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Membrane progesterone receptors in human regulatory T cells: a reality in pregnancy

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Objective To provide evidence of the existence of membrane progesterone receptor alpha (mPRα) on regulatory T cells (Treg) in peripheral blood during pregnancy, postulating a possible explanation for the effect of progesterone on preterm birth.

Design Cross-sectional study.

Setting Tertiary Obstetric Department in a University Hospital.

Population Healthy pregnant women.

Methods Treg cells from peripheral blood samples were studied by flow cytometry using multiple monoclonal antibody expression.

Main outcome measures Evaluate the number and percentage of $CD4^+CD25^{high}CD127^{low}$, the number and percentage of Treg cells among the total $CD4^+$ T cells, and the percentage and mean fluorescence intensity (MFI) of mPRα in that population, using several gating strategies.

Results 43 peripheral blood samples were collected from healthy women during pregnancy, whose median gestational age was 28.7 ± 7.1 (16–40) weeks. The percentage of $CD4^+$ in the total lymphocytes was 43% (32–51) and the percentage of $CD4^+CD25^{high}CD127^{low}$ was 4.8% (1.6–5.9), with only 45% (16–72) of those cells expressing the intracellular marker FoxP3 (Treg cell pool). We confirmed the existence of mPRα in that specific population because 8.0% (2.02–33) of the Treg cells were marked with the specific monoclonal antibody, with an $mPR\alpha^+$ MFI of 719 (590–1471).

Conclusions This research shows that Treg cells express mPRα during pregnancy, which might play an important role in immune modulation by progesterone.

Keywords Pregnancy, progesterone receptor, T regulatory cells.

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Introduction

It is commonly accepted that the act of giving birth is the final step in a pro-inflammatory signalling cascade. Consequently, the inflammatory process plays a pivotal role in the triggering of human labour both in term and in preterm birth (PTB).[1]

Maternal acceptance of the fetus during pregnancy results from T-cell tolerance rather than immunosuppression. However, there is strong evidence that maternal T cells are not exposed to fetal alloantigens and that changes in the production of progesterone play a major role in modulating local immunosuppression.[2]

The abundance or modulation of systemic regulatory T cells (Treg) could be involved in pregnancy complications.[3,4] However, it is not known whether the Treg suppressive mechanism is specific to PTB or if it is also involved in spontaneous normal term birth.[5] Progesterone has a major role in pregnancy maintenance and its secretion has been demonstrated in the amnion, chorion and decidua in humans.[6,7]

In the 1st trimester, progesterone is critical to pregnancy preservation until the placenta takes over this function. In later pregnancy, however, its function is less clear.[8] Although progesterone levels in the maternal circulation do not change significantly in the weeks or days preceding labour, the onset of labour is associated with a functional withdrawal of progesterone activity.[8,9]

There are several unanswered questions surrounding the role of progesterone in human pregnancy. Of these, the questions of what molecular mechanisms support progesterone action during pregnancy and what molecular

changes turn off progesterone signalling and allow parturition, are the most intriguing.[10] In the quest for a novel agent in PTB treatment, progesterone emerges as a good candidate due to its immunomodulatory action.[11] Although the exact mechanism of its immunomodulatory role is still unknown, reports demonstrate its rapid effects on human T cells.[2,12]

The extranuclear activity of progestins was identified to be mediated by an alternative membrane-localised progesterone receptor (mPR), which may be responsible for the rapid cell activation prompted by progesterone[11,13] and progesterone interaction with the immune system.[14]

The function of one of these receptors, mPRα, has been investigated[15] but its expression on specific subsets of immune cells has hardly been demonstrated.[12,16]

It is thus tempting to infer that mPRα is the mechanism by which progesterone regulates Treg cells, explaining progesterone actions during pregnancy and PTB. The aim of this investigation is to ascertain whether mPRα is present on Treg cells in peripheral blood during pregnancy.

