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Differentiation of ICOS$^+$ and ICOS$^-$ recent thymic emigrant regulatory T cells (RTE T_{regs}) during normal pregnancy, pre-eclampsia and HELLP syndrome

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Summary

Two different subsets of naturally occurring regulatory T cells (nT_{regs}), defined by their expression of the inducible co-stimulatory (ICOS) molecule, are produced by the human thymus. To examine the differentiation of ICOS$^+$ and ICOS$^-$CD45RA$^+$CD31$^+$ recent thymic emigrant (RTE) T_{regs} during normal pregnancy and in the presence of pre-eclampsia or haemolysis elevated liver enzymes low platelet (HELLP)-syndrome, we used six-colour flow cytometric analysis to determine the changes in the composition of the ICOS$^+$ and ICOS$^-$ T_{reg} pools with CD45RA$^+$CD31$^+$ RTE T_{regs}, CD45RA$^+$CD31$^-$ mature naive (MN) T_{regs}, CD45RA$^-$CD31$^+$ and CD45RA$^-$CD31$^-$ memory T_{regs}. With the beginning of pregnancy until term, we observed a strong differentiation of both ICOS$^+$ and ICOS$^-$CD45RA$^+$CD31$^+$ RTE, but not CD45RA$^+$CD31$^-$ MN T_{regs}, into CD45RA$^-$CD31$^-$ memory T_{regs}. At the end of pregnancy, the onset of spontaneous term labour was associated with a significant breakdown of ICOS$^+$CD45RA$^-$CD31$^-$ memory T_{regs}. However, in the presence of pre-eclampsia, there was a significantly increased differentiation of ICOS$^+$ and ICOS$^-$CD45RA$^+$CD31$^+$ RTE T_{regs} into CD45RA$^-$CD31$^+$ memory T_{regs}, wherein the lacking differentiation into CD45RA$^-$CD31$^-$ memory T_{regs} was partially replaced by the increased differentiation of ICOS$^+$ and ICOS$^-$CD45RA$^+$CD31$^-$ MN T_{regs} into CD45RA$^-$CD31$^-$ memory T_{regs}. In patients with HELLP syndrome, this alternatively increased differentiation of CD45RA$^-$CD31$^-$ MN T_{regs} seemed to be exaggerated, and presumably restored the suppressive activity of magnetically isolated ICOS$^+$ and ICOS$^-$ T_{regs}, which were shown to be significantly less suppressive in pre-eclampsia patients, but not in HELLP syndrome patients. Hence, our findings propose that the regular differentiation of both ICOS$^+$ and ICOS$^-$CD45RA$^+$CD31$^+$ RTE T_{regs} ensures a healthy pregnancy course, while their disturbed differentiation is associated with the occurrence of pre-eclampsia and HELLP syndrome.

Keywords: HELLP syndrome, ICOS$^+$ and ICOS$^-$ T_{regs}, immune suppression, pre-eclampsia, pregnancy

Introduction

In mammalian organisms, the maternal immune system must tolerate the semi-allogeneic fetus during pregnancy. Numerous murine and human studies have shown that immunosuppressive regulatory T cells (T_{regs}) have an important role in protecting the fetus from immune-mediated rejection [1–3]. Interference with respect to their number, differentiation and suppressive function was shown to be responsible for certain pathological conditions, such as infertility [4,5] and the occurrence of recurrent spontaneous abortions [6–9]. Meanwhile, aberrant T_{reg} cell homeostasis was also linked to the pathophysiology of most human gestation-associated diseases that occur in the third trimester and manifest as preterm labour [10–12], pre-eclampsia [13–16] and gestational diabetes [17].

Currently, two different T_{reg} populations are described in the literature. The naturally occurring T_{regs} (nT_{regs}) are induced in the thymus and were shown to suppress immune

responses to self- and alloantigens [18]. The peripherally induced T_{regs} (iT_{regs}) are generated by conversion of conventional naive T cells under certain tolerogenic conditions and have the capacity to suppress immune responses towards foreign antigens [19,20]. As it is currently not possible to distinguish between nT_{regs} and iT_{regs} phenotypically, it is extremely difficult to recognize the contribution of each T_{reg} population to tolerance induction during pregnancy and to understand their potential roles for the pathogenesis of the different gestation-associated diseases. However, functional analysis of different T_{reg} subsets, which differs with respect to their degree of differentiation, shows that naive $CD45RA^+$ T_{regs} have lower suppressive activity than non-activated human leucocyte antigen D-related (HLA-DR)$^-CD45RA^-$ memory T_{regs} or activated HLA-$DR^+CD45RA^-$ memory T_{regs} [21]. Astonishingly, this relation was found to be reversed in healthy pregnant women, whose naive T_{reg} subset had very high suppressive activity, while the HLA-DR^+ memory T_{regs} were less suppressive [5]. These findings suggest that the increase of the suppressive activity of the naive $CD45RA^+$ T_{reg} population may represent a key event in tolerance induction during pregnancy. This assumption is strengthened further by the fact that naive $CD45RA^+$ T_{regs} were shown to be expanded in the periphery during the normal pregnancy course, but were found to be reduced significantly in the periphery of pregnant women suffering from most common pregnancy complications (preterm labour, pre-eclampsia, gestational diabetes) [11,12,17]. As naive $CD45RA^+$ T_{regs} always represent nT_{regs}, it seems that nT_{regs} are crucial for a successful pregnancy course.

Recently, two different nT_{reg} populations were detected in the human thymus, which differ concerning their expression of the inducible co-stimulatory (ICOS) molecule. Their induction by dendritic cells, proliferation and survival were shown to be regulated differentially. It was confirmed that the murine as well as the human T_{reg} pool consists of a predominant $ICOS^-$ T_{reg} subset, which is much more sensitive to apoptosis, and a minor highly proliferative $ICOS^+$ T_{reg} subset which is resistant to activation-induced cell death [22,23].

Like conventional naive $CD45RA^+$ T cells, both $ICOS^+$ and $ICOS^-$ T_{regs} are released from the thymus as $CD31^+$ recent thymic emigrants (RTE T_{regs}) and develop in a way comparable to conventional RTE T cells. It is known that RTE T cells undergo peripheral post-thymic proliferation to form a long-living $CD31^-$ mature naive (MN) T cell population, which has the capacity to maintain the naive T cell pool in elderly people. Thereby, both RTE and MN T cells can differentiate into memory T cells after stimulation with appropriate antigens [24]. Recently we showed that the normal differentiation of RTE T_{regs} is changed with the onset of pregnancy. We found that RTE, but not MN T_{regs}, differentiated increasingly into memory T_{regs} and that these emerging memory T_{regs} were maintained during the whole pregnancy course, but disappeared with the onset of spontaneous term labour [25]. In the presence of pre-eclampsia, this differentiation seemed to be impaired, but replaced partly by the increased differentiation of MN T_{regs} into memory T_{regs}. Obviously, this phenomenon leads to diminished suppressive activity of the total T_{reg} pool, which is reduced significantly in pre-eclampsia patients [11]. Currently, it is neither known whether the differentiation of $ICOS^+$ and $ICOS^-CD45RA^+CD31^+$ RTE T_{regs} is regulated differentially during pregnancy, nor whether aberrant differentiation of $ICOS^+$ or $ICOS^-CD45RA^+CD31^+$ RTE T_{regs} is involved in the pathogenesis of pre-eclampsia. Therefore, we examined the thymic output and the differentiation of $ICOS^+$ and $ICOS^-CD45RA^+CD31^+$ RTE T_{regs} during the normal pregnancy course and in the presence of gestation-associated diseases such as pre-eclampsia and haemolysis elevated liver enzymes low platelet (HELLP) syndrome.

In this study, we show that normal pregnancy is characterized by the increased differentiation of both $ICOS^+$ and $ICOS^-CD45RA^+CD31^+$ RTE but not $CD45RA^+CD31^-$ MN T_{regs} into $CD45RA^-CD31^-$ memory T_{regs}. Spontaneous term labour was associated mainly with a sharp decline of $ICOS^+CD45RA^-CD31^-$ memory T_{regs} within the total $CD4^+CD127^{low+/-}$ forkhead box protein 3 $(FoxP3)^+$ T_{reg} pool. Compared to normal pregnancy, the differentiation of $ICOS^+$ and $ICOS^-CD45RA^+CD31^+$ RTE T_{regs} into $CD45RA^-CD31^-$ memory T_{regs} was impaired strongly in pre-eclampsia patients. In contrast, the differentiation of $ICOS^+$ and $ICOS^-CD45RA^+CD31^+$ RTE T_{regs} was relatively normal in patients with HELLP syndrome. However, there was a significantly increased generation of $CD45RA^-CD31^-$ memory cells within the $CD4^+ICOS^+CD127^+FoxP3^-$ responder T cell (T_{resp}) pool.

Materials and methods

Patient collectives and healthy volunteers

Peripheral blood samples were collected from 31 non-pregnant fertile female volunteers (group 1), 128 pregnant women during the normal pregnancy course (groups 2–5), 41 women with spontaneous term labour (group 6), 45 women 1 day postpartum (group 7), 42 pregnant women affected by pre-eclampsia (group 8) and 18 women affected with HELLP syndrome (group 9). The diagnosis of pre-eclampsia was made in the case of blood pressure of more than 140/90 mm Hg occurring on two separate occasions, 6 h apart, along with significant proteinuria (>300 mg/l in a 24-h collection or a dipstick reading of >1 on a voided random urine sample in the absence of urinary tract infection) in previously normotensive women. The diagnosis of HELLP syndrome was made on the basis of haemolysis, elevated liver enzyme levels (aspartate and alanine aminotransferase >30 U/l) and thrombocytopenia thrombocyte count <150 000/μl). The blood samples from healthy

pregnancies (groups 2–5) were collected from women who had routine ultrasonography to exclude fetal malformations (groups 2–4), from women delivering by term elective caesarean section in the absence of labour (group 5), from women in the presence of spontaneous term labour before delivery (group 6) and from women who had delivered spontaneously 1 day before (group 7). The blood samples from women affected by pre-eclampsia or HELLP syndrome (groups 8 and 9) were taken during their hospitalization. The clinical characteristics of all these women (groups 1–9) are summarized in Table 1. The study was approved by the Regional Ethics Committee. All women were fully informed of the aim of the study and informed consent was obtained from all participants.

