Aus der Klinik und Poliklinik für Frauenheilkunde und Geburtshilfe der

Ludwig-Maximilians-Universität München

Direktor: Prof. Dr. med. Sven Mahner

Characterization and evaluation of immunological factors in the placenta of spontaneous and recurrent miscarriages

Dissertation
zum Erwerb des Doktorgrades der Medizin an der Medizinischen Fakultät der Ludwig-Maximilians-Universität zu München

vorgelegt von Elisabeth Johanna Rogatsch
aus Innsbruck
2022

Mit Genehmigung der Medizinischen Fakultät
der Universität München

Berichterstatter:	Prof. Dr. Udo Jeschke
Mitberichterstatter:	PD Dr. Roxana Popovici PD Dr. Robert Ochsenkühn
Mitbetreuung durch den promovierten Mitarbeiter:	PD Dr. Thomas Kolben
Dekan:	Prof. Dr. med. Thomas Gudermann
Tag der mündlichen Prüfung:	17.02.2022

Widmung

Meinen drei größten Unterstützern
Meiner Mama, meinem Papa und meinem Bruder

CONTENTS

INHALTSVERZEICHNIS

1. INTRODUCTION

1.1 The innate and adaptive immune system

Humans are in a permanent interaction with the surrounding environment. Within this interaction, there is the constant threat of pathogens streaming into the body and harming the organism.[1] To protect human beings, a complex immune system responds via various mechanisms.[2] This synergy of processes plays a pivotal role in the survival of both humans and animals.[3] Protection against infection depends on a close interaction between the innate and the adaptive immune system. The diverse cells within the immune system have the ability, through the innate and adaptive immunity, to fight pathogens such as bacteria, viruses, fungi, and toxins.[1, 2, 3]

The innate immune system exists at birth and is based on physical, chemical, cellular and humoral protective mechanisms against pathogens.[1] In general, it combines the elements of the immune system (neutrophils, macrophages, monocytes, acute phase proteins, complement, and cytokines) to provide an immediate defense.[4] The innate immune system reacts directly, without adaption or imprinting, and is non-specifically activated upon first contact with the pathogen.[1] Whereas the innate immune system responds rapidly but may damage normal tissue through lack of specificity - the adaptive immune system responds precisely and develops slowly within several days or weeks.[4]

The specific immune response benefits from its memory. As a result, subsequent exposure to the same pathogen leads to a more effective and rapid response.[5, 6] The adaptive immunity plays a unique role. It has the ability to lead a two-stage targeted effector response using antigen-specific receptors on T and B cells. Firstly, in the lymphoid tissue, the recognition and presentation of the antigen by the specific T or B cell starts a reaction of cell priming, activation, and differentiation. Secondly, there are two effector response pathways. Either the activated T cell leaves the lymphoid tissue for the focus of infection or the activated B cell releases antibodies into the blood, which then reach the site of disease.[4] Studies show that there is a strong association between intrauterine bacterial or viral infections and pregnancy disorders such as abortion, preterm labor, pre-eclampsia and intrauterine growth retardation (IUGR).[7, 8, 9]

Therefore, it can be surmised that the immediate immune response at the feto-maternal interface may have a significant influence on a successful pregnancy. During gestation, this innate immunity plays an essential role in establishing an adequate microenvironment at the feto-maternal

interface by distinguishing between "infectious non-self" (bacteria, virus, etc.) and "non-infectious self" (mother, placenta, and fetus).[10]

1.2 Potential factors causing spontaneous and recurrent miscarriage

Miscarriage, either spontaneous or recurrent, is a common complication during pregnancy.[11] The term clinical miscarriage is used to describe a miscarriage following an ultrasound or histopathologically confirmed intrauterine pregnancy. Clinical miscarriages can be distinguished into early clinical pregnancy loss (before gestational week 12) and late clinical pregnancy loss (gestational weeks 12 to 21).[12] It is estimated that 30% of pregnancies are dismissed prior to implantation. Another 30% of pregnancies are terminated at the stage of implantation but prior to the missed menstruation.[13] Approximately 15% of conceptions end in early clinical pregnancy loss. However, it has been proven that there is a significant difference in the incidence of miscarriage according to age. The incidence rises from 10% in women aged 20 to 24 years to 51% in women aged 40 to 44 years.[14] Late losses between 12 and 21 gestational weeks are less frequent and equate to around 4% of pregnancies.[15] In spontaneous miscarriages, well-known risk factors include fetal chromosomal or endocrine disorders. Antiphospholipid syndrome, thrombophilia or maternal anatomical malformations have also been established as causes for recurrent pregnancy loss.[16] Clinical studies state that there is a strong causality between miscarriages and parental chromosome abnormalities[17,18] acquired thrombophilia[19, 20] and thyroid autoimmunity[21, 22,23] during pregnancy. Meanwhile, autoantibodies[24,25], hereditary thrombophilia[19,26], sperm DNA fragmentation[27,28] and alcohol consumption[29] are considered to only have a moderate impact. It is further believed that natural killer (NK) cell dysfunction[30,31,32], abnormal HLA-G expression[33], PCO syndrome[34], uterine malformations[35,36], hCG gene polymorphisms[37,38] and obesity[39,40] have a weak to moderate influence on the occurrence of a miscarriage.[12]

Recurrent miscarriage is defined as the occurrence of two or more failed clinical pregnancies before the 20th week of pregnancy.[41] The incidence of recurrent pregnancy loss is approximately 1–2% and affects 5% of families who have the desire to have a child.[42] Until now, only 50% of the pathogenesis of recurrent miscarriage causes are known. These known factors include immunological, endocrinological, genetical and infectious components. Metabolic and anatomical abnormalities, and other unexplained factors are also recognized. So far, it is hypothesized that recurrent pregnancy loss derives from multiple interactions of the afore mentioned factors. About 50% of couples who have undergone an entire medical investigation remain clueless as to the reason why they are unable to carry out the process of pregnancy and deliver a healthy child.[17]

1.3 Pregnancy: immune cells and the maternal immune system in the feto-maternal interface

The overall aim and intention of the study concerning Papers 1 and 2 was the evaluation of immunological factors and cytokines in placental tissue that are involved in recurrent and spontaneous abortions. The human placenta and the process of pregnancy is a complex system. Various mechanisms need to function in order to maintain a successful pregnancy and to nourish the fetus until birth. The placenta has multiple processes to protect the fetus such as immune activity, production of diverse chemokines, cytokines and antimicrobial peptides.[43,44] The three main functions of this multiplex organ are to develop and enable the trophoblast invasion and growth, to secure the placental angiogenesis and to establish the immunomodulation within the feto-maternal interface.[45,46] The feto-maternal interface is defined as the interaction between the uterus, particularly the decidua and the inner third of the myometrium, and the invasive extravillous trophoblast cells (EVT). The decidua, which is the decidualized endometrium, consists of cells like stromal cells, lymphatic cells, leukocytes, luminal and glandular epithelium, spiral arteries and fetal derived EVT.[46] The expression of markers within the surface of maternal immune cells leads to the recognition of cells as trophoblasts or fetus.[47,48,49,50,51] The maternal immune system, including maternal lymphocytes such as CD4+ T cells, CD8+ T cells, NK cells and regulatory T cells (Treg cells), plays an important part in the development of a healthy pregnancy. Treg cells are involved in tissue homeostasis and provide immune tolerance. Moreover, Treg cells are also linked to tumor growth and organ transplantation tolerance. They are also augmented systemically and locally during gestation. In miscarriages, not yet fully discovered factors are presumed to be related to the failure of implantation in humans. Treg cell impairment and maldistribution disturbs the Treg cell-mediated tolerance and may be one of the etiologies of miscarriage.[52] Former studies state that Treg cells are diminished in peripheral blood and the decidua in miscarriage cases. In recurrent miscarriages, it has been observed that the suppressive capability of Treg cells was impaired.[53]

1.4 Immune response during normal pregnancy and spontaneous and recurrent pregnancy loss

During gestation, the maternal immune system provides protection to the the fetus. However, definite components and pathways remain incompletely understood and still need to be investigated. Distinct immunological mechanisms have been considered.[54,55] Cytokines are proposed to play an essential role in embryo–maternal signaling. The majority of T-helper (Th) cells can be subdivided into Th1 and Th2 cells. Th1 cells mostly produce pro-inflammatory cytokines, such as Interferon-γ (IFN- γ), tumor necrosis factor-α (TNF-α) and Interleukin-2 (IL-2), and operate to induce abortion.[38] Commonly, Th2 cells secrete anti-inflammatory cytokines, such

as IL-4, IL-6 and IL-10, and appear to be necessary in maintaining pregnancy via their immunosuppressive effects.[56] It is believed that an imbalance of cytokines caused by exogenous or endogenous factors can support pregnancy complications and even spontaneous abortion.[57, 58] The normal range of the Th1/Th2 cell ratio is considered to be less than 10.3.[59] Previous studies show that Th1 and Th2 cells are key players in immune responses, especially in immune rejection and tolerance.[60, 61] It has been detected that a major portion of helper T cells (up to 30%) are Th1 cells associated with cellular immunity. In contrast, only up to 5% of helper T cells are Th2 cells, which are part of the humoral immunity. Th2 dominance is important and connected to a normal pregnancy. Meanwhile, up to 2% of Th17 cells can be found during a normal pregnancy.[62] The T cell population fluctuates during pregnancy: decreasing in early pregnancy but elevating again at term. This leads to an overall increase in their relative percentages in the third trimester.[63, 64] The appropriate balance of Th1, Th2, Th17 cytokines, and Treg cells leads to a successful pregnancy.[65] We speculate that a shift in the local Th1/Th2 balance may result in an unwilling abortion.[121] In the case of recurrent miscarriage, the immune response is generally divided into immune suppression and immune tolerance.[66] In contrast, certain obstetric disorders, including habitual abortion, may be due to an increase in Th1-dependent cytokines, especially TNF-α.[59]

