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"Science knows no country, because knowledge belongs to humanity"

Louis Pasteur

International Congress of Sericulture, Milan, 1876

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GENERAL INTRODUCTION

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1 GENERAL INTRODUCTION

Pregnancy is a fascinating immunological paradox. During pregnancy the semi-allogeneic fetus generally grows without any complications. In any other situation contact with non-self-antigens would generate an immune reaction aimed to destroy the foreign invaders, such as with pathogens, solid organ transplantation, and cancer. However, in pregnancy the maternal immune system adapts to accept the fetus with its paternally inherited antigens.

It is believed that either inaccurate or inadequate adaptations of the maternal immune system to the pregnancy situation could lead to problems with the placenta function. Since the main function of the placenta is to provide oxygen and nutrients to the developing fetus, inadequate placental development could lead to complications with the fetus during pregnancy such as miscarriage or fetal growth restriction.

THE CONCEPT OF MATERNAL-FETAL IMMUNE TOLERANCE

The first to show that the existence of immunological tolerance in pregnancy was Sir Peter Medawar in 1953 [1]. Medawar recognized the paradoxical nature of the maternal immune system accepting an antigenically foreign body to grow for months during pregnancy [2]. Medawar proposed some possible explanations: *"The reasons why the foetus does not habitually provoke an immunological reaction from its mother may be classified under three headings: (a) the anatomical separation of foetus from mother; (b) the antigenic immaturity of the foetus; and (c) the immunological indolence or inertness of the mother"[2].* These three proposals have spearheaded many scientific endeavors resulting in the current field of reproductive immunology.

During more than half a century that followed it has conclusively been shown that the three hypotheses of Medawar are invalid [2]. Firstly, there is no anatomical separation of the fetus from the mother. There is direct contact between maternal immune cells and fetal cells at several sites in the placenta, as will be discussed below. Furthermore, immune cells travel from the mother to the fetus and visa-versa: this is referred to as microchimerism [3, 4]. Secondly, the fetus is not antigenically immature, and the maternal immune system is able to respond adequately to fetal cells [5]. The maternal immune system can recognize paternally inherited antigens, including human leukocytes antigens (HLA) [6]. Thirdly, the maternal immune system during pregnancy is not inert, as she still is able to mount an immune response against pathogenic infections and fetal cells can be recognized [5].

INTERACTION SITES IN THE PLACENTA

Maternal-fetal cell interactions during pregnancy mainly occur in the placenta. This is where fetal trophoblast cells encounter maternal immune cells. Throughout gestation interaction sites with the fetal cells change (Figure 1).

During implantation the blastocyst adheres to the uterine lining and invades into the endometrium, which subsequently transforms into the decidua [7]. The developing embryo

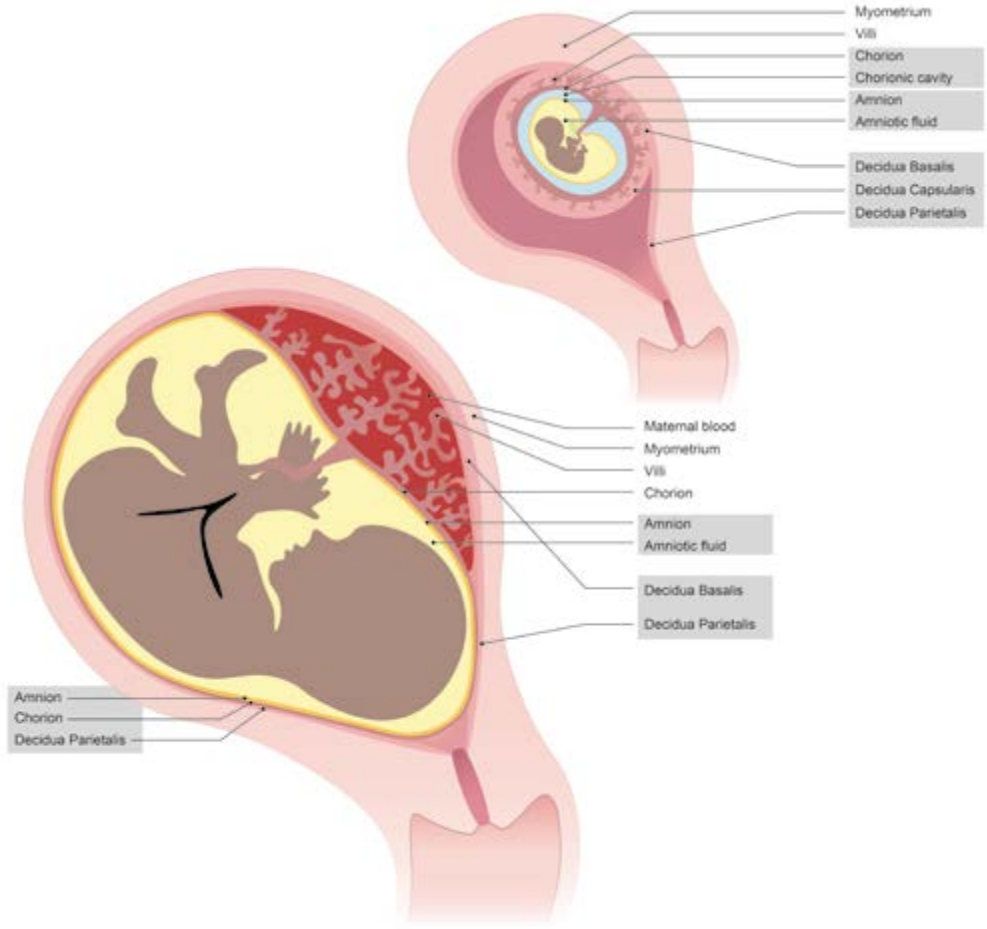

Figure 1. Schematic overview of anatomical changes of the maternal-fetal interaction sites. From (A) late first trimester (~8 weeks) to (B) term.

becomes completely encapsulated by the maternal decidua that had contact with the fetal syncytium, which will become the trophoblast cells. At the site of implantation, the decidua basalis will develop, where on the other side it becomes thinner and is named decidua capsularis (Figure 1A). The decidua parietalis is the uterine lining, which at the beginning of pregnancy is not yet in contact with fetal cells. During growth of the fetus the decidua capsularis and parietalis fuse together (now named decidua parietalis). (Figure 1B). The decidua parietalis is in direct contact with fetal trophoblast cells from the chorion leave. The last layer is the amnion, within this jelly the fetus resides in the amniotic fluid. Later in pregnancy this layer fuses with the chorion, combining the decidua parietalis, chorion and amnion in a single membrane (Figure 1 and 2).

During the early encapsulation phase at the site of implantation lacunae (fluid filled spaces) are formed within the syncytium cell mass. Cytotrophoblast will form a shell around the syncytium, creating a barrier between maternal decidua and syncytium. Within the syncytium villi are formed

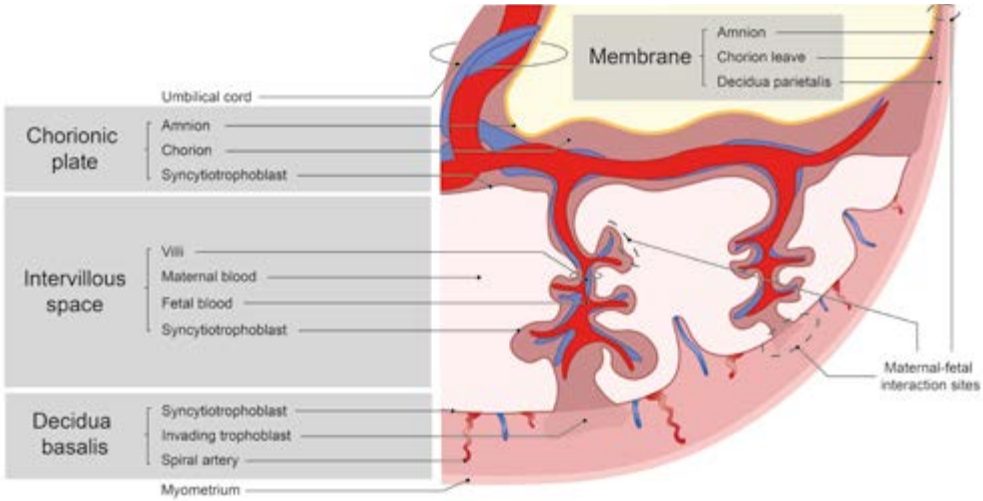

Figure 2. Overview of the structure of a term placenta and maternal-fetal interaction sites.

and the cytotrophoblast cells (CTBs) invade into the decidua as extravillous trophoblast cells (EVTs). These EVTs also invade maternal vessels and widen the spiral arteries, for proper blood supply. Early in gestation the EVTs also plug the spiral arteries, when the plug is removed maternal peripheral blood flows into the intervillous space, bringing peripheral blood into contact with the syncytiotrophoblast cells (SCTs) of the villi and the chorionic plate.

At term there are three interactions sites: decidua parietalis (maternal) with chorion leave (fetal), decidua basalis (maternal) with invading fetal trophoblast cells, and the maternal blood being in contact with fetal syncytiotrophoblast cells on the villi and the chorionic plate (Figure 2). The placenta at the implantation site consists of three layers where different types of trophoblast cells and immune cells encounter one another.

1. Decidua: mainly maternal cells and invading fetal extravillous trophoblasts
    a. Basalis: decidua at site of implantation
    b. Capsularis: grows around the blastocyst
    c. Parietalis: membrane lining the uterus
2. Intervillous space: location of nutrient exchange; maternal blood is in contact with the fetal syncytiotrophoblast.
3. Chorionic plate: mainly fetal cells; maternal blood is in contact with the fetal syncytiotrophoblast

Human leukocyte antigens

All nucleated cells of the body and platelets express HLA class I, while HLA class II can be expressed by antigen presenting cells, activated T cells and activated endothelial cells. HLA class I can present peptides derived from intracellular proteins to CD8+ cells, whereas HLA class II can take up, process and present peptides derived from self-antigens and foreign antigens from the extracellular milieu

to CD4+ T cells [17]. Whereas in normal circumstances the peptides presented will originate from self-proteins, not giving rise to an immune response. However, in case of for instance a viral infection, these peptides are derived from non-self proteins, and are recognized as foreign.

HLA class I includes the polymorphic HLA-A, -B and -C and the non-polymorphic HLA-E, -F and G molecules. HLA class II consists of HLA-DR, -DQ and -DP molecules. HLA is co-dominantly expressed and is inherited as haplotypes, where one set of genes (including all class I and class II molecules) is inherited from mother, and one is inherited from father. While in a given individual the number of different HLA alleles is restricted, the variation within the population is immense. This polymorphism in HLA molecules between individuals is essential to prevent the entire population to succumb due to a new or mutated pathogen. The inheritance of haplotypes means that the fetus is considered semi-allogeneic in the context of the maternal immune system.

When cells present foreign peptides within HLA molecules to T cells, this can lead to T cell activation, which is desirable in case of a pathogenic infection. However, when during pregnancy the maternal cells presents a fetal paternally inherited peptide, which can be an HLA molecule or other peptides in their own HLA molecule, T cells can get activated and attack the fetal cells, this is indirect recognition. However, T cells and B cells of the mother can also recognize directly the foreign paternal HLA antigens, which may lead to destructive immune response. This challenge is partly evaded in pregnancy since the fetal trophoblast cells do not present the polymorphic HLA-A and -B molecules and no HLA class II molecules. EVTs do express HLA-C, -E, -F and -G, and interestingly SCT do not express any HLA molecules.

THE IMMUNE SYSTEM

As described above maternal immune cells encounter fetal trophoblast cells during pregnancy. The main immune cells present (apart from granulocytes) are discussed in this thesis and are introduced below.

Monocytes are present in the peripheral blood and recognized by the expression of CD14. They can phagocytose and present antigens, secrete chemokines and travel to tissues. When they extravasate to tissues, they become macrophages or dendritic cells. Dendritic cells are present in very low numbers in the decidua.

Macrophages are recognized by the expression of CD68. They have similar functions to monocytes and represent their counterpart in tissues. Next to protecting against pathogens they are important for maintaining tissue homeostasis. Decidual macrophages have shown to be important for tissue homeostasis by exhibiting regulatory and suppressive properties and by cleaning up and repairing of damaged tissues. This form of scavenging is crucial for a rapidly developing organ such as the placenta. Furthermore, macrophages play a role in spiral artery remodeling by interacting with uterine NK cells and EVTs.

NK cells are prominently present in the first-trimester decidua and are thought to play a key role in supporting trophoblast invasion, spiral artery remodeling and protection against pathogens. In the peripheral blood they are recognized by the expression of CD16, whilst in the decidua they do not express CD16 but do express CD56. NK cells can kill cells missing HLA expression (missing

self-hypothesis) and cells bound by IgG antibodies (Antibody Dependent Cellular Cytotoxicity). Furthermore, NK cell functions such as cytotoxicity and cytokine production can get suppressed or enhanced by KIR or NKG2 receptor binding to HLA-C, -G or -E, or by responding to its cytokine environment [8, 9].

T cells can be distinguished in CD8+ and CD4+ T cells and show increasing frequencies in the decidua throughout gestation. **CD8+ T cells** are described as cytotoxic T cells because of their capacity to kill target cells when intracellular peptides from pathogens are presented via HLA class I molecules. Decidual virus-specific CD8+ T cells are described to be HLA-A and -B restricted [10]. Furthermore, CD8+ T cells specific for the HY-antigen (male specific antigen) are found in women with male pregnancies [11]. Indicating that fetal cells during pregnancy can be recognized [12]. A tight immunological balance is needed to prevent the killing of fetal cells by CD8+ T cells while keeping the capacity to fight of infection.

CD4+ T cells are described as T helper cells (Th) and play an important role in the activation and suppression of other immune cells. They generally do this by cytokine production but can also do this by cell-cell interaction (reviewed in [13]). During cell-cell interaction the CD4+ T cell recognizes peptides presented in the HLA class II molecule and can use co-stimulatory, co-inhibitory molecules and cytokines to then activate or inhibit other immune cells. There are several subsets of Th cells that can give different types of signals, this is referred to as effector functions. The most studied Th effector cell subsets are Th1, Th2, Th17 and regulatory T cells (Tregs). In general, **Th1 cells** are known as a pro-inflammatory subset that produces IFNγ [14], stimulates cellular immune response and targets bacteria and protozoa. **Th2 cells** stimulate humoral immune responses important in parasitic infection. They produce pro-inflammatory cytokines (IL-5), but also anti-inflammatory cytokines (IL-4 and IL-10) and can thereby suppress Th1 response [14]. **Th17 cells** get their name from the pro-inflammatory cytokine they produce (IL-17) and are known for their function to inhibit Treg cell differentiation [15]. **Treg cells** in the CD4+ T cell lineage are classically recognized by the expression of FOXP3 and CD25 (IL-2R) [16]. Tregs produce anti-inflammatory cytokines (IL-10, TGFβ and IL-35), are important for immune regulation and play a key role in healthy pregnancy as will be discussed in chapter 3.

IMMUNE SYSTEM IN THE PLACENTA

In both the maternal blood and the decidua, maternal immune cells are present that come in direct contact with fetal cells. The decidua basalis during first trimester contains many monocyte/macrophage cells and NK cells, and few T cells. B cells numbers are very low and granulocyte numbers are low, but they increase toward term. Throughout gestation the myeloid compartment remains stable, while the NK cell compartment decreases, and the T cell compartment increases [18]. Next to changing frequencies, their function also varies throughout the different developmental stages of the placenta [18]. In the different chapters of this thesis their roles will be explained in detail.

To achieve proper trophoblast invasion, the trophoblasts need to be recognized by maternal immune cells. The maternal immune cells (NK cells, T cells and macrophages) can bind to the HLA

molecules present on the EVT with either inhibitory or activating receptors. A tight balance of activation and regulation is required to ensure that there is sufficient trophoblast invasion, but infections can still be resolved.

EVTs express HLA-G: this molecule can create an immune regulatory environment, since most receptors that can bind to it are inhibiting inflammatory properties of immune cells. For instance, binding of HLA-G to Ig-like transcript 2 (ILT2) and ILT4 on macrophages causes inhibition of macrophage toxicity and binding on NK cells inhibits their secretion of IFN- , a compound that can activate macrophages [19, 20]. HLA-G can also be secreted (sHLA-G) into the circulation where it can bind to inhibitory receptors (e.g. ILT2 and ILT4) on several types of immune cells (T, NK, B, dendritic cells, and monocytes) resulting in inhibition of their effector functions [21]. Interestingly SCT do not express any HLA molecules. However, they are not target by NK cells (missing self-hypothesis), this could be due to missing activating ligands and/or expression of inhibitory molecules on SCT.

PREGNANCY COMPLICATIONS

Adverse pregnancy outcomes may occur for multiple reasons of which one could be inaccurate or inadequate adaptations of the maternal immune system to the pregnancy. When this occurs, it could cause the placenta to be less functional, thereby possibly affecting the growth of the fetus. This thesis focuses on two pregnancy syndromes: unexplained recurrent pregnancy loss (uRPL) and chronic intervillositis of unknown etiology (CIUE).

uRPL is defined as two or more spontaneous pregnancy losses (miscarriages) without a known cause [22, 23]. About 1-2% of couples who are trying to conceive experience uRPL [24]. Since the cause is unknown, no specific treatment can be given and the psychological burden upon affected couples is severe. There are many studies exploring the immune system of women with uRPL in peripheral blood and of the placenta. Keller et al. wrote a systematic review in 2020 on 18 studies that focused on Treg in RPL [25]. The results showed that Tregs in both the decidua and peripheral blood of women with RPL are decreased in numbers and are less functional at time of the unsuccessful pregnancy. Interestingly, many of these women will get an ongoing pregnancy at some point [24]. The immunology in the ongoing pregnancies of these women has not frequently been studied. Craenmehr et al. found in the women with an ongoing pregnancy after uRPL that HLA-G was upregulated on the EVTs compared to controls [26]. This finding suggests the presence of an increased immune tolerance during pregnancy in these women compared to controls without a history of uRPL.

CIUE is a poorly understood condition associated with adverse pregnancy outcome such as recurrent pregnancy loss and fetal growth restriction. Diagnosis can only be made after pregnancy based on the placenta pathology were an infiltrate of CD68+ cells can be found [27]. There is a need to find a clinical biomarker to be able to treat patients, but also a need to understand what causes the adverse pregnancy outcomes. It is known that there are CD68+ cells in the intervillous space, which previously have phenotypically been characterized as anti-inflammatory macrophages [28]. Furthermore, increased numbers of regulatory T cells compared to controls were found [29]. On

the other side, cytotoxic T cells directed against paternal antigens are also increased and there are signs of complement activation on the SCT [30, 31]. These findings suggest there is immune activation as well as immune modulatory mechanisms at play.

AIMS AND OUTLINE OF THIS THESIS

It has been shown that the maternal immune system adapts to the pregnancy situation and that when immune regulatory factors are not adapting enough pregnancy complications can occur. However, there is incomplete knowledge on how immune cells interact at the maternal-fetal interface and what exactly is needed to maintain a successful pregnancy. The aim of the studies in this thesis was to visualize the immune cells phenotype and interactions at the maternal-fetal interface. Moreover, the role of immune regulatory factors needed to maintain a successful pregnancy after uRPL is explored.

To study the maternal immune cell composition in the decidua throughout healthy gestation IMC was used **(Chapter 2)**. This study gives insight into the immune cell changes that are required during the changing demands and needs of the placenta during development. Furthermore, combining the data with SMC results gave the ability to show limitations and strengths of different types of techniques.

In **Chapter 3, 4 and 5** immune regulatory factors were studied in the context of uRPL. **Chapter 3** gives a detailed overview of different type of Tregs and their possible roles during pregnancy and uRPL. As we sought to understand what is needed to maintain an ongoing pregnancy, **Chapter 4** describes the Treg composition in women with a successful pregnancy after a history of uRPL compared to controls. Furthermore, in **Chapter 5** sHLA-G levels in blood plasma of women with a history of uRPL with either a successful or unsuccessful pregnancy outcome are compared.

Lastly, the pregnancy syndrome chronic intervillositis of unknown etiology was studied in **chapter 6 and 7**. CIUE is thought to be a disorder that is driven by the maternal immune system. However, in **Chapter 6**, three dizygotic twin cases were used to investigate if there is a fetal contribution to CHI. In **chapter 7**, we used IMC for in-depth visualization of the maternal immune cells that are present in the intervillous space in the dizygotic twin cases. In this chapter we aimed to gain insight in the different types of macrophages present in CHI.

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2

IMAGING MASS CYTOMETRY REVEALS THE PROMINENT ROLE OF MYELOID CELLS AT THE MATERNAL-FETAL INTERFACE

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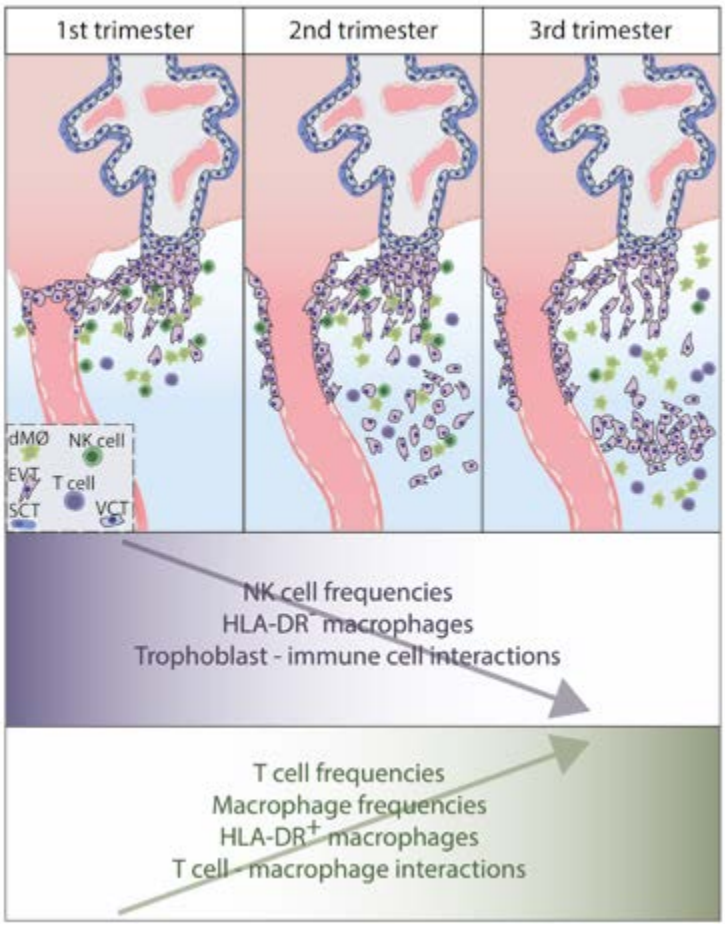
ABSTRACT

While the immunological complexity of the maternal-fetal interface is well appreciated, the actual interaction of maternal immune cells and fetal trophoblasts is insufficiently understood. To comprehend the composition and spatial orientation of maternal immune cells and fetal extravillous trophoblasts, we applied imaging mass cytometry on decidua basalis of the three trimesters of healthy pregnancy. Within all trimesters, we observed considerably higher frequencies of myeloid cells in the decidua than is seen with single-cell suspension techniques. Moreover, they were the most pronounced cell type in the microenvironment of other decidual cells. In first trimester, HLA-DR$^-$ macrophages represented the most abundant myeloid subcluster and these cells were frequently observed in the vicinity of trophoblasts. At term, HLA-DR$^+$ macrophage subclusters were abundantly present and frequently observed in the microenvironment of T cells. Taken together, our results highlight the dynamic role of myeloid cells at the human maternal-fetal interface throughout gestation.



Graphical abstract

INTRODUCTION

The placenta is essential in providing nutrients and oxygen to the developing fetus during pregnancy. During early placentation, fetal trophoblasts invade into the maternal endometrium. When this invasion occurs, fetal trophoblasts encounter maternal immune cells which may recognize the fetal cells and affect invasion. Disturbed trophoblast invasion likely occurs in several pregnancy complications and is associated with alterations in the composition of the maternal immune cells at the decidua [1-4]. Since pregnancy complications can be divergent in their time of onset, it is important to analyze the maternal decidual immune cell composition throughout gestation. However, before studying pregnancy complications, it is essential to gain understanding in how trophoblasts and immune cells interact with one another at different trimesters during healthy pregnancy.

Most research on phenotyping of decidual maternal immune cells during gestation has been performed using suspension techniques. Hereby, the tissue is mashed over a filter, usually followed by an enzymatic digestion step. Based on the results of single cell suspension techniques the most prominent maternal immune cells in the first trimester at the maternal-fetal interface have been described as NK cells (~60%), followed by macrophages (~20%), and T cells (~10%) [5, 6]. Later in gestation the NK cells decrease and T cells increase in frequency, whereas macrophage proportions remain constant, suggesting altering functions for NK cells and T cells at different trimesters [6-9]. Based on these observations extensive phenotyping using single cell suspension techniques of decidual maternal immune cells has mainly focused on NK cells and T cells using specifically designed panels, largely neglecting the myeloid compartment [6, 10]. Besides phenotypic analysis, the functional role of decidual maternal immune cells has been studied frequently by using *in vitro* assays or by using prediction algorithms based on suspension scRNA-seq data [11]. In these types of studies, however, information on spatial orientation of the cells is missing: if particular cells do not encounter each other *in situ*, effects observed in culture assays with these cell types might not be relevant.

In situ techniques, such as immunohistochemistry, have been scarcely used to determine phenotypes, or to quantify the distribution of the major immune cell lineages over different trimesters [9, 12, 13]. Nonetheless, *in situ* techniques can provide a more accurate quantification, as there is no specific cell loss due to the isolation process. Furthermore, it provides the spatial information, which can be used for designing relevant cell culture assays. Imaging mass cytometry (IMC) provides the possibility for in-depth studies on immune cell interactions in the decidua.

Macrophages and other myeloid cells appear to be stably present during gestation and are an essential part of the intricate immune network at the maternal-fetal interface [6]. Phenotypically, macrophage populations are often subdivided into two subpopulations, namely M1 and M2 [6, 14, 15]. However, this terminology is misrepresenting the broader spectrum of macrophage variation. Macrophages have a high plasticity, as their phenotype is continuously being altered in response to environmental cues [16-18]. In-depth phenotyping could help visualize the dynamic changes in the different subtypes and maturation/activation stages of macrophages. Because of the near-absence of B cells and dendritic cells at the maternal-fetal interface, macrophages represent

the main type of antigen-presenting cells in the decidua [6]. Decidual macrophages have generally been described to express HLA-DR [7, 15, 19]. Therefore, they likely play a crucial role in antigen presentation to $CD4^+$ T cells and may thereby contribute to the establishment of maternal-fetal immune tolerance, as well as regular immune surveillance. Macrophages have also been described to secrete a wide range of growth factors and cytokines, which suggests that they may have important functions throughout gestation, including during blastocyst implantation, trophoblast invasion, and for tissue homeostasis [20-22].

Here, we applied IMC on the human decidua to define the spatial orientation of immune cells in relation to each other and to fetal trophoblasts cells. First, we phenotypically determined the immune cell clusters present in the decidua and assessed their distribution over the different trimesters. Next, we determined the microenvironment profiles of both immune cells and trophoblast cells. Hereby, we focused on the myeloid cell compartment to gain knowledge of its potential role throughout different trimesters. Overall, our 42-marker IMC panel provides a baseline for immune cell phenotype and distribution, as well as their direct microenvironment at the decidua basalis during first, second, and third trimester.

METHODS

Tissue Material

Placental samples were obtained from elective abortions of first (n=3) and second trimester (n=5), primary c-sections (n=2), secondary c-sections (n=1) and spontaneous delivery (n=2) with informed consent (Table 1). The study was carried out according to the guidelines issued by the Medical Ethics Committee of the Leiden University Medical Center (LUMC; protocols P08.087 and P11.196), and in accordance with the Declaration of Helsinki. Tonsil samples were used as controls for IMC staining and taken along in every staining to check the consistency in antibody labelling (data not shown). Tonsil samples were obtained from the department of Pathology of the LUMC and were anonymized and handled according to the medical ethical guidelines described in the Code of Conduct for Proper Secondary Use of Human Tissue of the Dutch Federation of Biomedical Scientific Societies. All tissues were cut into 4-µm sections and placed on glass slides (Superfrost plus, Thermo scientific, USA).

Immunohistochemistry and immunofluorescence

Formalin fixed, paraffin embedded (FFPE) tissue samples were Haematoxylin and Eosin (H&E) stained according to standardized protocol. Consecutive slides were used for IMC staining within 24 hours. Additionally, for correct determination of decidua location of all placentas samples an HLA-G immunohistochemical (IHC) staining and H&E staining was performed. For validation of HLA-DR expression or absence on myeloid cells immunofluorescence (IF) staining was performed, with HLA-DR (clone in Table 2) and CD14 (clone in Table 2). Slides of three term samples were stained for Collagen IV (clone M0785), Fibronectin (clone F3648) and Laminin (clone L9393) In short, FFPE sections were deparaffinized by a series of xylene and ethanol after which IHC sections were incubated 20 minutes in 0.3% H_20_2. Next, antigen retrieval was performed by microwaving 10

Table 1. Patient characteristics and measurement details.

	SampleID	Trimester	Gestational age (weeks+days)	Delivery mode	Maternal age	ROIs measured	area measured (mm2) *	# of immune cells detected/analysed	# of trophoblasts detected/analysed	Ratio immune cells/trophoblasts
1	2307	First	10	Suction curettage	unknown	5	4.62	5926	5225	1.134
2	2317	First	11	Suction curettage	unknown	5	6.71	7326	6680	1.097
3	2331	First	10	Suction curettage	unknown	7	4.5	3939	3612	1.091
4	2177	Second	21	Surgical abortion	unknown	8	4.76	1511	6243	0.242
5	2268	Second	18	Surgical abortion	unknown	8	6.87	2974	4739	0.628
6	2280	Second	18	Surgical abortion	unknown	7	3.95	2983	2898	1.029
7	2297	Second	16	Surgical abortion	unknown	7	6.87	10190	4390	2.321
8	2324	Second	15	Surgical abortion	unknown	7	5.82	6489	5202	1.247
9	2366	Term	39	primary c-section	30	6	2.25	1041	489	2.129
10	2374	Term	39	primary c-section	32	12	4.85	2514	1003	2.506
11	2394	Term	41+1	spontaneous	36	9	8.93	4244	2772	1.531
12	2427	Term	42+1	secondary c-section	37	8	6.28	1912	2819	0.678
13	2398	Term	41+4	spontaneous	32	9	6.7	1820	5856	0.311

* area of region of interest (ROI) including tissue and background.

Table 2. Imaging mass cytometry antibody panel.

	Target	Clone	RRID	Metal	Incubation time	Temperature	Dilution
1	Pan-Keratin	C11 and AE1/AE3		^{106}Pd	Overnight	4°C	50
2	Collagen I	EPR7785		^{115}In	Overnight	4°C	50
3	**HLA-DR**	TAL 1B5		^{141}Pr	5 hours	RT	100
4	EGF-R	D38B1		^{142}Nd	Overnight	4°C	50
5	CD68	D4B9C		^{143}Nd	Overnight	4°C	100
6	**CD11b**	D6X1N		^{144}Nd	5 hours	RT	100
7	**CD4**	EPR6855	AB_2864377	^{145}Nd	Indirect	4°C	50
8	**CD8α**	D8A8Y		^{146}Nd	5 hours	RT	50
9	CD31	89C2		^{147}Sm	Overnight	4°C	100
10	CD73	D7F9A		^{148}Nd	5 hours	RT	100
11	**CD69**	EPR21814	AB_2891140	^{149}Sm	Overnight	4°C	100
12	Granzyme B	D6E9W		^{150}Nd	5 hours	RT	100
13	CD66b	G10F5		^{151}Eu	5 hours	RT	100
14	Ki-67	8D5		^{152}Sm	Overnight	4°C	100
15	**CD3**	EP449E		^{153}Eu	Overnight	4°C	50
16	TIM3	D5D5R(TM)		^{154}Sm	5 hours	RT	100
17	CD141	E7Y9P		^{155}Gd	Overnight	4°C	50
18	NKG2A	LS-C165590		^{156}Gd	5 hours	RT	50
19	CD39	EPR20627		^{157}Gd	5 hours	RT	100
20	CD1c	EPR23189-196	AB_2884015	^{158}Gd	5 hours	RT	50
21	FOXp3	D608R		^{159}Tb	Overnight	4°C	50
22	**PD-1**	D4W2J		^{160}Gd	5 hours	RT	50
23	DC-SIGN	NBP1-77284		^{161}Dy	Overnight	4°C	50
24	IDO	D5J4E(TM)		^{162}Dy	Overnight	4°C	100
25	**CD14**	D7A2T		^{163}Dy	5 hours	RT	100
26	CD204	J5HTR3		^{164}Dy	5 hours	RT	50
27	**CD45RO**	UCHL1	AB_2563752	^{165}Ho	Overnight	4°C	100
28	D2-40	D2-40		^{166}Er	Overnight	4°C	100
29	**CD56**	E7X9M		^{167}Er	5 hours	RT	100
30	**CD103**	EPR4166(2)		^{168}Er	5 hours	RT	50
31	**CD38**	EPR4106	AB_2864383	^{169}Tm	Overnight	4°C	100
32	**CD45RA**	HI100		^{170}Er	5 hours	RT	100
33	**CD15**	BRA-4F1		^{171}Yb	Overnight	4°C	100
34	Cleaved caspase-3	ASP175		^{172}Yb	5 hours	RT	100
35	**CD163**	EPR14643-36		^{173}Yb	5 hours	RT	50
36	CD7	EPR4242	AB_2889384	^{174}Yb	5 hours	RT	100
37	**CD45**	D9M8I		^{175}Lu	5 hours	RT	50
38	**CD11c**	EP1347Y	AB_2864379	^{176}Yb	5 hours	RT	100
39	Vimentin	D21H3		^{194}Pt	Overnight	4°C	50
40	HLA-G	MEM-G2		^{198}Pt	5 hours	RT	100
41	αSMA	D4K9N		^{209}Bi	5 hours	RT	100
42	βcatenin	D10A8		^{89}Y	Overnight	4°C	100

In bold are the markers that are overlapping with the SMC panel.

minutes with 10 mM citrate solution (pH 6.0). Slides where incubated with superblock (Invitrogen) for 20 minutes at room temperature (RT) prior to primary antibody staining (clone in Table 2) for 1 hour at RT. Binding of primary antibody in IHC was visualized using a goat-anti-mouse or rabbit Ig-HRP (DAKO envision) and 3,3'-Diaminobenzidine as a chromogen. For IHC hematoxylin was used for nuclear counterstaining before slides were dehydrated and covered using mounting medium. For IF goat-anti-rabbit IgG-AF546 (Invitrogen) and goat anti-mouse IgG1-AF488 (Invitrogen) where used to visualize the primary antibodies before the slides were dehydrated and covered using mounting medium.

Mass cytometry antibodies

Heavy metal isotope-tagged monoclonal antibodies are listed in Table 2. All, but keratin, α-smoot muscle actin (SMA), CD4, EGF-R, Vimentin, and HLA-G antibodies were conjugated with heavy metal isotopes in-house using the MaxPar X8 Polymere Antibody Labeling Kit according to the manufacturer's protocol (Fluidigm, Californië, USA). EGF-R was pre-conjugated by Fluidigm. Conjugation of two keratin antibody clones to 106Pd, was performed using a protocol adapted from Schulz et al. [23]. Conjugation with 209Bi to α-SMA was performed using a protocol adapted from Spitzer et al. [24]. Cisplatin 194 and 198 were conjugated to Vimentin and HLA-G using a protocol adapted from Mei et al. [25]. CD4 was stained using a secondary staining step with α-mouse-145Gd. All 43 primary antibodies and the one secondary antibody were titrated to determine the optimal labelling concentration. Additionally, all antibodies unconjugated and conjugated to a metal were tested by immunohistochemistry before being used in IMC as described above. For each of the 42 metal-tagged primary antibodies the best of two incubation options was used: 5 hours at room temperature or overnight at 4°C.

Imaging mass cytometry antibody staining

Imaging mass cytometry antibody staining was performed as previously described by Ijsselsteijn et al. [26]. In short, 4-µm FFPE sections were deparaffinized by a series of xylene and ethanol after which citrate-microwave antigen retrieval was performed. The sections were blocked with superblock solution, after which they were stained with anti-CD4 (mouse IgG1, dilution in table 2) overnight at 4°C. After washing, the sections were stained with the secondary anti-mouse-145Gd antibody for 1 hour at room temperature. The sections were washed again and stained with the metal labeled antibodies for 5 hours at room temperature (table 2). Slides were then stained with the second antibody mix for overnight incubation at 4°C. Lastly, the slides were stained with Iridium nuclear staining, washed with demineralized water, and dried under an air flow.

Imaging mass cytometry data acquisition

The Hyperion was autotuned, using a three-element tuning slide according to the manufacturer's protocol (Fluidigm). Using the consecutive HE-stained slides the Regions of interest (ROIs) (areas varied from 0.26 mm^2 to 1.84 mm^2) on the IMC slides could accurately be set on the decidua. This resulted in five to twelve ROIs per placenta sample. The selected ROIs were ablated at 200 Hz.

Data analysis

Creating a single cell mask using cell segmentation

For each ROI a single cell mask was created as previously described, with some adjustments [27]. First, based on the consecutive H&E staining and IMC trophoblast markers the villi and extravillous space were removed from all ROIs in the exported DNA file to analyze the decidua basalis only. Ilastik (v1.3.3) was used to create three probability maps based on the DNA signal ($193^{Iridium}$), myeloid markers (CD14, CD68, and CD163), other immune cells (CD45 and CD56). These three probability maps were together loaded in Cellprofiler (v2.2.0), with DNA as primary object, their size was increased by 2 pixels (2um) to make sure membrane marker expression was included in all types of cells and myeloid markers and other immune cells were loaded as secondary object. Then a single cell mask per ROI was exported from Cellprofiler. For each ROI the mask was compared to the original IMC data. Trophoblast- and maternal stromal cell markers were not used to create the mask because of the large cytoplasm size. Therefore, the signal often does not belong to a nucleus or is too close to other nuclei to mask correctly. Since all objects (DNA signal) in the mask were expanded by two pixels, membrane staining besides nuclear staining was included for all types of cells.

Background removal and data normalization

To improve data recovery and reduce noise, semi-automated thresholding, a machine learning algorithm in Ilastik, was used to separate true signals from noise as was described earlier [27, 28]. For each individual marker, the algorithm was trained to separate noise from signal which resulted in binary pixel values.

