Received: 22 October 2018 | Revised: 16 January 2019 | Accepted: 3 February 2019

DOI: 10.1111/sji.12754

HUMAN IMMUNOLOGY

WILEY Scandinavian Journal of Immunology

Kinetics of Helios(+) and Helios(−) T regulatory cell subsets in the circulation of healthy pregnant women

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Funding information
This work was supported by Project SVV 244-260435 from Charles University, Prague and Projects LO1309 and LO1508 from the Ministry of Education, Youth and Sports of the Czech Republic and RVO VFN 64165/2012 from General University Hospital in Prague.

Abstract

Regulatory T cells (Tregs) play a critical role in the maintenance of a pregnancy. While the kinetics of the number of peripheral blood Tregs has been satisfactorily described in mouse models, analysis of these cell populations in human pregnancy is complicated by high variability in the quantity of Tregs and inconsistencies in the markers used for detecting different types of Treg. In the light of this, we set out to investigate the kinetics of various types of Treg, including CD45RA, GARP and PD-1(+) Tregs, in the peripheral blood of pregnant women in the first, second and third trimester, and at the time of delivery. Tregs, defined as a CD4(+)CD25(++)CD127(dim)Foxp3(+) population of leucocytes, were detected using flow cytometry. Natural thymus-derived Tregs and induced Tregs in the peripheral blood were distinguished by the expression or absence of a Helios marker, respectively. Our results showed that during normal pregnancy the sizes of various Treg subpopulations varied across women and also in an individual woman did not remain constant but varied significantly, most notable being the decrease observed at the time of delivery. Helios(−) cells were significantly less frequent in the peripheral blood of healthy pregnant women than Helios(+) cells, and the majority of Tregs were Helios(+)PD-1(+) Tregs.

1 | INTRODUCTION

Pregnancy is a biological process that requires continuous modulation of the mother's immune system, enabling the semi-allogeneic foetus to be carried. Throughout gravidity, the mother's immune cells must maintain a state of immunological tolerance, while at the same time the capacity to protect the host from pathogens and to control any resulting inflammation must not be compromised. A balance between immune tolerance and immune activation is thus required.

Several mechanisms have been proposed to be responsible for the host's tolerance of the foetus, including a network of various immune cell populations and molecules. Among them, $CD4^+CD25^+Foxp3^+$ T regulatory cells (Tregs) play an essential role. The importance of the Treg population for successful pregnancy has been confirmed in human as well as animal models. For example, the level of both local and systemic $CD4^+CD25^+$ Tregs has been observed to be lower in spontaneous abortion cases compared with normally

developing pregnancies.[1] Furthermore, Jasper et al[2] detected a significantly reduced expression of Foxp3 in the endometrial tissue of infertile women.

In mouse models, the number of peripheral blood Tregs increases during pregnancy, reaching a peak at midgestation, and subsequently decreases to almost background levels in the final stages, reaching non-pregnant levels at term or shortly thereafter.[3–5] The number of Tregs in the peripheral blood of pregnant women has been reported to be similar to that observed in animal models.[4,6] However, methodological inconsistencies—in particular in the use of markers for the detection of Treg populations—and the high variability in Treg numbers even in non-pregnant women complicate the analysis of Treg kinetics during human pregnancy.[7] Some studies have even reported insignificant changes in the frequency of circulatory Tregs during pregnancy.[6,8,9]

Neither the origin of Tregs during pregnancy nor the precise mechanism of their generation is yet clear. Discriminations can be made between naturally occurring thymic-derived Tregs (nTregs) and inducible Tregs (iTregs) generated in the periphery by the expression of Helios, a member of the Ikaros transcription factor family.[10] Samstein et al[11] reported the key role of iTregs for maternal-foetal tolerance in placental mammals, and others have stressed the importance of nTregs.[12,13] It is probable that both iTregs and nTregs play an important role in a successful pregnancy,[14,15] although how exactly they do remains to be determined. Furthermore, both iTregs and nTregs can be defined by the expression of CD45RA, which is present only in naïve Tregs and not in effector Tregs.[16]

The induction of $CD25^+Foxp3^+$ cells and development of the characteristic properties of Tregs require the transforming factor beta (TGF-β).[17] TGF-β is secreted by the latent form in which a mature TGF-β protein is bound to the latency-associated peptide. Activated Tregs, but not other $CD4^+$ clones, express glycoprotein A repetitions predominant (GARP), a transmembrane protein containing leucine-rich repeats. Binding TGF-β to GARP may be necessary for Tregs to be able to activate TGF-β upon TCR stimulation.[18] GARP expression is thus correlated with the regulatory activity of Tregs.[19]

