Asian Research Journal of Gynaecology and Obstetrics

5(1): 1-14, 2021; Article no.ARJGO.65503

Assessment of Quantitative and Functional Aspects of Dendritic Cell Subsets in Early Onset Pre-Eclampsia Patients

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Authors' contribution

This work was carried out in collaboration among all authors. Authors AS, KV and SN participated in the conception of this study. Author AS wrote the manuscript. All authors have read and approved the manuscript.

Article Information

Editor(s):
(1) Dr. Rajbala Singh, Siddhartha Institute of Pharmacy, India.
(2) Dr. Eghon Guzman B., Hospital Dr. Sotero del Rio, Chile.
Reviewers:
(1) Ricardo Leon Sanchez Consuegra, Universidad Libre Seccional Barranquilla, Colombia.
(2) Rayah Sulaiman Baban, Al-Nahrain University, Iraq.
Complete Peer review History: http://www.sdiarticle4.com/review-history/65503

Study Protocol

Received 10 December 2020
Accepted 14 February 2021
Published 20 February 2021

ABSTRACT

Background: Pre-eclampsia (PE) is a pregnancy associated pro-inflammatory disorder. The only known treatment is to deliver the placenta and fetus. PE is clinically identified by hypertension and proteinuria post-20th week of gestation. Early onset pre-eclampsia (EOPE), a severe form of PE is defined as when the clinical symptoms are observed before 34 weeks of gestation. There are no definite biomarkers available for early diagnosis of EOPE. Human dendritic cell (DC) subsets ($CD1c^+$, $CD141^+$ myeloid DCs and plasmacytoid DCs) are intricately involved with the process of inflammation and are significantly altered (quantitatively and functionally) in several proinflammatory disorders. These changes offer value for monitoring DC subsets as potential biomarker(s) and as targets for immunotherapeutic treatment. DC subsets play a critical role in normal pregnancy by mediating efficient migration and invasion of trophoblasts and maintaining anti-inflammatory environment of immunotolerance. In contrast, the status of DC subsets in the proinflammatory microenvironment of EOPE pregnancy requires thorough evaluation. In this direction, the designed study protocol aims to understand how DC subsets are altered (quantitatively and functionally) in EOPE patients, compared to normal pregnant women.

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Methods: Study is observational, designed to determine changes in the profile of DC subsets in the blood and decidua of EOPE diagnosed patients (n=30) by multiparametric flow cytometry approach. Normal pregnant women (n=30) are included as controls.
Results: The study participants enrolment started in December 2020 and this study protocol describes the methodology being employed.
Discussion: Human DC subsets are altered both quantitatively and functionally in the pro-inflammatory microenvironment. EOPE is a pro-inflammatory disorder and changes in the composition and function of DC subsets in these patients, compared to normal pregnant women is unclear. Understanding changes in the profile of DC subsets in blood samples from EOPE patients will provide the foundation for identification of potential biomarkers. Similarly, changes in the profile of DC subsets in the decidua of EOPE patients will provide the basis for developing novel immunotherapeutic strategies targeting distinct DC subsets or their products for the treatment of EOPE. Overall, the current study protocol and subsequent findings will help develop future large scale, prospective design clinical trials focussing on formulating strategies for early diagnosis and treatment of EOPE among pregnant women.

Keywords: Pre-eclampsia; dendritic cell subsets; plasmacytoid dendritic cells; cd1c+ myeloid dendritic cells; cd141+ myeloid dendritic cells; flow cytometry.

ABBREVIATIONS

PE : Pre-eclampsia
EOPE : Early-onset Pre-eclampsia
DC : Dendritic cells
mDCs : Myeloid Dendritic cells
pDCs : Plasmacytoid Dendritic cells
ILT-3 : Immunoglobulin-like Transcript-3
TLR : Toll-like Receptors
IL-12 : Interleukin-12
TNF-A : Tumor Necrosis Factor-Alpha
IFN-A : Interferon-Alpha

1. INTRODUCTION

Pre-eclampsia (PE) is a pro-inflammatory, pregnancy- associated disorder and is one of the major causes of maternal and fetal associated morbidity and mortality in India and worldwide [1]. In addition, there is an increased risk for the mother and her child to develop serious complications later in life including cardiovascular diseases and diabetes mellitus [2]. PE pathogenesis suggests a bias towards pro-inflammatory immune response triggering impaired trophoblast invasion and incomplete spiral arteries remodeling in the uterus, ultimately resulting in placental ischemia. Therefore, placental vascularity and blood supply to the fetus is reduced. The placental oxidative stress leads to the release of placental factors that trigger more inflammatory cytokine release and endothelial dysfunction causing downstream clinical symptoms such as hypertension [3].

Currently, the only known definitive treatment for PE is to deliver the placenta and the fetus [4]. Diagnosis of PE is made on the basis of clinical symptoms that appear after 20 gestational weeks; characterized by a) hypertension with a systolic blood pressure of 140mm Hg and a diastolic blood pressure of 90mm Hg, measured twice over four hours apart and b) proteinuria (>0.3g per 24 hours) [3]. Based on the onset of clinical symptoms, PE is divided into early onset (EOPE), if symptoms develop before 34 weeks of gestation, and late onset preeclampsia if symptoms develop at or after 34 weeks of gestation. Although EOPE is more severe and high risk for mother and fetus [5], currently, there are no definite biomarkers for early diagnosis.