1.5 Polarization of macrophages and the Th response in the placenta (Paper 1)

Pregnancy is a unique biological event which leads to significant changes in the maternal immune system.[67] The interplay of functioning immune cells in the placental tissue is important for both mother and fetus. Various other immune cells are also located in the placental bed. In early pregnancy, macrophages, Treg cells, uterine NK cells, and dendritic cells are the most prominent immune cells in the placenta.[68] In the human decidua, NK cells are generally the most dominant group. They are responsible for up to 70% of the immune cell population. Meanwhile, macrophages are the second largest group with up to 20% of the leukocyte population.[63] It is common knowledge that macrophages develop out of circulating monocytes.[69,70] During gestation, the phagocytic function of monocytes is relatively diminished. This decrease in function may be a supportive mechanism in protecting allogeneic fetuses from monocyte activation.[71] Monocytes stay in the bloodstream for 1-2 days. They then migrate from the blood vessels into tissue where they become macrophages. Macrophages play a crucial role in the regulation of tissue repair and remodeling, host defense, immune homeostasis and angiogenesis.[70,72,73] It is generally believed that macrophages can be distinguished into two types: classically activated macrophages (M1) and alternatively activated macrophages (M2).[74,75] These two categories vary in their effector function and surface markers, as well as their pattern of cytokine secretion.[76] M1 macrophages are driven by Th1-type responses and are part of the inflammatory response due to their

secretion of IL-12, IL-23 and reactive oxygen species.[77,78,79] M2 macrophages are involved in promoting Th2-type responses and demonstrate immunosuppressive qualities by participating in tissue remodeling, angiogenesis, and tumor progression.[80,81] The Th2-type response leads to antibody production, plasma cell maintenance, and enhanced phagocytic activity.[82, 83, 79]

IL-4 and IL-10 are predominately produced by M2 phenotypes. IL-4 is part of the differentiation of naive CD4-T cells into the Th2 phenotype.[84] It is believed that the M2 polarization through the immunomodulatory pathway is associated with a healthy pregnancy. M2 macrophages can be categorized into M2a, M2b, M2c, and M2d subsets. These subsets can be further subdivided according to the occurrence of various stimuli, markers, surface molecules. They also provide immunological functions by producing different chemokines and cytokines.[85, 78, 79] In contrast, bacterial components like gram-negative lipopolysaccharide (LPS), a ligand for Toll-like-receptor 4 (TLR4), induce M1 polarization.[86] Therefore, external pathogens activate Toll-like-receptor signaling which then shifts decidual macrophage polarity. It changes the immunosuppressive M2 dominancy into the inflammatory M1 phenotype.[87, 88, 89]

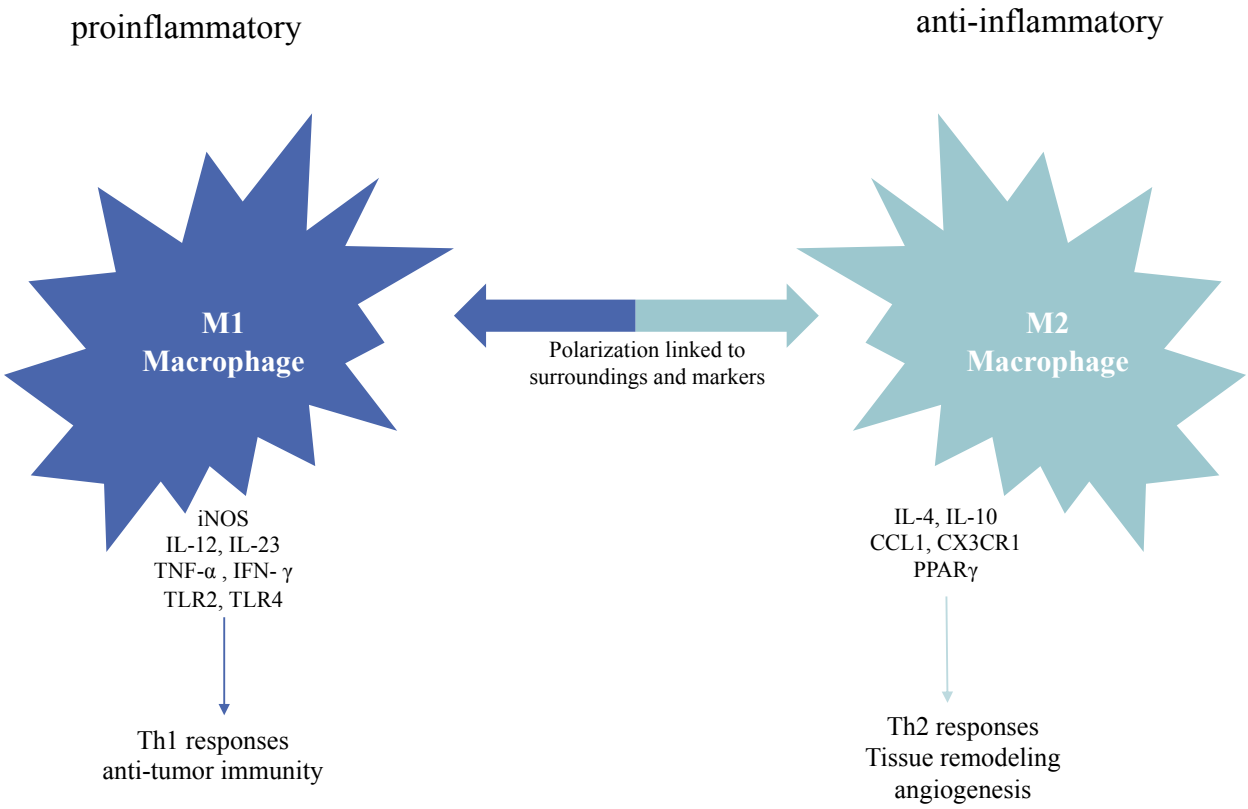


FIGURE 1: M1 / M2 POLARIZATION OF MACROPHAGES
adapted and inspired by s.LIU et al, The role of decidual immune cells on human pregnancy Journal of reproductive medicine 2017[67]

Macrophages are extraordinarily flexible and can remain in a suspended state, this enables them to respond to other signals, rather than stay committed to only one activated phenotype. For instance, tumor-associated macrophages can undergo a bidirectional transformation between M1 and M2 phenotypes to assist the process of anti-inflammation and immunosuppression.[90] Furthermore, the phenotype and function of macrophages relies on their residential environment, which has a strong influence on them.[82] It is recognized that there is an increase in the number of M2-polarized macrophages in the placental bed during normal pregnancy.[91] Considering those with complications, such as recurrent miscarriage and preeclampsia, the M2-polarized macrophages then lead to M1 polarization and the mediation of inflammatory responses.[92,93] Decidual macrophages are highly versatile and express a particular combination of cell surface markers: CD14, CD68, and MHC class II antigen HLA-DR. However, when it comes to activation they can be differentiated by the expression of CD11c and CD86.[94] The number of decidual macrophages varies with the gestational age of the fetus. Although macrophages are present within the placenta at all points during the pregnancy, the highest populations are found within the first and second trimester.[95,96,97] These macrophages guide adaptive T cell responses, monitor innate NK cell responses and produce anti-inflammatory substances such as IL-10, prostaglandin E2 (PGE2), and IDO.[46]

Peroxisome proliferator-activated receptor-gamma (PPARγ) is part of the superfamily of nuclear hormone receptors. Together with the other subtypes, PPARα and β, it is relevant in lipid metabolism and also recognized to be involved in the process of placentation. There is evidence that all three subtypes are expressed in the placenta. The expression of the subtype of α and β diminishes as the cells differentiate, whereas PPARγ stays expressed potently in these cells [98, 99, 100, 101, 102].[103] *I*n Paper 1 we focused on PPARγ. The expression of PPARγ is predominantly found in adipose tissue as well as in trace amounts in colon and immune cells. PPARγ is recognized especially in macrophages and has been found to guide macrophage differentiation and to participate in adipogenesis.[104,105,106] The versatile nuclear receptor PPARγ is suspected to be important in the development of activating alternatively M2 macrophages in the placenta.[107] Within a normal pregnancy, PPARγ regulates multiple key functions such as cell differentiation, nutrient balance and response to anti-inflammatory and oxidative stress. A pathological pathway often leads to decreased EVT invasion, altered lipid metabolism, increased inflammation and oxidative stress and ultimately to placental disorders including miscarriage. Nevertheless, the fact that all of these elements are partly controlled by PPARγ validates the crucial role it plays in the physiology and pathology of gestational tissue.[103] Overall PPARγ is essential for its anti-inflammatory effects and it has been demonstrated to downregulate the expression of pro-inflammatory cytokines like IL-6, IL-8, and TNFα in the placenta. [108,109] A decreased number of

PPARγ expressing decidual macrophages may lead to M1 polarized macrophages and a proinflammatory response and, at last, to miscarriage.

1.6 Regulation of the immune cell markers TLR4 and IL4Rα in the trophoblast (Paper 2)

Trophoblast differentiation plays an essential guiding role in the growth, functionality, and maintenance of the placenta during pregnancy. The trophoblast is of strong relevance and governs key functions in establishing the process of pregnancy. It provides the blood-flow to the implantation site, which then supports the maturation of the fetus with nutrients and oxygen. The main maternal-fetal exchange surface is formed by the villous trophoblast. It is considered to be crucial for fetal development and therefore must be able to adjust to environmental changes in order to ensure placental growth throughout the whole pregnancy. Trophoblast differentiation is a strictly regulated process and abnormalities are linked to placental dysfunction disorders.[103, 110, 111] It is proposed that trophoblast cells are also educators of various immune cells. The trophoblast can regulate consecutive immunological behaviors with its signals.[112] The appropriate immune cell cross-talk may be fundamental for a normal pregnancy and outcome. The occurrence of defects or changes in this sensitive system can lead to complications or loss. The trophoblast cells can be further distinguished into the EVT, which invades the decidua, or the intermediate villous trophoblast (IVT) and villous trophoblast (VT) cells, which reside in the placenta and are covered by the syncytium. The IVT is an individual type of trophoblast that beholds some of the morphologic and functional qualities of both cytotrophoblasts and syncytiotrophoblasts. Furthermore, this heterogeneous cell group owns characteristic immunohistochemical markers and different functions. Multiple forms of trophoblastic diseases arise primarily out of the IVT.[113,114,115]

Toll-like receptors (TLR) are a group of transmembrane proteins, which are evolutionarily able to recognize pathogen-associated molecular patterns in bacteria, viruses, fungi and parasites. Their expression ranges from immune cells to non-immune cells, such as trophoblasts, decidual cells and amniotic epithelium. At the feto-maternal interface, TLRs are broadly found and they guide and influence local and systemic immune responses in the placental tissue.[112] In our research in Paper 2, we focused on TLR4, which is an important receptor of the innate immune system and effectively guides host cell responses to LPS.[116] Studies depict already that TLR4 is associated with the occurrence of miscarriages.[117] TLR4 activates the NF-kB transcription factor by recognizing LPS. It regulates the expression of related factors, such as TNF-α and IL-12, all of which can lead to habitual abortion.[118] It is contemplated that the trophoblast and its cells are modulators of the immune system within the feto-maternal interface and may regulate multiple

immune cell mechanisms. The binding of TLR4 in the trophoblast stimulates the production of pro-inflammatory cytokines and chemokines, which then initiates the activation of macrophages, NK cells, and neutrophils.[112,119,120] We speculate that inadequate signaling of TLR4 may disturb immunologic pathways.

