First-trimester trophoblasts obtained by chorionic villus sampling maintain tolerogenic and proteomic features in successful pregnancies despite a history of unexplained recurrent pregnancy loss

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Original publication available at:
https://doi.org/10.1111/aji.13314

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Title: First trimester trophoblasts obtained by chorionic villus sampling maintain tolerogenic and proteomic features in successful pregnancies despite a history of unexplained recurrent pregnancy loss.

Running head: Immune profile of ex vivo trophoblasts in RPL

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Abstract

Problem. While there are several known causes for recurrent pregnancy loss (RPL), about 50% are unexplained (uRPL), and in these cases an aberrant immune regulation seems to be involved. Although fetally derived trophoblast cells have a key role in immune regulation, it is difficult to study their immune function during pregnancy, and it is not known if trophoblast function may be an inherent aberration in uRPL or if it is associated with the outcome of the current pregnancy.

Method of study. Chorionic Villus Sampling (CVS) was performed for clinical indications at 12 weeks of gestation. Superfluous materials, divided in small explants, were cultured for 20-24h, and supernatants (conditioned medium) were collected from 36 women with singleton normal pregnancies, of whom 9 women had a history of RPL. The secreted immune protein profile was measured by proximity extension assay and the conditioned medium was further used in functonal *ex vivo* models to assess ability to polarize blood monocytes and CD4+ T cells into immune regulatory phenotypes, as detected by flow cytometry.

Results. Conditioned medium from chorionic villi, human fetally derived placental tissue, was able to induce a decidual-type of M2 like macrophages, as well as an expansion of Treg cells *ex vivo*, both in women with uRPL and in control women. The preserved immunological properties were confirmed by a maintained immune protein profile in RPL compared with controls.

Conclusions. Trophoblasts in an *ex vivo* model maintain tolerogenic and proteomic profile features in successful pregnancies, despite a previous history of RPL.

Keywords: Recurrent pregnancy loss, chorionic villus sampling, proteomics, fetal-maternal immune tolerance, macrophages and regulatory T-cells

Introduction

Recurrent pregnancy loss (RPL) is a very distressing early pregnancy complication, which affects 1-3% of pregnancies (1). There are several known causes for RPL, including parental chromosomal and uterine abnormalities, hormonal factors, major thrombophilia, autoimmune diseases and antiphospholipid antibody syndrome. However, 50-60 % of RPL are still unexplained (uRPL), and in these cases an aberrant immune regulation is thought to be involved (2). It is not known if the aberrant immune regulation is an inherent general and constant property, or if it is associated with the immune regulation of each pregnancy.

Immune regulation in pregnancy is an interplay between fetal and maternal factors. Fetally derived trophoblast cells infiltrate the decidualized endometrium and have a key role in initiating and maintaining tolerance (3). In order to regulate trophoblast invasion, and to promote fetal tolerance and homeostasis, the decidua is composed of a unique set of immune cells with specialized properties (4). Macrophages and regulatory T (Treg) cells are both enriched in the decidua, and hold a central position in fetal tolerance establishment (4–6). We have previously demonstrated that fetally derived placental explants were able to induce M2-like macrophages of a decidual phenotype, as well as immunosuppressive Treg cells (3). We further showed that the trophoblasts were the responsible cells for these effects, in part by secretion of M-CSF, IL-10, TGF-β and TRAIL. The *in vitro* model was based on placental tissues from voluntary termination of pregnancy in normal pregnancies. However, it is not known if these properties are maintained when using placental tissue from ongoing pregnancies, and whether the properties may be altered in a pathological condition. In uRPL, a compromised trophoblast function has been suggested (7,8), mainly involving multiple aberrant signal transduction pathways (7,9–21), while their potential immunological dysfunction in uRPL has not been explored.

Chorionic villus sampling (CVS) offers a possibility for *ex vivo* studies of trophoblast functions. CVS is an invasive prenatal diagnosis technique, which allows in a very early stage of pregnancy to diagnose potential fetal chromosomal abnormalities, by collecting and analyzing chorionic villi. The superfluous tissue, not used for clinical purposes, can be used for research aims.

This study aims to use CVS as a source of placental tissue from ongoing pregnancies to; (1) evaluate if CVS can be used as an *ex vivo* model of trophoblast functional aspects; (2) assess whether or not the immune modulatory properties of trophoblasts are maintained in successful pregnancies, despite a history of uRPL; and (3) characterize the secreted proteomic profile of placental tissues obtained by CVS.