Methods

We undertook a cross-sectional study of healthy women attending normal prenatal appointments at our Obstetrics Unit between December 2013 and May 2014. Exclusion criteria consisted of multiple gestation, pre-existing disease, preterm rupture of membranes, chorioamnionitis, placenta *praevia*, placental abruption, clinical signs of infection (maternal temperature ≥37.5°C, white blood cells ≥15 000 cells/mm^3 in maternal blood) or use of hormone therapies within 3 months before enrolment.

Gestational age was assessed by date of last menstrual period or by ultrasound performed in the first trimester.

The investigation was approved by the Ethical Committees of Coimbra University and Coimbra University Hospital and informed consent was obtained from each participant.

Specimen collection

Peripheral venous blood samples were obtained and collected in lithium heparin tubes. Samples were kept in a cool environment until being processed within 1 hour of collection.

Flow cytometry staining

In brief, 100 ml of whole blood containing 0.5–1 × 10^6 white blood cells was placed in a clean test tube and stained to localise the mPRα receptor on the cell surface, using the N-terminal mPRα antibody described by Thomas et al.[17] Cells were first incubated in a blocking solution [0.5% bovine serum albumin {BSA}], in phosphate-buffered saline (PBS) for 30 minutes to 1 hour and then incubated with the mPRα antibody (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at room temperature for a further 30 minutes to 1 hour. Cells were washed with PBS 0.5% BSA and incubated for 30 minutes with Cruz Fluor 488 goat anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology, Inc.) at room temperature in the dark. Cells were washed with the blocking solution, and the surface was stained with PB conjugated anti-CD4, PE-Cy7 conjugated anti-CD25, and PerCP-Cy 5.5 conjugated anti-CD127.

Subsequently, intracellular staining for detection of FoxP3 was performed using AF647 labelled anti-human FoxP3 (Biolegend, San Diego, CA, USA) and the staining set (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions. Flow cytometry data were acquired on a FACS Canto II instrument (BD Biosciences, San Jose, CA, USA) equipped with three lasers to allow multicolour detection with different fluorophors, using FACS DIVA software (BD Biosciences).

Lymphocyte populations were selected according to the forward angle (FSC-A) and side angle (FSC-H) scattering signal, and at least 50 000 gated lymphocyte cells were detected for each sample. Dead cells were excluded by forward and side scatter characteristics and an FSC-A versus FSC-H dot plot was used to discriminate doublets, detecting disparity between cell size versus cell signal.

Isotype control antibodies were used to help assess the level of background staining, as well as samples without staining and single stain, for each antibody.

Treg analysis and mPRα expression

Gating strategies were employed to evaluate the percentage of $CD4^+CD25^{high}CD127^{low}$ cells, the percentage of Treg cells in total $CD4^+$ T cells, and the percentage and mean fluorescence intensity (MFI) of mPRα in that population.

Our gating strategy for identifying the Treg population was based on a total lymphocyte gate based on a FSC/Side light scatter (SSC) dot plot followed by doublet discrimination with an FSC-A versus FSC-H dot plot. Accordingly, CD4-positive cells were gated over SSC characteristics; depending on CD25 and CD127 expression, $CD4^+$ cells were gated based on the expression of $CD25^{high}$ and $CD127^{low}$ markers, and the $CD4^+CD25^{high}CD127^{low}$ population was detected. As the literature varies as to the markers for the exact phenotype for a Treg cell population, we moved on to the $CD4^+CD25^{high}CD127^{low}$ population, and also searched for $FoxP3^+$ cells. In the $CD4^+CD25^{high}CD127^{low}FoxP3^+$ (regulatory T-cell population), the $mPR\alpha^+$ subset was identified and characterised by percentage and mean fluorescence intensity (MFI).

The statistical analysis was based on at least 15 000–20 000 gated $CD4^+$ cells. FLOWJO software (Tree Star Data Analysis Software, Ashland, OR, USA) was used for the flow cytometry analysis.