As part of our investigation into the kinetics of circulating Tregs during pregnancy, the aim of this study was to determine how the distinct populations of naïve Tregs ($CD45RA^+$) and of $GARP^+$ Tregs and the level of TGF-β varied in the serum of pregnant women, by monitoring these quantities at three points in time during pregnancy and then during labour. We also monitored programmed cell death 1 (PD-1) positive Tregs, as the PD-1/PD-L1 pathway regulates maintenance of foetus tolerance.[20]

For this study, a cohort of 22 women over the course of their pregnancy donated blood in the first (6-12 weeks), second (20-25 weeks) and third (30-35 weeks) trimesters, and at the time of giving birth. Using FACS analysis, we determined the expression of markers defining various degrees of Treg maturation, activation and inhibition, and determined the quantity of TGF-β, a cytokine polarizing Treg cell induction in the serum of healthy pregnant women.

2 | MATERIALS AND METHODS

2.1 | Patients

The study was approved by the Ethics Committee of the First Faculty of Medicine, Charles University, Prague. All women included in the cohort read, dated and signed an Informed Consent Form and were fully apprised of the nature, significance and implications of the study. Women with an untreated allergy, autoimmune disease or cancer were excluded. The cohort consisted of 22 healthy pregnant women between 26 and 38 years old (mean age 31 years). Pregnancies were physiological, without complications, and no developmental defect or intrauterine growth restriction was diagnosed. All deliveries were without complications, and each baby was born alive.

Peripheral blood samples were to be collected from each donor at four time points: at 8-12 weeks of gestation, at 20-25 weeks of gestation, at 30-35 weeks of gestation and at the time of delivery. From our cohort, 10 women donated blood at all four time points, 8 at three time points and the remaining 4 at two time points.

2.2 | Flow cytometry

Peripheral blood samples (3-mL tubes; Vacutainer®Becton Dickinson, San José, CA, USA) were collected in tubes containing ethylenediaminetetraacetic acid (EDTA) and were analysed within 4 hours. The following monoclonal antibodies (mAbs) were used in this study: anti-CD45 (Krome Orange; Beckman Coulter, Brea, CA, USA; clone B61840AA), anti-CD4 (Alexa Fluor 700; eBioscience, San Diego, CA, USA; clone OKT4), anti-CD25 (PE; Beckman Coulter; clone B1.49.9), anti-CD127 (PE-Cy7; Beckman Coulter; clone B61544), anti-PD-1 (APC-eFluor 780; eBioscience; clone eBioJ105), anti-CD45RA (FITC; Beckman Coulter; clone ALB11) and anti-GARP (PE-eFluor 610; eBioscience; clone 614D9) as cell surface markers; and anti-Foxp3 (eFluor660; eBioscience; clone PCH101) and anti-Helios (eFluor 450; eBioscience; clone 22F6) as intracellular markers. 100 μL of the obtained peripheral blood sample was used for analysis. Leucocytes were first stained with mAbs cell surface markers for 30 minutes on ice. Then red blood cells were lysed by a two-step protocol. The cells were first incubated with lysing solution

EXCELLYSE I (Exbio, Vestec, Czech Republic) for 5 minutes, after which deionized water was added for 5 minutes. After washing with PBS, the cells were fixed and permeabilized by incubation for 30 minutes with a fixation/permeabilization buffer (eBioscience), and were then stained with anti-Foxp3 and anti-Helios mAbs. Data were collected on a Navios cytometer, and the results were analysed using Kaluza 5.1 software (both Beckman Coulter). Lymphocytes were gated based on both forward and side scatter parameters. Only singlet cells were used for the analysis. After gating on CD45 and CD4, a proportion of Tregs were identified as $CD25^{++}CD127^{dim}Foxp3^{+}$ cells. The presence or absence of Helios was used to detect the population of nTregs and iTregs, respectively. $Helios^{+}$ and $Helios^{-}$ populations were further discriminated by positivity to CD45RA, PD-1 and GARP. The data were expressed

