Role of T cells in women with recurrent pregnancy loss

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IN
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SUBMITTED BY:

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CERTIFICATE

I hereby certify that the thesis entitled "***Role of T cells in women with recurrent pregnancy loss***", which is submitted by Mr. Priyajeet Dey, in fulfillment of the requirement for the award of Masters in Biotechnology in Department of Biochemistry, All India Institute of Medical Science, New Delhi, India is a record of candidate's own independent and original research work carried out by him under the supervision and guidance of Dr. D.N. Rao, Professor & Head, Department of Biochemistry, All India Institute of Medical Science, New Delhi, India and Dr. Manju Anand, Associate Professor, Department of Biotechnology, Thapar University, Patiala, Punjab, India. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any degree.

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PRIYAJEET DEY

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ABBREVIATIONS

RPL	Recurrent Pregnancy Loss
RM	Recurrent Miscarriage
Th cell	T-Helper cell
DC	Dendritic cell
PBMC	Peripheral blood mononuclear cells
Treg	T-Regulatory cell
Th17	Type of T-cell
Th1	Type of T-cell
Th2	Type of T-cell
IL	Interleukine
PHA	Phytohaemagglutinin
BSA	Bovine serum albumin
CD	Cluster of Differentiation
DCHFDA	2'-7'-Dichlorodihydrofluorescein diacetate
TGF-beta	Transforming Growth Factor- Beta
IFN-gamma	Interferon-gamma
HLA-C	Polymorphic histocompatibility antigen
µl	micro liltre
pg	Picogram
ml	millilitre
HLA-G	Human leukocyte antigen G
CCR6	Chemokine receptor 6
FOXP3	Forkhead box P3
RPMI	Roswell Park Memorial Institute medium
RPM	Revolutions per minute
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate

APC	Allophycocyanin
PE	Phycoerythrin
PerCP	Peridinin-chlorophyll-protein complex
ELISA	Enzyme linked Immunosorbent Assay

Introduction

Recurrent Pregnancy loss or recurrent miscarriage, the most common complication of pregnancy, is the spontaneous loss of a pregnancy before the fetus has reached viability. The term therefore includes three or more consecutive spontaneous loss of pregnancy from the time of conception until 24 weeks of gestation in the UK, but may be 20 weeks of gestation in other parts of the world.

Miscarriage occurs in 15-20% of recognised pregnancies. It affects about 1% of couples attempting to conceive and thus, associated with psychological morbidity. While several mechanisms or causes have been described that account for the 50% etiology in RM females, the most important ones has been attributed by chromosomal & genetic abnormalities and immune dysregulation including autoimmunity. Also, it has been found that a woman may develop recurrent miscarriage after a successful pregnancy.

Etiology

Genetic Abnormalities:

- Recurrent miscarriage may be linked to chromosomal abnormality in 2-5% of couples. A balanced reciprocal or Robertsonian translocation is the most common type.
- Increasing maternal age affects ovarian function and increases rates of aneuploidy in association with older oocytes.
- There are a higher number of conceptions that are chromosomally abnormal and do not develop.
- As the number of miscarriages increases, the risk of chromosomal abnormalities decreases and the risk of underlying maternal cause increases.

Introduction

Uterine anomalies:

- Uterine anomalies (arcuate or septate) are seen in between 10-25% of cases of recurrent miscarriage. Only 50% of pregnancies where there is a uterine structural abnormality achieve term delivery.
- Uterine fibroids are present in up to 30% of women, but the way they affect reproductive loss is unclear.
- Cervical incompetence (late miscarriage preceded by spontaneous rupture of membranes or painless cervical dilatation) may often be a cause of mid-trimester recurrent miscarriage.
- Second-trimester miscarriages may be linked to uterine malformations such as the presence of a uterine septum or a bicornuate uterus.

Endocrine:

- Women with polycystic ovarian syndrome are at higher risk of miscarriage, which may be related to insulin resistance and hyperinsulinaemia.
- There is insufficient evidence to support the use of metformin during pregnancy to reduce this risk.
- Uncontrolled diabetes mellitus is risk factors for recurrent miscarriage.

Inherited thrombophilia:

- Inherited thrombophilia, such as protein C and S deficiency may have a role in recurrent miscarriage, because of an increased risk of thrombosis in the uteroplacental circulation.
- Women with second-trimester miscarriage should be screened for inherited thrombophilia.

Introduction

Infections:

- It is now believed that in order for an infective agent to be responsible, it must be capable of persisting in the women's genital tract undetected and must cause few symptoms.
- Tuberculosis (pelvic): affects mostly fertility
- Listeriosis: rarely associated with fetal loss
- Little evidence for role of chlamydia
- Syphillis seroreactivity associated with spontaneous abortion, perinatal morbidity and morbidity to viable infant
- Bacterial vaginosis 2nd to role on inhabitation of uterus and role in premature delivery (but mostly 2nd trimester loss and evidence inconsistent)
- TORCH infections (Toxoplasmosis, Rubella, CMV and herpes simplex)
    - Can be associated with individual pregnancy loss but as they are only contracted once, unlikely to be associated with RPL
    - Routine screening for these disease no longer recommended by most
    - HIV : protease inhibitors can cause hyperglycemia
    - Parvovirus : associated with 2nd trimester miscarriage or pre-term birth

Bacterial vaginosis in the first trimester is a risk factor for second-trimester miscarriage and preterm delivery.

Antiphospholipid syndrome (APS):

- This is the most important treatable cause of recurrent miscarriage.
- The antiphospholipid antibodies, lupus anticoagulant, anticardiolipin antibodies and anti-B2-glycoprotein I antibodies may be associated with recurrent miscarriage before ten weeks.

Antiphospholipid antibodies are present in 15% of women with recurrent miscarriage. APS is the only proven thrombophilia that is associated with adverse pregnancy outcomes.

