Visualization of the maternal immune system at the maternal-fetal interface

Krop, J.

J. Krop[1], J.M. Kapsenberg[1], C. van der Keur[1], F. Koning[1], S. Heidt[1],
F.H.J. Claas[1], M.L.P. van der Hoorn[2], M. Eikmans[1]

[1]Department of Immunology, Leiden University Medical Center, Leiden, The Netherlands
[2]Department of Obstetrics, Leiden University Medical Center, Leiden, The Netherlands

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A SPECIFIC SUBSET OF REGULATORY T CELLS WITH MULTIPLE CO-SIGNALLING MOLECULES IS LESS PREVALENT IN THE DECIDUA BASALIS OF WOMEN WITH A HISTORY OF UNEXPLAINED RECURRENT PREGNANCY LOSS

In preparation

ABSTRACT

Recurrent pregnancy loss (RPL) occurs in 1%-2% of couples trying to conceive. Unexplained recurrent pregnancy loss (uRPL) is a heterogeneous condition affecting 50%-70% of RPL cases, of which disruption in maternal immune tolerance is thought to be a contributing factor. Previous data show that Treg frequencies and function are reduced in women with uRPL. Some women do experience a successful pregnancy after a history of uRPL. In this study we hypothesized that Treg frequencies are comparable to a control group of women without a history of RPL. Using a 42-marker CyTOF panel focussing on T cell markers we measured term decidua basalis and parietalis single-cell digests of women with a history of uRPL and now a successful pregnancy (case group) and from pregnant women without a history of RPL (control group). We found that Treg phenotypes do not significantly differ between these groups. Interestingly, we identified a subset of Tregs expressing multiple co-stimulatory molecules and co-inhibitory molecules simultaneously; 4-1BB, OX40, ICOS, CD27, CD28, CD95, CTLA-4, PD1 and TIGIT. This Treg subset was present in a significantly lower frequency in the basalis in cases compared to controls. The results suggest that decidual Treg frequencies in women with a history of uRPL during term pregnancy 'normalized' toward those seen in controls, instead of being lower, as previously described in 1st trimester decidua during pregnancy loss. The Treg subset we identified needs to be further studied to better understand the clinical implication.

INTRODUCTION

Recurrent pregnancy loss (RPL) affects 1%-2% of couples trying to conceive. In 50%-70% of these couples no underlying cause can be found, and this is indicated as unexplained RPL (uRPL) [1-3]. As of 2018, RPL is defined as two or more pregnancy losses before 24 weeks of gestation [4]. Some couples with a history of uRPL do get an ongoing pregnancy with a healthy live born child as result.

Pregnancy is a semi-allogeneic situation in which maternal immune regulation is instrumental to sustain a healthy pregnancy. Indeed, Treg cells are shown to be essential for healthy pregnancy in allogeneic mice opposed to mice with a syngeneic pregnancy[5]. Furthermore, the maternal-fetal interface represents an anti-inflammatory environment: M2 macrophages, uterine natural killer (NK) cells, and the Th2 pathway predominate, and fetal extravillous trophoblast (EVT) cells do not express the classical HLA antigens but they do upregulate the immune regulatory HLA-G molecule. Imbalance in this environment could lead to uRPL and therefore immunomodulatory treatments have been proposed for women with uRPL. However, efficacy of different treatments is not yet clear [6, 7]. Providing a better understanding of what is needed to sustain a successful pregnancy in women with uRPL aids in selecting suitable treatment candidates.

A systematic review by Keller et al. shows that the frequency and functionality of Tregs is decreased in women with uRPL compared to controls, both in peripheral blood and the decidua [8]. However, some women after uRPL have a successful pregnancy which led us to the question if their decidual Treg frequencies 'normalized' to those observed in healthy controls and if their phenotype is similar.

Previously we showed that women with a history of uRPL and a subsequent successful pregnancy demonstrate increased HLA-G expression locally at the maternal-fetal interface [9], but reduced soluble HLA-G levels in their plasma [10]. HLA-G is an important immune checkpoint molecule that can inhibit the effector function of T cells, NK cells, and myeloid cells [11]. This indicates that there are immunological differences between women with a history of uRPL and subsequent successful pregnancy and a control group of pregnant women with no history of uRPL.