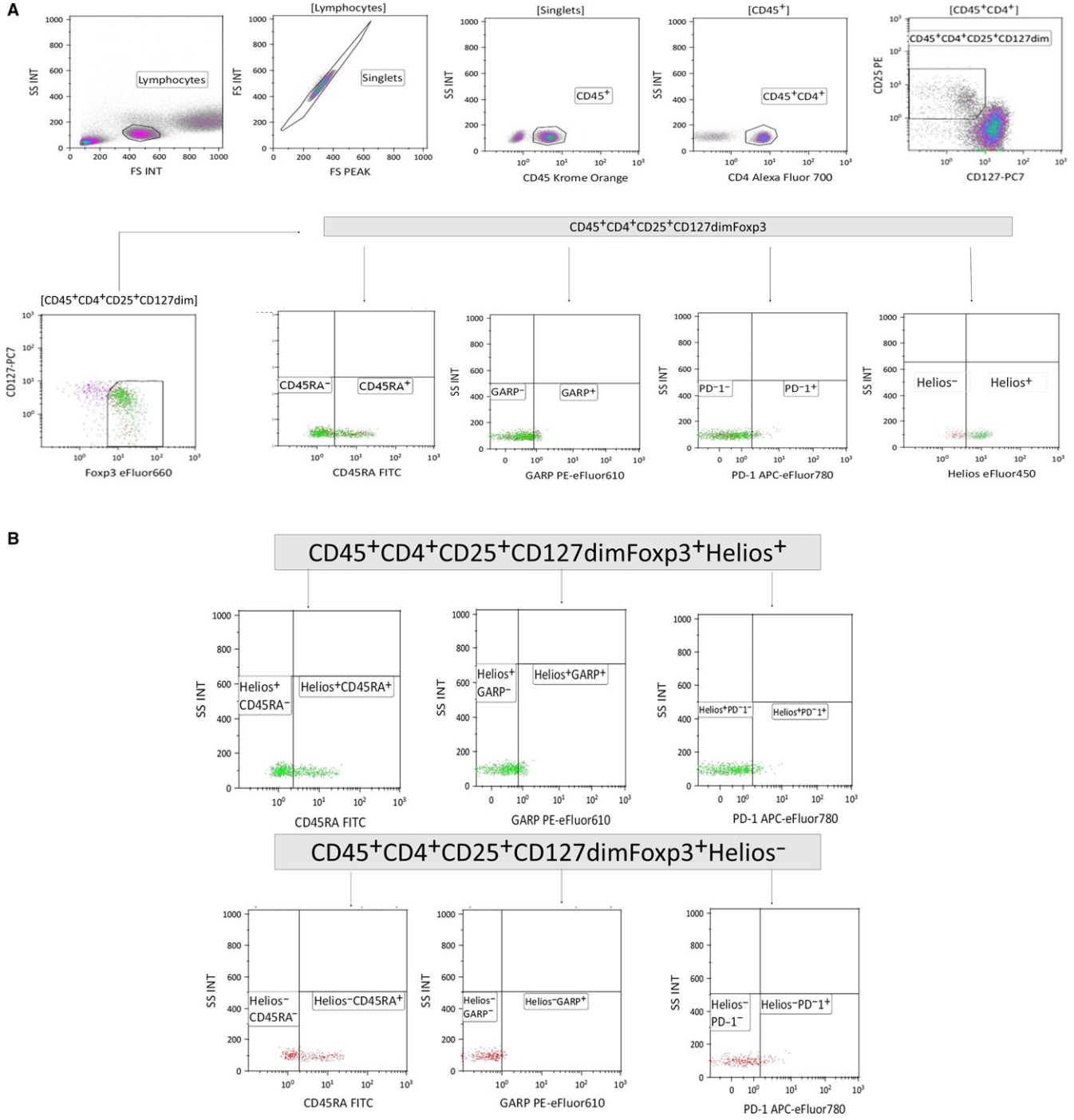

FIGURE 1 Gating strategy for the identification of Tregs. Lymphocytes (30 000 events) were gated on FSC/SSC, and after selecting singlets, Tregs were gated as $CD45^{+}CD4^{+}CD25^{+}CD127dimFoxP3^{+}$ Tregs. CD45RA, GARP, PD-1 and Helios-positive and Helios-negative populations were detected from this population (Figure 1A) or from $CD45^{+}CD4^{+}CD25^{+}CD127dimFoxP3^{+}Helios^{+}/Helios^{-}$ cells (Figure 1B)

as absolute values (ie 10^6/L). Representative flow cytometry dot plots illustrating the gating strategy for detection and enumeration of subsets are shown in Figure 1.

2.3 | ELISA

Whole blood was collected from the cubital vein into Vacutainer© tubes, and serum from each sample was prepared by centrifugation 15 minutes by 1000 *g* and was stored at −80°C until use. A human TGF-β1 Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA) was used to quantify the amount of TGF-β1. Acid activation of serum was performed according to the manufacturer's instructions. The activated samples were diluted before measurement; 10 μL of serum was mixed with 190 μL Calibrator Diluent RD5-53. Quantities were estimated based on a standard curve generated with recombinant TGF-β1.

2.4 | Statistical analysis

The Prism 5 program (GraphPad Software, San Diego, CA, USA) was used for statistical analysis, and the data were analysed using the Kruskal-Wallis test with Dunn's multiple comparison post-test. All results are expressed as median values. A value of $P < 0.05$ was considered statistically significant.