Human dendritic cell (DC) subsets are intricately involved with the mechanism of inflammation. Human DCs are broadly divided into two types: a) Myeloid DCs (also called as conventional or classical DCs: $CD11c^+$ and b) Plasmacytoid DCs (pDCs): $CD11c^-$ $CD123^+$. The myeloid DCs (mDCs) are of 2 subtypes: a) $CD1c^+$ and b) $CD141^+$. These three subsets of DCs can be identified by the differential expression of 3 surface molecules: CD1c (BDCA-1), CD123 and CD141 (BDCA-3) [6] (Fig. 1).

Human DCs are identified as $Lineage^-$ $HLA\text{-}DR^+$. Human DC subsets are subdivided into myeloid DCs ($CD11c^+$ $CD123^+$) and plasmacytoid DCs ($CD11c^-$ $CDD123^+$). The myeloid DCs are further subdivided into $CD1c^+$ and $CD141^+$.

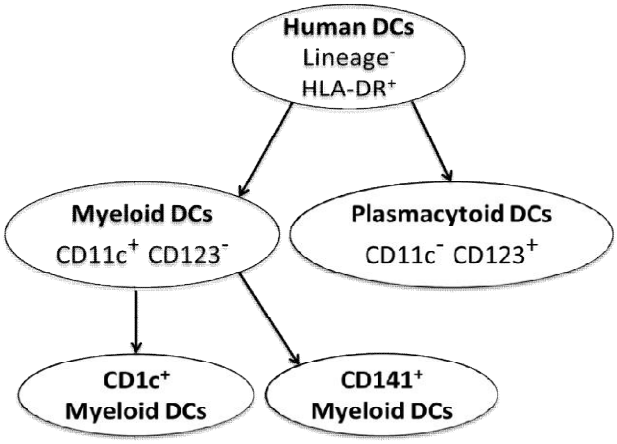

Fig. 1. Human dendritic cell (DC) subsets identification through flow cytometry

Pro-inflammatory disorders including lupus, Crohn's disease, systemic sclerosis, atopic dermatitis, rheumatoid arthritis, inflammatory bowel disease and multiple sclerosis are associated with drastic changes in circulating DC subsets that suggest evaluation of their value as biomarkers [7,8]. In addition to diagnostic potential, monitoring circulating DC subsets can offer insight into the progression of inflammatory disease and response to treatment [9,10]. Along these lines, several tolerogenic DCs based clinical trials for treating pro-inflammatory diseases are ongoing [11]. Other treatment strategies include targeting the pathogenic cytokines produced by DC subsets [12].

DC subsets have been well characterized in normal pregnancy [13,14]. $CD141^+$ mDCs predominate in the decidua of first trimester of pregnancy, with reduction in $CD1c^+$ mDCs and no changes in pDCs [13]. $CD141^+$ mDCs are known to mediate differentiation of anti-inflammatory T-helper 2 (Th2) type of cells [15] and are tolerogenic in nature-expressing immunoglobulin like transcript-3 (ILT-3) [13]. Such functions of DCs are critical for a) efficient invasion and remodeling of spiral arteries by trophoblasts, subsequent successful establishment of feto-placental unit and b) maintenance of immune tolerance towards fetus [16].

In contrast, it is unclear how the pro-inflammatory microenvironment in EOPE patients alters the profile (quantitative and functional) of DC subsets in circulation and decidua. Identification of changes in the profile of DC subsets in EOPE could offer novel strategies for identification of biomarkers and immunotherapeutic treatment. In support of this thought process, studies indicate both quantitative and functional changes in DCs among PE patients. For example, in preeclamptic decidua, the total DCs are increased and the chemokines involved in DC migration to the decidua are also increased [17]. In addition, GM-CSF expression (mediator of DC differentiation and activation) has been shown to increase in preeclamptic decidua [18] causing enhanced local differentiation and activation of DCs. Density of DCs drastically affects antigen presentation influencing the outcome of T cell responses. The CD4 T cell profile in PE patients is altered as the number of Th2 cells is decreased in the pre-eclamptic decidua, compared to normal pregnancy associated decidua [19]. Along these lines, the ability of DCs to induce regulatory T cells is impaired in PE patients [20], disrupting the tolerogenic environment characteristic of a normal pregnancy. There is a bias towards the generation of pro-inflammatory Th1 and Th17 cells, and pro-inflammatory cytokine production in DCs isolated from PE patients suggesting functional alterations in DC subsets [21-23].

Overall, the main objective of the study is to delineate how the three DC subsets are altered quantitatively and functionally in blood and decidua of EOPE patients, compared to normal pregnant women. This study will provide significant insight into the role of DC subsets in EOPE pathogenesis. Importantly, the findings of this observational study will determine feasibility for designing future prospective studies focussing on a) identifying blood-based DC subsets associated

biomarkers for early diagnosis of EOPE and b) developing immunotherapeutic interventions targeting distinct DC subsets or their products in decidua (placenta) for the treatment of EOPE.