Changes in the composition of the total $CD4^+$ $CD127^{low+/-}FoxP3^+$ T_{reg} pool/$CD4^+CD127^+FoxP3^-$ T_{resp} pool with ICOS$^+$ and ICOS$^-$ T cells were examined during the normal pregnancy course, in the presence of spontaneous term labour and in the presence of pre-eclampsia or HELLP syndrome. To examine whether the differentiation of ICOS$^+$ and ICOS$^-$ T_{regs}/T_{resps} was regulated differentially during normal pregnancy, or regulated aberrantly in the presence of pre-eclampsia and HELLP syndrome, we calculated the percentages of $CD45RA^+CD31^+$ RTE cells, $CD45RA^+CD31^-$ MN cells, $CD45RA^-CD31^+$ memory cells and $CD45RA^-CD31^-$ memory cells, both within the total ICOS$^+$ and ICOS$^-$ T_{reg}/T_{resp} pools and within the total T_{reg} pool. These measurements were performed by six-colour flow cytometric analysis for all patient groups. Moreover, comparative analyses concerning the suppressive activity of separated ICOS$^+$ and ICOS$^-$ T_{regs} were performed for non-pregnant women (group 1), healthy pregnant women (groups 4 and 5) pre-eclampsia patients (group 8) and women affected with HELLP syndrome (group 9).

Fluorescence activated cell sorter (FACS) staining

Venous blood samples (10 ml) were collected from all participants into ethylenediamine tetraacetic acid (EDTA)-containing tubes. Whole peripheral blood mononuclear cells (PBMCs) were isolated by Lymphodex (Inno-Train Diagnostik GMBH, Kronberg, Germany) gradient centrifugation and analysed by six-colour flow cytometric analysis. Briefly, the PBMCs (8×10^6 cells) were surface-stained with 10 μl peridinin chlorophyll (PerCP)-conjugated anti-CD4 (BD Bioscience, Heidelberg, Germany), 20 μl phycoerythrin (PE)-conjugated anti-ICOS (BD Bioscience), 5 μl PE cyanin (Cy)7-conjugated anti-CD127 (eBioscience, Frankfurt, Germany), 5 μl Alexa Fluor 647-conjugated anti-CD31 (BD Bioscience) and 5 μl allophycocyanin (APC)-conjugated anti-CD45RA (BD Bioscience) mouse monoclonal antibodies. Subsequently, intracellular staining was performed for the detection of FoxP3 using a fluorescein isothiocyanate (FITC)-labelled anti-human FoxP3 staining set (clone PCH101; eBioscience), according to the manufacturer's instructions. Negative control samples were incubated with isotype-matched antibodies. Dead cells were excluded by forward- and side-scatter characteristics. Cells were analysed by a FACS Canto cytometer (BD Bioscience). Statistical analysis was based on at least 100 000 gated $CD4^+$ T cells.

Positive selection of $CD4^+CD127^{low+/-}CD25^+$ T_{reg} cells

Whole peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood (50 ml) drawn in EDTA tubes by Lymphodex (Inno-Train Diagnostik GMBH) gradient centrifugation. $CD4^+CD127^{low+/-}CD25^+$ T_{reg} cells were purified using the Regulatory T Cell Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's instructions. First, $CD4^+CD127^{low+/-}$ T cells were isolated by magnetic depletion of non-$CD4^+$ and $CD127^{high+}$ cells. In the second step, the $CD4^+CD127^{low+/-}CD25^+$ T_{regs} were isolated by positive selection over two consecutive columns. The $CD4^+CD127^{low+/-}CD25^-$ T cells were obtained in the flow-through fraction and used as T_{resps}. The $CD4^+CD127^{low+/-}CD25^+$ T_{regs} were subsequently retrieved from the columns.

Sorting and functional testing of T_{reg} subsets

For the sorting of the isolated $CD4^+CD127^{low+/-}CD25^+$ T_{regs} into ICOS$^+$ and ICOS$^-$ T_{regs}, cells were stained with 20 μl PE-conjugated anti-ICOS (BD Bioscience) and 20 μl FITC-conjugated anti-CD4 (BD Bioscience) mouse

Table 1. Clinical characteristics

Group	Number	Age, median (range)	Weeks' gestation, median (range)	Diagnosis
1 (non-pregnant)	31	25 (18–38)	–	Healthy
2 (1st trimester)	31	32 (32–39)	13 (13–14)	Healthy
3 (2nd trimester)	31	31 (21–41)	22 (20–23)	Healthy
4 (3rd trimester)	33	31 (19–42)	31 (27–35)	Healthy
5 (Term, caes. section)	33	32 (20–39)	38 (37–39)	Healthy
6 (Term, spont. delivery)	41	32 (19–38)	40 (38–42)	Healthy
7 (1 day postpartum)	45	30 (19–41)	–	Healthy
8 (Pre-eclampsia)	42	33 (21–45)	35 (23–41)	Pre-eclampsia
9 (HELLP syndrome)	18	32 (31–43)	36 (30–40)	HELLP syndrome

caes. = caesarean; spont. = spontaneous; HELLP = haemolysis elevated liver enzymes low platelet.

monoclonal antibodies. Total $CD4^+CD127^{low+/-}CD25^+$ T_{regs} were obtained from 11 non-pregnant fertile women, 10 healthy pregnant women (groups 4 and 5), 11 pregnant women affected by pre-eclampsia (group 8) and six pregnant women affected by HELLP syndrome (group 9). In all experiments, dead cells were excluded, while the remaining $CD4^+CD127^{low+/-}CD25^+$ T_{regs} were sorted using a FACS Vantage SE Sorter (BD Bioscience).

To analyse the suppressive activity of each isolated T_{reg} population, 2×10^4 T_{resps} were co-cultured with the purified T_{reg} subsets at ratios of 1 : 2 to 1 : 256 in 96-well U-bottomed plates. Suppression assays were performed in a final volume of 100 μl/well of X-VIVO15 medium (Lonza, Verviers, Belgium). For T cell stimulation, the medium was supplemented with 1 μg/ml anti-CD3 and 2 μg/ml anti-CD28 antibodies (eBioscience). As controls, $CD4^+CD127^{low+/-}CD25^+$ T_{regs} and T_{resps} alone were cultured both with and without stimulus. Cells were incubated at 37 °C in 5% CO_2. After 4 days, 1 μCi $[^3H]$-thymidine (Hartmann Analytic, Braunschweig, Germany) was added to the cultures and cells were incubated for a further 16 h. Then, the cells were harvested and $[^3H]$ incorporation was measured by scintillation counting. According to the cell numbers achieved after cell sorting, the assays were performed as single or multiple determinations. In order to compare the suppressive activity of the different T_{reg} subsets in non-pregnant women, healthy pregnant women and pre-eclampsia patients, the maximum suppressive activity (ratio of T_{regs} to T_{resps} 1/2) and the minimum ratio of T_{regs} to T_{resps} at which a suppression of at least 15% could be achieved were calculated [11].

Statistical analysis

Statistical comparison of the percentages of $ICOS^+$ and $ICOS^-$ T_{regs}/T_{resps} within the total T_{reg}/T_{resp} pool between the different patient groups was performed using the non-parametric Wilcoxon–Mann–Whitney *U*-test. This test was also used for statistical comparison of the percentages of $CD45RA^+CD31^+$ RTE cells, $CD45RA^+CD31^-$ MN cells, $CD45RA^-CD31^+$ and $CD45RA^-CD31^-$ memory cells within the $ICOS^+$ and $ICOS^-$ T_{reg}/T_{resp} pool. Comparison of the suppressive activity of the different T_{reg} subsets between non-pregnant women, healthy pregnant women and pre-eclampsia or HELLP syndrome patients was also performed using the non-parametric Wilcoxon–Mann–Whitney *U*-test. $P < 0{\cdot}05$ was considered significant. For all tests, the software package BiAS for Windows (version 10·06) was used.

Results

$ICOS^+$ and $ICOS^-$ T_{regs} show similar differentiation during the normal pregnancy course

In this study we examined whether there were differences in the differentiation of $ICOS^+$ and $ICOS^-$ T_{regs}/T_{resps} during the normal pregnancy course and whether the presence of pre-eclampsia or HELLP syndrome was associated with deficient differentiation of either $ICOS^+$ or $ICOS^-$ T_{regs}/T_{resps} compared to healthy pregnancies. Therefore, we first divided the total $CD4^+CD127^{low+/-}FoxP3^+$ T_{reg} pool/ $CD4^+CD127^+FoxP3^-$ T_{resp} pool into $ICOS^+$ and $ICOS^-$ T_{regs}/T_{resps} and then determined the percentages of $CD45RA^+CD31^+$ RTE and $CD45RA^+CD31^-$ MN T_{regs}/T_{resps}, as well as $CD45RA^-CD31^+$ and $CD45RA^-CD31^-$ memory T_{regs}/T_{resps} within both the total $ICOS^+$ and $ICOS^-$ T_{reg}/T_{resp} pools. Figure 1a–h shows the gating strategy that was used in all experiments. These measurements were performed with venous blood samples of non-pregnant fertile women (group 1), healthy non-labouring pregnant women during the normal pregnancy course (groups 2–5), spontaneously term labouring women (group 6) and women having delivered spontaneously 1 day before (group 7). The data obtained from healthy non-labouring third-trimester women (groups 4 and 5) were compared with third-trimester women affected by pre-eclampsia (group 8) or HELLP syndrome (group 9). Table 1 summarizes the clinical characteristics of all participants in this study.