3 | RESULTS

3.1 | Concentration of TGF-β in serum and number of Foxp3$^+$ cells and Helios$^+$ or Helios$^-$ cells in peripheral blood

As Tregs are differentiated under the influence of TGF-β, we measured the amount of this cytokine in serum prepared from

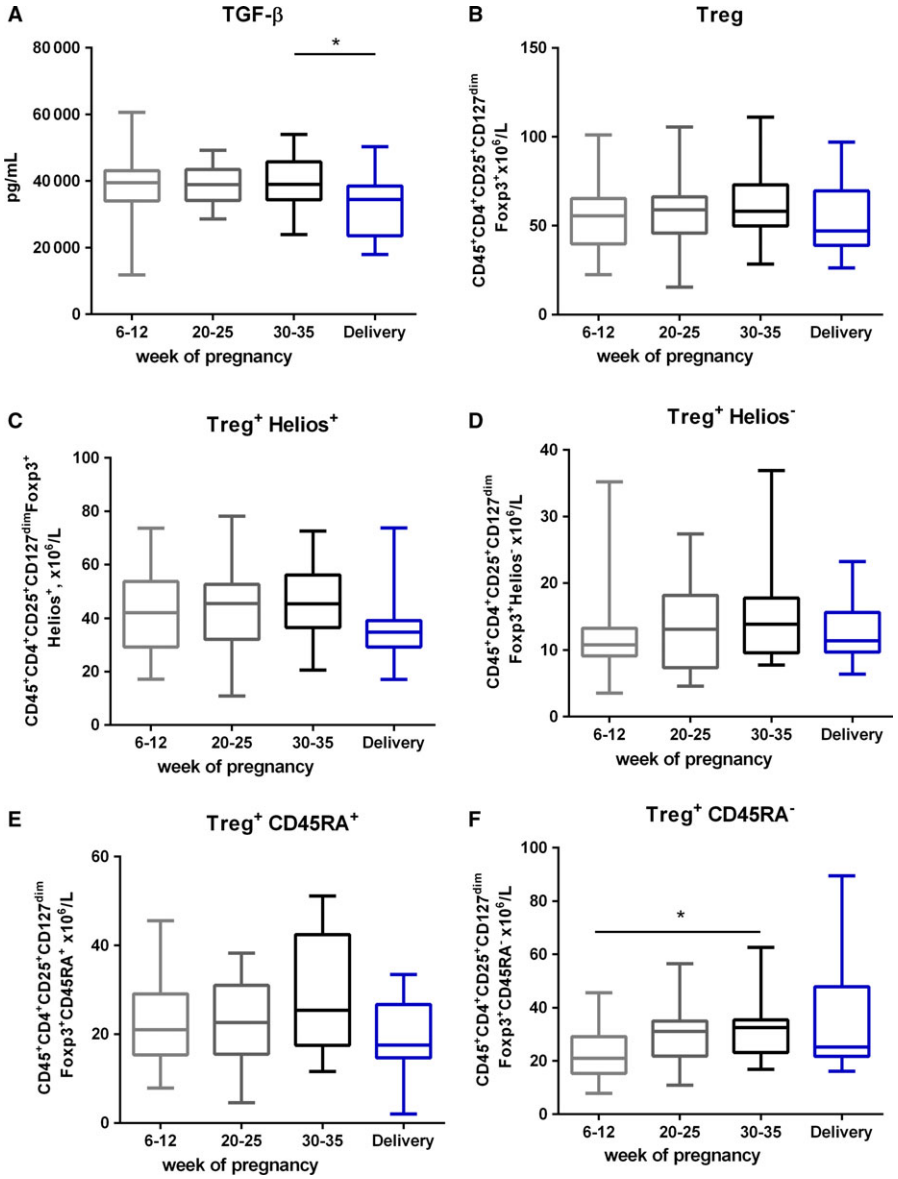

FIGURE 2 Box plots representing changes in TGF-β production measured by ELISA and number of Helios$^+$/Helios$^-$ Tregs detected by flow cytometry during pregnancy. TGF-β production (A), absolute counts of Treg cells (B), Helios$^+$ (C) Helios$^-$ (D), CD45RA$^+$ (E) and CD45RA$^-$ (F) Treg subsets in peripheral blood samples from healthy women in the first (6-12 wk), second (20-25 wk) and third (30-35 wk) trimester of pregnancy, and at the time of delivery. Horizontal line inside box represents median, bottom whiskers represent minimum value, and top whiskers represent maximum value. *$P < 0.05$

the blood of women from our cohort at each of the selected time points. As shown in Figure 2A, the concentration of TGF-β showed a significant decrease during the time of delivery. This finding correlates with the kinetics of the number of Tregs defined as the $CD4^+CD25^{++}CD127^{dim}Foxp3^+$ population of leucocytes (Figures 2B and 3B).

