peritoneal macrophages treated with LPS from *E. coli*; this effect was mediated through activation of TLR4; this receptor was also detected in stromal and gland cells from eutopic or ectopic endometrium from endometriosis patients (120). The authors suggested that resident Gram-negative bacteria of the vagina, including *E. coli*, could migrate to the upper reproductive tract and have an involvement in endometriosis by the accumulation of endotoxin in the menstrual/peritoneal fluid that would unchain pelvic inflammation, leading to growth of lesions. High concentrations of PGE_2 in peritoneal or menstrual fluids could be a factor that promotes bacterial survival since it stimulates bacterial growth *in vitro* and simultaneously causes impairment of the function of lymphocytes (121).

Taking these results as antecedent, using a mouse model of endometriosis, it was demonstrated that simultaneous administration of blood (simulating menstrual reflux that reaches pelvic cavity during retrograde menstruation) with LPS in mice, which were previously injected with endometrial implants, induces the growth of endometriotic lesions and the production of TNF-α, IL-6, and MIP-2 in peritoneal fluid (122), which in turn is related with NF-κB expression (123). An additive effect has been observed with the simultaneous treatment of macrophages with LPS and E2, which induced secretion of pro-inflammatory factors and proliferation of endometriotic cells (55). The above suggests a role of LPS in coordination with steroid hormones in the first stages of endometriosis development through induction of inflammatory responses and recruitment of neutrophils to endometriotic lesions, which in turn possibly participate in the release of angiogenic factors, unchaining a process of inflammatory angiogenesis (122). These observations have to be taken carefully since findings can be caused by an asymptomatic or subclinical vaginal infection or contamination with bacteria from cervicovaginal microbiota that contribute to bacterial contamination of analyzed samples (124).

On the other hand, the role of the microbiome in the pathogenesis of endometriosis has also been suggested; however, the role of this microbial community in endometrial function and the development of its pathologies remain unclear. In contrast to the general acknowledgment about uterine cavity as a sterile environment, recently by the usage of next-generation sequence tools in combination with culture techniques, it has been reported that endometrium has its microbiota (125); however, it is still debated whether the bacterial presence is due to contamination from the vaginal or cervical microbiome (126).

Most of the studies have reported the presence of members of phyla Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria, among others, as part of "health" uterine microbiota (127–129), and that changes of this diversity and its abundance are related to alterations in endometrial function (127, 130, 131), benign uterine pathologies (128, 132, 133), or endometrial cancer. In the case of endometriosis, changes of taxonomic bacterial composition to potentially pathogen bacteria in patients diagnosed with the disease have been detected in peritoneal fluid, cervical mucus, and cervicovaginal microbiota (129, 134, 135). However, studies that detect the real changes in endometrial microbiota related to endometriosis are lacking; furthermore, studies carried out to date in general lack a control group with healthy women (without any benign pathology) and face difficulties to obtain endometrial samples, among other technical complications (126, 134, 136).

On the other hand, although the mechanisms used by microbiota of the upper reproductive tract to regulate host functions are still unidentified, it has been suggested that there is a relation between uterine microorganisms and the uterine immune system, and it is speculated that bacteria interact with endometrium and control the expression of endometrial factors involved in inflammatory response, proliferation, apoptosis, expression of leukocyte subsets, infiltration of plasma cells, and secretion of immunoglobulins, which in turn regulates processes of decidualization, embryo implantation, pregnancy development, and protection against infections (116). More studies are necessary to confirm and characterize the composition and the role of microbiota on the physiology of the human female reproductive system, particularly in the endometrium, and its relationship with components of uterine microenvironment, as hormonal levels and metabolites are produced at the endometrium. In the future, this knowledge could enable the potential use of bacterial microbiota as a diagnostic tool and as a therapeutic target.

ASSOCIATION OF INFLAMMATION WITH INFERTILITY IN PATIENTS WITH ENDOMETRIOSIS

Chronic inflammation is strongly linked to alterations in female fertility, as occurs with infertility-associated endometriosis, where the produced "hostile" environment alters the sperm transport, tubal motility, and oocyte development (through impaired uterine peristalsis), as well as embryo implantation (137). At the uterine level, inflammation induces an impairment of endometrial decidualization as a response to progesterone resistance due to the alteration in the function of PR. Therefore, the expression of progesterone-responsive genes is dysregulated during the implantation window, which comprises a period of 8–10 days after the luteinizing hormone (LH) surge (mid-secretory phase) (11). Some of these altered genes encode biomarkers of endometrial receptivity (138), which are involved in embryo attachment and stimulation of decidua invasion, such as adhesion molecules (CAM family), cytokines, growth factors, and prostaglandins (11).

Decidualization occurs during the secretory phase of the menstrual cycle in response to ovarian hormones, conducive to differentiation and specialization of the endometrium, independent of the presence of a fertilized oocyte, initiating a series of profound changes that allow embryo implantation and is finished when the development of the placenta is complete (139). At the molecular level, changes reflected in gene expression, proteome, and secretome occur. In particular, two robustly secreted molecules are the hallmark of decidualization: insulin-like growth factor binding protein-1 (IGFBP-1) and prolactin (PRL) (140), whose expression is related to PR activity (141, 142). Both IGFBP-1 and PRL are drastically downregulated in the eutopic endometrium of women affected by endometriosis (143). In addition, IGFBP-1 and PRL also show a lower secretion by cultured endometrial stromal cells from women with endometriosis than those from healthy women (144).

CAM encompasses integrins, cadherins, selectins, and immunoglobulins, which typically are glycoproteins involved in cell-to-cell adhesion; during the implantation window, they lead to firm attachment of the blastocyst to the endometrial pinopods to guarantee successful implantation (138). Between CAMs is αvβ3 integrin, a molecule that suffers dynamic alterations over the course of the menstrual cycle, with a peak of expression at the mid-secretory phase. αvβ3 is activated upon binding to its ligand osteopontin, and forms aggregates that recruit cytoskeletal proteins and components of cell signaling pathways required for attachment of embryo. Endometrial αvβ3 integrin expression was significantly reduced in patients with endometriosis during the window of implantation (145), probably in response to local estrogen (146). The diminished αvβ3 expression is related to impaired production of the HOXA10 transcription factor (member of family homeobox, which is involved in the development and physiology of the uterus) that regulates expression of subunit β3 (147, 148). Reduced expression of HOXA10 is related to epigenetic changes, and there exists evidence of hypermethylation of its promoter and its consequent silencing in the eutopic endometrium of endometriosis patients, in comparison to healthy endometrium (149). Also, HOXA10 regulates IGFBP-1 expression, making it a key transcription factor during the process of uterine receptivity maintaining (150).

HOXA11 is another member of the homeobox family that is involved in diverse processes associated with embryo development and aspects of female reproductive tract physiology such as endometrial growth, differentiation, receptivity, and embryonic development. HOXA11 is detected at significantly lower levels (mRNA and protein) in the eutopic mid-secretory endometrium of infertile patients with endometriosis than in endometrium of fertile women. HOXA11 promoter is hypermethylated; this can be one of the possible molecular mechanisms causing a decrease in its expression. Obtained results indicate the relation of HOXA11 with infertility in endometriosis (151). A previous report supports this observation since HOXA11 seems to regulate PRL expression during decidualization (152). It is to be noted that regulation of expression of HOXA genes in healthy endometrium is mediated by sexual hormone action, mainly P4, showing a significant increase at the secretory menstrual phase (153, 154), which suggests that its dysregulation in endometriosis is also related to alteration in hormonal pathways.

Further transcriptional regulators involved in uterine receptivity are down-regulated in eutopic endometrial tissue from endometriosis patients. These include GATA Binding Protein 2 (GATA2), SRY-Box 17 (SOX17), Indian hedgehog signaling molecule (IHH), COUP transcription factor 2 (COUPTFII), Wingless-type MMTV integration site family (WNT4), Forkhead box O1 (FOXO1), AT-rich interaction domain 1A (ARID1A), and histone deacetylase 3 (HDAC3), most of which are involved in regulation of P4-responsive pathways (17).

Among other alterations observed in CAMs of eutopic endometrium, there is an altered regulation of the E-cadherin and β-catenin expression in epithelial cells of endometrium from infertile women with endometriosis during the mid-secretory phase, since both are expressed at increased levels (155). The β-catenin expression is associated with the Wnt pathway, which could be activated in endometriosis. In the fertile endometrium, increased E2 levels induce Wnt/β-catenin signaling to enhance proliferation, whereas P4 inhibits this signaling pathway during the secretory phase, reducing proliferation and promoting cell differentiation (156); however, in endometriosis, it is probable that P4 resistance and hyper-estrogenic milieu prevent inactivation of this pathway, thus promoting cell proliferation while inhibiting decidualization (155).

Glycodelin A, an immunomodulator glycoprotein regulated by P4 during the window of implantation, is downregulated in endometrium of women with endometriosis (143, 157). However, there are inconsistencies between published reports; for example, an analysis of expression reveals an increase in glycodelin A levels in the eutopic endometrium of patients with endometriosis in contrast to those from the endometrium of control group (158). This coincides with its elevated concentrations found in peritoneal fluid and serum of endometriosis patients compared to controls, in both proliferative and secretory cycle phases; importantly, values of concentration correlate with level of pain intensity (159). Differences could be attributed to the type of endometriotic lesion and the severity of the disease or ethnicity of the study population, even though general evidence suggests that glycodelin A has a significant participation in uterine receptivity and could be useful as a non-invasive biomarker of endometriosis.

Among altered cytokines, leukemia inhibitory factor (LIF), a member of IL-6-like family E2-responsive, essential for blastocyst implantation, also shows reduced expression in infertile patients with endometriosis, especially at moderate stages of the disease (160, 161). Another IL-6-like family member required for embryo implantation, IL-11, and its receptor, are absent in the glandular epithelium of endometrium from endometriosis patients, in contrast to fertile women (161). Another cytokine involved in endometrial infertility is IL-1β, which can alter the differentiation of stromal endometrial cell by causing disruption of decidual function through cellular depletion of ER-α, PR, and gap junction

alpha-1 protein (also known of connexin 43 or Cx43); inhibition of IL-6-mediated extracellular signal-regulated kinase (ERK)1/2 pathway recover decidualization markers as well as production of steroid receptors (162).

Among other receptivity markers altered in endometriosis are the transcriptional gene repressor BLC6 and the histone deacetylase sirtuin 1 (SIRT1), whose co-expression is promoted by pro-inflammatory environment in eutopic endometrium from endometriosis patients; both are used for the diagnosis of disease. BLC6 and SIRT1 overexpression (induced by Kirsten rat sarcoma viral oncogene, KRAS) alter the actions of P4 by suppression of the promoter of *GLI1*, a critical mediator of progesterone action in the Indian Hedgehog pathway, contributing to the pathophysiology of this disease and infertility through the increase of P4 resistance (163).

Overall, receptivity endometrial markers show a strong interaction with components of pro-inflammatory and hormonal environment, which influence negatively their expression and function and hence contribute to impede a successful pregnancy in endometriosis patients. In order to preserve women's fertility and to promote effectiveness of *in vitro* reproduction techniques, it is necessary to restore the adequate expression and production of molecules involved in uterine receptivity, through safe and personalized immunomodulatory therapies that reduce or avoid surgical interventions and their adverse effects. Moreover, it is necessary to establish an integral management of the disease according to individual requirements of each patient, which encompasses non-invasive diagnosis and medical and psychological therapies, through the interactions of researchers in basic molecular biology with distinct clinical specialists.

CURRENT AND FUTURE TREATMENTS FOR INFLAMMATION ASSOCIATED WITH ENDOMETRIOSIS

The treatment more extensively used for endometriosis is hormone modulation by progestins that can bind PR and regulate its activity, thus suppressing ovulation, provoking amenorrhea, and a hypo-estrogenic environment; however, not all the patients that receive the treatment have benefited from it. Another common treatment is the inhibition of production of prostaglandins, through a COX or an aromatase inhibitor, for example, a non-selective and non-steroidal anti-inflammatory drug such as ibuprofen or naproxen, which significantly reduce symptoms of disease, mainly pelvic pain. However, they can produce secondary effects such as the development of osteopenia, osteoporosis and bone fractures, increased cardiovascular risk, and negative gastrointestinal effects (6, 53). To ensure the efficiency of treatment and avoid undesirable secondary effects, the compound used should be effective on specific targets of distinct affected pathways simultaneously and ideally originated from natural sources. In this section, we describe selected potential drugs and alternative treatments with these characteristics, which are recently reported in the literature.

Resveratrol, a polyphenolic compound with anti-proliferative and anti-inflammatory actions, found in many dietary sources, is an alternative for endometriosis treatment (164). In a rat model with experimentally induced endometrial implant, resveratrol significantly reduced implant areas, while it decreased serum and peritoneal levels of VEGF and MCP-1. For this reason, resveratrol is considered a potential novel therapeutic agent that acts by inhibition of angiogenesis and inflammation (165).

Crocin is a vegetal compound found in some flowers and saffron, and it possesses anti-inflammatory and anti-proliferation properties. In a mice model of endometriosis with crocin treatment, aspects of inflammation, angiogenesis, growth of lesions, as well as endothelial apoptosis and proliferation were evaluated. Crocin avoids the growth of the lesion through inhibition of proliferation without causing apoptosis, as well as reducing the expression and secretion of inflammatory cytokines INF-γ, TNF-α, and IL-6, as well as VEGF and proliferating cell nuclear antigen (PCNA) (166).

Another natural compound is nobiletin, a flavonoid isolated from citrus peels with capacity for inhibiting NF-κB activation. A recent study in mice shows that administration of nobiletin significantly reduced lesion size and expression of PCNA, VEGF, E-cadherin, IL-6, IL-1β, TNF-α, MMP-1, and MMP-3; this observation was related with the inactivation of NF-κB through targeting the activity of IκB kinases (167).

Recently, molecules isolated from sources used in traditional medicine have also been reported; this is the case of dehydrocostus lactone from the roots of *Aucklandia lappa*, used in Asiatic traditional medicine for the treatment of diseases associated to inflammation and pain. This compound inhibited Akt and NF-κB pathways in endometriotic cells and macrophages. Dehydrocotus lactone caused apoptosis in endometriotic cells by activation of caspase-3, -8, and -9, and decreased the production of PGE_2 and neurotrophins, while that in macrophages induced the decrease of IL-10, VEGF, MMP-2, and MMP-9 (168).

On the other hand, therapies can be implemented by using existent drugs used to treat other inflammatory diseases. Simvastatin, which belongs to the statins family, was assayed in a baboon model of endometriosis. Treatment with simvastatin induced ER-α expression while provoking a reduction of ER-β in lesions and eutopic tissue. Furthermore, simvastatin significantly reduced the expression of neopterin, a marker of inflammation, oxidative stress, and immune system activation. Collectively, the present findings indicate that the inhibition of the mevalonate pathway by simvastatin reduces the risk of developing endometriosis in the primate model of this disease by decreasing the growth of endometrial lesions, by modulating the expression of genes encoding for estrogen receptors, and by reducing inflammation (169).

Regardless of the finding of new potential therapies for endometriosis treatment, it is necessary to perform more studies to determine the effectiveness of therapeutical agents in women and to assess possible side effects on reproductive tract function.

Additionally, these discoveries can enhance the activity of traditional drugs if used simultaneously with them.

CONCLUSION

Endometriosis is a complex gynecological disease characterized by a chronic inflammatory process that is strongly linked to altered sex hormonal-dependent pathways, mainly through steroid hormone receptor function (**Figure 1**). According to current evidence, its origin is multifactorial, involving molecular, biochemical, and cellular alterations that in turn are interconnected and possibly related to responses to the external stimuli; however, it is not yet determined whether these alterations are the consequence or the effect of the disease. It is necessary to elucidate the key components of each of the involved pathway in the development of endometriosis, particularly those related to inflammation, responsible for the activation of other important cellular pathways involved in the development and maintaining of the endometriotic lesions, which in turn contribute to the maintenance of the inflammatory milieu. Further studies are still necessary for elucidating the pathogenesis of the disease, by translating animal and *in vitro* models to clinical studies that mimic the disease in humans the nearest. This knowledge will allow to determine the ideal targets for developing novel therapies to treat endometriosis effectively, through recovery of altered cell functions and that at the same time avoid recurrence of the implants or undesirable secondary effects. In turn, these targets ideally must be accurate non-invasive biomarkers for the diagnosis and classification of the disease. For this end, it is imperative to develop biomarker panels that ideally must contain individual molecules involved in interconnected pathways, as well as molecular complexes or bacterial signatures, which in turn will contribute to the selection of personalized treatments according to disease particularities and/or fertility desire in each patient, looking to improve her life quality.

AUTHOR CONTRIBUTIONS

EG-G, EV-M, and MC designed the concept. EG-G and CR-M wrote the first draft of the manuscript. IC-A and OC-O revised and corrected the first version of the text. All authors read, revised, and approved the manuscript for publication.

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REVIEW
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Role of the NLRP3 Inflammasome in Preeclampsia

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Reproduction involves tightly regulated series of events and the immune system is involved in an array of reproductive processes. Disruption of well-controlled immune functions leads to infertility, placental inflammation, and numerous pregnancy complications, including preeclampsia (PE). Inflammasomes are involved in the process of pathogen clearance and sterile inflammation. They are large multi-protein complexes that are located in the cytosol and play key roles in the production of the pivotal inflammatory cytokines, interleukin (IL)-1β and IL-18, and pyroptosis. The nucleotide-binding oligomerization domain, leucine-rich repeat-, and pyrin domain-containing 3 (NLRP3) inflammasome is a key mediator of sterile inflammation induced by various types of damage-associated molecular patterns (DAMPs). Recent evidence indicates that the NLRP3 inflammasome is involved in pregnancy dysfunction, including PE. Many DAMPs (uric acid, palmitic acid, high-mobility group box 1, advanced glycation end products, extracellular vesicles, cell-free DNA, and free fatty acids) are increased and associated with pregnancy complications, especially PE. This review focuses on the role of the NLRP3 inflammasome in the pathophysiology of PE.

Keywords: NLRP3 inflammasome, pregnancy, preeclampsia, interleukin-1β, inflammation

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INTRODUCTION

Reproduction, including development of oocyte and sperm, ovulation, corpus luteum function, fertilization, implantation, placentation, maintenance of pregnancy, and parturition, is essential for species maintenance, and reproductive events for next generation are tightly regulated (1). Pregnancy has been studied extensively over the years (2). From the perspective of the maternal immune system, a conceptus is a semi-allogeneic tissue that must be rejected; however, that does not generally happen. It was quickly ruled out that the fetus is shielded from the maternal immune system via the placenta acting as a physical barrier because the fetal extravillous trophoblast cells deeply penetrate the uterine mucosa and directly communicate with various maternal immune cells to avoid rejection (3).

Inflammation is basically a complex protective immune response to harmful stimuli such as pathogens, damaged or dead cells, and irritants (4). This response is tightly regulated by the host, enabling survival after infection or injury and maintaining tissue homeostasis. However, excessive inflammation may cause chronic or systemic inflammatory diseases. On the other hand, the immune system also contributes to the regulation of reproductive function and pregnancy (5). Immune-mediated processes such as tissue growth, remodeling, and differentiation are crucial to maintain pregnancy (1, 5). Disruption of well-controlled immune functions leads to infertility, placental inflammation, and numerous pregnancy complications, such as preeclampsia

(PE), obesity during pregnancy, gestational diabetes mellitus (GDM), spontaneous abortion, and recurrent pregnancy loss (6–8).

There is an increasing body of evidence to suggest that inflammation and immune cells are involved in both physiology and pathophysiology of pregnancy. Since infection is not involved in the majority of the phenomena related to pregnancy physiology and pathology, it remains unclear why inflammation is involved. Recently, there have been numerous reports of inflammasome mechanisms that control sterile inflammation involved in pregnancy pathologies. Inflammasomes are large multi-protein complexes found in the cytosol that play key roles in the production of the pivotal inflammatory cytokines, interleukin (IL)-1β and IL-18, and pyroptosis (inflammatory cell death) [(9–11); **Figure 1**]. In particular, nucleotide-binding oligomerization domain, leucine-rich repeat-, and pyrin domain-containing 3 (NLRP3) inflammasome is a key mediator of sterile inflammation. Excessive activation of the NLRP3 inflammasome contributes to the pathogenesis of a wide variety of diseases, such as diabetes, atherosclerosis, and obesity-induced insulin resistance (12–17). The present review focuses on the role of the NLRP3 inflammasome in placental inflammation and pregnancy complications, especially PE.

IMMUNE CELLS INVOLVED IN PREGNANCY

The most important immune cells that induce pregnancy immune tolerance is CD4+ regulatory T cells (Tregs) (18). The transcription factor, forkhead boxP3 (Foxp3), is a master regulator of the development and function of Tregs (19). The frequency of Foxp3+Tregs increases during normal pregnancy in the decidua and peripheral blood in humans and mice (20–22). Shima et al. (23) used an animal model to demonstrate that $CD4^+CD25^+Foxp3^+$ Tregs play a critical role in regulating immune tolerance at the implantation site to support implantation and successful pregnancy. The frequency of Tregs is lower in human pregnancy complications such as PE or miscarriage (24). In addition, seminal fluid induces and accumulates paternal-specific Tregs that are involved in the preimplantation uterus, and insufficient expansion of Tregs against paternal antigens may trigger spontaneous abortion (25).