We found that the onset of normal pregnancy was associated with a significant decrease of $CD45RA^+CD31^+$ RTE (Fig. 2a,e), as well as $CD45RA^-CD31^+$ memory T_{regs} (Fig. 2c,g) within both the $ICOS^+$ and the $ICOS^-$ T_{reg} pools. The percentages of $CD45RA^+CD31^-$ MN T_{regs} did not change, neither within the $ICOS^+$ nor within the $ICOS^-$ T_{reg} pool (Fig. 2b,f). However, there was a strong increase in the percentage of $CD45RA^-CD31^-$ memory T_{regs} within both T_{reg} pools (Fig. 2d,h). During the further pregnancy course (groups 2–5) this situation was almost maintained until term, when with the onset of normal spontaneous term labour (group 6) the original distribution of $CD45RA^+CD31^+$ RTE T_{regs}, $CD45RA^-CD31^+$ and $CD45RA^-CD31^-$ memory T_{regs} was restored according with that of non-pregnant women (group 1). Surprisingly, after spontaneous term delivery (group 7), there was a new, significant, decrease of $CD45RA^+CD31^+$ RTE and $CD45RA^-CD31^+$ memory T_{regs} (Fig. 2a,c) and a complementary increase of $CD45RA^-CD31^-$ memory T_{regs} within the $ICOS^+$ T_{reg} pool (Fig. 2d). A similar decrease of $CD45RA^+CD31^+$ RTE and $CD45RA^-CD31^+$ memory T_{regs} was also observed within the $ICOS^-$ T_{reg} pool (Fig. 2e,g), but instead of the $CD45RA^-CD31^-$ memory T_{regs}, the $CD45RA^+CD31^-$ MN T_{regs} increased complementary postpartum (Fig. 2f). Supporting information, Fig. S1 also shows the confidence intervals for the median for these results.

In summary, these data show that the onset of pregnancy is associated with a decreased thymic distribution of both $ICOS^+$ and $ICOS^-CD45RA^+CD31^+$ RTE T_{regs} and that the already distributed $ICOS^+$ and $ICOS^-CD45RA^+CD31^+$ RTE T_{regs} differentiate increasingly into $CD45RA^-CD31^-$ memory

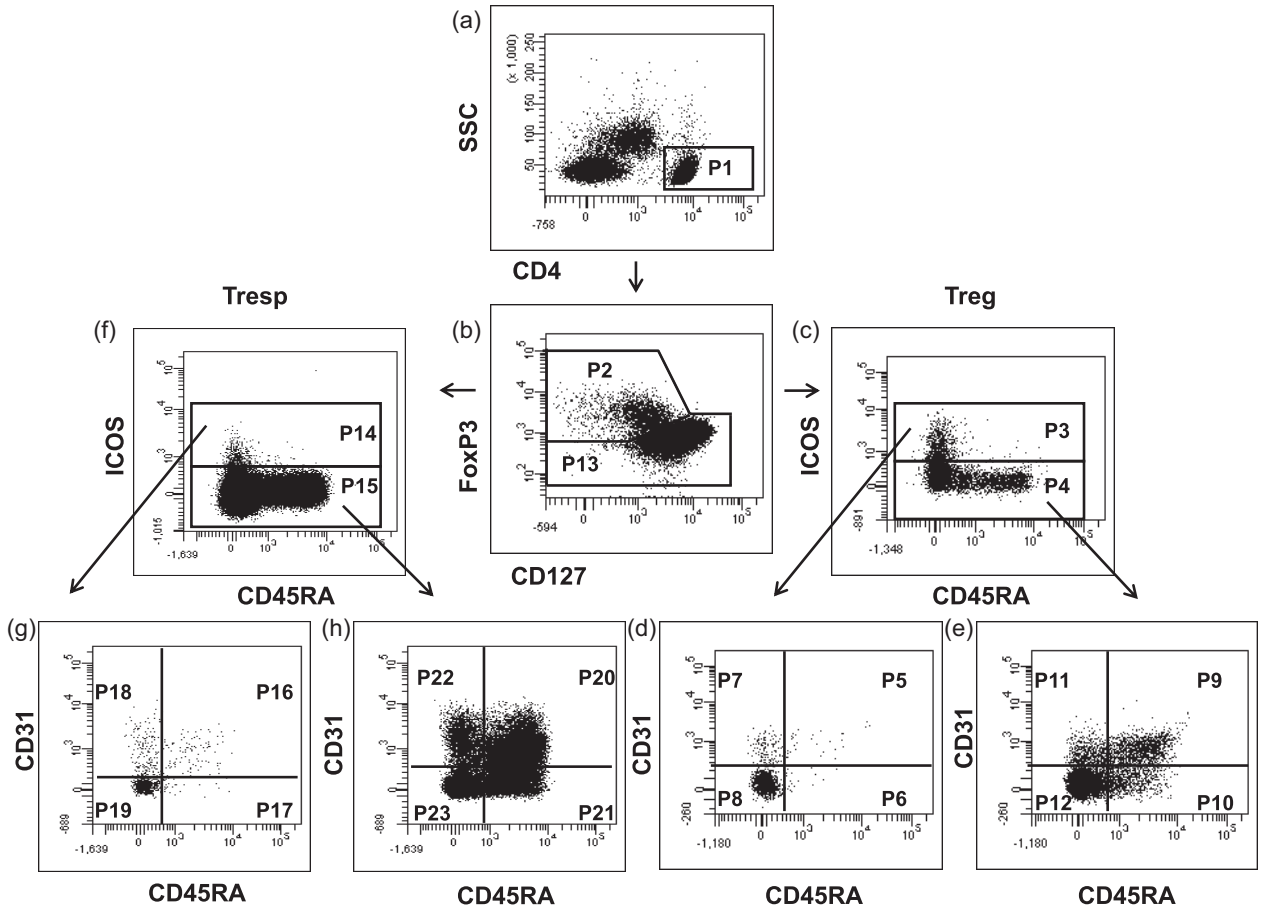

Fig. 1. Gating strategy for six-colour flow cytometric detection of CD45RA$^+$CD31$^+$ recent thymic emigrant (RTE), CD45RA$^+$CD31$^-$mature naive (MN) T_{regs}, CD45RA$^-$CD31$^+$ memory T cells and CD45RA$^-$CD31$^-$ memory T cells within the inducible co-stimulatory (ICOS)$^+$ and ICOS$^-$ regulatory T cell (T_{reg}) pool, as well as within the ICOS$^+$ and ICOS$^-$ responder T cell (T_{resp}) pool. At first, CD4$^+$ T cells (P1) were gated by fluorescence intensity of CD4 *versus* side-scatter (SSC) (a). The CD4$^+$CD127$^{low+/-}$ forkhead box protein 3 (FoxP3)$^+$ T_{regs} (P2) and the CD4$^+$CD127$^+$FoxP3$^-$ T_{resps} (P13) were gated by fluorescence intensity of FoxP3 *versus* CD127 (b). The ICOS$^+$ (P3) and ICOS$^-$ T_{regs} (P4) were gated by fluorescence intensity of CD45RA *versus* ICOS (c). The percentages of CD45RA$^+$CD31$^+$ RTE T_{regs} (P5, P9), CD45RA$^+$CD31$^-$ MN T_{regs} (P6, P10), CD45RA$^-$ CD31$^+$ memory T_{regs} (P7, P11) and CD45RA$^-$ CD31$^-$ memory T_{regs} (P8, P12) were estimated by analysing the total ICOS$^+$ (P3) and ICOS$^-$ T_{reg} pool (P4) for its expression of CD45RA and CD31 (d,e). Moreover, the ICOS$^+$ (P14) and ICOS$^-$ T_{resps} (P15) were gated by fluorescence intensity of CD45RA *versus* ICOS (f). The percentages of CD45RA$^+$CD31$^+$ RTE T_{resps} (P16, P20), CD45RA$^+$CD31$^-$ MN T_{resps} (P17, P21), CD45RA$^-$ CD31$^+$ memory T_{resps} (P18, P22) and CD45RA$^-$ CD31$^-$ memory T_{resps} (P19, P23) were estimated by analysing the total ICOS$^+$ (P14) and ICOS$^-$ T_{resp} pool (P15) for its expression of CD45RA and CD31 (g,h).

T_{regs}. This situation seems to be maintained during the entire healthy pregnancy course. With the onset of spontaneous term labour, the percentage of CD45RA$^-$CD31$^-$ memory T_{regs} declined, while the percentage of CD45RA$^-$CD31$^+$ memory T_{regs} increased strongly. Furthermore, there was a significant redistribution of CD45RA$^+$CD31$^+$ RTE T_{regs}, both within the ICOS$^+$ and ICOS$^-$ T_{reg} pools.

The decrease of ICOS$^+$CD45RA$^-$CD31$^-$ but not ICOS$^-$CD45RA$^-$CD31$^-$ memory T_{regs} is crucial for the onset of spontaneous term labour

To examine whether the onset of normal spontaneous term labour was associated primarily with a loss of either ICOS$^+$ or ICOS$^-$ T_{regs}, we estimated their percentages within the total CD4$^+$CD127$^{low+/-}$FoxP3$^+$ T_{reg} pool during the whole pregnancy course. In addition, we examined whether there were considerable changes in the composition of the total CD4$^+$CD127$^{low+/-}$FoxP3$^+$ T_{reg} pool with CD45RA$^+$CD31$^+$ RTE and CD45RA$^+$CD31$^-$ MN T_{regs}, as well as CD45RA$^-$CD31$^+$ and CD45RA$^-$CD31$^-$ memory T_{regs}, each of ICOS$^+$ and ICOS$^-$ T_{regs} (Fig. 3). We found that total T_{regs} decreased continuously (Fig. 3a) and that the percentages of ICOS$^+$ and ICOS$^-$ T_{regs} did not change markedly during the normal pregnancy course (Fig. 3b,c). However, the presence of spontaneous term labour was associated with a significant decrease of ICOS$^+$ T_{regs} and a complementary increase of ICOS$^-$ T_{regs} (Fig. 3b,c).