We measured the quantity of $Helios^+$ and $Helios^-$ a marker suggested to determine the proportion of nTregs and iTregs, respectively, during pregnancy. The median number of Foxp3 $Helios^+$ cells was greater than that of Foxp3 $Helios^-$ cells over the course of the whole pregnancy (42.13 vs 10.79×10^6/L in the first trimester; 45.41 vs 13.12×10^6/L in the second trimester; 45.37 vs 13.89×10^6/L in the third trimester and 35.27 vs 11.39×10^6/L at the term of pregnancy) (Figure 2C,D), but the kinetics of cell numbers was similar in both populations. A slight increase in the second and the third trimester was not significant while there was a significant decrease in $Helios^+$ cells at the time of delivery.

3.2 | Kinetics of the production of TGF-β and the number of $Foxp3^+$ cells and $Helios^+$ or $Helios^-$ cells in the peripheral blood of individual donors

We further analysed the kinetics of TGF-β production during pregnancy. The kinetics of TGF-β in the serum differs from donor to donor, and also concentrations of this cytokine detected in the serum vary in an individual donor over time. Examples of typical curves are shown in (Figure 3A). As shown in Figure 3A,B, the individual lines representing kinetics of TGF-β in the serum were not correlated with the number of Tregs in the peripheral blood of an individual donor. Also, no correlation was found between the kinetics of $Helios^+$ and $Helios^-$ Tregs (Figure 3C,D). The quantities of positive or negative CD45RA, PD-1 and GARP cells did not display any distinctive kinetic pattern during pregnancy, although the median number of $CD45RA^+$, $CD45RA^-$, $PD\text{-}1^+$, $PD\text{-}1^-$ and $GARP^-$ Tregs decreased at the time of delivery, while the mean number of $CD45RA^+$ Tregs increased in the third trimester and the mean number of $CD45RA^-$ Tregs increased in the second and the third trimester (Figures 2E,F).

3.3 | Comparison of $Helios^+$ and $Helios^-$ cell populations

The size of Helios(+)/Helios(−) populations revealed by positive expression of CD45RA marker was similar to that revealed by negative expression of the same marker. With the exception of $Helios^-CD45RA^-$, these cell populations tended to decrease in size at the time of delivery (Figures 4A,B and 5A,B).

Most $Helios^-$ cells were $PD\text{-}1^-$, whereas $Helios^+$ cells were 10 times more frequently $PD\text{-}1^+$ at each observed time point (Table 1, Figures 4C and 5C). A decrease in the entire $Helios^+/Helios^-$ $PD\text{-}1^+$ population was also detected at the time of delivery. The highest quantity of $Helios^-PD\text{-}1^+$ was in the first trimester, with decreasing observations at subsequent time points (Table 1, Figure 4D), whereas the level of $Helios^+PD\text{-}1^+$ remained steady during

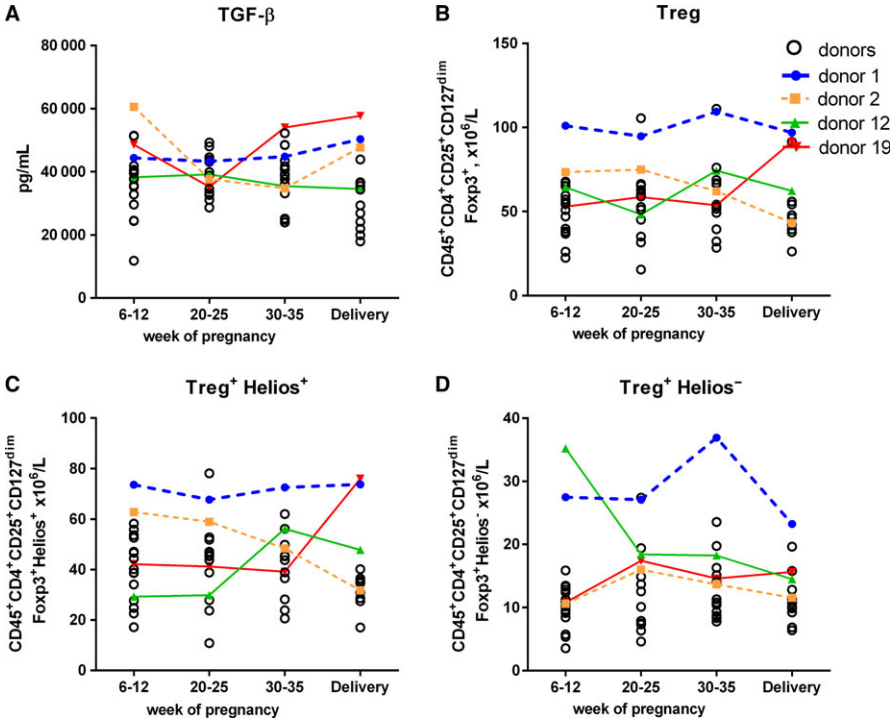

FIGURE 3 Examples of kinetics of TGF-β production and number of cells in Treg subsets during pregnancy of four individual donors. Kinetics of TGF-β production (A), absolute counts of Tregs (B), $Helios^+$ (C) and $Helios^-$ (D) Treg subsets by individual blood donors (donor 1 ●, 2 ■, 12 ▲ and 19▼) in first (6-12 wk), second (20-25 wk) and third (30-35 wk) trimester of pregnancy, and at the time of delivery