Natural killer (NK) cells, particularly decidual NK cells, are also essential immune cells involved in establishing pregnancy; they are the most abundant leukocyte population during the first trimester of human pregnancy (1, 26). Decidual NK cells directly communicate with extravillous trophoblast cells and other immune cells in the fetal-maternal boundary area, and promote fetal tolerance and pregnancy progression (26).

Monocytes also accumulate in the decidua, in a process that involves communication with trophoblast cells (1, 27). They can differentiate into dendritic cells (DCs) in the decidua during murine and human pregnancy (28, 29). DCs regulate immune tolerance by inducing effector T cell apoptosis and expansion of Tregs due to reduced antigen presentation, reduced expression of co-stimulatory molecules, or enhanced production of anti-inflammatory IL-10 (1, 30). Monocytes also differentiate into macrophages depending on the tissue, and polarization of macrophages is well-understood (inflammatory M1 and anti-inflammatory M2 type macrophages). It has been suggested that dysfunction of decidual macrophages and dysregulation of M1/M2 balance are critical events in the pathogenesis of PE. Moreover, activation of NLRP3 inflammasome in the reproductive organs including placenta is known to occur by these macrophages.

MECHANISMS OF NLRP3 INFLAMMASOME ACTIVATION

Inflammasomes recognize various inflammation-inducing stimuli, such as endogenous danger/damage-associated molecular patterns (DAMPs) and exogenous pathogen-associated molecular patterns (PAMPs). They tightly regulate the production of proinflammatory cytokines such as IL-1β and IL-18 (9, 13, 31). The NLRP3 inflammasome is the most widely studied and is activated in response to a wide array of stimuli, including exogenous and endogenous danger signals [(9, 11); **Figure 1**]. The NLRP3 inflammasome is typically composed of NLRP3, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), and caspase-1 as an IL-1β-converting enzyme (32). Activation of NLRP3 in response to danger signals leads to nucleation of ASC into prion-like filaments via pyrin domain (PYD)–PYD interactions (33). ASC is then linearly ubiquitinated for NLRP3 inflammasome assembly, followed by procaspase-1 interaction with ASC using caspase recruitment domain (CARD)-CARD interactions, forming its own prion-like filaments (34). Activated caspase-1 (a cysteine protease) cleaves the precursor cytokines, pro-IL-1β and pro-IL-18, generating the biologically active cytokines, IL-1β and IL-18, respectively (9–11). Moreover, active caspase-1 is able to induce pyroptosis as an inflammatory form of cell death due to cleaved gasdermin D (GSDMD) (35, 36). Caspase-1 proteolytically cleaves GSDMD into a N-terminal domain and C-terminal domain. Cleaved N-terminal domain of GSDMD binds to phosphatidylinositol phosphates and phosphatidylserine in the cell membrane, forming a 10–20 nm pore and induces a lytic form of cell death, pyroptosis (36). Another feature of gasdermin D-dependent pyroptosis is the release of IL-1β and IL-18 via GSDMD-forming cell membrane pore.

The production and secretion of mature IL-1β are regulated via two steps, including the transcription of pro-IL-1β and proteolytic processing into a mature form IL-1β by inflammasomes (9–11). Prior to its activation, NLRP3 must be primed in most cell types. Nuclear factor κB (NF-κB)-activating stimuli, such as lipopolysaccharide (LPS), upregulate mRNA expression of *NLRP3* and *IL-1β*, resulting in elevated expression of NLRP3 and pro-IL-1β protein (9–11). On the other hand, another priming step facilitates the rapid induction of the NLRP3 inflammasome via deubiquitination of NLRP3 (37, 38).

The upstream mechanisms of NLRP3 activation have been elucidated by many studies, and include the release of cathepsins into the cytosol after lysosomal destabilization,

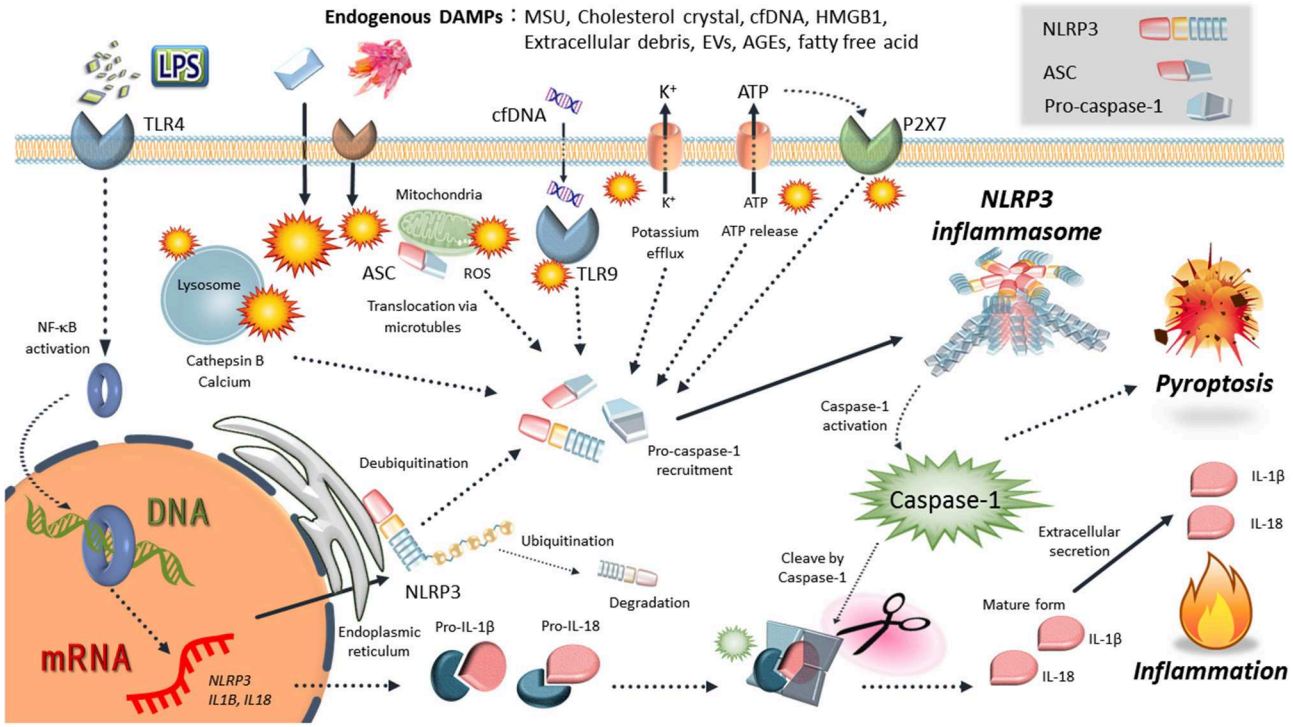

FIGURE 1 | Schematic mechanisms of NLRP3 inflammasome activation. NLRP3 is activated by various endogenous DAMPs: uric acid crystals (MSU), cholesterol crustal, cell-free DNA (cfDNA), high-mobility group box 1 (HMGB1), extracellular debris, extracellular vesicles (EVs), advanced glycation end-products (AGEs), and free fatty acid. Various events such as intracellular ATP release, NLRP3 deubiquitination, relocalization, reactive oxygen species (ROS) generation, mitochondrial dysfunction, lysosome rapture, and cathepsin release occur depending on the effects of damage-associated molecular patterns (DAMPs). Then, inflammasome components, including NLRP3, ASC, and procaspase-1, form the NLRP3 inflammasome complexes. Finally, activated caspase-1 induces the inflammatory form of cell death known as pyroptosis and cleaves the precursor cytokines pro-IL-1β and pro-IL-18, generating the biologically active cytokines IL-1β and IL-18.

potassium efflux, generation of mitochondrial reactive oxygen species (ROS), and release of mitochondrial DNA (39, 40). Cytosolic leakage of cathepsin B via lysosomal rupture is essential for NLRP3 inflammasome activation, especially by endogenous DAMPs (41). Leakage of cathepsin B also leads to potassium efflux and mitochondrial damage. Potassium efflux and reduced potassium concentration within cells result in NLRP3 inflammasome activation (10). In response to potassium efflux, NEK7 (a member of the family of mammalian NIMA-related kinases) directly interacts with NLRP3 inflammasome (42, 43). Cellular and mitochondrial ROS production also act as NLRP3 inflammasome activators (44, 45). Furthermore, recent studies have demonstrated that the NLRP3 inflammasome is tightly regulated by multiple mechanisms, including ubiquitination, phosphorylation, nitrosylation, microRNAs, and endogenous regulators (e.g., pyrin-only proteins and CARD-only proteins) (9, 46–48).

Following NLRP3 activation through the above mentioned regulatory mechanisms, NLRP3 relocates from endoplasmic reticulum to the mitochondria, where it forms complexes with ASC (49). IL-1β and IL-18 secretion is regulated by caspase-1 activation by many NLRP3 inflammasome activators, including monosodium urate (MSU) crystals, silica crystals, asbestos, and cholesterol crystals (12, 13, 31, 50). Additionally to the canonical pathway of the NLRP3 inflammasome, the inflammasome activation can also be indirectly triggered by caspase-11 in mice (or the homologs caspase-4 and caspase-5 in humans), which has been termed the non-canonical inflammasome pathway (51). In this non-canonical pathway, caspase-11 directly recognized and binds to intracellular LPS, resulting in its oligomerization and activation by autoproteolytic cleavage (35). Then, caspase-11 can directly induce the cleavage of GSDMD to induce pyroptosis (35, 36). Details of the structure and activation mechanism of the NLRP3 inflammasome are refer to following great reviews (10, 17, 39, 40, 52).

PREECLAMPSIA AND THE NLRP3 INFLAMMASOME

PE is a pregnancy-specific hypertensive syndrome that complicates around 5–10% of all pregnancies worldwide (53), and is a leading cause of maternal and fetal morbidity and mortality. It is characterized by the onset of hypertension and proteinuria in the third trimester of pregnancy, and is associated with 12% of infants with fetal growth restriction (FGR) and approximately 20% of preterm deliveries (54). The clinical manifestations of PE reflect widespread systemic inflammation

and endothelial dysfunction, resulting in vasoconstriction, end-organ ischemia and increased vascular permeability (55). The placenta has been shown to play a central role in the pathogenesis of PE due to the rapid disappearance of disease symptoms after delivery. Thus, placenta-derived circulating factor(s) may induce excessive inflammation and endothelial defects, leading to PE (56).

During normal pregnancy, trophoblast cells invade, and remodeling of maternal spiral arteries and the fetoplacental unit produce angiogenic factors, such as vascular endothelial growth factor (VEGF) and placental growth factor (PlGF), to support the developing placenta (57, 58). Inadequate trophoblast remodeling of spiral arteries, which is a key feature of PE, is believed to result of dysregulation in placental angiogenesis and maternal immune response (55). Following that, various inflammatory factors are produced by the diseased and hypoxic placenta, which activates systemic inflammatory responses (27, 59). It is widely recognized that antiangiogenic factors, including soluble endoglin (sEng; a coreceptor for transforming growth factor β) and soluble fms-like tyrosine kinase (sFlt-1; a receptor for VEGF), induce PE-like phenomena (57, 60). Indeed, overexpression of sEng and sFlt-1 in pregnant rats leads to severe PE symptoms including hypertension, proteinuria, renal and endothelial dysfunction, hemolysis, elevated liver enzymes, and FGR (60).

Pathophysiological changes of PE include inflammation and immune cell activation (61–63). The main pathological features of PE include a general inflammatory response by cytokines, such as IL-1β, IL-6, IL-8, and tumor necrosis factor-α (TNFα) (7, 64, 65). Siljee et al. (66) reported that IL-1β has a potential to improve prediction of PE during the first trimester. A decreased frequency of peripheral Tregs is characteristic immune cell dynamics seen in PE patients (6). On the other hand, M2-like immunomodulatory macrophages are abundantly present in the decidua in healthy pregnant women and participate in spiral artery remodeling via the angiogenic factors, VEGF and PlGF (27). Increased numbers of M1-like inflammatory macrophages are observed in PE patients and may be associated with increase in inflammatory cytokines, decreased spiral artery remodeling, and increased production of sFlt-1 and sEng (27).

In recent years, there has been a rapid increase in reports that the NLRP3 inflammasome is involved in the pathogenesis of PE (**Figure 2**). Higher expression of components of the NLRP3 inflammasome has been reported in peripheral blood mononuclear cells and placental tissue from PE patients compared with that of healthy normal pregnant women (67–69). In addition to immune cells, human trophoblast cells express NLRP3, ASC and caspase-1 that are components of the NLRP3 inflammasome (70–72). IL-1β secretion is induced in response to nigericin or nano-silica crystals, typical activators of the NLRP3 inflammasome, in human trophoblast cells (71, 72).

HYPERTENSION AND THE NLRP3 INFLAMMASOME IN PE

Maternal hypertension is a characteristic of PE and the renin–angiotensin system has been implicated in its pathogenesis of PE (73, 74) generated a mouse model of PE-like symptoms by mating females expressing human angiotensinogen with males expressing human renin, resulting in mice exhibiting maternal hypertension, proteinuria, and FGR. Angiotensin II (AngII) is a strong vasoconstrictor that contributes to hypertension and stimulates sFlt-1 production and secretion from the placenta in mice (75). Infusion of AngII in pregnant mice can lead to high maternal blood pressure, proteinuria, and FGR (75, 76). Deficiency of NLRP3 inflammasome components attenuates the development of AngII-induced hypertension, but does not affect FGR, proteinuria, or sFlt1 levels (76).

Furthermore, during non-pregnant conditions, infusion of AngII induces hypertension with activation of NLRP3 inflammasome in the aorta, and NLRP3 deficiency attenuated AngII-induced hypertension via inhibition of NLRP3 inflammasome activation in mice (77). A murine experimental hypertension model (uninephrectomy and treatment with deoxycorticosterone acetate and 0.9% NaCl in the drinking water) induced activation of the NLRP3 inflammasome in kidney and specific NLRP3 inhibitor, MCC950, inhibited the NLRP3 inflammasome and inflammation, resulting in improvement of hypertension in mice (78). In rats, salt-induced hypertension occurs partly due to the role of NLRP3 inflammasome activation in the hypothalamic paraventricular nucleus, while blockade of brain NLRP3 attenuates the hypertensive response (79). An absence of ASC also reduces pulmonary hypertension induced by hypoxia (80). These findings suggest that the NLRP3 inflammasome contributes to the development of hypertension in both pregnant and non-pregnant situations. On the other hand, NLRP3 inflammasome has been shown to contribute to a wide range of acute and chronic kidney diseases (81); the importance of NLRP3 inflammasome in renal pathologic abnormalities in PE pathology is not well-understood.

ACTIVATION OF NLRP3 INFLAMMASOME BY DAMPS IN PE

Release of DAMPs from various cells during stress has been implicated in pregnancy complications. In PE patients, many DAMPs, such as, cholesterol, uric acid crystals (MSU), extracellular DNA, high-mobility group box 1 (HMGB1), extracellular cell debris, advanced glycation end-products (AGEs), and free fatty acids, have been detected in higher levels in the peripheral blood and placenta (**Figure 2**) and act as NLRP3 inflammasome activators.

CHOLESTEROL AND THE NLRP3 INFLAMMASOME IN PE

Cholesterol crystals activate inflammatory responses and promote inflammatory cell infiltration, resulting in progression of atherosclerosis and development of cardiovascular disease (16, 82). Cholesterol crystals also cause lysosome rupture, resulting in the release of cathepsin B to the cytosol, and are a candidate activator of the NLRP3 inflammasome (82, 83).

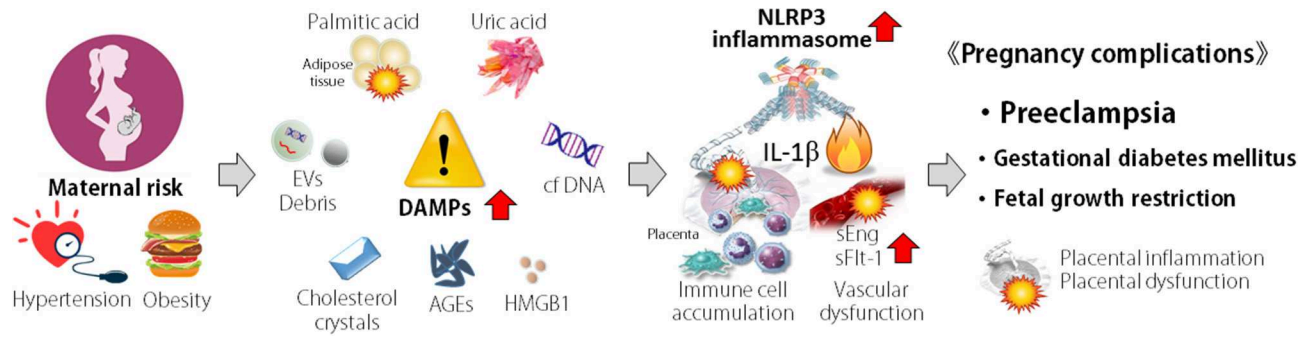

FIGURE 2 | Concept of association of NLRP3 inflammasome in pathogenesis of pregnancy complications. Maternal risk such as hypertension and obesity are associated with the elevation of damage-associated molecular patterns (DAMPs). DAMPs activate NLRP3 inflammasome, accumulate immune cells, and induce inflammatory cytokine production and vascular dysfunction in placenta. These events result in placental inflammation and dysfunction, leading to pregnancy complications, such as preeclampsia, spontaneous abortion, recurrent pregnancy loss, and fetal growth restriction.

Maternal cholesterol serum levels are elevated in PE and cholesterol accumulates in placenta of PE patients, along with increased levels of NLRP3 and IL-1β expression (84, 85). In an *in vitro* human placental explant experiment, treatment with cholesterol crystals significantly increased the release of IL-1β, and cholesterol crystal-induced IL-1β secretion was suppressed by treatment with MCC950, as a specific inhibitor of the NLRP3 inflammasome (84). Cholesterol crystals also strongly activated the NLRP3 inflammasome in macrophages and induced IL-1β secretion, dependent on activation of the NLRP3 inflammasome (82, 83, 86). In addition to macrophages, cholesterol crystals markedly increase the formation and activation of NLRP3 inflammasome in endothelial cells, as demonstrated by increased colocalization of NLRP3 with ASC or caspase-1, enhanced caspase-1 activity, and elevated IL-1β secretion in mice (87). These findings indicate that cholesterol induces placental inflammation via the NLRP3 inflammasome pathway in human placenta, suggesting the contribution of enhanced NLRP3 inflammasome activation to harmful placental inflammation in PE.

MSU AND THE NLRP3 INFLAMMASOME IN PE

Saturation of uric acid in body fluids results in the formation of MSU crystals. These are identified as danger signals from dying cells, resulting in an acute and/or chronic inflammatory response known as gout, which is associated with the deposition of MSU crystals (41, 88) demonstrated that MSU crystals activate the NLRP3 inflammasome, resulting in the production of active IL-1β and neutrophil accumulation in mice, suggesting a pivotal role for inflammasomes in inflammatory diseases. In terms of the mechanisms of NLRP3 inflammasome activation, MSU crystals are taken up by phagocytosis and lysosomal damage is induced, resulting in the release of cathepsin B and stimulation of ROS production from mitochondria (89).

Elevated levels of MSU in the maternal circulation have been shown in many pregnancy complications, especially PE (69, 84, 90, 91). In human first trimester trophoblast cell lines, IL-1β was produced in response to MSU crystals via the NLRP3 inflammasome (91). Brien et al. (91) reported that MSU crystals induce a proinflammatory profile with predominant secretion of IL-1β and IL-6 in human placental explants, and these effects were IL-1-dependent, as confirmed using a caspase-1 inhibitor and IL-1 receptor antagonist. In addition, administration of MSU crystals to pregnant rats induced placental inflammation (increase IL-1β, IL-6, and TNFα production, and macrophage accumulation) and FGR. Indeed, MSU crystals elicit an increase in the recruitment of macrophages and neutrophils with IL-1β secretion in the NLRP3 inflammasome-dependent manner (41, 92). These findings suggest that higher levels of MSU in PE patients trigger placental inflammation via NLRP3 inflammasome activation, resulting in the pathogenesis of PE.

EXTRACELLULAR DNA AND THE NLRP3 INFLAMMASOME IN PE

Extracellular released cell-free DNA (cfDNA) circulating in the blood, which is considered a product of apoptosis and/or necrosis, acts as a DAMP and is related to many types of inflammatory diseases (93, 94). Toll-like receptor 9 (TLR9), originally identified as a sensor of exogenous DNA fragments, contributes to cfDNA-mediated inflammatory processes (95). It is activated by bacterial DNA rich in unmethylated CpG motifs, and can also be activated by DNA from mammalian cells such as nucleic and mitochondrial DNA. Therefore, TLR9 signaling is of interest as a candidate molecule responsible for the first signal in sterile inflammation (96). It was previously reported that cfDNA released from apoptotic hepatocytes activates TLR9 systems, which in turn triggers a signaling cascade that increases transcription of the genes encoding pro-IL-1β and pro-IL-18. Furthermore, mice lacking components of the NLRP3 inflammasome showed reduced amounts of cfDNA and improved liver injury (96). Pan et al., reported that mitochondrial

DNA is directly recognized and binds with NLRP3, resulting in the formation of NLRP3 inflammasome complex and its activation (97).