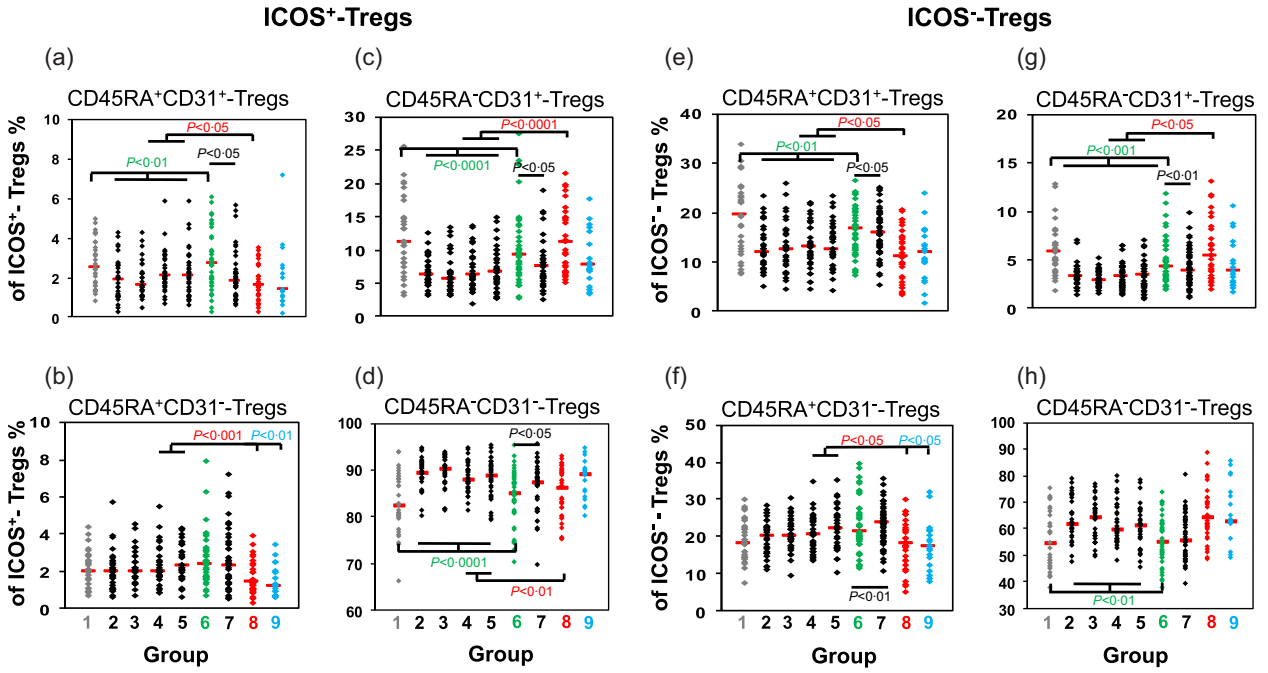

Fig. 2. Changes in the differentiation of inducible co-stimulatory (ICOS)$^+$ and ICOS$^-$ regulatory T cells (T_{regs}) during the normal pregnancy course and in the presence of pre-eclampsia and haemolysis elevated liver enzymes low platelet (HELLP) syndrome. The percentages of CD45RA$^+$CD31$^+$ recent thymic emigrant (RTE) T_{regs} (a,e), CD45RA$^+$CD31$^-$mature naive (MN) T_{regs} (b,f), CD45RA$^-$CD31$^+$ memory T_{regs} (c,g) and CD45RA$^-$CD31$^-$ memory T_{regs} (d,h) within the ICOS$^+$ and ICOS$^-$ T_{reg} pool were estimated in healthy non-pregnant fertile women (group 1, ◆), healthy pregnant women during their pregnancy course (groups 2–5, ◆), spontaneously term labouring women (group 6, ◆), 1 day postpartum (group 7, ◆) and in women with pre-eclampsia (group 8, ◆) or HELLP syndrome (group 9, ◆). Significant differences concerning the percentages of the different T_{reg} subsets were detected between women during the normal pregnancy course (groups 2–5) and non-pregnant women (group 1) or women with spontaneous term labour (group 6). Significant differences were also observed between spontaneously term labouring women (group 6) and women 1 day postpartum (group 7). In addition, significant differences were detected between healthy third-trimester women (groups 4 and 5) and women with pre-eclampsia (group 8) or HELLP syndrome (group 9).

Thereby, the percentage of ICOS$^+$CD45RA$^-$CD31$^-$ memory T_{regs} decreased strongly (Fig. 3g), while the percentage of ICOS$^-$CD45RA$^-$CD31$^-$ memory T_{regs} did not change perceptibly (Fig. 3k). Instead, there was a significant increase in the percentages of ICOS$^-$CD45RA$^+$CD31$^+$ RTE and ICOS$^-$CD45RA$^-$CD31$^+$ memory T_{regs} (Fig. 3h,j). Supporting information, Fig. S2 also shows the confidence intervals for the median for these results. Therefore, it seems that the occurrence of normal spontaneous term labour is characterized mainly by a significant decrease of ICOS$^+$CD45RA$^-$CD31$^-$ memory T_{regs} and a significant redistribution of ICOS$^-$CD45RA$^+$CD31$^+$ RTE T_{regs} within the total CD4$^+$CD127$^{low+/-}$FoxP3$^+$ T_{reg} pool.

Pre-eclampsia patients show a significantly increased differentiation of both ICOS$^+$ and ICOS$^-$CD45RA$^+$CD31$^+$RTE T_{regs} into CD45RA$^-$CD31$^+$ instead of CD45RA$^-$CD31$^-$ memory T_{regs}

To examine whether ICOS$^+$ or ICOS$^-$ T_{regs} show deficient differentiation in the presence of pre-eclampsia or HELLP syndrome, we determined the percentages of CD45RA$^+$CD31$^+$ RTE and CD45RA$^+$CD31$^-$ MN T_{regs}, as well as CD45RA$^-$CD31$^+$ and CD45RA$^-$CD31$^-$ memory T_{regs}, within the ICOS$^+$ and the ICOS$^-$ T_{reg} pool of both healthy third-trimester women (groups 4 and 5) and patients affected with pre-eclampsia (group 8) or HELLP syndrome (group 9). We found that the differentiation of ICOS$^+$ and ICOS$^-$ T_{regs} was disturbed similarly in pre-eclampsia patients (Fig. 2a–h). Compared to healthy pregnancies, the percentages of CD45RA$^+$CD31$^+$ RTE and CD45RA$^+$CD31$^-$ MN T_{regs} (Fig. 2a,b,e,f) were reduced significantly, while the percentages of CD45RA$^-$CD31$^+$ memory T_{regs} (Fig. 2c,g) were increased significantly in both T_{reg} pools. The only difference was found in the ICOS$^+$ T_{reg} pool, as the percentages of CD45RA$^-$CD31$^-$ memory T_{regs} were decreased significantly in pre-eclampsia patients (Fig. 2d), but unchanged in the ICOS$^-$ T_{reg} pool (Fig. 2h). Surprisingly, compared to healthy pregnancies, HELLP syndrome patients showed only significantly decreased percentages of CD45RA$^+$CD31$^-$ MN T_{regs} within the ICOS$^+$ T_{reg} pool as well as within the ICOS$^-$ T_{reg} pool (Fig. 2b,f). The percentages of CD45RA$^+$CD31$^+$ RTE T_{regs}, CD45RA$^-$CD31$^+$ and CD45RA$^-$CD31$^-$ memory T_{regs}