Real time PCR and Western blot analysis

For mPRα assessment by RT-PCR and Western blot, blood samples were submitted to Ficoll-hypaque density gradient centrifugation to obtain peripheral blood mononuclear cells (PBMCs). PBMCs were then collected under optimal conditions to ensure high purity samples. Part of the PBMCs were lysed in RNeasy RLT lysing buffer (Qiagen, Austin, TX, USA) and frozen at −80°C until RNA extraction. The rest of the cells were treated with RIPA buffer and completed with protease inhibitors, which enables rapid and efficient cell lysis and solubilisation of proteins until subsequent Western blot assays. Both techniques were performed based on protocols previously described by Ndiaye et al.,[18] Thomas et al.[17] and Dosiou et al.[16] RT-PCR results were analysed in a Light Cycler 480 (Roche Instruments).

Statistical analysis

Data were analysed by IBM® SPSS 21 Statistics software (IBM Corporation, Armonk, NY, USA) and data are expressed as mean ± standard deviation (SD) or median and interquartile range (IQR) values, as appropriate for the type of distribution.

Using the nonparametric Mann–Whitney *U*-test, statistical comparison were made between groups of the total number and percentages of $CD4^+CD25^{high}CD127^{low}$ of $CD4^+$ T cells, the total number and percentages of the Treg cell subset within the total $CD4^+CD25^{high}CD127^{low}$ population, and the total number and percentages of $mPR\alpha^+$ Treg cells, and the MFI between the different women's characteristics (parity and gestational age) was determined. Statistical significance was considered for a P value <0.05. There were no missing data in our population sample.

Results

A total of 43 peripheral venous blood samples were extracted from healthy pregnant women with a median gestational age of 28.7 ± 7.1 (16–40) weeks, divided between 2nd trimester (42%; $n = 18$) and 3rd trimester (58%; $n = 25$). Clinical data of the population are shown in Table 1.

First, $CD4^+$ T cells were gated and analysed for the expression of CD25 and CD127; subsequently, the number and percentage of $CD4^+$ $CD25^{high}$ $CD127^{low}$ cells were estimated for all participants. Afterwards, Treg cells were characterised by the expression of FoxP3 and mPRα to estimate both the percentage and absolute number of Treg cells and the $mPR\alpha^+$ expression on those Treg cells. The MFI of $mPR\alpha^+$ on Treg cells was estimated for all participants.

Figure 1 (A,B in the main article, Figure S1a–d) shows our flow cytometric gating strategy for the $CD4^+CD25^{high}CD127^{low}FoxP3^+$ population (regulatory T-cell population) in peripheral blood.

Table 1. Clinical data

Variable	Value
Age (Years)	
Mean ± SD (min–max)	30 ± 4.8 (21–37)
Gestational age (weeks)	
Mean ± SD (min–max)	28.7 ± 7.1 (16–40)
$n = 43$	
Nullipara (*n*, proportion)	29 (67%)
2nd Trimester (*n*, proportion)	18 (42%)
3rd Trimester (*n*, proportion)	17 (39%)
Delivery (*n*, proportion)	8 (19%)