Materials and Methods

Subjects

This prospective study involved 36 pregnant women at 12 weeks of gestation referred to the Prenatal Diagnosis Unit of Tor Vergata University Hospital between 2014 and 2017 in order to undergo CVS. Gestational age was calculated on the basis of the last menstrual period and was confirmed by ultrasonography.

Women were divided into two groups: 27 healthy women with singleton normal pregnancy and without obstetrics complications in their past obstetrical history, and 9 women with a history of RPL, now presenting with a singleton normal pregnancy. RPL was defined as 3 or more consecutive pregnancy losses before 20th week of gestation. Clinical characteristics are presented in Table 1. Since 2 women had minor thrombophilia and 3 had hypothyroidism, these cannot in a strict sense be considered as uRPL, although it is uncertain whether these conditions were causing the RPL (22). In the remaining 4 women in the RPL group, no explanation to RPL was found despite thorough investigation using an extended protocol (23). Among the 27 control women, all samples were used

in the proteomics assay, while 9 samples were used for functional studies, selected to match samples

from the 9 RPL women with regards to age. The total groups (Table 1) were matched for age, BMI,

smoking behavior and consistently nullipara were excluded from the study control group.

Main indications for CVS in our cohort were: advanced maternal age, defined as >35 years of age

(n = 26), maternal/paternal increased risk for genetic inheritable diseases (n = 2), balanced

chromosomal abnormalities (n = 3) and genetic disease carriers (n = 3), ultrasonographic soft markers

suspected for chromosomopathies (n = 2). No woman in our study had a previous pregnancy with

fetal chromosomal abnormalities. All fetuses from pregnancies included in the study had a normal

chromosomal map and were negative in a genetic disease test carried out based on the family risk

history.

The pregnancies were followed prospectively to assess their outcomes: all women from the two

groups had an uncomplicated, at term pregnancy. The study was approved by the Institutional Review

Board (protocol number: R.S. 11/16) of Tor Vergata University Hospital, Rome, Italy, and a written

consent was obtained before performing CVS from each eligible patient. The healthy control women

also signed a written consent before donating blood as approved (M39-08) by the ethics committee in

Linköping, Sweden.

CVS technique

An ultrasound Eidos Hitachi equipped with a transabdominal 3.5-MHz Biopsy Convex Probe was

used to guide CVS. All the procedures were done transabdominally by the same expert operator (AP)

with the patient placed in a supine position. An 18 G needle was used based on a common clinical

protocol approved to obtain a proficient material quality assay. The skin was sterilized with topical

application of povidone-iodine. The needle was flushed with 0.5 ml heparin before the procedure. An

appropriate site was selected during a preliminary ultrasound examination, and then a needle was

introduced under ultrasound guidance until the placenta was reached. Once the needle tip was in

place, the stylet was withdrawn and a 10-ml syringe with 5 ml of culture medium was attached to the

needle hub. Aspirations of chorionic villi were performed in a manual mode. A tissue sample was

obtained by moving the plunger of the syringe upward in order to create the discontinuous negative

pressure necessary, while the needle was advancing through the chorion. Then the needle was moved

forward and backward to obtain adequate specimens from various sites. A check for fetal well being

was performed at the end of each procedure. Villous sampling was performed in all cases with a

single needle insertion and an amount of about 50 mg of sample (range 40–60) was sent for

cytogenetic analysis. Superfluous material, which remains after the routinely clinical investigations,

has been used as explants to collect the conditioned medium.

CVS explants culture

Immediately after CVS collection, the decidua was removed, and the fetal placental tissue was

processed. The fetal part of the placenta (simply called "placenta" in this paper) was rinsed with

sterile saline to remove traces of maternal blood, and washed in sterile PBS. The CVS specimens

were handled according to our previous protocol for placental explants (3), thus dissected and placed

in 24-well plates with culture medium consisting of RPMI 1640 (Life Technologies-Invitrogen,

Burlington) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (PAA Laboratories,

Paris, France) and 1% PEST/L-glutamine (Life Technologies- Invitrogen). A total of ∼50–100 mg of

wet tissue was added to each well, pre-filled with 10 µl culture medium/mg tissue. The CVS explants

were incubated for 20–24 h at 37 °C and 5% CO_2. The conditioned medium (CM) was collected,

centrifuged, and stored in aliquots at −70 °C.

Functional studies of macrophages and T cells.