Therefore, we studied if women with a history of uRPL and subsequent successful pregnancy have similar decidual Treg frequencies and phenotype compared to controls.

METHOD

Study material

Placentas of 3rd trimester pregnancies were obtained after uncomplicated pregnancy delivered by either spontaneous vaginal delivery (at home or in the Leiden University Medical Center; LUMC) or primary or secondary cesarean section in the LUMC. All samples were obtained after informed consent and the study was carried out in accordance with the Declaration of Helsinki and with the guidelines issued by the Medical Ethics committee of the LUMC (protocol P11.196). Exclusion criteria for uRPL group and control group were BMI > 35, gestational diabetes, preeclampsia, fetal growth restriction, early ruptured membranes. Criteria for uRPL were at least 3 consecutive miscarriages and exclusion of all risk factors as stated in ESHRE guideline 2018 [4].

Decidual leukocytes from both the decidua basalis and parietalis were isolated as previously described [12]. In short, the decidual layers (basalis and parietalis) were macroscopically dissected from the placenta. Peripheral blood was mostly washed away from the tissue with PBS and finally with RPMI 1640 medium (Life Technologies), after which the tissue was minced and resuspended with Accutase (on RT, Gibco Life technologies). The tissue with Accutase was transferred to a C tube, homogenised, and incubated for 1 hour at 37°C. After digestion, cells were filtered through a 250-µm and 70-µm filter and washed with RPMI. Lastly, the leukocytes were isolated using Percoll (GE Healthcare) gradients and washed with RPMI, after which they were counted and kept overnight (4 to 12 hours) at 4°C before staining for mass cytometry. Before staining a reference sample was thawed and stained along in every staining round, to confirm marker stability and have a control staining differences.

Suspension mass cytometry

Cells were incubated with 20 U/ml Benzonase Nuclease (Sigma-Aldrich) for 5 minutes, filtered over a 70-µm filter, and counted. A maximum of 3.5 million cells were stained per tube. Next to that, an aliquot from the reference sample containing PBMCs, PHA stimulated PBMCs, IL-15 stimulated PBMCs was thawed and taken along in every staining to account for marker variation over time, staining mixes, and machine variations. The samples were stained for mass cytometry with the panel in **Supplementary Table 1.** Most antibodies were pre-conjugated from Fluidigm or self-conjugated using the MaxPar X8 Polymere Antibody Labeling Kit according to the manufacturer's protocol (Fluidigm, California, USA). 193Pt, 198Pt, 209Bi were conjugated as described before [13]. Antibody staining and data acquisition was performed as previously described (Van Unen, Immunity 2016) adding intracellular staining. In short, cells were incubated with 1 uM Cell-ID Intercalator-103Rh (Fluidigm) for 15' at room temperature (RT), washed taken-up in 45 µl and incubated with 5 µl of Fc receptor block (Biolegend) for 10'. Next, 50 µl of the antibody mix was added to incubate for 45' at RT. After staining, 2 ml of Stain buffer (Fluidigm) with 10U/ml Bezonase Nuclease was added and centrifuged after 5'. The cells were washed once more before continuing intracellular staining. For intracellular staining cells were incubated with 1 ml of FoxP3 Fix/Perm solution (Ebio) for 45' at 4°C. Next, cells were washed with perm buffer (Ebio) and taken up in 50 µl perm buffer after which 50 µl of the intracellular antibody mix was added for incubation of 30' at RT. Cells were washed once with perm buffer and twice with Stain buffer and incubated with 42 nM Cell-ID Intercalator in MaxPar Fix-Perm buffer for 60' at RT. Prior to acquisitions cells were washed three times with Stain buffer, once with milliQ (shortly before acquisition) and counted. Cells were taken up in a dilution of $7*10^5$ cells/ml in milliQ and EQ calibration beads (Fluidigm) for normalization were added 1:10 diluted and measured with the Helios mass cytometer (Fluidigm).