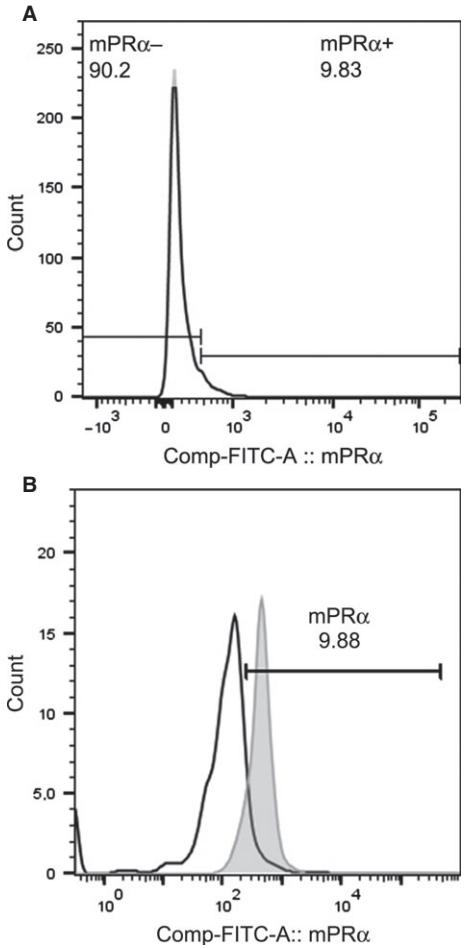

Figure 1. Flow cytometric gating strategy for $CD4^+CD25^{high}CD127^{low}FoxP3^+$ Treg analysis in peripheral blood. Peripheral blood lymphocytes were stained with FITC-labeled anti-mPRα, APC-labelled anti-FoxP3, PE-Cy7-labeled anti-CD25, PerCP-Cy 5.5-labeled anti-CD127 and PE-labelled anti-human CD4 antibodies. A, FITC anti-mPRα histogram: percentage of $mPR\alpha^+$ subset within the total $CD4^+CD25^{high}CD127^{low}FoxP3^+$Treg cell pool. B, Isotype control for mPRα.

Table 2 shows the absolute number and percentage of the different populations studied, in the normal course of pregnancy.

As the results show, the percentage of $CD4^+$ in the total lymphocytes was 43% (32–51) and the percentage of $CD4^+CD25^{high}CD127^{low}$ was 4.8% (1.6–5.9), with only 45% (16–72) of those cells expressing the intracellular marker FoxP3 (Treg cell pool).

We were able to verify the expression of mPRα in that specific population, as 8.0% (2.0–33) of those Treg cells were positive for this marker, with an $mPR\alpha^+$ MFI of 719 (590–1471).

To ascertain whether the number or percentage of $CD4^+$ cells, $CD4^+$ $CD25^{high}$ $CD127^{low}$, Treg cells and $mPR\alpha^+$ Treg cells varied with different clinical characteristics, a subgroup analysis was done, as shown in Supporting Information Table S1. The clinical characteristics analysed compared with others were as follows: parity (nullipara if it was the first pregnancy); 2nd trimester (14–27 weeks); 3rd trimester (≥28 weeks); delivery date.

The percentage and absolute number of $CD4^+CD25^{high}CD127^{low}$ was elevated in women in the 3rd trimester or at delivery date ($P = 0.001$), with the highest levels at delivery date ($P = 0.04$ and $P = 0.007$, respectively).

The percentage and absolute number of Treg cells were higher in women in the 3rd trimester, with the strongest difference shown in the percentage of Treg cells ($P = 0.02$).

Finally, the percentage of $mPR\alpha^+$ Treg cells was higher in the nulliparas ($P = 0.026$) and there was an increase in the absolute number of $mPR\alpha^+$ Treg cells from the 2nd to the 3rd trimester of pregnancy, although this was not statistically significant (0.25 versus 1.22, $P = 0.08$, respectively). No other comparisons between groups had statistical significance, although a trend towards a higher number of $CD4^+$ cells could be perceived in the date of delivery ($P = 0.058$).

Western blot experiments designed to examine mPRα protein expression showed the presence of a protein about 40 kD in size in our PBMC samples. Representative results of two independent experiments are shown in Figure 2.

Moreover, expression of mPRα mRNAs was detected by RT-PCR using mPRα-specific primers.

Table 2. Absolute number and percentage of the different populations studied

n = 43	$CD4^+$ (total lymphocytes)	$CD4^+$ $CD25^{high}$ $CD127^{low}$ (in $CD4^+$ T lymphocytes)	Treg cells (in $CD4^+$ $CD25^{high}$ $CD127^{low}$)	$CD4^+$ $CD25^{high}$ $CD127^{low}$ $FoxP3^+$ $mPR\alpha^+$
% Cells				
Median	43	4.8	45	8.0
IQR	(32–51)	(1.6–5.9)	(16–72)	(2.0–33)
Absolute number*				
Median	959.9	42.91	11.5	0.98
IQR	(302.1–1517.4)	(3.23–86.2)	(1.07–36.5)	(0.08–2.55)
MFI				
Median	–	–	–	719
IQR				(590–1471)

IQR, interquartile range; MFI, mean fluorescence intensity.
*number cells/μl blood.