2. METHODS

2.1 Study Design

This is an observational study to determine quantitative and functional differences among DC subsets of early onset pre-eclampsia patients based on well-established experimental protocols.

2.2 Sample Size

We aim to recruit 30 early onset pre- eclampsia patients and 30 normal pregnant women based on the feasibility of budget and time. Enrolment of these women started in December 2020 and will be completed by the end of 2021. Both blood and placenta samples are collected from each subject.

2.3 Participant Recruitment and Study Sites

Pregnant women already being enrolled/ admitted at Civil hospital, Gandhinagar are first assessed for the eligibility criteria. This is followed by sample collection and follow up with a case report form to collect information about the participant's medical condition, their family medical history and other clinical information. The collected samples (blood and placenta) are brought to the research laboratory at the Indian Institute of Public Health Gandhinagar (IIPHG) where further sample processing procedures are carried out. As the final step, the processed samples are analysed by flow cytometry (Thermofisher Attune Nxt) at the Institute of Science, Nirma University, Ahmedabad. An overview of the study sites is shown in Fig. 2. SOPs are developed for collection, transfer and processing of samples.

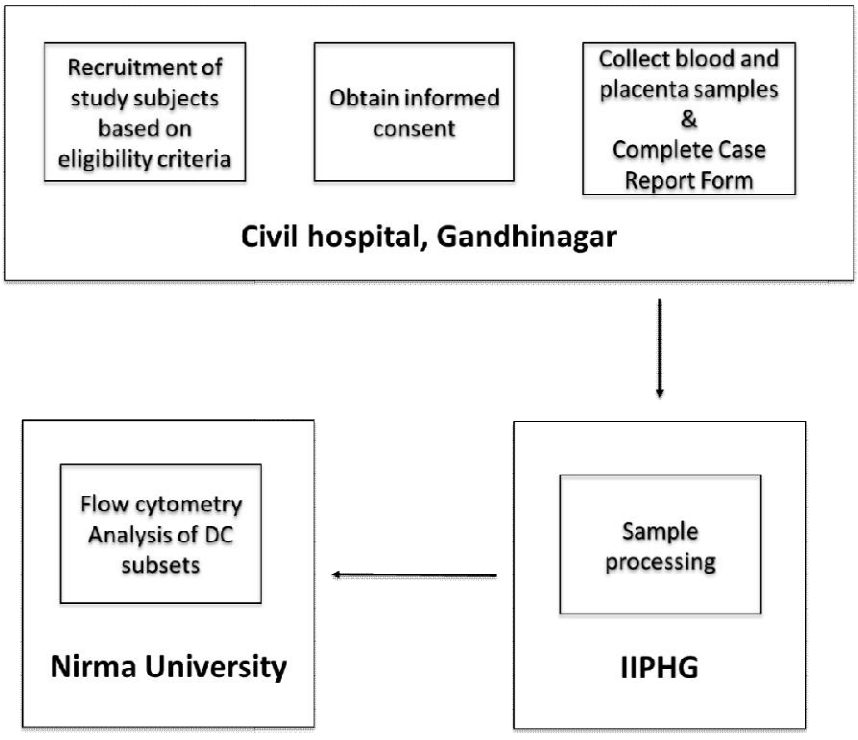

Fig. 2. An overview of processes involved in each study site

The recruitment of study subjects based on eligibility criteria, obtaining informed consent, collection of blood and placenta samples and completion of case report form for each participant is being done at the Civil hospital, Gandhinagar. The blood and placenta samples are processed at the Indian Institute of Public Health Gandhinagar (IIPHG), followed by acquisition of samples on flow cytometer (Attune Nxt, ThermoFisher Scientific) and data analysis at the Institute of Science, Nirma University, Ahmedabad

2.4 Recruitment of Study Subjects Based on Eligibility Criteria

An eligibility criteria checklist is being utilized for the recruitment of participants. This form is completed and signed by the obstetrician at Civil hospital, Gandhinagar. There are two sections (A & B) for eligibility assessment. Patients answering 'NO' to 'ANY' of the section A criteria are further assessed by section B criteria in order to be eligible for the study. In section B, patients answering 'YES' are classified as early-onset pre-eclampsia (EOPE) group and those answering 'NO' are classified as normal pregnant women.

Section A criteria: Patients answering 'NO' for the following conditions will be ELIGIBLE for the study.

1. Multiple pregnancy (pregnancy with more than one fetus).
2. Women pregnant for the second or more time.
3. Late & new onset hypertension and proteinuria developing at or after 34+0 weeks of gestation (Late-onset pre-eclampsia).
4. Chronic hypertension (≥140/90mm Hg) diagnosed before pregnancy or in the first half of pregnancy (<20 weeks) and continued for >12 weeks after delivery.
5. Atypical pre-eclampsia (pre-eclampsia symptoms <20 weeks of gestation or > 48hrs after delivery.
6. Positive for SARS-CoV-2 infection (currently or in the past).
7. Medical complications: Urinary tract infections, HIV+, Hepatitis B+, Hepatitis C+, Infectious diseases, Diabetes mellitus, Collagen disorders, Autoimmune disorders (SLE), Thrombocytopenic purpura, antiphospholipid antibody syndrome, Hemolytic uremic syndrome, Acute fatty liver of pregnancy, Fetal malformations, Premature rupture of membranes, Chorioamnionitis/ Chronic villitis, Inflammatory diseases, Renal diseases, Severe extragenital pathology, Post transplantation state, Cancer history, Heart failure/Ischemic heart disease.
8. Smoking.
9. Any "other" obstetric complications. "Other" term for pre-eclampsia patient group includes obstetric complications except for the early-onset pre-eclampsia condition. Examples include hemorrhage, obstructed labor, amniotic fluid embolism.
10. Maternal age >35 years.
11. BMI > 27Kg/m2.
12. Pregnant via assisted reproductive technology (ART).