TABLE 1 Absolute counts (10^6/L) of Helios$^+$/Helios$^-$Tregs in peripheral blood of women in first (6-12 wk), second (20-25 wk) and third (30-35 wk) trimester of pregnancy, and at the time of delivery. Statistical analysis showed differences between first trimester and time of delivery ($P = 0.016$) in Helios$^-$PD-1$^+$Tregs; first trimester and time of delivery ($P = 0.021$) and second trimester and time of delivery ($P = 0.020$) in Helios$^+$PD-1$^-$ Tregs; third trimester and time of delivery ($P = 0.017$) in Helios$^+$PD-1$^+$Tregs

	Helios$^-$		Helios$^+$	
	PD-1$^-$	PD-1$^+$	PD-1$^-$	PD-1$^+$
6-12 wk	9.37	1.27	5.37	38.19
20-25 wk	12.12	1.20	5.94	37.85
30-35 wk	12.36	0.90	4.13	40.96
Delivery	10.44	0.68	3.38	32.31

pregnancy, with a decrease only observed at the time of delivery (Table 1, Figure 5D).

Populations positive for the GARP marker consist of a relatively small quantity of cells (1-3 × 10^6/L) and did not display any tendency to change during pregnancy or to decrease at the time of delivery (Figures 4E,F and 5E,F). The median of both Helios$^+$/Helios$^-$ populations negative for GARP was larger than for those positive for GARP (Helios$^+$GARP$^-$ approximately 40 times larger and Helios$^-$GARP$^-$ approximately 10 times larger), but these populations also did not show any significant change during pregnancy.

4 | DISCUSSION

Many studies suggest that Treg subsets play a significant role in maintaining a pregnancy and that human labour may be