For the *ex vivo* assays, blood samples were collected at Linköping University Hospital (Linköping,

Sweden) from 9 healthy non-pregnant female volunteers, between 18–45 years of age, not taking

hormonal contraceptives or any other medication. A written informed consent was obtained from all

subjects. All experiments were performed in accordance with the Helsinki Declaration ethical principles for medical research.

Isolation of blood cells

Peripheral blood mononuclear cells (PBMCs) were isolated on a LymphoPrep gradient (Axis-Shield, Dundee, Scotland, UK), according to the manufacturer's instructions, followed by washing in HBSS (Invitrogen). Isolated PBMCs were used for isolation of CD14$^+$ monocytes or CD4$^+$ T cells by positive selection using immunomagnetic cell sorting. PBMCs were resuspended in sterile MACS buffer (phosphate buffer saline (PBS) supplemented with 2 mM EDTA (Sigma-Aldrich, Saint Louis, Missouri, USA) and 0.5% fetal bovine serum (FBS)), and the CD14$^+$ or CD4$^+$ cells were isolated with anti-CD14 or anti-CD4 mAb-coated MicroBeads, according to the manufacturer's protocol, using MS MACS columns (all from MiltenyiBiotec, BergischGladbach, Germany).

Cell cultures of macrophages and CD4$^+$ T helper cells

To analyze the effects of CVS-CM on Treg induction and macrophage polarization, CVS-CM was added to either CD4$^+$ Th cells at the concentration of 12.5% or macrophage cultures at two different concentrations: 12% and 25%. The models of macrophage and Th cell activation and polarization have been optimized and used in previous studies from our group (3,24,25).

Macrophages were generated from CD14$^+$ monocytes, as previously described (3,24) with the exception of using 96-well instead of 24-well plates, and corresponding adjustments of cell numbers. Recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF, 5 ng/ml) or macrophage colony-stimulating factor (M-CSF, 50 ng/ml) (both from PeproTech, Rocky Hill, United States) were used as basic growth factors with or without adding CVS-CM. Cells were cultured at a density of 100,000 cells/well in 100 μl cell culture medium for 6 d at 37 °C and 5% CO_2. TrypLE enzyme was used to detatch macrophages from the bottom of the plates. In macrophage cultivation

experiments, cells from 9 RPL women and 9 healthy controls were used, and exposed to GM-CSF, M-CSF, 12% CVS-CM and 25% CVS-CM in the same experiment.

To analyze the effects of CVS-CM on Th cells, isolated CD4$^+$ T cells were cultured as previously described (3), in the presence or absence of CVS-CM, at a density of 100,000 cells/well in 100 µl T cell culture medium, consisting of IMDM (Invitrogen) supplemented with L-glutamine (292 mg/ml; Sigma-Aldrich), sodium bicarbonate (3.024 g/l; Sigma-Aldrich), penicillin (50 IE/ml), streptomycin (50 mg/ml) (Cambrex Corporation, East Rutherford, NJ, USA), 100× nonessential amino acids (10 ml/l; Invitrogen), 5% heat-inactivated FBS, for 3 d at 37 °C and 5% CO_2. In T cell experiments, cells from 9 RPL patients and 9 healthy controls were used.

Flow cytometry staining and analysis

Cells were resuspended in PBS supplemented with 0.1% FBS (PBS 0.1% FBS) and stained with Abs for extracellular staining and their corresponding isotype controls (for Ab details, see Supplemental Table I) for 30 min at 4 °C in the dark. PBS 0.1% FBS was added, followed by centrifugation at 500 g for 5 min. The cell pellet was resuspended in PBS 0.1% FBS for final flow cytometric analysis. LIVE/DEAD™ Fixable Aqua Dead Cell Staining (TermoFisher Scientific) was used to assess cell viability, according to manufacturer´s instructions. After extracellular staining, cells were permeabilized by using the Foxp3 staining kit (eBioscience), followed by staining with anti-human Foxp3 (for Ab details, see Supplemental Table I) for 30 min at 4 °C. After washing, cells were resuspended in PBS 0.1% FBS. Data were acquired using a FACSCanto II and analyzed with FACSDiva software version 6.1.2 (BD Biosciences) or Kaluza software version 1.1 (Beckman Coulter, Fullerton, California). Isotype controls were used to set the cut-off for macrophage markers. The CD25^{high} gate was set according to a slightly lowered expression of CD4 on CD4$^+$ cells (CD4^{dim}) (25).