Review of Literature

Mammalian pregnancy is an unique immunological state that requires a balance of immune tolerance and suppression to maintain the pregnancy. Abnormal maternal immune response has been proposed to be one of the mechanisms underlying RM (Saini et al. 2011). Several immune mechanisms are involved in the establishment of the active multifactorial maternal-foetal tolerance (Mincheva-Nilsson 2006): deviation of the systemic maternal immune system toward T helper type 2 (Th2 type) (Wegmann et al. 1993), expression of the non-classical HLA-G molecules by trophoblasts thus inhibiting maternal NK cell attack (Rouas-Freiss et al. 1997), promoting apoptosis of activated Fas+ maternal lymphocytes through FasL expression by the syncytiotrophoblast (Frängsmyr et al. 2005; Abrahams et al. 2004), down-regulation of NKG2D receptor on maternal peripheral blood mononuclear cells (PBMC) by placental exosomes carrying NKG2D ligands (Mincheva-Nilsson et al. 2006) and Indoleamine 2,3-dioxidase-mediated tryptophan degradation that suppresses the immune response by inhibition of T lymphocyte proliferation(Munn et al. 2002). Maternal tolerance toward foetal alloantigen was explained by the predominant Th2-type immunity during pregnancy, which overrules Th1-type immunity, therefore protecting the foetus from maternal Th1-cell attack (Dealtry, O'Farrell, and Fernandez 2000). Indeed, predominant Th1-type immunity has been observed in recurrent spontaneous abortion (Piccinni et al. 1998). It has been known that inhibitors of Th1 cytokines or by administering the Th2 cytokine, IL-10, the effect of Th1 cytokines such as IL-2, IFN gamma and TNF alpha can be reversed in mice undergoing miscarriage (Chaouat et al. 1995) (Clark et al. 1998). Hence, the balance between effector cells and regulator cells is necessary to maintain an immune homeostasis.

Helper T (Th) cells play a critical role in regulating immune responses. Now, the Th1/Th2 paradigm has been expanded into the Th1/Th2/Th17 and regulatory T (Treg) cells paradigm (Saito et al. 2010). Th cell are regulated by the Treg cell. Their capacity to produce cytokines is suppressed by the immunoregulatory cytokines such as TGF-beta and IL-10. The Th17 cells which produce the proinflamatory cytokines,IL-17 play important role for the induction of inflamation , has pathogenic mechanism in autoimmune disease

and acute transplant rejection. The Treg cells has a major role in immune regulation and induction of tolerance. Treg cells are known to inhibit proliferation and cytokine production in both CD4+ and CD8+ T cells, immunoglobulin production by B cell, maturation of DC and cytokine activity of NK cells.

An inverse relationship between the numbers of Th17 cells and Treg cells is observed in peripheral blood and decidua of unexplained recurrent spontaneous abortion cases. IL-6 is a key cytokine that blocks the development of Treg cells and induces the differentiation of Th17 cells.

Several reports have demonstrated that regulatory T cells decreased in the peripheral blood and/or deciduas in women with RPL. In 2004, Sasaki et al.61 first reported the association between regulatory T cells and spontaneous abortion. CD4+ CD25high regulatory T cells in the peripheral blood and deciduas decreased in spontaneous abortion group as compared to induced abortion group. Furthermore, the percentage of circulating CD4+ CD25+ regulatory T cells significantly increased in early pregnancy comparing to non-pregnant state. However, women with spontaneous abortions did not demonstrate the increase in regulatory T cells during pregnancy. In addition, decidual CD4+ CD25high T cells were significantly lower in women with spontaneous abortion than women undergoing induced abortion. They also observed that decidual and peripheral blood CD4+ CD25+ regulatory T cells were anergic and suppressed the proliferation of CD4+ CD25 T cells via cell contact manner. Arruvito et al.52 have published a wonderful regulatory T-cell study comparing women with RPL with fertile controls. Opposite to fertile controls, in women with RPL, CD4+ CD25+, CD4+ CD25high, and Foxp3+ regulatory T cells did not show any significant fluctuation during a menstrual cycle. CD4+ CD25high and Foxp3+ T cells regulatory T cells in women with RPL not only significantly decreased as compared to those of controls, but also were as low as those of postmenopausal women. Moreover, regulatory T cells from women with RPL showed suppressive, but significantly lower in function as compared to those of fertile controls. Lymphocyte immunotherapy (LIT) with paternal or third-party lymphocytes has been demonstrated to increase CD4+ CD25bright T cells. The proportion of these CD4+

CD25bright T cells was higher in women with a successful pregnancy than in women with pregnancy loss after LIT. The presence of intravenous immunoglobulin with human CD4+ CD25+ regulatory T cells in culture significantly increased the expression of Foxp3, TGF-b, and IL-10. These findings suggest that deceased number and defective function of regulatory T cells in women with RPL results in reproductive failure, and immunotherapy may reverse the decreased number and function of regulatory T cells.

Th17 cells have been studied in women with reproductive failure during the past few years. Nakashima et al. showed the accumulation of IL-17+ T cells in the deciduas in women with inevitable abortion. Decidual IL-17+ T cells were mostly CD4+ T cells and a few CD8+ cells also expressed IL-17 in this study. In addition, the number of decidual IL-17+ cells was positively correlated with the number of decidual neutrophils. However, they could not find any difference in the number of decidual IL-17+ T cells between women with missed abortion and normal pregnancy. From these results, the authors concluded that decidual IL-17+ cells might be involved in the inflammation of the late stage of abortive process, not the causative factor of abortion. 48 Because their data of IL-17+ cells were limitedto inevitable abortion, not to RPL, it may be difficult to generalize the results as the immunologic mechanism of RPL.

How can the maternal immune system both induce fetal tolerance and at the same time sustain a strong anti-microbial response?

Taken together, there is now ample evidence that the circulating Treg population is not increased in size during pregnancy and circulating Treg cells show normal suppressive function during pregnancy. An increase in circulating Treg numbers or suppressive capacity would indeed be dangerous considering their unspecific suppressive capacity and the importance of protecting the mother and her fetus from infections. On the other hand there is a need for fetal tolerance, which would benefit from an increase in Treg cell mediated suppression. This paradox could, at least in part, be explained by differential Treg cell composition and function in the different compartments. While Treg cells in decidua are frequent and show a stable suppressive phenotype the circulating Treg cell pool, on the other hand, is not increased in size and circulating Treg cells show a “normal”

phenotype and suppressive capacity. However, a state of fetal tolerance should also be present systemically, for example when considering the presence of fetal cells in the maternal circulation. One smart solution would be if circulating Treg cells showed a high degree of plasticity and flexibility. Indeed, the unpublished data point in this direction and recent knowledge in T helper cell plasticity has changed the view on T helper lineage commitment and plasticity.