Discussion

Progesterone has been known to play an important role in the reproductive tract for the initiation and continuation of pregnancy, with good results in the prevention of spontaneous abortion and recently in preterm labour. Nonetheless, progesterone-mediated responses are complex because they are mediated by multiple types of receptors.[19]

Undoubtedly this steroid is able to prevent the maternal immune system from activating effector T-cells capable of attacking fetal cells, resulting in a T-cell tolerance during pregnancy.[2] Recent data suggest that progesterone may be important in maintaining uterine quiescence in the latter half of pregnancy by limiting the production of stimulatory prostaglandins and inhibiting the expression of contraction-associated protein genes within the endometrium.[8] However, the exact route by which this is accomplished is still being researched.

Regulatory T cells were shown to expand during human pregnancy, with functional studies finding that they create a tolerant microenvironment through regulation of immune cell responses at the fetal–maternal interface.[20]

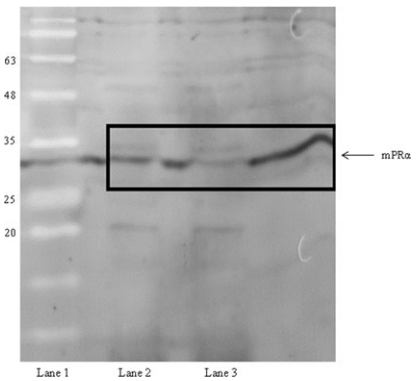

Figure 2. Representative results of Western blot analysis. Western blot analysis of mPRα expression in peripheral blood mononuclear cells (PBMCs): 20 μg/protein/lane; mPRα antibody concentration (1 : 2000). Lane 1 – molecular weight marker; kD. Lanes 2 and 3 – mPRα in PBMCs.

Since 1980, some groups have tried to identify expression of progesterone receptors during pregnancy, although with contradictory results.[21,22] Nevertheless, the gathering of scientific effort has enabled not only the presence of lymphocytic progesterone receptors[23] to be verified, but also validation of the existence of progesterone-induced blocking factor and its role in pregnancy.[24] Recently, some authors have attempted to demonstrate that the actions of progesterone on T lymphocytes are mediated by one or more putative membrane receptors, but all experiments were done in non-pregnant animal models.[18] Moreover, although receptors for oestrogens have been confirmed in Treg cells,[25] to the best of our knowledge progesterone receptors have not been studied in this subset of human cells.

Main findings

This research postulates a primordial role for mPRα in the intertwining between Treg cells and progesterone in human pregnancy.

In our work, we have shown the existence of mPRα in the Treg cell pool, with 8.0% of Treg cells being $mPR\alpha^+$, with an MFI of 719.

Some authors have indicated a significant decrease in $CD4^+$ T cells within the total leucocyte pool in spontaneous labour, which could indicate that a strong immune stimulation and subsequent apoptosis of the activated $CD4^+$ T cells may occur during labour.[5] When comparing our results with those published in the literature, this population remained almost unchanged throughout the whole pregnancy, with a slight increase on delivery day, which contrasts with published literature.

$CD4^+CD25^{high}CD127^{low}$ isolated Treg cells appear to be the best Treg population achieved regarding purity, function, stability and *in vitro* expansion capacity, promising isolation of pure Treg populations with high suppressive activity.[26] Our results were similar to published ones, with $CD4^+CD25^{high}CD127^{low}$ cells making up 4.8% (1.6–5.9) of the $CD4^+$ T-cell population.

However, the most widely accepted phenotype for Treg cells is the co-expression of CD4, CD25 [α-chain of the interleukin (IL)-2 receptor] and FoxP3.[27] We therefore assumed that the $CD4^+CD25^{high}CD127^{low}$ $FoxP3^+$ is the phenotype of the Treg cell pool.