Factors such as multiple pregnancy, chronic hypertension diagnosed before pregnancy, atypical pre-eclampsia, stated medical complications and infections (including COVID-19), smoking, higher maternal age, increased BMI and other obstetric complications can introduce biological mechanisms unrelated to the true representation of pre-eclampsia pathogenesis. Therefore, subjects with these scenarios are excluded from the study. Additionally, women who got pregnant via ART procedure are excluded from this study as these women demonstrated increased risk of PE, compared to women with spontaneous pregnancy [24]. It is not clear if the technique of assisted reproductive technology itself influences the placental biology [25]. Among spontaneous pregnancy, the risk of pre-eclampsia is much lower in women who are getting pregnant for the second or more time [26]. Therefore, these women are excluded from the study. As the study focus is on early-onset pre-eclampsia patients, pregnant women with late-onset pre-eclampsia are excluded from the study.

Section B criteria: Patients answering 'YES' are eligible for enrolment as early-onset pre-eclampsia participants. Patients who answer 'NO' are eligible for enrolment as normal pregnant women participants.

1. Early & new onset hypertension (≥140/90mmHg) developing before 34+0 weeks of gestation.
2. Early & new onset proteinuria (≥0.3g/24hr) developing before 34+0 weeks of gestation.

Based on the eligibility criteria, 2 groups of pregnant women are created: a) Early-onset pre-eclampsia (EOPE) patients and b) Normal pregnant women. These pregnant women are given the patient information sheet and sample collection details are clearly explained to them by the obstetrician.

2.5 Sample Collection

2.5.1 Blood collection

Around 2 ml of blood is collected from each pregnant woman at the time of parturition. Experienced hospital staff/phlebotomy team are performing the blood collection by venepuncture. The sample is collected in sterile blood collection

tubes coated with EDTA, stored at room temperature (25-28)□C, and brought to research lab at IIPHG for further processing.

2.5.2 Placenta collection

Placenta is collected during parturition, placed in a sterile tissue collection bag enclosed in an ice box and brought to the research lab at IIPHG for further processing. Proper and approved biosafety practices for handling and disposal of biological materials is followed [27,28].

2.6 Case Report form (CRF) Preparation

Clinical data is collected from the pregnant women recruited into the study. These data are entered into the CRF during sample collection (blood and placenta) and post-partum. Briefly, general details of the subject are entered into CRF, including name, date of birth and BMI. In addition, participant and their family history are collected. Clinical parameters are recorded in the CRF; including gestation age, diastolic & systolic blood pressure, mean arterial pressure, proteinuria, any ongoing medical treatments, presence of HELLP/IUGR/atypical pre-eclampsia, recently taken hemoglobin levels, platelet levels and complete blood counts, doppler examination of uterine arteries and any other obstetric complication. Other parameters such as delivery date and gestation, placenta weight, type of delivery, systolic and diastolic blood pressure, any medical treatments done, and any other obstetric complications are also recorded.

Additionally, post-partum clinical parameters are entered into the CRF: Infant birth weight, systolic and diastolic blood pressure, presence of atypical pre-eclampsia, APGAR score, neonatal outcomes (example: Perinatal/fetal death, delivery<34 weeks, fetal distress syndrome, necrotizing enterocolitis, intraventricular haemorrhage) and maternal outcomes (example: Death, Pulmonary edema, acute renal failure, cerebral thrombosis, disseminated intravascular coagulation).