Immunofluorescence

FFPE sections of parietalis and basalis were deparaffinized by a series of xylene and ethanol after which antigen retrieval was performed by microwaving for 10 minutes with 10 mM citrate solution (pH 6.0). Slides were incubated with primary antibodies mouse-IgG1 anti-human FoxP3 (clone

236A/E7, eBioscience) and rabbit-IgG anti-human CD3 (clone ab828, Abcam). Lastly, goat-anti-rabbit IgG-AF546 (Invitrogen) and goat anti-mouse IgG1-AF488 (Invitrogen) where used to visualize the primary antibodies. Slides were covered using DAPI-ProLong Gold (Invitrogen, P36941) and a coverslip. Slides were scanned using the Panoramic MIDI2 scanner (Sysmex).

Data analysis

Statistical analysis was Performed using GraphPad Prism Version 9.3.1. Mann-Whitney test was used to assess statistical differences between the case and control group. P-values < 0.05 were considered to denote statistically significant differences.

Suspension mass cytometry

Reference samples between every measurement were compared to identify problems with marker expression. Living single CD45+ cells were selected using FlowJo, as described before [12]. Next, the single cell data were loaded in Cytosplore, hyperbolic-arcsinh transformation with cofactor 5 was performed and a five-level HSNE was performed, here the frequency of T cells within the CD45+ cells were calculated. Within the HSNE T cells were selected and zoomed into to level three HSNE, where CD4, CD8 and TCRyd T cell frequencies were calculated. All T cells were exported and uploaded to OMIQ. In OMIQ the frequency of Tregs was calculated within the CD3+CD4+ T cells. Furthermore, Treg numbers, CD45RO+ CD4+ cell excluding Tregs numbers (CD4 central memory/effector memory T cells; CD4-cm/em) and CD8+ T cell numbers were exported, and ratios were determined.

Immunofluorescence

CaseViewer (3DHistech) version 2.4 was used to select decidual area and in that area cells were counted that are positive for both the DAPI (nucleus staining), CD3 and FoxP3 and had a size between 4-10 µm. Area and Treg cell numbers in that area were exported and the number of Tregs per mm^2 decidual area was calculated.

RESULTS

Patient Characteristics

Pregnancy from women with a history of uRPL (case group) had a mean gestational age (GA) of 40 weeks and 2 days, whereas pregnancy from women without a history of uRPL (control group) had an average GA of 41 weeks and 4 days (Table 1). Both the case and control group had a median parity of 1. The case group had a median of three miscarriages in their history and the control group none. Average age for the women in the case and control group was 31 and 32, respectively. BMI was not significantly different between cases (mean 24.5) and controls (mean 23.7).

T cell frequencies do not differ between cases and controls

Using HSNE we could identify the T cell population within the CD45 compartment. T cell frequencies did not significantly differ between the case and control group in both the basalis and

Table 1. Medians (min-max) of patient characteristics, Mann Whitney test.

Parameter (medians)	History uRPL successful pregnancy (n=9)	Control (no history of RPL) (n=5)	p-Value
Gestation age (wks+days)	40+2 (37+3 – 41)	41+4 (39 – 42+1)	p = 0.134
Parity	1 (1-3)	1 (1-3)	p = 0.706
Previous miscarriages	3 (3-8)	0	p<0.001
Maternal age	31 (26 – 39)	32 (26 – 37)	p = 0.629
Maternal BMI	24.5 (18.7 – 26.2)	23.7 (21.1 – 33.5)	p> 0.999

parietalis (Figure 1A). We did observe heterogeneity within both the cases and controls for CD4 and CD8 T cell frequencies within the CD45 compartment (data not shown).

Decidual CD4, CD8, and γδ T cell frequencies within total T cells did not significantly differ between the case and control group (Figure 1B, C and D), neither was there a difference between basalis and parietalis T cell frequencies.

Women with a history of uRPL have similar decidual Treg frequencies compared to controls

Within the CD4 T cell compartment the frequencies of CD25highFoxP3+ Tregs was determined and the ratio between Treg and CD4-Tcm/em and between Treg and CD8 was calculated. No significant difference was observed for the Treg frequencies, Treg:CD4-Tcm/em ratio and Treg:CD8 ratio between cases and controls (Figure 2A). Both groups showed a considerable heterogeneity. To confirm our findings, we counted the CD3+FoxP3+ cells of the same samples in situ using IF and calculated the Treg cell counts per mm^2 decidual surface area. Again, we did not observe a significant difference between cases and controls (Figure 2B).

