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

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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

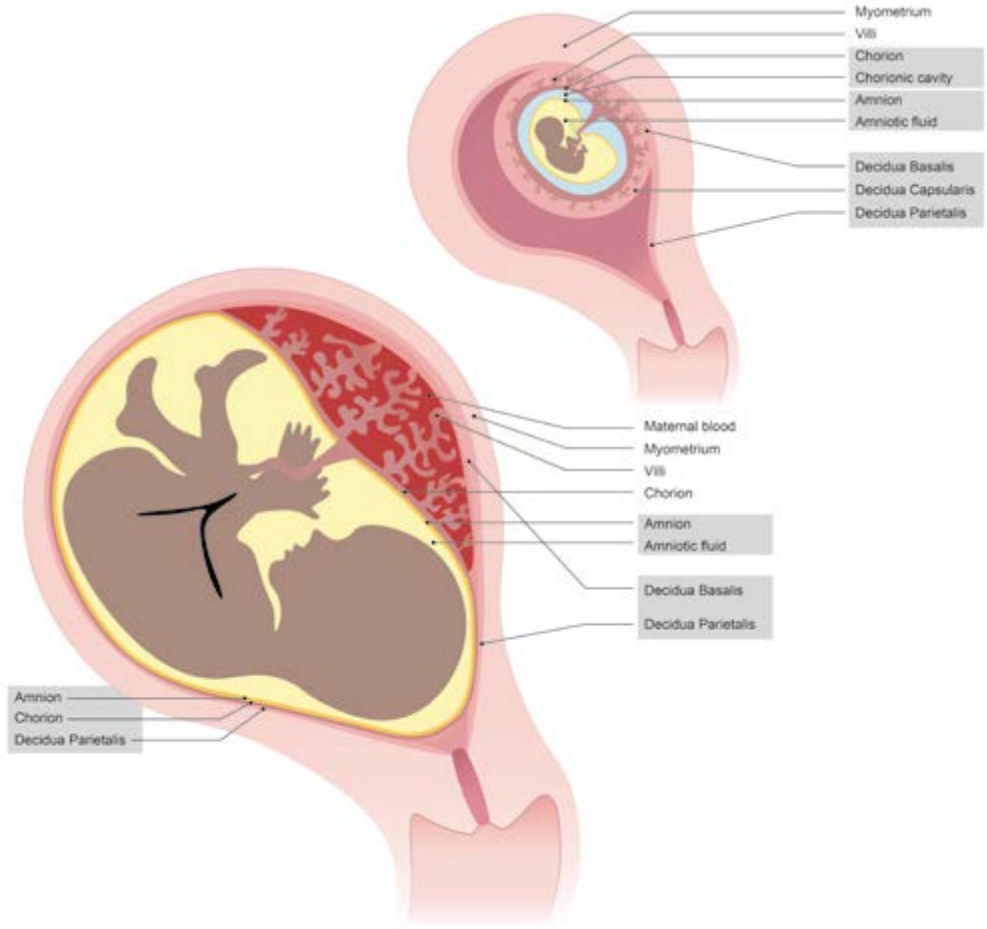

Figure 1. Schematic overview of anatomical changes of the maternal-fetal interaction sites. From (A) late first trimester (~8 weeks) to (B) term.

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

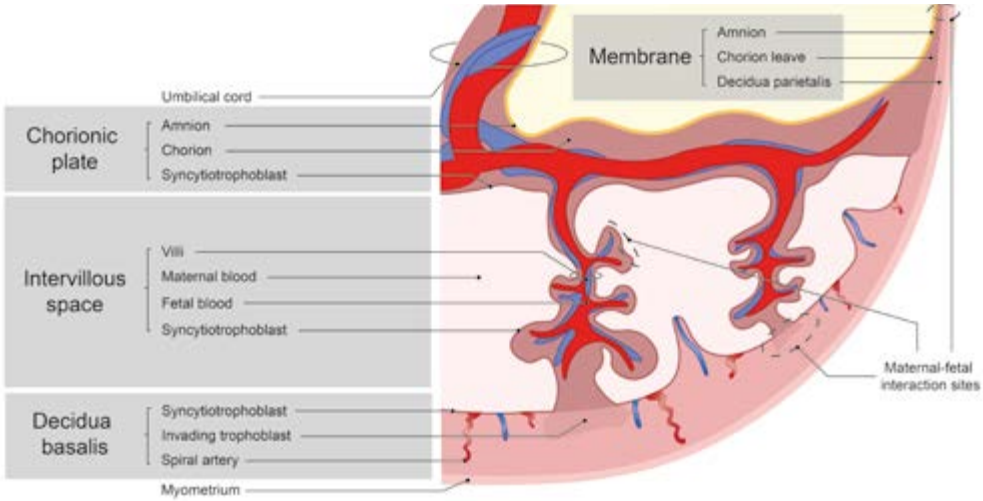

Figure 2. Overview of the structure of a term placenta and maternal-fetal interaction sites.

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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