Phenotyping of segmented cells

All masks together with all binarized thresholded ROIs were loaded in ImaCytE [29]. Each cell in the mask was combined with its corresponding thresholded pixel intensity file, and FCS files could be exported with the marker expression per cell as relative frequency of positive pixels. These single-cell FCS files were analyzed by t-SNE in Cytosplore (v2.3.1). Seven t-SNEs were performed. First a t-SNE was made on absence or presence of marker expression, where a cluster with no marker expression (background1) and a cluster with marker expression was determined. On the cells with marker expression another t-SNE was performed where immune cells were separated from other tissue cells (Supplementary Figure S3). Next a separate tSNE was made on immune cells and on other tissue cells. For tissue cells a distinction could be made between trophoblasts, maternal stromal cells, and unknown cells with not enough markers to phenotype precisely. For the immune cells four major immune cell lineages could be identified, namely myeloid cells, NK cells, granulocytes, T cells, and unknown, with the latter not having enough positive markers to phenotype accurately. Another tSNE was performed on the myeloid cells, NK cells, and T cells to determine smaller subclusters (minimum of 100 cells per cluster). All cell subclusters can be found in Figure 2A and tSNE clustering in Supplementary Figure S3.

Comparing SCM and IMC data

SCM and IMC data were compared by using Cytosplore. Both single cell FCS files were loaded separately in Cytosplore after which CD45 positive and DNA positive cells where selected (54.351 cells for IMC, 7.198.273 cells for IMC (down sampled to 54.351 cells for visualization)). Next, the 17 overlapping markers between panels (CD45, CD14, CD15, CD69, CD4, CD8a, CD163, CD103, CD11c, HLA-DR, CD45RA, CD3, CD38, CD45RO, PD1, CD56 and CD11b) were used to generate major immune lineage clusters. Frequencies were exported and analyzed in Graphpad Prism (V8).

Microenvironment analysis

The assigned phenotypes were loaded back into ImaCytE with the masks to localize the phenotypes in the tissue [29]**.** The microenvironment analysis was done per sample, combining all ROIs per sample (Table 1). The distance (in pixels) that determines cell proximity was set to 10 (1 pixel = 1 μm^2). We found with this setting that the first cells surrounding the cell of interest were identified without another cell in-between, meaning that two neighboring cells may directly affect each other by contact and/or by secreted products (Supplementary Figure S6A, B and C). Next, the microenvironments per phenotype were exported per sample for further data analysis including permutation z-scores.

The percentages of the microenvironment of each cell with all other cells were calculated per sample by using the count of at least one co-localized cell in a 10-pixel radius divided by the absolute number of cells in that cluster of that sample (observed microenvironment). Next, the frequency of the immune cell clusters was calculated (within the total of all immune cells and trophoblast cells). These percentages were used to determine the chance of random cells in the microenvironment. This was done by multiplying the frequency of the cell cluster of interest with the frequency of the cell cluster it could interact with. The expected percentage was subtracted from the percentage of the observed microenvironment (Supplementary Figure S6D).

For data visualization, heatmaps show z-scores per row based on the percentages of the corrected microenvironment analysis. The z-score shows the amount of SD the sample value is above or below the mean of the specific row. Other graphs show percentages of the corrected microenvironmental analysis data.

Permutation z-scores were used to confirm that the calculated interactions we find do not occur at random. A permutation z-score of 1.96 (probability of <0.05) was used as cutoff.

Statistics

IMC and SMC data ware plotted in a box and whiskers plot visualizing min to max points using GraphPad Prism (V8). The two groups were compared by Mann-Whitney test corrected for the three trimesters by Bonferroni correction. The microenvironment analyses were visualized by their median and interquartile range. When first, second, and third trimester were compared the Kruskal-Wallis test and Dunn's multiple comparisons test was used. When multiple clusters were compared over the same trimester the Friedman test and Dunn's multiple comparisons test was used.

RESULTS

Differential decidual immune cell frequencies in tissue sections versus digested tissue suspensions

For analysis of frequencies and visualization of immune cells in their spatial context, we designed and optimized a 42-marker IMC panel for application on human decidual samples (Table 2). In our previous work, we visualized the immune cell compartment of the decidua basalis at first, second, and third trimester using suspension mass cytometry (SMC) [6]. We compared results of SMC and IMC to determine the possible effect of sample processing on cell frequencies in the suspension technique. Frequencies of the major immune cell populations within the $CD45^+$ cell compartment, using the 17 overlapping markers in both panels, were evaluated (Figure 1 and Supplementary Figure S1 and S2, and Table 2). The four major immune cell lineage markers, CD14, CD56, CD3 and CD15 present in both panels are visualized in Figure 1A for IMC. Using a cell mask to segment all cells into single cells the IMC data could be quantified similarly to SMC data using tSNE (Figure 1B).

To compare the IMC tissue approach with the suspension technique, the frequency of granulocytes, other myeloid cells (monocytes, macrophages, DCs), NK cells, and T cells were determined at first trimester, second trimester, and term. SMC only stains extracellularly located proteins while IMC also stains intracellular proteins. For that matter, decidual NK cells may express intracellular CD3 as observed in Figure 1B [11]. Kinetics for the majority of the cell types were comparable over time between IMC and SMC (Figure 1C and Supplementary Figure S2). The main difference observed was the high frequency of myeloid cells in IMC compared to SMC (1^{st} trimester: median 35.8% vs 17.6%, p=0.004; 2^{nd} trimester: median 52.5% vs 26.8%, p=0.004; Term: median 60% vs 9.4%, p=0.001) (Figure 1C). Furthermore, we found a significant increase in IMC of the myeloid compartment represented 35.8%, 52.5%, 60% of the $CD45^+$ cell compartment at first, second, and third trimester, respectively (first trimester vs term: p=0.012). Consequently, frequencies of NK cells within the $CD45^+$ compartment by IMC were lower compared to SMC at term (median 11% vs. 25.7%, p=0.019). A similar finding applied to T cells, where the frequency was significantly lower in first trimester and term (1^{st} trimester: median 0.9% vs. 11.4%, p=0.004; Term: median 14.9% vs. 37.3%, p=0.001) (Figure 1C). For NK cells determined by IMC at first, second, and third trimester the median frequency within the $CD45^+$ compartment was 41.6%, 28.1%, and 11%, respectively and for T cells 0.9%, 4.5%, and 14.9%, respectively.

In situ identification of 16 phenotypically distinct immune cell clusters present in the decidua basalis at different trimesters

On basis of the immune cell markers (n=33) of the complete IMC panel, we were able to identify 16 phenotypically different immune cell clusters (Figure 2A). These clusters were found in all samples, but were differently distributed over the trimesters (Figure 2B, C, Supplementary Figure S3). Six phenotypically distinct subclusters were identified in the myeloid compartment by density-based clustering using Cytosplore (Figures 2B, D and Supplementary Figure S3) [30]. Four of the six subclusters could clearly be distinguished from one another by their expression of HLA-DR and DC-SIGN, referred to as dMØ1, dMØ2, dMØ4 and dMØ5 (Figures 2B, D). Multiple other markers

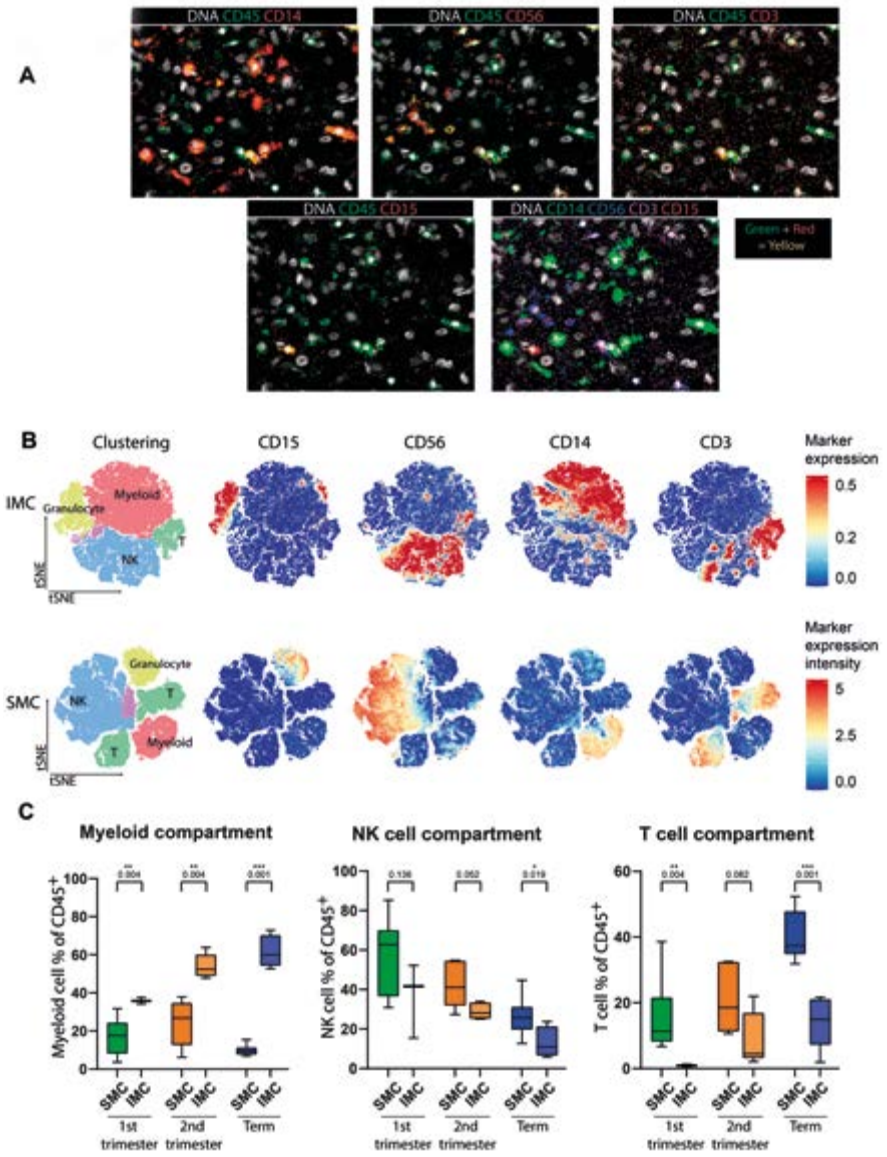


Figure 1. Comparison of major immune cell lineage frequencies at the maternal-fetal interface by IMC and SMC. (A) Raw IMC data show that all major immune lineages can be distinguished. Using a mask, the IMC data is segmented in single cells with DNA as primary object. (B) tSNE of the major immune cell lineages within the CD45$^+$ compartment of IMC and SMC by automatic clustering using 17 overlapping markers (Table 2). For visualization but not for quantification, SMC is randomly down sampled to the same number of cells as IMC (54.351 cells). For IMC, the marker expression is binarized from 0 to 1, meaning one cell with 0.5 expression has 50% of pixels in the cell being positive for the marker. Whereas in the SMC data the marker expression visualizes the intensity of a marker (arcsin transformed to 5) such as bright or dim expression of a marker. The red cluster is the myeloid cluster, green the T cell cluster, blue the NK cell cluster, yellow the granulocyte cluster and purple non-defined CD45+ cells (debris and B cells). (C) Comparison between SMC and IMC of the frequency (as percentage of CD45$^+$ cells) of the myeloid cell- (without granulocytes), NK cell- and T cell compartment at

all three trimesters (1st trimester SMC n=12, IMC n=3; 2nd trimester SMC n=6, IMC n=5; term SMC n=9, IMC n=5). Myeloid cells where significantly higher frequent in IMC than SMC at all three trimesters (1st trimester: median 17.6% vs 35.8%, p=0.004; 2nd trimester: median 26.8% vs 52.5%, p = 0.004; Term: median 9.4% vs 60%, p= 0.001). NK cells showed a trend toward a decreased frequency in IMC compared to SMC, this decrease was significant at term (1st trimester: median 62.8% vs 41.6%, p=0.136; 2nd trimester: median 41.1% vs 28.1%, p = 0.052; Term: median 25.7% vs 11%, p= 0.019). T cells showed significantly decreased frequency by IMC compared to SMC in first trimester and term (1st trimester: median 11.4% vs 0.9%, p=0.004; 2nd trimester: median 18.5% vs 4.5%, p = 0.082; Term: median 37.3% vs 14.9%, p= 0.001). Data are represented as Min to Max boxplots, Mann-Whitney test, *P < 0.05; **P < 0.01; ***P < 0.001. IMC, imaging mass cytometry; SMC, suspension mass cytometry; tSNE, t-distributed stochastic neighbor embedding; HSNE, Hierarchical Stochastic Neighbor Embedding.

besides HLA-DR and DC-SIGN were differentially expressed between subclusters, as shown in Figure 2A and Supplementary Figure S3. Two of the HLA-DR$^-$ subclusters (dMØ1 and dMØ4) were especially prominent in first trimester samples (Figure 2B). The two HLA-DR$^-$ subclusters showed no detectable HLA-DR expression in the IMC analysis (Figure 3A). We validated the low expression or absence of HLA-DR on a subset of CD14$^+$ cells by applying immunofluorescence (IF) staining using a secondary antibody for amplification of the signal intensity (Figure 3B). Despite this amplification, a subset of HLA-DR$^-$ myeloid cells was still observed. Furthermore, we also found HLA-DR dim and HLA-DR negative myeloid cells in the SMC data [6].

One myeloid subcluster (dMØ3) expressed both NK cell markers and myeloid markers, which has not previously been described in the decidua. Besides the myeloid markers CD14, CD68, and CD163, this population expressed NK cell markers CD56 and CD7 (Figure 2D and Supplementary Figure S3). To exclude an artefact of IMC, we cross-referenced our SCM data [6], whereby we also found this cluster (Supplementary Figure S4A). In further support of our finding, we also identified this cluster in an independent dataset previously published by Vento-Tormo et al. [11] (Supplementary Figure S4B). Additionally, we found one myeloid subcluster (MoDC-like) that does not seem to express CD14, while expressing CD204, HLA-DR, and CD11c. Furthermore, we identified granulocytes, which were CD66b$^+$CD15$^+$. This subset was not included in further analysis.

T cells are known to be present in relatively low frequencies in the decidua. Based on our previous data [6] we found that a reliably defined cluster consists of >100 cells and we therefore merged all T cells into three subclusters: CD4$^+$ T cells, CD8$^+$ T cells, and CD3$^+$CD4$^-$CD8$^-$ T cells.

We found 6 different NK cell subclusters. dNK1 were characterised by CD69 expression (dNK1a, dNK1b). The dNK2 subcluster was identified based on absence of CD69, CD11c or CD103 expression, as recently characterized by Vento-Tormo et al. [11] (Figure 2A and Supplementary Figure S3). Finally, dNK3 were characterized by CD11c (dNK3a, dNK3b) or CD103 (dNK3c) expression. In concordance with previous literature the dNK1 subclusters were the most prominent dNK cells present in first trimester [11], whereas in second trimester and term most dNK cell subclusters were distributed more equally (Figures 2D).

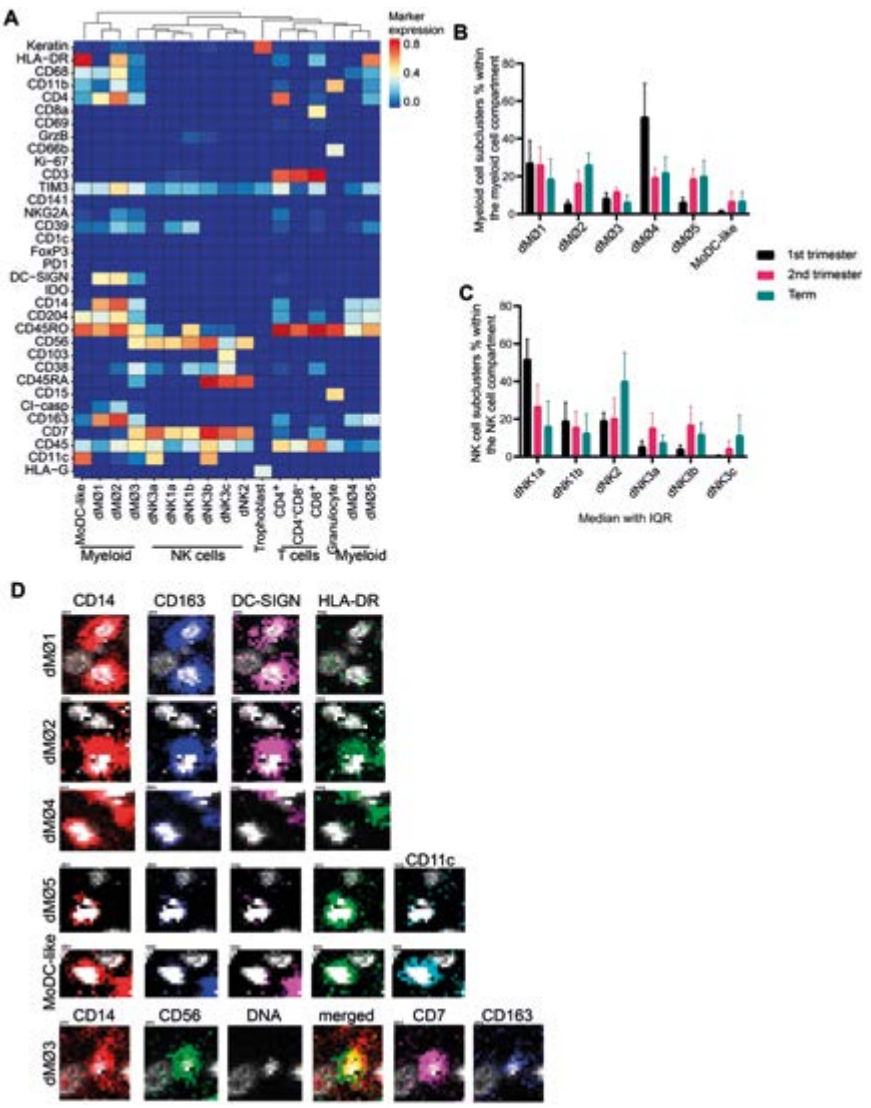


Figure 2. Identification of immune cell subclusters and trophoblasts at the maternal-fetal interface. (A) All 16 identified subclusters of different immune cell lineages and trophoblasts of all trimesters are visualized in a heatmap showing marker expression (white to red) or absence thereof (blue) (13 samples, 92855 cells). Cluster IDs are displayed at the bottom of the heatmap, marker names are on the left. (B) Myeloid cell subcluster frequencies are visualized within the myeloid compartment over the different trimesters. The overall distribution over the different subclusters is significantly different in first trimester ($p<0.001$), second trimester ($p=0.020$), and term ($p=0.010$). In first trimester DR^-SIGN^+ and DR^-SIGN^- represent the most prominent subclusters. (C) NK cell subcluster frequencies are shown within the NK cell compartment over the different trimesters. The overall distribution over the different subclusters is significantly different in first trimester ($p<0.001$), second trimester ($p=0.027$), but not at term ($p=0.080$). At first trimester $CD45RO^-RA^-$ represents the most prominent subcluster. (D) Raw IMC data showing positive (pos), dim or negative (neg) expression of the markers with the biggest variation between the different myeloid cell subclusters. (B+C) Data are represented as medians with IQR, Friedman test per trimester.

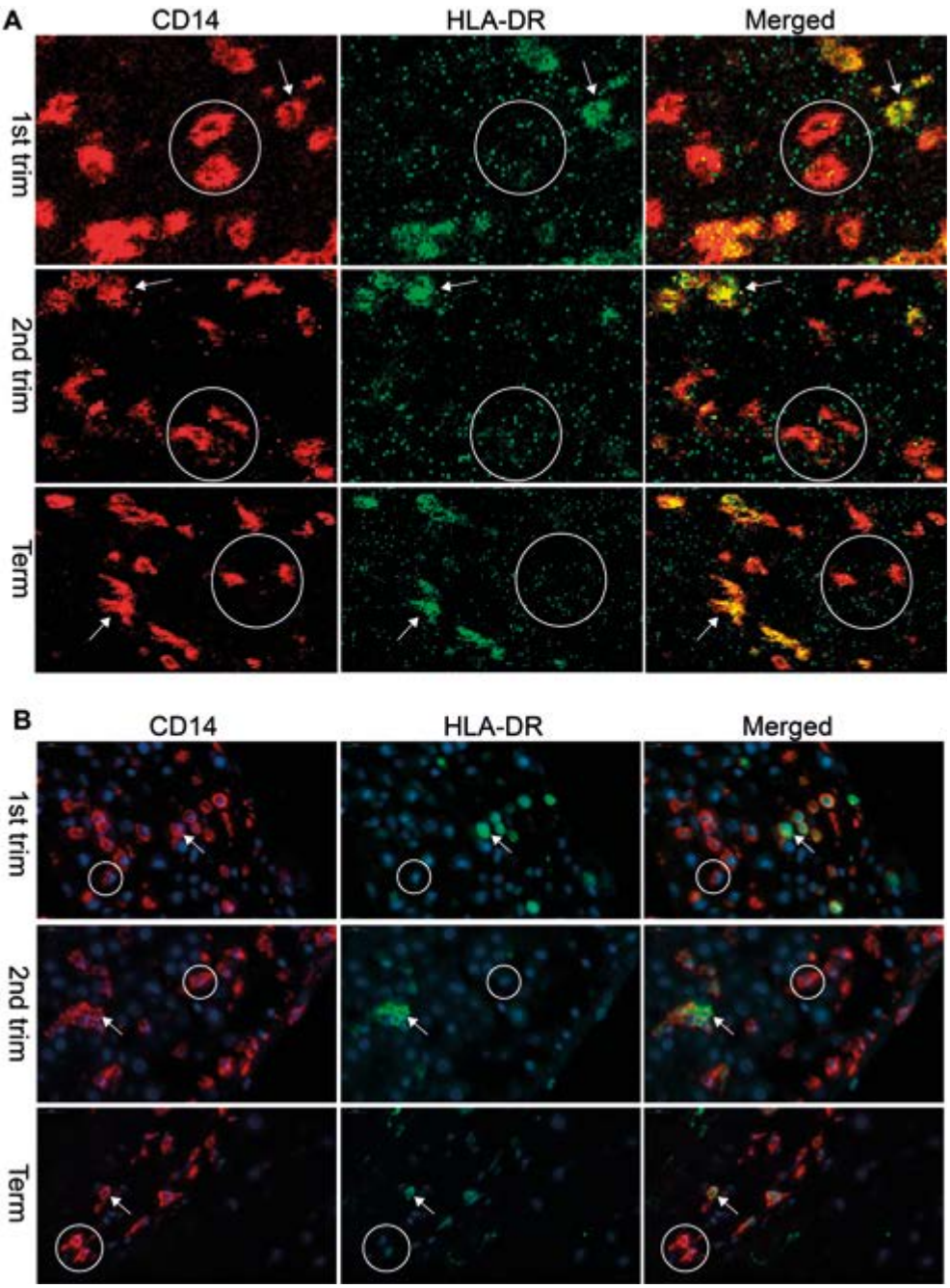


Figure 3. Verification of absence of HLA-DR on myeloid cell subclusters at the maternal-fetal interface by immunofluorescence (IF) staining. (A) In all trimesters (and all samples), myeloid cells ($CD14^+$) that lack HLA-DR expression (circle), and myeloid cells that do have HLA-DR expression (arrow) are observed. (B) Confirmation by IF that not all $CD14^+$ cells in the decidua express HLA-DR (circle), while others do (arrow).

Myeloid cells are the most prominent immune cells in the microenvironment of trophoblasts at all three trimesters

In addition to immune cells, we visualized trophoblasts *in situ* based on expression of Keratin and HLA-G (Figure 2A, Supplementary Figure S5A). At first trimester, trophoblasts were present in equal numbers to immune cells (Table 1). In second trimester and term there was more variation in the ratio of immune cells over trophoblasts between samples.

Next, we systemically analysed the microenvironment of trophoblasts and immune cells in the tissue within a 10-pixel (10 µm) radius and corrected for random co-localisation based on cell frequencies (Supplementary figure S6). This approach is specifically oriented at identifying cells that might influence neighbouring cells by direct contact and/or secreted products. When analysing the microenvironment of trophoblasts, we observed a steady decrease in all immune cells in the vicinity of trophoblasts at term (Figure 4A and Supplementary Figure S7A), as can be observed in representative images of first trimester (Figure 4B) and at term (Figure 4C).

Since many studies have focused on the role of dNK cells in first trimester we used that information to confirm our microenvironment analysis methodology. The presence of dNK cells in the vicinity of trophoblasts during early invasion has been described to be important for proper trophoblast invasion and spiral artery remodelling (reviewed in [31]). Recently Vento-Tormo suggested that dNK1 specifically interact with trophoblasts [11]. Using our IMC data we could indeed confirm that at first trimester NK cell interactions with trophoblasts frequently occur, and specifically this is mediated by dNK1s (Figure 4D).

When focusing on myeloid subclusters in the trophoblast cell microenvironment, we observed at first trimester that myeloid cells were primarily represented by the two HLA-DR$^-$ subclusters (dMØ1 and dMØ4) (Figure 4E). Interestingly, at term relatively low frequencies of myeloid cells were detected in the microenvironment of trophoblasts, despite the relative frequency of myeloid cells at term being higher than in first trimester (Supplementary Figure S2B). This could be the result of deposition of fibrinoid encompassing trophoblast cells [32]. Indeed, we observed fibrinoid deposition to be increased in term decidua in both IMC and consecutive haematoxylin and eosin stained slides (Supplementary Figure S5A). The fibinoid deposition stained positive for collagen IV, fibronectin and at some locations for pan-Laminin (Supplementary Figure S5B). T cells were nearly absent in the trophoblast cell microenvironment at all trimesters (Supplementary Figure S7).

Myeloid cells represent the most dominant cell type in the microenvironment of other immune cells at all three trimesters

The presence of the major immune cells in the microenvironment of other immune cells was also studied. Myeloid cells were the most abundantly present immune cell in the microenvironment of all other immune cells (Supplementary Figure S7A, blue lines). The frequency of myeloid cells in the microenvironment of other immune cells remained relatively stable over time (Supplementary Figure S7A), even though the relative cell frequency of myeloid cells increased during gestation (Supplementary Figure S2). This indicates that we used an appropriate correction method for random co-localisation based on cell frequencies. For further verification we used permutation

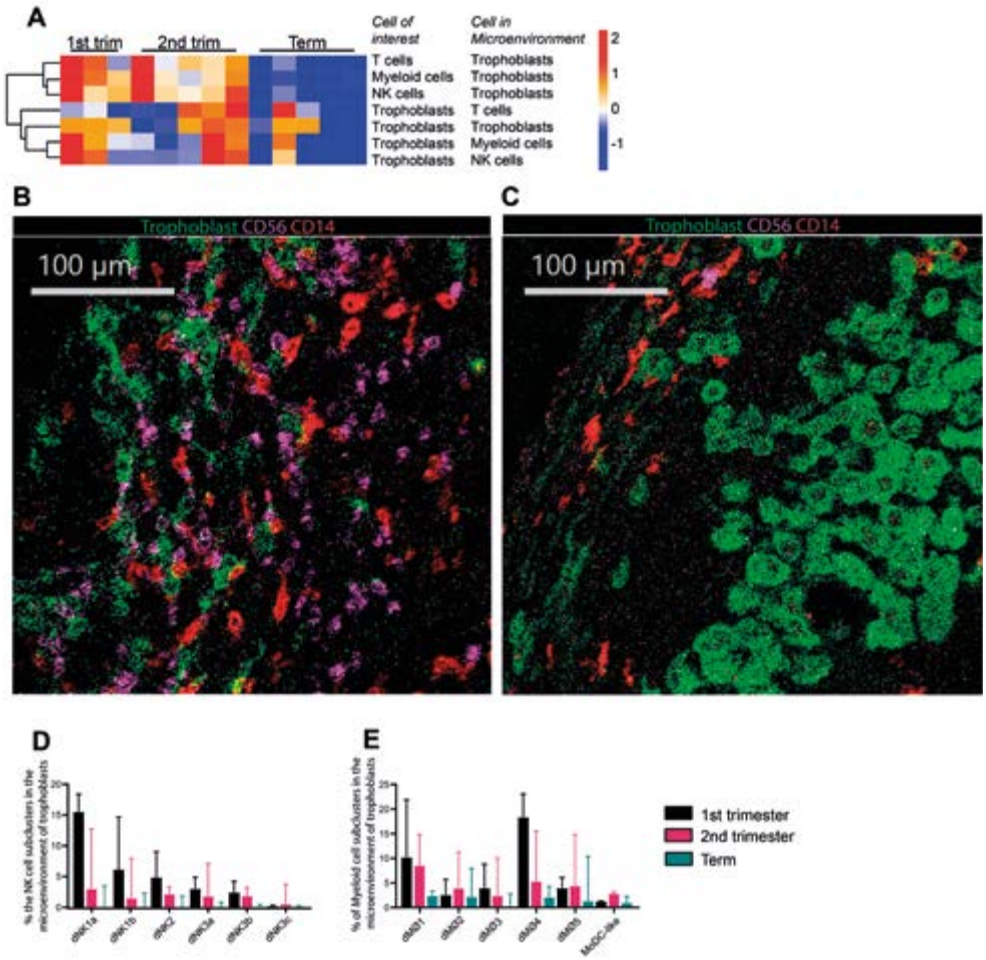


Figure 4. Microenvironment profile of trophoblasts. (C) Z-score hierarchical clustering heatmap visualization of trophoblast interactions with major immune cell lineages at first trimester, second trimester, and term. Orange/red indicates a higher amount of interactions and blue a lower amount of interactions than the mean of corrected microenvironment analysis frequency per row. (B+C) Visualization of myeloid cell (red) and NK cell (magenta) interactions with trophoblasts (green) in (B) first trimester and (C) term decidua. (D) Frequency of the six NK cell subclusters (of all immune cells and trophoblasts) that are within the microenvironment of trophoblasts. A Friedman test of first trimester samples indicates a differential distribution of the subclusters in the microenvironment of trophoblasts ($p<0.001$). A Kruskal-Wallis test of the three trimesters and subcluster dNK1a shows a significant decrease of dNK1a cells in the microenvironment of trophoblasts (Kruskal-Wallis $p=0.020$; multiple comparisons first trimester vs term $p=0.033$). (E) Frequency of the six myeloid subclusters (within both immune cell and trophoblast compartment) that are within the microenvironment of trophoblasts. A Friedman test of first trimester samples indicates a differential distribution of the subclusters in the microenvironment of trophoblasts ($p<0.001$), similarly for second trimester ($p=0.014$), but not for term ($p=0.115$). A Kruskal-Wallis test of the three trimesters and subcluster dMØ4 showed a significant decrease of dMØ4 cells in the microenvironment of trophoblasts (Kruskal-Wallis $p=0.014$; multiple comparisons first trimester vs term $p=0.024$). (B+C) Data are represented as medians with IQR.

testing and found that the interactions identified do not occur at random (Supplementary Figure S7B). In contrast to the stable presence of myeloid cells in the microenvironment of other immune cells over time, we observed an increase of T cells in the microenvironment of other immune cells from first trimester to term, accompanied by a decrease of NK cells in the microenvironment of other immune cells (Figure 5A).

Next, we analysed the subclusters within the microenvironment of the myeloid compartment. First, we observed a trend towards an increase in the amount of T cells in the microenvironment of myeloid cells from first trimester to term (Figure 5A and Supplementary Figure S7). This was both the case for $CD4^+$ T cells and $CD8^+$ T cells (Figure 5B). At term, there was an uneven distribution of T cells in the microenvironment of myeloid subclusters (Friedman test p=0.001). Three HLA-DR^+ macrophage subclusters (dMØ2, dMØ3, dMØ5) had a higher amount of $CD4^+$ T cells in their microenvironment at term than the HLA-DR^- macrophage subclusters (dMØ1, dMØ4) (Figure 5C). Figure 5D shows a representative image were HLA-DR^- macrophages (green and yellow) are less often in the microenvironment (circles) of CD4+ T cells (red and magenta) than HLA-DR^+ macrophages (white and cyan)

Lastly, we observed that the decrease of NK cells in the microenvironment of myeloid cells from first trimester to term could not be ascribed to one specific myeloid subcluster or NK cell subcluster (Figure 5E). In first trimester, the NK cell dNK1a and dNK1b subclusters were prominently present in the microenvironment of macrophages (Figure 5E, dashed box). In second trimester, the NK cell subset dNK3b became increasingly present in the microenvironment of all myeloid subclusters (Figure 5E, black box). At term, there was no particular NK subset highly present in the microenvironment of myeloid subclusters.

DISCUSSION

To comprehend the composition and spatial orientation of maternal immune cells and fetal extravillous trophoblasts, we applied in-depth immune profiling and microenvironment analysis on tissue sections of first trimester, second trimester, and term decidua from healthy pregnancies. We demonstrated that myeloid cells are present in high numbers in the decidua and that these cells are also highly abundant in the microenvironment of other cells. Furthermore, we showed that the distribution of different myeloid subclusters changes throughout the subsequent trimesters, which may go hand in hand with a functional change, as suggested by the microenvironment analysis.

Suspension techniques can be subject to bias because of incomplete tissue digestion, peripheral blood contamination and contamination by non-decidual tissue such as fetal villi and peripheral blood. In contrast to suspension techniques, we observed a higher frequency of macrophages throughout all three trimesters. Myeloid cells constituted ~35% of the immune cell compartment in the decidua during first trimester and ~60% during second and third trimester, as compared to ~20% within all trimesters as determined using suspension techniques [5-7]. Our combined results of IMC and SMC suggest a selective loss of myeloid cells when using suspension techniques for phenotyping. Consequently, the importance of myeloid cells in the decidua during all trimesters in healthy pregnancy may previously have been underestimated. In addition, in the IMC data

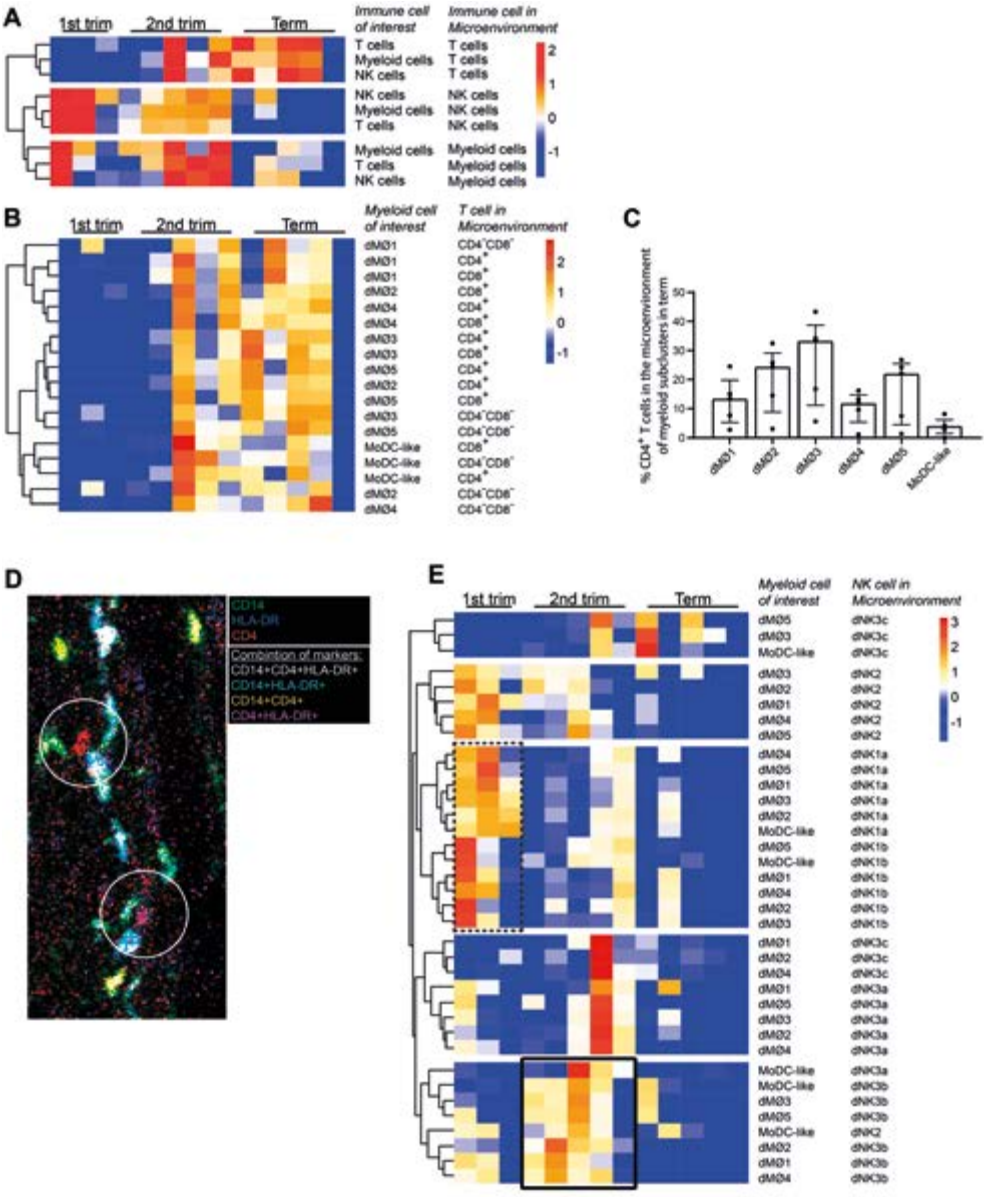


Figure 5. Microenvironment profile of immune cells. In the Z-score hierarchical clustering heatmaps, orange/red indicates a higher amount of interactions and blue a lower amount of interactions than the mean of corrected microenvironment analysis frequency per row. (A) Visualization of the changes between trimesters of major immune cell lineages with other major immune cell lineages in their microenvironment. (B) Visualization of the changes between trimesters of myeloid subclusters with T cells clusters in their microenvironment. (C) Frequency of the amount of $CD4^+$ T cells (of all immune cells and trophoblasts) that are in the microenvironment of the six myeloid cell subclusters at term. In the microenvironment of the six myeloid subclusters at term there is an unequal distribution $CD4^+$ T cells in their microenvironment (Friedman test $p<0.001$). (D) Visualization of HLA-DR^+ (white and cyan) and HLA-DR^- (green and yellow) myeloid cells

in the microenvironment of (circles) CD4+ T cells (red and magenta) in a term placenta. (E) Visualization of the changes between trimesters of myeloid subclusters with NK cell subclusters in their microenvironment. The dotted box highlights that the most prominent NK cell subclusters present in the microenvironment of all six myeloid subclusters at the first trimester are dNK1a and dNK1b. The black box highlights that the most prominent NK cell subcluster in the microenvironment of all six myeloid subsets at the second trimester is the dNK3b subcluster.

the distribution of major immune cell lineages within the $CD45^+$ compartment of the first trimester consisted of ~40% NK cells and ~1% (T cells). Importantly, our data are not contradicting current dogmas, since consistent with literature we observed decreased NK cell frequencies and increased T cell frequencies throughout gestation [6-9]. Additionally, our IMC data are in line with previous studies using immunohistochemical staining to determine the distribution of decidual leukocyte populations throughout gestation [9, 13].