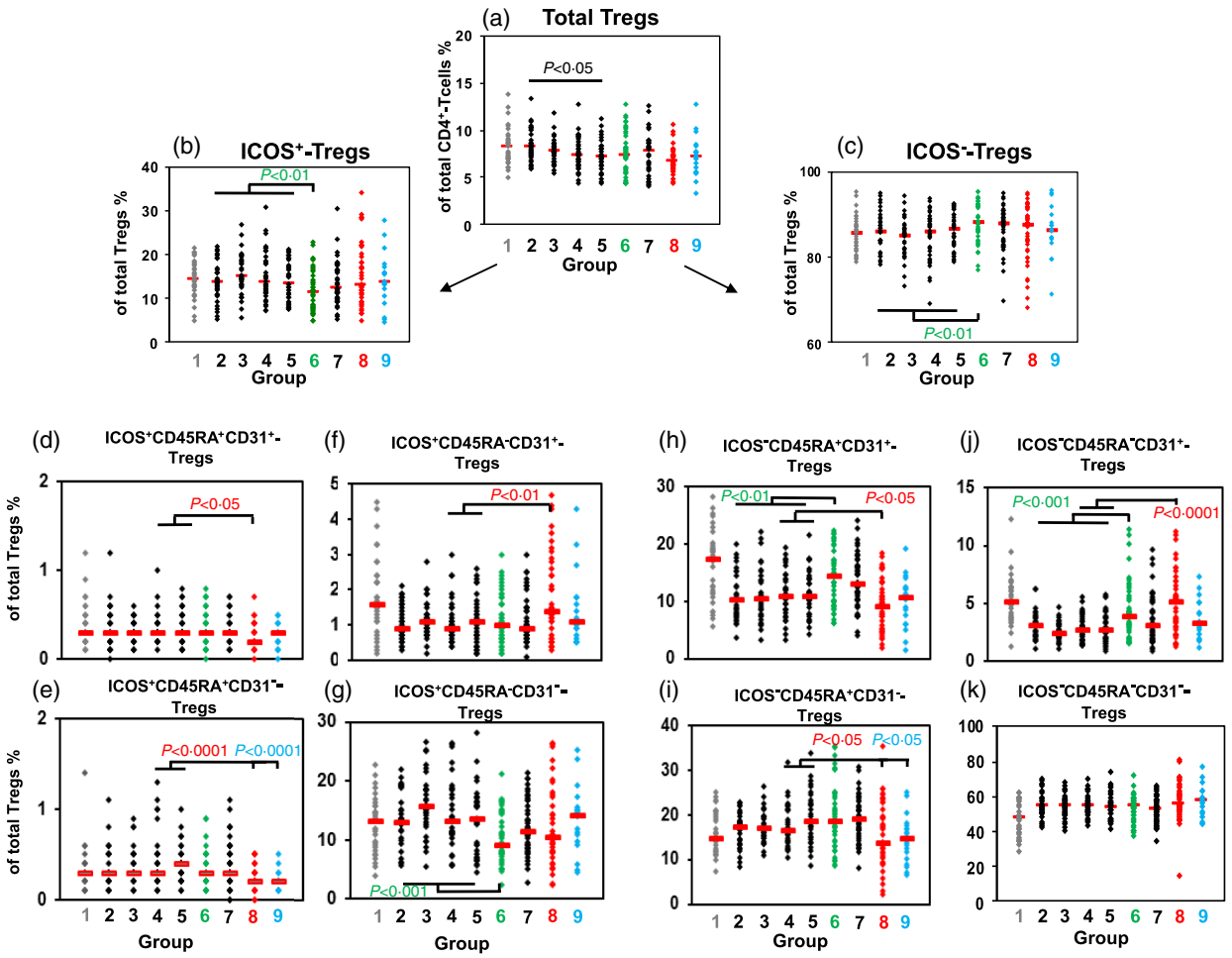

Fig. 3. Detection of inducible co-stimulatory (ICOS)$^+$ and ICOS$^-$CD45RA$^+$CD31$^+$ recent thymic emigrant (RTE) regulatory T cells (T_{regs}), ICOS$^+$ and ICOS$^-$CD45RA$^+$CD31$^-$ mature naive (MN) T_{regs}, ICOS$^+$ and ICOS$^-$CD45RA$^-$CD31$^+$ memory T_{regs} and ICOS$^+$ and ICOS$^-$CD45RA$^-$CD31$^-$ memory T_{regs} within the total CD4$^+$CD127$^{low+/-}$forkhead box protein 3 (FoxP3)$^+$ T_{reg} pool during the normal pregnancy course and in the presence of pre-eclampsia and haemolysis elevated liver enzymes low platelet (HELLP) syndrome. The percentage of total CD4$^+$CD127$^{low+/-}$FoxP3$^+$ T_{regs} within CD4$^+$ T cells (a), the percentages of ICOS$^+$ (b) and ICOS$^-$ T_{regs} (c), ICOS$^+$ and ICOS$^-$CD45RA$^+$CD31$^+$ RTE T_{regs} (d,h), ICOS$^+$ and ICOS$^-$CD45RA$^+$CD31$^-$ MN T_{regs} (e,i), ICOS$^+$ and ICOS$^-$CD45RA$^-$CD31$^+$ memory T_{regs} (f,j) and ICOS$^+$ and ICOS$^-$CD45RA$^-$CD31$^-$ memory T_{regs} (g,k) within the total CD4$^+$CD127$^{low+/-}$FoxP3$^+$ T_{reg} pool were determined in healthy non-pregnant fertile women (group 1, ◆), healthy pregnant women during their pregnancy course (groups 2–5, ◆), spontaneously term labouring women (group 6, ◆), 1 day postpartum (group 7, ◆) and in women with pre-eclampsia (group 8, ◆) or HELLP syndrome (group 9, ◆). Significant differences concerning the percentages of the different T_{reg} subsets were detected between women during the normal pregnancy course (groups 2–5) and non-pregnant women (group 1) or women with spontaneous term labour (group 6). In addition, significant differences were detected between healthy third-trimester women (groups 4 and 5) and women with pre-eclampsia (group 8) or HELLP syndrome (group 9).

again reached values in the range of healthy pregnancies in both T_{reg} pools.

The total CD4$^+$CD127$^{low+/-}$FoxP3$^+$ T_{reg} pool was not decreased in the presence of pre-eclampsia and HELLP syndrome (Fig. 3a). When detecting total ICOS$^+$ and ICOS$^-$ T_{regs} within the total CD4$^+$CD127$^{low+/-}$FoxP3$^+$ T_{reg} pool, we found that the relationship between these two T_{reg} subsets was not changed (Fig. 3b,c). Rather, we could see that the deviations in the differentiation of the ICOS$^+$ and ICOS$^-$ T_{reg} pool in pre-eclampsia and HELLP syndrome patients were also observable when detecting ICOS$^+$ and ICOS$^-$ T_{regs} as CD45RA$^+$CD31$^+$ RTE, CD45RA$^+$CD31$^-$ MN, CD45RA$^-$CD31$^+$ memory and CD45RA$^-$CD31$^-$ memory T_{regs} within the total CD4$^+$CD127$^{low+/-}$FoxP3$^+$ T_{reg} pool (Fig. 3d–k). The percentages of both ICOS$^+$ and ICOS$^-$CD45RA$^+$CD31$^+$ RTE T_{regs} (Fig. 3d,h), as well as ICOS$^+$ and ICOS$^-$CD45RA$^+$CD31$^-$ MN T_{regs} (Fig. 3e,i), were reduced significantly, while the percentages of ICOS$^+$ and ICOS$^-$CD45RA$^-$CD31$^+$ memory T_{regs} (Fig. 3f,j) were increased significantly in the presence of pre-eclampsia. However, the percentages of ICOS$^+$ and ICOS$^-$ CD45RA$^-$CD31$^-$ memory T_{regs} remained unchanged within the total CD4$^+$CD127$^{low+/-}$FoxP3$^+$ T_{reg} pool. In the presence of HELLP syndrome, we only detected significantly

reduced percentages of $ICOS^+$ and $ICOS^-CD45RA^+CD31^-$ MN T_{regs} within the total $CD4^+CD127^{low+/-}FoxP3^+$ T_{reg} pool (Fig. 3e,i). All other T_{reg} subsets again reached values in the range of healthy pregnancies. Supporting information, Figs S1 and S2 show the confidential intervals for these results.

These data suggest that the occurrence of pre-eclampsia is associated with an increased differentiation of both $ICOS^+$ and $ICOS^-CD45RA^+CD31^+$ RTE T_{regs} into $CD45RA^-CD31^+$ memory T_{regs} which presumably do not proliferate enough to increase $CD45RA^-CD31^-$ memory T_{regs}. As the $CD45RA^-CD31^-$ memory T_{regs} are not reduced in these patients, it seems that there is an alternatively increased differentiation of both $ICOS^+$ and $ICOS^-CD45RA^+CD31^-$ MN T_{regs} into $CD45RA^-CD31^-$ memory T_{regs}in the presence of pre-eclampsia. With the occurrence of HELLP syndrome, this increased differentiation of $ICOS^+$ and $ICOS^-CD45RA^+CD31^-$ MN T_{regs} into $CD45RA^-CD31^-$ memory T_{regs} seems to be exaggerated, and presumably induces an increasingly enhanced differentiation of previously non-activated $ICOS^+$ and $ICOS^-CD45RA^+CD31^+$ RTE T_{regs} into $CD45RA^-CD31^+$ memory T_{regs} with stronger proliferative capacity. Thus, the percentages of $CD45RA^+CD31^+$ RTE T_{regs}, $CD45RA^-CD31^+$ and $CD45RA^-CD31^-$ memory T_{regs} seem to adjust again to normal values detected in healthy pregnancies.

$ICOS^+$ and $ICOS^-$ T_{regs} have similar suppressive activity that is reduced strongly in patients affected with pre-eclampsia, but not HELLP syndrome

To examine whether the suppressive activity of $ICOS^+$ and $ICOS^-$ T_{regs} decreases in the presence of pre-eclampsia or HELLP syndrome, the total $CD4^+CD127^{low+/-}CD25^+$ T_{reg} pool obtained from 11 non-pregnant fertile women (group 1), 10 healthy pregnant women (groups 4 and 5), 11 pregnant women affected by pre-eclampsia (group 8) and six women affected by HELLP syndrome (group 9) was isolated by magnetic activated cell sorting (MACS) and sorted into $ICOS^+$ and $ICOS^-$ T_{regs} (Fig. 4a). Subsequently, both $ICOS^+$ (Fig. 4a, P1) and $ICOS^-$ T_{regs} (Fig. 4a, P2) obtained from each women were analysed separately for their suppressive capacity. Figure 4b–e shows the results of the maximum suppressive activity (ratio $T_{regs}/T_{resps} = 1/2$) and the ratio of the T_{regs}/T_{resps} up to which the T_{regs} could be diluted to achieve a minimum suppressive activity of at least 15%. Depending on the number of the separated $ICOS^+$ and $ICOS^-$ T_{reg} cells the suppression assays were performed as single or multiple determinations. Both parameters did not differ between $ICOS^+$ and $ICOS^-$ T_{regs}, either in non-pregnant or healthy pregnant women. There were also no differences concerning these parameters between non-pregnant women and healthy pregnant women. However, $ICOS^+$ and $ICOS^-$ T_{regs} showed a significantly decreased suppressive activity regarding both parameters in pre-eclampsia patients compared to non-pregnant and pregnant women. In contrast, both $ICOS^+$ and $ICOS^-$ T_{regs} obtained from patients with HELLP syndrome showed a similar high suppressive activity as detected in healthy pregnant and non-pregnant women (Fig. 4b–e).

These findings suggest that the increased differentiation of $ICOS^+$ and $ICOS^-CD45RA^+CD31^+$ RTE T_{regs} into $CD45RA^-CD31^+$ instead of $CD45RA^-CD31^-$ memory T_{regs} has a negative influence on the suppressive activity of both the $ICOS^+$ and $ICOS^-$ T_{reg} pools. As the percentages of all T_{reg} subsets, with the exception of the $CD45RA^+CD31^-$ MN T_{regs}, were found to be in the normal range in patients with HELLP syndrome, it seems that the reinforced differentiation of $ICOS^+$ and $ICOS^-CD45RA^+CD31^+$ RTE T_{regs} into sufficiently proliferating $CD45RA^-CD31^+$ memory T_{regs} causes the restoration of the suppressive activity of both T_{reg} pools.

$ICOS^+$ and $ICOS^-$ T_{resp} cells differentiate similar to $ICOS^+$ and $ICOS^-$ T_{regs} during the normal pregnancy course

Regarding the differentiation of T_{resps}, we also could not detect any shift in the composition of the total $CD4^+CD127^+FoxP3^-$ T_{resp} pool with $ICOS^+$ or $ICOS^-$ T_{resps} during the normal pregnancy course (Fig. 5a,b). We could even see a similar differentiation of the $ICOS^+$ and $ICOS^-$ T_{resps}, whose $CD45RA^+CD31^+$ RTE and $CD45RA^-CD31^+$ memory T cells also decreased strongly with the beginning of pregnancy (Fig. 5c,g,e,i). Their percentages of $CD45RA^+CD31^-$ MN T cells either did not change (Fig. 5d) or even rose (Fig. 5h), while their $CD45RA^-CD31^-$ memory T cells increased significantly (Fig. 5f,j). Similar to T_{reg} cell differentiation, this condition was maintained during the entire pregnancy course. However, with the onset of spontaneous term labour, the original distribution of $CD45RA^+CD31^+$ RTE, $CD45RA^-CD31^+$ and $CD45RA^-CD31^-$ memory T cells was restored for $ICOS^-$ T_{resps} (Fig. 5g,i,j), but not for $ICOS^+$ T_{resps} (Fig. 5c,e,f). These findings reveal that beside the increased generation of $ICOS^+$ and $ICOS^-CD45RA^-CD31^-$ memory T_{regs}, there is also an increased generation of $ICOS^+$ and $ICOS^-CD45RA^-CD31^-$ memory T_{resps} during the normal pregnancy course. With the beginning of spontaneous term labour, $ICOS^+$ and $ICOS^-CD45RA^-CD31^-$ memory T_{regs}, as well as $ICOS^-CD45RA^-CD31^-$ memory T_{resps}, diminish while the $ICOS^+CD45RA^-CD31^-$ memory T_{resps} do not decrease. Therefore, it appears that the continued existence of $ICOS^+CD45RA^-CD31^-$ memory T_{resps} may account for the induction of normal spontaneous term labour.