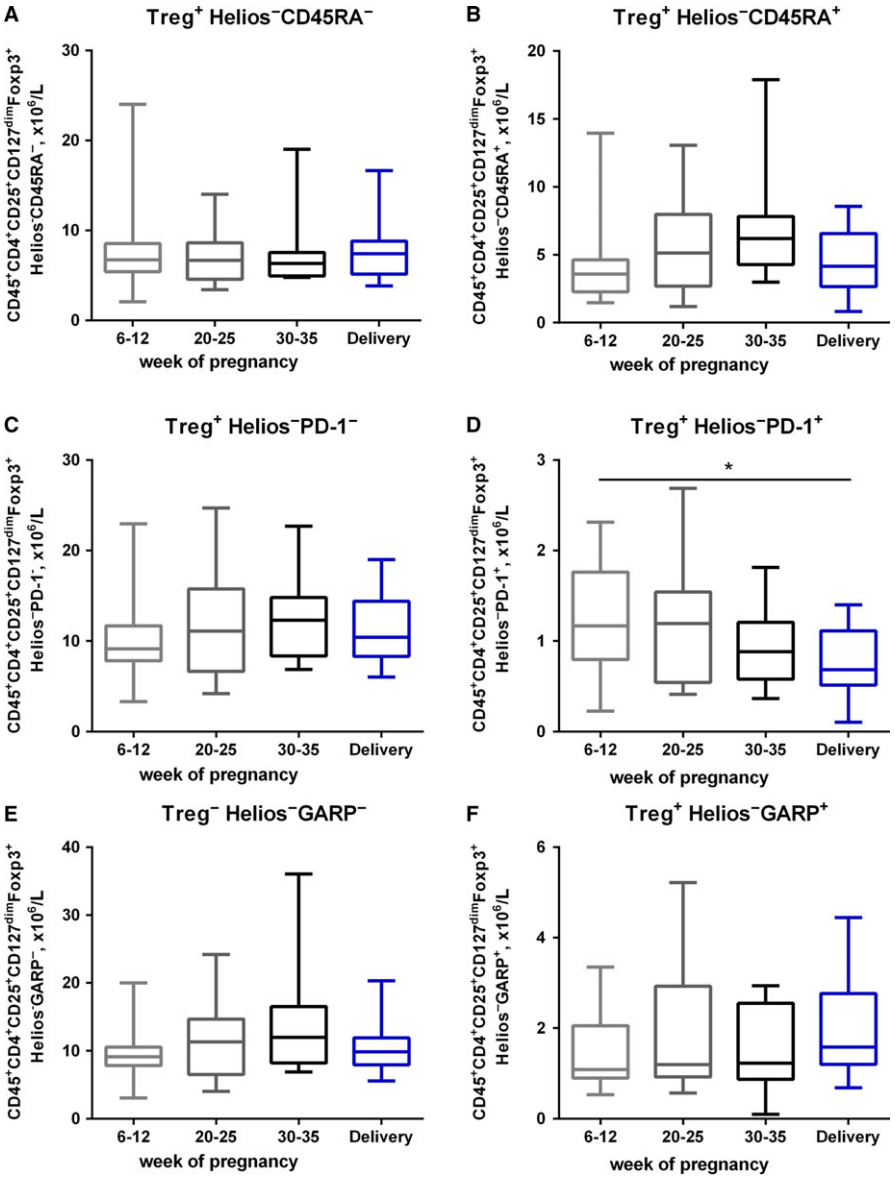

FIGURE 4 Box plots representing changes in number of cells in Helios$^-$ Treg subsets during pregnancy, detected by flow cytometry. Absolute counts of Helios$^-$ Treg cells negative for CD45RA (A), PD-1 (C) GARP (E) and positive for CD45RA (B), PD-1 (D) and GARP (F) subsets in peripheral blood samples from healthy women in first (6-12 wk), second (20-25 wk) and third (30-35 wk) trimester of pregnancy, and at the time of delivery. Horizontal line inside box represents median, bottom whiskers represent minimum value, and top whiskers represent maximum value. *$P < 0.05$

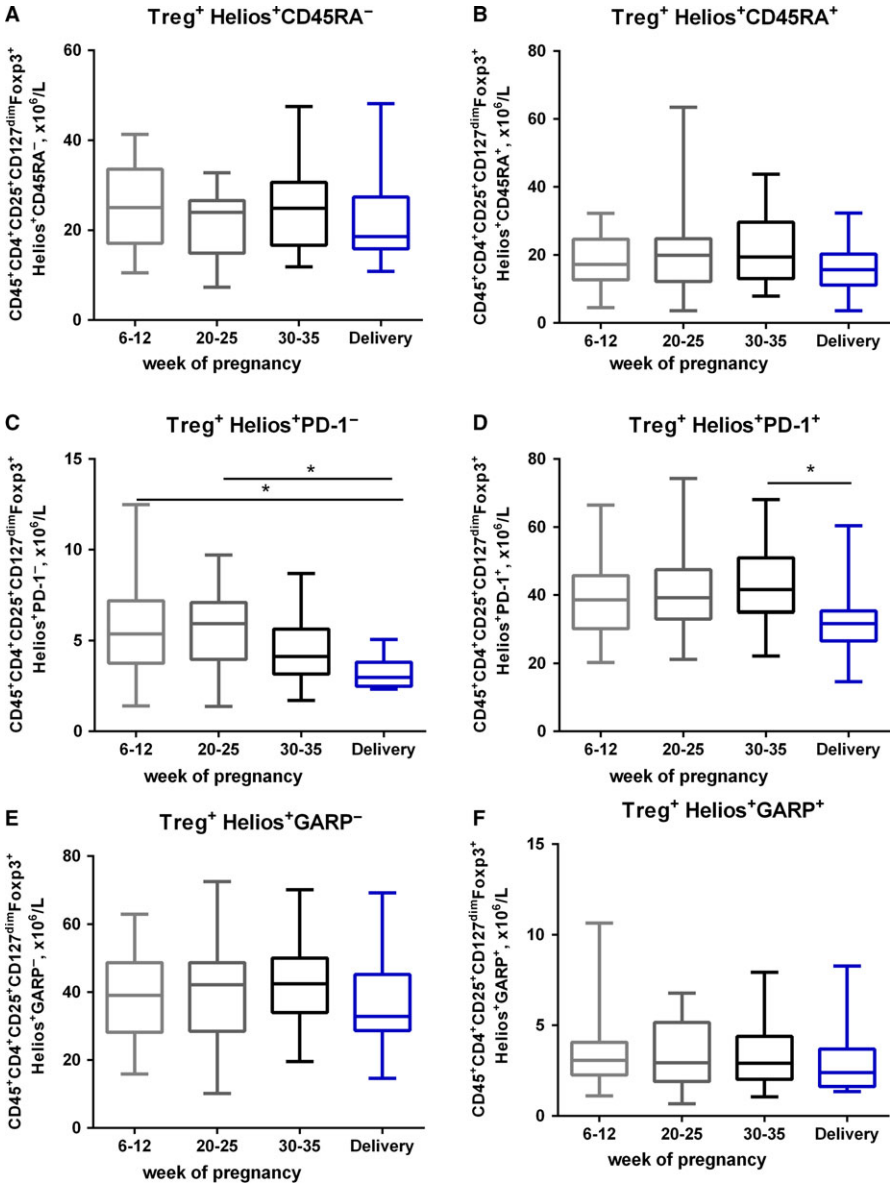

FIGURE 5 Box plots representing changes in number of cells in Helios$^+$Treg subsets during pregnancy, detected by flow cytometry. Absolute counts of Helios$^+$Tregs negative for CD45RA (A), PD-1 (C) GARP (E) and positive for CD45RA (B), PD-1 (D) and GARP (F) subsets in peripheral blood samples from healthy women in first (6-12 wk), second (20-25 wk) and third (30-35 wk) trimester of pregnancy, and at the time of delivery. Horizontal line inside box represents median, bottom whiskers represent minimum value, and top whiskers represent maximum value. *$P < 0.05$