The IL4, IL5, IL9, IL13- knockout mice showed normal pregnancy in allogeneric pregnancy, suggesting that Th2 type immunity might not be essential for the successful pregnancy. High Th1 cytokine IL2, IFN-gamma and stimulation of toll like receptor , induce abortion. Two recent articles, shows that the frequency of circulating Th17 cells to CD4+ is low. The IL17 producer is CD4+ T cell and the IL 17 expressing CD8+ T cell are rare. A study is observed in acute renal rejection suggesting that increase th17 cells in pregnancy might not be good for the pregnancy. Unexpectedly the frequency of Th17 cells in the decidua is significantly higher compared to the peripheral blood. The uterine cavity is not completely sterile and so the Th17 cells might play a role in the response. IL 17 increase the progesterone secretion by JEG-3 human choriocarcinoma cells, this show that IL17 play role in pregnancy .The Treg cells in allogenic pregnancy is much higher compared to syngenic. In human extravillous trophoblast express polymorphic histocompatibility antigen, HLA-C, which can elicit an allogenic T cell response. The pregnancy with HLA-C mismatched child induces an increased percentage of activated T cells in decidual tissue. The HLA-C mismatched pregnancy exhibit significant increased suppressive capacity in one away MLR reaction to umbilical mononuclear cells suggesting the Treg play a role in regulation of successful pregnancy.

Aim and Objectives

AIM: To determine the proportion of T cells in the females undergoing the recurrent pregnancy loss or recurrent Miscarriage.

Objectives:

- To determine the proportion of classical helper T cells (Th1 and Th2 cells) in females undergoing recurrent miscarriage and healthy pregnant females.
- To determine the proportion of regulatory T cells and Th17 cells in recurrent miscarriage females and their ratio in Indian population.
- To evaluate the cytokines levels in these females and compare it with healthy pregnant females.

Materials and Methods

Instrument/Equipment used in this study:

- Centrifuge , Sigma, USA
- CO_2 Incubator , Thermo Fisher Scientific, USA
- Pipettes , Thermo Scientific, Eppendorf
- BD Bioscience caliber, Flow cytometer.

All the chemicals were of Analytical Reagent/Molecular Biology grade.

Isolation of Peripheral blood mononuclear cells (PBMCs):

Reagents:

- Histopaque-1077 (Sigma-Aldrich, St. Louis, MO, USA)
- RPMI-1640 media complete and plain.
- Phytohemagglutinin (Sigma-Aldrich)

Media preparation: Preparation of RPMI 1640:

- 16.4 gm - RPMI powder (Sigma-Aldrich)
- 2.0 gm – Sodium bicarbonate ($NaHCO_3$).
- Double distilled water – 1 litre.

Concentration of antibiotics:

50 µg/ml – Streptomycin = 100 µl

Gentomycin = 1250 µl

For penicillin, 50 µl for 1000 ml (100 unit/ml).

For 1 liter media.

- 16.4 gm – RPMI powder
- 2.0 gm – Sodium bicarbonate

- 100 µl - Streptomycin
- 1250 µl - Gentamycin
- 50 µl - Penicillin

RPMI complete media:

- RPMI-1640 media
- 1 % penicillin, streptomycin, and gentamycin
- 10% fetal calf serum

Sample collection:

A total of 20 patients with history of idiopathic RPL (n=10) and females with history of successful pregnancy without any miscarriage (n=10) were recruited from the Out-Patient Department of Obstetrics & Gynecology, AIIMS, New Delhi, India. All the patients recruited for the study were pregnant during sampling and they were sampled after the documentation of fetal heart beat within 12 weeks of gestation. Human ethical committee approval was obtained from the institute and written consent was taken from the patients.

PBMC Isolation:

Ten ml of heparinized venous blood was collected from the females who were enrolled for this study.

Method:

- RPMI media and Histopaque-1077 (of density 1.077g/l) were brought to room temperature at least for one hour prior to PBMC isolation.
- Two millilitre of Histopaque-1077 were poured into the 15 millilitre centrifuge tube then in equal ratio of 1:1 the blood sample were taken and layered above the histopaque by the side of the tube wall.

- The tubes were centrifuged at 2000 rpm for 15 minutes at room temperature.

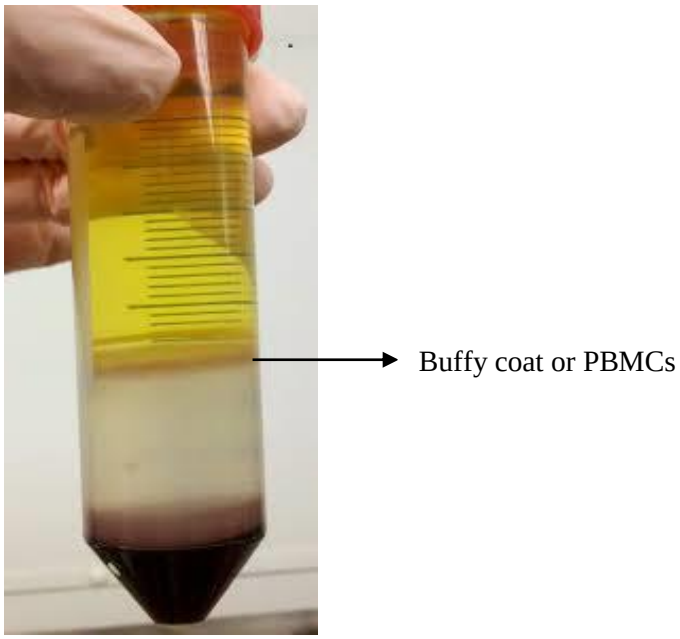


Fig 1: Isolated PBMC in a fifty milliliter tube.

- The buffy coat layer containing the PBMCs were collected and poured into a fresh tube containing 3 ml of RPMI media.
- The tubes were centrifuged at 2000 rpm for 10 minutes (first washing).
- The pellet obtained was again washed with RPMI media (second washing).
- After washing, the pellet was collected and resuspended in 2 ml of RPMI containing 10% fetal calf serum.