Proteomics assay

The Olink® Inflammation panel kit (https://www.olink.com) was used for the proteomics assay of CVS-CM. This panel includes 92 inflammation-related protein biomarkers, involved in several biological processes. as categorized by GO terms; apoptotic processes, cell activation, cell adhesion, cellular response to cytokine stimulus, chemotaxis, extracellular matrix organization, inflammatory response, MAPK cascade, regulation of immune response, response to hypoxia and secretion.

The Olink® technology is based on the proximity extension assay (PEA). In the first step, 92 oligonucleotide labeled antibody pairs bind their target proteins. If the pair of antibodies bind their protein in close proximity, a hybridization of the reporter oligonucleotides takes place. The product is amplified and detected by real-time qPCR. Thus, the requirement of dual antibody binding secures a high specificity and the PCR amplification secures a high sensitivity of the PEA.

Protein levels are linearized on a log2 scale and expressed as Normalized Protein eXpression (NPX) units. The detection limit was defined as 3 SD above the background. For proteins under the lower limit of detection, half of the detection limit value was assigned to the samples. Proteins with more than 50% of values under the detection limit were excluded from the analysis (CCL11, CCL19, CCL25, CD6, CX3CL1, FGF-5, FGF-23, IL2, IL4, IL5, IL7, IL-10RA, IL13, IL-15RA, IL-17A, IL-17C, IL-20, IL-22RA1, NRTN, NT-3, SLAMF1, TRANCE). Included proteins, which were detected in more than 50% of the samples, are listed in Figure 3. All samples were analyzed by the Clinical Biomarker Facility Unit at SciLifeLab in Uppsala, Sweden. In the proteomics assay, CM from 9 RPL patients and 27 healthy controls were used.

Data analysis and statistics

Kolmogorov-Smirnov test was used to analyze data distribution. The majority of the flow cytometry and proteomics data was normally distributed and therefore analyzed with parametric tests. One-way ANOVA followed by the Sidak's post hoc test for paired data was used to compare flow cytometry data, and two-tailed Student´s t-test was used to compare protein levels, in women with and without a history of RPL. The Pearson's test was used for testing of correlations between parameters.

Differences were considered statistically significant for p value < 0.05. Data are expressed as mean

and standard deviation (SD). All data were analyzed using GraphPad Prism version 8.2.0 (La Jolla,

CA, USA).

Results

Effects of CVS-CM on macrophages polarization

To assess the effects of CVS-CM on macrophage polarization, CD14$^+$ blood monocytes, isolated

from non-pregnant healthy women, were cultured with GM-CSF in the presence or absence ofCVS-

CM. GM-CSF was used to induce an M1-like phenotype (mainly pro-inflammatory), in order to asses

if CVS-CM can reverse the M1 phenotype and increase expression of M2 phenotype markers. GM-

CSF-macrophages generated in the presence of 12% and 25% CVS-CM, both from controls and RPL

patients, acquired an M2 decidual macrophage-like phenotype with increased proportions of cells

expressing CD14, CD163, CD209, while the proportion of CD86$^+$ cells was lower (statistically

significant at 25% CVS-CM in the control group), compared with GM-CSF-macrophages not

exposed to CVS-CM. The proportions of cells expressing CD206 and HLA-DR were not affected by

CVS-CM. The polarization effect was more prominent with 25% CVS-CM (Figure 1). However, no

differences were found when comparing CVS-CM from women with RPL to CVS-CM from controls.

No differences were found in the viability of the macrophages across the different groups (data not

shown). M-CSF was used as a control for M2 polarization, and always induced a CD14^{high}, CD163$^+$,

CD209$^+$ population (data not shown).

Effects of CVS-CM on Treg cell induction

To assess the effects of CVS-CM on Th cells, we cultured CD4$^+$ T cells from healthy non-pregnant

women in the presence or absence of CVS-CM. CD4$^+$ T cells exposed to CVS-CM showed an

increased proportion of CD25^{high}Foxp3$^+$ Treg cells (Figure 2), both from controls and RPL patients. No differences were found in CD25^{high}Foxp3$^+$ Treg cells proportions, when exposed to control CVS-CM versus RPL CVS-CM. No differences were found in Treg cell viability across the different groups (data not shown). Furthermore, since there was a variation in induction level, we tested but found no correlations between induction levels of CD25^{high}Foxp3-expressing Treg cells and CD163-expressing macrophages, indicating that there is no general defect in quality of the tissues since they are able to influence at least one of the two cell types.

Proteomics data

For 22 of the 92 proteins, undetectable levels were observed in more than 50%, as described above (Methods). Among the remaining 70 proteins, no differences were found between CVS-CM from controls and women with RPL. The levels of the measurable proteins are presented in Figure 3.