During pregnancy, the amount of total cfDNA and cf-fetal DNA (cffDNA) is significantly increased in the maternal blood depending on the stage of pregnancy (98). There are also significant associations between elevated cfDNA and cffDNA with pregnancy complications such as PE and FGR (98–104). We recently showed that expression levels of TLR9 and the amount of cffDNA from the placenta were higher in PE patients compared with that in women with normal placenta (NP), and PE-derived cffDNA stimulated levels of inflammatory cytokine, including IL-1β and sEng secretion depend on TLR9 signaling, compared with NP-derived cffDNA (105). Moreover, a synthetic TLR9 ligand activated inflammatory responses including IL-6 secretion together with stimulation of sFlt1 secretion, while inhibition of TLR9 reduced sFlt1 secretion in human trophoblast cells (106). In mice, administration of a TLR9 ligand induces PE-like symptoms, such as hypertension, proteinuria, placental inflammation, and FGR. Moreover, injection of human fetal DNA, but not adult DNA, induces placental inflammation, fetal resorption, and preterm birth in pregnant mice, and notably, these adverse effects are improved in TLR9-knockout mice (107). These findings suggest that excessive extracellular DNA acts as a DAMP and causes pregnancy complications, especially PE, via TLR9 signaling.

In trophoblast cells, cfDNA is also capable of detecting danger signals via the intracellular DNA sensor, interferon-inducible protein 16 (IFI16). Indeed, IFI16 agonist poly(dA:dT) stimulates sFlt-1 and sEng production in human trophoblast cells in an IFI16-dependent manner (108). Extracellular DNA plays an essential role in the induction of inflammatory responses; however, further research is required to clarify the role of extracellular DNA in NLRP3 inflammasome activation in pregnancy complications.

HMGB1 AND THE NLRP3 INFLAMMASOME IN PE

HMGB1 is an important DAMP that acts as an architectural chromatin-binding factor and is generally located in the nucleus of most cell types under physiological conditions (109). When cells are exposed to stress, HMGB1 is translocated into the extracellular milieu and elicits inflammatory responses via the production of proinflammatory mediators and accumulation of inflammatory cells. HMGB1 interacts with TLR2, TLR4, and receptor for AGE (RAGE), resulting in elevated levels of HMGB1 in tissues and serum associated with the development of inflammation during pathological conditions (110). It is reported that HMGB1 induces the formation of the NLRP3 inflammasome (111). HMGB1 also activates the NLRP3 inflammasome since that stimulation with HMGB1 induces the release of IL-1β with increase in NLRP3 inflammasome component, these effects can be attenuated by inhibition of the NLRP3 inflammasome (112). In addition, Deng et al. (113) demonstrated that HMGB1 directly binds LPS and targets its internalization into the lysosomes of cells via the RAGE, resulting activation of caspase-11-dependent non-canonical inflammasome signaling. On the contrary, NLRP3 inflammasome activation accelerates atherosclerosis induced by HMGB1 secretion, indicating that HMGB1 is a key downstream signaling molecule of NLRP3 inflammasome activation (114). Therefore, the vicious cycle of HMGB1 and the NLRP3 inflammasome may exacerbate inflammation and pathological conditions.

In peripheral blood, HMGB1 concentrations are significantly elevated in PE patients compared with those of healthy pregnant and non-pregnant women (115, 116). Compared with healthy placenta, protein and mRNA expression of HMGB1 and its receptor RAGE, are increased in severe PE placentas (116). In human trophoblast cells, HMGB1 stimulates inflammatory cytokine production dependent on NF-κB activation and ROS signaling via TLR4 (117). In human placenta, treatment with PE serum increased the expression and release of HMGB1, which induced endothelial cell activation (118). In addition, HMGB1 treatment increased NLRP3 protein expression and activation of caspase-1, resulting in an increase of mature IL-1β secretion in human chorioamniotic membranes (119). These findings indicated that HMGB1 contributes to placental inflammation and NLRP3 inflammasome activation as endogenous DAMPs, leading to PE. Interestingly, the expression levels of HMGB1 in the uterus are lowest during the expected time of implantation, and exogenous administration of HMGB1 leads to pregnancy failure accompanied by induction of inflammatory responses in rats, indicating a role of excessive extracellular HMGB1 in PE as well as infertility (120).

PLACENTAL DEBRIS AND THE NLRP3 INFLAMMASOME IN PE

The outer layer of the placenta is covered by a single syncytiotrophoblast that forms the maternal-fetal interface (1). When portions of the syncytiotrophoblast become damaged, cellular debris is extruded into the maternal blood in membrane-enclosed vesicles (121). During normal healthy pregnancy, trophoblastic debris is produced by programmed cell death/apoptosis in the placenta. This extracellular debris is believed to induce a tolerogenic response in maternal endothelial and immune cells (122). On the other hand, extracellular debris from PE placenta mainly originates from necrotic cell death, and exposing endothelial cells to necrotic trophoblastic debris leads to their activation (123). The amount of trophoblastic debris shed into the maternal blood is greatly increased in PE patients compared with that in healthy pregnant women (108).

It is likely that trophoblastic debris includes various types of danger signals, such as DNA, RNA, adenosine, HMGB1, and MSU (118, 124). The degree of trophoblastic debris from human placenta is increased by treatment with PE serum and antiphospholipid antibodies, resulting in the activation of endothelial cell activation and induction of immune cell adhesion (118). Interestingly, necrotic, but not apoptotic, trophoblastic debris contains IL-1β protein, whereas much of the trophoblastic debris is dead cell corpses that might not be able to produce new

proteins (124). On the other hand, adenosine in trophoblastic debris and cell surface adenosine receptor A2B signaling also contributes to the pathogenesis of PE (125). Iriyama et al. (125) demonstrated that chronically elevated placental adenosine leads to the hallmark features of PE (hypertension, proteinuria, and FGR) in a mouse model. Moreover, elevated adenosine in PE patients is correlated with Th1/Th2 imbalance, and adenosine directly induces sFlt-1 production from placenta (126). Baron et al. (127) showed that extracellular adenosine activates the NLRP3 inflammasome and IL-1β secretion by interaction with adenosine receptors and through adenosine cellular uptake using nucleotide transporters. These findings suggest that adenosine signaling in debris activates NLRP3 inflammasome in placenta, resulting in PE.

EXTRACELLULAR VESICLES AND THE NLRP3 INFLAMMASOME IN PE

Extracellular vesicles (EVs) are also produced and released by living cells and can be detected in all biological fluids, including blood. EVs are nanosized particles that are traditionally classified into subtypes, such as exosomes, microvesicles, and apoptotic/necrotic bodies (debris). EV cargo includes bioactive molecules such as protein, lipids, and nucleic acid (DNA, mRNA, microRNA, and non-coding RNA) (128). Significantly higher levels of syncytiotrophoblast-derived EVs are found in the peripheral blood of women with PE compared with women with normal pregnancies (129). EVs isolated from PE patients differ phenotypically and functionally from those isolated from healthy pregnant women (130). Indeed, syncytiotrophoblast-derived EVs (including exosomes) from patients with PE contain higher levels of sFlt-1, sEng, and neprilysin, and treatment with EVs from PE patients impairs angiogenesis of endothelial cells and changes the characteristics of monocytes *in vitro* (131, 132). In addition, exosomes from PE patients cause vascular dysfunction and directly result in adverse PE-like birth outcomes in mice (131). Kohli et al. (133) demonstrated that administration of EVs led to accumulation of activated platelets and induced activation of NLRP3 inflammasome within the placenta, resulting in a PE-like phenotype in pregnant mice. Intriguingly, genetic deletion of NLRP3 inflammasome or pharmacological inhibition of inflammasome abolished this PE-like phenotype, indicating the pathogenesis of PE by EVs was dependent the NLRP3 inflammasome.

FREE FATTY ACID AND THE NLRP3 INFLAMMASOME IN PE

Obesity is a major risk factor for PE and FGR (134, 135). Obesity represents low-grade chronic systemic inflammation (136), and maternal obesity increases the risk of the offspring developing obesity and insulin resistance in the later stages of life (137–141). The NLRP3 inflammasome is involved in the pathogenesis of obesity-related inflammatory diseases, including metabolic syndrome, type 2 diabetes, and cardiovascular diseases (12, 13, 31, 50). There are many common mechanisms between PE and obesity-related pregnancy complications, and obesity accelerates the systemic features of PE.

Free fatty acids levels are elevated in the plasma of obese humans (142), and it has been proposed that they act to promote inflammatory responses by directly engaging TLRs and inducing the NF-κB-dependent production of inflammatory cytokines (143, 144). In particular, one of the major saturated fatty acids, palmitic acid (PA), causes intracellular crystallization, which in turn activates the NLRP3 inflammasome via lysosomal dysfunction in macrophages (145). PA also induces NLRP3 inflammasome activation by generating ROS and inducing autophagy dysfunction, resulting in secretion of mature IL-1β (144, 146, 147). Similar to other crystalline molecules, intraperitoneal administration of PA crystal induces neutrophil recruitment in an IL-1β-dependent manner (145).

Serum PA levels are increased in women with PE and FGR (148–150). Treatment with free fatty acid solution to mimic the plasma of PE patients induces lipid droplet accumulation, mitochondrial dysfunction, and apoptosis in human umbilical vein endothelial cells (149). In addition, PA induces activation of the NLRP3 inflammasome, resulting in the secretion of mature IL-1β by human trophoblast cells (147). NF-κB activation and IL-6 production are associated with higher levels of lipid accumulation in the placenta of obese women compared with those of lean women (151). These findings suggest that saturated fatty acids directly induce placental inflammation, resulting in PE.

AGEs AND THE NLRP3 INFLAMMASOME IN PE

AGEs are heterogeneous, reactive, and irreversibly crosslinked molecules formed from the non-enzymatic glycation of proteins, lipids, and nucleic acids (152, 153). They interact with RAGE and/or TLR4 to induce inflammatory responses (154, 155). AGE-RAGE interactions may increase and perpetuate the inflammatory condition, leading to obesity, diabetes mellitus, and cardiovascular and kidney diseases. Both *in vivo* and *in vitro* experiments have demonstrated that AGEs stimulate NLRP3 inflammasome activation and IL-1β secretion in human umbilical vein endothelial cells, kidney, and pancreatic islets (117, 156, 157). Ablation of the NLRP3 inflammasome improved AGE-induced abnormal insulin sensitivity, pancreatic islet damage, and inflammatory responses (158). These findings suggest that consumption of AGEs increases obesity-related dysfunction via NLRP3 inflammasome activation.

Increasing evidence indicates that AGEs and IL-1β are associated with PE and obesity in pregnant women (134, 135, 159–161). In human placenta, AGEs increase *in vitro* release of IL-1β, IL-6, IL-8, and TNFα depend on NF-κB activation (162). We also demonstrated that in human placental tissues, AGEs directly increase both the transcription and secretion of IL-1β (117). In addition, AGEs stimulate pro-IL-1β production, resulting in the acceleration of mature IL-1β secretion by NLRP3 inflammasome activation in human trophoblast cells. AGEs also induce sFlt-1 production through RAGE signaling, suggesting a

direct link with the pathology of PE (163). Antoniotti et al. (164) reported that AGEs led to activated inflammatory responses in endometrial cells, impaired decidualization, compromised implantation of blastocyst, and suppressed trophoblast invasion. Therefore, AGEs adversely may impact not only PE but also endometrial function and embryo implantation.

OTHER PREGNANCY COMPLICATIONS ASSOCIATED WITH THE NLRP3 INFLAMMASOME

GDM is also classed as an obesity-related pregnancy complication. In GDM, high levels of serum glucose are associated with increased inflammation in blood as well as placenta (165). Excess glucose induces IL-1β secretion from human trophoblast cells depending on the NLRP3 inflammasome (166). In addition to the placenta, caspase-1 activation and mature IL-1β secretion are higher in the adipose tissue of pregnant patients with GDM compared with healthy pregnant women (167), and treatment with caspase-1 inhibitor suppresses IL-1β secretion, suggesting the contribution of NLRP3 inflammasome activation in GDM.

Inflammation of the maternal-fetal interface such as intra-amniotic inflammation or chorioamnionitis, which can be induced by intra-amniotic infection or DAMPs, is a causal link to spontaneous preterm birth, which is a leading cause of perinatal mortality and morbidity (168). In a non-primate rhesus macaques chorioamnionitis model induced by intra-amniotic injection of LPS, the amnion upregulated neutrophil accumulation via the chemoattractant IL-8 in an IL-1-dependent manner (169). In a mouse model of intra-amniotic inflammation-induced preterm birth, the NLRP3 inflammasome was activated following IL-1β secretion in the fetal membranes and decidua basalis (170). In addition, IL-1β blockade decreased inflammation-induced preterm labor in mice (171). These findings suggest that the NLRP3 inflammasome plays a pivotal role in inflammation of the maternal-fetal interface associated with preterm birth, and IL-1 is a potential therapeutic target for these conditions.

To understand the role of the NLRP3 inflammasome in normal pregnancy and pregnancy complications, please refer the essential review (172).

CONCLUSION

Accumulating evidence suggests that the NLRP3 inflammasome plays an essential role in the pathogenesis of pregnancy inflammatory complications. Various types of DAMPs act as danger signals to activate the NLRP3 inflammasome in reproductive organs, resulting in pregnancy inflammatory complications (**Figure 2**). Once activated, the NLRP3 inflammasome drives the robust release of mature IL-1β, initiating a positive feedback loop that results in the accumulation of other immune cells (neutrophils and macrophages) and an increase in the "danger" cytokines and chemokines. Considering the potential for excessive NLRP3 inflammasome and IL-1β production, it is not unexpected that several negative regulatory mechanisms exist in nature to control inflammasome function. Understanding how the NLRP3 inflammasome regulates pregnancy complications and how to control excessive NLRP3 inflammasome activation is essential for the identification of new targets for the treatment of reproductive dysfunction.

AUTHOR CONTRIBUTIONS

KS and TK wrote the manuscript. MT critically revised the manuscript. All authors read and approved the final manuscript.

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The Association Between Previous TORCH Infections and Pregnancy and Neonatal Outcomes in IVF/ICSI-ET: A Retrospective Cohort Study

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Objective: This study aimed to investigate the associations between previous TORCH infection (cytomegalovirus, toxoplasmosis, herpes simplex virus, and rubella) with pregnancy and neonatal outcomes in couples undergoing IVF/ICSI-ET.

Materials and Methods: A total of 18,074 couples underwent fresh IVF/ICSI-ET (*in vitro* fertilization/intracytoplasmic sperm injection–embryo transfer) cycles were included in our analyses. TORCH infection status was determined by serological confirmation of cytomegalovirus, toxoplasmosis, herpes simplex virus, and rubella IgG in the absence of IgM antibodies. Clinical pregnancy, ectopic pregnancy, miscarriage, live birth, preterm birth, congenital malformation, and perinatal death were evaluated in both infection and non-infection group. Multivariate logistic regression was applied to calculate odds ratio.

Results: Previous toxoplasmosis infection is associated with a significantly decreased preterm birth rate [$P = 0.045$, OR = 0.755 (95% CI, 0.571–0.997), Adjusted OR = 0.749 (95%CI, 0.566–0.991)]. No differences in clinical pregnancy, ectopic pregnancy, miscarriage, and perinatal death were observed between the corresponding TORCH infection group [IgM (–) IgG(+)] and the non-infection group [IgM (–) IgG (–)].

Conclusions: Previous TORCH infections were not associated with adverse pregnancy and neonatal outcomes in IVF/ICSI-ET overall, and toxoplasmosis infection might be associated with a lower preterm birth rate in patients underwent IVF/ICSI-ET. The necessity of TORCH IgG screening in IVF procedure might need re-evaluation, and further cost-effective analysis might be helpful for the clinical management strategy.

Keywords: TORCH, previous infection, pregnancy outcome, neonatal outcome, IVF/ICSI-ET

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INTRODUCTION

TORCH infections classically comprise cytomegalovirus (CMV), toxoplasmosis, rubella, herpes simplex virus (HSV), and other commonly seen intrauterine infections, such as varicella and enteroviruses. The diseases caused by these pathogens can be transmitted from mothers to offspring (1) and are the main contributors to prenatal and neonatal abnormality and mortality (2).

CMV is a common viral pathogen which represents significant health concerns, as it could remain dormant in the host body over a long time, and may transmit from a mother to her developing fetus (3). It has been documented that congenital CMV infection affect ∼0.3–2.0% of all newborns (4). The relationship between the CMV viral load and pregnancy and live-birth outcomes were suspected, with several previous studies showing that there was a positive correlation between high viral load and adverse clinical outcomes of the fetus (5, 6). *Toxoplasma gondii* infection during pregnancy may also result in severe fetal damage, which manifests as the classic triad of chorioretinitis, hydrocephalus, and intracranial calcifications with parasites transmitting through the placenta (7). Rubella virus infection during pregnancy predisposes the fetus to developing a constellation of congenital deformities known as congenital rubella syndrome (CRS) secondary to maternal infection, especially during the first trimester (8). Congenital HSV infection shares clinical features with other congenital infections, such as microcephaly, hydrocephalus, and chorioretinitis, and usually presents with clinical symptoms at birth, mainly due to exposure to HSV during delivery (9). Other pathogens like spirochete *Treponema pallidum* will cause Syphilis syndrome (10). Most of the pathogens mentioned above have common clinical features of rash and ocular abnormalities that bring a huge burden on healthcare system and the society (11).

The life cycle of TORCH agents are different from each other, and the infections of TORCH are believed to have lifelong influences. For CMV infection, lifelong latency is established after acute infection in infected hosts (12). The natural cycle of initial infection is related to an increased IgG level and decreased IgM level, while women with IgG-seropositive CMV infection could not be absolutely protected against reactivation or reinfection of the same pathogen (13). Besides, it has been reported that more children in the United States acquire congenital CMV infection from non-primary maternal infection than from primary maternal infection (14). Meanwhile, several studies have found that there is a link between human behavior, personality, or mental disorders and toxoplasmosis IgG seropositivity (15). A nested case-control study with age-matched participants found that fetal gastroschisis was associated with maternal HSV IgG reactivity (16). Rubella IgG is considered as a protective antibody from recurrent Rubella infection, so WHO and other guidelines strongly recommend individuals to take the Rubella vaccination reaching an IgG titer of 10 IU/mL anti-Rubella antibodies in serum (17). However, the influence of previous TORCH infection on long-term health, especially pregnancy and neonatal outcomes, is yet to be confirmed.

Since the infection rate of TORCH was relatively high among women at child-bearing age in Asia (18, 19), women are routinely checked for TORCH infection status, namely, IgM and IgG, before commencing IVF/ICSI cycles in our center. Nevertheless, there is no reliable information about the impact of previous TORCH infections on the outcomes of pregnancy and live birth in women undergoing IVF-ET. Hence, our study aims to investigate the association between past TORCH infections with IVF-ET outcomes.

MATERIALS AND METHODS

This retrospective, hospital-based cohort study was approved by the Hospital Ethics Committee, Women's Hospital, Zhejiang University School of Medicine. Since it is a retrospective chart review study with only de-identified information collected, the Ethics Committee of Women's Hospital, Zhejiang University School of Medicine had determined exemption for informed consent of the study participants.

Patients admitted to our hospital for IVF treatment from January 1, 2010, to December 31, 2016, were enrolled. Inclusion criteria are as follows: (1) *in vitro* fertilization with fresh embryo transfer; (2) TORCH lab tests obtained within 6 months prior to transfer, with negative serum TORCH IgM. Exclusion criteria were (1) age more than 42 years old; (2) number of embryos transferred <1; (3) lost to follow-up; (4) incomplete data; and (5) data error. A total of 31,377 couples were recruited, and 18,074 were included in our final analysis. Flow chart was as in **Figure 1**.

Patients were divided into two groups, according to TORCH IgG seropositivity or not, clinical outcome data were collected and evaluated between the two groups. Primary outcomes were clinical pregnancy and live birth rate. Secondary outcomes were ectopic pregnancy, miscarriage, preterm birth, major malformation, minor malformation, and perinatal death. Clinical pregnancy was defined as the visualization of at least one gestational sac on ultrasound. Live birth was defined as baby born alive after 28 weeks of gestation. Ectopic pregnancy is defined as the development of a fertilized egg elsewhere than in the uterus. Miscarriage was defined as clinical pregnancy that was subsequently spontaneously miscarried. Preterm birth is defined as baby born alive before 37 weeks of gestation. Major malformations were defined as anomality that generally cause functional impairment or require surgical correction, and minor malformations were defined as the remaining malformations (20). Perinatal death was defined as infant death occur at less than 7 days of age or fetal death after 28 weeks of gestation. IVF and pregnancy outcomes were obtained from routine telephone follow-up by staff in our IVF center. Baseline characteristics were obtained from the running database in our IVF center. The rate of the primary and secondary outcomes were all calculated based on number of all cycles respectively.