By combining multiple myeloid markers (e.g. CD14, CD68, CD163, CD11b), our IMC panel was well suited to accurately determine the frequency of the myeloid population within the $CD45^+$ compartment. Additionally, the quantification strategy we used in our analysis is highly accurate since all markers were measured simultaneously on the same tissue slide. We binarized our data to correct for staining intensity differences between samples caused by the time till fixation and the fixation procedure [27]. Moreover, we used machine learning methods to identify cells, instead of manually counting cells or by using subjective grading strategies [33]. For the microenvironment analysis, we compared different pixel distances between cells. We chose a 10-pixels distance between cells, since it most optimally represented the direct cellular microenvironment: the first cells adjacent to the cell of interest were identified without another cell in-between, meaning that two neighboring cells may directly affect each other by contact and/or by secreted products. Furthermore, we confirmed by permutation testing that the interactions we found do not occur at random.

Within the myeloid compartment (excluding granulocytes), we identified six phenotypically distinct subclusters. We detected the presence of a CD14+CD56+ (dMØ3) subcluster, which has not previously been described in the decidua. Upon unsupervised clustering, these cells cluster together with the myeloid cell compartment, rather than the NK cell compartment. They are positive for NK cell markers CD56 and CD7, and for the myeloid markers CD68, CD4, CD163, CD204, and DC-SIGN. Additionally, some cells are positive for NKG2A and CD11c, which can be present on both NK cells and myeloid cells. Furthermore, they are positive for the activation markers CD39, TIM3, and CD38. This cluster does not seem to directly interact with trophoblasts. Upon cross referencing we also identified this subcluster in our previous SMC data [6], and in the first trimester scRNAseq data set from Vento-Tormo et al. [11], where these cells similarly cluster together with other myeloid cells rather than NK cells. Currently, there is limited information on $CD14^+CD56^+$ cells. Some studies showed that progenitors expressing myeloid markers can develop into NK-like cells [34-36]. Furthermore, Sconocchia et al. found $CD14^+CD56^+HLA\text{-}DR^+CD11b^+CD33^+$ cells in peripheral blood from healthy individuals that were capable of inducing more T cell proliferation than their $CD56^-$ counterpart [37]. Furthermore, they produce detectable levels of IL-6 and IL-1β besides

the typical monocyte cytokines *in vitro* [37]. Future functional analyses of the decidual CD14$^+$CD56$^+$ population may help to point out more specifically its function in the placenta.

The two dominant myeloid subclusters in first trimester were lacking expression of HLA-DR (dMØ1, dMØ4). Based on the reduced expression of several markers besides HLA-DR (e.g. CD45, CD4, NKG2A, CD38, and CD39), these cells likely have a different activation status than the HLA-DR$^+$ subclusters [38-42]. It has been described that most myeloid cells in the decidua are HLA-DR positive [7, 15, 19], and are thereby capable of presenting antigen to CD4$^+$ T cells. The HLA-DR$^-$ cells appear to represent a genuine population, as we observed CD14$^+$ subsets that abundantly expressed HLA-DR in the same tissue slides, excluding the possibility of a technical error. To substantiate our results, we confirmed by immunofluorescence that indeed considerable numbers of decidual myeloid cells can be detected having no or diminished surface expression of HLA-DR. Interestingly, the two HLA-DR$^-$ subclusters represented the only myeloid subset to be highly present in the microenvironment of first trimester trophoblasts. This may suggest that HLA-DR$^-$ myeloid cells play a role in trophoblast invasion during early pregnancy. In support of this notion, Shimada et al. described in a study on first trimester decidua samples from electively terminated pregnancies and from miscarriages with normal or abnormal fetal chromosomes that CD68$^+$CD163$^+$HLA-DR$^-$ macrophages favored the maintenance of pregnancy at an early stage [43]. In concert with the notion that HLA-DR$^-$ myeloid cells (expressing low levels of activation markers) may be involved in trophoblast invasion, it has been shown that activated macrophages can inhibit trophoblast invasiveness *in vitro*, which might be disadvantageous during early placentation [44, 45]. *In vitro* analysis of the two HLA-DR$^-$ subclusters would need to point out how they may influence trophoblast invasion, and whether trophoblasts and/or their secreted products may influence myeloid cell phenotype and function.

The role of myeloid cells at term appears to be different compared to first trimester, as there no longer is intimate contact with trophoblasts, and their immune cell microenvironment has changed. In first trimester, we detected mostly dNK1a and dNK1b NK subclusters in the microenvironment of all myeloid subclusters. Thereby we strengthen the current hypothesis that dNK1 represents the main NK cell subset that is important for spiral artery remodeling in first trimester [11]. Interestingly, both HLA-DR$^-$ subclusters, as well as the dNK1a NK subcluster, were highly abundant in the microenvironment of trophoblasts during first trimester. Further studies need to point out if these cells work in harmony and if disturbance of either one is related to improper trophoblast invasion. As expected, during gestation the microenvironment of the myeloid subclusters changed from being NK cell-rich to being T cell-rich.

When evaluating the microenvironment of trophoblasts it is clear that there are less immune cells in their vicinity at term compared to first trimester. This decrease is likely caused by the production of fibrinoid tissue secreted by the trophoblasts, whereby they embed themselves. The matrix-type fibrinoid produced by the trophoblast cells is thought to be needed to anchor the placenta to the uterine wall, to prevent bleedings when the placenta detaches at delivery, and to regulate trophoblast invasion by cell surface integrins [46]. Integrins are important in regulating all aspects of immune cell functioning, both when maintaining homeostasis and during inflammation and regulation [47]. From the perspective of the trophoblasts the matrix-like fibrinoid

may create a physical barrier that helps prevent allo-recognition by immune cells. Furthermore, it may help limiting the invasion by trophoblasts to go any further at a time point where implantation and spiral artery remodeling have already been achieved.

The IMC technique has low throughput and is suitable as an explorative approach to generate new concepts. Hence, one of the limitations of this study is the small sample size, which also limited the statistical analyses. For first trimester we have included 3 samples. Therefore, we performed statistical testing on the cluster frequencies and found that the differences (median distance) between 1st trimester samples (3.51) is smaller than 1st trimester against 2nd trimester (5.43) or term samples (5.55) (data not shown). Next to that, we observed a clear difference to the interaction profiles in the different trimesters and therefore did not increase the sample size. Despite the limited sample size, the immune cell distribution was relatively homogeneous over all samples of the different trimesters, except for one term sample (2398). This sample did not show many immune cells in its microenvironment, possibly due to the relative low frequency of immune cells compared to trophoblasts (ratio: 0.311) present in this sample compared to other samples. Furthermore, findings of kinetics for cell types over time were in concordance with previous literature showing that NK cell frequencies decreased, and T cell frequencies increased throughout gestation. In addition, we added an extra layer of information by in-depth immune profiling combined with the study of spatial cell orientation. It needs to be mentioned that cells found to be in each other's microenvironment do not necessarily have interaction. Hence, further functional analyses will need to be performed to determine the exact role of each cell in its microenvironment and by what mode it is affecting any surrounding cells.

In summary, we phenotypically determined immune cell subclusters and trophoblasts present in the decidua throughout different trimesters and studied their microenvironment. During healthy pregnancy, myeloid cells show a high abundance at the maternal-fetal interface. At the three trimesters of gestation these cells are dynamic in their phenotype and interactions. In future studies, the results can be used to set up substantiated *in vitro* culture experiments to identify the consequences of cell interactions as found in the current study. Furthermore, once the role of specific maternal decidual immune cells is clear in healthy pregnancy, any deviations in their characteristics may give insight in the role they play in pregnancy complications.

LIMITS OF THE STUDY

Here we visualize the spatial orientation of maternal immune cells and foetal trophoblasts within the maternal-fetal interface, however we did not study the maternal stromal cells, which are also present in high numbers as can be observed in supplemental figure 5A. When making the single cell mask that is necessary to analyse that many different markers simultaneously, the nucleus of the cells needs to be visible. When cutting 4-µm slides, cells with large cytoplasm might lead to a gap in the mask and these cell interactions will be missed in the microenvironment analysis. This could possibly lead to an underestimation of the number of interactions of cells with large cytoplasm (e.g. macrophages and trophoblasts). Furthermore, the microenvironment analysis is limited to one cell next to another and does not include multiple different cells surrounding one cell, which would give a more complete image of the microenvironment. However, this type

of analysis remains challenging to perform as this IMC technique is relatively new and analysis techniques are still under development.

This study is broad, focussing on and giving an overview on many different cell's microenvironments. Therefore, no functional test was performed because that might take away the overview purpose of the study. Because of this we can only speculate on the effect that cells in each other's microenvironment may have. Future studies with functional testing may address currently forwarded hypotheses.

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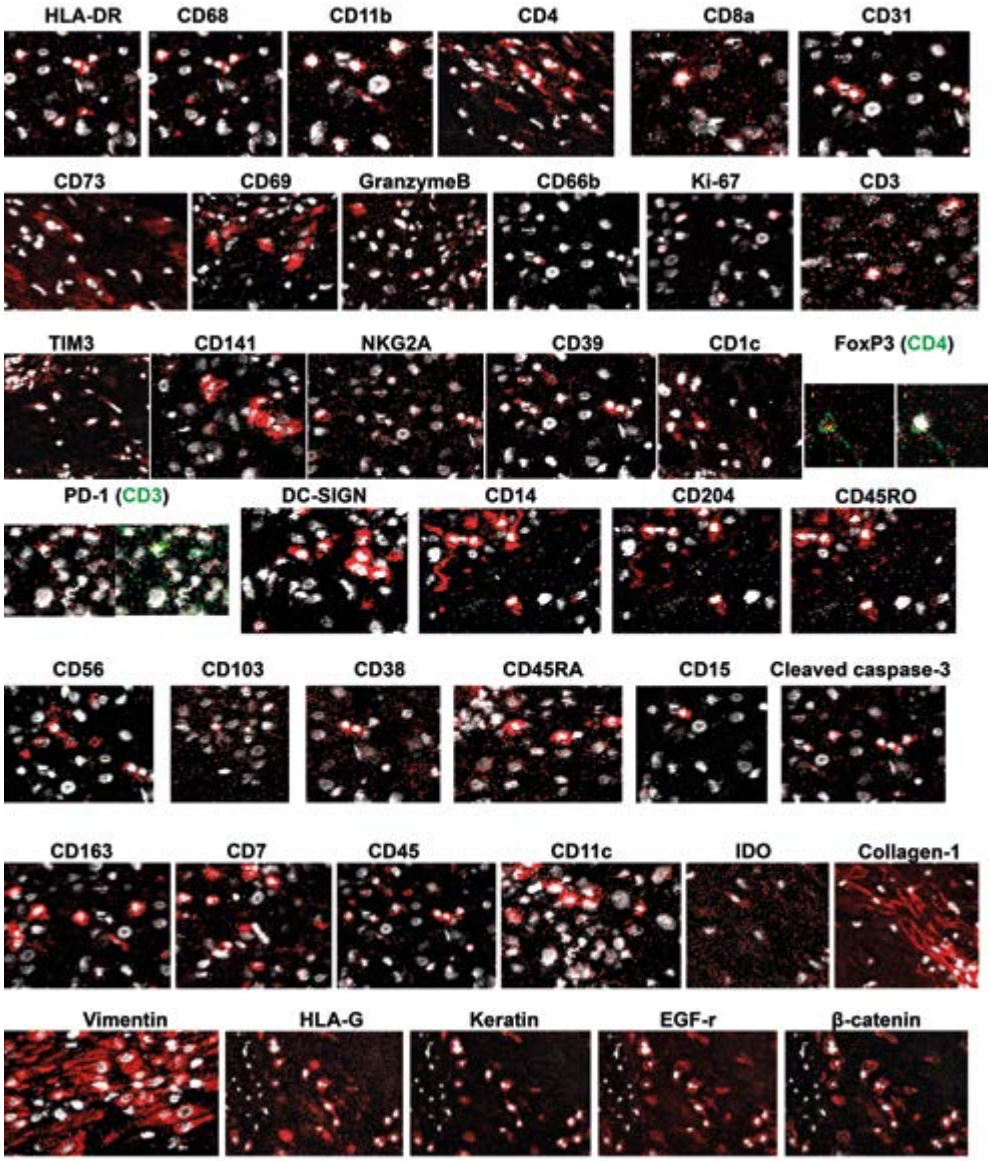
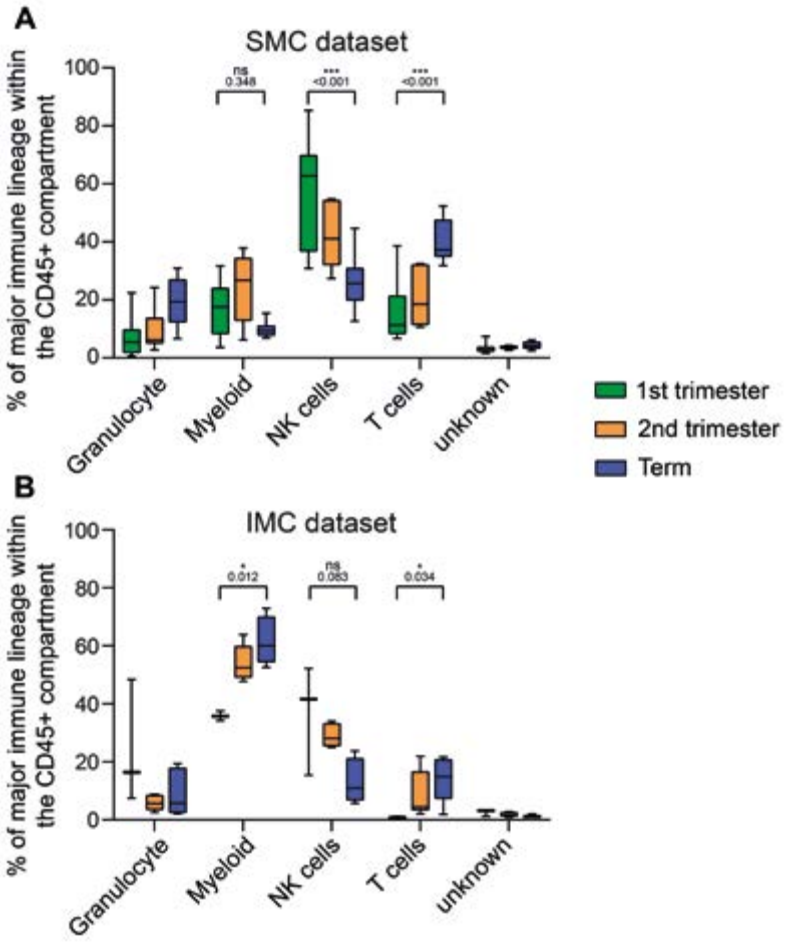
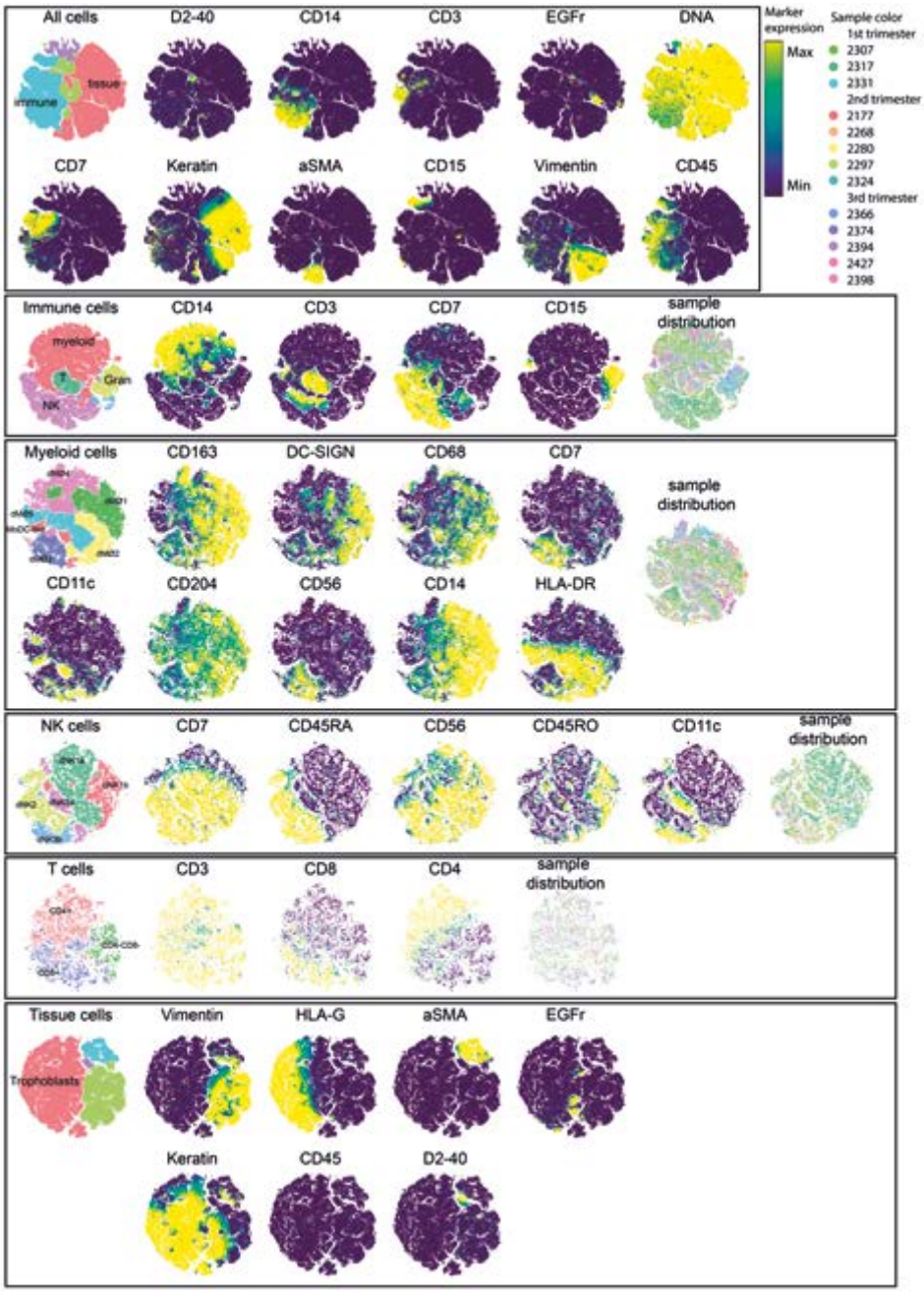
SUPPLEMENTARY DATA



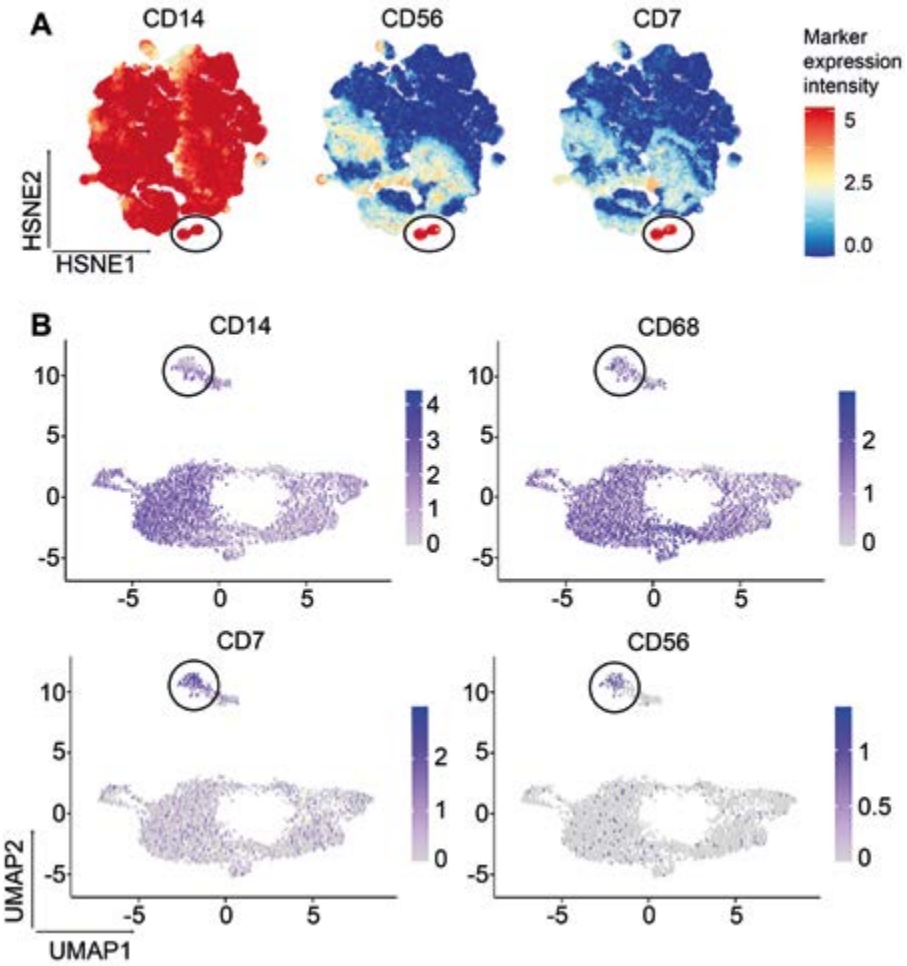
Supplementary Figure S1. Marker expression of each marker used in IMC. Visualization of all 42 markers in the IMC panel, exported from MCD viewer. Markers were individually thresholded for improved visualization. Marker expression is depicted by a red signal, DNA expression by a white signal. FoxP3 is hard to visualize with DNA and therefore is visualized with CD4 (green) co-expression. PD-1 is a dim marker and visualized with CD3 (green) co-expression.



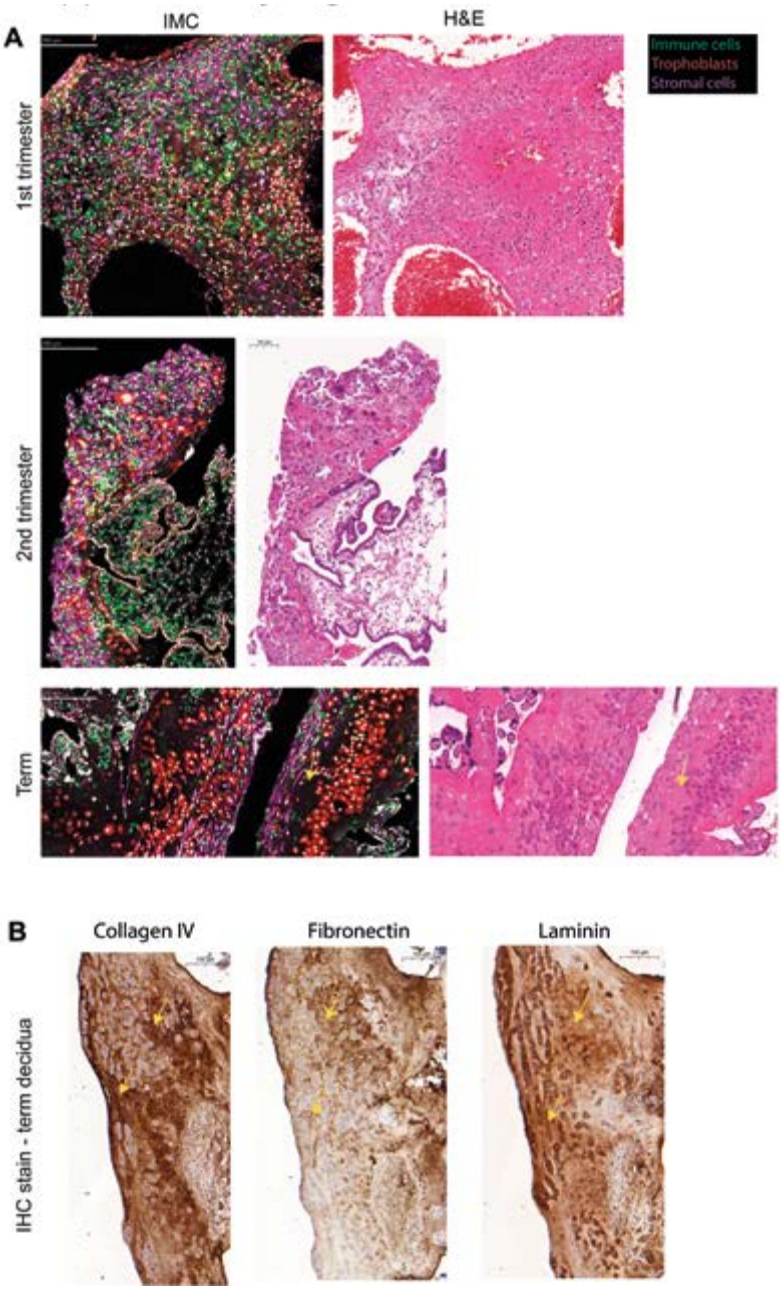
Supplementary Figure S2. Comparison of major immune cell lineages by IMC and SMC. Frequency of major immune cell lineages including granulocytes and unassigned cells (unknown) in the $CD45^+$ compartment by both SMC and IMC in first and second trimester and term samples. Data are represented as Min to Max boxplots, Kruskall-Wallis and Dunn's multiple comparisons test, *P < 0.05; **P < 0.01; ***P < 0.001.



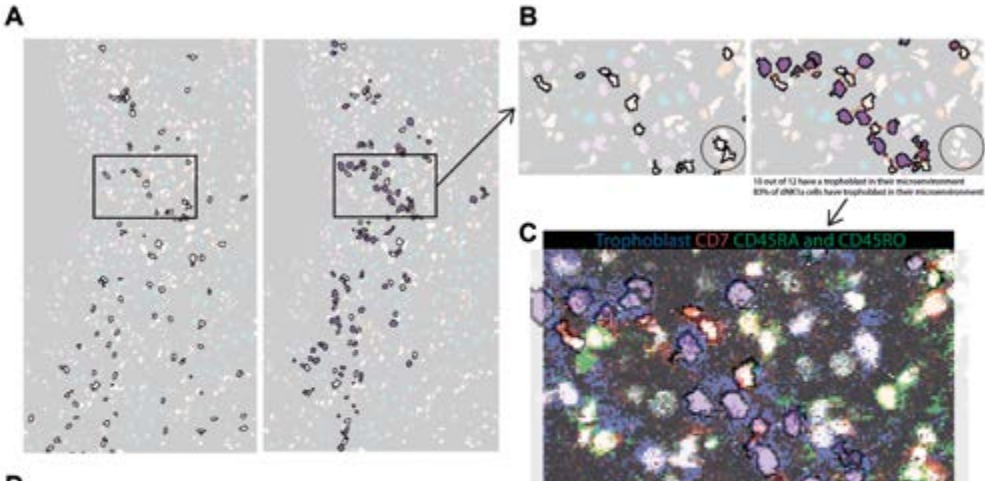
Supplementary Figure S3. tSNE plots visualizing clusters and individual marker expression. tSNE visualization of all clusters identified by IMC using all 42 markers in the panel for clustering. Clusters contain at least 100 cells, if during automatic clustering a cluster is smaller than 100 cells it is merged with the most similar cluster according to the dendogram created by the stochastic neighborhood analysis. Clusters only differing from dim to bright marker expression are merged, since dim and bright expression, due to previous binarization of signal intensity, is irrelevant.



Supplementary Figure S4. dMØ3 population found in SMC dataset and single cell RNA sequencing dataset. Identification of the dMØ3 cluster (circled) in (A) the suspension mass cytometry dataset [6], and (B) the single cell RNA sequencing dataset from Vento-Tormo [11]. HSNE, Hierarchical Stochastic Neighbor Embedding; UMAP, Uniform Manifold Approximation and Projection.



Supplementary Figure S5. Representative overview IMC image of trophoblasts, decidual stromal cells, and immune cells in all three trimesters. (A) Overview image from raw data from MCD viewer of first and second trimester and term samples. DNA is visualized in white; trophoblasts are visualized in red using Keratin and HLA-G; decidual stromal cells are visualized in magenta using vimentin; and immune cells are visualized in green using CD45 and CD14. Consecutive Haematoxylin and Eosin slides of the similar location as in A for overview purposes and for visualization of fibrinoid-like tissue, indicated by a yellow arrow. (B) IHC staining of term decidua with Collagen IV, Fibronectin and Laminin, fibrinoid-like tissue is indicated by yellow arrows.



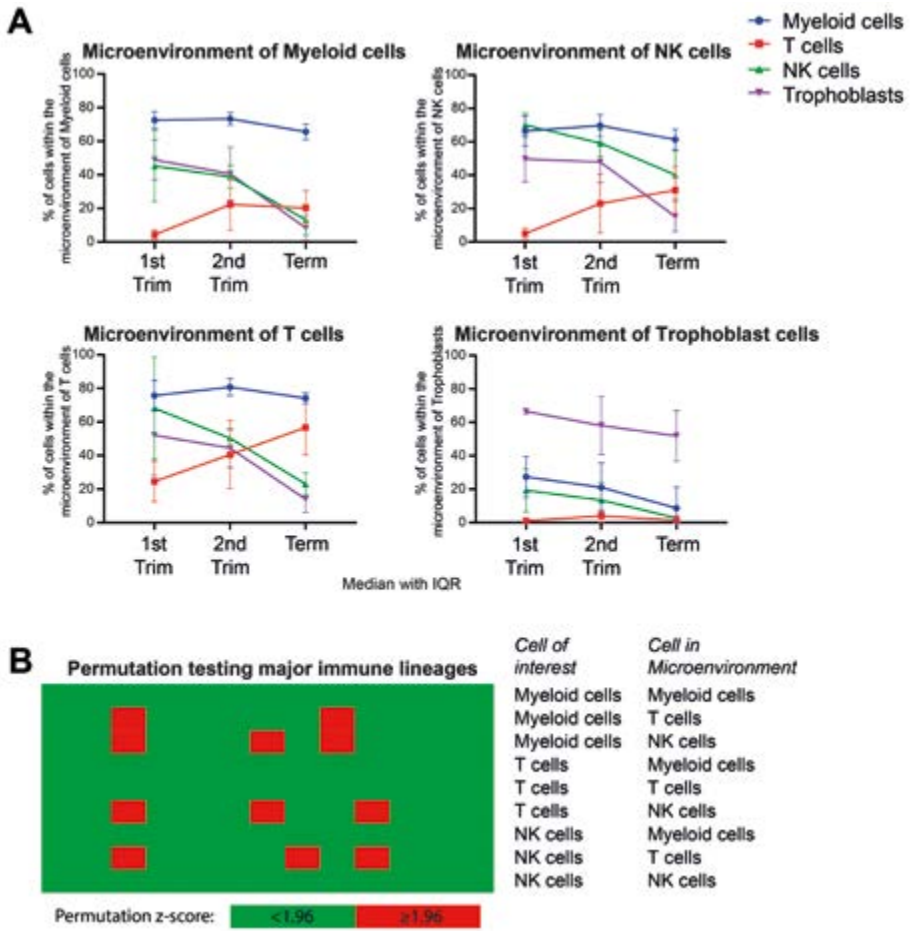
1st trim_ROI10_small area

population	cell count	frequency
rest immune	38	52,05%
dNK1a	12	16,44%
Trophoblast	25	34,25%

1st trim_ROI10_small area

Phenotype of interest	Phenotype of microenvironment	# of at least one colocalized cell	# of celltype of interest	% observed interaction	% expected interaction	% interaction
dNK1a	Trophoblast	10	12	83,33%	5,63%	77,70%

Supplementary Figure S6. Microenvironment analysis explained After mask design and phenotype notation, cells were loaded back into ImaCytE where interaction analysis was performed. In this example the frequency of trophoblasts (purple) in the microenvironment of dNK1a cells (white) is calculated (small region of 1st trim placenta) including correction for cell frequency. (A) visualization of dNK1a and (B) dNK1a cells with a trophoblast in their microenvironment trophoblasts cells. (B) zooming in to our example region we observed 12 dNK1a cells. Ten of those 12 have a trophoblast in their microenvironment (circle on the two that do not) which is 83%. (C) We find this is correct when comparing to raw data. (D) We calculated the cell frequency of dNK1a (16.44%) and trophoblasts (34.25%) within one sample and corrected for that by multiplying it with one another (16.44%*34.25% = 5.63%). We subtract the expected interaction from the observed interaction giving a corrected frequency for the amount of trophoblasts in the microenvironment of dNK1a cells. This was done per sample for all cell clusters.



Supplementary Figure S7. Frequency and permutation z-score of the microenvironment analysis. (A) Frequency of major immune lineages and trophoblasts in the microenvironment of other major immune lineages and trophoblast. Other than z-scores of (Figures 4A and 5A) the percentages of immune cells in the microenvironment of other cells can amongst others visualize the constant high frequency of myeloid cells in the microenvironment of other cells. (B) Permutation z-scores of major immune lineages interactions shows that most interactions are not at random (≥1.96; probability of <0.05).

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REGULATORY T CELLS IN PREGNANCY: IT IS NOT ALL ABOUT FOXP3

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ABSTRACT

In pregnancy, the semi-allogeneic fetus needs to be tolerated by the mother's immune system. Regulatory T cells (Tregs) play a prominent role in this process. Novel technologies allow for in-depth phenotyping of previously unidentified immune cell subsets, which has resulted in the appreciation of a vast heterogeneity of Treg subsets. Similar to other immunological events, there appears to be great diversity within the Treg population during pregnancy, both at the maternal-fetal interface as in the peripheral blood. Different Treg subsets have distinct phenotypes and various ways of functioning. Furthermore, the frequency of individual Treg subsets varies throughout gestation and is altered in aberrant pregnancies. This suggests that distinct Treg subsets play a role at different time points of gestation and that their role in maintaining healthy pregnancy is crucial, as reflected for instance by their reduced frequency in women with recurrent pregnancy loss. Since pregnancy is essential for the existence of mankind, multiple immune regulatory mechanisms and cell types are likely at play to assure successful pregnancy. Therefore, it is important to understand the complete microenvironment of the decidua, preferably in the context of the whole immune cell repertoire of the pregnant woman. So far, most studies have focused on a single mechanism or cell type, which often is the FoxP3 positive regulatory T cell when studying immune regulation. In this review, we instead focus on the contribution of FoxP3 negative Treg subsets to the decidual microenvironment and their possible role in pregnancy complications. Their phenotype, function, and effect in pregnancy are discussed.

PLACENTAL DEVELOPMENT AND IMMUNE EVASION BY TROPHOBLASTS

The most striking feature of pregnancy is that a semi-allogeneic fetus is tolerated by the maternal immune system. This is in sharp contrast with solid organ transplantation, where an allograft will be rejected by the patient's immune system unless the patient takes immunosuppressive drugs. Since direct contact between maternal and fetal cells occurs at the maternal-fetal interface in the placenta, it is thought that maternal immune cells in the placenta do not attack the fetal cells (trophoblasts) because of the tolerogenic microenvironment created by regulatory T cells (Tregs) and other immune cells.

Trophoblast development

The main function of the placenta is to provide oxygen and nutrients to the developing fetus. In the first-trimester, nutrients are mainly provided by uterine glands in a hypoxic environment as no active maternal blood flow has been established yet. Once active maternal blood flow in the placenta has commenced around weeks 11-12 of gestation, oxygen and nutrients are exchanged over a thin lining of fetal cells. Since the fetus is semi-allogeneic, as it inherits both maternal and paternal antigens, the fetal trophoblast cells may potentially be recognized as foreign by maternal immune cells. Three main types of trophoblasts can be distinguished: cytotrophoblasts (CTBs), syncytiotrophoblasts (SCTs), and extravillous trophoblasts (EVTs). At the beginning of the first trimester, the maternal-fetal interface consists of the maternal parenchymal cells in the decidua and the fetal SCTs (Figure 1A). Later in pregnancy, this interface is mainly represented by maternal decidual cells and the EVTs (Figure 1B), where a distinction is made between decidua basalis and decidua parietalis. Importantly, a second maternal-fetal interface is established when active

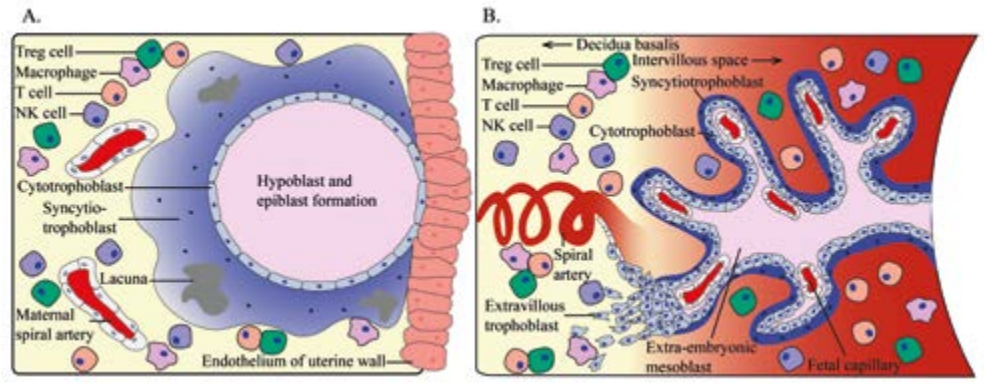

Figure 1. Schematic overview of the maternal-fetal interface at different trimesters. **(A)** During first-trimester, the maternal immune cells in the decidua can come into contact with fetal syncytiotrophoblasts, when around weeks 11-12 the maternal blood flow commences **(B)** a second maternal-fetal interface occurs. The maternal immune cells in the periphery can come into contact with fetal syncytiotrophoblasts, while the maternal decidual immune cells are in contact with the fetal extravillous trophoblasts. Indicating immunotolerance needs to adapt during the shift in gestation.

maternal blood flow in the placenta has commenced. The maternal peripheral blood then comes into contact with the SCTs lining the fetal villi. From the moment these maternal-fetal interfaces have been established, it is of utmost importance for maternal immune cells to keep the balance between tolerizing the semi-allogeneic fetus, and at the same time maintaining the ability to form a robust immune response against pathogens upon infection.

Mechanisms by trophoblasts for avoiding and modulating immune responses

The classical human leukocyte antigen (HLA) class I molecules HLA-A, -B, and -C are normally present on virtually all nucleated cells in the body and present intracellular antigens to surveilling T cells. Non-classical HLA molecules are selectively present, and have initially been described on trophoblasts in the placenta [1] and later also in other tissues [2-4]. HLA class II is mainly expressed by antigen-presenting cells (APCs), including dendritic cells (DCs), macrophages, and B cells. Since the fetus inherits half of its genes from the father, it also inherits half of the paternal HLA alleles, which can potentially be recognized as foreign by the maternal immune system.