In contrast, IL4 receptor alpha (short IL4Rα) acts as a generally known receptor in the IL4 and IL13 pathways.[122] These two interleukins, common anti-inflammatory cytokines, can downregulate the LPS-induced inflammatory response by placental tissues depending on the IL4Rα. The receptor activation of IL4 and IL13 leads to the transcription of the factors STAT6 and GATA3 which ultimately induces Th1 and Th2 responses.[123] IL4 and 13 also stimulate various cytokines and chemokines, such as IL1B, TNFα, IL8, and the macrophage inflammatory protein MIP1A.[123,124,125,126,127] One of the main functions of IL4 is to induce the differentiation of naïve CD4 positive T cells into Th2 cells.[84] Therefore, a decrease of IL4 receptor density can lead to an enhanced Th1 cell response and altered levels of Th1 related cytokines. Together, this may result in the rejection of the fetus and ultimately in pregnancy loss. The induction of IL4/13 leads to suppression of Th1 and enhancement of Th2 immunity.[123,128,129] The response to an increased pro-inflammatory environment is the counteraction of multiple anti-inflammatory molecules. Maintaining homeostasis within the immune system is essential for the outcome of pregnancy.

1.7 Overview of results and achievements

The study revealed differences in the expression of the immune cell markers PPARγ, IL4Rα and TLR4 in the decidua and the trophoblast cells within the placenta of spontaneous miscarriage, recurrent miscarriage, and the healthy control group. Furthermore, we aimed to show changes in the macrophage polarization in the abortive placenta tissue. As described in Paper 1, the expression of PPARγ in the placenta was evaluated by RT-PCR. We found a significant downregulation of PPARγ in spontaneous miscarriages ($p=0.010$) and recurrent miscarriages ($p=0.004$) compared to the control cases. The tissue was additionally examined by immunohistochemistry. With this method, we could show a significantly lower expression of PPARγ in the intermediate villous trophoblast (IVT) in the group of spontaneous ($p=0.001$) and recurrent ($p=0.01$) miscarriages than in the healthy placenta group. Immunohistochemical staining of CD68, a marker for macrophages, demonstrated that decidual macrophages were increased in recurrent ($p=0.181$) and significantly upregulated in spontaneous ($p=0.0013$) miscarriages compared to the control. Through the use of double immunofluorescence, we identified PPARγ expressing cells in the decidua basalis and CD68 positive macrophages. We found a very large CD68 positive macrophage population and co-expressing PPARγ cells in the samples of spontaneous miscarriages as well as the control cases. In contrast, in recurrent

miscarriages, we could demonstrate a nearly total absence of PPARγ in macrophages. We found that only 5-8% of macrophages were PPARγ-positive. Moreover, we characterized the small macrophage population in the recurrent miscarriage group with M1/M2 markers and CD68. The expression of the M1 marker iNOS is found in 2-5% of macrophages in the healthy placentas and in 90% of the recurrent miscarriage placentas. Another M1 marker, TLR2, is highly expressed in the normal decidua but without co-expression in macrophages. We concluded that 80-90% of the recurrent miscarriage placentas co-express TLR2 together with macrophages. Furthermore, we used the chemokine receptor 1 CX3CR1 as a M2 marker, which is broadly expressed (>95%) by the control decidual macrophages. In comparison, in the recurrent miscarriages, just 40-50% of macrophages presented a co-expression with CX3CR1. Finally, we used the chemokine CCL1, a marker for the subtype M2b of M2 polarized macrophages, to compare the macrophages in recurrent miscarriage placentas with the control placentas. In the control group, 40-50% of the macrophages presented a co-expression with CCL1. Whereas, in the recurrent miscarriage group we found only 5-10% of macrophages co-expressing CCL1.

In Paper 2, we examined the expression of receptors TLR4 and IL4Rα in the trophoblast and the decidua. Immunohistochemistry was used to analyze the samples. IL4Rα was proven to be significantly downregulated in the syncytiotrophoblast of recurrent ($p=0.001$) and spontaneous miscarriage placentas ($p=0.001$) compared to the healthy tissue. Additionally, in the decidual tissue a significant downregulation of IL4Rα was detected in the group of spontaneous miscarriages ($p=0.021$) in comparison to the control. The double immunofluorescence using IL4Rα and HLA-G was used to determine the expression of IL4Rα in the decidua of all three groups. In the healthy decidual tissue, both markers were found. In the miscarriage groups, no IL4Rα positive cells could be observed. Therefore, we discovered a stronger expression of IL4Rα positive cells in the healthy placenta than in the spontaneous and recurrent miscarriage group. On the mRNA level, we observed a significant downregulation of IL4Rα in recurrent miscarriages ($p=0.002$), whereas in spontaneous miscarriages there was no significant decrease in comparison to the control group.

When focusing on the trophoblast using immunohistochemistry, the intermediate villous trophoblast (IVT) presented a significantly lower expression of TLR4 in spontaneous miscarriages than in healthy placentas ($p=0.04$). TLR4 tends to be downregulated in the IVT of recurrent miscarriages ($p=0.389$) compared to the control. TLR4 expression showed no significant changes in the miscarriage groups either in the syncytiotrophoblast or in the cytotrophoblast. In decidua of abortive tissue, we recognized a significant difference in TLR4 between the healthy placenta and the spontaneous miscarriages ($p=0.003$). In the RT-PCR a significant downregulation of the mRNA expression of TLR4 in the group of recurrent miscarriages ($p=0.004$) compared to the

healthy tissue was found. However, we could not detect a significant downregulation of TLR4 in spontaneous miscarriage samples.

1.8 Conclusion: Immune regulation during and after pregnancy

The immune regulation is in a constant flow in human beings, especially before and during pregnancy. As mentioned earlier, it has been discovered that the macrophage population density in the spontaneous miscarriage changes when compared to habitual abortions and healthy placenta. We speculate that the immune answer might be over-regulating. Ultimately, this could be the reason why trophoblastic tissue is not accepted by the mother. In contrast, it is believed that in the case of recurrent miscarriages, the immune system has already experienced a certain immunological imbalance. Therefore, the immune cells in the habitual miscarriage placenta respond in a different way to the immunological changes. Especially in Paper 1, we demonstrated that PPARγ, a M2 macrophage marker, was diminished significantly in the placentas of recurrent miscarriages. The macrophages were examined further. Characterizing the status of polarization using the M1 markers TLR2 and iNOS and the M2 markers CCL1 and CX3CR1 proved the loss of M2 macrophages in the recurrent miscarriage placentas (Figure 1). Hence, we found that there was a shift towards the M1 polarization, which might support the rejection of the fetus. We assume that the inflammatory response is suppressed and modified in the recurrent miscarriage placentas.

During pregnancy, we discovered that the decidual macrophage population is altered significantly in the spontaneous and in the recurrent miscarriage group. In the latter group, we hypothesize that there are no elevated levels of macrophages due to a recurring process in a dysfunctional immune system. Studies showed that CD68 positive macrophages were increased significantly after pregnancy, but the number did not diverge between normal pregnancy, spontaneous or recurrent miscarriages. This indicates that decidual macrophages are recruited for the preparation and maintenance during pregnancy.[92] The population of decidual macrophages plays an important role as a key immunoregulator at the feto-maternal interface, depending on the local environmental actions, resulting in fetal antigen tolerance throughout gestation.

In Paper 2 we discovered that IL4Rα was found in the trophoblast of spontaneous, recurrent miscarriages and normal placentas. We could identify a significant downregulation of IL4Rα expression of the trophoblast within the syncytiotrophoblast in spontaneous and recurrent miscarriages compared to the healthy control. The invasion of the trophoblast is strictly regulated. Studies showed that the silencing of IL4Rα may affect this sensitive process. The decreased expression of IL4Rα was reported to stimulate trophoblast cells to invade.[130] We hypothesize that

a downregulated IL4Rα expression may be a form of recovery in order to promote the invasion of trophoblast cells and save them from apoptosis.

There is the growing belief that the kind of response originated by the placenta might define the immunologic response of the mother and consequently the pregnancy outcome. Multiple studies stated that the placenta and decidua embody important immunological components. They modify and affect the global response of the mother to protect the fetus from infections. The interaction of these complex cellular and molecular aspects need to be understood in order to make appropriate decisions related to treatment and prevention of severe pregnancy complications.[131] Further investigations focusing on the interplay of various immunological factors need to be performed in order to further elucidate this quite complex but common phenomenon.

1.9 Contribution to publications included in this thesis

Elisabeth Johanna Rogatsch (EJR) performed the experiments for the publications 1 and 2 and made substantial contributions to acquisition of all sample and the evaluation of data. EJR majorly contributed to the data analysis and completed the statistical work. EJR took the images included in the publications, drafted and wrote the material and methods parts of Paper 1 and Paper 2.