The Treg subset with co-signalling molecules in basalis is less prevalent in cases compared to controls

tSNE analysis on the Tregs show that these cells were FoxP3+, CD25+, CD127dim, and Helios+ (Figure 3A). Interestingly, we found a Treg subset that expresses the co-stimulatory immune checkpoint molecules 4-1BB, OX40, ICOS, CD27, CD28, and CD95. Furthermore, this subset also expressed the inhibitory checkpoint molecules CTLA-4, PD-1, and TIGIT, but lacked expression of TIM3 and LAG3. Lastly, they expressed CD39 and CD69 (Figure 2A). There were significantly more of these cells present in the control basalis Treg compartment than in the basalis of cases (p = 0.012) (Figure 3B).

DISCUSSION

We aimed to get a better understanding of the decidual maternal immune system needed for an ongoing pregnancy. We found that women with a history of uRPL and subsequent successful pregnancy have comparable decidual Treg frequencies to controls. However, we do find a Treg subset which expresses multiple costimulatory and co-inhibitory molecules. This subset is less prevalent in the case basalis compared to controls.

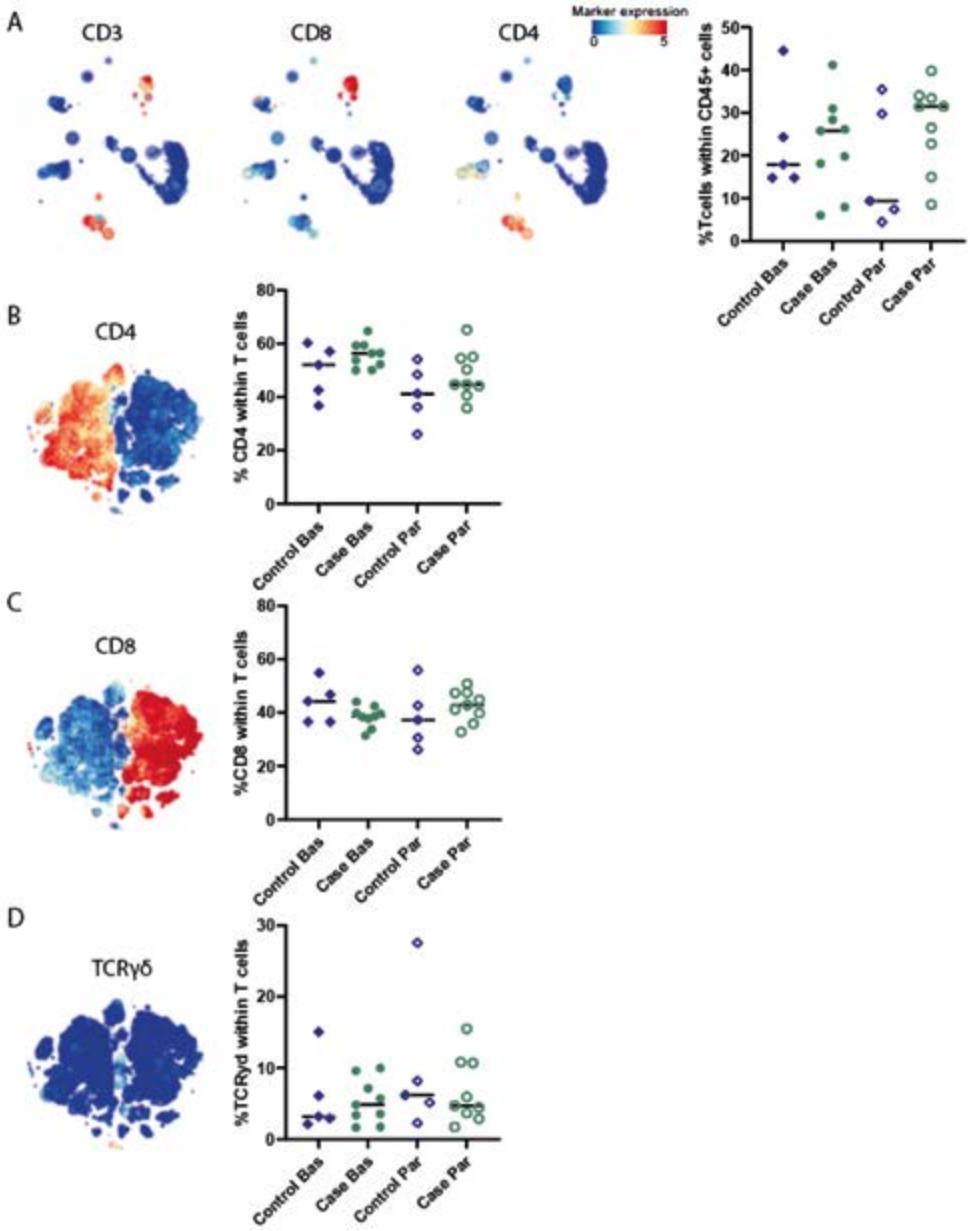

Figure 1. Decidual T cell frequencies in decidua parietalis (Par) and basalis (Bas) of women with a successful birth after uRPL (case) and controls. HSNE visualization and frequencies of T cells within the total CD45+ decidual immune cells show a wide spread between T cell frequencies between individuals (A). HSNE visualization and frequencies within the total T cells for CD4 T cells (B), CD8 T cells (C) and TCRγδ T cells (D) shows no significant differences between cases and controls.