One way for the trophoblasts to evade recognition by the maternal immune system is lack of the polymorphic HLA-A, -B, and HLA class II molecules on their cell surface. Interestingly, EVTs do express polymorphic classical HLA-C molecules. The regular function of these molecules is to present a wide variety of pathogen-associated peptides to surveilling $CD8^+$ T cells [5]. Since HLA-C is polymorphic, its presence on trophoblasts can possibly also lead to allorecognition of the inherited paternal HLA-C by maternal T cells [6]. EVTs may help to tip the local maternal immune balance towards tolerance by their expression of non-classical HLA-E and HLA-G [7], and possibly also HLA-F [8]. The mechanisms responsible for the presence or absence of the specific HLA class I types on trophoblasts have not fully been elucidated yet [5]. Expression of HLA molecules on trophoblasts allows them to escape natural killer (NK) cell recognition [9]. HLA-G was first described on CTBs and has been shown to induce immune tolerance [10, 11] (described below). HLA-E also has tolerogenic properties as it can bind to the NK cell receptor CD94/NKG2A upon which NK cell activity is inhibited [12]. SCTs, which are in direct contact with the maternal blood, do not express any HLA molecules [13], which would potentially render them sensitive to NK cell-mediated killing [13]. However, for NK cells killing an activating ligand needs to be present on the target cell, which is likely missing on trophoblasts [14].

Trophoblasts express several molecules that are thought to dampen alloimmune reactivity, including PD-L1, PD-L2, CD200, and FasL [15-19], some of which are differentially expressed throughout gestation [19]. Trophoblasts are also known to produce soluble factors with an immune-modulatory action, such as soluble HLA-G (sHLA-G), transforming growth factor-beta (TGF-β), and indoleamine 2,3-dioxygenase (IDO). TGF-β is known to have various functions and will be extensively discussed below. Since IDO causes local tryptophan deprivation [20], which is an essential amino acid required for T cell activation, elevated local IDO levels lead to inhibition of T cell activation. Recently, the role of galectins in pregnancy has become more apparent, as they were found to play an important role in suppressing the maternal immune system [21]. Galectins on human trophoblasts modulate a number of regulatory mechanisms [22], such as induction of T cell apoptosis [23] and induction of Treg cell development [24].

Maternal immune cells in the decidua

Not only the composition of fetal cells in the placenta but also the composition of maternal immune cells changes throughout gestation. Already before conception, as early as seminal plasma exposure, activation and proliferation of fetus-specific maternal T cells in uterine draining lymph nodes have been observed in murine models [25]. In humans, maternal APCs and $CD8^+$ T cells seem to get recruited to the ectocervix upon coitus, but their specificity remains unknown [26]. In the first trimester of human pregnancy, maternal leukocytes account for 30-40% of all cells in the decidua [27]. During this period, the most prominent immune cells are decidual NK (dNK) cells (~60%), macrophages (~20%), and T cells (~10%) [27-29]. During gestation, dNK cell frequencies decrease, macrophage frequencies remain relatively stable, and T cell frequencies increase [28]. Next to these main immune cell populations, innate lymphoid cell (ILCs) other than NK cells, DCs, B cells, NKT cells, granulocytes, and mast cells are found in the decidua [30-32].

Despite the many mechanisms that trophoblasts have to evade an alloimmune response, fetus specific immune recognition has been observed in mice [33]. Furthermore, fetus-specific $CD8^+$ T cells [34, 35] and inherited paternal antigen (IPA)-specific antibodies are found in maternal peripheral blood during pregnancy [36-38]. Both HLA-C and HLA-E restricted $CD8^+$ T cells, specific for viral and bacterial peptides, are present in humans [39]. However, maternal $CD8^+$ T cells could recognize the paternally inherited HLA-C from the fetus or fetal minor histocompatibility antigens, and if not suppressed are likely to attack the fetal trophoblasts [34, 35]. Besides this, approximately 30% of pregnancies result in the formation of paternal HLA-specific IgG antibodies [38, 40]. Allo-antibodies directed against HLA-C of the fetus do not necessarily appear to be detrimental to pregnancy outcome [41], but some studies do show that they are associated with spontaneous preterm deliveries and recurrent pregnancy loss (RPL) [42, 43]. Therefore, to inhibit the effect of maternal immune components, it is thought that local immune regulation is required to prevent anti-fetal immunity.

MATERNAL TREG CELLS DURING GESTATION

To prevent a detrimental immune reaction against the fetus, maternal immune cells need to be regulated. The level of both $FoxP3^+$ and $Foxp3^-$ Tregs is increased in the peripheral blood of pregnant women compared to non-pregnant control women [44, 45]. While the proportion of total T cells in the decidua is low during the first trimester (~10%), of which 10-30% of the $CD4^+$ T cells are Tregs [28, 29, 46, 47], later in pregnancy the proportion of Tregs significantly increases in the decidua [46, 48]. In mice the importance of Tregs during implantation and for maintenance of a healthy pregnancy is evident. This was shown in murine studies by injecting abortion prone mice with $CD25^+$ Tregs from wild-type pregnant mice, which led to a significantly increased litter size [49]. Alternatively, depleting $CD25^+$ Tregs during the implantation period of non-synergistically mated mice caused high fetal resorption [50]. Depleting Tregs in the mid-gestation phase in non-sterile mice also resulted in high fetal resorption [51]. In a systematic review of 17 studies on human pregnancy, it has been shown that the number and functionality of Tregs are diminished in women experiencing RPL, both in the peripheral blood and in the decidua, compared to control women

[52]. Similarly, in women with pre-eclampsia decreased Treg frequencies in both the periphery and the decidua and impairment in the signaling of peripheral blood Tregs have been found [45, 53, 54].

Using extensive mass cytometry panels containing more than 38 immune cell markers, we have previously shown that there is great heterogeneity in immune cell subsets among the different trimesters [48]. Interestingly, five Treg-like clusters were found to be differently distributed over the three trimesters. This could be attributed to the developmental changes in the placenta, causing a constant change in the possible cell-cell interactions between immune cells and different EVT subsets that seem to exist over different trimesters [55]. Apart from that, a deficit in Treg presence and functionality has been observed in pregnancy complications such as PE, infertility, and RPL [56]. Such complications arise at different periods of pregnancy, i.e., during implantation, <22-24 weeks of gestation or throughout gestation [57, 58]. Taken together, as both Treg subsets and the initiation of complications can be prominent in a particular time frame of gestation, it might be that disbalances in different Treg subsets could play a role in the onset of different complications. Therefore, it is important to investigate the presence and functioning of the wide range of Treg subsets present during pregnancy.

ADVANCES IN TREG IDENTIFICATION

Regulatory T cells were originally named suppressor cells [59]. Ideas and insights changed over time, and suppressor cells have endured much debate. In 1983 it was shown in mice that both CD8 (Lyt-2$^+$) and CD4 (Lyt-2$^-$) suppressor cells were present that expressed the I-J molecule [60]. When the I-J molecule turned out not to exist and suppressor cells could not be identified in any other way, interest in these cells waned. The arrival of novel molecular technologies propelled new knowledge, which made immunological tolerance become more evident and revived interest in T suppressor cells, now referred to as Tregs [61]. In 2001, the *FoxP3* gene was identified in scurfy mice and later as a key transcription factor for Treg cell development and function in both humans and mice [62, 63]. Subsequently, several FoxP3$^-$ Treg subsets were identified, as will be discussed below. Initially, it was hypothesized that Tregs could only be generated in the thymus (tTregs), but in the 2000s this concept was challenged by studies showing that Tregs could be induced from conventional T cells in the periphery (pTregs) [64, 65]. It is thought that tTregs and pTregs function in distinct ways, recognize different types of antigens (autoantigens vs. foreign antigens), and are needed in different immunological events such as preventing T cell trafficking to an organ and preventing T cell priming by APC, respectively [66].

Because tTregs and pTregs can have different roles, there is a need for phenotypic markers to distinguish the two. While Helios and Nrp-1 have been proposed as markers for tTregs in mice [67, 68], it has been shown that Helios deficiency or Nrp-1 deficiency does not impede tTreg development [66, 69]. Consequently, there is no consensus on which markers can distinguish tTregs from pTregs [66, 70, 71]. Helios is associated with the promoter regions of apoptosis/cell survival genes, and Helios deficient FoxP3$^+$ Tregs show increased inflammatory cytokine expression, which suggests the importance of Helios in suppressing the production of effector cytokines [72]. Even though Nrp-1 is not essential for tTreg development, it seems to increase Treg immunoregulatory

properties, such as an increased capacity for tumor infiltration [71, 73]. When comparing Nrp-1 and Helios there is no consistent overlap in expression of these markers [66]. In humans, Helios is found on Tregs, but Nrp-1 is not found on peripheral blood Tregs and can, therefore, be excluded as tTreg marker [67, 74]. More recently CNS1 has been suggested to distinguish between tTregs and pTregs. However, since CNS1 is a *FoxP3* enhancer, it is debatable whether this marker distinguishes FoxP3$^-$ tTreg and pTreg populations [75, 76].

Treg subsets are often identified by their co-signaling molecules. Many Treg subsets express co-signaling molecules, such as ICOS, PD-1, TIGIT, and TIM-3, which upon interaction with their ligand can alter their function to either activation or senescence [77-79]. These co-signaling molecules, which can be present on both FoxP3$^+$ and FoxP3$^-$ Tregs, have widely been discussed in several reviews [80-82]. Similarly, the heterogeneity within FoxP3$^+$ Tregs, generally described as CD4$^+$CD25$^+$CD127$^-$ in functional assays, has been extensively reviewed elsewhere [83-88]. However, the heterogeneity within the FoxP3$^-$ compartment has not been elaborated on and will be discussed here in the context of pregnancy. Besides co-signaling molecules, several soluble factors affect the action of Tregs and are produced by these cells to mediate their immune regulatory effects. These will first be briefly reviewed.

SOLUBLE FACTORS

IL-10

IL-10 is an immunomodulatory cytokine that is produced by many immune cells in the decidua, including most known Treg subsets. It has an effect on trophoblasts and innate- and adaptive immune cells within the decidua [89]. Single nucleotide polymorphisms (SNPs) in the promoter region of IL-10 correlate with adverse pregnancy outcomes in humans [90]. Next to that, the administration of recombinant IL-10 or IL-10 producing B cells to mice leads to reduced incidence of fetal resorption [91]. Concomitantly, IL-10 null mice in sterile cages showed normal litter size, whereas administration of a danger signal in the form of a low dose of LPS to these mice resulted in increased fetal resorption [92, 93]. These data suggest that IL-10 is an important mediator of immune regulation during pregnancy. In human pregnancy, decreased serum IL-10 levels or IL-10 production by PBMCs are associated with the occurrence of PE and RPL [94-99]. This suggests that IL-10 producing immune cells are important for maintaining an uncomplicated pregnancy.

IL-10 induces expression of HLA-G on trophoblasts, which has direct and indirect immune suppressive effects (described below) [100]. IL-10, together with HLA-G, can induce monocyte-derived DCs *in vitro* to differentiate into tolerogenic DCs (DC-10) that have immunosuppressive properties [101, 102]. They exert their immunosuppressive properties by the production of IL-10, expression of HLA-G, and upregulation of inhibitory receptors for HLA-G (namely ILT2, ILT3, and ILT4). Furthermore, these tolerogenic DCs downregulate co-stimulatory molecules CD80 and CD86, as well as HLA-DR [102-105]. DC-10s induce Tregs by their expression of ILT4 and by IL-10 production [106]. Macrophages are also regulated by IL-10 [107]. It has been shown that IL-10 acts on macrophages by controlling their metabolic pathways, causing activation, proliferation, and inflammatory responses to be inhibited [107, 108]. Next to that, CD4$^+$ T cell proliferation is

suppressed by IL-10, antigen-experienced specific $CD4^+$ T cells can be induced into an anergic state, and conventional T cells can be induced to convert to Tregs [105, 109-111].

TGF-β

TGF-β is produced by and has an immunomodulatory effect on multiple cell types present in the decidua [112-121]. In the early implantation phase, TGF-β is important for trophoblast invasion in the endometrium [122, 123]. In humans, TGF-β serum levels are elevated in pregnant women compared to non-pregnant women, and serum levels are higher in early pregnancy compared to late pregnancy [124]. However, women experiencing RPL display a decrease in TGF-β serum levels compared to women undergoing elective termination for non-medical reasons [125]. Interestingly, there are indications from mouse studies that TGF-β induced Tregs could prevent spontaneous abortion, but this effect needs to be elucidated further [121, 126].

TGF-β can inhibit NK cell and T cell activation and proliferation by repressing the mammalian target of rapamycin (mTOR) signaling pathway [127, 128], and similarly, suppress activation of dNK cells [119]. Since dNK cells are important contributors to angiogenesis at the maternal-fetal interface, their cytotoxicity needs to be suppressed but they should still be able to execute their role in angiogenesis. A balanced TGF-β level may, therefore, be important to maintain correct functioning of dNK cells [119]. Furthermore, TGF-β can affect T cells directly by inhibiting their proliferation and differentiation [129, 130], and indirectly by its inhibitory effect on APCs. HLA-class II on APCs is downregulated, activation of macrophages is downregulated, and maturation of DCs is prevented by TGF-β [118, 131-135]. Next to that, the presence of TGF-β is needed for the induction of several $FoxP3^+$ and $FoxP3^-$ Treg subsets by APCs [136-139].

HLA-G

As discussed above, HLA-G was first described on trophoblasts [1]. Interestingly, also myeloid and lymphoid cells, such as the below described $FoxP3^-HLA\text{-}G^+$ Treg, can express HLA-G and secrete sHLA-G [140-142]. HLA-G is oligomorphic and has seven isoforms, of which some are membrane-bound (HLA-G1 to -G4), and others are secreted as a soluble form (sHLA-G5 to -G7) [143]. Several polymorphisms in the untranslated region (UTR) of the *HLA-G* gene have been associated with lower sHLA-G levels in both blood and seminal plasma [144, 145]. In both PE and RPL, a reduction in serum sHLA-G levels has been observed compared to healthy control women [146-149]. Together these observations highlight the possible importance of (s)HLA-G during pregnancy.

(s)HLA-G exerts its immunoregulatory effects on a wide variety of immune cells because of its interactions with several inhibitory receptors, of which ILT2 seems to be most prominent [150]. Other receptors for (s)HLA-G are ILT4, KIR2DL4, and CD8. The ITL2 receptor is expressed on monocytes/macrophages, DCs, B cells, and some NK and T cells [151], while the ILT4 receptor is mainly present on macrophages, NK cells, and neutrophils [151, 152]. Upon ILT2 or ILT4 binding to HLA-G, NK cells and T cells receive a signal that leads to inhibited killing capacity [153-155]. In $CD8^+$ T cells, this inhibited killing capacity is reflected by the down-regulation of granzyme B expression [156]. KIR2DL4 has been identified on dNK cells and some T cell subsets. Engagement

of this receptor with sHLA-G results in activation and secretion of different types of cytokines and chemokines, but does not result in direct cytotoxicity [157]. Binding of sHLA-G with KIR2DL4 on NK cells results in the upregulation of a restricted set of chemokines and cytokines that can promote vascular remodeling [157]. CD8 is not only expressed by cytotoxic T cells but also by some NK cell subsets [82, 158]. When sHLA-G binds to CD8, this interaction inhibits cytotoxic activity and triggers FasL-mediated apoptosis in both the $CD8^+$ T cells and $CD8^+$ NK cells [159]. Besides effector cells, APCs can also be affected by HLA-G. For example, in concert with IL-10, HLA-G induces DCs to differentiate into tolerogenic DC-10 cells [101, 102]. Additionally, macrophages obtain a tolerogenic phenotype upon binding to HLA-G with their ILT2 or ILT4, and subsequently show reduced expression of HLA class II, CD80, and CD86. Such macrophages have been described to be similar to decidual macrophages as they also express IDO [160]. Together this suggests that decidual macrophages are under the constant influence of HLA-G, produced by either trophoblasts or $HLA\text{-}G^+$ Tregs.

$FOXP3^-$ REGULATORY T CELLS

$FoxP3^-$ $HLA\text{-}G^+$ Tregs

In the lymphoid compartment, HLA-G expressing $CD4^+$ and $CD8^+$ cells show reduced proliferation in response to allogeneic and polyclonal stimuli [142]. $CD4^+HLA\text{-}G^+CD25^-FoxP3^-$ Tregs (Figure 2, Figure 5, Table 1) suppress T cell proliferation through the expression of membrane-bound HLA-G1 and secretion of IL-10 and sHLA-G5 in a reversible, cell-contact independent and cell-contact dependent manner [142, 161]. They have functionally been compared to other Treg populations such as $FoxP3^+$ Tregs and Tr1 Tregs (discussed below), and represent a population that is distinct from tTregs [161-163]. Interestingly, $CD4^+$ and $CD8^+$ T cells can also acquire a similar $HLA\text{-}G1^+$ phenotype *in vitro* through trogocytosis [164], meaning the uptake of membrane fragments from another cell. Resting and activated $CD25^+$ T cells that acquire HLA-G1 expression by trogocytosis differ functionally from the $HLA\text{-}G^+$ tTregs, and they do not secrete sHLA-G5 and IL-10. They have been shown to exert their immune-suppressive capacity in a cell-contact dependent manner only [164], and will not be discussed further.

$HLA\text{-}G^+$ tTregs accumulate at sites of inflammation to regulate immune responses [165] and importantly, have also been found in the decidua [140, 166]. $CD4^+HLA\text{-}G^+$ Treg frequencies are increased in peripheral blood throughout pregnancy compared to non-pregnant controls [45, 140]. Interestingly, sHLA-G serum levels are also increased during pregnancy, while these levels are decreased in complicated pregnancies compared to healthy pregnancies [146-149]. However, it is unlikely that a direct correlation between $CD4^+HLA\text{-}G^+$ Treg frequencies and serum sHLA-G levels exists, since other cells (in the placenta) produce sHLA-G as well. $CD4^+HLA\text{-}G^+$ Treg frequencies within the $CD4^+$ T cell compartment are even higher in the decidua compared to those in peripheral blood [140, 166], suggesting a role in local immune regulation. In women with PE, decidual $CD4^+HLA\text{-}G^+$ Treg frequencies within the $CD4^+$ T cell compartment are even higher in the decidua compared to those in peripheral blood [140, 166], suggesting a role in local immune regulation. In women with PE, decidual $CD4^+HLA\text{-}G^+$ Tregs are decreased, whereas in the peripheral blood their

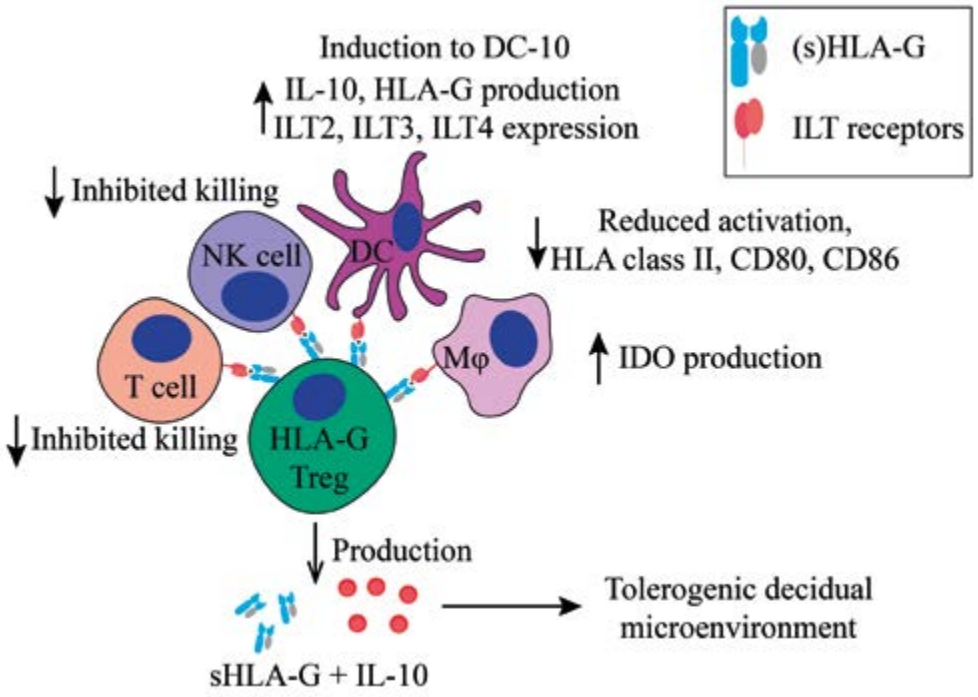

Figure 2. Main tolerogenic mechanisms of the FoxP3$^-$HLA-G$^+$ regulatory T cell. HLA-G$^+$ regulatory T cells can suppress in a cell-contact dependent manner via HLA-G1. It inhibits the killing capacity of T cells and NK cells, downregulates HLA class II, CD80 and CD86 in DCs and macrophages, and makes them tolerogenic by inducing IDO production in macrophages and the induction of DCs to DC-10 cells. HLA-G$^+$ regulatory T cells produce IL-10 and soluble HLA-G5 that helps to create a tolerogenic decidual microenvironment. HLA, human leukocyte antigen; NK, natural killer cell; DC, dendritic cell; IDO, indoleamine 2,3-dioxygenase; IL-10, interleukin-10; DC-10, tolerogenic DCs.

numbers remain unchanged compared to healthy control pregnancies [45, 166], indicating that in a healthy pregnancy these cells are induced locally, but to a lesser extent during PE.

Tr1 Treg

Tr1 Tregs (Figure 3, Figure 5, Table 1) suppresses T cell proliferation mainly through IL-10 and TGF-β production. They also produce low amounts of IFN-γ, IL-5, and IL-2, and express granzyme B [109, 112, 175]. Next to cytokine production, they can suppress other immune cells in a cell-contact dependent manner by using their KIR receptors or ectoenzymes [167]. Tr1 Tregs are peripherally induced upon chronic antigen stimulation in the presence of IL-10 [176]. Both HLA-G and IL-10 provided by APCs, like DC-10 cells, play a role in Tr1 Treg induction [105], indicated by their reduced induction by DC-10s when anti-HLA-G is added *in vitro*. Additionally, their induction is reverted when agonistic anti-ILT4 antibodies are added, but not when agonistic anti-ILT2 antibodies are added [105]. Interestingly, EVTs are also able to induce Tr1-like cells via HLA-G directly [113].

TABLE

Table 1. Overview of FoxP3$^-$ immune regulating T cells discussed in this review, how they are induced or activated, their main suppressive mechanism and how they function, their localization, animal models depletion assays, master genes for differentiation, and cell volume changes in complicated pregnancies.

Subset	Induction/ activation	Suppressive mechanism	Function	Localization	Depletion in animal models	Master genes of differentiation	Cell volume changes in complications
CD4$^+$HLA-G$^+$ Treg[142, 164]	Natural occurring [142]	Secretion of sHLA-G and IL-10 [142], and cell interaction with HLA-G [164]	Induction of HLA-G expression by trophoblasts, DC-10s and Tregs by IL-10. Inhibition of macrophages, NK cells and T cell killing.	Found in peripheral blood [45] and decidua [140]	Has not been performed	Not known	Found to be increased in peripheral blood of pre-eclampsia patients [45]
Tr1-(like) Treg [109, 113]	Via trogocytosis [164]	Secretion of IL-10 and TGF-β, and cell interaction [137, 167]	Induction of HLA-G expression by trophoblasts, DC-10s and Tregs by IL-10. Lysis of APCs, disruption of metabolic state of T cells.	Found in peripheral blood and decidua [113]	Has not been performed	Not known	Has not been described
Th3 Treg [168]	By APC in an IL-10 dominant microenvironment [110]	Secretion of TGF-β and IL-10 [168]	Induction of HLA-G expression by trophoblasts, DC-10s and Tregs by IL-10. Inhibition of NK cell and T cells and APC by TGF-β.	Found in the decidua [169]	Has not been performed	Not known	Has not been described
CD8$^+$ Treg [60]	By APC in presence of TGF-β and IL-4 [115, 138]	Suppress the secretion of immunoglobulins [170]	Prevent formation and suppressing production of IPA-specific antibodies.	Found in peripheral blood (CD8$^+$HLA-G$^+$ Treg) [45] and decidua [170]	Has not been performed	Not known	CD8$^+$HLA-G$^+$ Treg are increased in peripheral blood of pre-eclampsia patients [45]

Table 1. (continued)

Subset	Induction/ activation	Suppressive mechanism	Function	Localization	Depletion in animal models	Master genes of differentiation	Cell volume changes in complications
NO-Treg [171]	$CD101^+CD103^+$ are induced by trophoblasts [170]	Secretion of IL-10 [171, 172]	Induction of HLA-G expression by trophoblasts, DC-10s and Tregs by IL-10.	Found in peripheral blood [171]	Has not been performed	Not known	Has not been described
$TIGIT^+$ Treg [113]	Depends on nitric oxide, p53, IL-2, and OX-40 [171]	Secretion of IFNγ and IL-2 [113]	Induction of IL-10 production by APCs. Suppression of $CD4^+$ effector T cells.	Found in decidua [113]	Has not been performed	Not known	Has not been described
$V\delta1^+$ γδ T cell [173]	Unknown	Secretion of IL-10 and TGF-β [114]	Induction of HLA-G expression by trophoblasts, DC-10s and Tregs by IL-10. Inhibition of NK cell and T cells and APC by TGF-β.	Found in peripheral blood and decidua [174]	Has not been performed	Not known	Decreased amount in an abortion prone mice model [121]

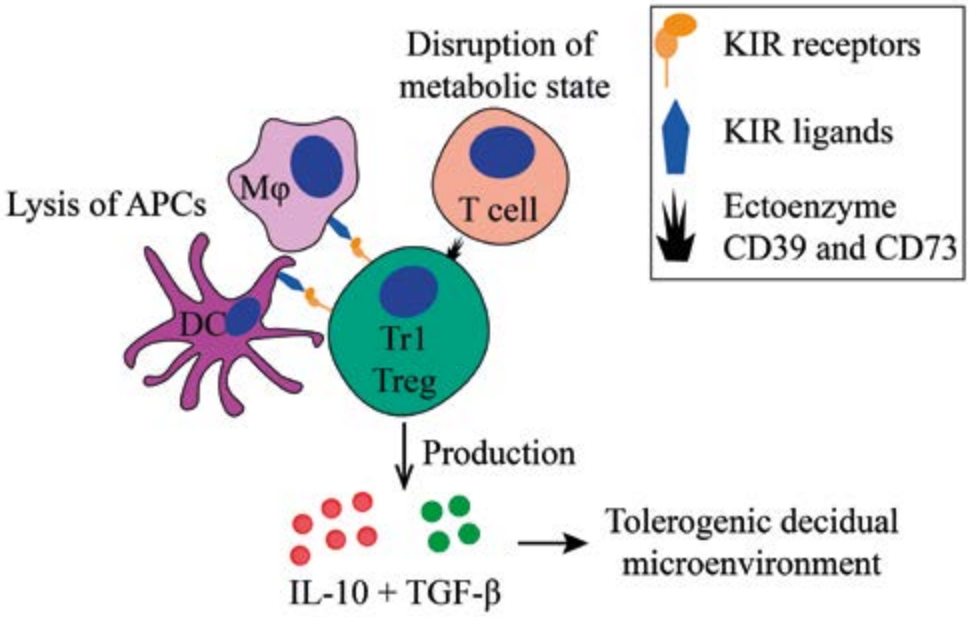

Figure 3. Main tolerogenic mechanisms of the Tr1 regulatory T cell. Tr1 regulatory T cells can in a cell-contact dependent manner lyse APCs via their KIR receptors and disturb the metabolic state of T cells. They produce IL-10 and TGF-β that helps to create a tolerogenic decidual microenvironment. APC, antigen-presenting cell; KIR, killer-cell immunoglobulin-like receptor; IL-10, interleukin-10; TGF-β, transforming growth factor-beta.

Recently, co-expression of CD49b and LAG-3 has been described as phenotypic markers for Tr1 Tregs in mice and humans [177]. This observation is under debate since a subsequent study only detected a small proportion of IL-10$^+$ Tregs co-expressing CD49b and LAG3 [178]. Due to their lack of a clear phenotype, Tr1 Tregs are often described as Tr1-like cells, as they have similar properties, such as IL-10 production. Tr1 Tregs can express the co-signaling molecules PD-1, CTLA-4, TIM-3, and ICOS [178-181], and several other molecules related to their function, including GARP, LAP, ectoenzyme CD39, and CD73 [182], as well as KIRs and ILT receptors. FoxP3 is only transiently expressed by Tr1 Tregs. Since functional Tr1 Tregs are found in patients who have a mutation in the *FoxP3* gene, FoxP3 appears not to be required for their development [110, 175].

Tr1-like Tregs have been identified in peripheral blood and various tissues [183], including the human decidua [113]. These Tregs express high levels of PD-1, express granzymes, and lack FoxP3. They produce IL-10 and IFN-γ, and thereby may have a similar suppressive mechanism as bona fide Tr1 Tregs [113]. Similar to Tr1 Treg, decidual Tr1-like Treg induction by EVTs can be partially reverted when agonistic anti-HLA-G antibodies are added, but not by anti-ILT2 [113]. Tr1 Tregs are able to selectively lyse APCs in a cell-contact dependent manner, but not B and T cells [167]. Lysis of APCs can cause amplification of the tolerogenic process since decreased numbers of activated APCs will generally lead to less activation of T cells. For this, the Tr1 Treg needs HLA-class I recognition of the APC through its KIR receptors, CD54/LFA-1 mediated adhesion, CD58/CD2 interaction, as well as CD155/CD226 ligation [167]. Furthermore, the Tr1 has been described to directly affect T cells by

their expression of ectoenzyme CD39 and CD73, which disrupts the metabolic state of effector T cells [182].

Th3 Tregs

The main suppressive effects of Th3 Tregs (Figure 4, Figure 5, Table 1) are mediated by TGF-β production, in a cell-contact independent manner [138]. Phenotypically these cells are $CD25^-$ and $FoxP3^-$, they are thought to express Helios, and express LAP and GARP, which can be used as surrogate markers for TGF-β production [184, 185]. Th3 cells also produce IL-10, but unlike Tr1 Tregs, they produce this in conjunction with IL-4 [115, 179]. Similar to Tr1 Tregs, Th3 Tregs are peripherally induced upon antigen stimulation [138]. The mechanism underlying the induction into either Th3- or Tr1 Treg remains poorly understood and is thought to depend on their microenvironment during priming [117, 186]. Another question that remains to be answered is whether Tr1 and Th3 Tregs truly represent different subsets or differentiation states and whether they differ depending on the microenvironment in which they reside.

With the limited markers identified so far, it is difficult to phenotypically identify Th3 Tregs, which may explain the limited number of articles describing the presence of the Th3 cell during pregnancy. Dimova et al. observed in paired decidua and peripheral blood samples mRNA cytokine profiles similar to Th3, the first description of a possible presence of Th3 cells in the decidua [169]. Importantly, no functional testing has been performed for Th3-like cells from the decidua, and their presence and role in pregnancy remains to be confirmed. Regardless, Th3 Treg was first described to have an important role in oral tolerance [184]. Interestingly, exposure to semen through oral sex has been proposed to be beneficial for subsequent pregnancy outcomes in couples experiencing PE or RPL [187-189], providing a possible mechanistic explanation for this effect.

Other Treg populations

Besides $FoxP3^-$ $HLA\text{-}G^+$, Tr1, and Th3 Tregs, other immune regulatory T cell populations that have been described, albeit to a lesser extent, include $CD8^+$ Tregs, nitric oxide (NO) induced $FoxP3^-$ Tregs, $TIGIT^+$ Tregs, $FoxP3^{dim}$ Tregs, and γδ T cells (Figure 5, Table 1).

$CD8^+$ Tregs are increasingly being recognized, even though they remain difficult to identify as there is no consensus on their phenotype. Both $FoxP3^+$ and $FoxP3^-$ $CD8^+$ Tregs have been described to have suppressive activities, indicating there also is heterogeneity in the $CD8^+$ Treg population

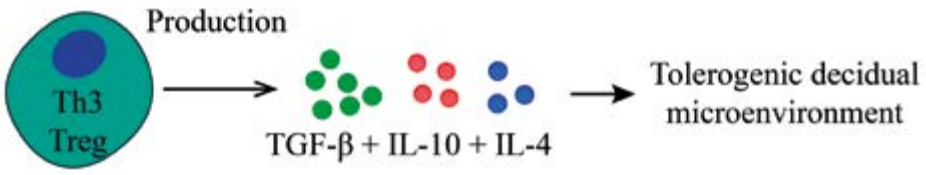

Figure 4. Main tolerogenic mechanisms of the Th3 regulatory T cell. Th3 regulatory T cells suppress in a cell-contact independent mechanism only by the production of TGF-β, IL-10 and differ here from the Tr1 regulatory T cell by the production of IL-4. TGF-β, transforming growth factor-beta; IL, interleukin.

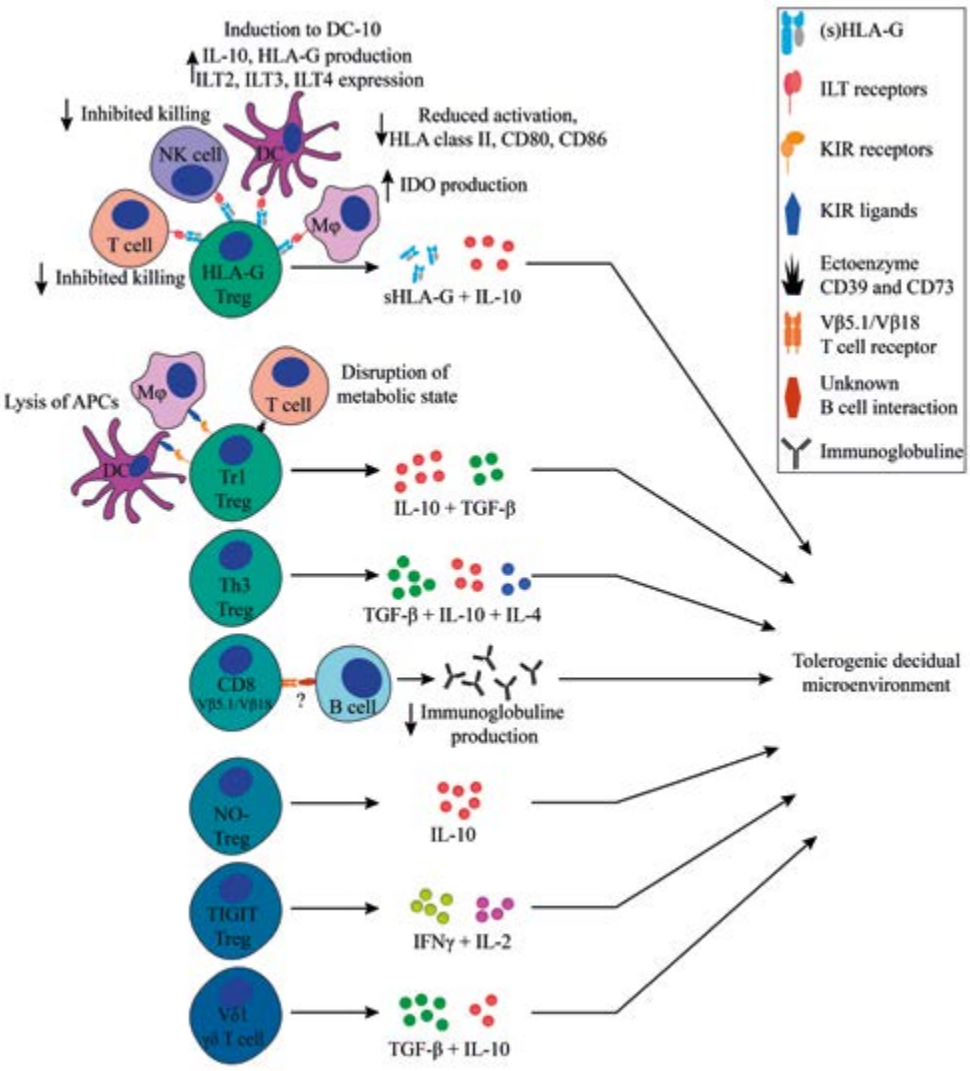

Figure 5. Overview of FoxP3⁻ immune regulating T cells discussed in this review and their main tolerogenic mechanisms in pregnancy. All Tregs described in this review can exert immunosuppressive properties in a cell-contact independent mechanism that together contributes to a tolerogenic decidual microenvironment. Next to that, the HLA-G⁺ Treg, Tr1 Treg, and CD8⁺ Treg can exert their immunosuppressive properties in a cell-contact dependent mechanism.

[190]. Shao et al. showed that a CD8⁺ Treg subset can be activated by trophoblast cells. This activation appears not to be HLA restricted since their expansion is unaffected when cultured in the presence of pan-HLA class I blocking antibodies [170]. When cultured with PBMCs, these CD8⁺ Tregs suppress the secretion of immunoglobulins in a cell-contact dependent manner, as shown using a trans-well system. While humoral immunity seemed to be dampened, these CD8⁺ Tregs did not have any suppressive effect on effector T cells. Phenotypically these cells can be identified as

being CD101$^+$ and CD103$^+$ [170]. Even though in a mixed lymphocyte reaction these CD8+ Tregs do not appear to suppress CD4$^+$ and CD8$^+$ T cells, they could potentially be important for preventing formation and suppressing production of IPA-specific antibodies.

Niedbala et al. described NO-induced Tregs (NO-Tregs) in mice [171]. These cells are characterized as CD4$^+$CD25$^+$GITR$^+$CD27$^+$T-bet^{low}, GATA3$^+$, and FoxP3$^-$, and they are induced from CD4$^+$CD25$^-$ T cells via p53, IL-2, and OX-40 [171]. Experimentally, the development of NO-Tregs was induced when using adoptive transfer of CD4$^+$CD25$^-$ T cells into SCID mice, together with application of an NO synthase inhibitor. NO-Tregs produce IL-4 and IL-10, but no IL-2, TGF-β, or IFN-γ. Addition of antagonistic anti-IL4 antibodies led to reduced proliferation of NO-Tregs, whereas blocking IL-10 blocked their suppressive effect on CD4$^+$CD25$^-$ cell differentiation [171, 172]. These data suggest that NO-Tregs suppress through IL-10, in a cell-contact independent manner. While NO-Tregs has not yet been studied in the context of pregnancy, NO appears to be involved in pregnancy with NO levels fluctuating throughout the different gestational ages and being lower during PE [191-194]. It would, therefore, be interesting to retrospectively study first-trimester blood samples of women who develop PE, to test if NO levels are already lower at this early time point of pregnancy, and to study NO-Treg formation in these patients in comparison to healthy controls.