FoxP3 is regarded as a lineage molecule for Treg cells and it is an intracellular marker. Consequently, it is very susceptible to degradation within a short space of time, and it is difficult to detetct and not really usable in large sample series. Moreover, $FoxP3^+$ T cells are phenotypically and functionally heterogeneous and involve both suppressive and non-suppressive T cells.[27]

Furthermore, CD127 was for a long period seen as an efficient tool to determine the phenotype and functional activity of Treg cells. Yet, there has been increasing controversy in comparisons with $CD4^+CD25^{high}FoxP3^+$ cells, particularly in the context of chronic infections.[27] However, no attention was given to it in the context of pregnancy. As such, it is currently accepted that CD127 expression inversely correlates with FoxP3 expression and suppressive activity of Treg cells .[27]

The data reported in the literature concerning the effect of pregnancy-specific hormones on FoxP3 expression by Treg cells are very contradictory.[5] The withdrawal of hormones at the end of pregnancy may affect FoxP3 expression by Treg cells, which was shown to be enhanced by progesterone in human studies.[3] Other authors postulate that progesterone, whose maximum levels are seen at the end of pregnancy, has the capacity to reduce FoxP3 expression by Treg cells *in vitro*.[25] Moreover, recent data indicate a significant decrease of Treg cells expressing FoxP3 in the 3rd trimester and women in labour at term.[5]

We therefore also determined FoxP3 expression in our research, making some comparisons feasible. Within $CD4^+CD25^{high}CD127^{low}$ cells, only 45% were Treg cells, corroborating the idea that Tregs in pregnant women have a reduced expression of FoxP3.[25]

Strengths

The strengths of our research lie in the fact that this is the first report on humans regarding the existence of mPRα in Treg cells, which is of utmost importance as it opens up a promising field in immunology and may help to explain more exactly the effects of progesterone on PTB, thereby allowing its more rational, widespread and effective use.

Moreover, as all methods are thoroughly described, the results are open to reproducibility studies by research groups.

Limitations

Nevertheless, there are limitations in our study that need to be taken into account. The different antibody panel chosen to characterise our populations is controversial (as discussed previously) and the small number of samples means our results should be considered with caution.

Interpretation

Controversies still persist that could not be solved by our results. The variation of the number of Treg cells during the three trimesters of human pregnancy is still under debate, with some authors reporting a rise in the 1st trimester with a peak in the 2nd trimester, and others reporting a reduction in the 2nd trimester.[28] In our study, the percentage and absolute number of $CD4^+CD25^{high}CD127^{low}$ cells was highest at delivery, suggesting the likely importance of activation of this population in the recrudescence of the inflammatory phenomenon nowadays believed to be labour. The results for Treg cells, with the highest

levels in the 3rd trimester, make this hypothesis even more reasonable.

Finally, the higher percentage of mPRα^{+}Treg cells in the nulliparas could be explained by the different physiological characteristics that differentiate circulating immune cells in pregnant women who have had a previous delivery.

Conclusions

We demonstrated the existence of mPRα in the circulating Treg cell pool. More information regarding its existence in the maternal–fetal interface (decidua) is necessary.

Further investigation should determine the functionality of this receptor and the mechanisms by which Treg cells modulate fetal protection and labour. Membrane progesterone receptor α might emerge as an instrument by which progesterone regulates Treg cells, allowing a rational recommendation for progesterone usage in PTB.

Disclosure of interests

The authors report no conflict of interest

Contribution to authorship

ALA and PM were responsible for recruiting the pregnant women and for data collection, evaluation and manuscript preparation. The other authors (SV-P, VA, PR-S, AM-P) were responsible for flow cytometry analysis, data collection, evaluation and manuscript revision.

Details of ethics approval

This is an original clinical research approved by the Ethical Committees of Coimbra University (2011) and Coimbra University Hospital (2013) and informed consent was obtained from each participant.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. (a) FSC versus SSC dot plot: lymphocyte gate. (b) SSC-A versus PE CD4 dot plot: $CD4^{+}$ population. (c) PerCP-Cy5.5 CD127 versus PE-Cy7 dot plot: $CD4^{+}CD25^{high}CD127^{low}$ population. (d) APC FoxP3 histogram to determine FoxP3 expression in $CD4^{+}CD25^{high}CD127^{low}$ population.

Table S1. Subgroup analysis of different blood populations in T cells among pregnant women. ■

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