2. PUBLICATIONS INCLUDED IN THE THESIS

2.1.Publication 1

Titel:

PPARγ expression is diminished in macrophages of recurrent miscarriage placentas

Authors:

Theresa Maria Kolben, Elisabeth Rogatsch, Aurelia Vattai, Anna Hester, Christina Kuhn, Elisa Schmoeckel, Sven Mahner, Udo Jeschke and Thomas Kolben

Journal:

International Journal of Molecular Science 2018 26 June;19(7). doi:10.3390/ijms19071872

Abstract:

Background: PPARγ belongs to the group of nuclear receptors which is expressed in the trophoblast and together with other factors is responsible for the maintenance of pregnancy. Apart from that PPARγ is also a main factor for macrophage polarization. The aim of this study was to investigate the combined expression pattern and frequency of PPARγ under physiological circumstances and in spontaneous and recurrent miscarriages in the trophoblast and in maternal macrophages of the decidua.

Methods: Human placental tissues of the first trimester (15 physiologic pregnancies, 15 spontaneous abortion and 16 recurrent miscarriage placentas) were analyzed for expression of the nuclear receptor PPARγ. Expression changes were evaluated by immunohistochemistry and real time PCR (RT-PCR) in trophoblast and in maternal macrophages of the decidua. Maternal macrophages were identified by double immunofluorescence using cluster of differentiation 68 (CD68) as marker for macrophages and further characterized regarding their M1/M2 polarization status.

Results: The intermediate villous trophoblast revealed a significantly lower PPARγ expression in spontaneous and recurrent abortion. Maternal macrophages express PPARγ Their number is significantly enhanced in the decidua of spontaneous miscarriages whereas in recurrent miscarriages maternal macrophages seem to express PPARγ only in very few cases.

Conclusion: PPARγ is associated with an M2 polarization state that is common for decidual macrophages. The lack of PPARγ in recurrent miscarriage decidual macrophages seems to be associated with a specific inflammatory response against the fetus.

International Journal of
Molecular Sciences

MDPI

Article

PPARγ Expression Is Diminished in Macrophages of Recurrent Miscarriage Placentas

Theresa Maria Kolben [1], Elisabeth Rogatsch [1], Aurelia Vattai [1], Anna Hester [1], Christina Kuhn [1], Elisa Schmoeckel [2], Sven Mahner [1], Udo Jeschke [1,*] and Thomas Kolben [1]

[1] Department of Obstetrics and Gynecology, University Hospital, LMU Munich, Marchioninistr. 15, 81377 Munich, Germany; Theresa.kolben@med.uni-muenchen.de (T.M.K.); elisabeth.rogatsch@icloud.com (E.R.); Aurelia.vattai@med.uni-muenchen.de (A.V.); anna.hester@med.uni-muenchen.de (A.H.); Christina.kuhn@med.uni-muenchen.de (C.K.); sven.mahner@med.uni-muenchen.de (S.M.); Thomas.kolben@med.uni-muenchen.de (T.K.)

[2] Department of Pathology, LMU Munich, Marchioninistr. 27, 81377 Munich, Germany; elisa.schmoeckel@med.uni-muenchen.de

* Correspondence: udo.jeschke@med.uni-muenchen.de; Tel.: +49-89-4400-74775

Received: 23 May 2018; Accepted: 19 June 2018; Published: 26 June 2018

check for updates

Abstract: PPARγ belongs to the group of nuclear receptors which is expressed in the trophoblast and together with other factors is responsible for the maintenance of pregnancy. Apart from that PPARγ is also a main factor for macrophage polarization. The aim of this study was to investigate the combined expression pattern and frequency of PPARγ under physiological circumstances and in spontaneous and recurrent miscarriages in the trophoblast and in maternal macrophages of the decidua. Human placental tissues of the first trimester (15 physiologic pregnancies, 15 spontaneous abortion and 16 recurrent miscarriage placentas) were analyzed for expression of the nuclear receptor PPARγ. Expression changes were evaluated by immunohistochemistry and real time PCR (RT-PCR) in trophoblast and in maternal macrophages of the decidua. Maternal macrophages were identified by double immunofluorescence using cluster of differentiation 68 (CD68) as marker for macrophages and further characterized regarding their M1/M2 polarization status. The intermediate villous trophoblast revealed a significantly lower PPARγ expression in spontaneous and recurrent abortion. Maternal macrophages express PPARγ. Their number is significantly enhanced in the decidua of spontaneous miscarriages whereas in recurrent miscarriages maternal macrophages seem to express PPARγ only in very few cases. PPARγ is associated with an M2 polarization state that is common for decidual macrophages. The lack of PPARγ in recurrent miscarriage decidual macrophages seems to be associated with a specific inflammatory response against the fetus.

Keywords: PPARγ; first trimester placenta; decidual macrophages; miscarriage

1. Introduction

Miscarriage, which is defined as either spontaneous or recurrent, is a common disorder in pregnancy [1]. It affects 25–50% of all reproductive-aged women. Immunologic, endocrine and metabolic mechanisms are involved in the success of human pregnancy and disturbances in any of these processes can lead to fetal loss [2]. Established risk factors are fetal chromosomal or endocrine disorders for spontaneous miscarriages and the antiphospholipid syndrome, thrombophilia or maternal anatomical malformations, especially for recurrent pregnancy losses [3]. In nearly 50% of affected patients, however, the cause of miscarriage remains unknown [4].

Peroxisome proliferator-activated receptor γ (PPARγ) belongs to the family of nuclear receptors [5] that are key players in maintaining pregnancy [6,7]. PPARγ, together with its heterodimer binding

partner retinoid X receptor alpha (RXRα), are involved in cell proliferation, cell differentiation, and organogenesis [8]. RXRα is upregulated in extravillous trophoblast in recurrent miscarriages in humans [9]. RXRα plays a pivotal role in the receptor family, due to its ability to form heterodimers with other nuclear receptors. Heterodimer partners include, e.g., peroxisome proliferator-activated receptor (PPAR), thyroid hormone receptor (TR), and liver X receptor (LXR) [2,10–12]. Especially the expression of the isoform PPARγ is linked to trophoblast invasion [13] and downregulation of the isoform RXRα seems to protect from apoptosis in human trophoblasts [9].

Not only trophoblast cells express PPARγ, but also macrophages [14]. Macrophages play a key role in immune response and they can respond to environmental stimuli by acquiring specific phenotypes [15]. In response to external cues they will undergo classical M1 activation with high levels of inflammation and microbicide as well as anti-tumor activity. Alternatively, the M2 pathway contains mostly parasite containment, tissue remodeling and most importantly in this case immunomodulatory functions like pregnancy [16–18].

Our former studies showed that the number of decidual macrophages is increased at the feto-maternal interface of preeclampsia placentas [19] and also in spontaneous miscarriage cases. An additional finding was the FasL-positivity of these macrophages [20]. Therefore, the aim of this study was a phenotype characterization of macrophage populations in abortive placental tissue, its PPARγ expression and the characterization of PPARγ expressing trophoblast sub-types.

2. Results

2.1. Immunohistochemistry

2.1.1. PPARγ-Expression in the Trophoblast

The expression of PPARγ in the nucleus and cytoplasm of trophoblast cells was analyzed in tissue from healthy pregnancies (15 cases), spontaneous miscarriages (SM, 15 cases), and recurrent miscarriages (RM, 16 cases; Figure 1a–d). The intermediate villous trophoblast (IVT) revealed a significantly lower expression in the group with recurrent abortions (Figure 1b) than in the group with healthy placentas (Figure 1a, IRS 8 vs. 12, $p = 0.01$). There was a significant downregulation of PPARγ in the IVT of spontaneous abortions (Figure 1c, IRS 9 vs. 12, $p = 0.001$) compared to the control group. Briefly, the staining results are shown in the boxplot in Figure 1d.

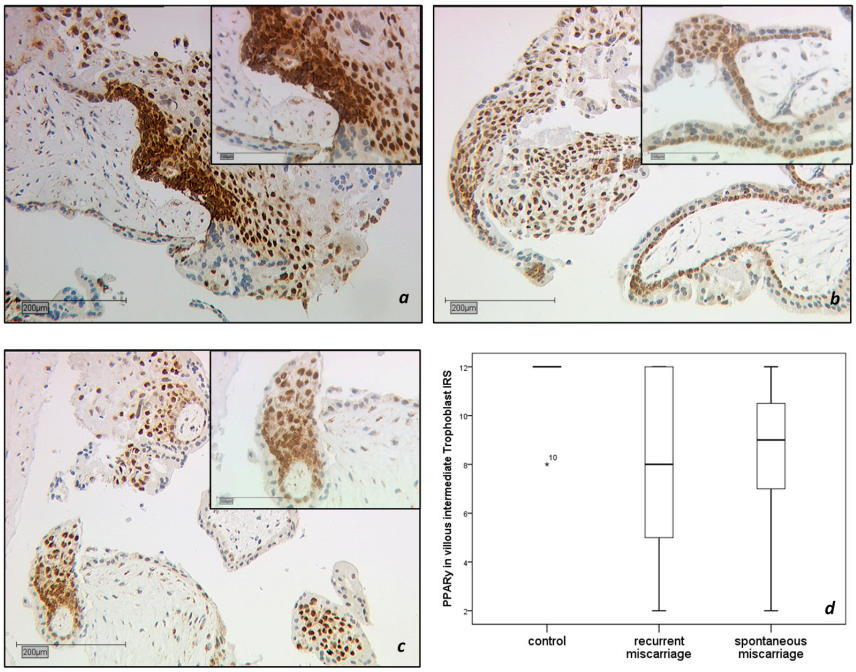


Figure 1. Immunohistochemical staining of Peroxisome proliferator-activated receptor gamma (PPARγ) in the villous trophoblast. PPARγ expression is found with high distribution and intensity in intermediate villous trophoblastic cells (IVT) of first-trimester placentas. The control group (15 cases) showed the strongest expression pattern (**a**). PPARγ was significantly downregulated ($p = 0.01$) in trophoblastic tissue of recurrent miscarriage (RM, 16 cases, (**b**). PPARγ expression in the IVT of spontaneous miscarriage tissue (SM, 15 cases), (**c**) was significantly decreased compared to the control ($p = 0.001$). The boxplot summarizes the statistical data of the immunohistochemical staining results (**d**). Scale is 200 µm. The insert picture is 100 µm scaled.

