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TREGS POPULATION IMPAIRMENT IS ASSOCIATED WITH AN INCREASED RISK OF SPONTANEOUS ABORTION

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Abstract: The regulatory T-cells, both natural (nTregs) and inducible (iTregs) are engaged in the immune tolerance establishment. One of the most intriguing tolerance-depending physiological conditions is the pregnancy where the embryo and placenta are not recognised as foreign. Among the extensively studied mechanisms supposed to ensure the maternal tolerance, Tregs are the object of a particular interest, being still not well characterised. The aim of the present study is to analyse the populations of peripheral regulatory T-cells in women with pregnancy failure in comparison to healthy subjects. PBMCs from two subject groups – controls - women with history of normal pregnancy and patients with pregnancy failure were isolated and stained with anti-CD3, -CD4, -CD45RA, -CD25, -FOXP3 antibodies. Their expression was evaluated by multicolour flowcytometry.

Our results show that the peripheral Tregs profile in patients with pregnancy failure is impaired. In contrast to the controls, in the patient group the entire CD4+FOXP3+Treg population is significantly decreased. It consists mainly of CD45RA- iTregs, while CD45RA+ nTregs represent a minority. In healthy subjects, both fractions are equally rich in CD25+ cells. Inversely, in the patient group, CD45RA- iTregs, but not CD45RA+ nTregs are more populated with CD25+ cells.

The immune tolerance development during pregnancy is based on a complex cellular network. Therefore, the impaired peripheral Tregs pool could have a significant impact on the tolerance development in patients with pregnancy failure.

INTRODUCTION

T regulatory cells (Tregs) are an object of intensive studies due to their role in controlling the immune responses and the suppression of autoimmune reactions. Treg cells are classified into two groups according to their origin. Natural Treg cells (nTregs) originating from the thymus are involved in the prevention of autoimmune reactions. The induced Treg cells (iTregs) are generated in the secondary lymphoid organs from naïve CD4 T cells under the simultaneous exposure to foreign antigens and/or specific cytokine milieu and play a specialized role in controlling the strength of immune reactions (Nancy and Erlebacher, 2014). Therefore, Tregs are recognized as an important element for the development of immune tolerance.

As a specific physiological condition, pregnancy is tightly linked to the establishment of immune tolerance against the semi-allogeneic foetus (Clark DA, 1999; Sharma, 2014; Medawar, P.B. 1953). Several mechanisms are in line to ensure the maternal tolerance, including the expression of non-classical MHC molecules by trophoblast cells (Ishitani, A. et al. 2003,), tryptophan catabolism by the enzyme IDO (Munn, DH et al. 1998), T cell apoptosis (Hunt, JS et al. 1997), the complement system (Hsi, BL et al. 1991) and the inhibitory costimulatory molecule, and programmed death ligand (PDL) 1.

Recent achievements argue that regulatory T cells have an important role in this process (Aluvihare, VR, 2004; Zenclussen, AC et al. 2005; Schumacher and Zenclussen, 2014). They are thought to mediate their immunosuppressive effects, through secretion of cytokines like IL-10 and TGF-b, as well as by acting as a sink for IL-2 (Nancy and Erlebacher, 2014). Tregs recruited into the uterus before the embryo implantation, allow the intimate associations between maternal cells and the embryo, foetus and placenta, which are required for reproductive success. (Robertson and Moldenhauer, 2014).

In animal studies an increase in the entire Tregs pool has been noted in the lymph nodes draining the uterus from as early as two days after mating together with accumulation of FOXP3+ cells and Foxp3 mRNA expression in the uterus. It was demonstrated that these events are independent from the foetal alloantigen (Aluvihare al., 2004; Zhao et al., 2007). In the absence of Treg cells, allogeneic foetuses are uniformly rejected, while syngeneic foetuses survived (Aluvihare al., 2004). Zenclussen et al. (2005) pointed out that the transfer of Tregs from normal pregnant mice prevents abortion, while the transfer of Tregs from non-pregnant normal mice to the abortion prone mice was ineffective and the transfer of Tregs on/or after day 4 of pregnancy as well.

However the studies in humans are limited, some fertility complications are linked to a Treg cell deficiency. It was found that in the decidua and peripheral blood of women with repeated miscarriages CD4+CD25^{high} Tregs are fewer and have lower suppressive capacity (Sasaki et al., 2004; Winger and Reed, 2011).

Similar results were obtained in women with preeclampsia (Hsu et al., 2012, Quinn et al., 2011, Sasaki et al., 2007). While most studies are focused on the role of the entire population of T regulatory cells for a successful pregnancy, little is known about the specific contribution of its major fractions – natural and inducible.