Salvany-Celades et al. identified three types of functional Tregs in the decidua, of which two subsets were negative or low for FoxP3 [113]. One of these is the PD-1^{high}, Tr1-like cell, which has been described above. The second is the TIGIT$^+$ Treg that is characterized by TIGIT positivity, low expression of CD25 and FoxP3, and intermediate expression of PD-1. TIGIT$^+$ Tregs express high levels of IFN-γ and IL-2, and low levels of IL-10. TIGIT$^+$ Tregs mainly suppress CD4$^+$ effector T cells in proliferation assays, but not consistently CD8$^+$ effector T cells. Interestingly, TIGIT$^+$ Tregs seem to vary in their characteristics, depending on the trimester in which they are encountered [113]: first-trimester TIGIT$^+$ Tregs show an increased expression of IL-10 compared to term TIGIT$^+$ Tregs. This difference in trimesters could be due to the microenvironment influencing their phenotype, or because they truly represent different subsets. TIGIT has been described to be expressed on multiple Treg subsets, and it can bind CD155 on APCs, which thereby increases their IL-10 production [195, 196]. Binding of TIGIT induces Tregs to produce IL-10 and fibrinogen-like protein 2 (Fgl2). By usage of Fgl2 the Tregs obtain the capacity to suppress Th1 and Th17 cells *in vitro*, but not Th2 cells [79, 196]. It would be interesting to determine the presence of TIGIT$^+$ Tregs during pregnancy complications and to investigate their possible role in providing a tolerogenic microenvironment in successful pregnancies.

In the first-trimester decidua, oγδ T cells produce high amounts of IL-10 and TGF-β [114, 197]. As described above, these cytokines are important for establishing an immune suppressive microenvironment in the decidua. Transfer of uterine γδ T cell culture supernatant, containing a high concentration of TGF-β, into the uterus of mice before pregnancy prevents fetal resorption [121]. Terzieva et al. identified the TCR repertoire from decidual γδ T cells and compared this to the repertoire of γδ T cells in peripheral blood. In 1st and 3rd trimester decidua they mostly found Vδ1$^+$ TCR, whereas this particular δ chain was hardly present in the peripheral blood [174]. Vδ1$^+$ T cells are described to have a tolerogenic effect [173, 198]. The possible role of γδ T cells in pregnancy is further suggested by another study showing higher numbers of γδ T cells in peripheral blood from

women experiencing RPL compared to controls. The specific presence of the Vδ1 chain was not investigated [199]. It would be interesting to determine the frequency and immune-suppressive effect of Vδ1$^+$ T cells in the decidua of women experiencing RPL compared to women with elective termination of pregnancy.

CONCLUDING REMARKS

In this review we have discussed several types of Tregs that may contribute to a tolerogenic environment in the decidua (Figure 5, Table 1) besides FoxP3$^+$ Tregs. Decidual Tregs seem to assist other cells in creating and maintaining a microenvironment where inflammatory signals are generally overruled by tolerogenic signals. Next to Tregs, this tolerogenic microenvironment is established and maintained by factors from paternal, maternal and fetal origin. Paternal contribution to this tolerogenic microenvironment comes early on from seminal fluid that contains tolerogenic factors such as TGF-β and paternal antigens for priming. Fetal trophoblasts contribute by their expression of tolerogenic HLA-G and HLA-E molecules, galectins, and PD-L1, and by their production of sHLA-G, IDO, and TGF-β. Next to this, the maternal contribution in maintaining a tolerogenic microenvironment in the decidua is provided by the decidual immune cells, which do not have an activated phenotype and produce IDO, TGF-β, IL-10 and sHLA-G.

It remains to be elucidated which mechanisms exactly attract Tregs to the decidua, if they are activated locally by APCs in the decidua or in the lymph nodes, where they proliferate, and if they are specific for fetal antigens. In mice, it has been shown that fetus-specific Tregs are already detectable in the uterine draining lymph nodes shortly after semen exposure and that their numbers increase upon pregnancy [200]. While this could be similar in the human situation, *in vitro* fertilization with donor semen, where there is no paternal semen exposure, often results in a healthy uncomplicated pregnancy, albeit at a lower rate than in naturally conceived pregnancies [201]. More information on the basic mechanisms of FoxP3$^-$ Tregs, as well as how they are initiated, is needed to provide insight in the deviations in frequencies or functionality of FoxP3$^-$ Treg subsets in pregnancy complications. Likewise, from a therapeutic point of view such basic mechanisms need to be clarified before possible novel therapeutic strategies can be developed. These therapies could be based on therapy designs similar to those proposed for FoxP3$^+$ Tregs, such as infusion of Tregs or application of the cytokines needed for induction of specific Treg subsets [202].

While it is clear that FoxP3$^+$ Tregs play a role in maintaining pregnancy, the relevance of the different types of FoxP3$^-$ Tregs herein needs to be established. FoxP3$^-$ Tregs with proven suppressive capacities are found in the decidua and are, therefore, likely to contribute to the tolerogenic microenvironment. However, studies such as depletion assays in mice need to be performed to confirm whether they play a non-redundant role in maintaining a healthy pregnancy. Since pregnancy is crucial for the existence of mankind, it is not surprising that there would be multiple mechanisms in play to establish a regulatory microenvironment to maintain a healthy pregnancy. Pregnancy complications for which no clear cause can be identified do occur, and it is plausible that many of these are related to a disbalance in maternal immune regulation. It would be helpful to get a better understanding of the function of all regulatory T cells present in the decidua,

to be able to recognize their relevance in healthy and complicated pregnancies. As such, the use of multiple omics techniques to identify the decidual microenvironment by a holistic approach could give insights in the presence, frequency, and distribution of the different types of Tregs in pregnancy [32, 48, 203, 204]. It is important to note that the time point of sampling is a crucial factor in such experiments, given the dynamic nature of the placental microenvironment.

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4

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.

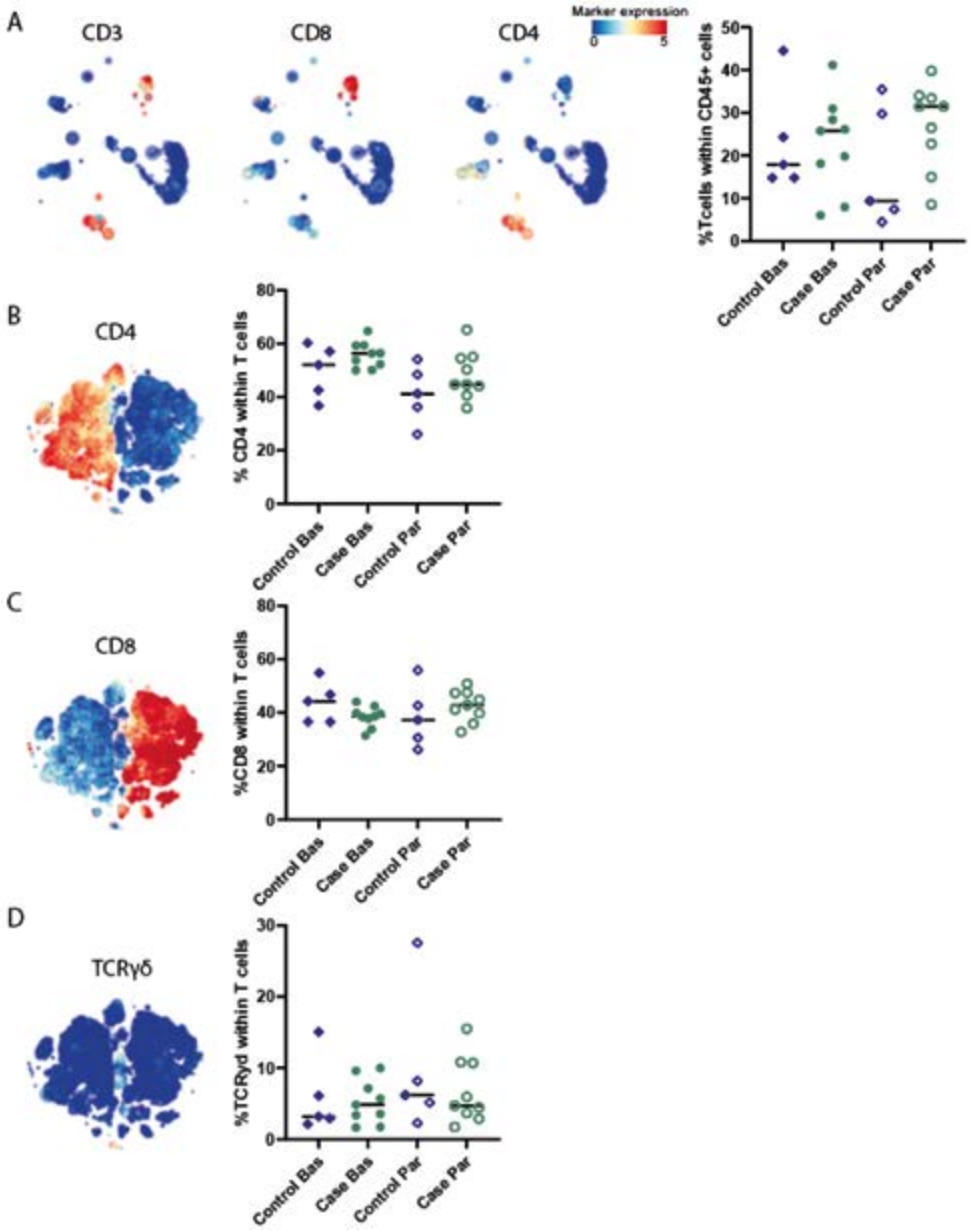

Figure 1. Decidual T cell frequencies in decidua parietalis (Par) and basalis (Bas) of women with a successful birth after uRPL (case) and controls. HSNE visualization and frequencies of T cells within the total CD45+ decidual immune cells show a wide spread between T cell frequencies between individuals (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.

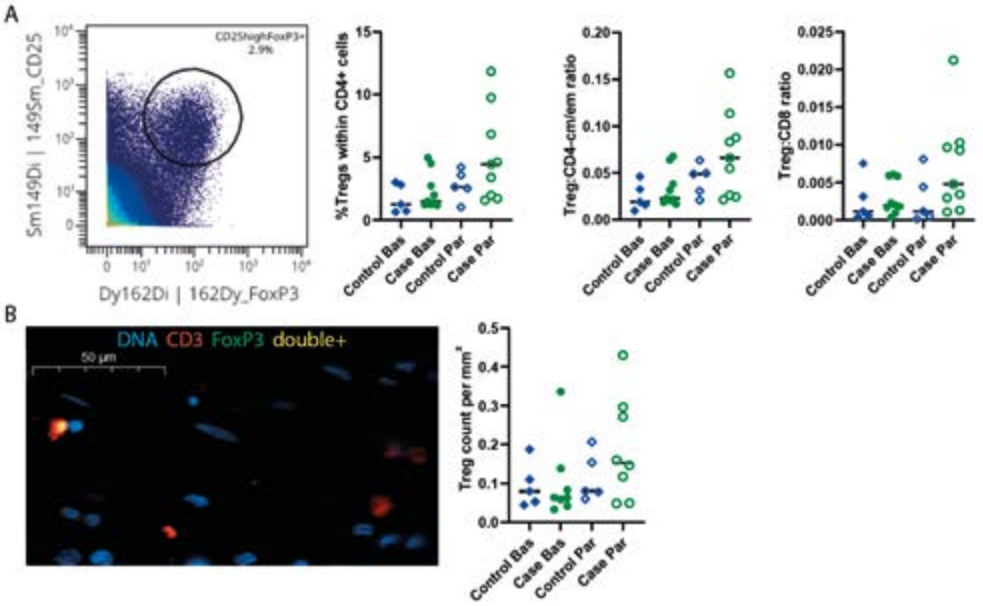

Figure 2. Decidual Treg frequencies in decidua parietalis (Par) and basalis (Bas) of women with a successful birth after uRPL (case) and controls. (A) Within the CD4+ T cell population CD25+FoxP3+ Treg cells are gated, Treg frequencies are plotted as are ratios between Treg cells and CD4-cm/em and Treg 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

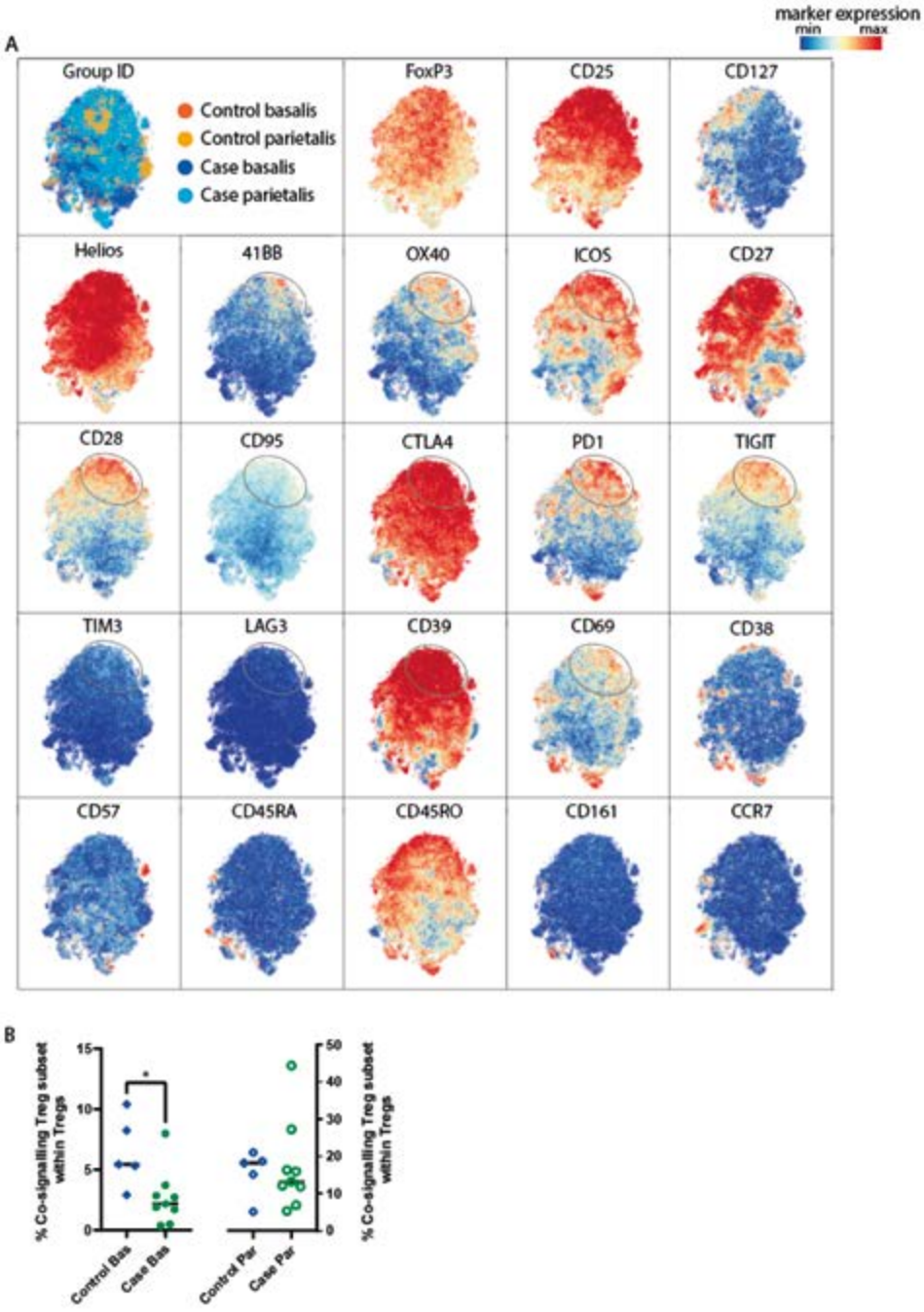

Figure 3. Treg phenotypes. (A) tSNE visualization of 23 markers from the T cells panel. Treg cells from the decidua basalis and parietalis, and cases and controls are equally distributed over the tSNE. Encircled is a cluster of cells that expresses several co-stimulatory as co-inhibitory (co-signaling 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

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SOLUBLE HLA-G BLOOD LEVELS ARE NOT INCREASED DURING ONGOING PREGNANCY IN WOMEN WITH A HISTORY OF RECURRENT PREGNANCY LOSS

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ABSTRACT

Recurrent pregnancy loss (RPL) affects 1-2% of couples who are trying to conceive. At some point, some couples do maintain a healthy pregnancy to term, but the underlying mechanism of RPL remains elusive. Human leukocyte antigen (HLA)-G is an immune modulatory molecule. Our group previously showed increased HLA-G levels in the decidua of term pregnancies after RPL, while other studies showed reduced soluble HLA-G (sHLA-G) blood levels in women with RPL. This led us to investigate sHLA-G levels in blood of women with RPL who had either a subsequent pregnancy loss (RPL-pregnancy loss) or a healthy term pregnancy (RPL-live birth), and compare these to healthy control pregnancies and non-pregnant controls. Soluble HLA-G concentrations were quantified by ELISA. Women with healthy term pregnancy had increased sHLA-G levels compared to non-pregnant controls. In contrast, RPL-live birth women at term did not have increased blood sHLA-G levels. Soluble HLA-G levels remained stable between first and third trimester. Interestingly, when comparing first trimester samples of RPL-live birth to RPL-pregnancy loss, sHLA-G levels also did not significantly differ. High sHLA-G levels in blood seem not to be crucial for an ongoing healthy pregnancy after RPL. However, since it was previously shown that women with RPL-live birth have increased HLA-G levels in term decidua compared to control pregnancies, the current data suggest that local and systemic immune regulation are not necessarily in concert. Further study of the contribution of fetus-derived HLA-G and HLA-G of maternal origin may provide more insight in the pathophysiology of RPL.

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, indicated as unexplained RPL [1-3]. As of 2018, RPL is defined as two or more pregnancy losses before 24 weeks of gestation [4]. No evidence-based therapeutic options are available and research on this pregnancy complication is confined due to the limited understanding of the pathophysiological mechanism. Furthermore, reliable predictive tools of pregnancy outcome are desirable [5]. Previously, our group showed increased HLA-G levels in the decidua of term pregnancies after RPL [6], while other studies showed reduced soluble HLA-G (sHLA-G) blood levels in women with RPL [7, 8]. This led to the hypothesis that women with a history of RPL and subsequent healthy ongoing pregnancy also have increased blood sHLA-G levels compared to women without a history of RPL; thereby leading to the notion that this could be a potential biomarker for pregnancy outcome. This study is the first to compare sHLA-G levels for pregnancy outcome of couples with a history of RPL.

During pregnancy it is important to maintain immunological tolerance towards the semi-allogeneic fetus. One of the factors associated with immune tolerance in pregnancy is human leukocyte antigen (HLA)-G.HLA-G is an immune checkpoint molecule as it binds inhibitory receptors (e.g. ILT2 and ILT4) on several types of immune cells (T, NK, B, dendritic cells, and monocytes) resulting in inhibition of their effector functions [9]. The efficiency of HLA-G to reduce $CD4^+$ T cell proliferation and cytokine production is similar to that of CTLA-4 [10]. It can also induce apoptosis of $CD8^+$ T cells [11]. Furthermore, HLA-G/ILT2 interaction on NK cells inhibits the polarization of lytic granules in the synapse and the production of IFN-γ [12, 13]. HLA-G levels have been shown to be low in blood of women with pregnancy loss [7, 14]. Taken together, reduced levels of HLA-G could lead to a more inflammatory immune profile, as is observed in women with RPL [15-17].

HLA-G was first described in the placenta and is expressed by fetal extravillous trophoblast cells, which lack expression of classical HLA-class I antigens except HLA-C [18]. Maternal immune cells like monocytes and macrophages can produce and secrete HLA-G as well [19, 20]. Therefore, soluble HLA-G (sHLA-G) can be measured in plasma and serum of non-pregnant healthy individuals [21, 22]. Blood sHLA-G levels are increased in pregnant women compared to non-pregnant women [21]. Throughout gestation some studies found stable sHLA-G levels [21, 23] whereas others found a decrease in sHLA-G levels [24-26]. However, when measuring levels of blood sHLA-G during pregnancy it is not known if the source is of fetal or maternal origin. Furthermore, the possible association with sHLA-G levels locally in the decidua, is not clear.

In contrast to studies showing reduced blood sHLA-G in RPL cases [7, 8], HLA-G levels in decidual tissue of women with RPL miscarriage are not reduced compared to elective abortion controls [6]. Furthermore, our research group showed that women with a history of RPL demonstrated increased decidual HLA-G levels in healthy term pregnancy compared to pregnant women without a history of RPL [6]. Taken together, these results lead to the question if women with a history of RPL and subsequent healthy ongoing pregnancy (RPL-live birth) also have increased blood sHLA-G levels compared to controls without a history of RPL. Secondly, we investigated if blood sHLA-G levels in

RPL-live birth women differ from those in women with RPL and a subsequent pregnancy loss (RPL-pregnancy loss). Herein, we tested if sHLA-G in blood during first trimester could be a potential biomarker for pregnancy outcome.

In the current study we found that RPL-live birth women at term do not significantly increase sHLA-G levels compared to non-pregnant controls, while pregnant control women without a history of RPL have increased sHLA-G levels. Next to that, we found that sHLA-G levels at first trimester do not differ between RPL-live birth and RPL-pregnancy loss women.

5

MATERIALS AND METHODS

Study material

A total of 101 sodium heparin plasma samples from 71 individuals were retrospectively included in this study to form three case groups and two control groups (Figure 1). For additional experiments we collected peripheral blood mononuclear cells for *HLA-G* typing. In this retrospective study, the case groups consisted of women with a history of at least three consecutive early pregnancy losses, following the international ESHRE guidelines from 2006 to define recurrent pregnancy loss (RPL)[27]. No underlying causes for RPL were found in the case groups after a full clinical workup. Exclusion criteria were age ≥40, BMI ≥35, smoking, and use of medication (e.g., progesterone). Of the 101 samples, 14 were from non-pregnant controls, 22 from pregnant controls, and 65 samples were from the cases. Of the cases, 9 samples were only used to study the influence of gestation age and reliability of sHLA-G measurement, since these 9 samples were from individuals of which another 1st trimester timepoint was included (e.g. 6 wks and 8 wks of gestation of the same pregnancy). The RPL-pregnancy loss group consists of 11 first-trimester samples from women with a history of RPL and another consecutive pregnancy loss at some point after sampling. This group consists of 9 individuals (samples come from different pregnancies). The RPL-live birth groups are women with a history of RPL and the samples are taken during an ongoing healthy pregnancy. The groups contain 23 first-trimester samples and 22 term samples from 26 individuals. From 19 individuals we had paired first-trimester and term samples (Figure 1). All pregnant women visited the Department of Obstetrics and Gynaecology at the Leiden University Medical Center (LUMC) between 2009 and 2019. Control samples were obtained from non-pregnant healthy females (n = 14). 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).

sHLA-G ELISA

The concentration of sHLA-G protein (soluble HLA-G5 and shed transmembrane HLA-G1) in plasma samples were measured using a commercial sHLA-G ELISA kit (MyBioSource, San Diego, USA; MBS2516229) with a detection range of 0.313 - 20 ng/mL and a sensitivity of 0.188 ng/mL. ELISA assays were performed according to the manufacturer's instructions. All samples were run in duplicate using several dilutions (1:5 to 1:40). The mean absorbance of duplicates of each sample

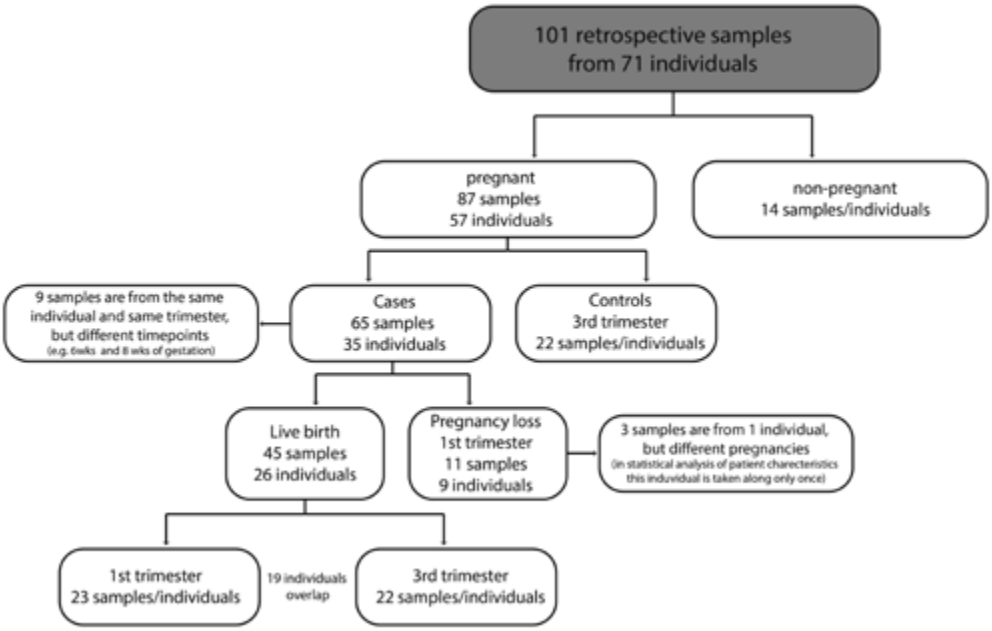

Figure 1.

was measured at a wavelength of 450 nm and sHLA-G concentrations were determined using the standard curve per plate provided in the ELISA kit.

HLA-G polymorphisms

Peripheral blood was used to isolate DNA in order to determine the *HLA-G* genotype and 3'UTR haplotype of the women using an in-house sequencing-based typing (SBT) assay developed [6] or next generation sequencing (NGS)-based assay [28] both in collaboration with GenDx. In short, in the SBT assay the 3'UTR of exon 8 was sequenced enabling the identification of polymorphisms including the 14-bp insertion/deletion (rs371194629), +3142C/G (rs1063320), +3187A/G (rs9380142), and +3196C/G (rs1610696). For the NGS-based assay the HLA-G gene was amplified with a PCR using HLA-G-specific primers, after which the PCR products were fragmented. The amplified and fragmented HLA-G gene was run on an Illumina Miniseq. The genotype of the samples was determined, and polymorphisms of interest were identified. The strength of the association between the 14-bp polymorphism and risk of RPL was calculated in three random effect models as previously done by Wang et al. [29]. These are the co-dominant (14-bp ins *vs* 14-bp del), dominant (14-bp ins/ins and 14-bp ins/del vs 14-bp del/del) and recessive (14-bp ins/ins vs 14-bp ins/del and 14-bp del/del) models.

Different combinations of 3'UTR polymorphisms from UTR haplotypes. UTR-1 and UTR-3 are associated with low sHLA-G expression, while URT-2, UTR-4, and UTR-7 are associated with high

sHLA-G expression [30-32]. Women with one 'low' and one 'high' haplotype were defined as 'medium' expressors.

Statistical analysis

Differences between groups were tested by Mann-Whitney U tests, chi-square tests or Wilcoxon test of paired samples. Correlation between BMI and concentration of sHLA-G was tested by a Spearman correlation. Values of $p<0.05$ were considered to indicate statistical significance.

5

RESULTS

Patient Characteristics

Soluble HLA-G (sHLA-G) concentrations were measured in 101 samples (Figure 1). To study the influence of gestational age (GA) at first trimester, from 9 individuals two timepoints during first trimester were measured. No significant correlation was found in the concentration of sHLA-G and GA in weeks (data not shown). In further analysis only one of the two first trimester timepoints was taken along, selected based on best GA match between the study groups.

Patient characteristics of the five study groups are shown in Table 1. Between the pregnancy groups there were no differences in parity, maternal age, and maternal BMI. The three RPL groups have a median of three pregnancy losses in their history whereas all the controls never experienced a pregnancy loss (p<0.001). Furthermore, the gestational age differs between the 1st trimester RPL live birth group and the 3rd trimester pregnancy control group (p<0.001).

Soluble HLA-G concentrations

We found that in the blood of RPL-live birth women the sHLA-G concentration was not significantly increased at 3rd trimester compared to non-pregnant controls (p=0.141), while this was the case in 3rd trimester control pregnancies without a history of RPL (p=0.002) (Figure 2). Furthermore, there was a trend towards a decrease of sHLA-G concentration in the blood of RPL-live birth women at 3rd trimester compared to 3rd trimester control blood (p=0.108) (Figure 2).

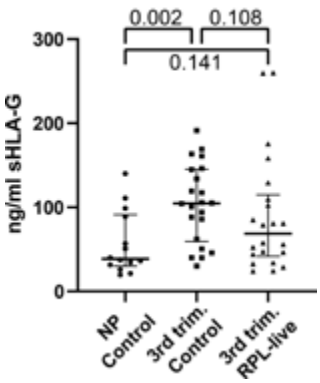

Figure 2.

Table 1. Patient characteristics of the individuals in the five study groups. Median (min-max), Mann-Whitney test.

	1st trim. RPL-live birth (n = 23)	3rd trim. RPL-live birth (n = 22)	p-Value[c]	1st trim. RPL-pregnancy loss (n = 9)	p-Value[d]	3rd trim. control pregnancy (n = 22)	p-Value[e]	NP control women (n = 14)
GA at time of blood collection (weeks+days)[a]	7+0 (4+0 – 12+3)	36+1 (35+0 – 36+4)	n.a.	5+0(4+0 – 9+3)	0.094	39+0 (37+0 – 39+0)	<0.001*	NK
Parity	0 (0-2)	0 (0-2)	0.996	1 (0-1)	0.406	1 (0-3)	0.067	NK
Previous miscarriages	3 (3-6)	3 (3-5)	0.293	3 (3-7)	0.818	0	<0.001*	NK
Maternal age[b]	35 (31-39)	34 (22-39)	0.667	36 (21-41)	0.670	33 (27-39)	0.251	NK
Maternal BMI	23.0 (19.2-34.1)	24.2 (19.2-34.1)	0.731	23.5 (21.8-25.2)	0.835	NK	n.a.	NK

[a] No exact GA of blood collection is known for 23 samples (1st trim. RPL live birth = 6, 3rd trim. RPL live birth = 13, 1st trim. RPL live pregnancy loss = 4).

[b] Maternal age is unknown for two samples (1st trim. RPL live birth = 1, 3rd trim. RPL live birth = 1).

[c] group 1st trim. RPL live birth and group 3rd trim. RPL live birth.

[d] group 1st trim. RPL live birth and 1st trim. RPL live pregnancy loss.

[e] 3rd trim. RPL live birth and 3rd trim. control pregnancy.

RPL: recurrent pregnancy loss. *NP:* non-pregnant. *GA:* gestational age. *NK*: not known

We further focused on the women with a history of RPL but with an ongoing term pregnancy, as we were able to also measure blood sHLA-G levels in these women at the first trimester. No difference of sHLA-G concentrations between first and third trimester was observed, neither when comparing group medians of all samples (p=0.424) (Figure 3A), nor when comparing samples in a pairwise fashion (p=0.165) (Figure 3B).

Next, we wondered whether blood sHLA-G levels were different during first trimester between women with RPL history who have ongoing pregnancy and those who suffer from pregnancy loss. We found no significant difference between the sHLA-G blood concentrations of RPL-live birth women compared to RPL-pregnancy loss women (p=0.513) (Figure 4).

Relationship of sHLA-G levels with BMI

We noticed a significant correlation between BMI and sHLA-G concentrations (r=0.651, p<0.001) (Figure 5A). All 70 individuals were included in this analysis. When an individual was in multiple study groups, the average concentration of sHLA-G was used. Therefore, we tested whether groups differed in sHLA-G levels after correcting for BMI. We found after correction no difference between in first trimester sHLA-G levels when comparing RPL-live birth to RPL-pregnancy loss (p=0.403) (Figure 5B). Additionally, we also found no difference between first- and third- trimester sHLA-G concentrations in the RPL-live birth women (p=0.451) (Figure 5C). Furthermore, we did not observe any other correlation between patient characteristics (e.g. parity) and sHLA-G levels.

Equal distribution of 3'UTR HLA-G gene polymorphisms among groups

Since decreased sHLA-G levels may be associated with certain polymorphisms in the *HLA-G* gene, we examined the distribution of *HLA-G* polymorphisms within the RPL and pregnant control group. No difference was observed in the distribution of the 14-bp insertion/deletion polymorphism between women with a history of RPL and the pregnant control group (Supplementary Table 1). Similarly, no difference was observed for three other polymorphisms (+3142C/G, +3187A/G and

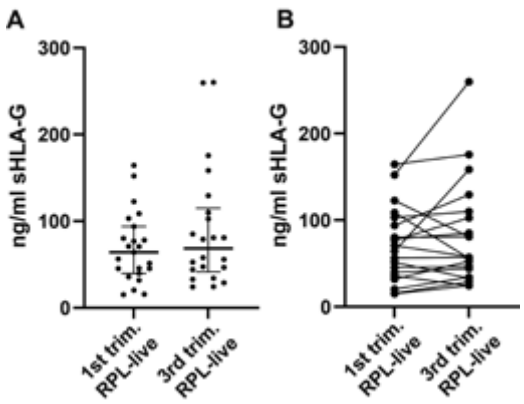

Figure 3.

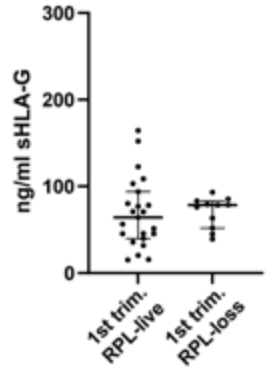

Figure 4.

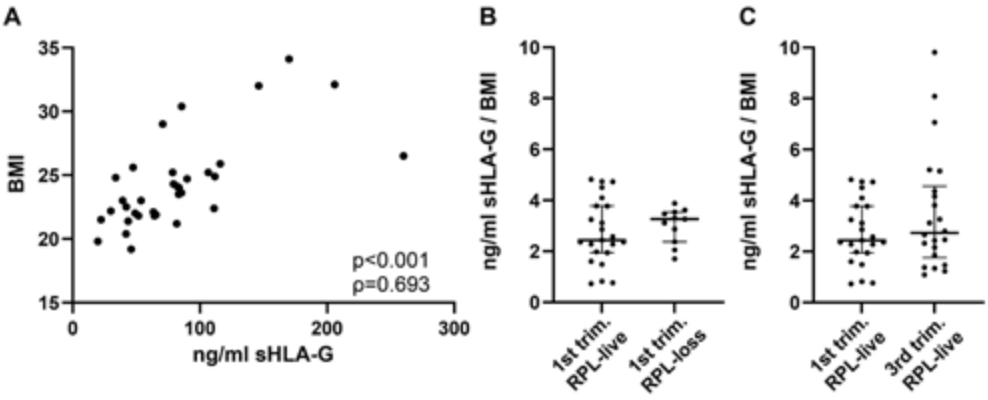

Figure 5.

+3196C/G) between the RPL and control group (data not shown). Next to genotype analysis also 3'UTR haplotypes were determined and analyzed. Due to low sample size, we made three groups referring to UTR haplotypes associated with low (UTR-1 and UTR-3), or high (UTR-2, UTR-4 and UTR-7) expression of sHLA-G or a combination of a low and high haplotype (medium) [30-32]. We found no significant differences between the RPL women and pregnant control women in the distribution of 3'UTRs described the be associated with low, medium or high HLA-G expression (data not shown). We also performed typing on the non-pregnant control group, but this group was too small to make comparisons with the RPL group or pregnant control group.

DISCUSSION

There are no substantiated therapeutic treatment options for unexplained RPL as long as the understanding of the pathophysiology of RPL is limited. Furthermore, no suitable biomarkers are available for pregnancy outcome after unexplained RPL. Comparison of women with a history of

RPL with subsequent ongoing pregnancy to those suffering from subsequent pregnancy loss may provide more insight into the underlying pathophysiology and may lead to the identification of predictive biomarkers for pregnancy outcome. Therefore, we compared sHLA-G levels between these two groups and found that sHLA-G is not a predictive marker for pregnancy outcome in the first trimester. In women with a history of RPL sHLA-G levels did not increase compared to non-pregnant controls and remained stable between the first and third trimester. At the same time, pregnant control women without a history of RPL did show increased levels of sHLA-G in the third trimester.

Previous literature shows an increase in sHLA-G levels in blood of pregnant women compared to non-pregnant individuals [21-23]. This is in concordance with results from the current study, where women at term with no history of RPL demonstrated an increase in sHLA-G concentration. In contrast, RPL-live birth women did not significantly increase their sHLA-G levels compared to non-pregnant controls and compared to pregnant controls. Our research group previously showed that local HLA-G levels at the maternal-fetal interface (decidua) at term are increased in women with a history of RPL compared to term decidua from women without a history of RPL [6]. Since HLA-G can be secreted into the circulation, we hypothesized that women with a history of RPL may also have high sHLA-G levels in their blood at the time of 3rd trimester of an ongoing pregnancy. However, we found an opposite trend of lower sHLA-G levels compared to term controls. These data show that HLA-G levels in the decidua are not reflected in the peripheral blood sHLA-G levels. Mechanistically, this suggests that when there is not sufficient sHLA-G systemically, the placenta might compensate by locally providing more HLA-G. Findings from Djurisic et al. strengthen this point as they showed that sHLA-G levels in uterine blood (comprising of maternal and fetal blood, intervillous and amniotic fluids) isolated from first-trimester abortion material contains higher levels of sHLA-G than peripheral blood [33]. Persson et al., however, did not observe a difference in placental blood and peripheral blood sHLA-G levels at term [34]. In both studies HLA-G expression in the placenta was studied (membrane bound MFI and mRNA expression, respectively), but not directly compared to sHLA-G peripheral blood levels. To test this hypothesis further, future studies should focus on comparing sHLA-G in blood to membrane bound HLA-G in paired decidua samples.

Studies on possible differences between local and systemic HLA-G may also confirm the importance of the local microenvironment to maintain pregnancy. Many studies focus on peripheral blood to study pregnancy-related immunology. However, it has been shown before that the immune cell composition in peripheral blood is vastly different from that in the decidua [35, 36]. The same probably holds true for several other immunological factors including HLA-G. Somewhat contradictory is the fact that an ideal biomarker would be obtained from the peripheral blood. Indeed, when we examined sHLA-G as a possible biomarker for pregnancy outcome at first trimester we found that it cannot be used as a biomarker for pregnancy outcome. As described above, we initially hypothesized sHLA-G levels to be relatively high in RPL-live birth women both at first trimester and term whereas we expected sHLA-G to be relatively low in women with RPL and subsequent pregnancy loss. However, there was no difference in the sHLA-G levels between the two groups. Bates et al. also did not find a difference in cytokine profiles between RPL-live birth and RPL-pregnancy loss women, but did find differences between pregnant and non-pregnant

controls [37]. Together, these results raise the question of whether the maternal condition changes to attain a healthy ongoing pregnancy after RPL and, if so, whether there is a role of the fetus in contributing to a healthy ongoing pregnancy in women with RPL.