Cell counting:

Ten microlitre of cell suspension were taken and 10 microlitre of trypan blue was added. The cells were counted using a Hemocytometer. The blue coloured cells were considered as dead. The unstained cells were counted and their population were calculated as follows:

Calculation:

Total cell count = Average number of viable cells x 2 x dilution factor $x10^4$

Cell Culture:

1×10^6 PBMCs per well were plated in a six well plate and the cells were stimulated with 10 µg/mL of brefeldin A (Sigma-Aldrich) (protein transport inhibitor) was added for 6 h at 37°C in a 5% CO_2-humidified incubator. After incubation, the cells were collected by centrifugation at 500 **g** for 5 minutes, and the culture media were collected and stored at -70°C for cytokine estimation.

Flow cytometry:

Flow cytometry is now a widely used method for analyzing expression of cell surface and intracellular molecules, characterizing and defining different cell types in heterogeneous cell populations, assessing the purity of isolated subpopulations, and analyzing cell size and volume. It allows simultaneous multi-parameter analysis of single cells. It is predominantly used to measure fluorescence intensity produced by fluorescent labeled antibodies detecting proteins or ligands that bind to specific cell associated molecules, such as DNA binding by propidium iodide.

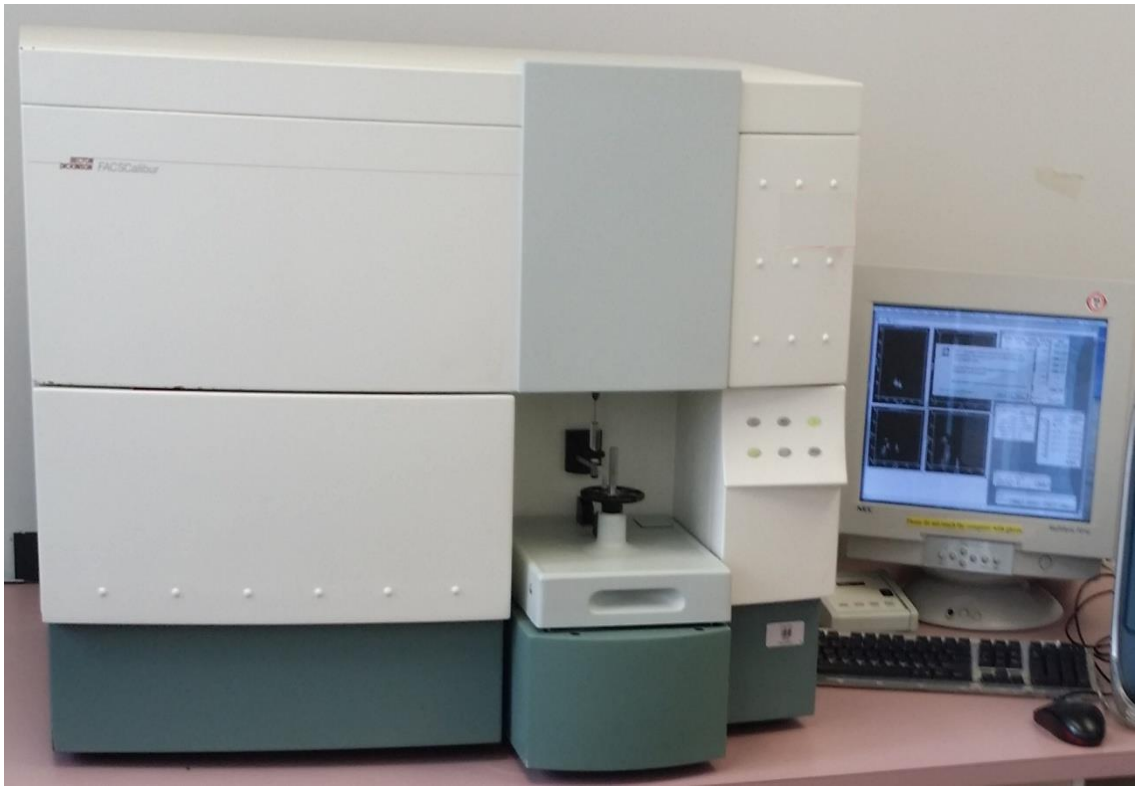


Fig2: Becton Dickinson FACS calibur flow cytometer

Principles of the flowcytometer:

One of the fundamentals of flowcytometry is the ability to measure the properties of individual particles. When a sample in solution is injected into a flowcytometer, the particles are randomly distributed in three dimensional spaces. The sample must therefore be ordered into a stream of single particles that can be interrogated by the machine's detection system. This process is managed by the fluidics system.

Essentially, the fluidics system consists of a central channel/core through which the sample is injected, enclosed by an outer sheath that contains faster flowing fluid. As the sheath fluid moves, it creates a massive drag effect on the narrowing central chamber. This alters the velocity of the central fluid whose flow front becomes parabolic with greatest velocity at its centre and zero velocity at the wall. The effect creates a single file

of particles and is called hydrodynamic focusing. Under optimal conditions (laminar flow) the fluid in the central chamber will not mix with the sheath fluid.

$$Re = pVD/\mu$$

D= tube diameter.

V= mean velocity of fluid.

p= density of fluid, and μ = viscosity of fluid.

Principles of fluorescence:

Fluorochromes are essentially dyes, which accept light energy (from a laser) at a given wavelength and re-emit it at a longer wavelength. These two processes are called excitation and emission. The process of emission follows extremely rapidly, commonly in the order of nanoseconds, and is known as fluorescence. Before considering the different types of fluorochrome available for flowcytometry, it is necessary to understand the principles of light absorbance and emission. Light is a form of electromagnetic energy that travels in waves. These waves have both frequency and length, the latter of which determines the colour of light. The light that can be visualized by the human eye represents a narrow wavelength band (380–700 nm) between ultraviolet (UV) and infrared (IR) radiation. Sunlight, for example, contains UV and IR light that, although invisible to the eye, can still be felt as warmth on the skin and measured scientifically using photodetectors. The visible spectrum can further be subdivided according to color, often remembered by the mnemonic 'ROY G BV' standing for red, orange, yellow, green, blue and violet. Red light is at the longer wavelength end (lower energy) and violet light at the shorter wavelength end (higher energy) When light is absorbed by a fluorochrome, its electrons become excited and move from a resting state to a maximal energy level called the 'excited electronic singlet state. The amount of energy required will differ for each fluorochrome and it's Excitation.