Surprisingly, the presence of pre-eclampsia and HELLP syndrome was associated with a clear shift in favour of $ICOS^-$ T_{resps} within the total $CD4^+CD127^+FoxP3^-$ T_{resp} pool (Fig. 5a,b). Thereby, it was striking that the changes concerning the composition of the total $ICOS^+$ T_{resp} pool were the same as

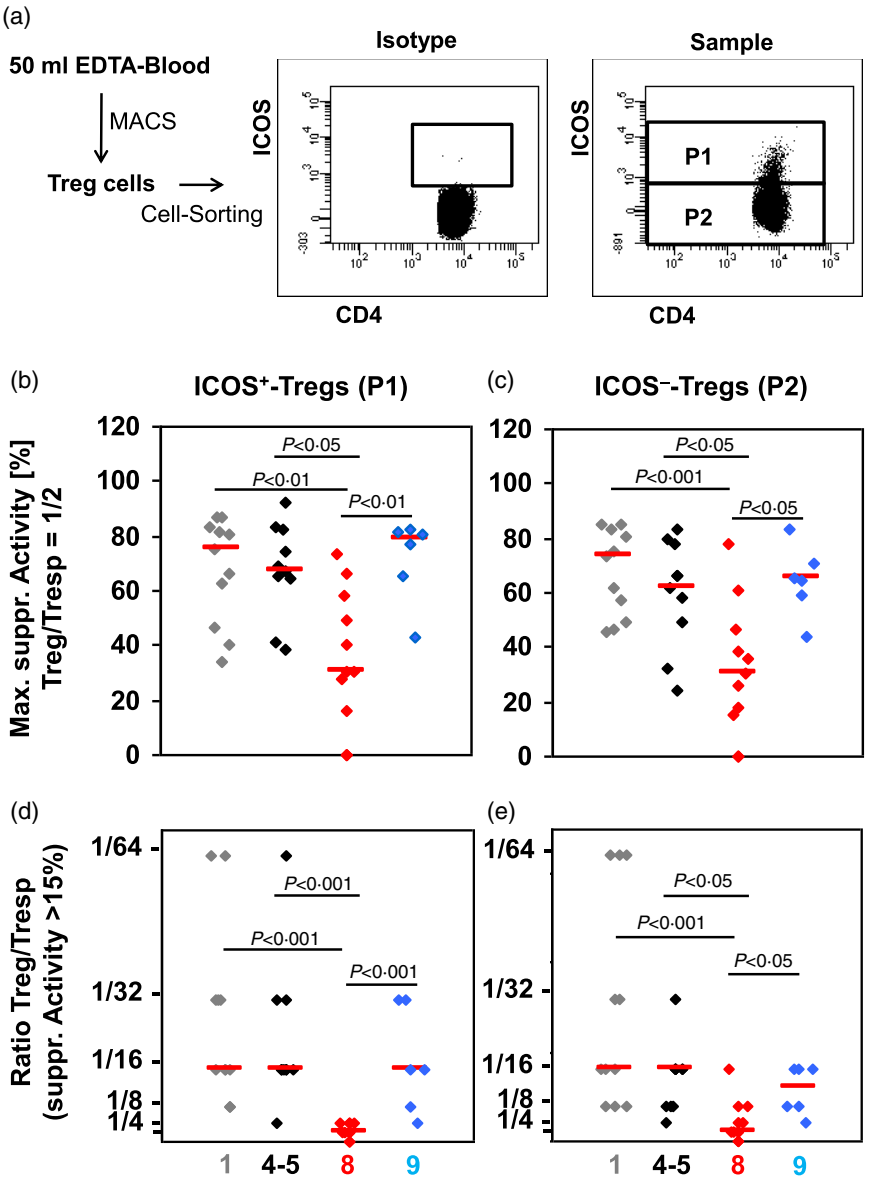

Fig. 4. Cell sorting and suppressive activity of inducible co-stimulatory (ICOS)$^+$ and ICOS$^-$ regulatory T cells (T_{regs}). CD4$^+$CD127$^{low+/-}$CD25$^+$ T_{regs} were isolated by magnetic activated cell sorting (MACS), stained with anti-CD4 and anti-ICOS-specific monoclonal antibodies and sorted into ICOS$^+$ (P1) and ICOS$^-$ T_{regs} (P2) (a). The suppressive activity of the different ICOS$^+$ and ICOS$^-$ T_{reg} subsets was estimated by suppression assays. Blood samples were obtained from non-pregnant women (◆), healthy third-trimester women (groups 4 and 5) (◆), pre-eclampsia patients (◆) and women with haemolysis elevated liver enzymes low platelet (HELLP) syndrome (◆). The figure shows the individual and median values of the maximum suppressive activity (T_{reg}/responder T cell (T_{resp}) = 1/2) (b,c) and of the ratio of T_{reg}/T_{resp} up to which the purified T_{reg} cells could be diluted to achieve a minimum suppressive activity of at least 15% (d,e). Compared to non-pregnant and healthy third-trimester women, the suppressive activity of both ICOS$^+$ and ICOS$^-$ T_{regs} was decreased significantly in pre-eclampsia patients, but not in HELLP syndrome patients.

those found in the ICOS$^+$ T_{reg} pool. In the presence of pre-eclampsia, the percentages of CD45RA$^+$CD31$^+$ RTE and CD45RA$^+$CD31$^-$ MN T cells decreased, while the percentages of CD45RA$^-$CD31$^+$ memory T cells increased significantly (Fig. 5c–e). An increased percentage of CD45RA$^-$CD31$^+$ memory T cells was also found within the ICOS$^-$ T_{resp} pool (Fig. 5i). Therefore, it seems that particularly the ICOS$^+$CD45RA$^+$CD31$^+$ RTE T_{resps} and presumably also the ICOS$^-$CD45RA$^+$CD31$^+$ RTE T_{resps} show an increased differentiation into CD45RA$^-$CD31$^+$ instead of CD45RA$^-$CD31$^-$ memory T_{resps} in the presence of pre-eclampsia. In contrast, there was a significantly increased differentiation of ICOS$^+$CD45RA$^+$CD31$^+$ RTE T_{resps} into ICOS$^+$CD45RA$^-$CD31$^-$ memory T_{resps} in the presence of HELLP syndrome (Fig. 5c,d,f). Supporting information, Fig. S3 also shows the confidential intervals for these results.

In summary, these findings suggest that an adequate differentiation of both T_{reg} and T_{resp} cells may be necessary for a successful pregnancy course. Obviously, the continued or enhanced generation of especially ICOS$^+$CD45RA$^-$CD31$^-$ memory T_{resps} plays an important role in spontaneous term delivery or HELLP syndrome.

Discussion

Our previous reports demonstrated that thymus-derived naturally occurring naive CD45RA$^+$ T_{regs} are of potential importance for successful pregnancy course [5,10–12,17]. Due to the fact that the human thymus contains two subsets of nT_{regs}, defined by their expression of the co-stimulatory molecule ICOS, we examined whether the