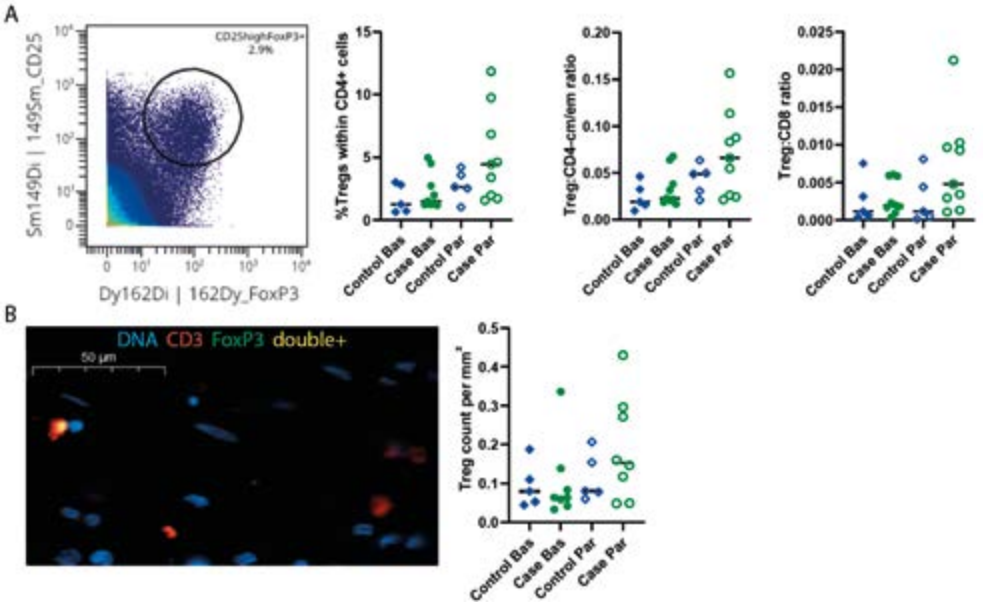

Figure 2. Decidual Treg frequencies in decidua parietalis (Par) and basalis (Bas) of women with a successful birth after uRPL (case) and controls. (A) Within the CD4+ T cell population CD25+FoxP3+ Treg cells are gated, Treg frequencies are plotted as are ratios between Treg cells and CD4-cm/em and Treg cells and CD8 cells. No significant differences are observed between cases and controls. (B) IF staining visualized CD3+ cells (red) and CD3+FoxP3+ double positive Treg cells (yellow). Treg counts per mm2 were not significantly different between cases and controls.

Literature studying women with a successful pregnancy after uRPL is limited and mainly focuses on peripheral blood rather than the local immune system. There are several studies focusing on decidual Tregs in women with uRPL: these studies have been put together in a meta-analysis by Keller et al. [8]. The meta-analysis shows that women with uRPL have less decidual Tregs compared to controls, both in the decidua and in peripheral blood. We found that women with a successful pregnancy after uRPL display similar Treg frequencies and numbers per mm^2 decidua tissue compared to controls. This may suggest that these women needed to establish normal (similar as controls) Treg frequencies to sustain a successful pregnancy.