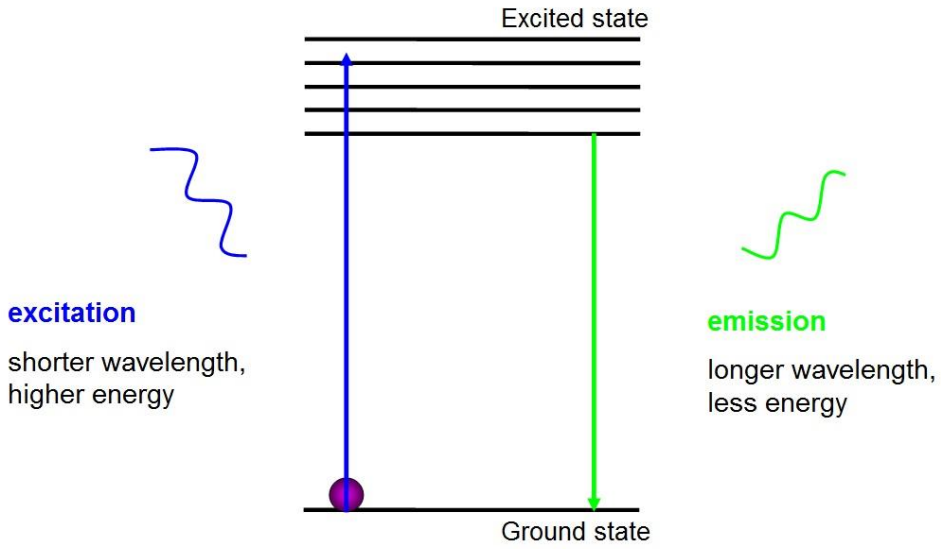


Fig3: Physical states of fluorochrome after excitation.

This state only lasts for 1–10 nanoseconds because the fluorochrome undergoes internal conformational change and, in doing so, releases some of the absorbed energy as heat. The electrons subsequently fall to a lower, more stable, energy level called the 'relaxed electronic singlet state. As electrons steadily move back from here to their ground state they release the remaining energy (Eemission) as fluorescence. As Emission contains less energy than was originally put into the fluorochrome it appears as a different color of light to Eexcitation.Therefore, the emission wavelength of any fluorochrome will always be longer than its excitation wavelength. The difference between Eexcitation and Eemission is called Stokes Shift and this wavelength value essentially determines how good a fluorochrome is for fluorescence studies. After all, it is imperative that the light produced by emission can be distinguished from the light used for excitation.

Dye	Laser excitation line (nm)	Maximal absorbance (nm)	Maximal emission (nm)	Fluorescence color
Alexa Fluor® 405	405, 407	401	421	
Alexa Fluor® 430	405, 407	433	541	
Alexa Fluor® 488	488	495	519	
Alexa Fluor® 633	633, 635, 647	632	647	
Alexa Fluor® 647	633, 635, 647	650	665	
Alexa Fluor® 660	633, 635, 647	663	690	
Alexa Fluor® 680	633, 635, 647	679	702	
Alexa Fluor® 700	633, 635, 647	702	723	Infrared
APC	633, 635, 647	650	661	
FITC	488	490	525	
Pacific Blue™	405, 407	410	455	
PerCP	488	490	675	
Phycoerythrin	488	490, 565	578	

Fig4: **Fluorochromes used in flowcytometry.**

Reagents:

- 2% BSA-PBS Buffer.

  2 grams of BSA was weight and 0.1 % Sodium azide for 100 millilitre of buffer in 1x PBS.

- Permeabilization Buffer.

  0.5 % Saponin in 2 % BSA-PBS buffer.

- 1x PBS.

- Fixation Buffer (2 % Paraformaldehyde).

- Fluorochrome-lebeled monoclonal antibodies (ebioscience, SAN Diego, CA, USA).

Flow Cytometry: Processing of the cell culture:

- After the culture, the PBMCs were then accounted to flow cytometry for the phenotyping of the Th1, Th2, Th17 and regulatory T (Treg cells) cells.
- The cells were incubated with PerCP-Cy5.5-CD3, APC-CD4 and FITC-CD25 anti-human monoclonal antibodies for 30 minutes at 4°C in dark in a staining buffer (1x PBS containing 2% BSA and 0.1% sodium azide).
- After washing, the cells were fixed with the fixation buffer (2% paraformaldehyde) for 10 minutes at the room temperature.
- For intracellular staining, the cells were washed with permeabilization buffer [(0.5% saponin (Sigma-Aldrich) in staining buffer] and are incubated with PECy7-IFNγ, PE-IL4 and PECy7-IL17A for 30 minutes at 4°C in dark.
- The cells were washed and the cells were fixed with the fixation buffer (2% paraformaldehyde) for 15 minutes at room temperature.
- After that, cells were transferred in FACS tubes and acquired on the FACSCanto II. 50,000 events were acquired for each sample and analysis was done by DIVA software (BD Bioscience, San Diego, CA, USA).

Determination of Cytokine levels:

The concentration of the proinflamatory cytokines: - TNF-α, TGF-β and anti-inflammatory cytokines:- IL-2 and IL-10 was determined in the culture supernatant from the RPL patient and the controls using ELISA (Ready-set-Go! ebioscience) as per the manufacturer's instruction.

ELISA – Enzyme linked Immunosorbent Assay combine the specificity of antibodies with the sensitivity of simple enzyme assays, by using antibodies or antigens coupled to an easily-assayed enzyme. ELISAs can provide a useful measurement of antigen or antibody concentration.