We found that in RPL-live birth there is no difference in blood sHLA-G levels between first trimester and third trimester. Some studies found a stable sHLA-G concentration [21, 23] while others found a decrease [24-26] throughout gestation. Yie et al. observed a decrease in healthy pregnancy but not in their preeclamptic group, where sHLA-G levels were decreased compared to controls but stable over gestation [24]. This is similar to our findings, where we observed in the RPL-live birth group a trend towards decrease compared to healthy pregnancy controls, but within the RPL-live birth group the sHLA-G levels were the same between first trimester and term.

Furthermore, we found a strong correlation between BMI and sHLA-G concentrations. In pregnancy it has been shown before that in obese women the sHLA-G levels are higher than in non-obese women [25]. However, Persson et al. did not find a correlation between BMI and sHLA-G levels [34]. Our cohort did include a few mildly obese women (n=8, BMI ≥30; n=61, BMI <30) but when these were excluded from the study, we still observed a significant correlation between BMI and sHLA-G concentrations ($p<0.001$, data not shown). High amounts of adipose tissue cause increased low-grade inflammation, which is described to be balanced by increased amounts of Treg cells [38]. Since HLA-G and Tregs are both immune regulatory, further studies could elucidate the role of HLA-G to balance inflammation mediated by adipose tissue. BMI was not documented for our control groups. Nevertheless, in the study groups with pregnant women BMI was no confounder, and after correcting for BMI in the other groups the main findings of the study were similar.

Lastly, we performed *HLA-G* typing for all women and focused on four polymorphisms in the 3'UTR of the *HLA-G* gene, which have been described to predispose to altered HLA-G expression, namely 14-bp insertion/deletion (rs371194629), +3142C/G (rs1063320), +3187A/G (rs9380142) and +3196C/G (rs1610696) [39-43]. It is believed that microRNAs bind differently to sites present in the 3'UTR of HLA-G mRNA depending on the polymorphisms in the 3'UTR region, thereby post-transcriptionally affecting the expression of HLA-G protein [44]. There are contradictory results where some studies described that women with RPL have *HLA-G* gene constitutions that predispose to reduced HLA-G expression and secretion [6, 45-47], while other studies found, similarly to the current study, no difference between RPL and control groups [8, 48, 49]. However, we cannot rule out that the difference in HLA-G levels between the women with RPL and pregnant control women is affected by genetic predisposition, as there seemed to be a slight trend in our study towards a higher frequency of the insertion phenotype in women with RPL, which is expected to be associated with lower sHLA-G production [40].

Our group size is too small to provide reliable results on the heterogeneity that is present in the HLA-G gene. Nevertheless, we have included these data to provide more insight into the study population. In this retrospective study we ideally aimed to have similar number is the RPL-pregnancy loss and RPL-live birth group. However, we obtained a limited amount of RPL-pregnancy loss samples. This could have to do with the fact that previously women were referred to our clinic when they had a history of three or more pregnancy losses. After three or more pregnancy losses the ongoing pregnancy rate is 80.3% [2]. Another limitation of the study is that sodium heparin was

used as blood anticoagulant. Previous studies have indicated that blood plasma prepared with EDTA anticoagulant have significantly higher sHLA-G levels than heparin prepared blood [50]. However, in the current study we used sodium heparin in all study groups, we confirmed reproducibility (data not shown) and can therefore accurately compare the groups. However, these concentrations cannot be compared with other studies where EDTA was used as blood anticoagulant. Also, no distinction can be made between different sHLA-G isoforms using the commercial ELISA kit (MyBiosource).

In summary, no significantly increased blood sHLA-G levels in RPL-live birth women were found, in contrast to pregnant women without a history of RPL. Furthermore, in women with a history of RPL blood sHLA-G levels at first trimester did not differ between RPL-live birth and RPL-pregnancy loss. Therefore, systemic blood sHLA-G levels cannot be used as biomarker for pregnancy outcome in women with a history of RPL. High sHLA-G blood levels are obviously not a prerequisite to ensure an ongoing pregnancy. Given that local HLA-G levels in the decidua are not directly reflecting systemic sHLA-G levels in maternal blood, it is important to incorporate the local environment in the placenta to investigate underlying mechanisms of RPL and subsequent live birth pregnancy.

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SUPPLEMENTARY DATA

Supplementary Table 1. Distribution of the 14-bp insertion/deletion in the 3' UTR region of *HLA-G* in women with a history of RPL and the control pregnancy group.

	RPL women (n = 35)	3rd trim. control pregnancy (n = 22)	OR	95% CI	p-Value#
Codominant genotype frequency					
Ins/ins	6 (17.14%)	3 (13.64%)	1.310	0.34 – 5.21	0.724
Ins/del	18 (51.43%)	9 (40.91%)	1.529	0.42 – 4.73	0.339
Del/del	11 (31.43%)	10 (45.45%)	0.550	0.18 – 1.69	0.285
Dominant phenotype frequency					
Ins/ins, ins/del	24 (68.57%)	12 (54.55%)	1.818	0.59 – 5.70	0.285
Del/del	11 (31.43%)	10 (45.45%)			
Recessive phenotype frequency					
Ins/del, del/del	29 (82.86%)	19 (86.36%)	0.763	0.19 – 2.95	0.724
Ins/ins	5 (17.14%)	3 (13.64%)			

Chi-square test.

OR, odds ratio; 95% CI, 95% confidence interval; del, deletion; ins, insertion.

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6

ONE-SIDED CHRONIC INTERVILLOSITIS OF UNKNOWN ETIOLOGY IN DIZYGOTIC TWINS: A DESCRIPTION OF 3 CASES

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ABSTRACT

Chronic intervillositis of unknown etiology (CIUE) is a rare, poorly understood, histopathological diagnosis of the placenta that is frequently accompanied by adverse pregnancy outcomes including miscarriage, fetal growth restriction, and intrauterine fetal death. CIUE is thought to have an immunologically driven pathophysiology and may be related to human leukocyte antigen mismatches between the mother and the fetus. Dizygotic twins with one-sided CIUE provide an interesting context to study the influence of immunogenetic differences in such cases. The main immune-cell subsets were investigated using immunohistochemistry. We identified three dizygotic twin pregnancies in which CIUE was present in only one of the two placentas. Two of the pregnancies ended in term delivery and one ended in preterm delivery. Presence of CIUE was correlated with lower placental weight and lower birthweight. Relative number of CD68, CD56, CD20, and CD3 positive cells were comparable between co-twins. The presence of one-sided CIUE in dizygotic twin pregnancy was associated with selective growth restriction in the affected twin. This suggests a unique fetal immunogenetic contribution to the pathogenesis of CIUE. Further study of dizygotic and monozygotic placentas affected by CIUE could identify new insights into its pathophysiology and into the field of reproductive immunology.

INTRODUCTION

Chronic intervillositis of unknown etiology (CIUE) is an uncommon histopathological lesion first described by Labarrere and Mullen in 1987 as massive chronic intervillositis [1]. The histiocytic intervillous infiltrate can occur in every trimester and is frequently accompanied by villitis of unknown etiology (VUE) and perivillous fibrin deposits of varying severities [2]. It has repeatedly been shown that CIUE is associated with adverse pregnancy outcomes [3] such as miscarriage, fetal growth restriction (FGR), pre-term birth, and intrauterine fetal death (IUFD) [3].

The pathophysiology of CIUE is poorly understood, but it appears to be immunologically driven [4, 5]. Factors that suggest an immune etiology include increased complement deposition in placentas with CIUE [6] and the detection of FOXP3-positive T-cells in the intervillous compartment [7]. CIUE is also associated with maternal auto-immune and alloimmune diseases including antiphospholipid syndrome, systemic lupus erythematosus, and fetal and neonatal alloimmune thrombocytopenia [8, 9]. Mixed lymphocyte cultures suggest a pathophysiological basis for human leukocyte antigen mismatches between the mother and the fetus in CIUE [8]. The proposed immune dysregulation in CIUE has led to several experimental treatments with prednisone, corticosteroids, aspirin, heparin, intravenous immunoglobulin, or different in combinations. Adverse outcomes, combined with the condition's high recurrence rate and the lack of evidence-based treatments for prevention of CIUE, highlight its clinical importance [3].

The phenomenon of dizygotic twins with one-sided CIUE, which occurs despite an identical intrauterine environment, suggests a significant contribution from fetal immunogenetics: the mother mounts an immune response against paternally inherited antigens present in only one of the two placentas. This series describes three cases of dizygotic twins with one-sided CIUE, reporting clinical outcomes, associated placental abnormalities and immunohistochemical findings.

MATERIALS AND METHODS

Case Selection

Patient samples were selected from the pathology department of the University Medical Center Utrecht (UMCU) between 2000 and 2015 using the hospital's pathology registry. During this period, approximately 13,300 placentas were studied, of which 1059 were dichorionic twin placentas. In total, 45 placentas with an intervillous infiltrate were identified, of which three were dizygotic dichorionic twin placentas (see: [10]). Slides were reviewed by experienced pathologists (PGJN and LEvdM) and the diagnosis of CIUE was based on our previously described criteria [3]. We defined CIUE as the presence of an infiltrate occupying 5% or more of the intervillous space with approximately 80% of mononuclear cells positive for CD68 [3]. Furthermore, clinical or histopathological signs of infection should be absent [3]. Patient characteristics and pregnancy outcomes were obtained from the medical records. This study was approved by the UMCU biobank committee (TC-BIO number: 16–434).

Clinical Definitions

Term delivery was defined as birth from 37 completed weeks of gestation onward, and preterm delivery was defined as birth between 24 and 37 completed weeks of gestation. Fetal growth

restriction (FGR) was defined as a birthweight below the third percentile. Discordant growth was defined as a birthweight discrepancy of >25%.

Histology and Immunohistochemistry

Tissue samples were taken from the umbilical cord, fetal membranes, and placental parenchyma according to the Amsterdam Placental Consensus Statement [11]. Formalin-fixed, paraffin-embedded (FFPE) tissues were H&E stained according to standard laboratory protocols and studied for routine diagnostics. When CIUE, VUE, or perivillous fibrin deposition was present, it was graded to mild, moderate, and severe/massive based on Benirschke et al. [12]. Fetal thrombosis was scored as either present or absent. For immunohistochemical analysis of the intervillous cell infiltrate in dizygotic twin pregnancies discordant for CIUE, four tissue blocks (two blocks per twin) were selected. Blocks were selected from equivalent locations close to the umbilical cord insertion and halfway between the umbilical cord and lateral edge of the placenta. Blocks from locations with macroscopic abnormalities were excluded. Sequential sections of 4 µm were placed on adhesive-coated glass slides and dried overnight at 37 °C. FFPE tissues were stained for CD3 (rabbit, DAKO A0452; DAKO solutions Ltd., Beverly HU17 0JW, United Kingdom), CD68 (mouse, Monosan NCL-CD-68-KP1; MONOSAN Antibodies and Reagents, Uden, The Netherlands), and CD56 (mouse, Monosan Mon3364; MONOSAN Antibodies and Reagents, Uden, The Netherlands) at the UMCU for routine diagnostics by Ventana (Roche, Tucson, AZ, USA). CD20 staining was performed at the Leiden University Medical Center. Slides were deparaffinized and antigen retrieval was performed with citrate (pH6). Peroxidase was blocked using H2O2 0.12%. Prior to incubation with the primary antibody, the slides were incubated for 1 h at room temperature with normal goat serum. The mouse monoclonal anti-CD20 (cy) antibody (1:400, DAKO M0755; DAKO solutions Ltd., Beverly HU17 0JW, United Kingdom) was incubated for 1 h at room temperature. Binding of the primary antibody was visualized using a PO-labelled goat-anti-mouse polymer (DAKO envision; DAKO solutions Ltd., Beverly HU17 0JW, United Kingdom) and diaminobenzidine as a chromogen. Hematoxylin was used for counterstaining before slides were dehydrated and covered using mounting medium.

Quantification of the Intervillous Infiltrate

The number of cells positive for CD3 (T cells), CD20 (B cells), CD56 (NK cells), and CD68 (macrophages) was quantified using the Philips IMS viewer system. The stained slides were aligned and annotations of approximately 0.75 mm2 were made per slide. Two annotations were placed subchorial, two were placed central, and two were placed basal (Supplementary Figure S1A). The number of positive cells in the intervillous space was counted for each marker in the aligned annotations (Supplementary Figure S1B–E). In a total of 36 annotations, 5002 CD68-positive cells, 1183 CD3-positive cells, 34 CD20-positive cells, and two CD56-positive cells were counted. All slides were scored with the same brightness settings and magnification. The observer was blinded for clinical outcomes.

Table 1. Patient characteristics.

Case	1		2		3	
Maternal characteristics						
Gravidity	3		1		1	
Parity	2		0		0	
Outcome previous pregnancy	FGR without complications		NA		NA	
Previous miscarriages	0		NA		NA	
Obstetric characteristics						
Artificial reproductive techniques					Ovulation-induction	
Gestational age [weeks]	37		40		35	
Mode of delivery	Spontaneous		Primary SC		Induction	
Indication Mode of delivery	NA		Twin 1 in breech		severe FGR	
Fetal characteristics						
Sex	Female	Male	Male	Female	Male	Female
Birthweight percentile	p<3	p10-50	p10-50	p50-90	p<3	p50-90
APGAR 5'	8	10	10	10	9	9
Admission NICU/NMCU	NMCU NMCU				NICU	
Placenta characteristics						
Placentaweight percentile	[1] p10-25		p75-90	p90	p10-25	p75-90
CIUE	Moderate	Absent	Moderate	Absent	Moderate	Absent
VIUE	Moderate	Absent	Severe	Moderate	Absent	Absent
Massive fibrin deposits	Absent	Absent	Moderate	Absent	Absent	Absent
Fetal thrombosis	Absent	Absent	Present, focal	Absent	Absent	Absent

NICU, neonatal intensive care unit; NMCU, neonatal medium care unit. [1] Placenta not weighted separately. Placenta weight percentile for total placenta.

RESULTS

Patient Characteristics

Clinical characteristics of the dizygotic twin pregnancies are described in Table 1 none of the three affected mothers had any significant past medical history. In case 1, the mother had had two previous pregnancies that both resulted in term delivery of a growth-restricted baby. In Cases 2 and 3, the mothers were primigravidae and in Case 3, became pregnant after ovulation induction. Two of the three pregnancies were delivered at term, and one was delivered pre-term after induction of labor for severe FGR in one of the fetuses. In two of the three dizygotic male-female twin-pregnancies, the fetal growth was discordant. Fetal weight discordance ranged from 9% to 50%. All six babies had an Apgar score above 7 at 5 min, two babies were admitted to the neonatal medium care unit (NMCU), and one baby was admitted to the neonatal intensive care unit (NICU). No IUFD or early neonatal death occurred in these cases. None of the pregnancies was treated (e.g., with prednisone, corticosteroids, aspirin, heparin, intravenous immunoglobulin) and none of the women in our case series had a subsequent pregnancy.

Placental Characteristics

Placental characteristics are described in Table 1. All three placentas were dichorionic diamniotic. The placental weight was within normal range for gestational age for all cases. In each of the three twin pregnancies, CIUE was only present in one of the two placentas. In two cases (1 and 2), CIUE was accompanied by villitis of unknown etiology (VUE). Concurrent fibrin deposits and fetal thrombosis were detected in the CIUE placenta in Case 2.

Quantification of the Intervillous Infiltrate

The number of cells positive for CD3, CD20, CD56, and CD68 in the intervillous space was quantified (Figure 1A, B). Most of the cells present in the intervillous space were CD68-positive cells, followed by CD3-positive cells. Few CD20-positive cells and CD56-positive cells were observed. The absolute number of cells present in the intervillous space was different between the pairs of co-twin fetuses (Figure 1C), however, the relative number of immune cells present in the intervillous space seems comparable between the pairs of co-twin fetuses (Figure 1D).

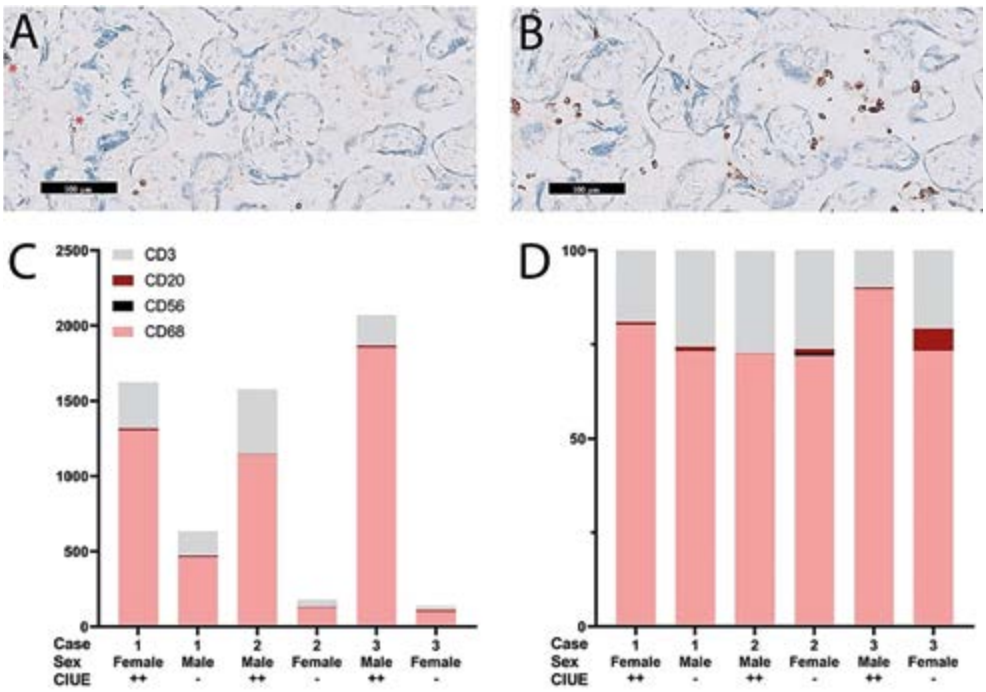

Figure 1. Quantification of the intervillous infiltrate. (A) Example of CD3-positive cells in the intervillous space. Two CD3-positive cells are also seen within the villi (see asterisk); (B) Example of CD68-positive cells in the intervillous space; (C) The absolute number of immune cells in the intervillous space was different among dizygotic twins with and without CIUE; (D) The relative number of immune cells in the intervillous space was comparable among co-twins.

Intervillous Cells in Relation to Clinical Outcomes

The fetuses with CIUE in their placenta had a lower birthweight compared to the fetuses without CIUE. In Case 1, the fetus with CIUE was admitted to NMCU; in Case 3, the fetus with CIUE in the placenta was admitted to NICU.

DISCUSSION

This case series describes three dizygotic twin pregnancies in which the placentas were one-sidedly affected by CIUE, the first published report of this phenomenon. Overall, FGR is more common in twin pregnancies than in singleton pregnancies [13]. Discordant growth is observed in both dichorionic and monochorionic twins, with an incidence of 10– 15% [13]. In monochorionic twins, who rely on a single placental circulation, selective FGR is usually driven by unequal placental sharing, in dichorionic twins, FGR has a broader etiology, including genetics, congenital infections, and placental dysfunction [14]. Several histopathological findings in the placenta are associated with FGR such as CIUE, VUE, massive peri-villous fibrin depositions, and fetal thrombosis [15]. One-sided VUE or MPFD in association with FGR have been described in previous studies of dizygotic twins [16-22]. In our twin cases, the presence of CIUE, with or without peri-villous fibrin deposits or VUE, was also related to a lower birthweight.

One-sided CIUE in the placenta of a dizygotic twin could suggest that CIUE is driven by maternal alloimmune sensitization against a particular fetoplacental histocompatibility antigen which differs between the twins. This is further supported by the presence of CIUE in cases of fetal and neonatal alloimmune thrombocytopenia, which is characterized by maternal allo-antibodies directed against paternally inherited antigens on the fetal platelets [23, 24]. The intervillous infiltrate contains predominately CD68-positive cells and CD3-positive cells. The CD68-positve cells represent a non-specific reaction of the maternal immune system. Boyd et al. showed that CD68-positive cells in the intervillous infiltrate express MRP14, which indicates that these cells are activated [25]. The CD3-positive cells resemble a reaction of the adaptive immune system and could detect a non-self-antigen on the fetoplacental cells. This has been confirmed using mixed lymphocyte cultures of peripheral blood mononuclear cells (PBMCs) from women experiencing CIUE and paternal PBMCs as target cells [8]. It could be valuable to ascertain the target antigen of the T-cell receptor on the fetal trophoblast membrane [26, 27]. This is particularly interesting in dizygotic twins, since the presented antigens differ between the co-twins. Remarkably, the relative amount of immune cells in the intervillous space of the co-twins is comparable.

One obvious limitation of this series is that it only describes three cases. However, given the rarity of CIUE, which affects approximately 1 in 10,000 pregnancies [28], and the incidence of twin pregnancies, increasing the number of cases is unlikely to overcome the presence of selection bias. As with many studies on placental histopathology, this study is limited by its retrospective design, which also encourages selection bias and makes it more difficult to exclude infectious causes for CIUE. Based on the clinical records, we were able to exclude the presence of clinical signs of infection and histopathological examination of these placentas did not reveal any signs for an infectious cause.

CONCLUSIONS

The presence of one-sided CIUE in dizygotic twin pregnancy suggests that CIUE is related to immunological incompatibility between the mother and the affected fetus. Studying one-sided histopathological changes in dizygotic twins and monozygotic twins could reveal novel insights into the pathophysiology of CIUE and other alloimmune gestational diseases.

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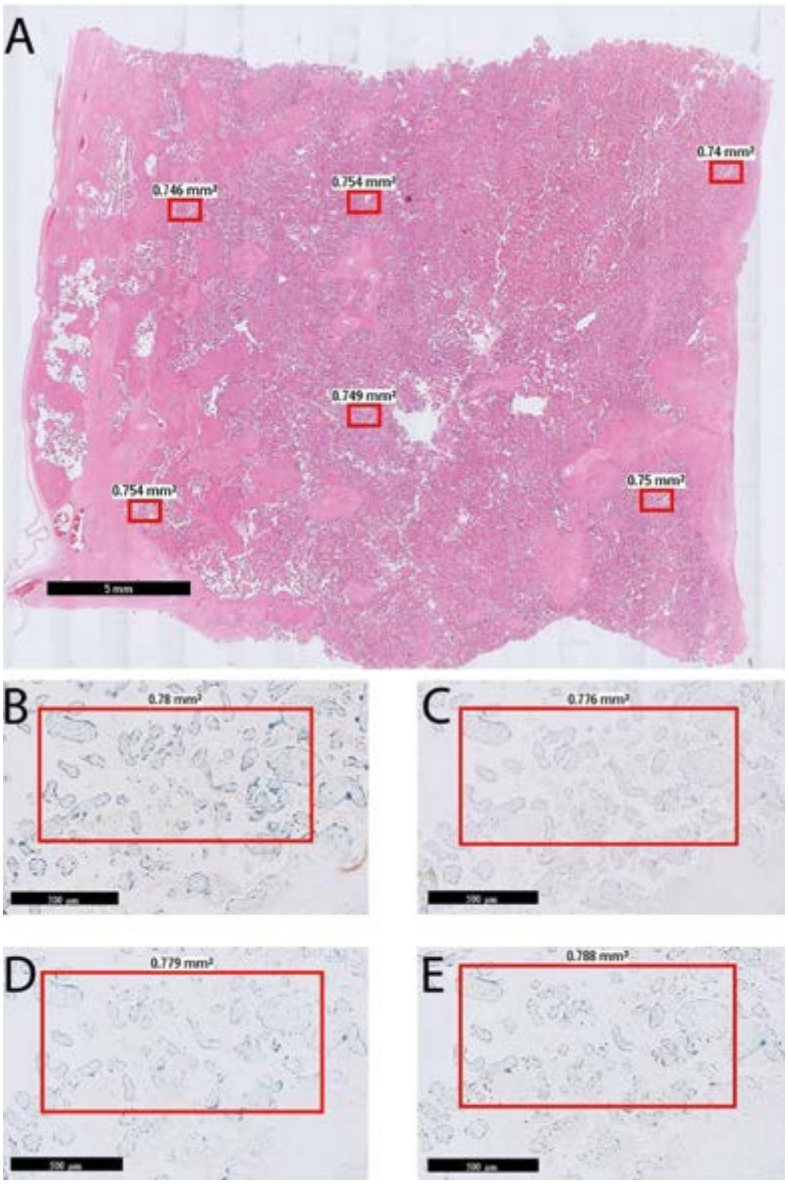
SUPPLEMENTARY DATA



Supplementary Figure S1. Annotations and alignment. (a) An example of selected annotations in a slide stained with H&E. (b–e) Examples of aligned annotations per staining: (b) CD3; (c) CD20; (d) CD56; (e) CD68.

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IDENTIFICATION OF A UNIQUE INTERVILLOUS CELLULAR SIGNATURE IN CHRONIC HISTIOCYTIC INTERVILLOSITIS

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ABSTRACT

Introduction

Chronic histiocytic intervillositis (CHI) is a rare histopathological lesion in the placenta characterized by an infiltrate of CD68+ cells in the intervillous space. CHI is associated with adverse pregnancy outcomes such as miscarriage, fetal growth restriction, and (late) intrauterine fetal death. The adverse pregnancy outcomes and a variable recurrence rate of 25-100% underline its clinical relevance. The pathophysiologic mechanism of CHI is unclear, but it appears to be immunologically driven. The aim of this study was to obtain a better understanding of the phenotype of the cellular infiltrate in CHI.

Method

We used imaging mass cytometry to achieve in-depth visualization of the intervillous maternal immune cells and investigated their spatial orientation in situ in relation to the fetal syncytiotrophoblast.

Results

We found three phenotypically distinct CD68+HLA-DR+CD38+ cell clusters that were unique for CHI. Additionally, syncytiotrophoblast cells in the vicinity of these CD68+HLA-DR+CD38+ cells showed decreased expression of the immunosuppressive enzyme CD39.

Discussion

The current results provide novel insight into the phenotype of CD68+ cells in CHI. The identification of unique CD68+ cell clusters will allow more detailed analysis of their function and could result in novel therapeutic targets for CHI.

INTRODUCTION

Pregnancy is a semi-allogeneic state where the paternally inherited antigens of the fetus are tolerated by the maternal immune system. When the maternal immune system fails to achieve immunological tolerance to fetal antigens pregnancy complications may occur, which could be the case in Chronic histiocytic intervillositis (CHI). CHI is a rare histopathological finding in the placenta. CHI is defined by maternal CD68+ cell infiltrate occupying at least 5% of the intervillous space, in the absence of clinical and histopathological signs of infection [1]. CHI can occur in any trimester and is associated with miscarriage, intrauterine fetal death (IUFD), fetal growth restriction (FGR), and preterm birth [2-5]. The severity of the infiltrate in CHI is correlated with adverse outcome [6-8]. Furthermore, the chance of recurrence in subsequent pregnancies is variable and estimated between 25%-100% [5, 8-10].

The pathophysiologic mechanism of CHI is unclear and current data is often based on small data sets due to the rarity of CHI. CHI appears to be immunologically driven as maternal CD68+ cells, C4d deposition [11] and FoxP3+ regulatory T cells (Tregs) are present [8] in placentas with CHI. High levels of complement-fixing fetus-specific HLA antibodies could be a pathophysiological basis for CHI as has been shown by Benachi et al. [12]. However, not all women with CHI develop fetus-specific HLA antibodies [7]. Reus et al. showed that women with CHI have increased paternal-specific cytotoxic T-lymphocyte precursor frequencies compared to controls with uncomplicated pregnancies [7]. Furthermore, CHI is associated with auto-immune and allo-immune diseases including antiphospholipid syndrome, systemic lupus erythematosus, and fetal-neonatal alloimmune thrombocytopenia [10, 13, 14].

Fetal syncytiotrophoblast (SCT) cells are damaged in CHI placentas: they express less CD200 and CD39 [6, 15] and more Intercellular Adhesion Molecule-1 (ICAM) [16] compared to unaffected placentas. These molecules are involved in immune cell recruitment and activation of myeloid cells [6, 16, 17]. Even though myeloid cell numbers are relatively high in CHI, their role and function are not clear and in-depth phenotypic data are lacking. These CD68+ cells have been reported to resemble M2-polarized macrophages [18]. They seem to express CD163 and the complement receptor 4 (CR4; CD11c/CD18), and to not express CD206 and CD209 which are important for endocytosis of pathogens and glycoproteins [18].

In a previous report we identified three dizygotic twin pregnancies with discordant CHI: each pregnancy had one affected and one unaffected twin [19]. These twins create a unique opportunity to study cases and controls exposed to the same maternal immune environment. In this study we enrolled these twin cases for in-depth phenotyping of the CD68+ cell compartment by imaging mass cytometry (IMC) in CHI. IMC allows for the measurement of 40 proteins simultaneously in situ. Furthermore, the spatial orientation of the immune cells was studied in the context of the SCT. This unprecedently detailed characterization of the intervillous infiltrate in CHI is the first step needed to uncover new therapeutic targets and prevent recurrence.

METHODS

Patient selection

CHI was diagnosed and scored according to the diagnostic criteria proposed by Bos et al; at least 5% of the intervillous space was occupied by an immune infiltrate, of which at least 80% were CD68+ cells, in the absence of clinical or histopathological signs of infection [1]. When CHI, villitis of unknown etiology (VUE), or perivillous fibrin deposition was present, it was graded to mild, moderate, and severe/massive based on Benirschke et al. [20]

Samples used for imaging mass cytometry studies

Three dizygotic twin cases were selected from the pathology department at the University Medical Center Utrecht between 2000 and 2015 [19] with UMCU biobank committee approval (TC-BIO number: 16–434) (Table 1). In two cases, one twin was affected by CHI (CHI twin), whereas his or her sibling was unaffected (control twin). Case 3 was a dizygotic twin pregnancy, but the unaffected placenta was excluded, as it did not fulfill the morphologic criteria of a healthy control. In this excluded placenta there were more CD68+ cells present than in healthy control, but less than 5% of the intervillous space was occupied, and therefore did not fit CHI criteria.

Validation cohort

All eleven CHI placentas available in our center and eight healthy term control placentas were included as a validation cohort for IMC results. Samples were selected from the pathology department at the Leiden University Medical Center between 2001 and 2022 (Table 2, Supplementary Table 1). CHI cases that were included were 3rd trimester (9/11) or late 2nd trimester (2/11) to enable comparison to healthy control placenta. This study was carried out in accordance with good practice code, the Declaration of Helsinki and was approved by the Medical Ethical Review Committee Leiden, Den Haag, Delft (study number: B21.034).

Imaging mass cytometry

Mass cytometry antibodies

IMC antibodies are listed in Supplementary Table 2. Most antibodies were conjugated with heavy metal isotopes in-house using the MaxPar X8 Polymere Antibody Labeling Kit according to

Table 1. Patient characteristics of twin cases

Case	1		2		3
Maternal age	24		38		31
Gravidity/Parity	1/0		1/0		3/2
Gestational age (weeks+days)	34+4		39+4		37+2
Birthweight/percentile	1380g/p<3	2750g/p50-90	3100g/p10-50	3390g/p50-90	1460g/p<3
chronic histiocytic intervillositis	Moderate	Absent	Moderate	Absent	Moderate
villitis of unknown etiology	Absent	Absent	Severe	Moderate	Moderate
Perivillous fibrin depositions	Absent	Absent	Moderate	Absent	Absent

Table 2. Patient characteristics of validation cohort and controls

	CHI (n=11)	Control (n=8)
Median (range) maternal age	34 (20 – 37)	34 (25 – 40)
Median (range) gravidity	3 (1 – 6)	2 (1 – 3)
Median (range) parity	1 (0 – 4)	1 (0 – 2)
Median (range) gestational age (weeks+days)	32+3 (23+3 – 39+1)	40+1 (38+4 – 41+2)
Median (range) birthweight percentile	<p3 (<p3 – p10)	P50 (p50 – p90)

the manufacturer's protocol (Fluidigm, California, USA). For five antibodies/isotopes a different protocol was used (Supplementary Table 2). All antibodies were titrated to determine the optimal labelling concentration. Additionally, all antibodies before and after conjugation to a metal were tested by immunohistochemistry before being used in IMC.

Imaging mass cytometry staining

IMC antibody staining was performed as previously described by IJsselsteijn et al. [21]. In short, 4-µm FFPE slides were deparaffinized, antigen retrieval with citrate was performed and the sections were blocked with superblock solution. Next, slides were stained with anti-CD4 (mouse-IgG1, dilution in Supplementary Table 3) overnight at 4°C. The next day the slides were stained with the secondary anti-mouse-145Gd antibody for 1 hour at room temperature (RT). Then the slides were stained with the first mix of metal-labeled antibodies for 5 hours at RT (Supplementary Table 3). Slides were washed and stained with the second mix of metal-labeled antibodies overnight at 4°C. Finally, the slides were stained with Iridium nuclear staining and dried.

Imaging mass cytometry data acquisition

Using a three-element tuning slide the Hyperion was autotuned according to the manufacturer's protocol (Fluidigm). Depending on the size of the intervillous space, 6 to 9 regions of interest (ROIs) from 1 mm^2 of villous tissue were randomly selected and ablated at 200 Hz. ROIs were equally placed 2/3 (CHI/control) subchorial, 2/3 (CHI/control) central and 2/3 (CHI/control) basal.

Data analysis

Creating a single cell mask using cell segmentation

Intervillous cells were manually selected for every ROI (Supplementary Figure 1). DNA staining was used to identify cells, CD141 to identify the SCT lining and consecutive hematoxylin-eosin (HE) staining was used for morphological guidance. After selecting the intervillous immune cells, Ilastik (v1.3.3) was used to create a probability map on the selected cells. The probability map was loaded in Cellprofiler (v2.3.1) and exported as single cell mask per ROI.

Background removal and data normalization

Semi-automated thresholding is an important step to correct for markers intensity changes due to sample workup procedures and time of storage, as there is a 15 year difference between the samples.

Semi-automated thresholding was performed in Ilastik to separate true signal from noise as described previously [22, 23]. For each marker, the algorithm was trained to separate noise (0) from signal (1) which resulted in binary pixel values. Therefore, in downstream analysis the marker intensity per cell indicates the proportion of positive pixels in that cell, rather than the marker intensity. Hereby 1 indicates all pixels in the cell are positive, 0.5 indicates half of the pixels in a cell are positive and 0 indicates none of the pixels in a cell are positive for the selected marker. Rarely all pixels are positive due to membrane or nuclear localization. Therefore, visualization of marker expression was set from 0 to 0.5 (all initially above 0.5 will become values of 0.5).

Phenotyping of segmented cells

As previously described [24], masks together with binarized thresholded ROIs were loaded in ImaCytE [25]. Each cell in the mask was combined with its corresponding thresholded pixel intensity file, allowing for FCS file exports with marker expression per cell as relative frequency of positive pixels. These single-cell FCS files were analyzed by tSNE in Cytosplore (v2.3.1). In the first tSNE all 5,735 cells were included and major immune lineages were determined. Non-immune cells ($CD45^-$, 522 cells) were excluded from analysis, as were duplicates (11 cells); Cells that have multiple markers belonging to different major immune lineages. In the second tSNE all 2,970 macrophages and monocytes were included. Based on the dendrogram and marker expressions some clusters were merged.

Immunohistochemistry and immunofluorescence

Consecutive sections of IMC slides were used for HE staining according to a standardized protocol.

Immunofluorescence staining

Triple immunofluorescence (IF) staining was performed, combining mouse-IgG2b anti-human CD68 (AMP41830, Abcam), rabbit-IgG anti-human CD38 (EPR4106, Abcam), and mouse-IgG1 anti-human HLA-DR (TAL1B5, Dako). In short, slides were deparaffinized and antigen retrieval was performed by microwaving for 10 minutes in 10 mM citrate solution (pH 6.0). Slides were blocked with Superblock (Thermoscientific, 37580) for 20 minutes after which the primary antibodies were added for incubation overnight at RT. The next day secondary antibodies were added for 1 hour at RT. For CD68/CD38/HLA-DR triple staining; goat-anti-mouse IgG2b-AF594 (Invitrogen), goat-anti-rabbit IgG-AF647 (Invitrogen), goat-anti-mouse IgG1-AF488 (Invitrogen). Slides were covered using 4',6-Diamidino-2-phenylindole (DAPI)-ProLong Gold (Invitrogen, P36941) and a coverslip. After scanning on the slides (Panoramic MIDI2 scanner, Sysmex), the coverslip and mounting medium were washed away. Finally, the same slides were stained with HE stain and scanned again.

CD39 immunohistochemical staining

Slides were deparaffinized and incubated for 20 minutes in 0.3% H_20_2. Antigen retrieval and blocking were performed as described above. Primary antibodies were added for incubation overnight at RT, after which goat-anti-mouse/rabbit Ig-HRP (DAKO envision) secondary antibody was added for

1 hour at RT. Lastly, slides were incubated with 3,3'-Diaminobenzidine (DAB), counterstained with hematoxylin, dehydrated and covered with a cover slip using mounting medium. Isotype controls were used to confirm that there was no non-specific background staining. Isotype controls were included for all immunohistochemistry (IHC) and immunofluorescence (IF) stains.

Semi-quantitative scoring of CD39

Since SCT consists of fused cells we could not quantify the number of SCTs expressing or not expressing CD39 and therefore choose for semi-quantitative scoring of CD39 expression on SCT. CD39 expression was scored by three authors (CK, HK and JK; level of agreement 82%). Triple immunofluorescence staining with CD68, CD38, and HLA-DR, together with the HE staining, identified the location of the CD68+ cell infiltrate (affected and unaffected areas) in CHI cases. From unaffected and affected areas a screenshot of three regions per placenta was taken and CD39 expression on SCT cells was scored on a scale from 0 to 2 (0: absent, 1: dim, and 2: bright expression) (scores 0 and 2 represented in Figure 4C). Scores of three regions from three researchers were averaged. Unaffected vs. affected areas were compared using the Wilcoxon matched-pairs signed-rank test, whereas comparison of unaffected areas from CHI placentas vs. healthy controls was performed using the Mann-Whitney U test.

RESULTS

Major immune lineages in the intervillous space

For exploratory in-depth phenotyping of the intervillous immune cells, we designed and optimized a 40-marker IMC panel (Supplementary Table 2) with a focus on the myeloid cell compartment. The clinical characteristics of the dizygotic twins are described in Table 1 and in more detail in van der Meeren et al. [19].