Next to Treg frequencies we compared the Treg:CD4-Tcm/em and Treg:CD8 ratios between the cases and controls, which did not show a significant difference. However, Treg frequencies and Treg:Tcm/em ratio were increased in the decidua parietalis in the cases compared to the decidua basalis. It could be due to sample size that this was not significant in the control group. Previous studies also found higher Treg frequencies in the decidua parietalis compared to the basalis [12, 14]. Our group previously showed a difference in immune cell networks in the decidua basalis and parietalis. The network for the parietalis showed Treg cells in the same network as CD4-Tem and CD4-Tcm, while this was less apparent in the decidua basalis where the innate immune cells were predominantly present [12]. This indicates that different immune cell interactions in the basalis

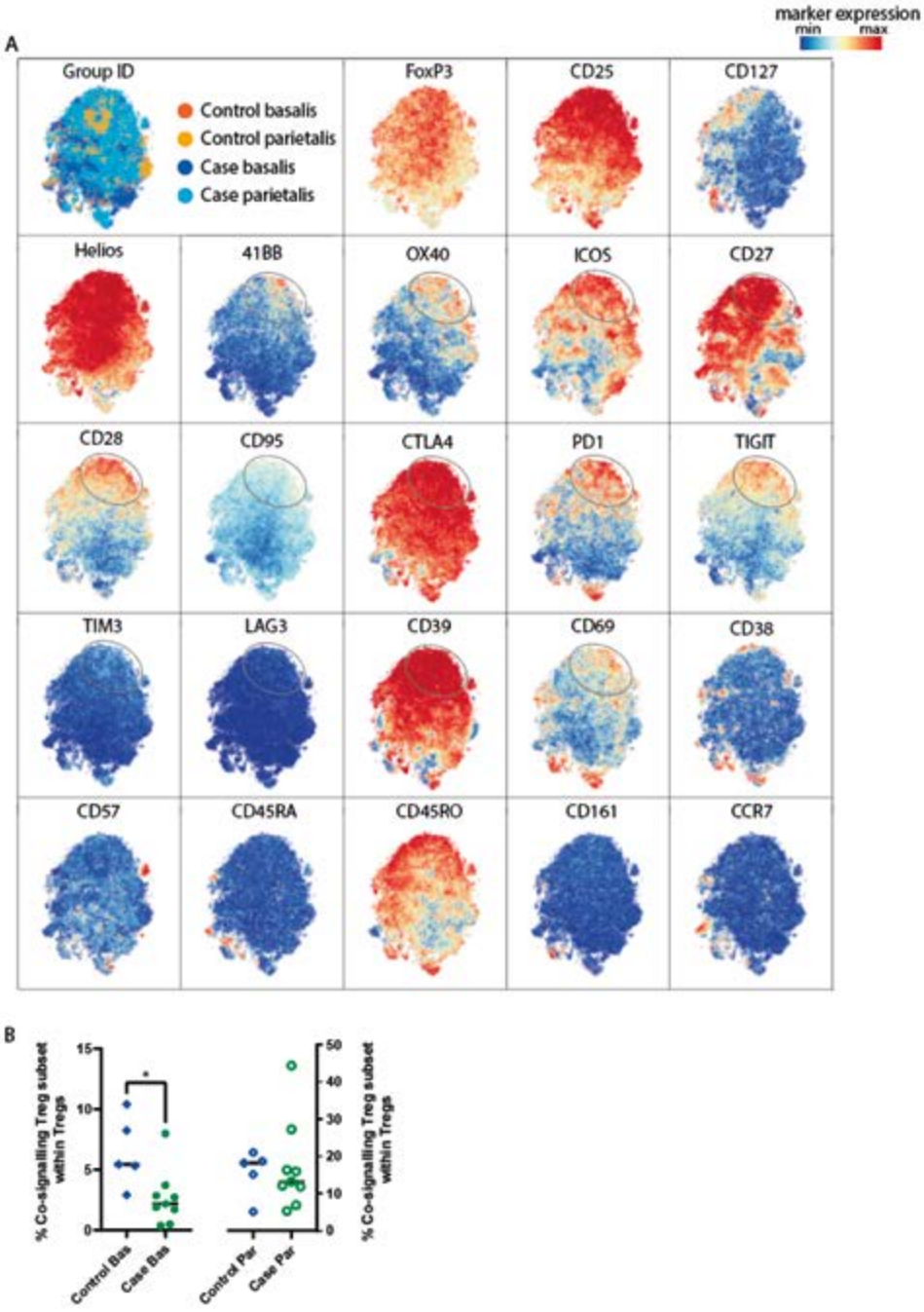

Figure 3. Treg phenotypes. (A) tSNE visualization of 23 markers from the T cells panel. Treg cells from the decidua basalis and parietalis, and cases and controls are equally distributed over the tSNE. Encircled is a cluster of cells that expresses several co-stimulatory as co-inhibitory (co-signaling Treg subset) molecules simultaneously. This co-signaling Treg subset shows significantly increased frequencies in the basalis of controls compared to the basalis of cases ($p = 0.012$, Mann-Whitney test).

compared to the parietalis contributes to the two different microenvironments. Other than a different immune cell composition [14], differences in collagen fibril composition [15], blood vessel presence, and density of lymphatic vessels [16] indicate that the basal landscape is vastly different from the parietalis.

When focusing on the Treg phenotype we noticed that the Tregs are Helios positive, which indicates they have a stable Treg phenotype [17, 18]. They express CD39 and CD69 suggesting they are highly active and suppressive [19-21]. Interestingly, we found a Treg subset expressing multiple co-stimulatory and co-inhibitory molecules. This Treg subset was present in lower frequencies in the decidua basalis in women with a history of uRPL compared to controls. Next to TCR recognition, co-stimulation is required for Treg development and function [22]. Initially, it was thought that