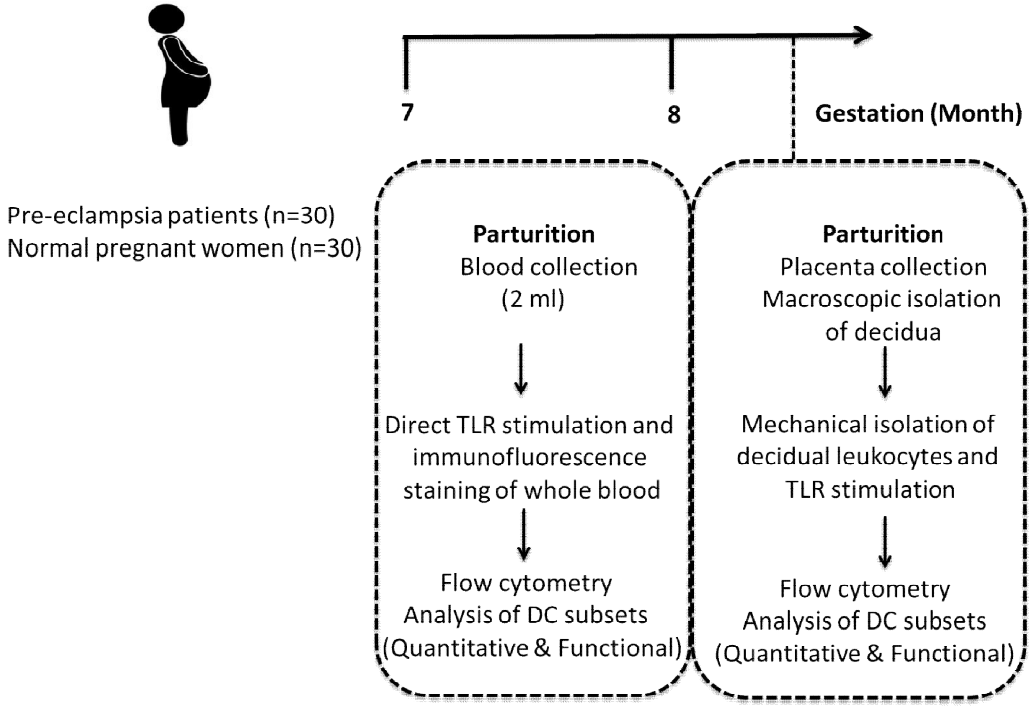

Fig. 3. An overview of research laboratory associated study protocol

At the time of parturition, blood and placenta are collected from EOPE patients (n=30) and normal pregnant women (n = 30). Blood samples are subjected to direct immunofluorescence staining and TLR stimulation to identify, quantitate DC subsets and to assess functional differences between EOPE and normal pregnant women

The collected clinical parameters are compared between pre-eclampsia patients and normal pregnant women. Data is presented as mean +/- standard deviation (SD) and range. Differences are considered significant when the p value will be equal to or less than 0.05. All statistical analyses assume a 2- sided significance level. Mann-Whitney U non-parametric test is used for comparisons between groups.

2.7 Experimental Work Plan

An overview of the complete experimental plan with blood and placenta samples is shown in Fig. 3.

2.7.1 Blood sample

A portion of blood sample is processed for direct immunofluorescence surface staining procedure and another portion for DC subset specific TLR stimulation.

2.7.1.1 Direct immunofluorescence staining of whole blood

This is a well-established methodology for directly detecting dendritic cell subsets in blood samples from normal pregnant women and healthy non-pregnant individuals [29-34]. This method is more efficient compared to other methods as it is shown to improve assay reproducibility and is less likely to show loss of lymphocyte subsets [35-38]. All monoclonal antibodies are titrated for determining optimum antibody concentration for usage.

Around 0.2ml of blood sample is stained with monoclonal antibodies against surface markers (Table 1), followed by RBC lysis. These samples are run on the flow cytometer (Thermo Fisher Attune Nxt) to characterize DC subsets. The 9-color multiparametric flow panel has been designed using FluoroFinder2.0 software (Table 1), with careful consideration given for minimal spectral spill-over values between fluorochromes so that automatic compensation can be easily performed using FlowJo software. Abc^{TM} Anti-Mouse Bead Kit (Thermo Fisher Scientific) is used to set up flow cytometry compensation.

The 9-color multiparametric flow panel is designed to identify the 3 DC subsets, simultaneously determining other phenotypic changes, such as activation (CD80), maturation (CD83) and tolerogenic properties (ILT-3). ILT3 (immunoglobulin- like transcript 3), also known as CD85K is highly expressed on myeloid DCs in the decidua of normal pregnant women [13]. ILT3 is involved in the induction of immune tolerance in DCs via interaction with HLA-G on extra villous trophoblasts (EVTs) [39]. Therefore, ILT-3, along with activation and maturation markers are included in the panel to monitor their expression changes in the pro-inflammatory environment of EOPE.

2.7.1.2 TLR stimulation of whole blood

This procedure has been well established for directly analyzing DC subsets functional responses in whole blood post stimulation/activation with TLR ligands [34]. Briefly, 0.5ml of blood sample is subjected to TLR stimulation, by using LPS 100ng/ml (for TLR-4 stimulation on CD1c+ mDCs) and CpG 2216 30ug/ml (for TLR-9 stimulation on plasmacytoid DCs) and poly I:C 30ug/ml (for TLR-3 stimulation on CD141+ mDCs) along with brefeldin A 10 ug/ml (protein transport inhibitor) for 5hrs at 37c, 5% CO_2. Selection of TLRs for each DC subset is based on differential expression of TLRs on these cells [40]. Thereafter, post surface staining, intracellular cytokine staining procedure (permeabilization and fixation) is performed- specifically for IL-12, TNF-A (for myeloid DCs) and IFN-A, TNF-A (for plasmacytoid DCs) (Tables 2-4). These samples are run on the flow cytometer (Thermofisher Attune Nxt). Production of these cytokines by DC subsets is drastically altered in several pro-inflammatory disorders [7,8]. Therefore, these cytokines are being included in the flow-panel to assess their production in EOPE patients.