Statistical analysis was performed using SPSS (Statistical Package for the Social Sciences Version 23.0, IBM Corp., Armonk, NY, USA). Chi-square test and Student's *t*-test were used to compare proportions and means respectively. Adjusted odds ratios (OR) with 95% confidence intervals (CI) were

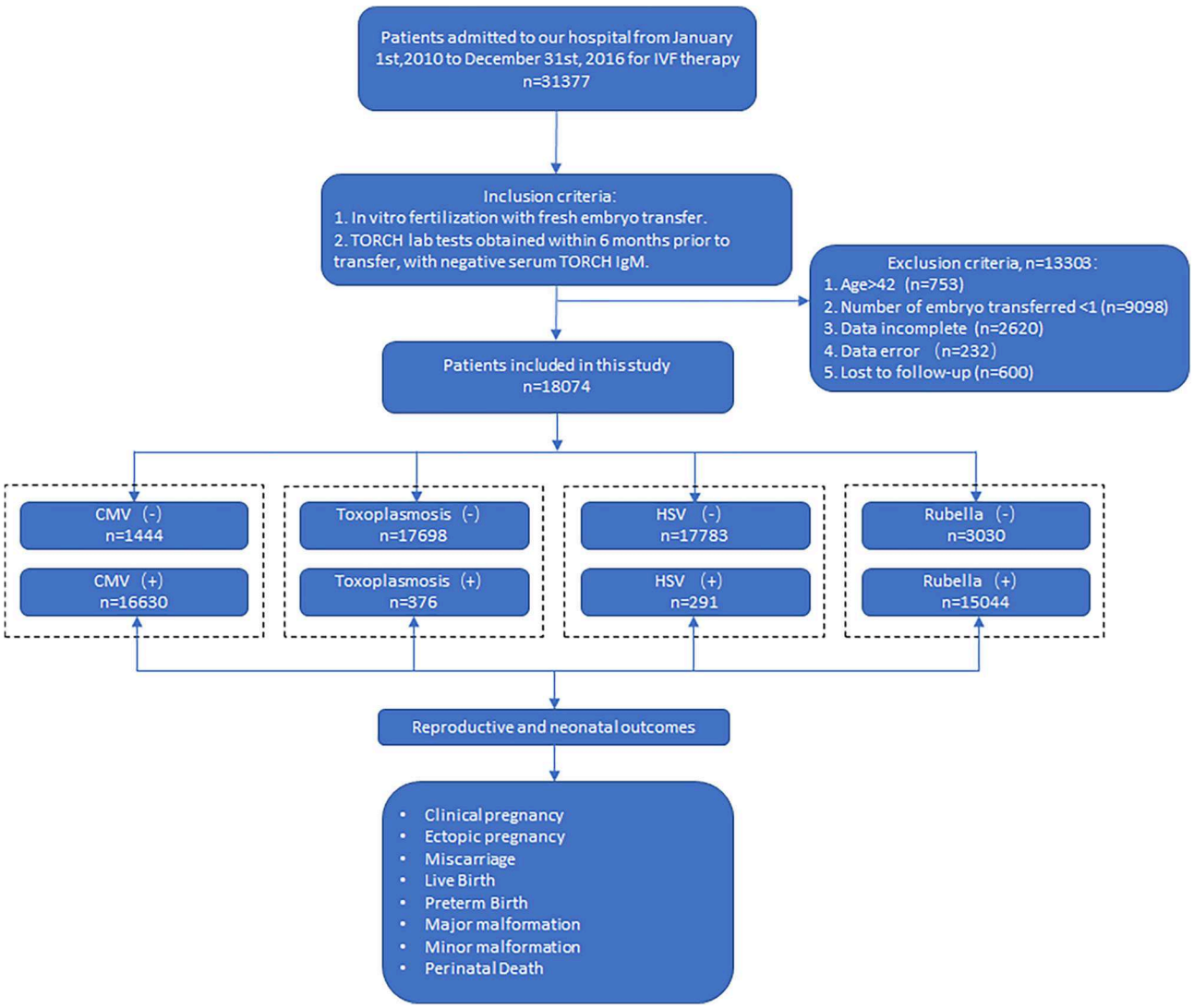

FIGURE 1 | Flowchart of the study. CMV (–): CMV IgM (–) IgG (–); CMV (+): CMV IgM (–) IgG(+). Toxoplasmosis (–): toxoplasmosis IgM (–) IgG (–); roxoplasmosis (+): toxoplasmosis IgM (–) IgG (+). HSV (–): HSV IgM (–) IgG (–); HSV (+): HSV IgM (–) IgG (+). Rubella (–): rubella IgM (–) IgG (–); rubella (+): rubella IgM (–) IgG (+).

calculated to approximate relative risks of adverse outcomes. ORs were estimated using multivariate logistic regression for control of confounding factors. CMV infection, maternal age, paternal age, baseline FSH, number of MII oocytes, number of oocytes, number of 2PN created, and number of embryos transferred were factors put into the logistic regression model for previous CMV infection effect analysis. Toxoplasmosis infection, maternal age, paternal age, number of embryos transferred were the factors put into the logistic regression model for previous Toxoplasmosis infection effect analysis, while HSV infection, maternal age, paternal age, duration of infertility, number of embryos transferred were the factors used in the multivariate logistic regression for previous HSV infection effect analysis. As for the previous rubella infection effect analysis, rubella infection, maternal age, paternal age, antral follicle count, endometrial thickness, number of MII oocytes, and number of embryos transferred were used as factors for multivariate logistic regression model. The method of backward testing was used for the selection of independent variables in logistic regressions with entry P value = 0.05 and removal P value = 0.1. $P < 0.05$ (two-tailed) was considered statistically significant.

RESULTS

Baseline Characteristics

Baseline characteristics of the study couples were shown in **Table 1**. There were differences in maternal age, paternal age, etiology of infertility, baseline FSH, number of MII oocyte, oocytes collected, 2PN created, and embryos transferred between the CMV IgM (–) IgG(+) group and CMV IgM (–) IIgG (–) group. All the baseline characteristics were similar between

TABLE 1 | Baseline characteristics of couples.

	CMV (–)	CMV (+)	*P*	Toxoplasmosis (–)	Toxoplasmosis (+)	*P*	HSV (–)	HSV (+)	*P*	Rubella (–)	Rubella (+)	*P*
No of IVF cycles	1,444	16,630		17,698	376		17,783	291		3,030	15,044	
Female Age	31.73 ± 4.42	31.22 ± 4.41	0.000	31.26 ± 4.41	31.36 ± 4.54	0.684	31.26 ± 4.41	31.38 ± 4.25	0.659	31.66 ± 4.54	31.19 ± 4.38	0.000
Duration of infertility (Years)	4.48 ± 3.29	4.30 ± 3.15	0.056	4.31 ± 3.16	4.46 ± 3.20	0.385	4.32 ± 3.16	3.98 ± 2.75	0.037	4.36 ± 3.18	4.31 ± 3.15	0.412
Female BMI	22.10 ± 2.75	22.18 ± 2.88	0.347	22.17 ± 2.88	22.20 ± 2.81	0.810	22.18 ± 2.87	21.77 ± 2.82	0.017	22.24 ± 2.81	22.15 ± 2.89	0.120
Male Age	33.99 ± 5.48	33.38 ± 5.28	0.000	33.41 ± 5.30	33.96 ± 5.61	0.049	33.42 ± 5.30	33.58 ± 5.51	0.621	33.88 ± 5.41	33.33 ± 5.28	0.000
Male BMI	23.69 ± 3.03	23.74 ± 3.20	0.543	23.73 ± 3.17	23.92 ± 3.59	0.304	23.74 ± 3.19	23.67 ± 3.03	0.746	23.81 ± 3.18	23.72 ± 3.18	0.160
Previous pregnancy in couple			0.495			0.284			0.524			0.001
Yes	818 (56.6)	9,266 (55.7)		9,864 (55.7)	220 (58.5)		9,927 (55.8)	157 (54.0)		1,772 (58.5)	8,312 (55.3)	
No	626 (43.4)	7,364 (44.3)		7,834 (44.3)	156 (41.5)		7,856 (44.2)	134 (46.0)		1,258 (41.5)	6,732 (44.7)	
Main reason of infertility			0.008			0.969			0.878			0.000
Tubal	745 (51.6)	9,377 (56.4)		9,905 (56.0)	217 (57.7)		9,962 (56.0)	160 (55.0)		1,651 (54.5)	8,471 (56.3)	
Anovulatory	50 (3.5)	558 (3.4)		598(3.4)	10(2.7)		597(3.4)	11(3.8)		89(2.9)	519(3.4)	
Male factor	296 (20.5)	3,257 (19.6)		3,482 (19.7)	71 (18.9)		3,601 (19.7)	52 (17.9)		593 (19.6)	2,960 (19.7)	
Endometriosis	154 (10.0)	1,462 (8.8)		1,573 (8.9)	33 (8.8)		1,578 (8.9)	28 (9.6)		336 (11.1)	1270 (8.4)	
Unexplained	79(5.5)	772 (4.6)		833(4.7)	18(4.8)		834 (4.7)	1 7(5.8)		130 (4.3)	721 (4.8)	
Others	130 (9.0)	1,204(7.2)		1,307(7.4)	27(7.2)		1,311(7.4)	23(7.9)		231(7.6)	1,103(7.3)	
Baseline FSH	6.88 ± 2.64	7.04 ± 2.45	0.020	7.02 ± 2.46	7.02 ± 2.61	0.990	7.03 ± 2.47	6.78 ± 2.28	0.083	6.99 ± 2.39	7.03 ± 2.48	0.416
AFC	10.72 ± 4.62	10.94 ± 4.61	0.104	10.92 ± 4.62	10.88 ± 4.32	0.843	10.91 ± 4.61	11.45 ± 4.75	0.055	10.74 ± 4.63	10.96 ± 4.61	0.023
Em Thickness(mm)	10.85 ± 2.97	10.80 ± 2.39	0.483	10.81 ± 2.45	10.79 ± 2.28	0.900	10.81 ± 2.44	10.79 ± 2.38	0.915	10.73 ± 2.34	10.82 ± 2.46	0.045
No of MII oocyte	1.07 ± 3.21	1.71 ± 3.88	0.000	1.66 ± 3.84	1.46 ± 3.77	0.324	1.65 ± 3.83	1.75 ± 4.13	0.664	1.51 ± 3.65	1.68 ± 3.87	0.024
Type of fertilization			0.528			0.588			0.338			0.358
IVF	1,021 (70.7)	11,775 (70.8)		12,536 (70.8)	260 (69.1)		12,585 (70.8)	211 (72.5)		2,115 (69.8)	10,682 (71.0)	
ICSI	423 (29.3)	4,855 (29.2)		5162 (29.2)	116 (30.9)		5,198 (29.2)	80 (27.5)		916 (30.2)	4,362 (29.0)	

(Continued)

TABLE 1 | Continued

	CMV (–)	CMV (+)	*P*	Toxoplasmosis (–)	Toxoplasmosis (+)	*P*	HSV (–)	HSV (+)	*P*	Rubella (–)	Rubella (+)	*P*
No of eggs collected	11.30 ± 5.88	10.91 ± 5.62	0.010	10.94 ± 5.64	10.68 ± 5.85	0.368	10.94 ± 5.65	10.71 ± 5.30	0.491	10.85 ± 5.66	10.96 ± 5.64	0.325
No of2PN created	6.78 ± 4.44	6.32 ± 4.10	0.000	6.36 ± 4.13	6.26 ± 4.28	0.621	6.36 ± 4.15	6.17 ± 3.85	0.434	6.34 ± 4.25	6.36 ± 4.11	0.774
No of embryo transfer	2.04 ± 0.61	1.94 ± 0.58	0.000	1.95 ± −0.59	1.96 ± 0.64	0.742	1.95 ± 0.59	1.97 ± 0.58	0.548	1.97 ± 0.60	1.95 ± 0.58	0.037
At least one good embryo transferred	815 (56.4)	9,433 (56.7)	0.835	10,047 (56.8)	201 (53.5)	0.200	10,074 (56.6)	174 (59.8)	0.283	1,677 (55.3)	8,571 (57.0)	0.099

Values are Mean ± Standard Deviation or N(%).
CMV (–):CMV IgM (–) IgG (–); CMV(+): CMV IgM (–) IgG(+).
Toxoplasmosis(–):toxoplasmosis IgM (–) IgG (–); toxoplasmosis (+): toxoplasmosis IgM (–) IgG(+).
HSV(–):HSV IgM (–) IgG (–); HSV (+): HSV IgM (–) IgG(+).
Rubella(–):rubella IgM (–) IgG (–); rubella (+): rubella IgM (–) IgG(+).

the toxoplasmosis IgM (–) IgG(+) group and toxoplasmosis IIgM (–) IgG (–) group. Baseline characteristics were similar between the HSV IgM (–) IgG(+) group and HSV IgM (–) IgG (–) group, except for duration of infertility, maternal BMI, and antral follicle count. As for the rubella IgM (–) IgG(+) group and rubella IgM (–) IgG (–) group, differences were found in maternal age, paternal age, previous pregnancy, etiology of infertility, endometrial thickness, number of MII oocytes, and whether at least one good embryo was transferred. All the factors with significant differences were put into the multivariate logistic regression analysis to adjust ORs.

Reproductive and Neonatal Outcome in the Presence of CMV IgG

We investigated the differences in reproductive and neonatal outcomes between the CMV IgM (–) IgG(+) group and CMV IgM (–) IgG (–) group. As shown in **Table 2**, CMV IgM (–) IgG(+) group showed a lower live birth rate compared with CMV IgM (–) IgG (–) group (31.8 vs. 34.2%), while no statistically significant difference was noticed [$P = 0.063$, OR = 0.948 (95% CI, 0.895–1.003), adjusted OR = 0.965 (95%CI, 0.911–1.023)]. There was no significant difference regarding the rate of clinical pregnancy, ectopic pregnancy, miscarriage, preterm birth, major malformation, minor malformation, and perinatal death between the two groups.

Reproductive and Neonatal Outcome in the Presence of Toxoplasmosis IgG

The comparison of the differences in reproductive and neonatal outcomes between the toxoplasmosis IgM (–) IgG(+) group and toxoplasmosis IgM (–) IgG (–) group were shown in **Table 3**. Previous toxoplasmosis infection significantly lowered the preterm birth rate [$P = 0.045$, OR = 0.755 (95% CI, 0.571–0.997), Adjusted OR = 0.749 [95%CI, 0.566–0.991)], while no significant differences were identified in clinical pregnancy, ectopic pregnancy, miscarriage, live birth, major malformation, neonatal minor malformation, and perinatal death between the two groups.

Reproductive and Neonatal Outcome in the Presence of HSV or Rubella IgG

Comparison between previous HSV or Rubella infection and non-infection group were shown in **Tables 4**, **5**, respectively. No differences in clinical pregnancy, ectopic pregnancy, miscarriage, live birth, preterm birth, offspring malformation, and perinatal death were noticed.

DISCUSSION

Infection during pregnancy has long been proven to cause adverse pregnancy outcomes, such as abortion and disastrous sequelae depending on the pathogens. The most well-known group of teratogenic pathogens are referred to as "TORCH" (*Toxoplasma gondii*, others like *Treponema pallidum*, rubella virus, cytomegalovirus, herpes simplex virus), for which, up to

TABLE 2 | Crude and adjusted ORs for reproductive and neonatal outcome by presence of IgG of CMV.

	CMV (–) N (%)	CMV (+) N (%)	*P*	Crude ORs (95% CI)	Adjusted ORs (95% CI)
Clinical pregnancy	612 (42.4)	7,009 (42.1)	0.862	0.995 (0.942–1.051)	1.016 (0.961–1.074)
Ectopic pregnancy	26 (1.8)	383 (2.3)	0.218	1.134 (0.928–1.386)	1.167 (0.954–1.427)
Miscarriage	80 (5.5)	997 (6.0)	0.484	1.043 (0.927–1.172)	1.070 (0.951–1.204)
Live Birth	494 (34.2)	5,294 (31.8)	0.063	0.948 (0.895–1.003)	0.965 (0.911–1.023)
Preterm Birth	90 (6.2)	970 (5.8)	0.535	0.965 (0.863–1.079)	0.996 (0.890–1.115)
Major malformation	6 (0.4)	69 (0.4)	0.997	0.999 (0.658–1.518)	1.011 (0.664–1.538)
Minor malformation	1 (0.1)	25 (0.2)	0.719	1.474 (0.542–4.006)	1.517 (0.557–4.130)
Perinatal Death	3 (0.2)	33 (0.2)	0.763	0.977 (0.541–1.766)	1.038 (0.574–1.879)

Clinical pregnancy, ectopic pregnancy, miscarriage, live birth, preterm birth, major malformation, minor malformation, and perinatal death were calculated based on number of all cycles. CMV (–):CMV IgM (–) IgG (–); CMV(+): CMV IgM (–)IgG(+).

TABLE 3 | Crude and adjusted ORs for reproductive and neonatal outcome by presence of IgG of Toxoplasmosis.

	Toxoplasmosis (–) N(%)	Toxoplasmosis (+) N(%)	*P*	Crude ORs (95% CI)	Adjusted ORs (95% CI)
Clinical pregnancy	7,474 (42.2)	157 (39.1)	0.223	0.937 (0.844–1.040)	0.937 (0.843–1.042)
Ectopic pregnancy	402 (2.3)	7 (1.9)	0.597	0.903 (0.620–1.317)	0.899 (0.617–1.312)
Miscarriage	1,059 (6.0)	18 (4.8)	0.332	0.889 (0.700–1.128)	0.884 (0.696–1.122)
Live Birth	5,676 (32.1)	112 (29.8)	0.348	0.948 (0.848–1.060)	0.948 (0.847–1.062)
Preterm Birth	1,047 (5.9)	13 (3.5)	0.045	0.755 (0.571–0.997)	0.749 (0.566–0.991)
Major malformation	75 (0.4)	0 (0)	0.411	–	–
Minor malformation	24 (0.1)	2 (0.5)	0.101	1.984 (0.963–4.089)	1.980 (0.960–4.083)
Perinatal Death	36 (0.2)	0 (0)	1.000	-	-

Ectopic pregnancy, miscarriage, live birth, preterm birth, major malformation, minor malformation, and perinatal death were calculated based on number of all cycles. Toxoplasmosis (–):toxoplasmosis IgM (–) IgG (–); toxoplasmosis (+): toxoplasmosis IgM (–)IgG(+).

TABLE 4 | Crude and adjusted ORs for reproductive and neonatal outcome by presence of IgG of HSV.

	HSV (–) N(%)	HSV (+) N(%)	*P*	Crude ORs (95% CI)	Adjusted ORs (95% CI)
Clinical pregnancy	7,495 (42.1)	126 (43.3)	0.693	1.024 (0.911–1.151)	1.019 (0.905–1.157)
Ectopic pregnancy	399 (2.2)	10 (3.4)	0.175	1.245 (0.905–1.713)	1.241 (0.902–1.709)
Miscarriage	1063 (6.0)	15 (4.8)	0.404	0.892 (0.680–1.168)	0.891 (0.679–1.167)
Live Birth	5,690 (32.0)	98 (33.7)	0.542	1.039 (0.919–1.174)	1.033 (0.912–1.170)
Preterm Birth	1,041 (5.9)	19 (6.5)	0.627	1.060 (0.838–1.340)	1.050 (0.829–1.329)
Major malformation	74 (0.4)	1 (0.3)	1.000	0.908 (0.338–2.441)	0.902 (0.336–2.424)
Minor malformation	26 (0.1)	0 (0)	1.000	–	–
Perinatal Death	36 (0.2)	0 (0)	1.000	–	–

Ectopic pregnancy, miscarriage, live birth, preterm birth, major malformation, minor malformation, and perinatal death were calculated based on number of all cycles. HSV (–):HSV IgM (–) IgG (–); HSV (+): HSV IgM (–) IgG(+).

now, the underlying mechanisms were still unclear (21). In our study, we found that there is no difference in clinical pregnancy, ectopic pregnancy, miscarriage, and perinatal death between the corresponding previous TORCH infection group and the non-infection group respectively. Previous toxoplasmosis infection is associated with a significantly decreased preterm birth rate.

Previous studies revealed that the crucial mechanisms of intrauterine infections leading to abortion, lowering live birth rate, and increasing risk of congenital malformations is possibly through upregulated oxidative stress and apoptosis pathways to inhibit placenta development and fetal growth (22–24). Patients admitted for IVF/ICSI-ET treatment will undergo a series of assisted reproductive technology to achieve pregnancy and are required to take a TORCH test before starting IVF/ICSI-ET cycles. With the number of infertile patients annually increasing, the cycles of IVF/ICSI-ET raised consequently, and ever since the administration of TORCH screening during or before pregnancy, more and more serologic positive patients have been identified (18). However, no preceding research has focused on investigating the effect of previous TORCH infection on patients undergoing IVF/ICSI-ET and the clinical significance of carrying out such screening in IVF centers. The lack of high-quality

TABLE 5 | Crude and adjusted ORs for reproductive and neonatal outcome by presence of IgG of Rubella.