only CD28 was the second signalling molecule next to TCR recognition. However, now it has become clear that there are many different co-signals that play a role in Treg activation and differentiation [23]. We measured multiple co-signalling molecules to get an idea of the potential functional capacity of the Treg cells. However, since the Treg subset we found expresses many different co-signalling molecules we cannot specify their potential functional capacity, as this can be very broad, depending on the cells they encounter, which ligands they have and other microenvironmental factors such as cytokine presence.

The procedure of counting the Treg cells in the IF slides highlighted the rarity of Treg cells in the decidua, and within this population only 5%-15% expresses a wide assortment of co-signalling molecules. Due to this low number, studying their functional capacity is paramount to determining their importance in creating an immune regulatory environment.

uRPL is a very heterogeneous group where likely not every couple has the same underlying cause. For that matter, high sample size could aid in defining different groups of women with uRPL. We acknowledge the limitations of our study, which include the few cases we studied, limiting the rigor of our findings based on environmental factors, mode of delivery and parity of our samples. Furthermore, in future studies paired miscarriage material could confirm if these women previously had reduced Treg frequencies which normalized in this pregnancy. A comment that needs to be made concerning the current study is that we looked at third trimester decidua, which has a different immunological environment than first trimester decidua [13]. Future research could use paired peripheral blood of women experiencing a pregnancy loss and following a subsequent successful pregnancy to see if Treg frequencies are already different in 1st trimester and if they increase in term pregnancy.

In summary, we found that Treg frequencies did not differ between cases and controls. We did observe a Treg subset expressing multiple co-stimulatory and co-inhibitory molecules, which has a lower abundance in decidua basalis of women with a history of uRPL compared to that of controls. Functional testing is needed to identify their functional capacity.