In the present study, we have analysed regulatory T cells, both nTregs and iTregs, in women with reproductive failure and compare them with healthy subjects. We found that in patient group the percentage of FOXP3+ cells was significantly lower than in the control group. An increase in the percentage of CD25+ nTregs, but not of CD25+iTregs according to age in healthy controls is also registered. In the patient group the percentages of both fractions remained relatively stable, being less dependent on the subject age. Our results show an impaired Treg expression profile in patients with reproductive failure.

MATERIALS AND METHODS

Study participants: 8 women (21-44 years) with unsuccessful pregnancies and 8 women (24-58 years) with history of normal pregnancy were included (Table1). Subjects with autoimmune, chronic inflammatory and proliferative diseases were not included in the study. Samples were obtained with the patient's informed consent. Blood samples were provided by the CRBM "Reprobiomed", Sofia.

Isolation of PBMCs: PBMCs were isolated from peripheral blood by Ficoll gradient centrifugation. Heparinized blood, collected by venipuncture, was diluted with RPMI 1640 medium and overlaid on Ficoll-Paque as previously described (Boyum, 1968). PBMCs were then carefully picked up, washed twice with serum-free medium RPMI 1640 and collected for further examinations.

Flow cytometry: The isolated PBMCs were stained with the following antibodies: anti -CD3-PerCP (clone SP34-2, BD Biosciences) and anti-CD4-AlexaFluor700 (clone OKT4), anti-CD45RA-eFluor450 (clone HI100), anti-CD25-APC (clone BS96), anti-FOXP3-PE (clone PCH101) (eBiosciences, San Diego, CA, USA). A rat IgG2a K-PE was used as an isotype control. Cells were incubated for 30 min at 4°C for the extracellular labelling. After the washing step, they were permeabilized and incubated with the anti-FOXP3 antibody, according to the manufacturer's instructions (eBiosciences). To prevent the nonspecific binding, the Normal Rat Serum was applied. Fluorescent labelling was acquired on BD FACS LSRII (BD Biosciences) and data were analysed with FlowJo V9 software (TreeStar).

Statistical analysis: Mann-Whitney t-test was used to determine the significance of differences between controls and patients. The non-parametrical Spearman correlation was applied in order to analyse the correlations between different lymphocyte fractions in the respective groups. For all statistical tests,

p values <0.05 were considered statistically significant. The data were analysed with GraphPad Prism version 6.

Table 1. Subject's characteristic.

Subjects (n)	**Age Median (range)**	**Pregnancy**
Healthy Controls (n=8)	31 (24-58)	successful
Patients* (n=8)	30 (21-44)	failure

*All patients had at least 2 incidences of spontaneous pregnancy loss in the first trimester. In 6 of them ART was unsuccessful due to unknown reason. In 2 women, no ART was applied before entry in the study.

RESULTS

Analysis of FOXP3+CD4+T cells

T regulatory cells are object of intensive studies directed to determine their role in a broad area of human pathology. They are characterised by the expression of FOXP3 (Fontenot, 2003). In the present study, the population of T regulatory cells, based on the expression of FOXP3+CD4+ was investigated in light of their suspected impact on pregnancy failure (Figure 1).

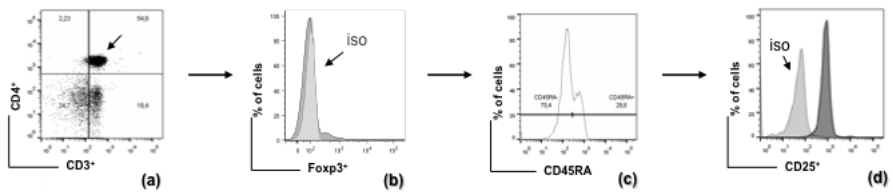

Figure 1: Flowcytometric evaluation of T regulatory cells. Isolated PBMCs were stained with a combination of extra- and intracellular antibodies directed to define them as Tregs. The gating strategy was based on the determination of CD4 T -cells as CD3+CD4+ (A); within CD4+ T-cells, the entire population of peripheral regulatory T-cells was defined as FOXP3+CD4+ (B). To specify natural and inducible Tregs, we examined the expression of CD45RA - CD45RA+ were considered as nTregs CD45RA-as iTregs (C). Both CD45RA+ and CD45RA- fractions were analysed for the expression of the classical Tregs molecule CD25 (D).