	Rubella (–) N (%)	Rubella (+) N (%)	*P*	Crude ORs (95% CI)	Adjusted ORs (95% CI)
Clinical pregnancy	1,281 (42.3)	6,340 (42.1)	0.891	0.997 (0.959–1.037)	0.982 (0.941–1.024)
Ectopic pregnancy	63 (2.1)	346 (2.3)	0.456	1.053 (0.919–1.206)	1.020 (0.886–1.174)
Miscarriage	188 (6.2)	889 (5.9)	0.531	0.974 (0.898–1.057)	0.975 (0.896–1.062)
Live Birth	962 (31.7)	4,826 (32.1)	0.722	1.008 (0.966–1.051)	0.992 (0.948–1.037)
Preterm Birth	175 (5.8)	885 (5.9)	0.819	1.010 (0.929–1.098)	1.003 (0.919–1.096)
Major malformation	15 (0.5)	61 (0.4)	0.659	0.937 (0.700–1.253)	0.931 (0.688–1.259)
Minor malformation	5 (0.2)	21 (0.1)	0.736	0.920 (0.564–1.498)	0.889 (0.544–1.453)
Perinatal Death	6 (0.2)	30 (0.2)	0.987	1.004 (0.647–1.556)	1.217 (0.722–2.053)

Ectopic pregnancy, miscarriage, live birth, preterm birth, major malformation, minor malformation, and perinatal death were calculated based on number of all cycles.
Rubella (–):rubella IgM (–) IgG (–); rubella (+): rubella IgM (–) IgG(+).

studies in this field places clinicians in a dilemma of patient management and decision making, which warrants more in-depth research.

It is reported that ∼30% of primary CMV infections presented a positive IgM, but there was a high false-positive rate. Thus, the IgG test was preferred as a diagnostic test 3 to 4 weeks after initial exposure. The results of serologic testing become meaningful when there is seroconversion from IgG negative to positive or the IgG titer rises greater than 4-fold from baseline (25). Hence, we want to explore the correlation between IgG seropositivity and pregnant and neonatal outcomes among infertile patients receiving IVF/ICSI-ET treatment.

CMV is the most prevalent congenital viral infection worldwide, influencing up to 2.0% pregnancies (26). The total birth incidence of congenital CMV infection is estimated to be 0.64%, and the risk of primary CMV infection in seronegative mothers ranges between 0.7 and 4.1% (27). In our study, we found patients with previous CMV infection had a lower live birth rate in comparison with those without CMV infection after IVF/ICSI-ET [$P = 0.063$, OR = 0.948 (95% CI, 0.895–1.003), adjusted OR = 0.965 (95%CI, 0.911–1.032)], indicating that previous CMV infection might have an adverse effect on live birth rate in patients undergoing IVF/ICSI-ET. Although the difference was not significant, the trend would remind clinicians to pay more attention to previous CMV infection. What's more, from the results, we can interpret that there are no statistically significant differences in clinical pregnancy rate or miscarriage rate between the CMV IgG(+) group and CMV IgG (–) group. It has been reported that the mechanism of intrauterine CMV infection causing pregnancy loss is mainly through placental inflammation. Scientists had detected higher levels of multiple cytokines and growth factors in amniotic fluid from those who with CMV infection than uninfected controls (21). The results observed in our study could possibly be caused by a similar pathophysiological effect of CMV IgG antibodies. Therefore, prevention of CMV infection seems to be more important during preparation for pregnancy, which incorporates hygiene precautions and behavioral interventions based on published findings, such as thoroughly washing hands with soap and water for 15 to 20 s (28). Further studies are needed to explore the effect of different levels of IgG avidity on pregnant and neonatal outcomes among patients underwent IVF/ICSI-ET.

Toxoplasma gondii is a similarly prevalent infection throughout the world. *Toxoplasma gondii* is a protozoan parasite that spreads through cat feces or through ingestion of uncooked meat. Infection of *Toxoplasma gondii* leads to fetal injury, with brain and ocular involvement (29). An Iranian survey showed overall seroprevalence of toxoplasmosis is 39.9% (95% CI, 26.1–53.7) among childbearing-age women (30). Identically, IgG of toxoplasmosis is the most sensitive test, because IgM has a high false-positive rate and remains elevated for up to 2 years after infection (31). Our study discovered previous toxoplasmosis infection resulted in a significant decrement in the preterm birth rate [$P = 0.045$, OR = 0.755 (95% CI, 0.571–0.997), adjusted OR = 0.749 (95% CI, 0.566–0.991)], indicating pre-pregnancy toxoplasmosis exposure might be a protective factor in infertile women. The reason for the decreased incidence of preterm birth is uncertain, but might be explained as the presence of antibody prevent individuals from further Toxoplasmosis infection (32). There are no significant differences found in clinical pregnancy, ectopic pregnancy, miscarriage, live birth rate, major malformation, neonatal minor malformation, and perinatal death between the toxoplasmosis IgM (–) IgG(+) group and IgM (–) IgG (–) group, which means patients with previous toxoplasmosis infection don't warrant more attention and further treatments.

Pregnant women without rubella immunity have a high risk of congenital Rubella infection. Fortunately, rubella vaccination has significantly reduced the prevalence of congenital rubella syndrome (CRS) in many countries (33). Serum IgG positivity of rubella implies there is an immune response from the past. No differences in clinical pregnancy, ectopic pregnancy, miscarriage, live birth rate, malformation rate, or perinatal death were noticed between patients with previous rubella infection [IgM (–) IgG(+)] and those without [IgM (–) IgG (–)], indicating rubella IgG may potentially be a protective factor during pregnancy.

HSV infection can be disastrous to newborns. However, neonatal HSV infections are uncommon, occurring in around 1 out of every 3,200 births in the United State considering the high prevalence of HSV infection in the overall population (1). Therefore, routine prenatal screening for HSV infection is not recommended (34). Our study confirmed no differences in clinical pregnancy, ectopic pregnancy, miscarriage, live birth rate, malformation rate, perinatal death between HSV IgM (–) IgG(+)

group, and the non-infection group, which is consistent with the advice mentioned above.

This study has limitations. First, despite the large quantity of 18,074 IVF/ICSI-ET cycles included, this study is retrospectively designed, which might have included unmeasured confounding factors and led to potential bias, such as information bias negatively impacting the veracity of the study. Further multi-center prospective studies are needed to provide more convincing evidence. Second, our study merely observed neonatal outcomes; the growth and development of the child were not evaluated. Long-term follow-up of offspring are needed to further explore the effect of maternal previous TORCH infections on offspring health. Third, the results are concluded from clinical data exhibiting a correlation between previous TORCH infection and the pregnancy and neonatal outcomes for couples underwent IVF/ICSI-ET, while we have not yet conducted further basic research to confirm the phenomenon and explore the underlying mechanisms. Last but not least, our study does not investigate the different TORCH pathogen co-infection effects, and further research is needed to distinguish the effect of different co-infected pathogens and to undertake subgroup analysis.

Previous studies have only paid attention to the effects of TORCH infection during pregnancy, and a lot of observations have demonstrated TORCH infection has accounted for several adverse prenatal and neonatal events, including miscarriage, malformation, and neurodevelopmental abnormalities. Our study focuses on a totally different timeframe and a special group of patients to explore whether past infection of TORCH before pregnancy will have an impact on maternal and neonatal outcomes in patients undergoing IVF/ICSI-ET. Our study indicated that previous TORCH infections were not directly associated with adverse pregnancy and neonatal outcomes, providing evidence for clinicians to reduce the screening frequency on this matter. Therefore, further cost-effective analysis might be helpful for clinical strategy of TORCH IgG screening in IVF procedure.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Hospital Ethics Committee, Women's Hospital, School of Medicine, Zhejiang University. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

DZ and GX designed the study and critically revised the manuscript. YL performed data analysis. YW, FW, WZ, LC, ST, YQ, YL, YH, and RZ collected data. YL, YW, FW, and SW drafted the manuscript. All authors reviewed the manuscript.

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Effect and Relationship of Seasons on the High Risk of Ovarian Hyperstimulation Syndrome After Oocyte Retrieval in Patients With Polycystic Ovary Syndrome

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Objective: To investigate the effect of seasons on the incidence of high risk of ovarian hyperstimulation syndrome (OHSS) after in oocyte retrieval in patients with polycystic ovarian syndrome (PCOS) and to establish a nomogram to predict the risk of OHSS.

Design: Single-center, retrospective study.

Setting: University-affiliated reproductive medicine center.

Patient(s): A total of 2,030 infertility patients with PCOS underwent the follicular phase long-acting long protocol IVF/ICSI in the reproductive medicine center from January 2017 to December 2019.

Intervention(s): None.

Main outcome measure(s): Logistic regression analysis was used to analyze the factors associated with a high risk of OHSS. We established a nomogram to predict the risk of OHSS in infertility patients with PCOS after oocyte retrieval.

Result(s): The incidence of patients at high risk of OHSS was significantly different from season-to-season and was especially higher in the summer and winter. Multivariate logistic analysis showed that gonadotropin dosage, number of retrieved oocytes, estradiol level, average bilateral ovarian diameter on the day human chorionic gonadotropin was administered, type of infertility, and average temperature were independent risk factors for OHSS after oocyte retrieval in PCOS patients. Based on the above independent risk factors, we constructed a prediction model for OHSS risk. To evaluate the efficiency of the prediction model, we calculated the C-index (0.849), area under the receiver operating characteristic curve (0.849), and internal validation C-index (0.846). Decision curve analysis suggested that the prediction model exhibited significant net benefits.

Conclusion(s): The incidence of PCOS patients at high risk for OHSS after oocyte retrieval fluctuated with seasonal temperature changes, and was significantly higher in

extreme climates. The prediction model had favorable predictive performance and clinical application value.

Keywords: polycystic ovary syndrome, ovarian hyperstimulation syndrome, temperature change, nomogram, receiver operating characteristic curve

INTRODUCTION

Seasonal changes affect the development of many diseases and are closely related to the morbidity and mortality rate of hospitalization (1, 2). For instance, seasonal variations in acute coronary syndromes have been reported, with incidence and mortality peaking in the winter because of the proportion of plaque rupture is highest in winter (3). However, infectious and respiratory diseases are more prevalent in the summer (4).

Ovarian hyperstimulation syndrome (OHSS) is a severe complication of controlled ovarian hyperstimulation (COH) that is related to age, body mass index, ovarian function, and the ovulation stimulation protocol. The risk of OHSS in polycystic ovary syndrome (PCOS) patients is significantly increased (5). The etiology of OHSS is complex. Emerging evidence has shown that OHSS is associated with inflammatory factors interleukin (IL)-6, tumor necrosis factor (TNF)-α, IL-8, vascular endothelial growth factor, and the local renin-angiotensin aldosterone system, which led to a series of pathologic changes, including increased capillary permeability, leakage of vascular fluid into the interstitial space to form pleural effusions or ascites, decreased effective circulating blood volume, blood concentration, and even thrombosis (6, 7).

The immune system changes significantly throughout the year. Specifically, it has been shown that serum concentrations of IL-6 and soluble IL-6 receptor exhibit seasonality with higher expression during cold climates. IL-6 is an inflammatory factor that increases capillary permeability and plays an important role in the pathogenesis of OHSS (8, 9). Extreme weather activates the hypothalamic-pituitary-adrenocortical (HPA) axis and the sympathetic nervous system, which resulted in a high level of aldosterone (10). Abnormal expression of aldosterone promotes renal tubular reabsorption and increases the levels of inflammatory mediators, both of which play a key role in the occurrence of OHSS. The purpose of this study was to evaluate the relationship between the incidence of patients with PCOS at high risk for OHSS after oocyte retrieval and season and to construct a prediction model for OHSS risk to provide a new strategy to reduce the incidence of OHSS.

MATERIALS AND METHODS

Study Design

A retrospective analysis of patients from Henan Province in China who were diagnosed with PCOS according to the Rotterdam criteria at The Center for Reproductive Medicine of The First Affiliated Hospital of Zhengzhou University, and underwent oocyte retrieval between 1 January 2017 and 31 December 2019. The follicular phase long-acting long protocol was used to stimulate follicles and *in vitro* fertilization (IVF)/ intracytoplasmic sperm injection (ICSI) was performed. The study was retrospectively, the access and processing of patient data was approved by the ethics committee under a protocol for retrospective studies.

The inclusion criteria were as follows: 1) diagnosis of PCOS according to the Rotterdam criteria; 2) first fresh cycle using follicular phase long-term protocol and recived oocyte retrieval; and 3) age < 40 years. The exclusion criteria were as follows: 1) cycle cancellation of fresh embryo transfer because of abnormal liver function tests, high serum progesterone levels, pre-implantation genetic diagnosis/pre-implantation genetic screening, personal reasons, and/or uterine factors; 2) a history of OHSS or OHSS following embryo transfer; 3) a history of endometriosis, adenomyosis, surgery for ovarian cysts, hydrosalpinx, and pelvic tuberculosis; and 4) The male suffers from severe oligozoospermia or teratozoospermia.

Patients with PCOS at high risk for OHSS (serum estradiol [E2] level on human chorionic gonadotropin [HCG] administration day > 11,010 pmol/L, number of retrieved oocytes ≥ 15 and perceived bloating, and/or symptoms, such as bloating, abdominal pain, chest tightness, oliguria, pleural effusion, blood hypercoagulability, and/or volume of ovaries increased before retrieval) were required to cancel fresh embryo transfer and freeze embryos.

Meteorological Data

Meteorological data for January 2017–December 2019 for Zhengzhou City, Henan Province, China were downloaded from the China Meteorological Data Net (http://data.cma.cn/) and included monthly minimum, maximum and average temperatures, and sunshine duration. Henan, China is located in the northern hemisphere with a warm temperate–subtropical monsoon climate. The dates of oocyte retrieval were divided into spring (March–May), summer (June–August), autumn (September–November), and winter (December–February).

Ovulation Stimulation Program and Embryo Transfer

On the 2^{nd}–3^{rd} days of menstruation, patients were given a long-acting gonadotropin releasing hormone agonist (Diphereline, 3.75 mg; Beaufour-Ipsen, Dreux, France) by subcutaneous injections. Thirty days later, we obtained blood from patients to determine serum follicle-stimulating hormone (FSH), luteinizing hormone (LH), E2, and progesterone (P) levels. At the same time, vaginal ultrasound was used to monitor the size of antral follicles. When the FSH level was < 5 IU/L, the LH level was < 3 IU/L, and the antral follicle was nearly 5 mm in diameter, COH was initiated. Based on patient age, anti-Mullerian

hormone level, antral follicle count, body mass index, and serum FSH level, we determined the individualized dosage of gonadotropin ([Gn] GONAL-f; Merck Serono, Darmstadt, Germany). When one dominant follicle was ≥ 20 mm in diameter and at least three dominant follicles were ≥ 17 mm in diameter, a trigger injection of HCG (recombinant human chorionic gonadotropin alfa for injection, Merck Serono) was administered the same night. After 36–37 h of the trigger injection, we performed transvaginal oocyte retrieval. The method of fertilization depended on semen quality. Fresh embryo transfer was performed 3–5 days after oocyte retrieval based on embryo quality, endometrial and patient's condition. The transplant was cancelled if patients were deemed at high risk for OHSS, the P level was > 3 ng/ml, or a uterine effusion was demonstrated.

Our primary outcome measure was the incidence of patients with PCOS at high risk for OHSS, calculated as the number of patients at high risk for OHSS per total number of patients. Secondary outcome measures were as follows. The fertilization rate was defined as the total number of fertilization oocytes per total number of retrieved oocytes. The cleavage rate was calculated as the total number of cleavages divided by the total number of fertilization oocytes. The high-quality embryo rate is expressed as the total number of high-quality embryos divided by the total number of cleavages. The clinical pregnancy rate was defined by the presence of a fetal heartbeat at 6–7 weeks of pregnancy. The live birth rate was defined as the total number of women with live births per total number of women with fresh embryo transfer.

Statistical Analysis

For comparison of continuous variables between multiple groups and when the variance was homogeneous among groups, one-way ANOVA or the Kruskal-Wallis non-parametric test was used. The LSD-t test was used for a pairwise comparison of continuous variables within the group, and a chi-square test was used for comparison of proportions (Bonferroni correction was used to account for multiple testing). Linear regression analysis was used when the outcomes were continuous variables. Multivariable logistic regression analysis was used when the outcomes were dichotomous variables. The features were considered as odds ratios (ORs) with 95% confidence intervals (CIs) and a P-value. According to the regression coefficient of the final variable, a personalized prediction model was constructed. All potential predictors were applied to develop the prediction model. To evaluate the accuracy and differentiation of the prediction model, the C-index and receiver operating characteristic curve (ROC) were measured. The prediction model was subjected to bootstrapping validation (1000 bootstrap resamples) to calculate a relatively corrected C-index. Decision curve analysis (DCA) was performed to determine the clinical usefulness of the prediction model by quantifying the net benefits at different threshold probabilities in the cohort. A $P \leq 0.05$ was considered statistically significant. Data analysis was conducted using SPSS 26.0 (Armonk, New York, USA) and R software (version 3.6.0; Miami, FL, USA). Delete sample objects with missing values under indicators.

RESULTS

A total of 2,030 patients with PCOS were included. Among them, 683 women with the high risk of OHSS cancelled fresh embryo transfer and frozen embryos, and the rest of the women served as controls, including 1,333 women who received fresh embryo transfer and 14 women without available embryos. We divided patients into four groups by season and compared various indicators of them. The results indicated that the number of retrieved oocytes, average bilateral ovarian diameter on the day of HCG administration, Gn dosage, incidence of patients at high risk for OHSS, and live birth rate were statistically different between seasons ($P < 0.05$; **Table 1** and **Figure 1**).

We analyzed the impact of season changes on the occurrence of patients at high risk for OHSS. Logistic regression analysis showed that Gn dosage, number of retrieved oocytes, serum E2 level, average bilateral ovarian diameter on the day of HCG administration, etiology of infertility, average temperature, average minimum temperature, and average maximum temperature were independent risk factors affecting OHSS risk in patients with PCOS ($P < 0.05$; **Table 2**).

The OHSS risk prediction model that incorporated the above independent risk factors was developed and is presented as a nomogram. We performed the precision, discrimination, stability, and application value of the model by the C-index, ROC curve, bootstrap internal validation method, and DCA. We deleted sample objects with missing values under some indicators. Ultimately, 1925 individuals were included in the model analysis. The C-index was 0.849, the AUC was 0.849, and the internal validation C-index was 0.846. DCA showed that the nomogram is clinically useful when the decision to intervene at an OHSS possibility threshold of 4% (**Figures 2** and **3**).

In different seasons, the live birth rate, number of retrieved oocytes, and high-quality embryo rate were significant differences. The relationships between the above three outcomes and the seasonal factor were analyzed by linear or logistic regression; there was no correlation between the live birth rate or number of retrieved oocytes and the seasonal factor ($P > 0.05$). The high-quality embryo rate was related with sunshine duration ($P < 0.05$; **Table 3**).

DISCUSSION

OHSS is a serious, iatrogenic complication of COH. Patients with PCOS are at an increased risk for OHSS due to the high ovarian responsivity to gonadotropin stimulation (11). Our results showed that the high risk for OHSS after oocyte retrieval in PCOS patients was associated with total Gn dosage, number of retrieved oocytes, E2 level, and average diameter of the bilateral ovaries on the HCG day of administration, which was consistent with previous studies (12–15). In addition, our study showed that PCOS patients with primary infertility had a greater incidence of OHSS, which may be related to more complex symptoms.

We found that the high risk for OHSS in PCOS patients was significantly different between the seasons. Correlation analysis showed that the monthly average temperature, average

TABLE 1 | Baseline characteristics and cycle outcomes in different seasons.

	Spring (Mar-May)	Summer (Jun-Aug)	Autumn (Sept-Nov)	Winter (Dec-Feb)	F	*P* value
Age, y	28.44 ± 3.69	28.8 ± 3.58	28.87 ± 3.64	28.91 ± 3.85	1.47	0.220
Fertilization method					—	0.382
IVF	85.8%(364/424)	86.9%(560/644)	83.4%(448/537)	85.8%(365/425)	—	
ICSI	14.2%(60/424)	13.1%(84/644)	16.6%(89/537)	14.2%(60/425)	—	
Type of infertility						0.109
Primary infertility	38.2%(162/424)	37.7%(243/644)	32.6%(175/537)	39.5%(168/425)	—	
Secondary infertility	61.8%(262/424)	62.3%(401/644)	67.4%(362/537)	60.5%(257/425)	—	
Infertility duration, y	3.9 ± 2.42	4.19 ± 2.76	4.11 ± 2.91	3.92 ± 2.57	1.38	0.247
BMI, kg/m2	24.39 ± 3.45	24.24 ± 3.21	24.48 ± 3.38	24.21 ± 3.39	0.73	0.532
Basal serum FSH level, U/L	5.85 ± 1.65	5.71 ± 1.5	5.92 ± 1.55	5.81 ± 1.65	1.83	0.139
Basal serum E2 level, pmol/L	46.24 ± 45.72	48.53 ± 40.88	52.80 ± 49.85	47.37 ± 41.20	0.152	0.118
Basal serum LH level, U/L	10.12 ± 7	10.29 ± 7.3	10.06 ± 7.87	9.99 ± 8.94	0.15	0.932
Basal serum AMH level, ng/ml	8.08 ± 4.25	8.46 ± 4.44	8.17 ± 4.27	8.38 ± 4.23	0.85	0.469
AFC	21.87 ± 5.43	22.17 ± 4.86	22.47 ± 4.64	22.2 ± 4.75	—	0.790
Number of retrieved oocytes	18.74 ± 7.91	17.65 ± 7.49[cd]	18.9 ± 7.97	19.13 ± 8.1	4.05	0.007
Endometrial thickness, mm	12.4 ± 2.62	12.195 ± 2.33	12.029 ± 2.53	12.287 ± 2.41	—	0.160
Serum E2 level on HCG day, pmol/L	1,059.88	1,006.95	962.95	1,015.23	—	0.086
Serum P level on HCG day, nmol/L	0.88 ± 0.58	0.81 ± 0.54	0.83 ± 0.51	0.84 ± 0.54	1.14	0.333
Average size of bilateral ovaries on HCG day, cm	5.44 ± 1.03	5.28 ± 0.99[d]	5.31 ± 0.93	5.45 ± 1.04	3.81	0.010
Gn dosage, U	2165.18 ± 914.91	2,082.16 ± 802.94[cd]	2,220.9 ± 878.52	2,253.56 ± 921.4	4.08	0.007
Incidence of high risk of OHSS	32.3%(137/424)	35.9%(231/644)	29.4%(158/537)	36.9%(157/425)	—	0.044
Fertilization rate	61.7%(4,903/7,947)	60.2%(6,839/11,364)	60.8%(6,166/10147)	60.7%(4,936/8,129)	—	0.211
Cleavage rate	98.9%(4,849/4,903)	98.7%(6,749/6,839)	98.8%(6,090/6,166)	98.9%(4,881/4,936)	—	0.675
High-quality embryo rate	60.2%[cd](2,917/4,849)	60.5%[cd](4,084/6,749)	64.4%[ab](3,920/6,090)	63.3%[ab](3,088/4,881)	—	0.000
Clinical pregnancy rate	76.0%(215/283)	76.7%(310/404)	74.5%(281/377)	74.7%(201/269)	—	0.887
Live birth rate	66.43%(188/283)	68.07%[c](275/404)	58.62%(221/377)	62.45%(168/269)	—	0.035

BMI, body mass index; AFC, antral follicle count; OHSS, ovarian hyperstimulation syndrome; FSH, follicle stimulatine hormone; LH, luteinizing hormone; AMH, anti-mullerian hormone; E2, estradiol; P, progesterone[a]Significantly different from spring.
[a]Significantly different from spring [b]Significantly different from summer [c]Significantly different from autumn [d]Significantly different from winter.

maximum temperature, and average minimum temperature were independent risk factors for OHSS, suggesting that effect of extreme climates. Extreme climates (heat and/or cold) could cause vasoconstriction, the release of inflammatory factors and the activation of the local renin-angiotensin aldosterone system, all of them are associated with the development of OHSS.