initiated by a decrease in the population of Tregs. However, data on the presence of maternal Tregs in peripheral blood during normal pregnancy are highly variable.[21–24] Furthermore, distinct subsets of Tregs can influence whether the course of a pregnancy is normal or pathological. It is not clear how exactly individual Treg populations are involved in the induction of immune tolerance, or what their potential role in pathogenesis might be during pregnancy.[6,8,13,25] In the present study, we investigated changes in various distinct Treg populations in healthy pregnant women. A cohort of pregnant women from the first trimester to the delivery was followed. Various markers of maturation, activation and functional properties of Tregs were used to characterize Treg subsets, and Tregs were defined as CD4$^+$ CD25$^+$CD127^{dim}Foxp3$^+$ cells.

A slight decrease in the total number of circulating Tregs on the day of delivery was detected. This finding is consistent with several reports.[23,24,26] However, the increase reported by some studies in the second trimester was not confirmed.[21,23] Furthermore, the quantity of Tregs in an individual woman does not remain constant but varies during the course of pregnancy, as shown in Figure 2.

We confirmed a correlation between the level of TGF-β and the number of Tregs, which supports the importance of TGF-β for the generation and/or action of Tregs during pregnancy[27,28] and suggests the importance of TGF-β as a predictive marker for pregnancy complication.

Helios$^+$ Tregs were 4 times more numerous than Helios$^-$ Tregs, suggesting that most, but not all, Treg cells might be of thymic origin, which is consistent with results obtained in a mouse model.[29] In vitro studies have shown that Helios$^+$ and Helios$^-$ populations of Tregs differ in their suppressive potential associated with the expression of surface molecules and production of cytokines.[30,31] We therefore tested these populations expressing various markers associated with their status and suppressive function.

The majority of naïve CD45$^+$ Tregs were Helios$^+$. However, according to Himmel et al[32] a lack of Helios expression does not exclusively identify human iTregs, so that Helios$^-$CD45RA$^+$ cells may also be thymic-derived Tregs. Based on the findings of several authors that the number of naïve CD45RA$^+$ Tregs is increased in the periphery of healthy pregnant women, while being significantly reduced in number in woman suffering from a pregnancy complication, it is reasonable to suggest a key role of this population for tolerance during pregnancy.[12,33,34] The total number of CD45RA$^+$ Tregs is highest in the third trimester of pregnancy, with a significant decrease occurring at the time of delivery (Figure 2E), and both Helios$^+$ and Helios$^-$ CD45RA$^+$ Tregs displayed similar kinetics.

GARP is a surface marker that maintains and increases the regulatory function of activated Tregs. It has been also shown that this molecule is involved in TGF-β activation by Tregs.[35] However, in our study the majority of Tregs were negative for GARP and did not show changes in their kinetics during normal pregnancy.

The suppressive capacity of Treg cells is increased by the interaction between PD-1 and its ligand PD-L1.[36] Taglauer et al[37] described the significant increase in PD-1 expression on Tregs in human decidua in comparison with the secretory phase endometrium. Our results show that the cell population most positive for PD-1 is the Treg subset Helios$^+$. This finding supports the importance of nTregs for the successful course of a pregnancy, as suggested by various authors.[9,12,13] However, some authors have stressed the crucial role of iTregs generated in the periphery after encountering paternal antigens.[38] Since our data indicated a significant increase in the number of Helios$^+$ cells during the later phases of pregnancy, it might be speculated that iTregs participate in maintaining the tolerance state initiated by nTregs.

In conclusion, we have presented new data on the kinetics of distinct subpopulations of Helios$^+$/Helios$^-$ Tregs showing that the sizes of these populations not only varied across individual donors but also varied in the same donor over the successive phases of pregnancy. The majority of Tregs during normal pregnancy were positive for markers Helios and PD-1, and the size of this population significantly decreased at the time of delivery.

CONFLICT OF INTEREST

None.

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How to cite this article: Kopřivová H, Hájková M, Koucký M, Malíčková K, Holáň V, Krulová M. Kinetics of Helios(+) and Helios(−) T regulatory cell subsets in the circulation of healthy pregnant women. *Scand J Immunol*. 2019;89:e12754. https://doi.org/10.1111/sji.12754