First, five major immune lineage compartments (CD45+) were visualized within the intervillous space: T cells (CD3+), innate lymphoid cells (ILCs) (CD3-CD7+), monocytes (CD68-CD14+), macrophages (CD68+), and granulocytes (CD15+) (Figure 1). Raw marker expression of major immune lineages in the intervillous space is visualized in Figure 1A and B, where the villi are marked with green dotted lines. We selected the intervillous cells to make a single cell mask after which marker expression per cell was extracted to obtain single cell data (Supplementary Figure 1). This allowed all intervillous cells and their marker expression to be visualized simultaneously in a single tSNE, and their numbers and frequencies to be determined (Figure 1C).

We identified 5,202 immune cells (146 to 2586 per sample). The placenta of the unaffected twin controls had a lower number of immune cells per mm^2 than their CHI twin counterparts (control twins: 46.5 and 19.3, vs CHI twins: 265.5 and 91.7). One cluster showed CD45 expression without any lineage markers, and equal distribution within the immune cell compartment (Supplementary Figure 2), which was excluded from further analysis. As expected, the CD68+ compartment was abundantly present per mm^2 in the CHI twins (control twins: 7.8 and 7.8, vs CHI twins: 195.6, 74.3 and 96.3). T cell numbers were also increased (control twins: 2 and 0.3, vs CHI twins: 12.8, 4.9, and

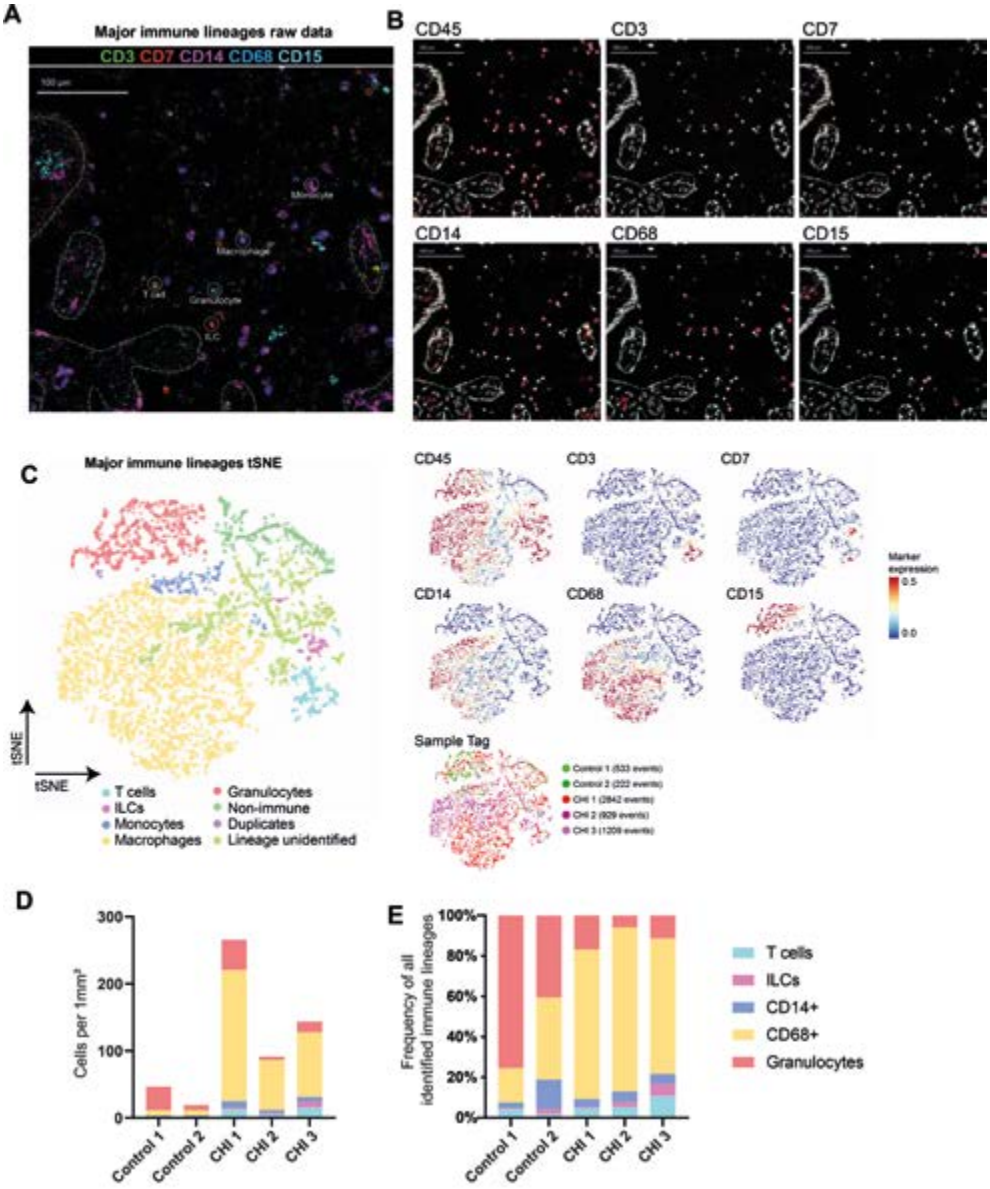

Figure 1. Major immune lineages in the intervillous space. Visualization of the major immune lineages in the intervillous space; T cells CD3+, ILCs CD7+CD3-, monocytes CD14+CD68-, macrophages CD68+, granulocytes CD15+. **(A)** Ex vivo visualization of the major immune lineage markers simultaneously in a CHI twin placenta. Within the green dotted lines are fetal villi, whereas the middle part with the depicted immune cells is the intervillous space. **(B)** Next, the major immune lineage markers are visualized one by one (red), with DNA staining (white). **(C)** Single cell phenotyping using tSNE analysis of the intervillous immune cells of the CHI twin placenta and their control twin placentae. Marker expression is visualized from 0 to 0.5, 0.5 indicating that at least 50% of the pixels in the cell are positive for that specific marker. **(D)** Absolute numbers of the different major immune cell types per 1mm^2 of tissue slide per sample. **(E)** Frequency distribution of the different major immune cell types per sample.

15.5) (Figure 1D). Granulocyte numbers per mm^2 were comparable, but their frequency was higher in the control twins compared to the CHI twins (Figure 1D, E).

Six phenotypically different immune cell clusters are identified within the macrophage and monocyte compartment

Next, the macrophage and monocyte compartment (excluding granulocytes) were combined for in-depth phenotyping of the myeloid compartment. Using 17 markers, six phenotypically different macrophage/monocyte clusters were identified (Figure 2A, B). Clusters 1, 2, 4, 5 and 6 were uniquely found in CHI twins, whereas in the control twin samples cluster 3 was also present. Clusters 1, 2, 4, 5 and 6 most resembled macrophages, with positive CD68 expression and low levels of CD14; whereas cluster 3 (CD14 positive and CD68 negative) showed the greatest phenotypic resemblance to monocytes (Figure 2A, lower graph).

Clusters 1 and 6 appeared to be sample-specific, since these clusters were mainly present in one CHI twin. Cells in cluster 1 do not express CD11b and CD11c but do express CD163. Cells in cluster 6 expresses CD68 and CD204. Clusters 2, 4 and 5 were observed in the three twins with CHI and absent in controls. Thus, are likely to be CHI specific clusters (Figure 2A, upper graph). Cluster 2 contains cells with both CD11b and CD11c which also express CD45RO, CD204, CD38, and HLA-DR. Cells in cluster 4 do not express CD11b and CD4, but most cells do express CD11c. Cluster 5 cells also do not express CD4 and CD204. Clusters 2, 4 and 5 have CD11c and HLA-DR expression in common and the three clusters contain cells expressing CD38.

CD68+HLA-DR+CD38+ cells are present in high numbers in CHI

When visualizing their spatial orientation, cells from clusters 2, 4 and 5 (Figure 2C) often co-localized with one another. At the specific locations where cells from cluster 2, 4 and 5 colocalize, raw data show that these clusters indeed express HLA-DR (green) and CD38 (light blue), as can also be observed in the tSNE and heatmap (Figure 2A).

To confirm our IMC data, immunofluorescence triple staining combining CD68, CD38, and HLA-DR was performed on a validation cohort consisting of 11 placentas with CHI and eight control placentas (Table 2, Supplementary Table 1). We could corroborate the presence of CD68+HLA-DR+CD38+ cells in the intervillous space of all 11 placentas with CHI, with complete absence in controls (Figure 3).

Reduced CD39 expression on syncytiotrophoblast cells is specific for regions with CD68+HLA-DR+CD38+ cell infiltrate

Subsequently, we focused on SCT and the localization of CD68+HLA-DR+CD38+ cells relative to SCT in CHI vs. control twins. Our IMC panel contains several markers that are expressed by SCT, including PD-L1, CD141 and CD39. No difference was found in the marker expression on SCT between CHI twins and control twins other than in CD39. The immunosuppressive enzyme CD39 has been reported to contribute to maternal-fetal tolerance via the adenosine pathway [26]. In the CHI twins CD39 expression was lower on SCT in regions where CD68+HLA-DR+CD38+ cell infiltrates

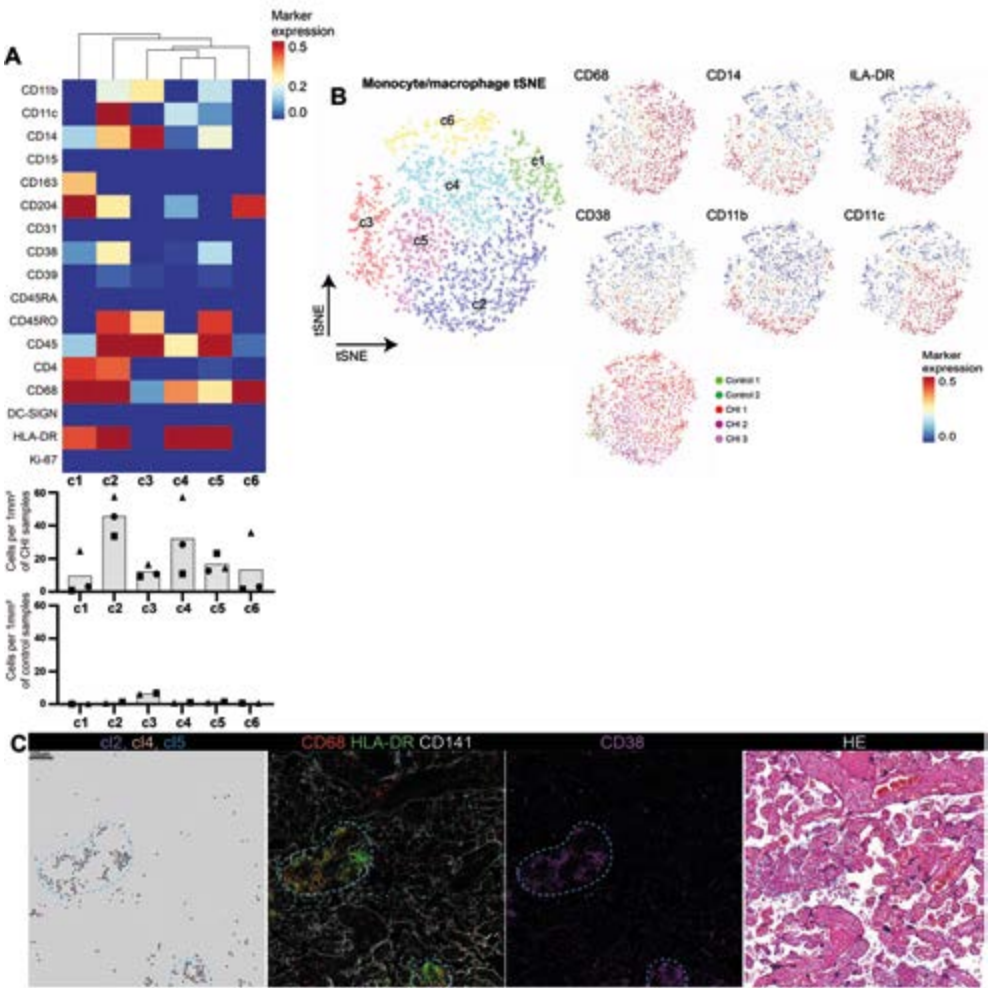

Figure 2. Phenotypic characterization of the different monocyte/macrophage cell clusters in the intervillous space. **(A+B)** tSNE on the monocyte/macrophage clusters using 17 markers. Simultaneous to marker expressions per cluster **(A)** absolute numbers and means of the 6 clusters per 1mm² of tissue slide is visualized for the CHI twins (upper graph) and the control twins (lower graph). **(C)** Cluster 2, 4 and 5 plotted back to the tissue location to visualize colocalization (blue dotted lines). Visualizing CD68 (red), HLA-DR (green) and CD38 (light blue) simultaneously (CD141 in white for SCT lining). With a consecutive HE slide.

were present (affected areas) compared to areas in the same placenta where these infiltrates were absent (unaffected areas) (Figure 4A).

Focally reduced CD39 expression was also confirmed in our validation cohort. A lower expression of CD39 on SCT cells was found in affected areas (mean grade: 1) compared to unaffected areas (mean grade: 1.83, p = 0.004) of the same sample. The unaffected areas in the CHI cases were graded similarly to control placentas (mean grade: 1.75, p=0.88) (Figure 4B, Supplementary Figure 3).

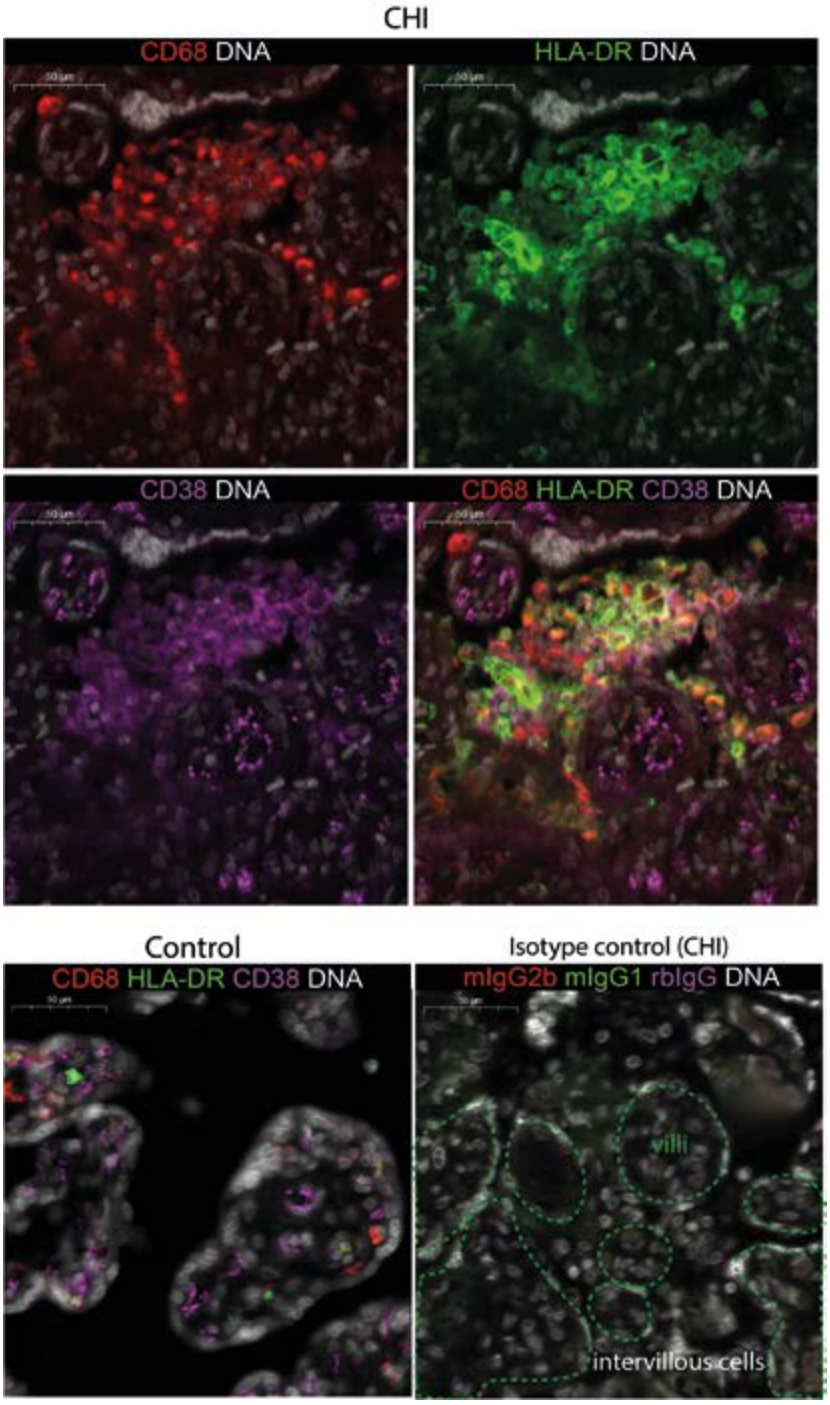

Figure 3. Immunofluorescence triple staining on validation cohort. Representative image of the 11 CHI samples that were stained by immunofluorescent staining (CD68 red, HLA-DR green, CD38 light blue, DNA white). Representative image of a healthy control and the isotype control.

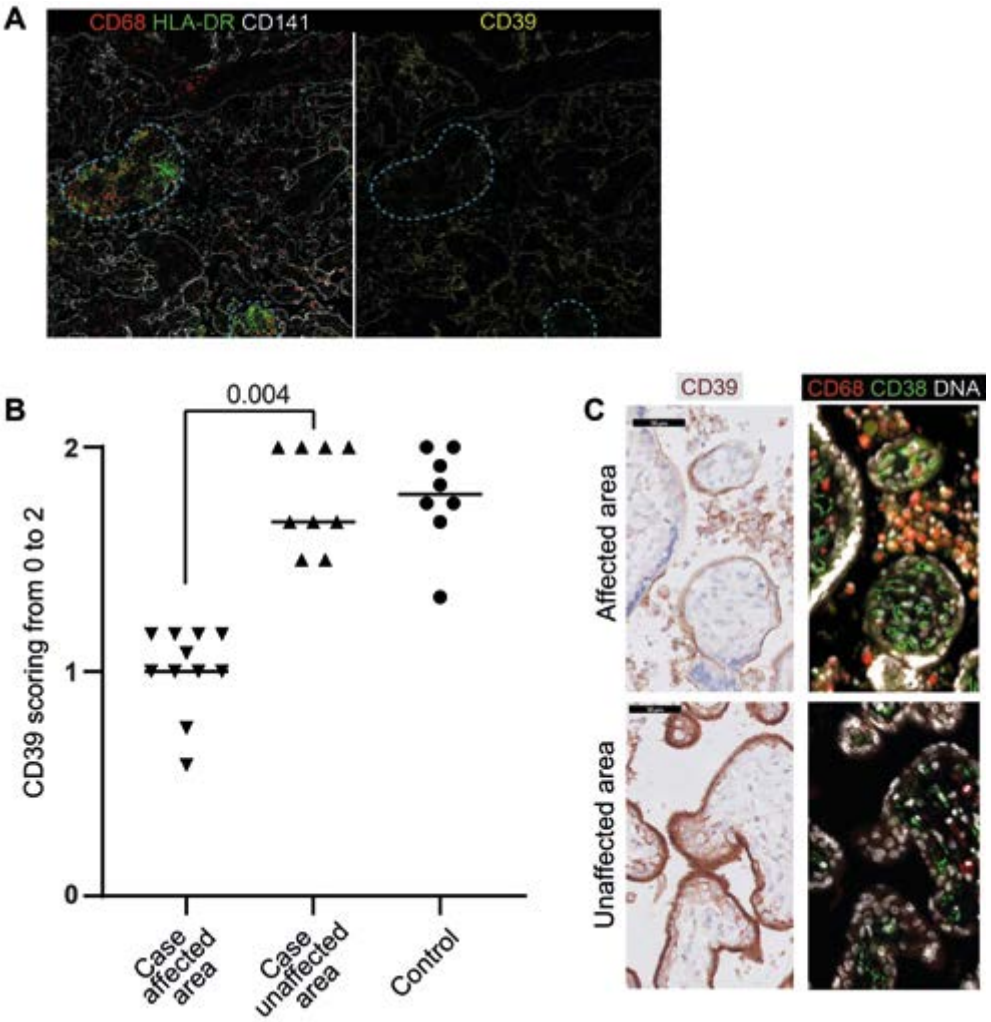

Figure 4. CD39 expression on SCT. **(A)** IMC data showing localization of CD68+HLA-DR+ and CD39 expression. **(B)** Semi-quantitative scoring of CD39 on SCT cells in CHI placenta validation cohort (Wilcoxon test, median and individual values are shown. **(C)** Visual representation of CD39 expression in one CHI case are visualized with consecutive slide stained for CD68 and CD38. In the affected area (with CD68+CD38+ cell accumulation) of this placenta reduced CD39 expression is seen on SCTs compared to the unaffected area (without CD68+CD38+ cell accumulation).

Figure 4C shows a representative image of affected and unaffected areas and the difference in CD39 expression between the two.

DISCUSSION

To gain a deeper understanding of the role of intervillous CD68+ cells in chronic histiocytic intervillositis, we applied in-depth immune profiling to tissue sections of CHI placentas and

matched (twin) controls using IMC. Five phenotypically distinct CD68+ cell clusters were found in the intervillous space of the CHI twin cases. Clusters 2, 4 and 5 were prominently present and unique for CHI. Cells in these three clusters expressed HLA-DR and CD38 and co-localized with areas where the CD39 expression on SCT was decreased.

The CHI-affected dizygotic twin cases showed a vast increase in placental CD68+ cells, both in absolute numbers and in frequencies compared to their control twin counterpart, which is the defining characteristic of CHI [1]. Besides the major immune lineages, non-immune cells were also found: these cells did not express CD45, nor any other lineage marker of our 40-marker panel. We also found unidentified immune cells expressing CD45 but no other lineage markers. While these cells may be B-cells (no specific B-cell marker was included in the IMC panel), our group previously showed that CD20+ cells are rare in the intervillous space of the placentas of the same twin cases [19]. Alternatively, these cells could be dimly expressing any of the lineage markers. Since the signal in IMC is not amplified by a secondary antibody and the resolution is 1 µm, low marker expression may be missed. The unidentified cells were present at the same frequency for both CHI and control twins, hence we did not characterize them in more detail.

When we specifically focused on the monocyte/macrophage compartment we found three phenotypically distinct clusters unique for CHI. Based on their phenotype these can be designated as macrophage, rather than monocytes. Clusters 2, 4 and 5 are phenotypically distinct from one another by the markers CD204, CD11b, CD45RO and CD4. CD45RO is upregulated in migratory monocytes [27]. CD11c, which all three clusters express, together with CD11b, forms the complement receptors (CR) 3 and 4 in conjunction with CD18. These play a role in phagocytosis, cellular adhesion, and migration within tissues [28]. Ligands for CR3 and CR4 include ICAM, which is found to be upregulated on SCT in CHI [16], and complement factor iC3b, which is likely to be present during CHI, as C4d deposition and membrane attack complex (MAC) complex formation (C5b-9) have been described on SCT in CHI [11, 12]. This suggests that the CD68+HLA-DR+CD38+ cells in the intervillous space may adhere specifically to SCT cells that express ICAM or iC3b. CD204 and CD68 are scavenger receptors, suggesting that cells expressing these markers have the potential to digest damaged/infected cells [29].

All cells of clusters 2, 4 and 5 express HLA-DR and most also express CD38. The CD38 signal is dim and thus could have been missed by IMC. The majority of CD68+ cells in CHI express both HLA-DR and CD38 in the validation cohort. Whereas CD38 can be present on monocytic myeloid-derived suppressor cells [30], in combination with HLA-DR expression it serves as a typical M1 marker [31, 32]. Previous literature showed that most intervillous cells express CD163, a typical M2 marker [18]. We found few cells expressing CD163, possibly due to low expression, dim expression can be missed in IMC. It could also be due to the difference in study cohort with Hussein et al., our samples have a higher gestational age compared to Hussein et al. [18]. However, we did observe many cells expressing CD204, a marker also associated with the M2 phenotype. The co-expression of both M1 and M2 markers on CD68+ cells suggests that M1/M2 polarization reflects a diverse spectrum of the myeloid cell population rather than representing a binary cell fate [33].

CD38 expression can be induced in monocytes and macrophages under inflammatory conditions [34], suggesting that the CD68+HLA-DR+CD38+ cells are in an inflammatory state

in CHI. In a murine arthritis study, synovial CD38+ macrophages were shown to expand during arthritis. Depletion of CD38+ macrophages by Clodrosome injection or blocking through TNF-α neutralization resulted in alleviated disease [35]. Another autoimmune disease with increased CD38+ mononuclear cells compared to healthy controls is systemic lupus erythematosus (SLE) [34, 36]. Interestingly, CD38-targeting treatments are candidates both for rheumatoid arthritis and SLE [37]. However, it should be acknowledged that CD38 is expressed on a variety of macrophages in different types of tissues, limiting its utility as therapeutic target.

Following phenotypic characterization of the CD68+ cell population we investigated whether SCT marker expression was affected in CHI. Our IMC data show that CD39 expression was altered in the CHI twins compared to the control twins. CD39 has been described as an immunosuppressive enzyme in tumor immunology [38]. In CHI pregnancy it previously has been shown that reduced SCT CD39 levels were associated with poor pregnancy outcome [6]. In our study, we found that CD39 expression was decreased in areas with CD68+HLA-DR+CD38+ cell infiltrate but not in unaffected areas of the same CHI placenta. It is not clear if CD39 gets downregulated before the CD68+ cells accumulate in the intervillous space or if this occurs as a consequence of increased numbers of CD68+ cells. Since it is unknown whether CD39 expression changes throughout gestation, as has been described for other markers like PD-L1 [39], we selected relatively high gestational ages for the CHI samples, to optimize the match with the controls. As a consequence of this selection, our CHI group has relatively good pregnancy outcomes (i.e. live birth) compared to other reports [40]. It has previously been described that the severity of infiltrate is associated with pregnancy outcome [41].

Interestingly, CD39 and CD38 come together in the adenosine pathway. Adenosine is an important immune regulatory molecule, as reviewed by Haskó et al. [42]. CD39 and CD38 are ectonucleotidase that are involved in the extracellular production of adenosine. Accordingly, reduced CD39 expression on SCT could result in a microenvironment with less adenosine. However, the presence of cells expressing CD38 could result in the formation of adenosine in the placenta with CHI. We found that both CD203a and CD73, two ectonucleotidases needed to produce adenosine via the CD38 pathway, are also present in the intervillous space of CHI placenta (data not shown). Further studies measuring levels of adenosine in placental blood could elucidate if placenta with CHI have more or less of this immune regulatory molecule.

Furthermore, future studies should focus on the functional capacity of the three macrophage clusters to elucidate their role in CHI. This could eventually result in novel therapeutics such as CD38-targeted treatments. Identification of a CHI-specific cell population could result in the identification of a diagnostic marker for the disease. Since these macrophages are in the intervillous space, it is conceivable that some might be detectable in maternal peripheral blood. Since these cells express scavenger receptors and likely digested placental debris locally, their cargo could potentially be identified as placenta-specific, as has been done previously for a glioma-specific protein [43]. These two characteristics combined could represent a possible detection pathway in which CD38-specific myeloid cells from maternal peripheral blood can be isolated and their cargo can be analyzed, to verify whether they are derived from the placenta. In this retrospective study, peripheral blood samples were not available from women with CHI.

This study is limited in the number of samples used for IMC. This limitation was addressed by confirming results in a validation cohort. Furthermore, since there is a detection limit of dim signals in IMC, corroboration using a different technique is imperative. We therefore used immunofluorescence and immunohistochemistry to confirm our results. Another limitation is that our samples from both the IMC and validation cohorts were of relatively advanced gestational age and therefore associated with disproportionately good pregnancy outcome [40]. However, as explained previously, this was necessary in order to maximize the validity of the comparison with healthy control samples. Therefore, we do not know if this cell cluster is present in early pregnancy losses caused by CHI. Lastly, during patient selection we did not exclude cases with concurrent VUE or perivillous fibrin deposition. Recently it has been hypothesized that CHI cases with or without concurrent VUE and/or perivillous fibrin deposition might have a different etiology. Therefore, we have included the grading of VUE and perivillous fibrin deposition in the patient characteristics tables. Our study cohort includes cases with and without VUE and/or perivillous fibrin deposition and we find the CD68+HLA-DR+CD38+ cells in all the cases.

Currently, there is great interest in the phenotypic characterization and function of the CD68+ cell infiltrate in the intervillous space in CHI, as a similar infiltrate is observed in cases of placental SARS-CoV-2 infection [44, 45]. It is not yet clear if the intervillous CD68+ cells in SARS-CoV-2-related CHI are phenotypically and functionally similar to those in idiopathic CHI, where no viral infection is present. Further research could help elucidate this.

In summary, we found three CD68+HLA-DR+CD38+ cell clusters that were distinct for CHI and express receptors that facilitate adherence to the SCT. The identification of these clusters provides an opportunity to study their function in more detail. Furthermore, if detectable in peripheral blood, the CD68+HLA-DR+CD38+ cells could potentially be used as diagnostic biomarkers and may lead to novel therapeutic targets for CHI.

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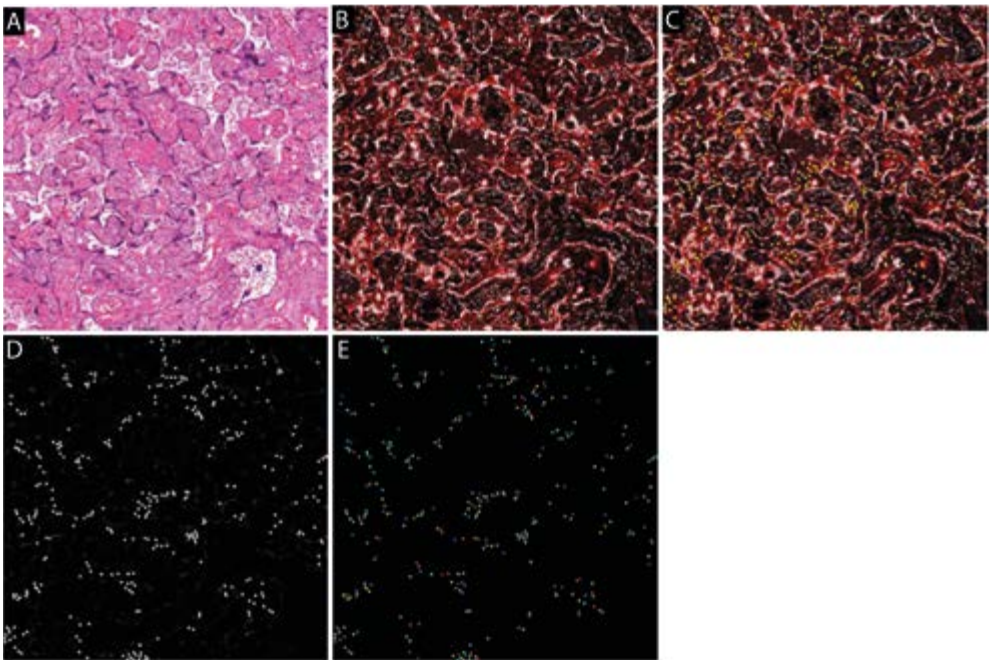
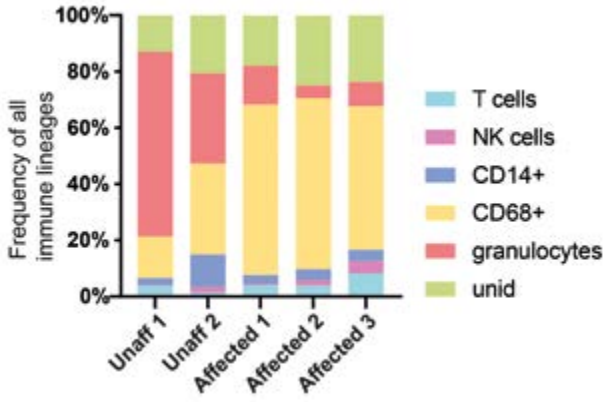
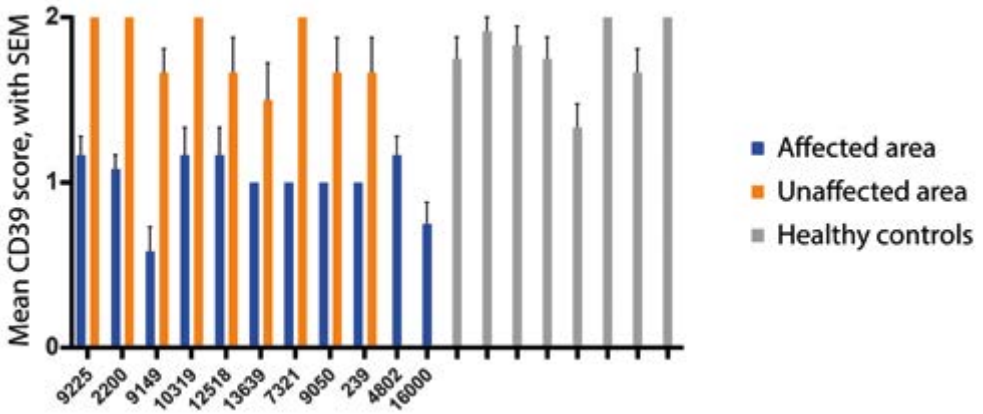
SUPPLEMENTARY DATA



Supplementary Figure 1. Making a single cell mask of intervillous cells. Using a consecutive HE stained slide **(A)** and DNA staining with CD141 marker expression **(B)** intervillous immune cells could be selected manually **(C)**. After manual selection the selected cells locations were extracted using ilastic **(D)** after which a single cell mask was made using CellProfiler **(E)**.



Supplementary Figure 2. Frequency distribution of the different major immune cell types per sample. Frequency distribution of the different major immune cell types per sample shows that unidentified immune cells are equally distributed over cases and controls.



Supplementary Figure 3. Paired CD39 scoring data. Paired semi-quantitative scores of CD39 expression on SCT in CHI placenta show that the areas with CD68+HLA-DR+CD38+ cell accumulation (affected) have reduced CD39 expression compared areas in the same placenta without CD68+HLA-DR+CD38+ cell accumulation (unaffected). Whereas controls showed similar scores to unaffected area in case samples.

Supplementary Table 1. Patient characteristics of validation cohort

	Gravidity	Parity	Maternal age	Gestational age (weeks+days)	Birthweight	Birthweight percentile	Placenta weight.	Placenta weight percentile	PE/HELLP	Pregancy outcome	Mode of delivery	CHI	VUE	Perivillous fibrin depositions
Case	2	0	27	30+1	1005	<p3	190	p3-p5	PE/HELLP	Live birth	SVD	Mild	Mild	Absent
Case	2	1	36	23+3	160	<p3	68	<p3	-	Fetal death	TOP IUGR	Severe	Absent	Mild
Case	4	2	36	34+3	1344	<p3	278	<p3	-	Live birth	PSC	Severe	Mild	Massive
Case	4	1	36	32+3	1372	<p3	313	p10-p25	PE	Live birth	SC	Mild	Absent	Mild
Case	1	0	31	33+4	1330	<p3	236	<p3	-	Live birth	SSC	Mild	Mild	Mild
Case	5	4	37	36+3	2509	<p3	298	<p3	-	Live birth	SVD	Mild	Mild	Mild
Case[a]	3	1	35	38+2	325	<p3 (at 25+4 GA)	NA	NA	-	IUFD at GA25+4	PSC	Severe	Absent	Mild
Case	1	0	20	39+1	2955	p3-p10	392	p3-p5	-	Live birth	SVD	Mild	Mild	Mild
Case	6	2	34	29+6	840	<p3	196	p5-p10	PE	Live birth	SSC	Severe	Absent	Massive
Case	1	0	27	29+2	870	<p3	160	p3-p5	PE	Live birth	SC	Mild	Absent	Absent
Case	3	2	30	24+6	370	<p3	80	<p3	PE	Intrapartum fetal death	IOL maternal PE, VD	Moderate	Absent	Absent
Control	1	0	25	39+4	3340	p50-p90	500	p10-p25	-	Live birth	SVD	Absent	Absent	Absent
Control	2	1	36	39+1	3830	p90	594	p75-p90	-	Live birth	SVD	Absent	Absent	Absent
Control	2	1	40	40+1	3670	p50-p90	404	<p10	-	Live birth	SSC	Absent	Absent	Absent
Control	3	2	30	40+0	3720	p50-p90	424	<p10	-	Live birth	SVD	Absent	Absent	Absent
Control	2	1	35	41+2	3845	p50-p90	390	<p10	-	Live birth	SVD	Absent	Absent	Absent
Control	2	1	33	40+1	3785	p50-p90	530	p50	-	Live birth	SVD	Absent	Absent	Absent
Control	2	1	39	40+2	3270	p50-p90	428	<p10	-	Live birth	SVD	Absent	Absent	Absent
Control	1	0	30	38+4	3475	p50-p90	458	p25	-	Live birth	SVD	Absent	Absent	Absent

a: Monochorionic twin case, with one affected with CHI and one unaffected. Affected side of the placenta is included in this study.

CHI; chronic histiocytic intervillositis. IOL; induction of labour, reason for IOL is provided. PSC; primary cesarean section. SSC; secondary cesarean section. TOP; Termination of pregnancy, the reason for TOP is provided. VD; vaginal delivery. VUE; villitis of unknown etiology.

Supplementary Table 2. Imaging mass cytometry antibody panel.