Extreme temperature exposure has adverse effects on the human body, especially females and elderly people are vulnerable to the potential adverse effects (16). Winter is the season with high incidence of cardiovascular and cerebrovascular diseases because of low temperature stimulates vasocontraction. When the outdoor temperature is higher than 5°C, systolic blood pressure increases by 6.7 mmHg and diastolic blood pressure increases by 2.1 mmHg for every 10°C decrease (17). Increased blood pressure speeds up the flow of fluid from the blood vessels into the interstitial space, facilitating the onset

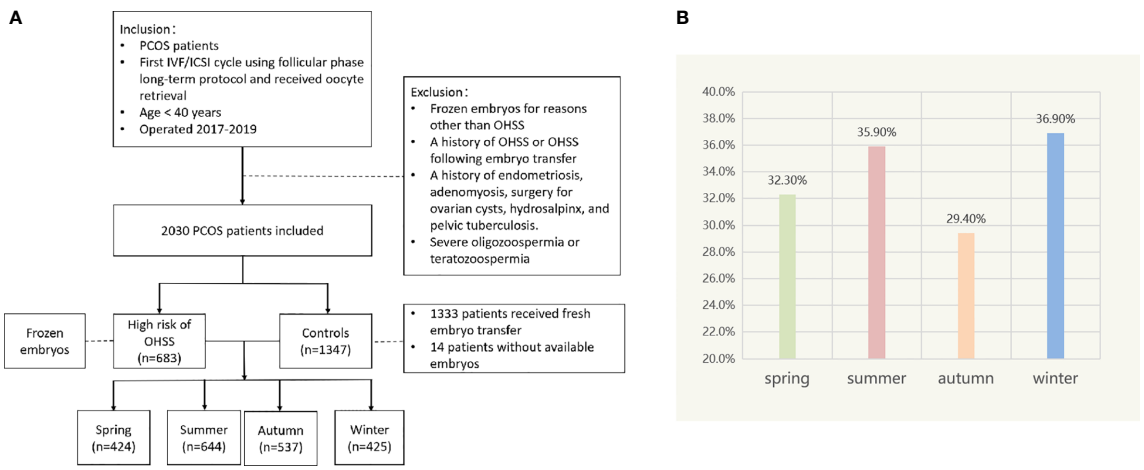

FIGURE 1 | **(A)** Flow chart depicting the patient selection. **(B)** Incidence of patients at high risk of OHSS in different seasons.

TABLE 2 | Association of high risk of ovarian hyperstimulation syndrome (OHSS) with meteorological factors.

	P value	OR	95% CI	
			Lower limit	Upper limit
Average temperatures, °C	0.003	0.395	0.214	0.727
Average minimum temperatures, °C	0.016	1.416	1.066	1.882
Average maximum temperature, °C	0.002	1.843	1.256	2.704
Sunshine duration, hr	0.153	0.996	0.991	1.001

OR, odd ratio; 95% CI, 95% confidence interval.

Calibration variables: age, infertility duration, BMI, basal serum FSH, LH, AMH level, AFC, Gn dosage, number of retrieved oocytes*, serum E2*, P level on HCG day, average size of bilateral ovaries on HCG day*, type of infertility*, average temperatures*, average minimum temperatures*, average maximum temperature*, sunshine duration.*

**P < 0.05.*

of OHSS. In the winter, the immune system is reinforced, which in turn promotes the serum concentrations of IL-6 and IL-6R. Studies have shown that the inflammatory factor, IL-6, is involved in the pathogenesis of OHSS (8). The IL-6R/IL-6 complex acts on ovarian vascular endothelium to promote endothelial cells to secrete VEGF by activating the STAT3/ERK signaling pathway, which increases vascular permeability (18). Cold temperature also stimulates the HPA axis to secrete adrenocorticotropic hormone (ACTH) (19) and aldosterone levels increase in a dose-dependent fashion with ACTH (10). At the same time, cold temperature also affects the SNS, leading to the activation of adrenaline receptors on the juxtaglomerular cells and the release of renin, thus activating the renin-angiotensin-aldosterone system, which increases the secretion of aldosterone, causes vasoconstriction and induce OHSS (20). Similarly, heat and dryness produce anxiety, irritability, and other negative emotions that activate the HPA axis and SNS and promote aldosterone secretion (21). Studies have confirmed that four consecutive days of mice housed at 35 ± 1°C led to a significant increase in cell volume and cell count in the adrenal cortex of mice, with a 16% increase in the serum levels of aldosterone ($P < 0.05$) (22). In addition, high temperatures can cause a series of physiologic changes including body heat dissipation and activity, blood redistribution, a large amount of blood flow to the skin and muscles, and water loss in the body that can subsequently contribute to the increases and aggravation of blood concentration (23).

We subsequently constructed an OHSS risk prediction model of the independent risk factors affecting the occurrence of high risk for OHSS after oocyte retrieval in patients with PCOS, and

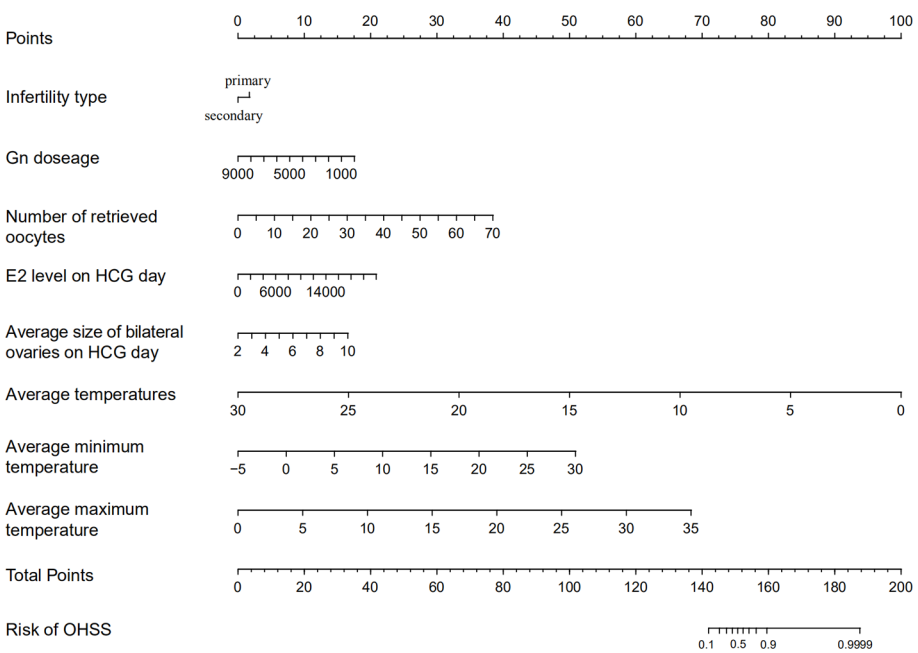

FIGURE 2 | Ovarian hyperstimulation syndrome (OHSS) risk nomogram. The scaled line to the right of each variable represents the value range of the variable, while the length of the line indicates the size of the variable's contribution to the outcome event. The value of each variable corresponds to the Points at the top of the figure, and all the scores add up to the Total points at the bottom. The risk of OHSS occurrence is represented by the Total points corresponding to the value of Risk of OHSS at the bottom of the figure.

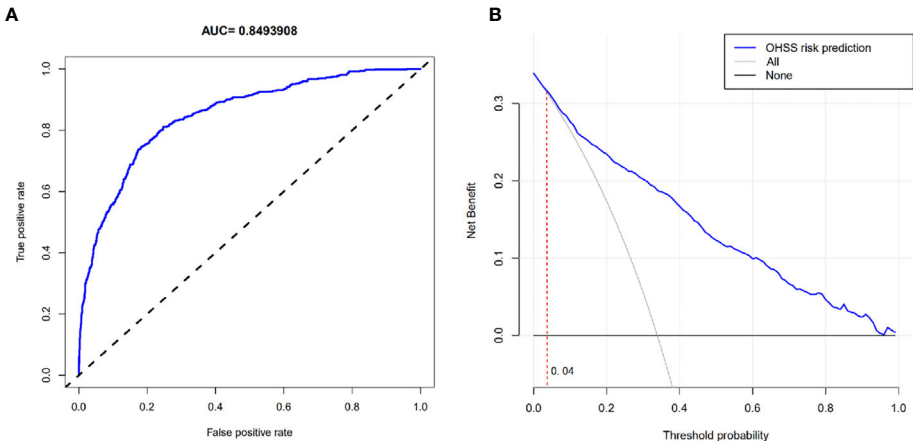

FIGURE 3 | **(A)** ROC curve. **(B)** Decision curve analysis. **(B)** The horizontal solid line represents the clinical net benefit when none patients are treated after oocyte retrieval, the backslash with a negative grey slope represents the net benefit when all patients are treated. When the blue curve was above the part of the two extreme curves where the threshold probability was >4%, using this nomogram in the current study to predict OHSS risk gets more clinical benefit.

evaluated and validated the predictive efficacy and clinical application value of this prediction model. The C-index was 0.849 and the AUC was 0.849, suggesting that the model has good precision and discrimination. The internal validation C-index was 0.846, which indicates that the prediction model is stable. The DCA revealed that the prediction model has a meaningful clinical effect when intervention was decided among nearly the entire range of threshold probabilities. The nomogram can help clinicians screen patients at high risk for OHSS and intervene as early as possible. For example, on the HCG trigger day, we can use the nomogram prediction model to screen for those at high risk for OHSS and assign a personalized trigger plan or prophylactic medication after retrieved oocytes (letrozole or cabergoline) to reduce the risk of OHSS.

Currently, the correlation between the high-quality embryo rate and season is inconsistent. The present study showed that the high-quality embryo rate was significantly lower in the spring and summer than the autumn and winter, which is consistent with the findings of Stolwik et al. (24), but in contrast to the conclusions of Rojansky et al. (25). Further studies have demonstrated that the high-quality embryo rate was correlated with sunshine duration and may be related to melatonin, which is present in follicular fluid and is involved in follicular development, ovulation, and oocyte maturation. Moreover, the high-quality embryo rate is inversely proportional to sunshine duration (26). Less sunshine in the autumn and winter leads to an increased secretion of melatonin, which has a positive effect on oocyte quality (27).

Our study first found that the incidence of PCOS patients at high risk for OHSS after oocyte retrieval in the cycle of IVF/ICSI fluctuated with temperature changes, and increased in hot or cold weather, suggesting that the dosage of Gn should be

TABLE 3 | Association of live birth rate, number of retrieved oocytes, and high-quality embryo rate with meteorological factors.

	Live birth rate[1]			Number of retrieved oocytes[2]			High-quality embryo rate[3]		
	P	OR	95%CI	*P*	β	95%CI	*P*	β	95%CI
Average temperatures, °C	0.516	0.817	(0.4431.504)	0.69	0.38	(-1.151 1.751)	0.69	0.46	(-0.049 0.074)
Average minimum temperatures, °C	0.519	1.099	(0.8251.464)	0.44	-0.33	(-0.937 0.410)	0.07	-0.94	(-0.055 0.002)
Average maximum temperature, °C	0.538	1.127	(0.771.649)	0.89	-0.08	(-0.978 0.848)	0.44	0.56	(-0.023 0.054)
Sunshine duration, hr	0.714	1.001	(0.9961.006)	0.13	-0.06	(-0.021 0.003)	0.00	-0.20	(-0.002 -0.001)

Calibration variables:

[1]age,infertility duration, infertility type, BMI*, basal serum FSH, E2, LH, AMH level, AFC, Gn dosage, number of retrieved oocytes, number of transplanted embryos, serum E2, P* level on HCG day, endometrial thickness*, average temperatures,*

average minimum temperatures, average maximum temperature, sunshine hours.

[2]age, infertility duration, BMI, basal serum FSH*, E2, LH, AMH level, AFC*, Gn dosage, serum E2*, P* level on HCG day, average size of bilateral ovaries on HCG day*, average temperatures, average minimum temperatures, average maximum temperature, sunshine duration.*

[3]age, infertility duration, BMI, basal serum FSH, E2, LH, AMH* level, AFC, Gn dosage, number of retrieved oocytes*, serum E2, P level on HCG day, average size of bilateral ovaries on HCG day, average temperatures, average minimum temperatures, average maximum temperature, sunshine duration*.*

**P < 0.05.*

considered to reduce the risk of OHSS in climate extremes. We constructed the prediction model of OHSS risk so that clinicians can conduct personalized and effective prevention and treatment measures for patients to reduce the risk of OHSS. Our study was limited by region and sample size. In the future, we will conduct a multi-center and large-sample size study to further explore the factors related to a high risk for OHSS and verify the predictive efficacy and clinical application value of the predictive model.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee for scientific research and clinical trials of the First Affiliated Hospital of Zhengzhou University. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

JZ contributed to the conception of study. YC, HS, and YM contributed to design work. YC was responsible for statistical analyses performing and manuscript writing. LM contributed to revising the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Endocrine Disruptor Compounds—A Cause of Impaired Immune Tolerance Driving Inflammatory Disorders of Pregnancy?

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Endocrine disrupting compounds (EDCs) are prevalent and ubiquitous in our environment and have substantial potential to compromise human and animal health. Amongst the chronic health conditions associated with EDC exposure, dysregulation of reproductive function in both females and males is prominent. Human epidemiological studies demonstrate links between EDC exposure and infertility, as well as gestational disorders including miscarriage, fetal growth restriction, preeclampsia, and preterm birth. Animal experiments show EDCs administered during gestation, or to either parent prior to conception, can interfere with gamete quality, embryo implantation, and placental and fetal development, with consequences for offspring viability and health. It has been presumed that EDCs operate principally through disrupting hormone-regulated events in reproduction and fetal development, but EDC effects on maternal immune receptivity to pregnancy are also implicated. EDCs can modulate both the innate and adaptive arms of the immune system, to alter inflammatory responses, and interfere with generation of regulatory T (Treg) cells that are critical for pregnancy tolerance. Effects of EDCs on immune cells are complex and likely exerted by both steroid hormone-dependent and hormone-independent pathways. Thus, to better understand how EDCs impact reproduction and pregnancy, it is imperative to consider how immune-mediated mechanisms are affected by EDCs. This review will describe evidence that several EDCs modify elements of the immune response relevant to pregnancy, and will discuss the potential for EDCs to disrupt immune tolerance required for robust placentation and optimal fetal development.

Keywords: endocrine disrupting compounds, reproduction, reproductive immunology, pregnancy, fetal tolerance, developmental origins of health and disease

INTRODUCTION

Endocrine disrupting compounds (EDCs) are defined by their potential to alter endocrine function through mimicking or blocking the actions of endogenous hormones (1, 2). Exposure to EDCs is considered a contributing factor in the increasing prevalence of common metabolic, neurological and inflammatory diseases. Male and female reproductive disorders, and a myriad of conditions including obesity, diabetes, non-alcoholic fatty liver disease, neurodevelopmental disorders, allergy, asthma, autoimmunity, and cancer, are all associated with EDC exposure (1). Alarmingly, the estimated human disease cost of EDCs in 2016 was 2.33% of GDP ($340 billion USD) in the USA and 1% of GDP ($217 billion USD) in Europe (3). Recent reports commissioned by the World Health Organization recommend greater investment in research to better understand the health impact of EDCs. Identified research goals include the development of comprehensive testing methods to detect EDCs, improved reporting mechanisms for chemical composition of products, and the need for more cross-disciplinary research to fully understand the impact on public and global health of EDCs contacted in everyday life (1).

EDCs are structurally and functionally diverse chemicals that can be natural or synthetic in origin (1). Natural forms include phytoestrogens found in widely-consumed food and animal products. These are likely less harmful than synthetic EDCs since they have generally low affinity for estrogen receptors (ER) (4), and exhibit low stability compared to many synthetic compounds that are engineered to be stable. However, given the high levels present in some foods, including infant formula, and the fact that the abundant phytoestrogen genistein binds ERβ with relatively high affinity, the potential health impacts of phytoestrogens need to be considered (4, 5).

Synthetic EDCs are far more diverse with several hundred identified and classified as persistent (exhibiting bioaccumulation) or non-persistent in the environment (2, 5). These compounds are present in many commonly used household and industrial products. They include chemicals used as solvents or lubricants, plasticizers, pesticides, fungicides, and pharmaceutical agents, that are present in plastics, detergents, household chemicals and building products, fire retardants, food, medicines, personal care products, perfume, and cosmetics (5).

EDCs interfere with the synthesis, biological actions, and metabolism of endocrine hormones, and disrupt hormone-regulated homeostatic processes in many tissues and physiological systems (2, 5). Through competitive interactions with hormone receptors, EDCs can act as agonists or antagonists and have a multitude of effects that range from enhancement, dampening, or blocking the action of endogenous hormones (2). Depending on the nature of the interaction, EDCs often exert non-monotonic dose responses characterized by low-dose effects, rather than linear dose responses like most other bioactive agents (6, 7). EDCs can also modulate synthesis of hormones and their respective receptors (2). Through these actions, they can interfere with physiological events and tissue homeostasis over the entire life cycle (2, 8–10). Depending on variables such as the duration, type, and dose of exposure, EDCs can exert transient or permanent impacts, to elevate long-term risk of chronic metabolic, neurological and immune diseases that may only become evident in later life (2, 9).

The events of reproduction, pregnancy, and fetal development are highly sensitive to EDCs because they involve a greater degree of tissue remodeling and hormone-dependence than other physiological processes. EDCs exert negative impacts on fertility and reproductive outcome, affecting gamete, embryo, and fetal development (2, 8, 11–13), with consequences that can cause fetal loss or attenuate offspring phenotype to impact lifetime health (2, 9, 14–19). The specific mechanisms by which different EDCs exert adverse developmental effects are not yet clear, and are likely to be complex and diverse. A large body of research has been generated in recent years to describe actions in different male and female reproductive tissue compartments. These actions are largely attributed to disruption of the hormone signaling that regulates most aspects of male and female reproductive physiology (2, 5).

In addition, EDCs are now understood to affect immune system development and function (20, 21), modulating many aspects of inflammatory and immune responses involving both the innate and adaptive immune compartments (22–29) (**Table 1**). Most reproductive processes are intimately dependent on a functional and appropriately balanced immune response (32, 33). Placental development and fetal growth are particularly dependent on adequate support from the maternal immune system (34), and a deficit in maternal immune cells and mediators that confer fetal tolerance is a central cause of poor gestational outcomes and impaired fetal development. An aberrant maternal immune response, that is insufficient in strength or skewed towards inflammation, can manifest as infertility, pregnancy loss, or a poor gestational outcome (32, 33, 35). Most often, these outcomes stem from failure of the maternal immune response to support embryo implantation and allow robust placental development (35–37).

These considerations raise the question of whether the adverse effects of EDCs on reproduction and pregnancy are at least partly due to mechanisms mediated by immune cells. Given the central role of the immune response in pregnancy, and the ubiquitous exposure of humans to environmental EDCs, it seems likely that EDC-induced immune disorders are a factor in the increasing incidence of fertility and gestational disorders.