There are three main types of ELISA

- Direct ELISA
- Sandwich ELISA
- Competitive ELISA

Sandwich ELISA is a less common variant of ELISA, but is highly efficient in sample antigen detection. The sandwich ELISA quantify antigens between two layers of antibodies (i;e the capture and detection antibody). The antigen to be measured must contain atleast two antigenic epitope capable of binding to antibody, since atleast two antibodies act in the sandwich. Either monoclonal or polyclonal antibodies could be used as the capture and detection antibodies in sandwich ELISA systems. The monoclonal antibodies recognize a single epitope that allow fine detection and quantification of small difference in antigen. A polyclonal is often used as a capture antibody to pull down as much as antigen as possible.

Method:

- The Corning Costar 9018 ELISA plate was coated with 100 µl/well of capture antibody in coating buffer. Then the plate was sealed and incubated overnight at 4°C.
- The wells were washed 5 times with >250 µl/well with wash buffer and allowing time for soaking during each wash step increases the effectiveness of washes.
- The dilution was made by 1 part 5X concentrated assay diluent with 4 parts DI water. Then the wells were blocked with 200 µl/well of 1X assay diluents and incubate at room temperature for 1 hour.
- The wells were washed with wash buffer for 5 times.
- Now by using 1X assay diluents, dilute standards.100 µl/well of the standard were added to the appropriate wells. 2-fold serial dilutions of top standards were performed to make the standard curve, then 100 µl/well of the samples were added

to the appropriate wells and the plate was sealed and incubate at room temperature for 2 hours or keep overnight at 4°C.

- The wells were washed with wash buffer for 5 times.
- 100 µl/well of detection antibody diluted in 1X diluents was added and the plate was sealed and incubate at room temperature for 1 hour.
- The wells were washed with wash buffer for 5 times.
- 100 µl/well of Avidin-HRP diluted in 1X diluents was added and the plate was sealed and incubated for 30 minutes.
- Again, the wells were washed with wash buffer for 7 times.
- Then,100 µl/well of substrate solution was added to each well and the plate was incubated at room temperature for 15 minutes.
- 50 µl of stop solution was added to each well.
- And the reading plate was taken at 450 nm.

Statistics:

Statistical analysis was done by student's t-test or alternatively by two-tailed Mann-whitney t test using Graphpad Prism 5 (Graphpad software ,La Jolla,CA,USA). $P < 0.05$ was considered significant.

Results

The recruitment was done from the Out-Patient Department of Obstetrics & Gynecology, AIIMS, New Delhi, India:-

Characteristics of the Patients Recruited for the study		
	Healthy Pregnant females (H/P)	RPL Pregnant females (RPL/P)
No. of patients (n)	10	10
Age (years)	27 (22-32)	26 (21-35)
Gravidity	2 (1-4)	4 (4-8)
Parity	1 (0-3)	0 (0-2)
Spontaneous abortion	_	3 (3-6)

Th 1 cells

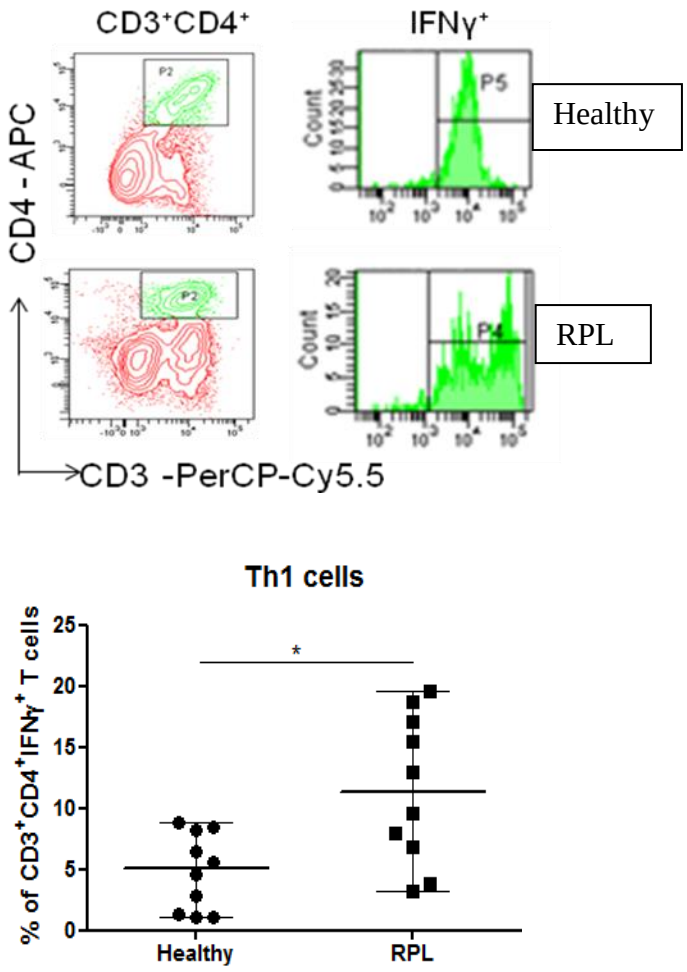


Fig1.a : Representative density dot plots showing the gating of CD3+CD4+ IFN-γ T cells of the PBMCs isolated from healthy pregnant (H/P), RPL pregnant (RPL/P). Data are expressed as singly dots with median. *P < 0.05.

The flow cytometric profiles of Th1 cell ($CD3^+$ $CD4^+$ $IFN\gamma^+$) and Th2 cells ($CD3^+$ $CD4^+$ $IL\text{-}4^+$) in the peripheral blood lymphocytes of the healthy pregnant (H/P) and RPL pregnant (RPL/P). Gates were set on $CD3^+$ $CD4^+$ T cells to estimate the percentage of IFN-γ and IL-4 producing cells. A significantly higher proportion of IFNγ-producing $CD3^+CD4^+$ T cells in the peripheral blood lymphocytes of RPL/P then those of healthy control (15.975 versus 4.35; P< 0.0015) (fig:1a).