2.1.2. CD68 Positive Decidual Macrophages in the Decidua

CD68 positive macrophages were investigated in the placenta of healthy pregnancies (15 cases), SM (15 cases), and RM (16 cases; Figure 2a–d). The number of CD68 positive macrophages was low in the decidua basalis of control specimens (Figure 2a). The macrophages were slightly increased in decidua basalis RM samples, but without statistical significance ($p = 0.181$) (Figure 2b; median number of macrophages = 21 vs. 16). Decidual macrophages were significantly increased in the decidua basalis SM group ($p = 0.013$) (Figure 2c; median number of macrophages 32 vs. 16). A summary of the staining results is shown in Figure 2d.

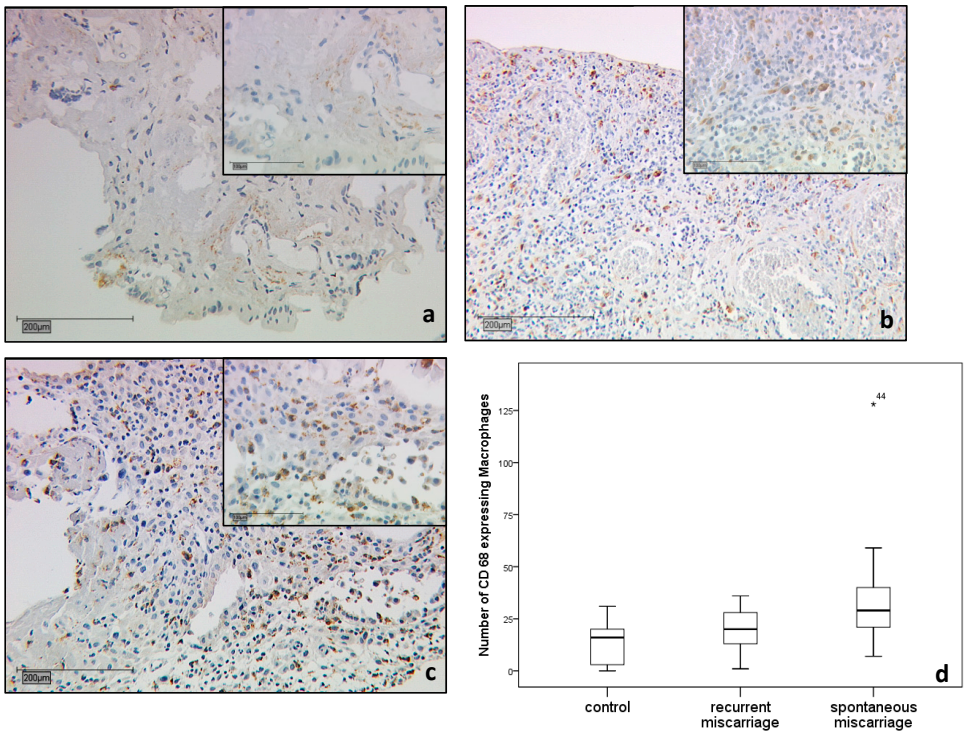


Figure 2. Immunohistochemical staining of decidual macrophages with CD68 as a marker for macrophage positivity. Decidual macrophages were increased in RM (16 cases) and SM samples (15 cases) compared to the control group (15 cases), (**a**). In recurrent miscarriage specimens, the decidual macrophages tended to be upregulated (p = 0.181) (**b**). In spontaneous miscarriage samples, the population of macrophages was significantly higher compared to the control (p = 0.013) (**c**). Summary of staining results of CD68 positive decidual macrophages (**d**). Scale is 200 µm. The insert picture is 100 µm scaled.

2.2. Double Immunofluorescence

Identification of PPARγ-Expressing Cells in the Decidua Basalis

Decidua basalis tissue of regular first trimester pregnancies (15 cases), SM (15 cases) and RM (16 cases) was double stained using antibodies against PPARγ (green staining), and CD68 (red staining). Nuclear staining appeared in blue. PPARγ + CD68 double immunofluorescence staining was performed to investigate the macrophage expression of PPARγ in RM, SM and control groups. CD68 staining in the cytoplasm of normal decidual cells is shown in Figure 3a. Figure 3b presents the cytoplasmic staining of PPARγ positive cells from the same area. The depiction of CD68 and PPARγ is represented as a co-expression by triple filter excitation in Figure 3c. Both markers are ubiquitously expressed in the healthy placenta (Figure 3a–c). A large number of CD68-positive macrophages was observed in RM samples (Figure 3d), although with almost no PPARγ expression (Figure 3e). Triple filter excitation demonstrates (Figure 3f) a near absence of PPARγ in macrophages of recurrent miscarriages. A large population of CD68 positive macrophages (Figure 3g) and PPARγ expressing cells (Figure 3h) was detected in SM samples. Figure 3i shows a strong co-expression of both markers. We identified CD68 positive macrophages also expressing PPARγ in the healthy

and in the spontaneous miscarriage placenta. In the group of recurrent miscarriages only very few PPARγ-expressing macrophages (5–8% of the macrophages in RM are PPARγ-positive) were detected.

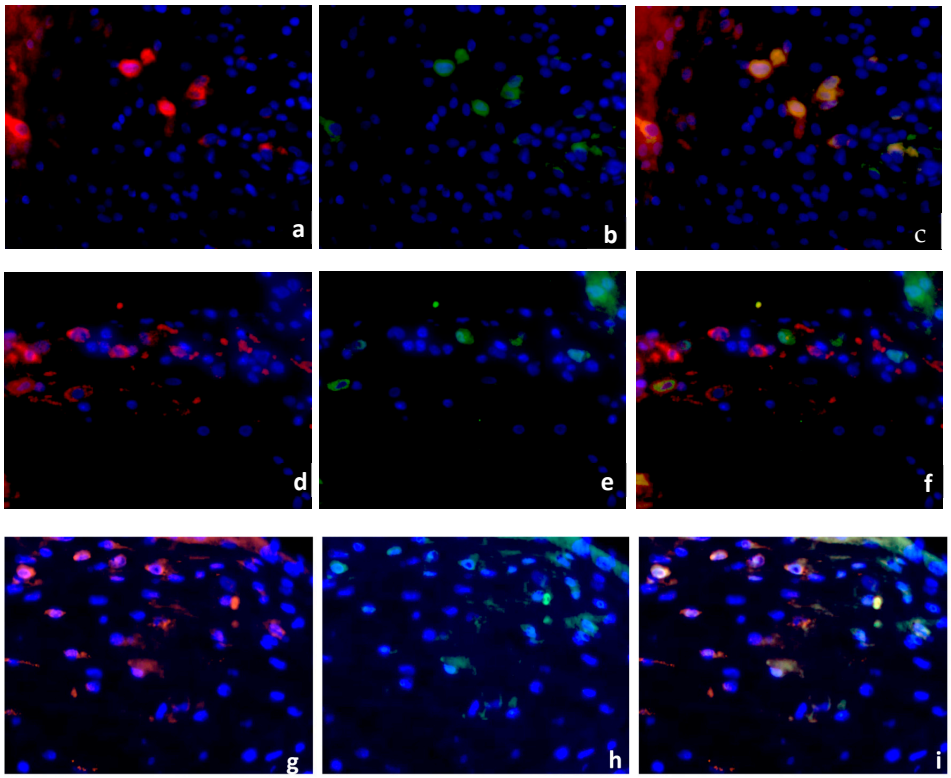


Figure 3. Double Immunofluorescence of CD68 & PPARγ. CD68 (red staining), PPARγ (green staining), nuclear staining (blue). CD68 (**a**) and PPARγ (**b**) are expressed in the decidua of healthy placenta (15 cases), co-expression presented as triple filter yellow (**c**). High distribution of CD68 positive macrophages was found in RM (16 cases), (**d**). PPARγ-positive cells are shown in (**e**). Triple filter excitation showed an absence of PPARγ positive macrophages (**f**). Both markers (CD68, (**g**) and PPARγ, (**h**)) are co-expressed in the group of SM (15 cases), (**i**). All pictures are 40× lens.

2.3. Characterization of the Macrophage Population in Recurrent Miscarriage Cases

Because macrophages in RM cases (16 cases) expressed PPARγ only in 5–8% of the total macrophage population compared to healthy control tissue (15 cases) and SM (15 cases), we further characterized these cells with a panel of M1/M2 markers and CD68. The M1 marker iNOS is expressed in 2–5% of the macrophages in healthy controls (CD 68 Figure 4a; iNOS Figure 4b, triple filter excitation Figure 4c). In RM cases, iNOS is expressed in 90% of the macrophages (CD68 Figure 4d, iNOS Figure 4e, triple filter excitation Figure 4f). TLR2 is intensely expressed in the healthy decidua but showed no co-expression with macrophages (CD 68 Figure 5a, TLR2 Figure 5b, triple filter excitation Figure 5c). In RM cases, TLR2 as M1 marker is co-expressed with macrophages in 80–90% of the cases (CD68 Figure 5d, TLR2 Figure 5e, triple filter excitation Figure 5f). The chemokine CCL1 is a marker for M2b polarized macrophages. In healthy controls, 40–50% of the macrophages showed co-expression with CCL1 (CD68 Figure 6a, CCL1 Figure 6b, triple filter excitation Figure 6c). In RM cases, only 5–10% of the macrophages showed co-expression with CCL1 (CD68 Figure 6d, CCL1 Figure 6e, triple filter excitation Figure 6f). CX3C chemokine receptor 1 (CX3CR1) as a M2 macrophage marker is widely

expressed (>95%) on healthy control decidual macrophages (CD68 Figure 7a, CX3CR1 Figure 7b, triple filter excitation Figure 7c). In RM cases, only 40–50% of the macrophages showed co-expression with C X3CR1 (CD68 Figure 7d, CX3CR1 Figure 7e, triple filter excitation Figure 7f).