This review will summarise evidence that common EDCs have capacity to interfere with pregnancy and fetal development through modifying maternal immune cells and mediators. We make the case that, given its critical importance to pregnancy outcome, and its sensitivity to perturbation by EDCs in other settings, the immune response warrants investigation as a mechanism by which EDCs affect reproductive success. Ultimately, devising strategies to protect humans and animals from the adverse reproductive effects of EDCs will require greater understanding of the how the immune system–EDC interaction contributes.

ENDOCRINE DISRUPTING COMPOUNDS AND REPRODUCTION

EDCs are well-documented to interfere with male and female reproductive hormone function, through genomic and non-genomic mechanisms that exert a wide range of endocrine

TABLE 1 | Common endocrine disrupting chemicals shown to impact the immune response.

Common Endocrine Disrupting Chemicals	Description/Sources
Bisphenol-A (BPA)	* most pervasive EDC * estrogen mimic * found in canned food, dental sealants and composites, and widely used in manufacture of epoxy, polycarbonate plastics and unsaturated polyester resins (30)
Phthalates	* widely used as plasticizers in polyvinyl chloride (PVC) products to impart flexibility and durability, including building materials, toys, personal care products and medical devices (31) * gained considerable attention due to specific concerns about pediatric exposure (31)
Alkylphenols * Nonylphenol (NP) * Octylphenol (OP)	* widely used as non-ionic surfactants in household applications, industrial and cosmetic products * undergo significant bioaccumulation due to their lipophilic properties and have weak estrogenic activity
Butyltins * Tributyltin (TBT) * Dibutyltin (DBT)	* found in plastic food containers, plastic water bottles, PVC pipes
Insecticides * Dichlorodiphenyltrichloroethane (DDT)	* agricultural and household use * persists in environment * estrogen mimic
Fungicides * Vinclozolin	* agricultural and household use
Herbicides * Atrazine	* agricultural use on crops * used on artificial turf
Parabens * Methylparaben	* common preservatives * used in food, cosmetic and pharmaceutical products * estrogenic effects
Brominated flame retardants * Polybrominated diphenyl ethers (PBDE)	* flame retardant used in household and industrial products * endocrine disrupter with carcinogenic properties
Synthetic hormones * 17α-ethinylestradiol	* used in oral contraceptive pills and found as a contaminant in wastewater * strong estrogenic properties

disturbances (5). In particular, these chemicals interfere with binding of hormones to their corresponding receptors, notably including estrogen receptor and androgen receptor to cause either agonistic or antagonistic effects. The net consequence is interference with physiologically normal signal transduction pathways, eliciting downstream changes to target gene expression and cellular function (38). Different EDCs exert a variety of effects on hormone signaling depending on the timing of exposure and the amount of EDC administered (2, 5).

Dysregulated hormone synthesis and signaling in reproductive tissues, and systemically in the hypothalamic-pituitary axis, thyroid, and other tissues influencing reproductive function, converge to have substantial consequences for sexual maturation and fertility (39–41). Emerging evidence indicates that sensitivity to EDCs is modulated by age and a range of environmental, lifestyle, and genetic factors that can exacerbate the impact of EDCs on reproductive health (3, 42, 43). These factors contribute to the difficulty in comparing studies and considerable discrepancies between study outcomes (3, 44).

Large clinical studies show correlations between EDC exposure and fertility disorders in women. Most notably, occupational exposure to EDCs, or consumption of EDC-laden foods, are associated with increased risk of infertility, time-to-pregnancy, and early pregnancy loss (14, 45). These effects may reflect early life and life course accumulated exposures. In particular, prenatal effects of EDCs are linked with later life incidence of reproductive conditions including polycystic ovarian syndrome, endometriosis, uterine fibroids, and reproductive cancers (9). In an IVF setting, women exposed to certain pesticides appear more likely to exhibit defects in oocyte maturation and developmental competence, leading to impaired fertility, embryonic defects, and poor IVF outcomes (41, 45).

Research in rodent models provides insight on how EDCs impact reproductive endocrinology (2, 40, 46). These manifest most obviously as altered timing of sexual maturation, impaired gamete development, and reduced fecundity (39, 46). For example administration to rodents or large animals of plasticizers such as phthalates and bisphenol A, (BPA), or pesticides including vinclozolin and glyphosate, all cause reduced ovarian weight, impaired follicle growth and oocyte viability, and reduced synthesis of ovarian sex steroid hormones (46–48). For detailed information on the specific impacts of EDCs on female reproductive physiology, the reader is directed to the following reviews (2, 5, 9, 40, 46, 49).

EDCs also exert considerable effects on male reproduction and gamete developmental competence. Direct or gestational exposure of male rats and mice to any of several EDCs leads to reduced reproductive capacity, characterized by decreased gonad weight, testosterone levels, and gamete quality, as well as increased likelihood of reproductive conditions including testicular cancer, cryptorchidism, and hypospadias (39, 40, 50). *In vitro* studies show in cattle that exposure to low doses of the herbicide atrazine reduces sperm viability and impairs capacity to undergo acrosome reaction in response to calcium signals (51). In men, epidemiological evidence shows a clear negative association between EDCs and male reproductive parameters, in association with reduced sperm concentration, motility, viability, DNA integrity, and altered sperm methylation patterns (40, 50, 52, 53). Various EDCs are also readily detectable in seminal plasma (54), and the seminal vesicles, which are the major source of seminal plasma, are an important target of EDCs including diethylstilbestrol that targets estrogen receptor-α (55). These changes are likely to compromise fertility, and alter reproductive outcomes beyond the fertilising capacity of sperm. In men utilising IVF clinics, exposure to phthalates was associated with differential methylation of specific DNA sequences in sperm, and was inversely associated with blastocyst quality (53). A wide range of specific effects of EDCs on male reproduction are reported, and these are reviewed in detail elsewhere (2, 5, 9, 39, 40).

ENDOCRINE DISRUPTING COMPOUNDS AND PREGNANCY

Fetal and placental development are highly hormone-dependent processes and are therefore particularly susceptible to endocrine

signaling disturbances (56–58). Reproductive-aged women are at high risk of EDC exposure, especially through everyday exposure to personal care products and household chemicals, and the events of pregnancy would reasonably heighten the health risks of EDC exposure in women (56). There is compelling evidence implicating EDC exposures as a risk factor in a range of pregnancy disorders (59–63). Several clinical and epidemiological studies link EDCs, notably pesticides and plasticizers, in common pregnancy complications that together affect around 20% of women, including recurrent miscarriage, fetal growth restriction, preeclampsia and related hypertensive disorders, and preterm birth (13, 56, 61, 64, 65). Many studies consistently show a wide array of EDCs are detectable in the urine, cord blood, plasma, amniotic fluid and breast milk of the vast majority of pregnant women (66–68). Patterns of exposure depend on geographic, socioeconomic, occupational and lifestyle factors, and fluctuate over the course of pregnancy, to occur in infinitely variable combinations (known as the 'exposome') that might have stronger relationships to adverse outcomes than any individual chemical exposure (69, 70). Nevertheless, while causal relationships are difficult to prove in humans, extensive studies show strong evidence of correlations between adverse clinical outcomes and serum or urinary levels of bisphenol A (BPA), phthalate metabolites, organophosphate pesticides, and other EDCs (61, 71–73).

EDCs may operate through pre-pregnancy exposures that affect organs systems critical for pregnancy health, through gestational exposures that interfere with hormone control of fetal and placental development and function, or other *via* systemic adaptations required to sustain pregnancy (63). There is clear evidence that pregnant women with existing health disparities, associated with low socioeconomic status, or certain racial groups such as non-white women in the US where levels of chemical toxicants are often higher, exhibit a disproportionate health burden associate with EDC exposures (3, 44).

The placenta is implicated as an important target for EDC actions. As a rapidly developing, dynamic organ the placenta is highly responsive to hormone regulation during its morphogenesis, and expresses a wide array of hormone receptors that control placental supply of nutrients to the growing fetus (57, 58). The placenta adapts to fetal and environmental cues to reconcile fetal demand for growth with nutrient availability, and disruption of hormone signaling interferes with this adaptive capability to disturb fetal growth and developmental programming (57).

Animal models document a range of potential mechanisms by which EDCs disrupt placental and fetal development. Some EDCs, notably including BPA and triclosan, accumulate directly in placental tissues, where they modulate placental hormone synthesis and metabolism (74, 75). *In vitro* experiments show that a range of EDCs can exert direct effect in trophoblasts including regulation of signaling pathways to cause genetic and epigenetic changes that impact cell survival and invasive capability (75). It seems likely that effects of EDCs are prominent in early pregnancy during placental morphogenesis, when the extent of invasion into maternal tissues, and interaction with the maternal vasculature, is rate-limiting for later gestation placental transport function (58). However because EDC effects in placental cells have not been well investigated to date, it is not yet possible to discern the contribution of direct effects in trophoblasts, versus mechanisms that involve the maternal compartment (58).

ENDOCRINE DISRUPTING COMPOUNDS AND OFFSPRING HEALTH

The effects of EDCs on the developing fetus have a lasting impact on offspring phenotype and susceptibility to later life health and disease (9, 76). The developmental defects caused by maternal EDC administration in pregnancy can have life-long and even transgenerational consequences (77). Maternal EDC exposures likely impart changes to offspring health and behaviour through direct effects in the placenta and fetus, as well as indirectly through maternal physiological adaptations required to support pregnancy.

Animal studies show EDCs including pesticides, phthalates and BPA act to decrease fertility, alter anogenital distance, cause early puberty, and disrupt testis/ovarian function in both male and female offspring (40). These exposures not only disrupt offspring reproductive capacity, but also alter aspects of development affecting brain and endocrine function (15, 16, 78). In humans there is compelling evidence that gestational exposure to a variety of EDCs during fetal life leads to decreased infant birth weight, reduced anogenital distance in male neonates, increased incidence of childhood obesity, and alterations to neurodevelopment and cognitive function, leading to reduced IQ and behavioral problems (9).

Concerningly, there is emerging evidence that EDCs can exert transgenerational effects, such that not only the immediate offspring, but also future generations may be impacted after maternal contact in pregnancy (9, 40). This may be mediated through epigenetic modifications to DNA methylation profiles in fetal gametes, caused by inappropriate timing or inhibition of activation signals during gamete development, or through DNA adduction induced by EDCs or their metabolites (40, 79).

The impact of paternal exposures and their mechanisms of action are less well defined, but emerging evidence points to effects on offspring phenotype mediated by altered epigenetic properties of sperm (80). Furthermore, EDCs present in seminal plasma (54), or altered seminal plasma composition resulting from EDC-attenuated ERα signaling (55), have potential to transmit effects of paternal exposures to offspring. This could occur by impaired capacity of seminal plasma to support sperm integrity, or by attenuating seminal plasma signals that modulate female reproductive tract gene expression and receptivity for pregnancy (81, 82). In mice, it has been reported that paternal contact with BPA prior to conception impairs offspring spatial memory (19), and alters social behaviour with increased anxiety in male offspring (83). These can begin very early in the life course – male fetuses exposed *in utero* to the fungicide vinclozolin or pesticide dichlorodiphenyltrichloroethane (DDT) exhibit later epigenetic changes in sperm that can be

transmitted to male offspring (84). In zebrafish, exposure to synthetic estrogen 17α-ethinylestradiol leads to an altered sperm and testicular transcript content, causing lymphodema in offspring (85). In human, recent evidence from a large-scale epidemiological study demonstrates a link between birth defects and fathers' occupational exposure to EDCs (17).

VIVIPAROUS REPRODUCTION AND THE IMMUNE RESPONSE

The embryo and the gestational tissues formed after implantation express antigens foreign to the mother, including transplantation antigens encoded by major histocompatibility complex (MHC) genes (33, 86). Both the innate and adaptive compartments are involved in the maternal immune adaptions required to avert effector immune responses to conceptus antigens (86, 87). Contrary to common assumptions, pregnancy requires a state of adaptive immune tolerance that depends on maternal lymphocytes being actively primed to recognise conceptus antigens (35, 86). Priming of the adaptive immune compartment must commence prior to implantation in order to initiate the necessary events of implantation, placental development and fetal growth, and ultimately to orchestrate on-time parturition and birth (35, 86).

Immune Mechanisms Essential for Implantation and Placental Development

Tightly controlled maternal immune regulation is important over the course of pregnancy, but the most critical period is the peri-conception phase spanning fertilization to embryo implantation (35). A series of dynamic changes in the uterine immune response determine whether or not embryo implantation can occur (88), and are instrumental in setting the trajectory of fetal development and shaping the offspring phenotype (35, 80, 89). Immune adaptation commences with sex hormone-induced changes in the ovulatory cycle followed by an inflammation-like response to seminal fluid components at coitus (90). Estrogen and seminal fluid together induce an influx of neutrophils, macrophages and dendritic cells (DCs), into the mucosal surface of the cervix and uterus (91–94). This is followed by transition to an anti-inflammatory and pro-tolerogenic immune environment in order to acquire embryo receptivity (34, 35, 86). Implantation only occurs if immune cells in the uterine endometrium exhibit a favourable, permissive response. In particular, expansion and recruitment of specialized immune cells known as regulatory T cells (Treg cells) must occur (95–98). Treg cells interact with dendritic cells and macrophages to promote decidualisation of uterine stromal cells, suppress inflammation, and inhibit effector immunity towards fetal antigens.

After implantation, an array of soluble mediators including cytokines, chemokines, steroid hormones, and prostaglandins released from placental trophoblasts are important for sustaining the developing fetal-placental unit (32). As well as Treg cells, abundant populations of uterine natural killer (uNK) cells act to mediate structural changes in the decidual vasculature that support placental invasion and development (99–101). Macrophages, DCs, and Treg cells each interact with uNK cells to facilitate the uterine vascular changes, while continuing to suppress inflammation and prevent immune effector cell activation (35, 100, 101) (**Figure 1**).

ENDOCRINE DISRUPTING COMPOUNDS AND THE IMMUNE RESPONSE TO PREGNANCY

Maternal EDC exposure is an identified risk factor in unexplained infertility and pregnancy complications, including preeclampsia, intra-uterine growth restriction, recurrent miscarriage, and spontaneous preterm birth (13, 61, 64, 65). Interference in hormone synthesis and signaling is implicated in the mechanisms by which EDCs contribute to pregnancy disorders (9), and there is a strong biological rationale to implicate inflammation, oxidative stress, and immune cells as local mediators of the pathophysiological changes induces by hormone dysregulation (59). That immune and inflammatory mechanisms are central to infertility and pregnancy disorders supports the prospect that EDCs act, at least in part, by driving an inappropriate maternal immune response (20, 21).

There is some evidence that EDCs are especially problematic in the peri-conception phase of pregnancy, when the maternal immune response is first established and the critical events of implantation and early placentation occur. Elevated phthalate metabolites in urine were shown to correlate with altered progression of embryo implantation, as indicated by a slower or faster rise in human chorionic gonadotrophin, with different metabolites appearing to be protective or adverse in their effects (102). Whether immune mechanisms are involved is not known, but seems biologically plausible. Others have shown that first trimester maternal peripheral blood cytokine levels correlate with the presence of several EDCs in urine, with a notable association between phthalates and pro-inflammatory interleukin (IL)-8 and interferon (IFN) (70). In another study, clear associations between polybrominated diphenyl ethers (PDBEs) and pro-inflammatory cytokines IL-6 and tumor necrosis factor (TNF), as well as between per- and poly-fluorochemicals (PFAS) and IL-6, were found in maternal peripheral blood in the second trimester (103). Similar associations between EDCs and pro-inflammatory cytokines were seen at term, in infant cord blood (70). These observations are consistent with EDCs acting to impair resolution of the inflammatory response in early pregnancy and compromise tolerance as pregnancy progresses, but additional studies would be required to prove this.

Only a small number of mechanistic studies have specifically explored the impact of EDCs on maternal or fetal immune parameters in pregnancy, but several point to a pro-inflammatory pathology that affects the vascular adaptations required for robust placental development. In mice, short-term oral BPA exposure in early pregnancy was shown to cause impaired spiral artery remodeling and intra-uterine growth restriction (11). Although the number of uNK and mast cells was not changed, this study

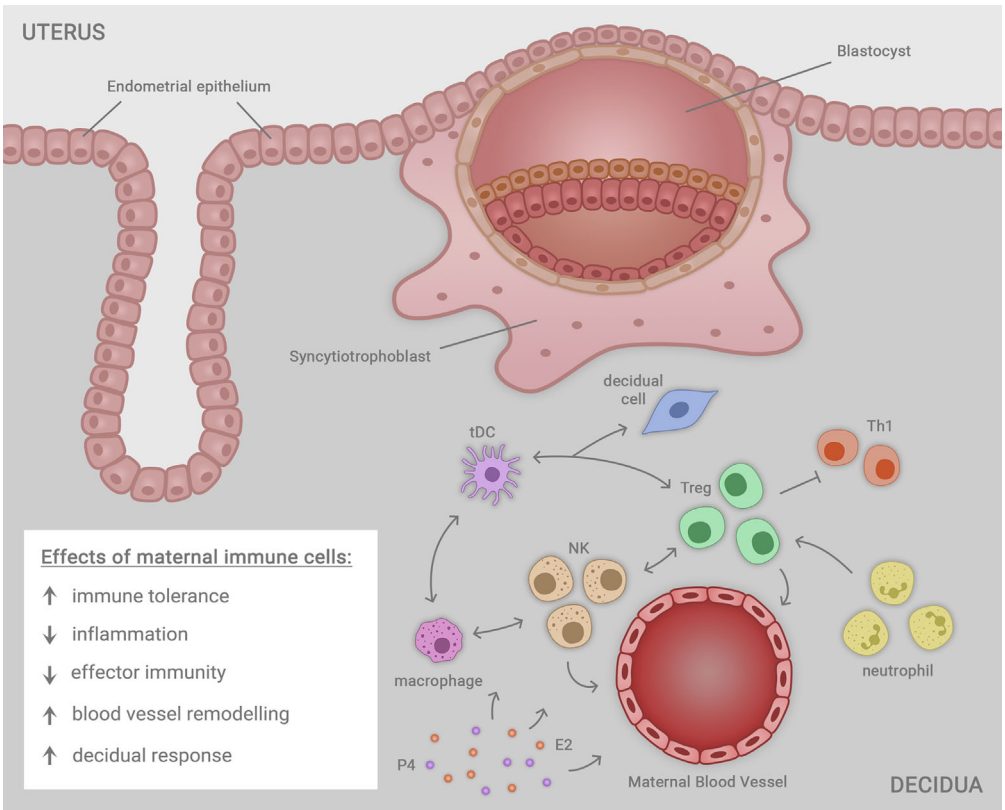

FIGURE 1 | Immune cells including macrophages, natural killer (NK) cells, regulatory T cells (Treg cells), neutrophils and tolerogenic dendritic cells (tDC) residing in the uterine decidua each contribute in a network of cellular interactions to facilitate embryo (blastocyst) implantation and trophoblast outgrowth, required for progression to healthy pregnancy. The decidual immune cells exert a range of regulatory effects on the local microenvironment that each contribute to the success of implantation, ensuring robust placental development that in turn supports healthy fetal growth and development in later gestation. The immune cells together act to mediate immune tolerance, suppress inflammation, inhibit effector immunity mediated by T helper type 1 (Th1) cells, promote uterine blood vessel remodeling, and facilitate transformation of uterine stromal cells in the decidual response. Ovarian sex steroid hormones estrogen (E2) and progesterone (P4) act to regulate immune cell populations through direct effects in immune cells, and indirect effects mediated by non-immune cell synthesis of immune-regulatory factors.

did not assess phenotypes of these cells, or the potential influence of other immune cell populations. Another study reported reduced trophoblast invasion and impairment of uterine vascular remodeling after low dose BPA administration in mice, along with preeclampsia-like features of maternal hypertension and elevated angiogenesis biomarkers and glomerular atrophy (104). Also consistent with an inflammatory mechanism, administration of polychlorinated biphenol (PCB) to mink resulted in uterine vascular changes and placental lesions, with degeneration of endothelial and trophoblast cells, particularly in the placental labyrinth zone (105). Low dose 17α-ethinylestradiol, used in oral contraceptive pills and prevalent in water supplies, caused impaired spiral artery remodeling, altered placental development, and fetal growth restriction (106). In non-pregnant mice, uterine expression of heat shock proteins (HSPs) that play important roles in antigen presentation and DC function are elevated in response to low dose BPA (107), but the impact of elevated HSPs on pregnancy is not clear.

Studies in other reproductive tissues are consistent with possible pro-inflammatory and immune-mediated effects of EDCs (108). In the mammary gland, BPA exposure in utero causes long term changes in expression of both pro- and anti-inflammatory cytokines, and this is postulated to be a potential mechanism for programming breast cancer risk (109).

As well as influencing the maternal immune compartment, EDCs likely elicit direct effects on immune cells in the placenta and fetus. The presence of EDCs in amniotic fluid and cord blood shows that many chemicals cross the placenta to access fetal tissues (58, 75). A wide range of EDCs including pesticides, plasticizers, fire retardants, and components of personal care products can be detected in the placenta (75). Compelling evidence of EDC effects on the developing fetal immune response is emerging (110, 111). In particular, phthalates and phenols are implicated as a factor in fetal programming of asthma and allergic airways disease, while heavy metals and air-borne particulates also contribute (21, 112). A wide range of immunomodulatory effects of EDCs on human immune cell development are reported, through mechanisms operating at the cellular, molecular, and epigenetic levels to alter innate and adaptive immune function in offspring (110).