2.7.2 Placenta (decidua) samples

Procedures for the isolation of decidua, decidual cells and leukocytes are being adapted from well-established studies [41,42]. Decidua basalis (part of decidua in contact with placenta) and decidua parietalis (rest of the decidua on the maternal myometrium end) are isolated from the placenta. Collected decidua are subjected to mechanical processing to obtain decidual cells. Decidual leukocytes are isolated by Ficoll-Paque density gradient centrifugation method [43]. The leukocytes settled at the interface are carefully collected and washed for immunophenotyping DC subsets. The total yield of leukocytes from this protocol is up to $30x10^6$ cells per decidual tissue per study participant.

Table 1. Surface markers for the generation of 9-color flow panel to determine quantitative and phenotypic differences among DC subsets in blood and decidua of EOPE and normal pregnant women

Excitation laser	Emission filter	Channel	Marker	Fluorochrome	Clone
Blue-488nm	530/30	BL1	Lineage	FITC	MØP9, NCAM-16.2, 3G8, SK7, L27, SJ25C1
	574/26	BL2	CD141	PE	1A4
	695/40	BL3	CD123	PerCP-Cy5.5	7G3
	780/60	BL4	ILT-3	PE-Cy7	ZM4-.1
Red- 637nm	670/14	RL1	CD1c	APC	AD5-8E7
	720/30	RL2	CD11c	AF-700	B-ly6
	780/60	RL3	HLA-DR	APC-Cy7	L243
Violet-405nm	440/50	VL1	CD80	V450	L307.4
	512/25	VL2	CD83	BV510	HB15e
	603/48	VL3	-	-	-
	710/50	VL4	-	-	-

Attune Nxt flow cytometer (Thermo Fisher Scientific) is being used to acquire fluorochrome labeled samples. The surface markers (Lineage, HLA-DR, CD11c, CD1c, CD141, CD123) are used to identify the 3 DC subsets. CD80, CD83, ILT-3 markers are used as representatives of activation, maturation and tolerogenic marker respectively. All antibodies are purchased from BD Biosciences, except for CD1c, respective isotype control (Miltenyi Biotec) and ILT-3, respective isotype control (BioLegend). The dashes indicate unused channels

Table 2. Surface markers and intracellular cytokine markers for determining functional differences in $CD1c^+$ myeloid DCs in blood and decidua of EOPE and normal pregnant women

Excitation laser	Emission filter	Channel	Marker	Fluorochrome	Clone
Blue-488nm	530/30	BL1	Lineage	FITC	MØP9, NCAM-16.2, 3G8, SK7, L27, SJ25C1
	574/26	BL2	-	-	-
	695/40	BL3	-	-	-
	780/60	BL4	TNF-A	PE-Cy7	MAb11
Red- 637nm	670/14	RL1	CD1c	APC	AD5-8E7
	720/30	RL2	CD11c	AF-700	B-ly6
	780/60	RL3	HLA-DR	APC-Cy7	L243
Violet-405nm	440/50	VL1	IL-12	V450	C11.5
	512/25	VL2	-	-	-
	603/48	VL3	-	-	-
	710/50	VL4	-	-	-

Blood samples and decidual leukocytes are subjected to stimulation with TLR-4 ligand, LPS. Intracellular cytokine staining procedure (permeabilization and fixation) is performed. Attune Nxt flow cytometer (Thermo Fisher Scientific) is being used to acquire fluorochrome labeled samples. The surface markers (Lineage, HLA-DR, CD11c, CD1c) are used to identify the $CD1c^+$ myeloid DC subset. Post-TLR4 stimulation with LPS, cytokines IL-12 and TNF-A are being measured. All antibodies are purchased from BD Biosciences, except for CD1c and respective isotype control (Miltenyi Biotec). The dashes indicate unused channels

2.7.2.1 Immunophenotyping and functional analysis of decidual DC subsets

A portion of freshly isolated decidual leukocytes is used for cell-surface antigen staining using monoclonal antibodies and another portion of cells will be subjected to specific TLR stimulation. Appropriate mouse anti-human isotype controls are included. In addition, appropriate fluorescence minus one (FMO) controls are used to eliminate any spill-over- induced background.

Around 2 $x10^6$ live cells are used per sample (dead cells are excluded by trypan blue counting with a hemocytometer). All monoclonal antibodies are titrated for determining optimum antibody concentration for usage. For surface staining, the cells are stained with appropriate monoclonal antibodies forming the 9-color panel

(Table 2). TLR stimulation of decidual cells are performed similar to blood samples and optimal concentration of TLR ligands to stimulate decidual DC subsets are being optimized. Similar to blood samples, surface staining (including activation markers) and intracellular cytokine staining (permeabilization & fixation) are performed for detecting IL-12, TNF-A (myeloid DCs) and IFN-A, TNF-A (plasmacytoid DCs) (Tables 2-4).