In healthy controls, a high individual variability in the percentage of FOXP3+CD4+ Tregs was observed. On the other hand, the patient group was homogeneous, but the values were found significantly lower (median value HC

= 5.94%, patients = 1.87%; range: 3.23-11.40 and 0.52 – 2.62, respectively, p=0.001). The fact that the percentage of CD4+T cells in both groups were almost identical (data not shown), an impairment of the FOXP3+ fraction might be suggested in women with reproductive failure (Figure 2A).

The peripheral Tregs pool consists of nTregs and iTregs. In the present study, both populations were evaluated according to the expression of CD45RA. The fraction of nTregs was considered as CD45RA+FOXP3+CD4+ T-cells and those of iTregs – as CD45RA-.

Both in controls and in patients, the portion of iTregs predominated, while nTregs were a minority (CD45RA+ Tregs median value HC=44.76%, patients=24.50%; range: 10.20-83.30 and 6.58 – 39.50, respectively, p>0.05; CD45RA-Tregs median value HC=77.10, patients=75.30; range: 25.40-91.30 and 60.50 – 93.40, respectively, p>0.05). In healthy subjects, no significant difference was detected between nTregs and iTregs, thus demonstrating that both fractions equally contribute to the arrangement of the peripheral Treg pool (p>0.05). On the contrary, in patients, the dichotomy between Tregs fractions was significant, indicating a leading role of iTregs (p=0.0002) (Figure 2B-D). In both patient and control groups, no correlation with age was found, neither for nTregs nor iTregs. In addition, nTregs and iTregs were found independent from CD45RA+ and CD45RA- CD4+ T-cells, suggesting that they are particular populations with a specific behaviour.

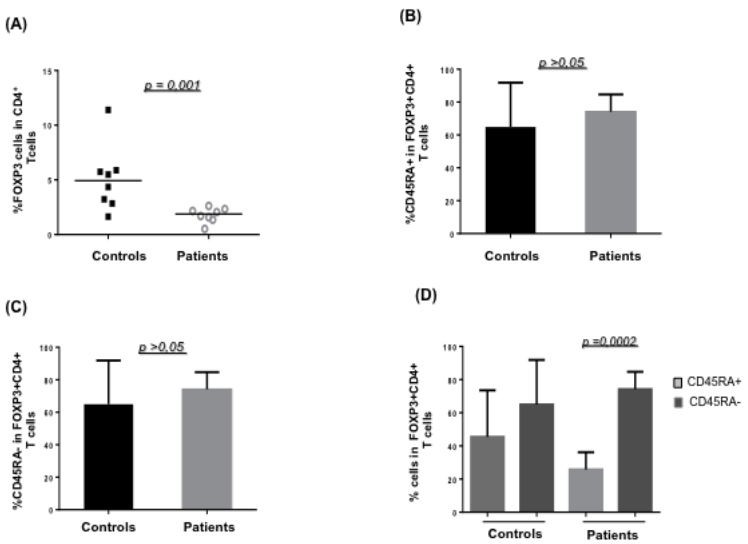

Figure 2: Analysis of the peripheral FOXP3+CD4+ T-cells. In patients with reproductive failure the percentage of FOXP3+CD4+ T-cells was lower as compared with the control group **(A)**; both in controls and patients it consisted of CD45RA+ **(B)** and CD45RA- **(C)** cells; in patients CD45RA- iTregs predominated, while in healthy subjects they equally participated in the formation of the peripheral Tregs pool **(D)**.

Analysis of CD25 expression

The regulatory T cells are recognised by the expression of the αIL-2R – CD25 even prior to the discovery of FOXP3 (Sakagushi 1995). Despite some controversies the expression of the CD25 molecule remains part of the fully developed, classical regulatory phenotype. In the present study, the expression of CD25 in both nTregs and iTregs was analysed. In the control group almost all of CD45RA+ and CD45RA-Treg cells were CD25+ (median value=77.65% and 69.45%; range: 37.2 – 98.9 and 16.70 – 93.30 respectively, p>0.05). That was not the case with patients, where CD45RA-Tregs, but not CD45RA+, were found more abundant in CD25+ cells (median value=52.00% and 91.15%; range: 12.00 - 75.00 and 49.60 – 95.10 for CD45RA+ and CD45RA- Tregs respectively, p=0.001), (Figure 3A-B). Then, we evaluated whether CD25+ cells might be associated with the subject age. Indeed, in controls but not in patients the CD25+nTregs were found to positively correlate with the subject age towards an increase (r=0.7, p=0.04) (Figure 3C).