EDCs AND HORMONE CONTROL OF IMMUNE CELLS

A clear mechanism for EDCs exerting significant influence on the maternal immune environment exists, as endocrine signaling in immune cells is an important aspect of normal immune regulation (113). Steroid hormones exert both direct and indirect influence on immune cells, the former through ligation of classical steroid hormone receptors for estrogen, androgens, and progesterone, to regulate a wide range of target genes. In addition, steroid hormones have rapid non-genomic effects in immune cells *via* binding to non-classical receptors on the cell membrane or in the cytoplasm (114). As well, steroid hormones control expression of a vast array of cytokines and chemokines in non-immune cell lineages in hormone-responsive reproductive tissues, to exert indirect effects on resident immune cell populations through this route (115).

It is well known that female sex steroid hormones exert potent regulatory effects on immune cells systemically and locally within the female reproductive tract over the course of the menstrual cycle and during pregnancy. In particular, estrogen and progesterone play important roles in the induction of maternal immune tolerance, both through direct signaling in immune cells and indirectly through actions on epithelial and stromal cells in the female reproductive tract (116, 117). Over the course of the estrous and menstrual cycle and after conception, estrogen and progesterone are key factors in driving expansion of Treg cells in readiness to accommodate embryo implantation (118–120).

Given this direct and indirect regulation by hormones, immune cells are highly susceptible to the effects of EDCs. EDCs broadly affect various immunological processes, including cellular and humoral responses, survival, differentiation and phenotypic maturation, as well as secretion of cytokines and other immune signaling mediators (22). Emerging evidence demonstrates substantial potential for EDCs to interfere with the endocrine signaling required for maternal immune adaptation to pregnancy (**Figure 2**). Below, we summarise the current evidence for EDC action on the innate and adaptive components of the immune response relevant to pregnancy, with a focus on immune cell types affected by EDCs and implicated in reproductive success. The argument that EDCs may act in pregnancy through influencing the maternal immune response is supported by studies of EDC effects on immune cells in other tissue settings and disease contexts.

Macrophages

Macrophages contribute to embryo implantation, placental development, and timing of birth (35, 121, 122). In the maternal compartment, immune-regulatory macrophages constrain inflammation, influence the adaptive immune response, and modulate uterine vascular function (123). EDC disruption of their functional phenotypes is likely to adversely impact placental morphogenesis, pregnancy progression and fetal development. In the placenta, a large population of fetus-derived macrophages known as 'Hofbauer cells' exert direct effects on placental development and transport function. These cells can respond to proinflammatory stimuli and contribute to

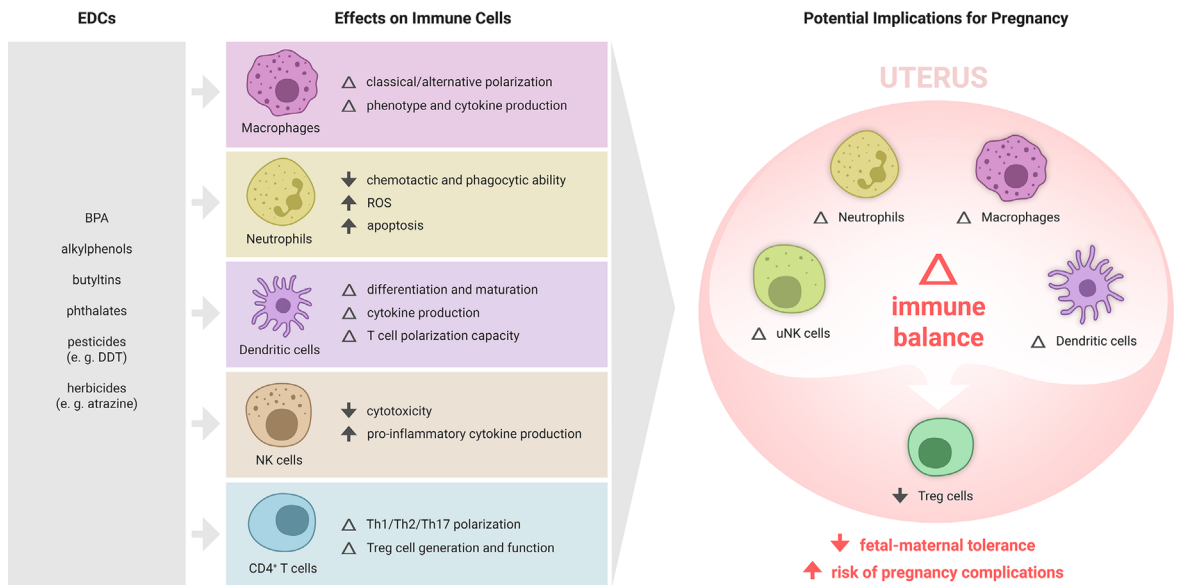

FIGURE 2 | Summary of EDC effects on immune cell subsets and potential implications for maternal immune adaptation to pregnancy. Various EDCs affect the differentiation, phenotype and function of specific immune cell subsets, each of which play important roles in maternal immune adaptation to pregnancy. While the effects of EDCs on the immune response to pregnancy are yet to be formally examined, there is substantial evidence from other settings showing that various ECs can modulate macrophages, T cells, NK cells, and dendritic cells. In particular, EDCs that impair the generation of regulatory T cells (Treg cells), key mediators of fetal-maternal tolerance that are essential for embryo implantation and placental development, are likely to elevate susceptibility to pregnancy complications, and warrant investigation as contributing risk factors in recurrent miscarriage, preeclampsia, preterm birth and related gestational disorders.

placental inflammation (124), so would also be susceptible to immune-modulatory effects of EDCs. Placental macrophages have been shown to upregulate production of prostaglandin E2 (PGE2) and cyclo-oxygenase-2 after exposure to mono-2-ethylhexyl phthalate (MEHP), the active metabolite of diethylhexyl phthalate (DEHP) (125).

Evidence from animal studies indicates that EDCs have capacity to alter macrophage phenotype and function, in a manner dependent on the polarization state of the macrophages at the time of exposure and the specific EDC (110). The most extensive evidence exists for effects of EDCs in M1-like classical macrophages. In murine macrophages, treatment with BPA, the alkyl phenols p-n-nonylphenol (NP) and p-n-octylphenol, or the chlorinated phenols 2,4-dicholophenol and pentachlorophenol, each lead to inactivation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling and suppression of TNF and nitric oxide (NO) following stimulation with lipopolysaccharide (LPS) (126–130). Interestingly, the capacity of BPA to suppress LPS-induced macrophage polarization is blocked by the ER antagonist ICI 182.780, suggesting BPA acts to regulate NF-κB signaling *via* ER (128, 129). Some of these effects occurred independently of classical ER signaling and were likely mediated by non-classical ER (129).

Other studies report that BPA and other EDCs have differing effects on macrophage production of pro-inflammatory cytokines and mediators, promoting a more activated, classical M1-like phenotype. For example in the mouse, benzo(a)pyrene (B(a)P) and hexachlorobenzene increase the production of NO in macrophage cell lines (127, 131). Similarly, human THP1 cell line-derived macrophages cultured with BPA exhibit increased pro-inflammatory TNF and IL-6 expression, dependent on classical ER signaling (132) Finally, treatment of mouse macrophages with EDCs including BPA, NP, dicyclohexyl phthalate and B(a)P causes cell death through apoptosis and necrosis pathways (127, 131).

Recent studies indicate impacts of various EDCs on M2-like alternative macrophages. In mice, exposure to polybrominated diphenyl ethers (PBDE) enhances estrogen mediated regrowth of mammary glands, in a manner potentially mediated by enhanced IL-10 expression and polarization of macrophages towards an M2-like state (133). Similarly, *in vivo* oral exposure of mice to BPA promotes the transition from ductal carcinoma *in situ* to invasive breast cancer through increases in pro-tumorigenic cluster of differentiation (CD)206^+ M2-like alternatively activated macrophages (134). In contrast, other studies demonstrate that *in vitro* treatment of NP to mouse bone marrow-derived macrophages decreases their polarization by IL-4 toward an M2-like phenotype, associated with reduced survival in LPS-induced sepsis (135).

These studies indicate EDCs at physiological doses may promote or inhibit several aspects of both classical and alternate macrophage activation and effector function. The differential effects observed are likely due to differences in dose, context and type of EDC, and therefore, further work is required to develop greater understanding of the effect of EDCs on macrophages in various settings in mice and humans.

Neutrophils

Neutrophils are important in preparing the female reproductive tract for embryo implantation, especially after coitus when they clear microorganisms, seminal fluid debris, and superfluous sperm, and help guard against sexually transmitted infection (90). Recent studies in mice and humans show that neutrophils are programmed by decidual signals to acquire an activated, pro-angiogenic phenotype (136, 137) akin to functions observed for tumor-associated neutrophils in cancer (138), suggesting a key role for neutrophils in establishing pregnancy. Given their importance in protecting from infection, and their emerging roles in regulating decidualization and placental development, studies to understand the impact of EDCs on uterine neutrophils may reveal novel pathways that exert long term influence on offspring health.

In both animal and human models, various EDCs impair neutrophil chemotactic and phagocytic ability and increase neutrophil apoptosis (139–142). In humans, chronic exposure to the pesticide DDT leads to a reduction in neutrophil chemotactic and phagocytic capacity that inversely correlates with incidence of infectious disease (143). BPA exposure is associated with increased reactive oxygen species (ROS) in human neutrophils *via* ER signaling, but does not cause changes in ROS-dependent formation of neutrophil extracellular traps (139).

Dendritic Cells

Several effects of EDCs on DC differentiation and maturation are reported, where EDCs have been shown to shift the polarization and expression of maturation markers on DCs. In murine models, *in vitro* atrazine exposure leads to phenotypic changes, causing a dose-dependent loss of DC surface MHC class 1, as well as decreased CD86, CD11b, CD11c and CD14 expression (144). Other studies show EDCs alter DC cytokine production in mice, eliciting increased TNF and decreased IL-10 (145, 146). In other studies, BPA and NP induce the differentiation of murine bone marrow cells into DCs, with BPA having a more substantial effect than NP in altering differentiation capacity (147).

Mechanistic studies show that EDCs exert effects on DCs through both ER-dependent and –independent pathways. In a model of ovalbumin-induced allergic lung inflammation, NP-treated mice developed more severe inflammation compared to the control, however this effect was eliminated in mice carrying an aryl hydrocarbon receptor (AhR) mutation, suggesting NP may affect DCs *via* AhR-dependent (ER independent) pathways (146). In humans, exposure to the alkylphenols NP enhance TNF and suppress IL-10 and type 1 IFN production in peripheral blood mononuclear cell (PBMC)-derived plasmocytoid DCs. The ER antagonist ICI 182.780 could reverse NP-induced TNF and IFN-β expression but was unable to reverse the suppressive effect of NP on IL-10 or IFN-α expression in plasmocytoid DCs, suggesting both ER-dependent and -independent pathways of alkyphenol regulation of DCs occur (145).

DCs are critical regulators of the strength and quality of an adaptive immune response through signals delivered at antigen presentation, and the impact of EDCs on DC antigen presentation has been explored. EDCs such as BPA influence

DC maturation and phenotype leading to an increased capacity to induce T-helper (Th)2 responses (148). Similarly, suppression of type 1 IFNs in human plasmocytoid DCs by phthalates programs a Th2 phenotype in T cells characterized by suppressed IFN-γ and enhanced IL-13 production (149). In contrast, BPA exposure increases CD1α expression in human PBMC-derived DCs, enabling them to drive polarization of naïve $CD4^+$ T cells towards a Th1 phenotype (150). While it is evident that EDCs can modulate DC phenotype, further studies are required to understand the specific effects of these DC changes for T cell phenotype, function, and maturation state.

Effects of EDCs on the DC contribution to pregnancy tolerance are unclear, but reasonably it would be expected that altered DC function and interaction with T cells could disrupt normal immune balance during pregnancy, and potentially skew permissive Treg cells towards destructive Th1 cells (151). In particular, an increase in TNF and IL-6 secretion by DCs in the peri-implantation period may create excessive inflammation that negatively influences embryo development. Whether EDC-exposed DCs inhibit Treg cells is an important question with substantial implications for fetal-maternal tolerance (151).

Natural Killer Cells

Natural killer (NK) cells are affected by a wide range of EDCs, all of which appear to decrease NK cell recognition of and cytotoxicity towards tumour cells, even after brief and low concentration EDC exposure (110). These functions are elicited through changes in NK cell surface markers and production of inflammatory cytokines, ultimately leading to changes in cellular function (110). For example, tributytlin (TBT) and DDT exposure significantly decrease the cytotoxic function of human NK cells *in vitro*, modulating their expression of cell surface proteins including CD16, CD18 and CD56, as well as cytolytic proteins such as perforin and granzyme B (152, 153). The loss of NK cell lytic function following exposure to these EDCs appears to result from activation of protein kinase C and the mitogen-activated protein kinase pathway (154–156). However, not all EDCs elicit the same functional effects in NK cells. *In vitro* atrazine exposure inhibits the ability of NK cells to lyse target cells through blocking lytic granule release, without impacting the release of perforin or granzyme proteins (157), demonstrating that EDCs have differing functional effects, presumably reflecting different mechanisms of action.

EDCs also have significant impact on the production of inflammatory cytokines by NK cells, with several studies clearly demonstrating NK cells exhibit non-monotonic dose responses (110). Inflammatory cytokines such as TNF, IL1-β, IL-6 and IFN-γ were increased in response to low-dose exposure to various EDCs including TBT and dibutylin (DBT) (158–160). In the case of TBT-induced pro-inflammatory cytokines this was mediated through the activation of extracellular-signal-regulated kinase 1/2 and p38 kinase pathways (158–160).

Overall, these studies demonstrate that EDCs have substantial capacity to modulate NK cells, in ways relevant to uNK cell function in pregnancy. uNK cells are highly regulated by ovarian E2 and P4, and contribute to cyclic remodeling of the uterus over the course of the menstrual cycle in preparation for embryo implantation (161). Indeed uNK cells are the most abundant immune cell population in the uterus, where they promote decidualization, facilitate spiral artery remodeling, and play critical roles in placental development (99–101). In particular, the effect of EDCs on NK cells is relevant to the common condition of endometriosis where exposure to phthalates and PCBs are implicated, and altered uNK cells are reported (162, 163). To date, there are no studies examining specific changes to the phenotype or function of the uNK cell subset following exposure to EDCs, although experiments investigating effects of BPA on the uterine vasculature point to a possible role for uNK cells and a target of BPA effects (11, 101). Further research is required to examine the effect of EDCs on uNK cells and their role in mediating EDC effects on fertility and fecundity.

$CD4^+$ T Cells

In addition to indirect effects on T cell differentiation through the impact of EDCs on antigen presenting cells, there is evidence that EDCs directly influence $CD4^+$ T cell differentiation and function. In studies of allergic disease, multiple EDCs have been shown to augment immunoglobulin (Ig)E-related responses through a common mechanism of enhancing T cell production of the Th2 inducing cytokine IL-4, *via* stimulation of nuclear factor of activated T-cells binding activity (30, 31, 164). Similar responses are observed in studies comparing adult versus prenatal exposure to BPA in male mice. In these studies, BPA promotes the antigen-stimulated production of Th2 cytokines (IL-10, IL-13 and IL-4) in adult mice, and both IFN-γ and IL-4 in adult offspring exposed to BPA prenatally (165).

In vitro studies of isolated mouse T cells exposed to EDCs from the alkylphenol family show suppression of Th1 development and enhanced Th2 development, in a manner independent of retinoic acid receptors, progesterone receptors, glucocorticoid receptor, retinoid x receptor, or ER (166). While this Th2 inducing capacity of EDCs is recapitulated in other studies (167, 168), it is notable that up-regulation of Th1 responses following either adult or prenatal exposure is also reported. Addition of BPA directly to splenocytes *in vitro* favours differentiation of Th1 cells, characterized by decreased IL-4 and increased IFN-γ production (169). The reason for variable effects of different EDC interventions on $CD4^+$ T cell polarization remains unclear, but likely reflects the effects of different chemical entities, dose, duration or *in vivo* timing of exposure. Further studies are therefore required to fully understand the range of impacts of EDCs on $CD4^+$ T cell differentiation.

Taken together, the published findings provide clear evidence that common EDCs such as BPA and phthalates can modulate T cell differentiation and function. The disturbance to Th1/Th2 polarization induced by EDCs may predispose to a range of inflammatory diseases (i.e. allergy, autoimmunity, and asthma) (110), and is also relevant to generation of maternal immune tolerance in pregnancy, where a specific suppression of Th1 cells is critical (34, 35).

As well as affecting Th1/Th2 polarization, BPA exposure influences the differentiation and functional phenotype of Treg cells. The elevated antigen-dependent induction of Th2 cytokines after BPA exposure seen in adult mice occurs in conjunction with

a shift away from Treg cell generation. A dose-dependent decrease in the $CD4^+CD25^+$ Treg cells among $CD4^+$ T cells with increasing BPA concentrations is reported (165). Similarly, BPA exposure during gestation and prior to weaning leads to a perturbed induction of oral tolerance characterized by a diminished accumulation of Treg cells (170). NP exposure in mice elevates Th2 and suppresses Treg cell numbers, which counteract the effects of ER agonists in the treatment of allergic rhinitis (171). In contrast, *in vitro* exposure of murine T cells to atrazine inhibits $CD4^+$ T cell proliferation and elicits increased $Foxp3^+$ Treg cells (172), again highlighting the variable effects of different EDCs on lymphocyte biology.

A key mechanism implicated in EDC modulation of Treg cells involves the transcription factor AhR. In mouse T cells, AhR is a key regulator of T cell differentiation into Treg and Th17 cells (173), and AhR activation promotes differentiation of functional Treg cells (174). EDCs such as dioxins can bind with high affinity and activate AhR, leading to the induction of functional Treg cells that suppress experimental autoimmune encephalitis (173). Activation of AhR by naturally occurring activators of the AhR signaling pathway, such as 6-formylindolo[3,2-b]carbazole, elicits an opposite effect where Treg development is suppressed and Th17 differentiation boosted (173). Since other EDCs including phenols and phthalates also affect immune processes *via* AhR modulation, the AhR pathway may be a central determinant of the differential impacts of different EDCs on T cell differentiation (175).

Given these effects, it seems highly plausible that BPA exposure affects the expansion of Treg cells in early pregnancy and has potential to compromise fetal-maternal tolerance. Other EDCs demonstrated to interfere with Treg cell populations could reasonably also impair maternal immune adaptation to pregnancy. Given that Treg cell insufficiency is implicated in a wide range of gestational disorders (35), this warrants investigation as a convergent mechanism by which EDCs contribute to elevated susceptibility and the rising incidence of these conditions.

CONCLUSIONS

There is mounting evidence pointing to a contribution of EDCs in adverse pregnancy outcomes, as well as infertility and subfertility. While many studies have assessed mechanisms involving endocrine impacts of EDCs on reproductive processes, there has been limited exploration of mechanisms involving immune cells. Given the critical significance of the maternal immune response in pregnancy and the now substantial literature demonstrating that common EDCs interfere with key elements of the immune response relevant to pregnancy (**Figure 2**), it is important to consider immune dysregulation amongst the effects that EDCs may exert. In particular, EDC exposures in women prior to or around the time of conception have potential to disturb generation of maternal immune tolerance required at embryo implantation (34, 35), with ongoing consequences for placental morphogenesis, and susceptibility to gestational conditions that arise from compromised placentation. Since seminal fluid factors contribute to priming immune tolerance towards paternal antigens in women, is possible that male EDC exposures can also interfere with maternal immune tolerance in the female partner.

Research to uncover the significance of immune effects of EDCs in reproduction and pregnancy is aligned with the World Health Organization's recommendation to improve knowledge on EDCs and human health (1). This research should span a range of approaches. Laboratory animal studies will be critical for demonstrating causal effects, elucidating mechanisms, and defining effects of frequency and strength of different EDC exposures. Future studies must be designed with a view to their translational impact for health and clinical relevance. For example, several studies to date have utilized supraphysiological doses of EDCs in order to demonstrate an impact, and these now need to be replicated using environmentally relevant doses (2). Nevertheless, there is compelling evidence from both reproduction (2) and immune (22, 110) studies that EDCs can exert substantial effects at low doses relevant to those in human environments. Building the evidence for environmentally relevant exposures is a priority, as is unravelling the complex biology of the U-shaped dose response curve typical of many EDCs (2).

Large scale human cohort studies will be important for investigating how EDCs interact with other environmental and lifestyle factors that attenuate their biological effects, and quantifying the relative risk attributable to EDC exposures. As noted in the US Endocrine Society's Second Scientific Statement (2), studies must be carefully designed to take into account variables that likely attenuate EDC risk, including genetic diversity, socioeconomic status, geographic variables, age at exposure, and occupation (3, 42, 43). Importantly, pregnant women must be included in population studies and future research must focus on pregnancy as a critical period for investigation (56). In turn this work will inform public policy and justify government regulations on environmental exposures that impact reproductive and pregnancy health. The benefits will extend to rare and endangered species and economically important livestock animals, where EDCs will otherwise exert accumulating harm.

AUTHOR CONTRIBUTIONS

JS, EG, and SR assembled information and wrote drafts of the manuscript. TO, CM, and DR assembled information, reviewed drafts, and provided specialist insight. All authors contributed to the article and approved the submitted version.

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