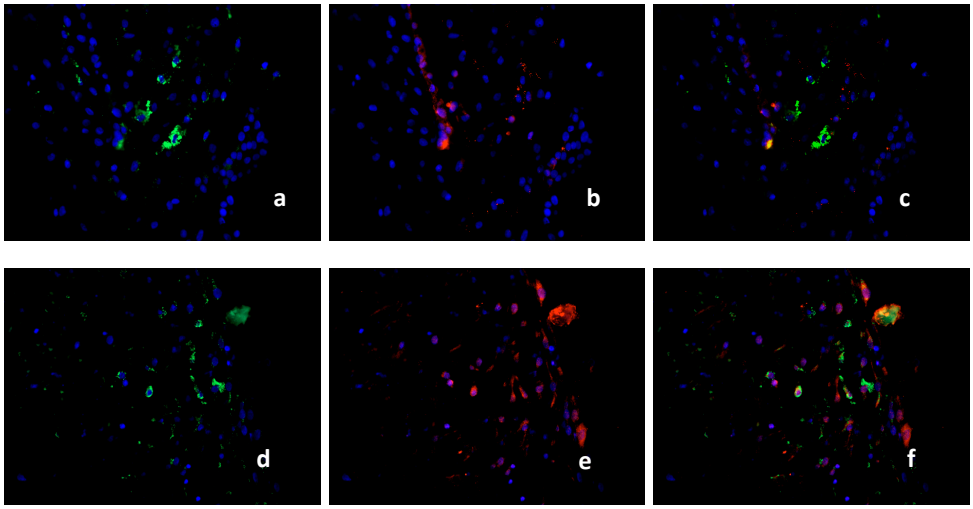


Figure 4. Double Immunofluorescence of CD68 and iNOS. CD68 (green staining), iNOS (red staining), nuclear staining (blue). CD68 (**a**) and iNOS (**b**) are co-expressed only in few macrophages in the decidua of healthy placenta, presented as triple filter yellow (**c**). High distribution of CD68 positive macrophages was found in RM (**d**). iNOS positive cells are shown in (**e**). Triple filter excitation shows a co-expression of iNOS and macrophages (**f**). All pictures are 40× lens.

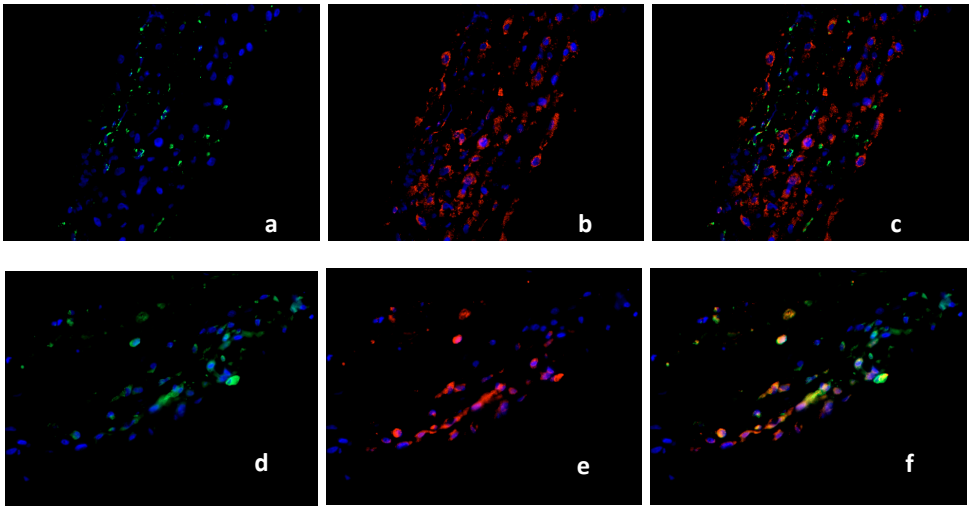


Figure 5. Double Immunofluorescence of CD68 and TLR2. CD68 (green staining), TLR2 showed strong expression in the healthy decidua (red staining), nuclear staining (blue). CD68 (**a**) and TLR2 (**b**) are not co-expressed in the decidua of healthy placenta, presented as triple filter yellow (**c**). CD68 positive macrophages in RM placenta (**d**). TLR2 positive cells are shown in (**e**). Triple filter excitation shows a co-expression of TLR2 and macrophages (**f**) in the RM placenta. All pictures are 40× lens.

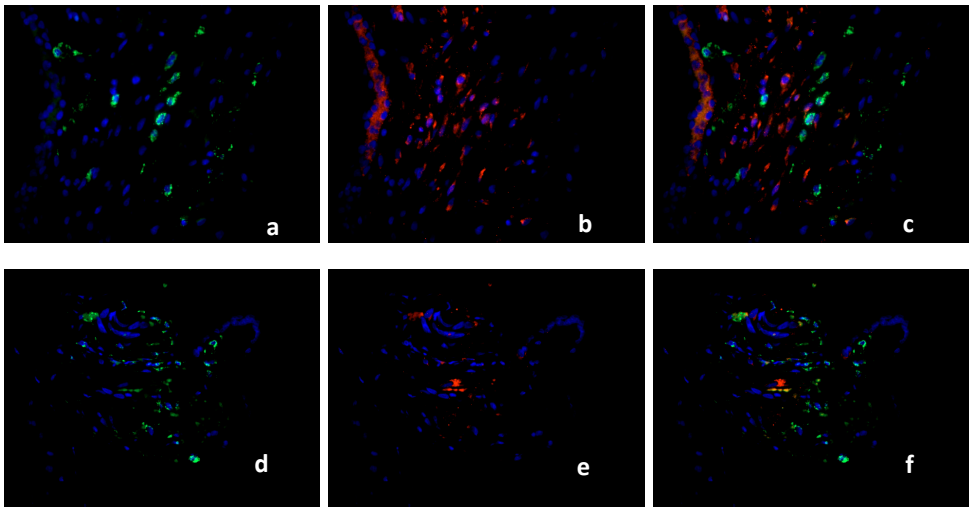


Figure 6. Double Immunofluorescence of CD68 and CCL1. CD68 (green staining), CCL1 showed intense expression in the healthy decidua (red staining), nuclear staining (blue). CD68 (**a**) and CCL1 (**b**) are co-expressed in the decidua of healthy placenta in a number of cells, presented as triple filter yellow (**c**). CD68 positive macrophages in RM placenta (**d**). Only few CCL1 positive cells are shown in (**e**). Triple filter excitation shows a diminished co-expression of CCL1 and macrophages (**f**) in the RM placenta. All pictures are 40× lens.

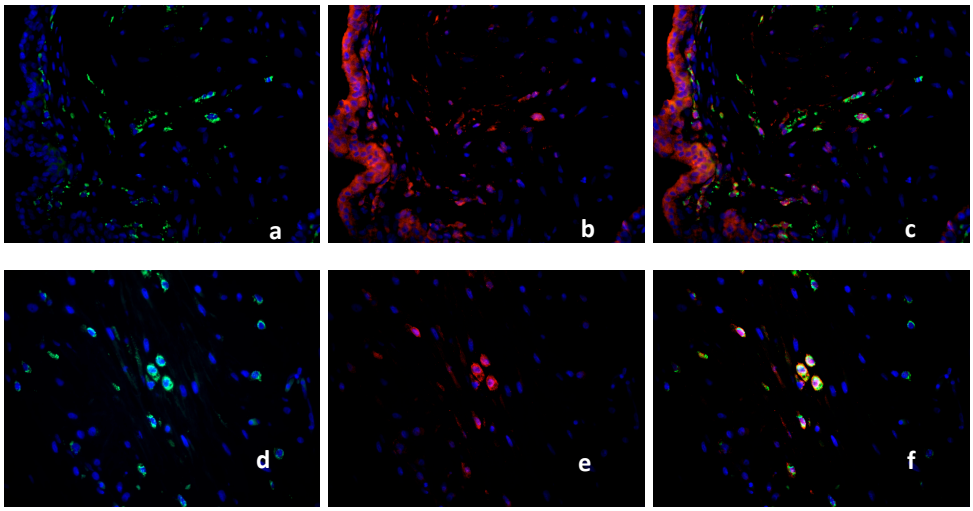


Figure 7. Double Immunofluorescence of CD68 and CX3CR1. CD68 (green staining), CX3CR1 showed expression in a variety of different cell types including endometrial glands and decidual stromal cells [21] in the healthy decidua (red staining), nuclear staining (blue). CD68 (**a**) and CX3CR1 (**b**) are co-expressed in the decidua of healthy placenta in almost all CD 68-positive cells (>95%), presented as triple filter yellow (**c**). CD68 positive macrophages in RM placenta (**d**). Only 40–50% CX3CR1 expressing cells (**e**) showed co-expression with CD68, as shown in (**f**). All pictures are 40× lens.

2.4. Evaluation of PPARγ Expression with Real-Time RT-PCR (TaqMan)

PPARγ mRNA (PPARG) expression was analyzed in placental tissue from SM, RM and healthy controls by quantitative RT-PCR. PPARγ was significantly downregulated in SM (15 cases, 1.8-fold; $p = 0.010$) and in RM (16 cases, 1.5-fold; $p = 0.004$) compared to the control group (15 cases, Figure 8).

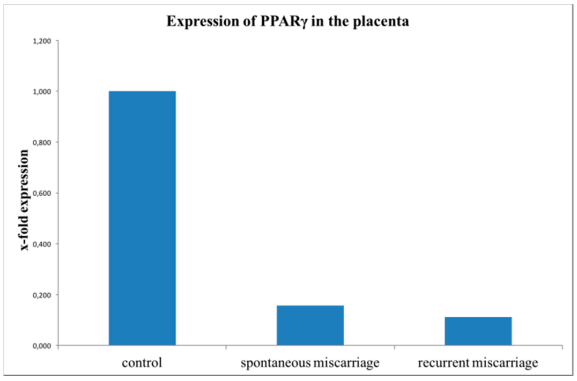


Figure 8. Results of PPARγ mRNA expression analysis with TaqMan RT-PCR from trophoblastic tissue. PPARγ mRNA expression was significantly downregulated in the miscarriage groups (SM, 15 cases, $p = 0.01$) and RM, 16 cases, $p = 0.004$)) compared to the healthy controls (15 cases). This bar graph shows the mean of relative PPARγ expression; therefore, the presentation of error bars is not appropriate.