2.8 Data Analyses and Statistics

At least 200 000 events within the combined lymphocyte-monocyte gate, based on the FSC and SSC parameters per sample is collected in the flow cytometer and data is analyzed using FlowJo software. Appropriate mouse anti-human isotype controls are included to rule out any non-specific background signal caused by primary antibodies. In addition, appropriate fluorescence

Table 3. Surface markers and intracellular cytokine markers for determining functional differences in CD141$^+$ myeloid DCs in blood and decidua of EOPE and normal pregnant women

Excitation laser	Emission filter	Channel	Marker	Fluorochrome	Clone
Blue-488nm	530/30	BL1	Lineage	FITC	MØP9, NCAM-16.2, 3G8, SK7, L27, SJ25C1
	574/26	BL2	CD141	PE	1A4
	695/40	BL3	-	-	-
	780/60	BL4	TNF-A	PE-Cy7	MAb11
Red- 637nm	670/14	RL1	-	-	-
	720/30	RL2	CD11c	AF-700	B-ly6
	780/60	RL3	HLA-DR	APC-Cy7	L243
Violet-405nm	440/50	VL1	IL-12	V450	C11.5
	512/25	VL2	-	-	-
	603/48	VL3	-	-	-
	710/50	VL4	-	-	-

Blood samples and decidual leukocytes are subjected to stimulation with TLR-3 ligand, poly I:C. Intracellular cytokine staining procedure (permeabilization and fixation) is performed. Attune Nxt flow cytometer (Thermo Fisher Scientific) is being used to acquire fluorochrome labeled samples. The surface markers (Lineage, HLA-DR, CD11c, CD141) are used to identify the CD141$^+$ myeloid DC subset. Post-TLR-3 stimulation with poly I:C, cytokines IL-12 and TNF-A are being measured. All antibodies are purchased from BD Biosciences. The dashes indicate unused channels

Table 4. Surface markers and intracellular cytokine markers for determining functional differences in plasmacytoid DCs in blood and decidua of EOPE and normal pregnant women

Excitation laser	Emission filter	Channel	Marker	Fluorochrome	Clone
Blue-488nm	530/30	BL1	Lineage	FITC	MØP9, NCAM-16.2, 3G8, SK7, L27, SJ25C1
	574/26	BL2	-	-	-
	695/40	BL3	CD123	PerCP-Cy5.5	7G3
	780/60	BL4	TNF-A	PE-Cy7	MAb11
Red- 637nm	670/14	RL1	-	-	-
	720/30	RL2	CD11c	AF-700	B-ly6
	780/60	RL3	HLA-DR	APC-Cy7	L243
Violet-405nm	440/50	VL1	IFNA-2b	V450	7N4-1
	512/25	VL2	-	-	-
	603/48	VL3	-	-	-
	710/50	VL4	-	-	-

Blood samples and decidual leukocytes are subjected to stimulation with TLR-9 ligand, CpG. Intracellular cytokine staining procedure (permeabilization and fixation) is performed. Attune Nxt flow cytometer (Thermo Fisher Scientific) is being used to acquire fluorochrome labeled samples. The surface markers (Lineage, HLA-DR, CD11c, CD123) are used to identify plasmacytoid DC subset. Post-TLR-9 stimulation with CpG, cytokines IL-12 and IFNA-2b are being measured. All antibodies are purchased from BD Biosciences. The dashes indicate unused channels

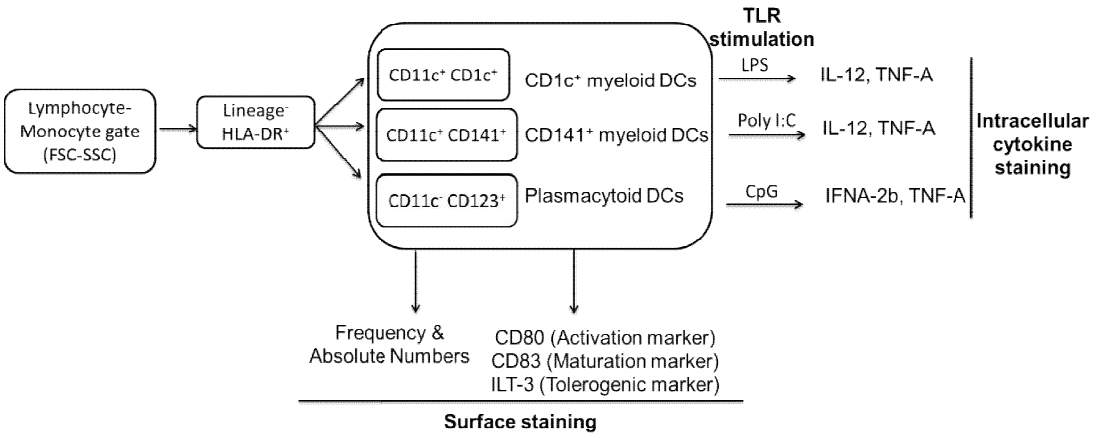

Fig. 4. Outline of data analysis for assessing quantitative and functional features of DC subsets in blood and decidua through flow cytomet

DCs are identified as lineage$^-$ HLA-DR$^+$ population within the lymphocyte-monocyte gate. These DCs are classified into 3 DC subsets based on the indicated combination of surface markers, followed by quantitative and functional analysis

minus one (FMO) controls is used to eliminate any spill-over- induced background. Data is analyzed using FlowJo software as follows (Fig. 4).

As the first step, based on FSC Vs SSC dot plots, cell debris and dead cells are excluded. This is followed by selection of lineage $(Lin)^-$ and major histocompatibility complex (MHC)- class II $(HLA\text{-}DR)^{hi/+}$ populations. Dendritic cells do not express lineage-specific markers ($CD3^+$ T cells, $CD14^+$ Monocytes, $CD16^+$ NK cells and granulocytes, $CD19^+/CD20^+$ B cells and $CD56^+$ NK cells). Therefore, the DCs in blood is identified as Lin^- $HLA\text{-}DR^{+/hi}$ [44].