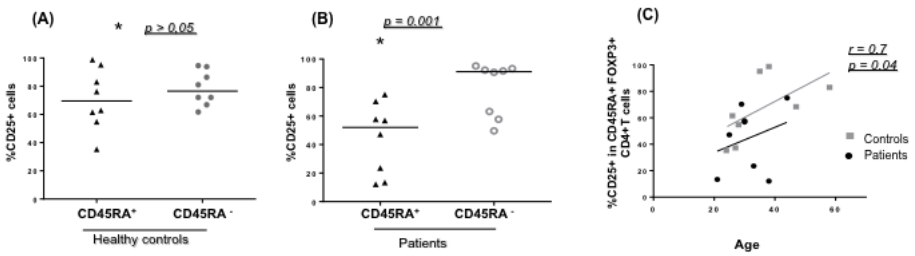

Figure 3: Differential expression of CD25 in CD45RA+ and CD45RA- Treg cells. While in controls both fractions had similar levels of CD25 expression with a slight prevalence in CD45RA+ (A), in the patient group CD45RA+Tregs were found to contain significantly lower percentage of CD25+ cells than CD45RA- did (B) and in comparison to the HC (*); in the control group the percentage of CD25+nTreg cells increased with age, while in patients no significant changes were found (C).

DISCUSSION

The regulatory T cells are of particular interest because of their proved and/or envisaged contribution to the pathogenesis of numerous medical conditions. Tregs per se, are committed to downmodulate the magnitude of immune reactions and prevent the self-antigen recognition. Consequently, deviations in the number and/or function of Tregs might be harmful to the establishment of the immune tolerance.

In our study we evaluated the peripheral regulatory T cells in women with reproductive failure. A significant decrease in the percentage of the entire Treg population described as FOXP3+CD4+ T-cells was found. Interestingly a high individual variability was observed in controls, but not in patients. Earlier, it was shown that the findings in the periphery reflect the cellular pattern in the decidua

and that in women with RPL Tregs are decreased (Sassaki, et.al. 2004, Munotz-Suano, 2011.). Therefore, one may suggest that shared immune deviation, found in the patient group, might result in an insufficient control towards foetal antigens.

The regulatory T cells in the periphery present a heterogeneous population. It consists mainly of nTregs and iTregs. However intensive the studies are, the role of each subpopulation remains unclear. To obtain a more precise picture regarding the contribution of each fraction, we evaluated both nTregs and iTregs. In controls, the percentage of CD45RA-Tregs slightly surpassed those of CD45+FOXP3+T-cells, indicating that either fraction has an equal contribution to the establishment of the entire peripheral Tregs pool. Contrarily in patients, a significant disproportion was determined with prevalence of iTregs. Although our results came from a small number of patients, the decrease in FOXP3+Treg population, more specifically nTregs in the periphery, could be associated with an impaired maternal tolerance. In another study, including 210 individuals undergoing assisted reproduction technologies (ART), the authors demonstrate that CD45RA+Tregs are of significant importance for the success of ART (Schlossberger, 2013). Regardless of some differences in the study design and the expression pattern, this study provides an indirect confirmation of our observations concerning the leading role of CD45RA+ Treg cells for the development of maternal tolerance.

From the early studies on Tregs, they have been characterised by the expression of the α-subunit of IL-2R-CD25. It is associated with IL-2, the principal surviving factor for Tregs. Although some authors assume the existence of other, non-conventional Treg subpopulations (Dimova, T., 2011; Terzieva V. 2014). Considering the pivotal role of CD25 for the development of mature FOXP3+CD4+ T-cells, we analysed its expression in both Tregs fractions. Unlike the control subjects, where both nTregs and iTregs were equally rich in CD25, patients' nTregs had fewer CD25+ cells than iTregs did. Also, our results showed that the patient CD25+ nTregs remain relatively stable and independent from the subject age. At the same time, in control subjects these cells were found to increase. Data from literature showed that the percentage of Tregs rises with aging in healthy individuals because of some particular features like down regulation of the pro-apoptotic Bim molecule expression, modified cytokine milieu, etc. (Chougnet, 2011). Consequently, the lack of correlation between Tregs and age, found in the patient group, indicate an impairment of the Treg population in women with reproductive failure. Another suggestion might be the effects of sex steroid hormones on the immune cells' development and function. It is evidenced not only by the *in vitro* studies (Mjosberg J. 2009), but also by epidemiologic data showing that autoimmune deviations are more frequent in women than in men and usually start early, at age 20-30 with the peak of the reproductive period.

In conclusion, our results demonstrate that the Treg population pattern in women with reproductive failure is significantly impaired. It might be due to

either a defect in Tregs development or hormonal influence on the Tregs' life cycle. Awareness of the dynamics of different Tregs populations during pregnancy will provide new knowledge about the mechanisms of pregnancy tolerance and will increase opportunities for new therapeutic interventions.

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