3. Discussion

Within this study we could show that PPARγ is downregulated in the intermediate villous trophoblast (IVT) in both spontaneous (SM) and recurrent miscarriage (RM) placentas. The downregulation of PPARγ was confirmed by RT-PCR in both miscarriage pregnancy cases.

In addition, we showed that in recurrent miscarriages, decidua basalis macrophages are nearly PPARγ-negative, whereas in normal controls and surprisingly also in SM decidua basalis macrophages are all PPARγ-positive. The additional characterization of the macrophage polarization status using the M1 polarization markers TLR2 and iNOS [22] and the M2 polarization markers CCL1 and CX3CR1 confirmed the loss of M2 polarized marcrophages [23] in recurrent miscarriages.

PPARγ as nuclear receptor is already known to be essential for the maturation of alternatively activated M2 macrophages [24]. M2 macrophages and decidual macrophages have mainly immune regulatory and homeostatic properties [25]. These macrophages have little in common with pro-inflammatory M1 macrophages, which is in line with the role for decidual macrophages in establishing and sustaining fetal tolerance [26].

In addition, rosiglitazone as a selective peroxisome PPARγ agonist has been shown to induce an M2 macrophage polarization via activating the PPARγ pathway [27,28]. The activation of PPARγ suppresses gene transcription by interfering with signal transduction pathways, such as the nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB), Signal transducer and activator of transcription (STAT), and Activator protein 1 (AP-1) pathways that are involved in pro-inflammatory immune responses [29–31]. It is a striking result of this study, that we could identify the loss of PPARγ in decidual macrophages of patients with recurrent miscarriages but not in patients with SM and of course in normal control placentas.

In spontaneous miscarriages, we identified a significant increase of decidual macrophages, although they were PPARγ positive. In former studies, we could show that these macrophages express FasL [20]. The expression of FasL on decidual macrophages had been already described before [32].

The role of the Fas/FasL system in the conditions of spontaneous abortion and pregnancy had been described for T cell apoptosis by decidual and trophoblast cells earlier [33,34]. Our group was able to describe an increased expression of FasL in decidual macrophages of spontaneous miscarriages [20]. Therefore, we speculated that FasL expression by macrophages could be a part of an M2-like polarization. FasL-expressing macrophages could induce apoptosis to Fas-bearing activated T-cells reducing potentially harmful immune responses against the semi-allogenic embryo. We further assumed that macrophages might mediate the T-cell triggered trophoblast apoptosis highlighting an alternative way of inducing apoptosis in SM [15].

Placental growth is exponential in the first trimester of pregnancy and involves coordinated events in trophoblast and mesenchyme, one of these is the differentiation of progenitor cytotrophoblast cells into intermediate villous trophoblast cells (IVT). These IVT are programmed to either fuse with the syncytium, or are transferred to extravillous trophoblast cells [35]. We found a downregulation of PPARγ in the IVT compartment in both spontaneous and recurrent miscarriage on protein as well as on mRNA-level. Already decades ago, the natural binding partner of PPARγ, the RXRα was described in this trophoblast compartment: RAR and RXR, both types of receptors were present in the proliferative intermediate villous trophoblast [36]. Later, RXRα was found to play a crucial role in pregnancy and is a key regulator of apoptosis in trophoblasts of patients with recurrent miscarriages [9]. PPARγ, on the other hand, was also described to be dysregulated in different trophoblast compartments of the miscarriage placenta [37,38], although PPARγ expression in the IVT was never investigated before. Interestingly, Fournier et al. described that activation of PPARγ induces accumulation of lipids, villous trophoblast differentiation and inhibits trophoblast invasiveness [39]. In addition, the expression of PPARγ is downregulated by stimulation of trophoblast cells with either arachidonic acid or 15d-PGJ2 [40]. Because we identified a downregulation of PPARγ in the IVT of miscarriages and a missing PPARγ expression in macrophages of RM cases, we might speculate that PPARγ ligands (e.g., prostaglandins) are released to a higher extent under these pathological circumstances.

4. Materials and Methods

4.1. Patient Data

The Institutional Review Board of the Ludwig-Maximilian-University, Munich, (Number of approval: 337-06, 29 December 2006) approved this study. All women signed an informed consent allowing analysis of all clinical and laboratory data mentioned in this study. Placental tissue from spontaneous miscarriages (SM) (n = 15) and recurrent miscarriages (RM) (n = 16) at gestational weeks 4 to 13 was obtained at the Department of Obstetrics and Gynecology, LMU Munich. Placental tissue from legal terminations of healthy pregnancies (n = 15) served as control group. The tissue was collected at a private practice clinic in Munich, Germany. The control group specimens were confirmed as healthy by a blinded independent pathologist. All placental material was acquired by dilatation and curettage, without any prior pharmaceutical induction. In cases of SM and RM, the operation was performed within 24 h after diagnosis. Instantly, after the uterine curettage, the obtained tissue was either frozen or formalin fixed for further analysis. All patients included had an inconspicuous family and medical history, which was obtained systematically. Patients with common disorders, autoimmune diseases, thrombophilia and microbiological infections (Bacteria and Chlamydia trachomatis) were excluded. Chromosomal abnormalities were ruled out by karyotype analysis in all samples, as described recently [11,41]. Table 1 summarizes the number of samples used for immunohistochemical staining for each gestational week. Table 2 shows the demographic and clinical characteristics of the study population.

Table 1. Number of slides used for immunohistochemical staining for each gestational week.

Gestational Age	Normal Pregnancy	Spontaneous Miscarriage	Recurrent Miscarriage
4th week	0	0	1
7th week	2	1	3
8th week	4	5	5
9th week	2	2	4
10th week	3	3	0
11th week	0	3	2
12th week	3	1	1
13th week	1	0	0
	n = 15	n = 15	n = 16

Table 2. Demographic and clinical characteristics of the study population.

Characteristics *	Normal Pregnancy n = 15	Spontaneous Miscarriage n= 15	Recurrent Miscarriage n= 16	p Value (Kruskal Wallis Test)
Maternal age (years)	31.18 ± 8.06 (18.7−43.3)	37.8 ± 4.51 (29.2–43.2)	35.76 ± 4.8 (29.5–46.9)	0.049
Gestational age (weeks)	9.53 ± 1.95 (7–13)	8.4 ± 1.89 (7–12)	9.3 ± 1.49 (4–12)	0.276
Gravidity	4 ± 1.8 (1–7)	1.6 ± 0.9 (1–4)	3.1 ± 1.1 (2–5)	0.001
Parity	2 ± 1.1 (0–4)	0.9 ± 0.8 (0–2)	0.3 ± 0.6 (0–2)	0.003

Values are Mean ± S.D. * Mean, standard deviation, range.

4.2. Immunohistochemistry

Formalin-fixed tissue slides were embedded in paraffin wax for immunohistochemistry. Samples were deparaffinized in xylol for 20 min and rinsed in 100% ethanol. Methanol/H_2O_2 incubation for 20 min was performed to inhibit endogenous peroxidase reaction. Afterwards, the specimens were rehydrated in deescalating alcohol gradients, starting with 100% ethanol and ending with distilled water. The samples were cooked in a pressure pot, containing a sodium citrate buffer (pH = 6.0), which consisted of 0.1 mM citric acid and 0.1 mM sodium citrate in distilled water. Subsequently, samples were washed in PBS twice and incubated with a blocking solution (reagent 1, ZytoChem Plus HRP Polymer System (Mouse/Rabbit), Zytomed, Berlin, Germany) for 5 min. Incubation with the primary antibody was performed with each section for 16 h at 4 °C. All antibodies used are listed in Table 3. Following every subsequent step, samples were washed twice in PBS (pH = 7.4). Blocking solutions, containing post block (reagent 2) for 20 min and HRP-Polymer (reagent 3) for 30 min, were applied. The chromogen-substrate staining was carried out using the Liquid DAB+ Substrate Chromogen System (Dako Scientific, Glostrup, Denmark), 1 min for CD68 and 2 min for PPARγ. The reaction was stopped by applying distilled water. Finally, tissue samples were counterstained with Hemalaun for 2 min and blued in tap water. Specimens were dehydrated in an ascending alcohol gradient and cover slipped with Eukitt® quick hardening mounting medium (Sigma Aldrich, St. Louis, MO, USA). Positive control (human colon tissue) as well as negative control staining was carried out as described previously [10,40]. All slides were analyzed using the microscope Leitz Wetzlar (Wetzlar, Germany; Type 307-148.001 514686). The immunoreactive score (IRS) was used for evaluation of the intensity and distribution pattern of antigen expression. This semi-quantitative score is calculated as follows: the optical staining intensity (grades: 0 = none, 1 = weak, 2 = moderate, 3 = strong staining) is multiplied by the total percentage of positively stained cells (0 = none, 1 $\leq$ 10%, 2 = 11–50%, 3 = 51–80% and 4 $\geq$ 81% of the cells). This multiplication has a minimum of 0 and a maximum of 12. Analysis of all slides was performed independently by two experienced staff members. Total number of macrophages

in a magnification field of 40× lens was calculated three times each in 3 different areas of the decidua basalis. The median number was calculated.

Table 3. Antibodies used for immunohistochemical characterization and double immunofluorescence of placental tissue samples.