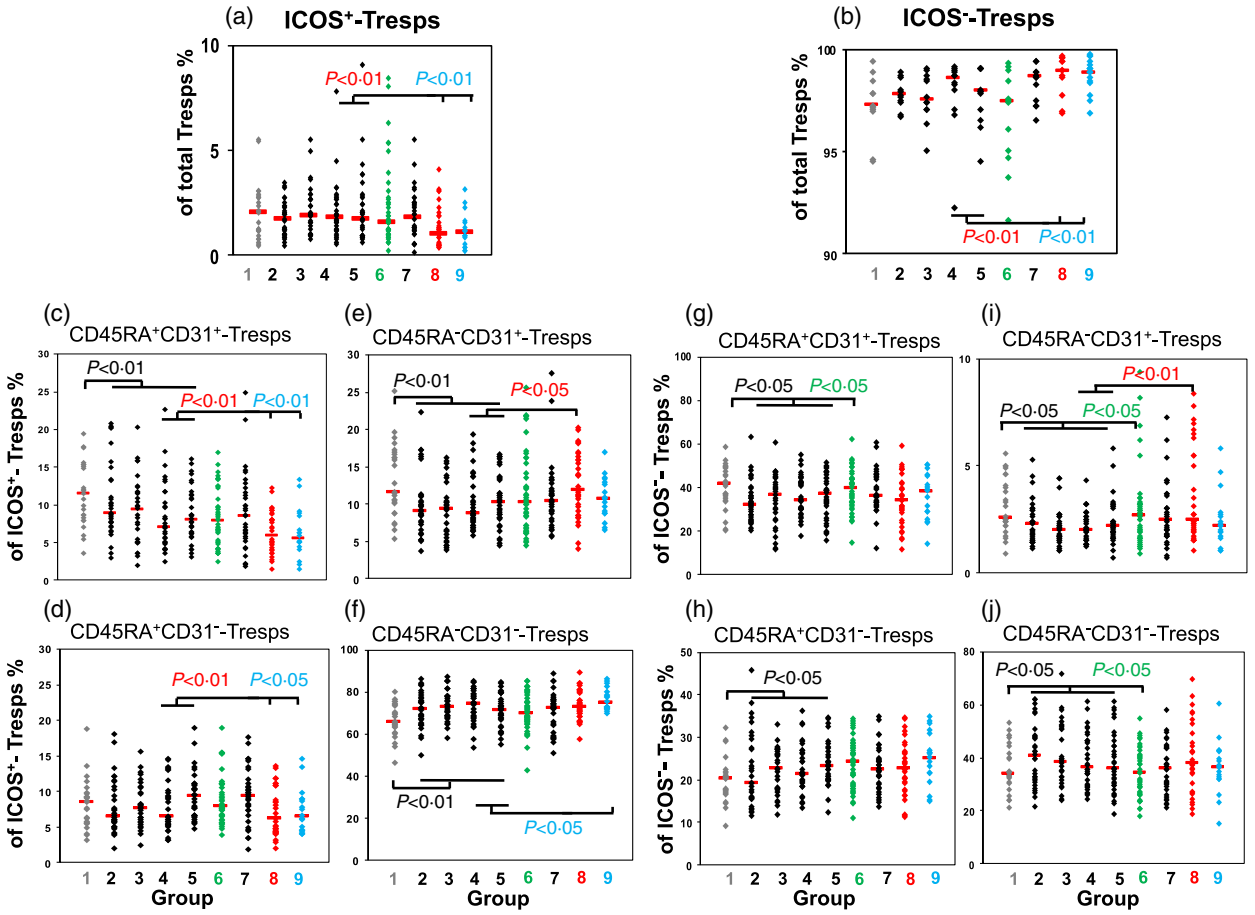

Fig. 5. Changes in the differentiation of inducible co-stimulatory $(ICOS)^+$ and $ICOS^-$responder T cells (T_{resps}) during the normal pregnancy course and in the presence of pre-eclampsia and haemolysis elevated liver enzymes low platelet (HELLP) syndrome. The percentages of $ICOS^+$ (a) and $ICOS^-$ T_{resps} (b) within the total T_{resp} pool, as well as $CD45RA^+CD31^+$RTE T_{resps} (c,g), $CD45RA^+CD31^-$MN T_{resps} (d,h), $CD45RA^-CD31^+$ memory T_{resps} (e,i) and $CD45RA^-CD31^-$ memory T_{resps} (f,j) within the $ICOS^+$ and $ICOS^-$ T_{resp} pool were estimated in healthy non-pregnant fertile women (group 1, ◆), healthy pregnant women during their pregnancy course (groups 2–5, ◆), spontaneously term labouring women (group 6, ◆), 1 day postpartum (group 7, ◆) and in women with pre-eclampsia (group 8, ◆) or HELLP syndrome (group 9, ◆). Significant differences concerning the percentages of the different T_{resp} subsets were detected between women during the normal pregnancy course (groups 2–5) and non-pregnant women (group 1) or women with spontaneous term labour (group 6). In addition, significant differences were detected between healthy third-trimester women (groups 4 and 5) and women with pre-eclampsia (group 8) or HELLP syndrome (group 9).

thymic output of $ICOS^+$ and $ICOS^-CD45RA^+CD31^+$ RTE T_{regs} and their subsequent differentiation in the periphery were regulated differentially during pregnancy and whether aberrant differentiation could be observed in the presence of pre-eclampsia or HELLP syndrome. For that, we compared the changes in the composition of the $ICOS^+$ and the $ICOS^-$ T_{reg} pool with $CD45RA^+CD31^+$ RTE, $CD45RA^+CD31^-$ MN, $CD45RA^-CD31^+$ memory and $CD45RA^-CD31^-$ memory T_{regs} during normal and complicated pregnancy and examined whether the characteristic changes observed within the $ICOS^+$ and the $ICOS^-$ T_{reg} pool could be confirmed when detecting the different $ICOS^+$ and $ICOS^-$ T_{reg} subsets within the total $CD4^+CD127^{low+/-}FoxP3^+$ T_{reg} pool. In addition, we examined whether there were similar changes in the composition of the total $CD4^+CD127^+FoxP3^-$ T_{resp} pool with the corresponding T_{resp} subsets.

We found that both $CD45RA^+CD31^+$ RTE and $CD45RA^-CD31^+$ memory T_{regs} decreased strongly with the onset of pregnancy, both within the $ICOS^+$ and $ICOS^-$ T_{reg} pools, indicating that the thymic output of both $ICOS^+$ and $ICOS^-$ RTE $T_{regs}$was greatly reduced. It is already known that the thymic activity is restricted severely during puberty in both men and women (thymic involution), due to the increased secretion of gonadal hormones [26]. Therefore, it seems likely that the excessive production of pregnancy hormones may cause an additional reduction of the thymic activity, which seems to be limited temporally for the duration of pregnancy. In mice, such transient thymic involution during pregnancy has already been shown and it was

demonstrated that progesterone and oestrogens had the capacity to decrease thymocyte proliferation at the DN stage without inducing apoptosis [26,27].

Our findings show that the decrease of CD45RA$^+$CD31$^+$ RTE and CD45RA$^-$CD31$^+$ memory T_{regs} at the onset of pregnancy was accompanied by a concurrent increase of CD45RA$^-$CD31$^-$ memory T_{regs}, while CD45RA$^+$CD31$^-$ MN T_{regs} did not change, both within the ICOS$^+$ and the ICOS$^-$ T_{reg} pools. Therefore, we propose that both ICOS$^+$ and ICOS$^-$CD45RA$^+$CD31$^+$ RTE T_{regs}, but not CD45RA$^+$CD31$^-$ MN T_{regs}, differentiate increasingly into CD45RA$^-$CD31$^-$ memory T_{regs}. Presumably, the restricted thymic release of RTE T_{regs} at the onset of pregnancy may cause their enhanced proliferation and force their direct differentiation into CD45RA$^-$CD31$^-$ memory T_{regs} during the entire pregnancy course. At the end of pregnancy, these newly generated populations of both ICOS$^+$ and ICOS$^-$CD45RA$^-$CD31$^-$ memory T_{regs} were found to break down with the onset of normal spontaneous term labour. This phenomenon may be explained by the fact that both naive ICOS$^+$ and ICOS$^-$CD45RA$^+$CD31$^+$ RTE T_{regs} may not have the capacity to proliferate indefinitely. Therefore, both ICOS$^+$ and ICOS$^-$CD45RA$^-$CD31$^-$ memory T_{regs} may decline at the end of pregnancy, when the ICOS$^+$ and ICOS$^-$CD45RA$^+$CD31$^+$ RTE T_{regs} lose the capacity to proliferate any longer.

Although ICOS$^+$ and ICOS$^-$CD45RA$^+$CD31$^+$ RTE T_{regs} differentiated similarly during the pregnancy course, we found a significant shift in the proportion of ICOS$^+$ and ICOS$^-$ T_{regs} within the total CD4$^+$CD127$^{low+/-}$FoxP3$^+$ T_{reg} pool in favour of ICOS$^-$ T_{regs} with the onset of normal spontaneous term labour. This was due mainly to a strong decrease of ICOS$^+$CD45RA$^-$CD31$^-$ memory T_{regs} and a potential increase of ICOS$^-$CD45RA$^+$CD31$^+$ RTE and ICOS$^-$CD45RA$^-$CD31$^+$ memory T_{regs}. As the ICOS$^+$ T_{regs} were shown to have a greater capacity for proliferation than ICOS$^-$ T_{regs} [22,23], the loss of ICOS$^+$CD45RA$^-$CD31$^-$ memory T_{regs} may have a greater impact on the composition of the total CD4$^+$CD127$^{low+/-}$FoxP3$^+$ T_{reg} pool than the loss of ICOS$^-$CD45RA$^-$CD31$^-$ memory T_{regs}. Therefore, our findings may propose that spontaneous term delivery may be induced primarily by the breakdown of ICOS$^+$CD45RA$^-$CD31$^-$ memory T_{regs}, but not ICOS$^-$CD45RA$^-$CD31$^-$ memory T_{regs}. Clearly, there is a significant thymic redistribution of ICOS$^+$ and ICOS$^-$CD45RA$^+$CD31$^+$ RTE T_{regs} with the onset of spontaneous term labour. Thereby, the redistribution of ICOS$^-$CD45RA$^+$CD31$^+$ RTE T_{regs} may be much more noticeable than that of ICOS$^+$CD45RA$^+$CD31$^+$ RTE T_{regs}, as these cells account for only a vanishingly small fraction within the total CD4$^+$CD127$^{low+/-}$FoxP3$^+$ T_{reg} pool.

Interestingly, after delivery, these newly released CD45RA$^+$CD31$^+$ RTE T_{regs} differentiated preferably into CD45RA$^+$CD31$^-$ MN T_{regs} in the ICOS$^-$ T_{reg} pool but into CD45RA$^-$CD31$^-$ memory T_{regs} in the ICOS$^+$ T_{reg} pool. Such findings may propose that placental hormones such as oestrogens and progesterone may be necessary for the direct differentiation of ICOS$^-$CD45RA$^+$CD31$^+$ RTE T_{regs} into ICOS$^-$CD45RA$^-$CD31$^-$ memory T_{regs} and that the postpartum loss of these hormones may, rather, favour the development of ICOS$^-$CD45RA$^+$CD31$^-$ MN T_{regs} instead of ICOS$^-$CD45RA$^-$CD31$^-$ memory T_{regs}. In contrast, it seems that there is a hormone-independent differentiation of ICOS$^+$CD45RA$^+$CD31$^+$ RTE T_{regs} into ICOS$^+$CD45RA$^-$CD31$^-$ memory T_{regs}. A hormone-dependent differentiation was already reported for conventional T cells, as it was shown that there is a profound loss of naive T cells during puberty, accompanied by a strong expansion of T cells with memory phenotype [28–31]. In addition, it was shown in mice that ovarian hormone ablation leads to phenotypical alterations in the major peripheral blood lymphocyte (PBL) and splenic T cell subsets by diminishing the peripubertal changes in the frequency of RTE, MN and memory T cells [32].