Discussion

We have shown that conditioned medium from normal pregnancy chorionic villi, human fetally derived placental tissue consisting of trophoblast cells, are able to induce an M2 like-phenotype and an expansion in the Treg population in an *ex vivo* model. These immunological properties are maintained in women with RPL. In line with this, the inflammation proteomic profile did not differ between the RPL and the control group.

The finding that CM from fetal tissues was able to induce an M2-like and Treg polarization profile is in agreement with our previous findings (3), showing the important function of trophoblast cells to induce a tolerant and homeostatic environment at the maternal side (4–6,24,26–33). Of note, CM from women with RPL did not differ compared with controls, neither in functional aspects, nor in the

proteomic profile. This may seem to be an unexpected finding since a dysregulation in trophoblasts function in RPL has been suggested in previous studies. However, there are several possible explanations for the lack of aberrations in RPL. It may be that the fetal/placental side is not dysfunctional in RPL, and from our study we cannot exclude dysfunction at the maternal side (34,35). It should also be noted that these patients have already gone beyond the 12 weeks of gestation, and therefore they succeeded in carrying on the pregnancy beyond the highest incidence period, since miscarriages in RPL mostly occur earlier, with peaks at 6 and 8 weeks (36,37). Accordingly, previous studies showing a dysregulation in trophoblast functions were carried out in tissues collected before the 12th of gestation. Furthermore, it has been shown that there is an immunological memory acquired for each pregnancy (38,39), and if no etiological causes can be identified for RPL, the general prognosis of subsequent pregnancies for unexplained RPL is good (40,41). It should be taken into account that unexplained/idiopathic RPL could be considered as a chance occurrence given the high general frequency of pregnancy losses (42). Saravelos et al. (43) stratify idiopathic RPL patients in two subgroups, women with RPL that probably has occurred predominantly by chance, without any specific underlying pathology, and women with uRPL that occur due to an underlying pathology. While the first group has a good prognosis for subsequent pregnancy live birth, conversely the second one has a poorer prognosis. It could therefore be that in our study we unwittingly selected patients included in the first group due to the study design, since CVS is performed after the 12 weeks of gestation, when the major incidence of miscarriage had already passed, and all of them has, therefore, an uncomplicated, at term pregnancy. Taken together, the similar functional and proteomic properties of trophoblast cells from RPL and control women show that beneficial functional effects are associated with a successful pregnancy, arguing against an inherent and constant trophoblast dysfunction in RPL.

The *ex vivo* model we used offers a possibility to investigate local immunological properties in an ongoing pregnancy, where the outcome of the pregnancy will be known. This is in contrast to previous

studies using materials from terminated pregnancies, both for controls in a setting of voluntary termination of pregnancies when the outcome of the pregnancy is not known, and in missed abortions when it is not known if aberrations occur primary or secondary to the miscarriage. Limitations in using the CVS model include: (1) it is not possible to perform this technique before the 12th week of gestation, when the majority of pregnancy losses occur; (2) CVS is an invasive prenatal diagnostic technique, with strict clinical indications and not widely used in clinical practice. Nevertheless, our *ex vivo* model has gained a deeper knowledge in local immune regulation and the secreted protein profile of trophoblast cells in successful pregnancies. A potential possibility would be to use CVS specimens from women that turn out to have a later complication during pregnancy, and thereby reveal aberrations in trophoblast function that could lead to subsequent immune pathology. Indeed, this approach was previously implemented, and revealed immunological aberrations in the first trimester of women that later developed gestational hypertension (35).

The protein profiling data support the preserved immunological trophoblast function in RPL condition, since surprisingly no differences at all were found in the inflammation proteomics panel between controls and RPL. For protein profiling, we used a new technique, PEA, which combines a high specificity with a high sensitivity (44), thus providing reliable mapping of secreted (and not secreted) proteins by trophoblast cells from normal pregnancy. As expected, several of the undetectable proteins are linked to T-cells; IL-2, IL-4, IL-5, IL-13, IL-17A, IL-17C, IL-22RA, and CD6. These results are in line with our previous study, in which IL-13 and IL-17A, associated with Th2 and Th17 cells, respectively, were not produced by fetally derived placental tissue (3). According to the traditional paradigm, a successful pregnancy is associated with a skewed immune response towards a Th2 and Treg associated profile (6,29–32). Nevertheless, in contrast to the traditionally proposed concept to consider pregnancy a Th2 phenomenon (45), the polarization of decidual macrophages and Treg cells seems to be driven by a general and Treg-associated tolerogenic microenvironment, rather than being driven by Th2 cells. This revised concept is supported by the