This is followed by determining frequencies and absolute numbers of a) Plasmacytoid DCs: $CD11c^-$ $CD123^+$, b) $CD1c^+$ Myeloid DCs: $CD11c^+$ $CD1c^+$ and c) $CD141^+$ Myeloid DCs: $CD11c^+$ $CD141^+$. In addition, the frequencies and mean fluorescence intensity (MFI) of the cytokines and activation, tolerogenic markers expressed by each of the 3 DC subsets is calculated. All the data are compared between normal pregnant women(n=30) and pre-eclampsia patients (n=30).

A standard non-parametric test (Mann-Whitney U- test) is used to determine statistical differences of blood and decidua derived DC subsets between the two groups of pregnant women. Differences at $P<0.05$ is considered statistically significant. IBM SPSS 20 software is used to perform statistical analyses.

3. RESULTS AND DISCUSSION

EOPE is a severe form of pre-eclampsia and currently, no definite biomarkers or treatment strategies are available. Human DC subsets are one of the key players in modulating the pro-inflammatory changes in the microenvironment. Towards this direction, the study is aimed at identifying changes in the profile (quantitative and functional) of DC subsets in EOPE patients, compared to normal pregnant women.

A prospective study design analyzing the DC subsets systematically through each trimester in pregnant women with normal birth outcome and those with EOPE outcome would be most effective in providing real prognostic values. However, analyzing all the described markers through flow cytometry approach in a prospective study design will impose logistical and resource constraints. Therefore, the described current study design is an essential first step that will be identifying only those specific DC markers that are significantly altered in EOPE diagnosed patients. These specific differences could then be examined in further detail in a large-scale prospective study, providing more insight in generating real multi-layered prognostic values. Furthermore, measurement of DC subsets at the time of birth in this study will enable a correlation analysis of the DC profile in blood and decidua tissue, that would re-enforce the significance of observed differences.

The experimental techniques chosen to characterize DC subsets in the study protocol are well established, reproducible and are being easily adapted. This study protocol allows the collection, processing, sample acquisition on flow cytometer and subsequent data analysis to be completed in the same day. An efficient collaboration of study sites has been established. However, due to the emergence of COVID-19 pandemic, there have been unanticipated delays in the process of recruitment and sample collection. These issues are being confronted, and it has been made mandatory among recruited pregnant women to be tested for SARS-CoV-2 before obtaining informed consent and sample collection. Appropriate changes have also been reflected in the eligibility criteria.

Human DC subsets present as ideal and novel cellular markers that could be monitored for pathogenic changes in early pregnancy as they are one of the key players involved in the decidualization and angiogenesis process leading to implantation and placentation. They play a critical role during pregnancy by modulating remodeling of decidual tissue and producing chemokines for the migration and invasion of trophoblast cells into the endometrium during placentation [16,45]. These features are significantly altered leading to the pathogenesis associated with EOPE patients. These immunological cellular changes could be combined with other risk factors to develop a comprehensive panel of biomarkers for early diagnosis of EOPE.

Multiparametric flow cytometry approach for diagnosis of pregnancy complications offers significant advantages. Multilevel changes in immune cells can be identified simultaneously in a small volume of biological sample within a short period of time. For example, one study identified significant quantitative and functional changes in DC subsets among pregnant women with intrauterine growth restriction (IUGR), without PE symptoms [46]. Therefore, timely intervention and care could be provided to women with pregnancy complications. However, the cost per test through flow cytometer could be higher, compared to other testing approaches. It is likely that the specific changes observed in DC subsets and their products through flow cytometry approach in EOPE patients in the study could be modified in future studies by detecting the same biomolecules through parallel lower cost methods such as ELISA assays.

4. CONCLUSION

Human DC subsets undergo quantitative and functional changes in response to a pro-inflammatory environment. EOPE is a proinflammatory pregnancy associated disorder and the profile of DC subsets in these patients is unclear. This observational study protocol puts forth a methodology based on multi-parametric flow cytometric approach for assessing the quantitative and functional features of DC subsets in blood and decidua of EOPE patients, in comparison to normal pregnant women. We expect that the study will identify specific DC-subsets associated changes in EOPE patients. Further large-scale prospective studies are needed that could translate the study findings into practical and tangible biomarker(s) and immunotherapeutic target(s) for early diagnosis and treatment or intervention strategies.

CONSENT AND ETHICAL APPROVAL

The study has been approved by institutional ethics committee (Indian Institute of Public Health Gandhinagar and Gujarat Medical Education and Research Society Medical College, Gandhinagar). Informed consent is being obtained from study participants.

AVAILABILITY OF DATA AND MATERIALS

Data is available from corresponding author upon reasonable request.

FUNDING

This study is being funded by the Women Scientist -A grant awarded to AS by the Department of Science & Technology, Government of India (No. SR/WOS-A/LS-497/2017).

ACKNOWLEDGEMENTS

We acknowledge the efforts of collaborating obstetricians and nurses for assisting with the ongoing recruitment, sample and clinical data collection from study participants.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sdiarticle4.com/review-history/65503