	Target	Clone	Company	Metal	Incubation time	Temperature	Dilution
1	Pan-Keratin*	C11 and AE1/AE3	Biolegend	^{106}Pd	Overnight	4°C	50
2	Collagen I	EPR7785	Abcam	^{115}In	Overnight	4°C	50
3	HLA-DR	TAL 1B5	ThermoScientific	^{141}Pr	5 hours	RT	100
4	EGF-R*	D38B1	Fluidigm	^{142}Nd	Overnight	4°C	50
5	CD68	D4B9C	Cell Signaling Technology	^{143}Nd	Overnight	4°C	100
6	CD11b	D6X1N	Cell Signaling Technology	^{144}Nd	5 hours	RT	100
7	CD4*	EPR6855	Abcam	^{145}Nd	Indirect	4°C	50
8	CD8	D8A8Y	Cell Signaling Technology	^{146}Nd	5 hours	RT	50
9	CD31	89C2	Cell Signaling Technology	^{147}Sm	Overnight	4°C	100
10	CD73	D7F9A	Cell Signaling Technology	^{148}Nd	5 hours	RT	100
11	CD69	EPR21814	Abcam	^{149}Sm	Overnight	4°C	100
12	Granzyme B	D6E9W	Cell Signaling Technology	^{150}Nd	5 hours	RT	100
13	Ki-67	8D5	Cell Signaling Technology	^{152}Sm	Overnight	4°C	100
14	CD3	EP449E	Abcam	^{153}Eu	Overnight	4°C	50
15	TIM3	D5D5R(TM)	Cell Signaling Technology	^{154}Sm	5 hours	RT	100
16	CD141	E7Y9P	Cell Signaling Technology	^{155}Gd	Overnight	4°C	50
17	NKG2A	LS-C165590	LSBio	^{156}Gd	5 hours	RT	50
18	CD39	EPR20627	Abcam	^{157}Gd	5 hours	RT	100
19	CD1c	EPR23189-196	Abcam	^{158}Gd	5 hours	RT	50
20	FOXp3	D608R	Cell Signaling Technology	^{159}Tb	Overnight	4°C	50
21	PD-1	D4W2J	Cell Signaling Technology	^{160}Gd	5 hours	RT	50
22	DC-SIGN	NBP1-77284	Novusbio	^{161}Dy	Overnight	4°C	50
23	IDO	D5J4E(TM)	Cell Signaling Technology	^{162}Dy	Overnight	4°C	100
24	CD14	D7A2T	Cell Signaling Technology	^{163}Dy	5 hours	RT	100
25	CD204	J5HTR3	ThermoScientific	^{164}Dy	5 hours	RT	50
26	CD45RO	UCHL1	Cell Signaling Technology	^{165}Ho	Overnight	4°C	100
27	D2-40	D2-40	BioLegend	^{166}Er	Overnight	4°C	100
28	CD56	E7X9M	Cell Signaling Technology	^{167}Er	5 hours	RT	100

Supplementary Table 2. (continued)

	Target	Clone	Company	Metal	Incubation time	Temperature	Dilution
29	CD103	EPR4166(2)	Abcam	^{168}Er	5 hours	RT	50
30	CD38	EPR4106	Abcam	^{169}Tm	Overnight	4°C	100
31	CD45RA	HI100	ThermoScientific	^{170}Er	5 hours	RT	100
32	CD15	BRA-4F1	Abcam	^{171}Yb	Overnight	4°C	100
33	CD163	EPR14643-36	Abcam	^{173}Yb	5 hours	RT	50
34	CD7	EPR4242	Abcam	^{174}Yb	5 hours	RT	100
35	CD45	D9M8I	Cell Signaling Technology	^{175}Lu	5 hours	RT	50
36	CD11c	EP1347Y	Abcam	^{176}Yb	5 hours	RT	100
37	Vimentin*	D21H3	Cell Signaling Technology	^{194}Pt	Overnight	4°C	50
38	HLA-G*	MEM-G2	ThermoScientific	^{198}Pt	5 hours	RT	100
39	αSMA*	D4K9N	Cell Signaling Technology	^{209}Bi	5 hours	RT	100
40	Bcatenin	D10A8	Cell Signaling Technology	^{89}Y	Overnight	4°C	100

* For 5 antibodies a different protocol was used; EGF-R was pre-conjugated by Fluidigm. Conjugation of two keratin antibody clones to 106Pd was performed using a protocol adapted from Schulz et al. [1]. Conjugation with 209Bi to α-SMA was performed using a protocol adapted from Spitzer et al. [2]. Cisplatin 194 and 198 were conjugated to Vimentin and HLA-G using a protocol adapted from Mei et al. [3]. CD4 was stained using a secondary staining step with α-mouse-145Gd.

Supplementary Table 3. Antibodies used in IF and DAB staining.

	Target	Clone	Cat#, Company	Company	Species	Dilution
1	HLA-DR	TAL 1B5	M0746	Dako	Mouse IgG1	2 ug/ml
2	CD68	LAMP4/1830	ab238094	Abcam	Mouse IgG2b	0,05 ug/ml
3	CD39	EPR20627	Ab223842	Abcam	Rabbit IgG	0.038 ug/ml
4	CD38	EPR4106	Ab108403	Abcam	Rabbit IgG	0,02 ug/ml
5	Mouse IgG1 isotype	DAK-GO1	X0931	Dako	Mouse IgG1	Same as target marker
6	Rabbit IgG isotype	Na	X0936	Dako	Rabbit IgG	Same as target markers
8	Mouse IgG2b isotype	DAK-GO9	X0944	Dako	Mouse IgG2b	Same as target marker
9	Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody. Alexa Fluor™ 647	Na	A21244	Invitrogen	Goat	5 ug/ml
10	Goat anti-Mouse IgG2b Cross-Adsorbed Secondary Antibody. Alexa Fluor™ 594	Na	A21145	Invitrogen	Goat	5 ug/ml
11	Goat anti-Mouse IgG1 Cross-Adsorbed Secondary Antibody. Alexa Fluor™ 488	Na	A21121	Invitrogen	Goat	5 ug/ml
12	EnVision Detection Systems Peroxidase/DAB.Rabbit/Mouse, HRP	Na	K5007	Dako	Na	Na

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8

SUMMARIZING DISCUSSION

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The main objective of this thesis was to investigate the role of the maternal immune system in maintaining a healthy pregnancy. In several chapters of this thesis, we have taken advantage of the advanced single cell technology of cytometry by time-of-flight (CyTOF) to achieve a better understanding to which immunological adaptations are needed to maintain a healthy pregnancy. By studying both healthy pregnancy **(chapter 2**) and pregnancies with adverse outcomes (**chapter 4 to 7)** more insight in the requirements for an optimal immune regulation can be obtained.

THE LOCAL AND SYSTEMIC MATERNAL IMMUNE SYSTEM

The focus of this thesis is on local maternal immune cells in the placenta (decidua and accumulation in the intervillous space) (**Chapter 2, 4, 6 and 7**). In a previous study from our group, we found that decidual and peripheral blood immune cells are phenotypically distinct from each other [1]. This may reflect the fact that the interaction between fetal cells and maternal decidual immune cells is different from that of fetal cells with maternal peripheral blood. For this thesis we chose to mainly focus on the local immune system. However, peripheral blood should not be ignored as trafficking between peripheral blood immune cells and local immune cells occurs while during pregnancy placental tissue is not accessible for immunological monitoring. Therefore, peripheral blood could contain biomarkers to predict adverse pregnancy outcomes as we studied in **Chapter 5** of this thesis. However, the observed discrepancy of soluble HLA-G levels in the peripheral blood with local HLA-G in women with a history of RPL showed us again that the local and systemic immune system often act differently during pregnancy.

UNEXPLAINED RECURRENT PREGNANCY LOSS

In **Chapter 5** we studied sHLA-G levels in pregnancies in the context of unexplained recurrent pregnancy loss (uRPL). HLA-G is a regulatory protein involved in the induction of immunological tolerance, which is of vital importance during pregnancy **(summarized in chapter 3)**. Opposite to what we expected, we found that women with successful pregnancy after a history of unexplained recurrent pregnancy loss (uRPL) have a similar, or a slightly reduced level of soluble HLA-G (sHLA-G) in peripheral blood compared to pregnant controls without a history of uRPL. In a previous study Craenmehr et al. found increased HLA-G levels locally in the decidua of women with a successful pregnancy and a history of uRPL compared to controls [2]. Therefore, we aimed to investigate whether increased sHLA-G levels were also present in the peripheral blood, which would provide a non-invasive clinically relevant assay. We hypothesized that similar results could be obtained by monitoring peripheral blood since HLA-G+ extravillous trophoblast cells (EVTs) are also a source for sHLA-G. One explanation of why local HLA-G levels and plasma sHLA-G levels show discrepant results could be that more sHLA-G is bound to its receptors in women with a successful pregnancy after a history of uRPL compared to controls. This could also be an explanation as to why no

difference was observed between 1st trimester sHLA-G levels of women with a history of uRPL of which the pregnancy resulted in a pregnancy loss or successful pregnancy.

In **Chapter 3** we described in more detail the effect that the interaction of (s)HLA-G with its receptors can have on different cell types and in addition the diversity of Tregs in the pregnancy setting is described. In **chapter 4** we studied Treg cells in women with successful pregnancy after uRPL and pregnant controls without a history of uRPL using suspension mass cytometry (SMC). We found equal numbers of T cells and Tregs between the groups, but the distribution of Treg subsets was different. Previously it was shown that women with ongoing uRPL have lower Treg frequencies in the decidua and less functional Tregs compared to controls [3]. Our results suggest that at least comparable Treg levels to those of controls without a history of uRPL are needed for a successful pregnancy in women with a history of uRPL. The biggest limitation of our studies is the lack of paired pregnancy loss samples. However, if our study cohort also had reduced Treg frequencies during their miscarriages, the question remains what triggered the increase of Treg frequencies in the successful pregnancy. To address this, we included ILT2 (or LILRB1) in the 42-marker SMC T cell panel in **chapter 4**. ILT2 is a receptor for HLA-G that can be present on myeloid cells, NK cells, and T cells. Binding of HLA-G to the ILT2 receptor on T cells can induce Tregs [4]. However, we observed ILT2 marker expression on only 0.1-0.5% of decidual CD4+ T cells, and not on FoxP3+ Tregs (data not shown). In conjunction, data from **chapter 4 and 5** suggest that reduced sHLA-G levels in uRPL are not caused by sHLA-G binding to the decidual CD4+ T cells via the ILT2 receptor.

Of note, there are many more HLA-G receptors on immune cells but also on placental stromal cells [4, 5]. NK cells and macrophages are important cell types during early implantation that can express HLA-G receptors (**chapter 2).** Further studies could point out if the HLA-G receptors on these cells are differently expressed in women with a successful pregnancy after uRPL compared to women with ongoing uRPL and controls. Binding of HLA-G to ILT2 or ILT4 on macrophages can polarize them to M2-macrophages, which may also lead to induction of increased Treg numbers and functionality [4, 6, 7]. Preliminary data of the SMC dataset from **chapter 4** show that 10-40% of CD3-CD56- cells (myeloid compartment and B cells) are ILT2 and HLA-DR positive (data not shown). However, we did not observe a difference between women with a history of uRPL and controls. Further analysis of the data could provide more information on the potential function of the decidual immune cells and if there are significant differences between women with a history of uRPL and controls. Next, functional testing, including HLA-G receptor blocking experiments will provide more information on how HLA-G could influence the decidual immune cell tolerance. **Chapter 2** provides a basis for designing *in vitro* experiments, as it shows which cells are likely to interact *in vivo* and should be cultured together to reveal their functional capabilities. To study 1st trimester recurrent pregnancy loss, ideally EVTs, macrophages, and NK cells isolated from the decidua should be cultured together and with different trophoblast cell lines [8]. Hereby trophoblast cell lines originating from recurrent pregnancy loss pregnancies and from elective termination (control) pregnancies should be used to study the fetal (e.g. HLA-G) contribution.

CHRONIC HISTIOCYTIC INTERVILLOSITIS

The IMC panels employed in **chapter 2** and **7** contain almost all the same markers (chapter 7 is missing CD66b and cleaved caspase 3). Therefore, we could identify that the CD68+ cells characterizing chronic histiocytic intervillositis (CHI) are phenotypically not similar to macrophages present in healthy decidua. This is not surprising as the interaction sites are different in the decidua and at the intervillous space, as described in the introduction of this thesis. The pathophysiology of CHI is poorly understood and the recurrence rate lies between 25 and 100% [9-12]. Interestingly, we identified one-sided CHI in three dizygotic twin cases **(chapter 6).** The mere existence of such cases suggests a significant contribution from the fetus' side while previously CHI has been described to be driven by maternal immunity [13, 14].

More data are emerging showing that CHI with unknown etiology could have different causes. It has been shown that women with CHI have fetus-specific HLA antibodies which could bind to the SCT in CHI [15]. However, not all women with CHI have fetus specific HLA antibodies [13]. Furthermore, C4d deposition on SCT is observed in CHI, indicating that antibodies able to activate complement may cause the inflammatory environment which is associated with CHI. Again, this is not the case in all CHI cases: some do not have any C4d deposition [16]. Additionally, not all women with fetus-specific HLA antibodies experience CHI. Lastly, Terry et al. observed in some, but not, all CHI cases IFN expression on SCT [17] (Terry et al. unpublished data), suggestive for a different, perhaps viral origin. Since the underlying mechanisms appear to be diverse, different treatment options should be considered for each case. However, what all cases have in common is the intervillous infiltrate of CD68+ cells **(chapter 7)**. Therefore, discovering their role in CHI could help to find a universal treatment option.

Currently there is no consensus on CHI treatment. After a pregnancy with CHI different types of treatment are given during a subsequent pregnancy to prevent CHI. In both a French and UK study by Mekinian et al. and Brady et al. aspirin, low-molecular weight-heparin (LMWH), prednisone and hydroxychloroquine alone or on combination were administered [12, 18]. Even though after treatment increased live births and less CHI was observed, this was not the case for all pregnancies. Indicating again the need to find other treatment options. Recently more specific antibody treatment has been applied with the TNF- antagonist Adalimumab as earlier applied progesterone, LMWH prednisone and hydroxychloroquine treatment did not result in successful pregnancy. Adalimumab was used as it showed to increase the success full pregnancy rate (71% versus 54%) in women with recurrent pregnancy loss while safety during pregnancy seemed good [19]. Adalimumab showed success in 2 CHI cases, however the study size should be increased before conclusions can be made on its efficacy in CHI cases [20].

It is currently not possible to properly isolate the maternal intervillous cells to study them. During tissue digestion, fetal Hofbauer cells are included, and by a procedure of leaking out the placenta to obtain the intervillous blood, the intervillous immune cells adhering to the SCT (which represents the cell population of interest) might stay behind. Therefore, we aimed to visualize the immune cells and SCT using IMC **(chapter 7)**. We found a unique phenotype of CD68+HLA-DR+CD38+ macrophages in the intervillous space. The number of T cells and NK cells was very low and

therefore we could not distinguish significant differences in their phenotype between the affected and unaffected twin. The CD68+ infiltrating cells in the intervillous space were previously described as anti-inflammatory, based on their phenotype [21]. We could not confirm this: using our IMC panel we could not identify them as clearly representing M1- or M2 macrophages. When analyzing these macrophages in the spatial context, we found that CD68+HLA-DR+CD38+ macrophages localize around the syncytiotrophoblast lining (SCT) which show signs of damage. It is currently not clear if the CD68+HLA-DR+CD38+ macrophages cause and/or enhance the damage to the SCT, or if they are present there to clean-up damaged SCTs, mediate and/or prevent inflammation. Now that we identified the CD68+HLA-DR+CD38+ phenotype, the macrophage infiltrate in CHI may be isolated at high purity after digestion of the placenta for functional testing.

ROLE OF MACROPHAGES DURING GESTATION

Both in **chapter 2** and **chapter 7** we phenotyped and visualized macrophages using imaging mass cytometry (IMC). IMC provides the opportunity to simultaneously measure M1 and M2 macrophage associated proteins and identify their micro-environment. Most clusters could not simply be defined as consisting of M1 or M2 macrophages, as most cells had marker expressions associated with both M1 and M2.

M1 and M2 macrophages have been characterized as two types of macrophages with a different metabolic program [22]. Further *in vitro* and murine studies helped to establish the role for these macrophages in human physiology (reviewed in [23]). M1 are described as inflammatory macrophages and are related to the Th1 response, whereas M2 are described as anti-inflammatory macrophage and are related to the Th2 response. However, attempts to translate *in vitro* results to the *in vivo* situation showed that *in vitro* models were unable to mimic the complex profiles observed in human disease (reviewed in [23, 24]). It has been extensively shown that macrophages can express a mixed M1/M2 phenotype in pathological conditions [25-27], as well as in pregnancy [28]**(Chapter 7)**. Therefore, macrophages should not be classified as purely M1 or M2. Metabolic pathway analysis, RNA sequencing and/or functional testing in well-defined *in vitro* setups could clarify on their specific function.

Since we could not with certainty identify if the decidual macrophages in **chapter 2** are M1 or M2 macrophages, we hypothesize on their potential function based on their microenvironment. In the first trimester we found that the two most prominent macrophage phenotypes (dMØ1 and dMØ4) colocalized with fetal EVTs and dNK1 cells **(figure 1A**). These two macrophage clusters did not express HLA-DR, which would prevent them from activating CD4+ T cells **(Figure 1B)**. Macrophages have previously been described to be present in the vessel wall during spiral artery remodelling and interact with dNK cells [29, 30]. Recently it has become clear that specifically dNK1 cells are involved in trophoblast invasion and spiral artery remodelling [31]. However, the phenotype for macrophages was not specified previously in as much detail, making it hard to study their roles with more precision. Previously, *in situ* experiments showed both NK cells and macrophages locally present during spiral artery remodelling, and express the matrix metalloproteinases-7 and -9, which could be involved in the degradation of vascular extracellular matrix [29]. To prove the role of NK cells and macrophages

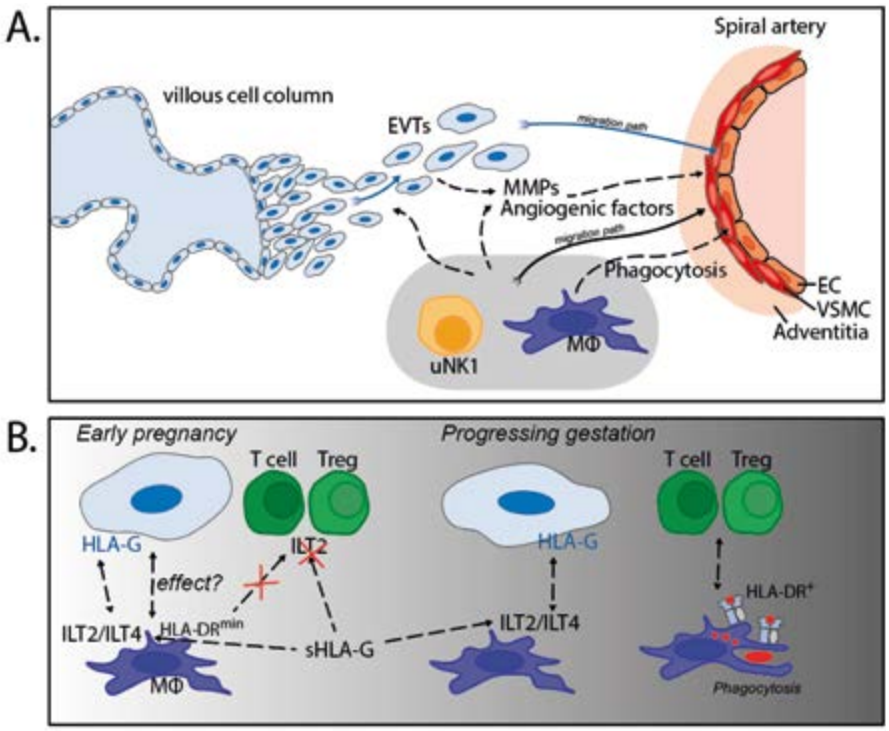

Figure 1. (A) Involvement of decidual macrophages during early pregnancy. (B) The role of macrophages during gestation and its interactions with trophoblasts and T cells with HLA-G and HLA-DR. EVTs=extravillous trophoblasts; EC=endothelial cells; VSMC= vascular smooth muscle cells; MMPs=matrix metalloproteases. black dotted lines: effect of macrophages on surrounding cells (adapted from Krop et al. *IJMS*. 2023).

in degradation of vascular extracellular matrix, using what we learned in **chapter 2,** *in vitro* models can be built using the correct macrophage subsets to confirm their function.

For NK cells and macrophages to be able to degrade vascular extracellular matrix, support trophoblast invasion but not kill other cells in the process a balanced immune regulation is required. The lack of HLA-DR expression on the macrophages interacting with EVTs at first trimester reduces their capacity to activate CD4+ T cells, including both inflammatory Th1, Th17, as well as anti-inflammatory Th2 and immune regulatory Tregs. Since these macrophages are the most prominent in first trimester and there is a lack of other APCs in the decidua (e.g. B cells and DCs) lack of CD4+ T cell activation signals could be the reason of the low decidual T cell numbers in early pregnancy **(Figure 1B)**.

Furthermore, **chapter 2** can be used as a basis for *in vitro* experiments since it shows the microenvironment of different NK cells, T cell and macrophages phenotypes. For example, it has been shown in preeclampsia that an excess of macrophages in and around spiral arteries is associated with reduced trophoblast invasion [32]. Rather than comparing them to all types of macrophages, these macrophages can be compared best to the dMØ1 and dMØ4 in healthy

pregnancy, since those are in the microenvironment of EVTs. This could help to confirm if these macrophages are positive, supporting spiral artery remodelling, or are negative, causing damage and thereby reduce proper spiral artery remodelling.

SUMMARY

In summary, in this thesis we show how multiple cell types (maternal and fetal) and other immune factors (e.g. sHLA-G) in the placenta that could be supporting each other to achieve a successful pregnancy **(Chapter 2, 3, 4 and 5)** or could potentially cause damage **(chapter 6 and 7)**. Furthermore, we created a basis for future studies to study the functional role of specific immune cells *in vitro* by co-culturing with cells that they encounter *in vivo* **(Chapter 2).**

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9

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Algemene introductie

Zwangerschap is een bijzonder proces waarbij één cel een volledig mens wordt. Uit diezelfde cel groeit naast een kindje ook de placenta, een uniek en essentieel orgaan dat één wordt met maternale cellen. Tijdens de zwangerschap kunnen immuuncellen zelfs van de moeder naar de foetus en vice versa reizen; dit proces wordt microchimerisme genoemd. In elke andere omstandigheid zijn cellen van verschillende individuen niet te combineren zonder een afstotingsreactie teweeg te brengen en is immuun onderdrukkende medicatie nodig zoals bij transplantatie. Het immuunsysteem bij zwangere vrouwen is echter wel functioneel: in geval van een infectie worden de ziekteverwekkers in principe gewoon opgeruimd. Het feit dat de lichaamsvreemde cellen van het kind niet afgestoten worden, wordt ook wel de immunologische paradox van zwangerschap genoemd. Het is nog steeds niet duidelijk wat er precies gebeurt met het immuunsysteem van de moeder tijdens de zwangerschap.

Veranderingen van het immuunsysteem zijn er wel degelijk, en in dit proefschrift hebben we de immunologische veranderingen tijdens gezonde en gecompliceerde zwangerschappen bestudeerd.

De algemene hypothese is dat onvoldoende aanpassingen van het maternale immuunsysteem tijdens zwangerschap kunnen leiden tot problemen met het functioneren van de placenta. Aangezien de belangrijkste functie van de placenta het leveren van zuurstof en voedingsstoffen aan de zich ontwikkelende foetus is, kan een onderontwikkeling van de placenta leiden tot complicaties tijdens de zwangerschap zoals miskraam of beperkte groei van de foetus.

Humaan leukocyten antigen (HLA)

Alle kern-houdende cellen van het lichaam en bloedplaatjes brengen HLA klasse I tot expressie, terwijl HLA klasse II enkel gevonden wordt op antigeen presenterende cellen (APC), geactiveerde T-cellen en geactiveerde endotheelcellen. De functie van HLA moleculen is waarschuwen dat er een infectie gaande is, door kleine stukken van eiwitten -peptiden- te presenteren aan T-cellen van het immuunsysteem. HLA klasse I kan peptiden die afkomstig zijn van intracellulaire eiwitten presenteren aan CD8+ T-cellen, terwijl HLA klasse II peptiden afkomstig van lichaamseigen eiwitten en lichaamsvreemde eiwitten uit het extracellulaire milieu kan opnemen, verwerken en presenteren aan CD4+ T-cellen. In normale omstandigheden zijn de gepresenteerde peptiden afkomstig van lichaamseigen eiwitten en geven geen aanleiding tot een immuunrespons. In het geval van een virale infectie of maligniteit zijn deze peptiden echter afkomstig van niet-eigen of tumor-specifieke eiwitten en worden ze als vreemd herkend. Hetzelfde kan gebeuren bij zwangerschap waarbij peptiden afkomstig van foetale HLA antigenen gepresenteerd kunnen worden in HLA moleculen van de moeder (indirecte herkenning), of het van de vader geërfde HLA eiwit als intact molecuul als lichaamsvreemd wordt herkend (directe herkenning). De twee types HLA moleculen kunnen onderverdeeld worden in verschillende groepen. HLA klasse I omvat de polymorfe HLA-A, -B en -C en de niet-polymorfe HLA-E, -F en G moleculen. HLA klasse II bestaat uit HLA-DR-, -DQ- en -DP-moleculen. HLA wordt co-dominant tot expressie gebracht en wordt overgeërfd als haplotypes,

waarbij één set genen (inclusief alle klasse I- en klasse II-moleculen) van moeder en één set genen van vader op de cellen van het kind tot expressie komen.

Contact tussen moeder en kind

Door de jaren heen zijn veel functies van de verschillende cellen in de placenta omschreven, maar het is nog steeds niet duidelijk hoe die cellen samenwerken om een gezonde zwangerschap tot stand te laten komen. Het is belangrijk om te weten dat er verschillende locaties van interacties zijn tussen maternale immuuncellen en foetale cellen. Het bloed dat door de placenta stroomt en de daarin aanwezige immuuncellen komen in contact met syncytiotrofoblast (SCT) cellen. De andere locatie waar interactie optreedt tussen immuuncellen en placentale cellen is de decidua: dit is de locatie waar de placenta en het chorionmembraan contact hebben met de uterine mucosa. Hier hebben de maternale immuuncellen contact met extravilleuze trofoblasten (EVT) en fibroblasten van foetale origine **(Hoofdstuk 1, Figuur 1 en 2)**. In principe brengt SCT geen HLA eiwitten tot expressie en kan dus ook niet herkend worden als lichaamsvreemd door het maternale immuunsysteem. De EVT brengen wel HLA moleculen tot expressie en kunnen wel degelijk worden herkend door het maternale immuunsysteem. EVT brengen enkel HLA-C, -E, -F en -G tot expressie, maar niet de polymorfe HLA-A en HLA-B, of klasse II moleculen. Het is beschreven dat HLA-G immuun tolerantie kan induceren. Dit fenomeen is omschreven in **hoofdstuk 3,** waarin de immuun regulatie door middel van regulatoire T-cellen (Tregs) wordt beschreven. EVT hebben een belangrijke rol bij het ontwikkelen van een gezonde placenta en dit proces wordt ondersteund door immuuncellen. NK cellen en macrofagen spelen hierbij een belangrijke rol **(Hoofdstuk 8, Figuur 1A)**. Zowel NK cellen als macrofagen produceren stoffen die belangrijk zijn voor hervorming van de maternale spiraal-arteriën. Daarnaast kunnen macrofagen beschadigde en dode cellen opruimen, wat essentieel is in een snel ontwikkelend orgaan als de placenta. Ook kunnen macrofagen als APC dienen in geval van een infectie.

Naast NK cellen en macrofagen zijn Tregs essentieel voor een gezonde zwangerschap. In zwangere muizen waarbij de Tregs experimenteel verwijderd zijn gaat een hoog percentage embryo's verloren. Eveneens is bij vrouwen met herhaald zwangerschapsverlies vastgesteld dat ze verlaagde Treg percentages hebben in vergelijking met vrouwen die een gezonde zwangerschap hebben doorgemaakt. Mogelijk zijn Tregs nodig als tegenbalans om na de benodigde activatie van het immuunsysteem voor de ontwikkeling van de placenta en verdediging tegen infecties herstel van homeostase te bewerkstelligen.

Wat op dit moment bekend is van de mogelijke functies van maternale immuuncellen in de placenta is wellicht nog maar het topje van de ijsberg. Vele cellen kunnen meerdere functies uitoefenen, afhankelijk van de signalen die zij op dat moment krijgen en de locatie waar ze zich bevinden. Dit kunnen zowel signalen zijn die ze van cel-cel contact krijgen, maar ook van oplosbare factoren. Meer informatie over de rol van de cellen in de placenta kan helpen bij de zoektocht naar de oorzaak en mogelijke behandeling van zwangerschapscomplicaties.

Het hoofddoel van dit proefschrift was het onderzoeken van de rol van het maternale immuunsysteem bij het in stand houden van een gezonde zwangerschap. Dit hebben we onder

andere gedaan door veel verschillende immuuncellen tegelijk in beeld te brengen met gebruik van de geavanceerde technologie van cytometrie (CyTOF) waarbij elke individuele cel gelijktijdig bestudeerd kan worden. Hierbij kan in beeld worden gebracht welke immunologische aanpassingen gepaard gaan met het behoud van een gezonde zwangerschap. Wij hebben zowel gezonde zwangerschappen **(hoofdstuk 2)** als zwangerschappen met ongunstige uitkomst **(hoofdstuk 4 tot 7)** bestudeerd.

Resultaten en discussie

Onverklaarbare herhaald zwangerschapsverlies (uRPL)

Onverklaarde herhaald zwangerschapsverlies (uRPL) is een situatie waarbij een vrouw een afbraak van twee of meer zwangerschappen voor de 24 weken draagtijd doormaakt zonder verklaarbare reden. Dit overkomt ongeveer 2% van de koppels.

Uit een eerdere studie van Craenmehr er al (*IJMS, 2019*) bleek dat HLA-G expressie in de decidua bij vrouwen met uRPL hoger was bij vrouwen met een succesvolle zwangerschap na uRPL, in vergelijking met vrouwen die een succesvolle zwangerschap hadden zonder voorgeschiedenis van uRPL (controles). Het doel van **hoofdstuk 5** in dit proefschrift was om te bepalen of de resultaten van Craenmehr et al te vertalen zijn naar oplosbare (s)HLA-G concentraties in het perifere bloed. Daarnaast bestudeerden wij bloed tijdens het eerste trimester van zwangerschap van vrouwen met een geschiedenis van uRPL om te bepalen of sHLA-G concentratie een indicator kan zijn voor zwangerschapsuitkomst. sHLA-G concentraties in het perifere bloed van vrouwen met een succesvolle zwangerschap na uRPL bleken vergelijkbaar met die van controles. Ook vonden we geen verschil in bloedconcentraties van sHLA-G tijdens eerste trimester tussen vrouwen met een doorgaande zwangerschap na uRPL en vrouwen met zwangerschapsverlies na uRPL. Dit is een indicatie dat het systemische immuunsysteem niet een directe afspiegeling is van het lokale immuunsysteem in weefsel. Onderzoek naar een biomarker in alternatieve biomaterialen zou hierbij een uitkomst kunnen bieden, bijvoorbeeld onderzoek naar immuunmarkers in een uitstrijkje of menstrueel bloed voorafgaand aan de zwangerschap.

Eerdere studies toonden aan dat vrouwen met uRPL lagere Treg-frequenties hebben in de decidua en minder functionele Tregs in vergelijking met controles. Het is echter niet duidelijk of Treg frequentie of functionaliteit verandert op het moment dat vrouwen met een uRPL voorgeschiedenis wel een doorgaande zwangerschap doormaken. Om dit te bestuderen hebben we in **hoofdstuk 4** een suspensie CyTOF panel samengesteld om gelijktijdig de frequenties en het fenotype van de Tregs te bepalen in de decidua van een doorgaande zwangerschap na uRPL in vergelijking met een controlegroep. We zagen minimale verschillen tussen de groepen, zowel in frequenties als in fenotypes. Echter, één specifieke Treg subset was wat betreft frequentie in mindere mate aanwezig in de uRPL groep. Mogelijk vertegenwoordigt deze subset een groep Tregs met een specifieke functionele capaciteit. Om deze veronderstelling te bevestigen zouden functionele testen moeten worden gedaan. De resultaten van deze studie suggereren dat op zijn minst vergelijkbare Treg-niveaus als in gezonde zwangerschap nodig zijn voor een succesvolle zwangerschap bij vrouwen met een voorgeschiedenis van uRPL.

Tregs kunnen receptoren voor HLA-G tot expressie brengen, één daarvan is ILT2. Verdere analyse van de data uit **hoofdstuk 4 (hoofdstuk 8)** toont aan dat ILT2-markerexpressie aanwezig is op slechts 0,1-0,5% van de deciduale CD4+ T-cellen en niet op FoxP3+ Tregs. Verder onderzoek is nodig om meer inzicht te krijgen in de rol van HLA-G bij het reguleren van immuuntolerantie tijdens de zwangerschap. Het uitvoeren van in vitro experimenten zou kunnen helpen bij het ontrafelen van de functionele eigenschappen van deze cellen en bij het achterhalen op wat voor manier HLA-G de immuunrespons kan beïnvloeden.

Chronische histiocytische intervillositis (CHI)

Chronische histiocytische intervillositis (CHI) is een zeldzame aandoening die zich tijdens de zwangerschap kan voordoen en die in de meeste gevallen leidt tot zwangerschapsverlies of vroeggeboorten. De oorzaak van CHI is niet bekend en er is momenteel geen marker beschikbaar die CHI op een vroeg moment tijdens de zwangerschap kan vaststellen . De diagnose kan pas na de geboorte worden gesteld door middel van pathologie-onderzoek op de placenta. Tevens is er geen consensus en wetenschappelijke onderbouwing voor de beste behandelmethode. Meer informatie over de oorzaak van CHI en wat er in de placenta gebeurt tijdens CHI is nodig om een gerichte behandelmethode te kunnen ontwikkelen.

In **hoofdstuk 6** identificeerden we bij drie dizygote tweelingen éénzijdige CHI: met andere woorden één van de tweelingen had een aangedane placenta en de ander niet. Dit suggereert dat naast een rol voor het maternale immuunsysteem de foetus ook een significante rol kan spelen bij de oorzaak van CHI. In **hoofdstuk 7** zijn we dieper ingegaan op het maternale immuunsysteem in de placenta's bij de dizygotische tweelingen. Door gebruik te maken van imaging CyTOF hebben we de macrofagen van de CHI-aangedane- en niet-aangedane tweeling met elkaar kunnen vergelijken. Hierbij ontdekten wij een uniek fenotype van CD68+HLA-DR+CD38+ macrofagen in de intervilleuze ruimte.

Het is onduidelijk of deze cellen de schade aan de placenta veroorzaken of verergeren, of juist aanwezig zijn om de ontsteking te bestrijden en/of schade in de placenta te herstellen. Met het vaststellen van het fenotype is het mogelijk om deze cellen in toekomstige studies te isoleren en hun functie te onderzoeken.

Macrofagen in zwangerschap

Veel onderzoeken waarbij deciduale macrofagen bestudeerd worden richten zich allereerst op isolatie van de macrofagen uit het weefsel. Echter, een belangrijke beperking bij deze benadering is dat macrofagen bij weefseldigestie achter kunnen blijven in het weefsel, sneller dood gaan en een instabiel fenotype hebben. Bij Imaging CyTOF is geen weefseldigestie nodig en het is daarom een goede techniek om op hoge resolutie en op betrouwbare manier de macrofagen in de decidua te bestuderen in de weefsel context. In **hoofdstuk 2** laten we ten eerste zien dat er meer macrofagen in de decidua aanwezig zijn dan voorheen werd gedacht. Daarnaast hebben we het fenotype van deciduale macrofagen en hun interacties met andere cellen tijdens eerste, tweede en derde trimester vergeleken.

De meest prominente macrofaagtypes (dMØ1 en dMØ4) in de decidua tijdens het eerste trimester co-lokaliseerden met foetale EVT en deciduale natural killer-1 (dNK1) cellen. Deze macrofagen brengen geen HLA-DR tot expressie, een molecuul dat normaliter nodig is voor activering van CD4+ T-cellen. Het gebrek aan CD4+ T-cel activeringssignalen kan mogelijk de reden zijn voor het lage aantal deciduale T-cellen in het vroege stadium van de zwangerschap. Macrofagen zijn eerder gevonden in de vaatwand tijdens de hervorming van spiraalarteriën en in interactie met dNK-cellen. De diversiteit aan fenotypes van macrofagen was echter niet eerder zo gedetailleerd gespecificeerd als in **hoofdstuk 2**, waardoor het voorheen moeilijk was om hun rol nauwkeuriger te bestuderen. Nu hun fenotype bekend is kunnen betere in-vitro modellen worden ontworpen om de rol van macrofagen bij de afbraak van vasculaire extracellulaire matrix en ondersteuning van trofoblast-invasie te bestuderen. Wij hebben aangetoond dat gedurende de zwangerschap andere macrofaag fenotypes (of dezelfde macrofagen met een alternatieve differentiatie status) aanwezig zijn in de decidua.

Samenvattend hebben we van het werk in dit proefschrift geleerd welk fenotypen de deciduale maternale immuuncellen hebben tijdens een gezonde zwangerschap, zowel zonder als met een voorgeschiedenis van uRPL, en ook bij zwangerschappen die gecompliceerd worden door CHI. Naast het fenotype is er meer informatie vergaard over welke interacties de cellen aan kunnen gaan, wat gezamenlijk een belangrijke basis vormt om goed onderbouwde in-vitro experimenten op te zetten. Deze in-vitro experimenten zijn essentieel om met zekerheid te bepalen wat de functie van de cellen is, en op grond hiervan markers te bepalen die een indicatie geven van klinische uitkomst en mogelijke interventies te ontwikkelen.

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ABOUT THE AUTHOR

Juliette Krop was born on September 3rd, 1993 in Amsterdam, the Netherlands. After graduating from the secondary school Montaigne Lyceum in The Hague, she studied Biomedical Laboratory Research at the University of Applied Sciences in Leiden. She became student tutor (2013) and she obtained her bachelor degree (2015) with her internship project entitled "A new tool for quantification of donor HLA-specific memory B cells", which she performed at the transplantation immunology group of the department of Immunology in the Leiden University Medical Center (LUMC). Afterwards she stayed in this group to work for four years as technician under supervision of Prof. Dr. Frans Claas and Dr. Sebastiaan Heidt studying HLA-specific memory B cells in solid organ transplantation. Because of her passion for pregnancy development, she became a PhD candidate on that topic in the group of Reproductive Immunology at the department of Immunology in the LUMC under the supervision of Prof. Dr. Frans Claas, Dr. Sebastiaan Heidt and Dr. Michael Eikmans (2018). As described in her PhD thesis, she used imaging mass cytometry to visualize several immune cell subsets in the placenta throughout healthy gestation and in chronic histiocytic intervillositis (CHI). Furthermore, she studied immune regulation in the context of recurrent pregnancy loss. She has visited many conferences during her PhD studies, of which she attended ten with an oral presentation. Furthermore, she has been an invited speaker at the CHI meeting in London and at the Institut Cochin Immunology-Axis in Paris. Additionally, she received a best abstract award at the EFI 2021 Amsterdam conference. During her time as a PhD candidate, she was also active as a chair of the PhD organization LEO, to represent PhD candidates at the Leiden university and organize social events and PhD-specific workshops. Furthermore, she co-established a PhD organization for the department of immunology, to bond PhDs from the department, improve the communication between the team leaders and PhDs and clarify career options after the PhD.

Currently, she works as a postdoctoral researcher in the Institut Cochin, Paris identifying immune cells important for labour onset and pre-term birth under the supervision of Dr. Céline Mehats. Furthermore, she continues her work on CHI collaborating with five different Parisian hospitals and research institutes after receiving a FHU grant for using spatial transcriptomics and spatial proteomics.