following: (1) IL4 and IL13-induced macrophages have a different phenotype from decidual macrophages (24), (2) Treg cells rather than Th2 cells are enriched in the first-trimester decidua (46), (3) IL-10 levels are higher at the fetal-maternal interface, compared with IL-4 and IL-13, as shown previously (3,47), and supported by the present findings Interestingly, IL-20, which has proinflammatory activities that are antagonized by IL-10, one of the key factors responsible for the establishment of a tolerant uterine environment (48), was undetectable in CVS-CM. Accordingly, IL-10 was detectable in CVS-CM in our analysis, which is in line with a IL-10- induced function in polarizing homeostatic macrophages and Tregs, in agreement with our previous functional studies (3). The trophoblast-induced macrophages phenotype is induced not only by IL-10, but also by M-CSF, which is to a high extent produced by trophoblast cells (3), confirmed in our present study. Furthermore, we previously reported (3) that placental explants/trophoblast cells from terminated 1st trimester pregnancies were able to expand Treg cells *in vitro* by a co-adjuvant action of IL-10, TGF-β and the apoptosis-inducing factor TRAIL (49,50). We here confirm the secretion of these factors in our ex-vivo model of trophoblast cells from CVS-CM. The mechanism of TRAIL-induced Treg expansion seems to be inhibition of the non-Treg cell pool expansion in a mouse model (50). In addition, LIF, another important factor for fetal-maternal tolerance and involved in the induction of Treg cells (51,52), was found to be present in our protein profiling of CVS-CM.

CCL2 was among the top secreted proteins. CCL2 is highly produced also by decidual macrophages (24), as well as by trophoblasts, as we showed previously (3). CCL2 is able to recruit and activate Th2 cells and is involved in M2-associated fetal-maternal tolerance, by reducing production of proinflammatory cytokines from macrophages (24,53). CCL20 and CXCL1 were also detectable in our proteomic CVS-CM profile; these chemokines are both induced by IL-17, and CCL20, in turn, is responsible for Th17 cells recruitment (24)(54–58). The Th17/Treg cells ratio paradigm is crucial also in a pregnancy immune setting, and enhanced Th17 or reduced Treg cells responses have been associated with pregnancy complications, such as recurrent pregnancy loss, preterm birth and

preeclampsia (32). Nevertheless, Th17 and Treg cells show a wide extent of plasticity in different

"inflammatory frameworks", so that they can switch to one another (32). At a lower level of detection,

also Th1-associated proteins were detected in the CVS-CM profile, including IFN-γ and CXCL9-11,

which emphasizes that controlled inflammation takes part in the fetal-maternal regulatory process.

Indeed, CXCL9-11 are involved in the recruitment of NK-cells (59), which are abundant in the

decidua, and in turn regulate trophoblast invasion (60). It should be pointed out that information

acquired by our CVS-CM proteomic panel offers a library for confirming presence of trophoblast-

derived proteins and further functional studies in fetal-maternal immune tolerance, both regarding

well-known, as well as new potential regulatory proteins in physiological and pathological condition

in pregnancy.

Conclusions

(1) An *ex vivo* model based on CVS-derived trophoblasts was established for evaluation of immune

properties and profiling of secreted proteins. (2) Trophoblasts maintained their tolerogenic and

proteomic profile features in successful pregnancies, despite a history of RPL. (3) The proteomic

profile confirmed the secretion of several central immune regulatory proteins and displayed new

candidate regulatory proteins.

expression in unexplained recurrent miscarriage). We would like to acknowledge the support of the

Clinical Biomarker Facility at SciLifeLab Sweden for providing excellent assistance in protein

analyses.

Declaration of interest statement

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Table 1. Demographic and clinical characteristics