Th 2 cells

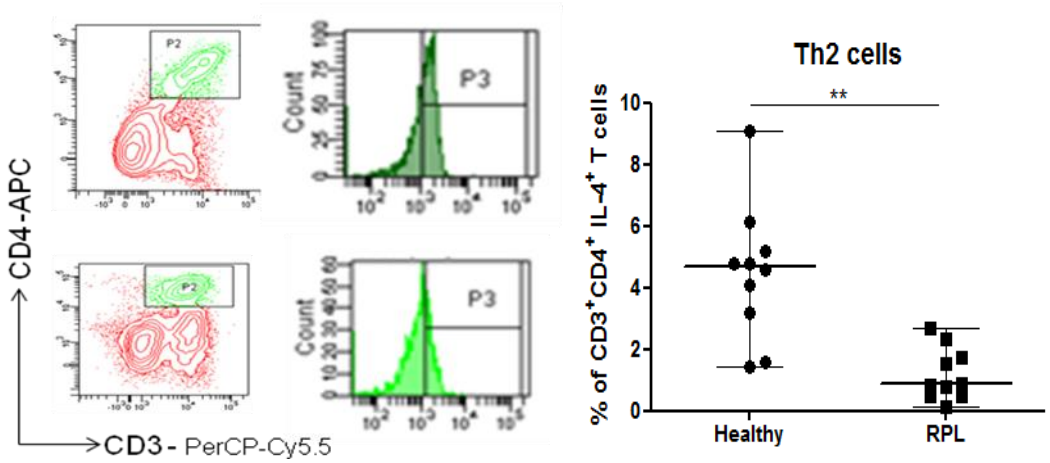


Fig1.b: Representative density dot plots showing the gating of CD3+CD4+ IL-4+ T cells of the PBMCs isolated from healthy pregnant (H/P), RPL pregnant (RPL/P). Data are expressed as singly dots with median. **P < 0.005.

In the fig5.b, significant lower proportion of IL-4 producing CD3+CD4+ T lymphocytes in the peripheral blood of pregnant females undergoing recurrent miscarriage was observed as compared to normal pregnant females (1.38 versus 4.685; P< 0.0004).

Th 17 cells

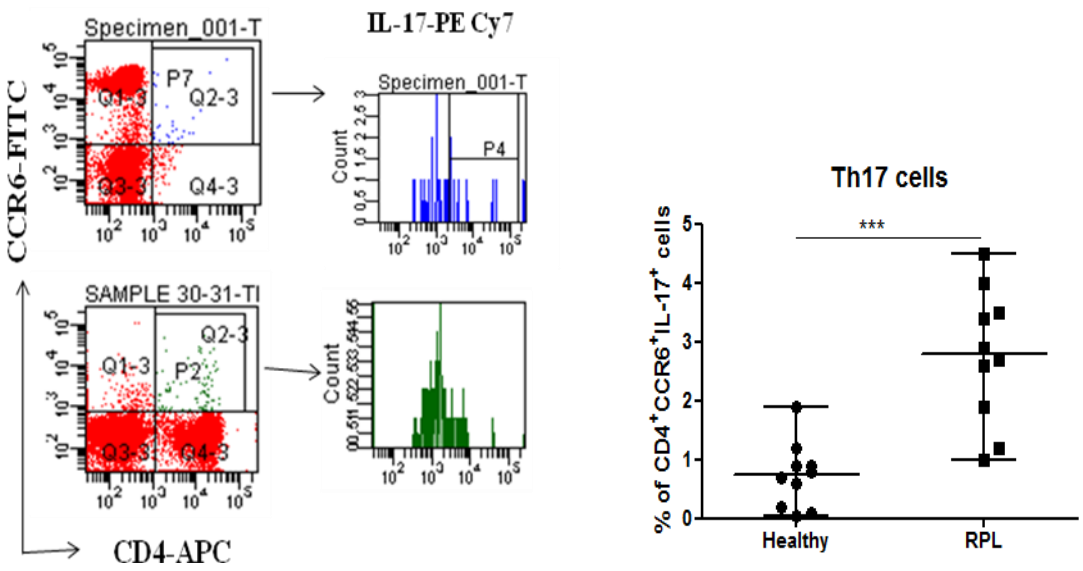
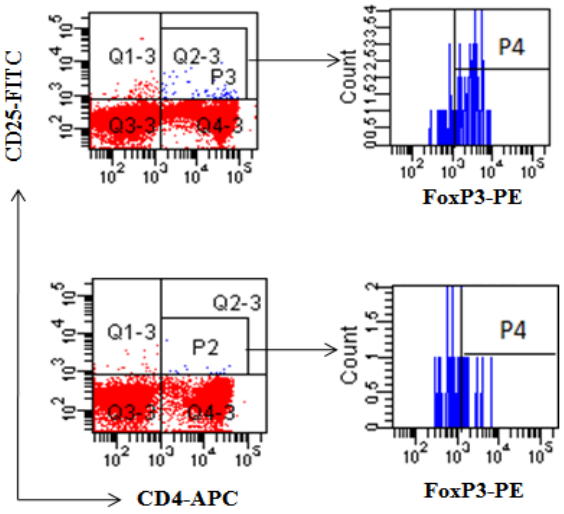


Fig1.c : Representative density dot plots showing the gating of CD4+CCR6+ IL-17+ T cells of the PBMCs isolated from healthy pregnant (H/P), RPL pregnant (RPL/P). Data are expressed as singly dots with median. ***P < 0.001.

The flow cytometric profiles of Th17 cell ($CD4^+$ $CCR6^+$ $IL\text{-}17^+$) in the peripheral blood lymphocytes of the healthy pregnant (H/P) and RPL pregnant (RPL/P). It was observed that the proportion of IL-17A producing $CD4^+$ T cells in the peripheral blood lymphocytes of pregnant RPL females were significantly higher than seen in the pregnant controls (2.795 versus 0.9; P<0.0002).