In contrast to spontaneous term delivery, the presence of pre-eclampsia or HELLP syndrome was not associated with a significant shift in the proportion of ICOS$^+$ and ICOS$^-$ T_{regs} within the total CD4$^+$CD127$^{low+/-}$FoxP3$^+$ T_{reg} pool. However, there was an increased differentiation of both ICOS$^+$ and ICOS$^-$CD45RA$^+$CD31$^+$ RTE T_{regs} into CD45RA$^-$CD31$^+$ memory T_{regs} instead of CD45RA$^-$CD31$^-$ memory T_{regs}, indicating that both ICOS$^+$ and ICOS$^-$CD45RA$^+$CD31$^+$ RTE T_{regs} may have deficiencies concerning their proliferative capacity. As these patients showed decreased percentages of both ICOS$^+$ and ICOS$^-$CD45RA$^+$CD31$^-$ MN T_{regs}, the deficient differentiation of CD45RA$^+$CD31$^+$ RTE T_{regs} into CD45RA$^-$CD31$^-$ memory T_{regs} may have been replaced partially by the increased differentiation of CD45RA$^+$CD31$^-$ MN T_{regs} into CD45RA$^-$CD31$^-$ memory T_{regs}. Nevertheless, pre-eclampsia patients showed decreased suppressive activity, both of their ICOS$^+$ and ICOS$^-$ T_{reg} pools. In contrast, the suppressive activity of both T_{reg} pools was in the normal range in patients with HELLP syndrome, a phenomenon which was confirmed when testing the suppressive activity of the total T_{reg} pool of these patients [11]. As these patients showed only decreased percentages of CD45RA$^+$CD31$^-$ MN T_{regs}, both within the ICOS$^+$ and the ICOS$^-$ T_{reg} pool and within the total CD4$^+$CD127$^{low+/-}$FoxP3$^+$ T_{reg} pool, we propose that the alternatively increased differentiation of CD45RA$^+$CD31$^-$ MN T_{regs} into CD45RA$^-$CD31$^-$ memory T_{regs} in pre-eclampsia patients may be exaggerated in patients with HELLP syndrome. The strong decrease of CD45RA$^+$CD31$^-$ MN T_{regs} could induce the additional differentiation of so far non-activated CD45RA$^+$CD31$^+$ RTE T_{regs} and could cause their further reinforced differentiation into CD45RA$^-$CD31$^-$ memory T_{regs} with stronger proliferation capacity. Presumably, this reinforced differentiation of ICOS$^+$ and ICOS$^-$CD45RA$^+$CD31$^+$ RTE T_{regs} into CD45RA$^-$CD31$^-$ memory T_{regs} ensures that the suppressive activity of both T_{reg}

pools is restored in patients with HELLP syndrome. Nevertheless, such mechanisms may constitute an emergency response causing a large consumption of functional RTE T_{regs}.

In addition, our results reveal that the reduction of thymic activity during pregnancy also affects the differentiation of $ICOS^+$ and $ICOS^-CD45RA^+CD31^+$ RTE T_{resp} cells. It was striking that similar to T_{reg} cells, both $ICOS^+$ and $ICOS^-CD45RA^+CD31^+$ RTE T_{resps}, but not $ICOS^+$ and $ICOS^-CD45RA^+CD31^-$ MN T_{resps}, differentiated into $ICOS^+$ and $ICOS^-CD45RA^-CD31^-$ memory T_{resps} during the normal pregnancy course. Currently, we are not able to explain how these mechanisms are realized. There are some indications in the literature that the CD31 molecule itself could play a role in the post-thymic homeostatic proliferation of conventional T_{resps}, and presumably of T_{regs} as well [24]. Similar to $ICOS^+$ and $ICOS^-CD45RA^-CD31^-$ memory T_{regs}, the population of $ICOS^-CD45RA^-CD31^-$ memory T_{resps}, but not that of $ICOS^+CD45RA^-CD31^-$ memory T_{resps}, broke down with the onset of normal spontaneous term labour. Such findings suggest that $ICOS^+CD45RA^+CD31^+$ RTE T_{resps} may have the ability to proliferate for longer than $ICOS^+$ and $ICOS^-CD45RA^+CD31^+$ RTE T_{regs} or $ICOS^-CD45RA^+CD31^+$ RTE T_{resps}. Presumably, at the end of pregnancy these conditions are responsible for the development of normal spontaneous term labour.

Regarding the presence of pre-eclampsia, our results propose that the proliferation capacity of $ICOS^+$ and $ICOS^-CD45RA^+CD31^+$ RTE T_{regs} as well as $ICOS^+$ and $ICOS^-CD45RA^+CD31^+$ RTE T_{resps} is impaired, so that both the suppressive effect of T_{regs} and the repellent effect of T_{regs} may be disturbed. However, in the presence of HELLP syndrome, there was a significant excessive production of $ICOS^+CD45RA^-CD31^+$ memory T_{resps}, whose effector functions could, presumably, have caused the reinforced differentiation of $ICOS^+$ and $ICOS^-CD45RA^+CD31^+$ RTE T_{regs} into $CD45RA^-CD31^-$ memory T_{regs}. Meanwhile, it is known that the ICOS molecule is expressed on follicular helper T cells (Tfh). These cells are necessary for B cell antibody isotype-switching, germinal centre (GC) formation and high-affinity antibody production [33]. Recently, the frequency of these cells was found to be increased significantly in patients with autoimmune diseases such as systemic lupus erythematosus (SLE) and ankylosing spondylitis [34,35]. In addition, ICOS signalling was shown to be required for the generation of both central and effector $CD4^+$ memory T cell populations [36]. Currently, it is not known whether $ICOS^+$ and $ICOS^-$ T_{resps} also differ concerning their induction, proliferation and survival. Further studies may be necessary to clarify the special role of $ICOS^+CD45RA^-CD31^-$ memory T_{regs}/T_{resps} for tolerance induction during healthy and affected pregnancies.

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Author contributions

M. W. and A. S. designed the study, M. W. and M. J. performed the study, J. S., M. S., K. M., S. M. and M. Z. contributed important methods and patients. M. W. and A. S. analysed the data and wrote the manuscript. All authors contributed to the final version of the manuscript and approved it.

Disclosure

None of the authors have any conflicts of interest related to this manuscript.

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Supporting information

Additional Supporting information may be found in the online version of this article at the publisher's web site:

Fig. 1. Changes in the differentiation of inducible co-stimulatory (ICOS)$^+$ and ICOS$^-$ regulatory T cells (T_{regs}) during the normal pregnancy course and in the presence of pre-eclampsia and haemolysis elevated liver enzymes low platelet (HELLP) syndrome. The percentages of CD45RA$^+$CD31$^+$ recent thymic emigrant (RTE) T_{regs} (a,e), CD45RA$^+$CD31$^-$ mature naive (MN) T_{regs} (b,f), CD45RA$^-$CD31$^+$ memory T_{regs} (c,g) and CD45RA$^-$CD31$^-$ memory T_{regs} (d,h) within the ICOS$^+$ and ICOS$^-$ T_{reg} pool were estimated in healthy non-pregnant fertile women (group 1, ◆), healthy pregnant women during their pregnancy course (groups 2–5, ◆), spontaneously term labouring women (group 6, ◆), 1 day postpartum (group 7, ◆) and in women with pre-eclampsia (group 8, ◆) or HELLP syndrome (group 9, ◆). The figures show the 95% confidence intervals and the median of all patient groups for each T_{reg} subset.

Fig. 2. Detection of inducible co-stimulatory (ICOS)$^+$ and ICOS$^-$CD45RA$^+$CD31$^+$recent thymic emigrant (RTE) T_{regs}, ICOS$^+$ and ICOS$^-$CD45RA$^+$CD31$^-$ mature naive (MN) T_{regs}, ICOS$^+$ and ICOS$^-$CD45RA$^-$CD31$^+$ memory T_{regs} and ICOS$^+$ and ICOS$^-$CD45RA$^-$CD31$^-$ T_{regs} within the total CD4$^+$CD127$^{low+/-}$forkhead box protein 3 (FoxP3)$^+$ T_{reg} pool during the normal

pregnancy course and in the presence of pre-eclampsia and haemolysis elevated liver enzymes low platelet (HELLP) syndrome. The percentage of total $CD4^+CD127^{low+/-}FoxP3^+$ T_{regs} within $CD4^+$ T cells (a), the percentages of $ICOS^+$ (b) and $ICOS^-$ T_{regs} (c), $ICOS^+$ and $ICOS^-CD45RA^+CD31^+$ RTE T_{regs} (d,h), $ICOS^+$ and $ICOS^-CD45RA^+CD31^-$ MN T_{regs} (e,i), $ICOS^+$ and $ICOS^-CD45RA^-CD31^+$ memory T_{regs} (f,j) and $ICOS^+$ and $ICOS^-CD45RA^-CD31^-$ memory T_{regs} (g,k) within the total $CD4^+CD127^{low+/-}FoxP3^+$ T_{reg} pool were determined in healthy non-pregnant fertile women (group 1, ◆), healthy pregnant women during their pregnancy course (groups 2–5, ◆), spontaneously term labouring women (group 6, ◆), 1 day postpartum (group 7, ◆) and in women with pre-eclampsia (group 8, ◆) or HELLP syndrome (group 9, ◆). The figures show the 95% confidence intervals and the median of all patient groups for each T_{reg} subset.

Fig. 3. Changes in the differentiation of inducible co-stimulatory $(ICOS)^+$ and $ICOS^-$ responder T cells (T_{resps}) during the normal pregnancy course and in the presence of pre-eclampsia and haemolysis elevated liver enzymes low platelet (HELLP) syndrome. The percentages of $ICOS^+$ (a) and $ICOS^-$ T_{resps} (b) within the total T_{resp} pool, as well as $CD45RA^+CD31^+$ recent thymic emigrant (RTE) T_{resps} (c,g), $CD45RA^+CD31^-$ mature naive (MN) T_{resps} (d,h), $CD45RA^-CD31^+$ memory T_{resps} (e,i) and $CD45RA^-CD31^-$ memory T_{resps} (f,j) within the $ICOS^+$ and $ICOS^-$ T_{resp} pool were estimated in healthy non-pregnant fertile women (group 1, ◆), healthy pregnant women during their pregnancy course (groups 2–5, ◆), spontaneously term labouring women (group 6, ◆), 1 day postpartum (group 7, ◆) and in women with pre-eclampsia (group 8, ◆) or HELLP syndrome (group 9, ◆). The figures show the 95% confidence intervals and the median of all patient groups for each T_{reg} subset.