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		RPL (n = 9)	CONTROLS (n = 27)	p VALUE*
Age (years)		37 ± 4.4	38 ± 3.2	0.45 [a]
BMI (Kg/m^2)		22 ± 1.4	23 ± 3.8	0.24 [a]
Number of previous miscarriages		3.1 ± 0.3	NA	NA
Week for previous miscarriages		7 ± 1.9	NA	NA
Previous pregnancy at term		3 (33)	20 (74)	0.04 [a]
Smoking		1 (11)	3 (11)	0.99 [b]
Minor trombophilia		2 (22)	1 (4)	0.15 [b]
Dysthiroidism		3 (33)	0	0.01 [b]
Celiac disease		0	1 (4)	0.99 [b]
Therapy during pregnancy	Corticosteroids	2 (22)	0	0.035 [b]
	Progesterone	2 (22)	0	
	Low dose aspirin	3 (33)	0	
	Thyroid hormones	3 (33)	1 (4)	
Clinical indications for CVS	Maternal age	5 (56)	21 (56)	<0.0001 [b]
	Previous chromosomal abnormalities	/	/	
	Genetic inheritable diseases	0	2 (7)	
	Balanced chromosomal abnormalities	3 (33)	0	
	Genetic disease healthy carriers	0	3 (11)	
	Ultrasonographic soft markers	1 (11)	1 (4)	
Gestational week of the delivery		39.3 ± 1.7	39.1 ± 1.4	0.60 [a]
Birth weight		3268 ± 278.1	3334.5 ± 335.9	0.28 [a]

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Data are expressed as Mean ± SD or n (%).
NA. Not Applicable
* Student´s t-test[a] or Fisher's exact test[b].

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Figure legends

Figure 1. Effects of Chorionic villi conditioned medium on macrophage polarization.

Blood monocytes from healthy non-pregnant women were differentiated to macrophages for 6 days with GM-CSF in the absence or presence of 12% or 25% chorionic villous sampling conditioned medium from control (ctrl) women (n=9) and from women with recurrent pregnancy loss (RPL, n=9). Phenotypic characterization by flow cytometry, expressed as % of macrophages. Data are shown as mean and S.D. One-way ANOVA and Sidak' s test: *$p < 0.05$; **$p < 0.005$; all p values refer to comparisons with GM-CSF only.

Figure 2. Effects of Chorionic villi conditioned medium on regulatory T cells.

a) CD4+ T-cells isolated from healthy non-pregnant women were cultured 5 days in tissue culture medium (TCM) in the absence or presence of 12% or 25% chorionic villous sampling conditioned medium (CVS-CM) from control (ctrl) women (n=9) and from women with recurrent pregnancy loss (RPL, n=9). Phenotypic characterization of Treg cells by flow cytometry, expressed as % of CD4+ T-cells. Data are shown as mean and S.D. One-way ANOVA and Sidak' s test: *$p < 0.05$; **$p < 0.005$; all p values refer to comparisons with tissue culture medium (TCM) only.

b) - c) Bivariate correlations between levels of CD25^{high}Foxp3-expressing Treg cells and CD163-expressing macrophages are shown in panel b) in presence of 12% of CVS-CM and in panel c) in presence of 25% of CVS-CM.

Pearson's test: n.s.

Figure 3. Proteomics assay data.