Regulatory T cells



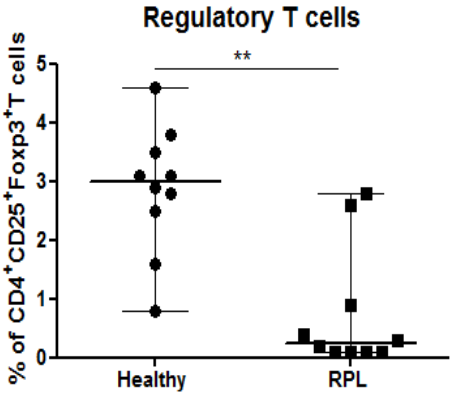
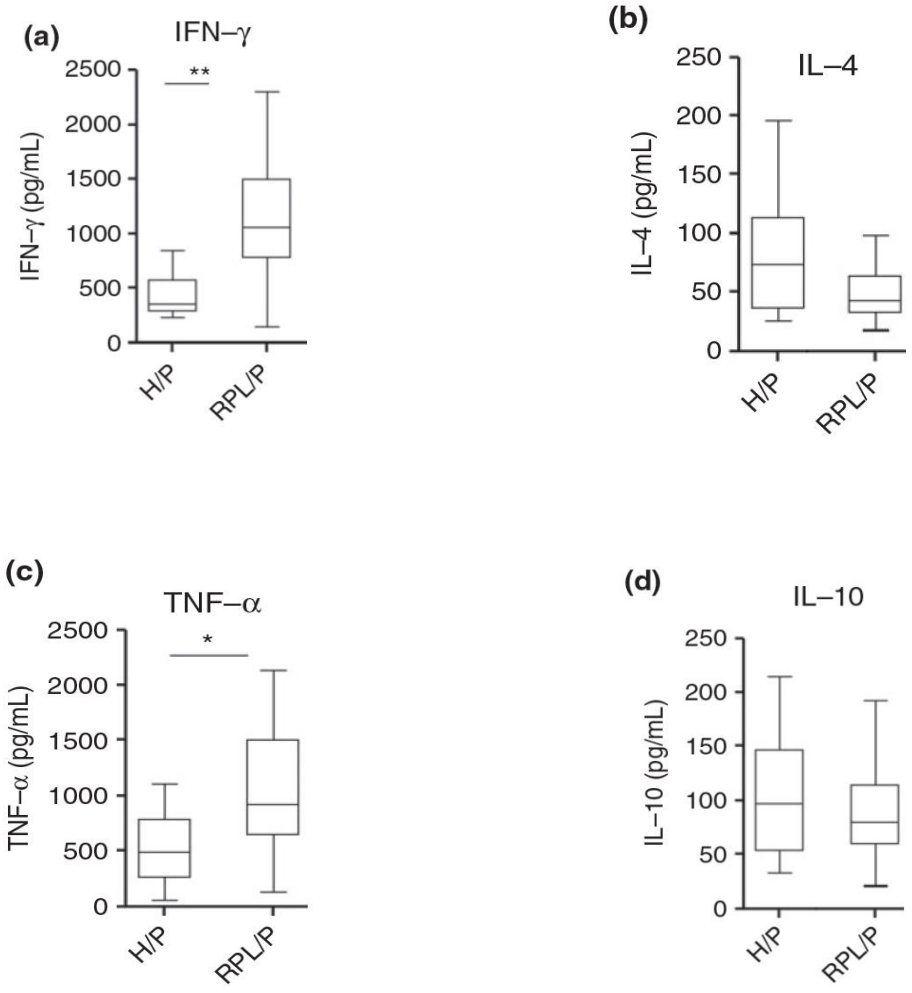


Fig5.d : Representative density dot plots showing the gating of CD4+CD25+ FOXP3+ T cells of the PBMCs isolated from healthy pregnant (H/P), RPL pregnant (RPL/P). Data are expressed as singly dots with median. **P < 0.005.

Gates were set on $CD4^+$ $CD25^+$ T cells to estimate the percentage of Foxp3 producing cells. A significantly, lower proportion of Foxp3-producing $CD3^+CD4^+$ T cells in the peripheral blood lymphocytes of RPL/P then those of healthy control, which having a much higher proportion of the regulatory T cells in the peripheral blood of the control compare to the RPL patient.

Cytokine levels in the women with the recurrent miscarriage:

We determined the levels of proinflammatory cytokines: IFN-γ and TNF-α and anti-inflammatory cytokines: IL-10 and IL-4. We observed a significant increase in the levels of IFN-γ and TNF-α in the RPL females as compared to controls whereas a significant decrease levels of IL-4 and IL-10 were observed in RPL females than controls. The decrease in anti-inflammatory cytokines in the serum might be a contributing factor for the miscarriage in these females.



Discussion

The key to successful pregnancy is to have a balanced immune tolerance whereas an inefficient modulation of T cell function may play a major role in recurrent pregnancy loss. Maintenance of pregnancy generally depends on the shift from the Th1-type of maternal immune response towards Th2-type. Earlier studies have shown that altered immunity in RPL women is mainly due to the dominance of the Th1/Th2 cytokines ratio in their peripheral blood(20). A predominant Th1-mediated immune response was observed in RPL pregnant patients than in those from healthy pregnant females. Even RPL women who were not pregnant at the time of recruitment also showed higher proportion of Th1-type immune response as compared to normal pregnant females.

A subpopulation of $CD3^{+}CD4^{+}$ T cells, Th17 cells, have been known that may modulate the tolerance during pregnancy by secreting the proinflammatory cytokine, IL-17A. Th17 cells play an important role in the induction of immunity against extracellular bacteria and fungal pathogens(21). We found increased proportion of Th17 cells ($CD3^{+}CD4^{+}$IL-$17A^{+}$) in women undergoing RPL. It has been known that the Th17 cell proportion remains stable during the pregnancy period in healthy individuals (22). An increase in Th17 cells in the peripheral blood and decidua of women with unexplained RPL as compared to normal pregnant women has been reported (7). Thus, their increased proportion may induce the rejection of the semiallogenic fetus.

Regulatory T cells play a role as a key regulator to counteract the effector cells such as Th17 cells. An elaborate immune balance between immune effectors and immune regulators is crucial to achieve implantation and maintain pregnancy until term. In addition to Th1 and Th2 immunity, it becomes evident that Th17 immunity and regulatory T cell-mediated immune regulation are deeply involved in pathogenesis of RPL. Further studies are needed to elucidate the immune mechanism operating during implantation and pregnancy.

The regulatory T cell are responsible for immune tolerance. These cells secrete anti-inflamatory cytokines like TGF-β, IL-10 which help in suppressing the effect of

proinflamatory cytokines like IFN-γ, IL-17. Hence, regulatory T cells are helpful in maintaining immune homeostasis and successful maintenance of pregnancy. Decreased level of regulatory T cells in the RPL females attributed to miscarriage as they are unable to maintain immune tolerance.

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