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SUPPLEMENTARY DATA

Supplementary Table 1. Suspension mass cytometry panel

intracellular	marker	metal	clone	cat#	Company	isotype	dilution	on reference sample
	CD45	89y	HI30	3089003B	Fluidigm	mouse	150	yes
	Live/death	103Rh			Fluidigm		500	yes
	CD45RA	114-qdot655	MEM-56	Q10069	Invitrogen	mouse	200	yes
yes	TGF-b	115In	TB21	MCA797	Bio-rad	mouse	40	no
	CCR6 (CD196)	141 Pr	G034E3	3141003A	Fluidigm	mouse	100	yes
	CD134 (OX40)	142 Nd	ACT35	350002	Biolegend	mouse	100	yes
yes	granzyme B	143 Nd	GB11	MA1-80734	Invitrogen	mouse	200	yes
yes	perforine	143 Nd	B-D48	MBS335051	mybiosource	mouse		yes
	CD69	144 Nd	FN50	3144018B	Fluidigm	mouse	300	yes
	CD4	145 Nd	RPA-T4	3145001B	Fluidigm	mouse	100	yes
	CD8a	146 Nd	RPA-T8	3146001B	Fluidigm	mouse	200	yes
	CD223 (LAG3)	147 Sm	11C3C65	369302	Biolegend	mouse	40	yes
	TIGIT	148 Nd	MBSA43	16-9500-82	Invitrogen	mouse	50	yes
	CD25	149 Sm	2A3	3149010B	Fluidigm	mouse	200	yes
yes	CD152 (CTLA-4)	150 Dy	BNI3	369602	Biolegend	mouse		yes
	CD278 (ICOS)	151 Eu	C398.4A	3151020C	Fluidigm	hamster	50	yes
	TCRgd	152 Sm	11F2	3152008B	Fluidigm	mouse	50	yes
	CD7	153 Eu	CD7-6B7	3153014B	Fluidigm	mouse	300	yes
	CD366 (TIM3)	154 Sm	F38-2E2	3154010B	Fluidigm	mouse	50	yes
	CD103	155 Gd	Ber-ACT8	350202	Biolegend	mouse	50	yes
	ILT2 (LILRB1)	156 Gd	GHI/75	3156020C	Fluidigm	mouse	100	yes
	CD20	157 Gd	2H7	302302	Biolegend	mouse	200	yes
	CD11c	157 Gd	Bu15	337202	Biolegend	mouse		yes
	CD137 (4-1BB)	158 Gd	4B4-1	3158013C	Fluidigm	mouse	50	yes
	CD197 (CCR7)	159 Tb	G043H7	3159003A	Fluidigm	mouse	100	yes

Supplementary Table 1. (continued)

intracellular	marker	metal	clone	cat#	Company	isotype	dilution	on reference sample
yes	Tbet	160 Gd	4B10	3160010C	Fluidigm	mouse	100	yes
	KLRG1	161 Dy	REA261	120-014-229	Miltenyi Biotec	human	50	yes
yes	FOXp3	162 Dy	259D/C7	3162024A	Fluidigm	mouse	75	yes
yes	EOMES	163 Dy	WD1928	14-4877-82	eBioscience	mouse	100	yes
	CD161	164 Dy	HP-3G10	3164009B	Fluidigm	mouse	200	yes
	CD127	165 Ho	AO19D5	3165008B	Fluidigm	mouse	400	yes
	CD39	166 Er	A1	328202	Biolegend	mouse	200	yes
	CD27	167 Er	O323	3167002B	Fluidigm	mouse	200	yes
yes	Helios	168 Er	22F6	137202	Biolegend	hamster	75	yes
yes	GATA3	169 Tm	REA174	130-108-061	Miltenyi Biotec	human	50	yes
	CD3	170 Er	UCHT1	3170001B	Fluidigm	mouse	150	yes
	CD28	171 Yb	CD28.2	302902 or 302937	Biolegend	mouse	150	yes
	CD38	172 Yb	HIT2	3172007B	Fluidigm	mouse	500	yes
	CD45RO	173 Yb	UCHL1	304239	Biolegend	mouse	150	yes
yes	RORyt	174 Yb	028-835	562197	BD Bioscience	mouse	40	no
	CD279 (PD-1)	175 Lu	EH 12.2H7	3175008B	Fluidigm	mouse	150	yes
	CD56	176 Yb	NCAM16.2	3176008B	Fluidigm	mouse	200	yes
yes	DNA1	191Ir			Fluidigm		2500	yes
yes	DNA2	193Ir			Fluidigm		2500	yes
	CD57	194 Pt	hcd57	322325	Biolegend	mouse	100	yes
	HLA-DR	198 Pt	L243	307602	Biolegend	mouse	150	yes
	CD95 (Fas)	209Bi	DX2	305602	Biolegend	mouse	100	yes