Levels of detectable immune-related proteins in chorionic villous sampling-conditioned medium (CVS-CM) from controls (c, n=27) and from women with recurrent pregnancy loss (rpl, n=9). Values are expressed as linearized log2 scale NPX (Normalized Protein eXpression) and sorted by size in the control group. Data are shown as mean and S.D. two-tailed Student´s t-test: n.s.
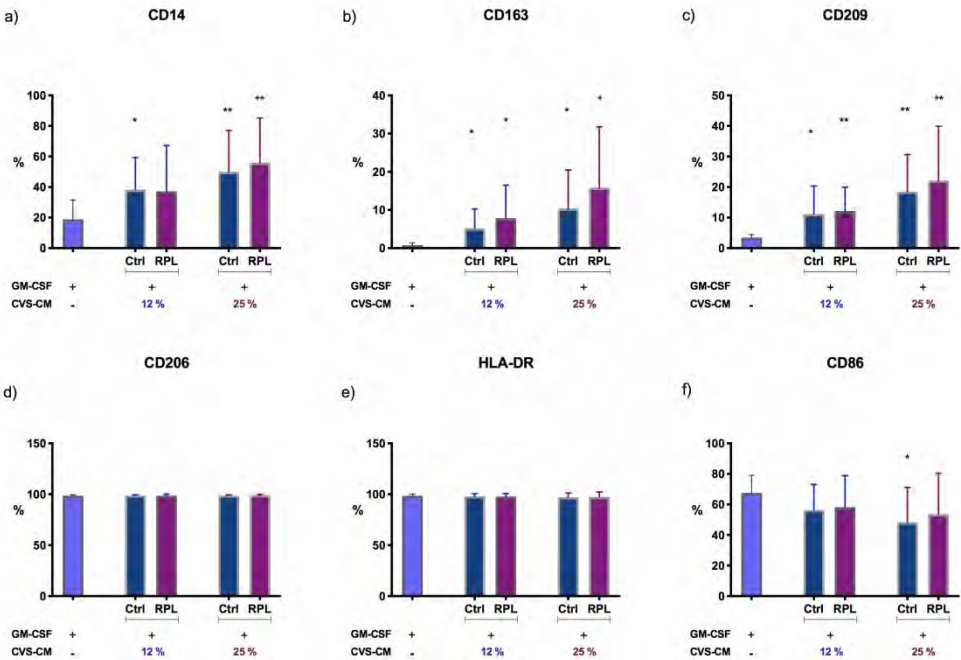
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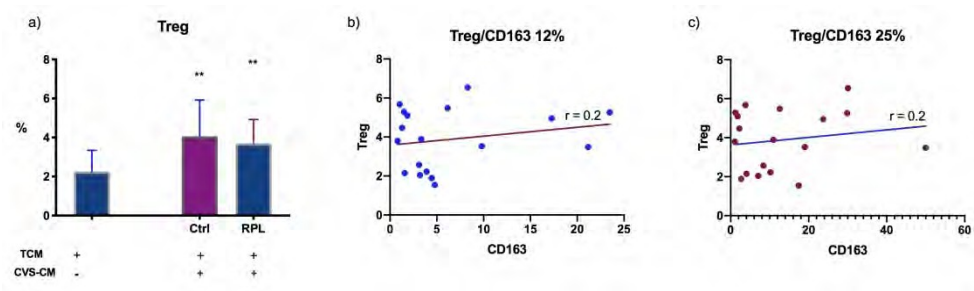
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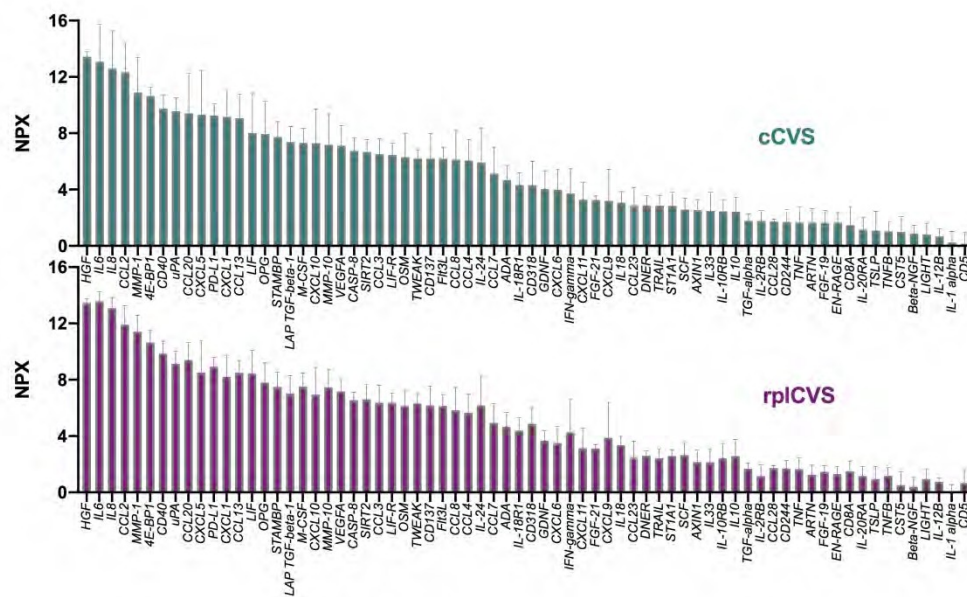
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Supplemental table I. Antibodies used for flow cytometry.

Cell type	Antigen	Isotype	Clone		Fluorochrome	Manufacturer	
			Antigen	Isotype		Antigen & Isotype	Clone
Macrophages	HLA-DR	Mouse IgG2b	L243	27-35	FITC	BD Biosciences	
	CD 163	Mouse IgG1	GHI/61	X40	PE	BD Biosciences	
	CD 206	Mouse IgG1	19.2	X40	APC	BD Biosciences	
	CD 209	Mouse IgG2b	DCN46	27 35	PerCP-Cy5.5	BD Biosciences	
	CD86	Mouse IgG1	2331/FUN-1	X40	V450	BD Biosciences	
T helper cells	CD4	/	SK3	/	FITC	BD Biosciences	
	CD25	/	B1.49.9	/	PC-7	BioLegend	Beckman Coulter
	Foxp3	/	PCH101	/	PE	eBioscience	