Antibody	Isotype	Clone	Dilution	Source
PPARγ [a,b]	rabbit IgG	polyclonal	1:500 in PBS [a] 1:500 in Dako [b]	Abcam Serotec, Cambridge, UK DAKO (S322); Carpenteira, CA, USA
CD 68 [a,b]	mouse IgG1	monoclonal	1:8000 in PBS [a] 1:8000 in Dako [b]	Sigma Aldrich (CL1346), St. Louis, MO, USA DAKO (S322); Carpenteira, CA, USA
iNOS [b]	Rabbit IgG	polyclonal	1:3000 in Dako	Thermo Scientific, (NPA3-030A) DAKO (S322); Carpenteira, CA, USA
TLR2 [b]	Rabbit IgG	polyclonal	1:750 in Dako	Sigma Aldrich, St. Louis, MO, USA Dako (S322); Carpenteira, CA, USA
CCL1 [b]	Rabbit IgG	polyclonal	1: 50 in Dako	Sigma Aldrich, St. Louis, , MO, USA Dako (S322); Carpenteira, CA, USA
CX3CR1 [b]	Rabbit IgG	polyclonal	1: 400 in Dako	Abcam Serotec, Cambridge, UK Dako (S322); Carpenteira, CA, USA
Cy-2 or -3 [b]	goat IgG anti-mouse	polyclonal	1:500 [b]	Dianova, Hamburg, Germany
Cy-2 or -3 [b]	goat IgG anti-rabbit	polyclonal	1:100 [b]	Dianova, Hamburg, Germany

[a] antibodies used for immunohistochemistry, [b] antibodies used for immunofluorescence.

4.3. Immunofluorescence

4.3.1. Evaluation of PPARγ-Expressing Cells as Macrophages

For the visualization of PPARγ-expressing cells in the trophoblast, tissue samples of SM, RM, both first-trimester abortion placentas, and healthy controls (first trimester) were used. The antibodies used are shown in Table 3. Double immunofluorescence staining for PPARγ and CD68 as a specific macrophage marker was performed to identify expression patterns in the nucleus and the cytoplasm.

4.3.2. Evaluation of M1/M2 Marker on Decidual Macrophages

In order to further characterize the macrophage polarization state, TLR2 and iNOS, as well as CCL1 and CX3CR1, were used for M1 and M2 polarization, respectively. Each specimen was incubated overnight at 4 °C with monoclonal anti-CD68 mouse IgG1 and one of the polyclonal IgG antibodies against PPARγ, TLR2, iNOS, CCL1, or CX3CR1. Polyclonal Cy-2- and polyclonal Cy-3-conjugated antibodies (Dianova, Hamburg, Germany) were used as secondary antibodies. Incubation was performed for 30 min at room temperature. Samples were fixed with Vectashield® mounting medium with DAPI (Vector Laboratories; Burlingame, CA, USA) and analyzed with the Axioskop fluorescent photomicroscope (Zeiss; Oberkochen, Germany). Images were taken with the Axiocam camera system (Zeiss CF20DXC).

4.4. Evaluation of PPARγ with Real-Time RT-PCR (Taq Man)

4.4.1. RNA Extraction from Placental Tissue

mRNA extraction was accomplished using the placental tissue of 15 women with SM, 16 women with RM and 15 healthy controls from the 7th to the 12th week of gestation. RNA extraction using 10 mg tissue of each sample was accomplished with RNeasy® Lipid Tissue Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

4.4.2. Reverse Transcription

According to the protocol reverse transcription (RT) was carried out with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, Fisher Scientific Company, Waltham, MA, USA) and placed in a mastercycler® gradient (Eppendorf, Hamburg, Germany). RT conditions were as follows: 10 min at 25 °C, 2 h at 37 °C, 5 min at 85 °C and continued by a hold step at −20 °C.

4.4.3. Real-Time Reverse Transcription PCR

After conversion of RNA to cDNA, PCR was performed on all samples individually. Real-Time Reverse Transcription PCRs were covered with optical caps in optical 96-well (Applied Biosystems™, Fisher Scientific Company, Waltham, MA, USA) reaction microtiter plates. Each reaction was accomplished with a volume of 20 µL, including 1 µL cDNA, 8 µL H_2O (DEPC treated DI water; Sigma, Taufkirchen, Germany) and 10 µL TaqMan® Fast Universal PCR Master Mix 2× (Applied Biosystems, Nr. 4367846; 50 mL). The total contained 1 µL TaqMan® Gene Expression Assay 20× (HS01115513_m1 for PPARγ, Applied Biosystems). The temperature protocol was as follows: 20 s at 95 °C, 40 cycles of amplification, denaturation for 3 s at 95 °C and denaturation plus annealing process for 30 s at 60 °C. Processing the PCR assays was performed using the 7500 Fast Real-Time PCR System (Applied Biosystems), and quantification was accomplished by the $2^{-\Delta\Delta Ct}$ method using β-actin as housekeeping gene (Applied Biosystems, Hs_99999903_m1).

4.5. Statistics

Analysis of the collection and statistical data was processed with the SPSS software version 24 (SPSS, Chicago, IL, USA) and Excel version 12.3.1 (Microsoft Windows 2016; Redmond, WA, USA). The Mann-Whitney U signed-rank test was used for the comparison of two independent groups. p-values < 0.05 were considered to be statistically significant.

5. Conclusions

Mouse knockout models showed that PPARγ is essential for placentation. PPARγ depletion leads to fetal loss in early pregnancy due to the missing PPARγ expression and extended placental defects [42]. In addition, decidual M1-like macrophage polarization events are associated with PPARγ modulation strategies [14]. Therefore, the PPARγ pathway is a new molecular target for future preventive strategies for the treatment of spontaneous and recurrent miscarriages.

Author Contributions: Conceptualization, T.M.K., T.K. and U.J.; Methodology, C.K.; Software, A.H.; Validation, A.V., C.K. and U.J.; Formal Analysis, C.K.; Investigation, E.R.; Resources, S.M.; Data Curation, E.S.; Writing-Original Draft Preparation, E.R.; Writing-Review & Editing, U.J.; Visualization, E.R.; Supervision, U.J.; Project Administration, T.K.; Funding Acquisition, S.M.

Acknowledgments: We thank Simone Hofmann for technical support.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

MDPI	Multidisciplinary Digital Publishing Institute
PPAR	Peroxisome-Proliferator-activated Receptor
SM	Spontaneous miscarriage
RM	Recurrent miscarriage

References

1. Makrigiannakis, A.; Petsas, G.; Toth, B.; Relakis, K.; Jeschke, U. Recent advances in understanding immunology of reproductive failure. *J. Reprod. Immunol.* **2011**, *90*, 96–104. [CrossRef] [PubMed]
2. Knabl, J.; Vattai, A.; Huttenbrenner, R.; Hutter, S.; Karsten, M.; Jeschke, U. Rxralpha is upregulated in first trimester endometrial glands of spontaneous abortions unlike LXR and PPARγ. *Eur. J. Histochem.* **2016**, *60*, 2665. [CrossRef] [PubMed]
3. Toth, B.; Jeschke, U.; Rogenhofer, N.; Scholz, C.; Wurfel, W.; Thaler, C.J.; Makrigiannakis, A. Recurrent miscarriage: Current concepts in diagnosis and treatment. *J. Reprod. Immunol.* **2010**, *85*, 25–32. [CrossRef] [PubMed]
4. Arck, P.C.; Rucke, M.; Rose, M.; Szekeres-Bartho, J.; Douglas, A.J.; Pritsch, M.; Blois, S.M.; Pincus, M.K.; Barenstrauch, N.; Dudenhausen, J.W.; et al. Early risk factors for miscarriage: A prospective cohort study in pregnant women. *Reprod. Biomed. Online* **2008**, *17*, 101–113. [CrossRef]
5. Pestka, A.; Fitzgerald, J.S.; Toth, B.; Markert, U.R.; Jeschke, U. Nuclear hormone receptors and female reproduction. *Curr. Mol. Med.* **2013**, *13*, 1066–1078. [CrossRef] [PubMed]
6. Guibourdenche, J.; Alsat, E.; Soncin, F.; Rochette-Egly, C.; Evain-Brion, D. Retinoid receptors expression in human term placenta: Involvement of rxr alpha in retinoid induced-hcg secretion. *J. Clin. Endoc. Metab.* **1998**, *83*, 1384–1387.
7. Toth, B.; Hornung, D.; Scholz, C.; Djalali, S.; Friese, K.; Jeschke, U. Peroxisome proliferator-activated receptors: New players in the field of reproduction. *Am. J. Reprod. Immunol.* **2007**, *58*, 289–310. [CrossRef] [PubMed]
8. Arck, P.; Toth, B.; Pestka, A.; Jeschke, U. Nuclear receptors of the peroxisome proliferator-activated receptor (PPAR) family in gestational diabetes: From animal models to clinical trials. *Biol. Reprod.* **2010**, *83*, 168–176. [CrossRef] [PubMed]
9. Pestka, A.; Toth, B.; Kuhn, C.; Hofmann, S.; Wiest, I.; Wypior, G.; Friese, K.; Jeschke, U. Retinoid x receptor alpha and retinoids are key regulators in apoptosis of trophoblasts of patients with recurrent miscarriages. *J. Mol. Endocrinol.* **2011**, *47*, 145–156. [CrossRef] [PubMed]
10. Knabl, J.; Pestka, A.; Huttenbrenner, R.; Plosch, T.; Ensenauer, R.; Welbergen, L.; Hutter, S.; Gunthner-Biller, M.; Jeschke, U. The liver x receptor in correlation with other nuclear receptors in spontaneous and recurrent abortions. *PPAR Res.* **2013**, *2013*, 575604. [CrossRef] [PubMed]
11. Vattai, A.; Ziegelmuller, B.; Kost, B.; Kuhn, C.; Hofmann, S.; Bayer, B.; Anslinger, K.; Jeschke, U.; Ditsch, N. The expression of thyroid hormone receptors (THR) is regulated by the progesterone receptor system in first trimester placental tissue and in bewo cells in vitro. *Eur. J. Obstet. Gynecol. Reprod. Biol.* **2015**, *195*, 31–39. [CrossRef] [PubMed]
12. Chen, Y.; Moradin, A.; Schlessinger, D.; Nagaraja, R. RXRα and LXR activate two promoters in placenta- and tumor-specific expression of plac1. *Placenta* **2011**, *32*, 877–884. [CrossRef] [PubMed]
13. Hutter, S.; Knabl, J.; Andergassen, U.; Jeschke, U. The role of ppars in placental immunology: A systematic review of the literature. *PPAR Res.* **2013**, *2013*, 970276. [CrossRef] [PubMed]
14. Xu, Y.; Romero, R.; Miller, D.; Kadam, L.; Mial, T.N.; Plazyo, O.; Garcia-Flores, V.; Hassan, S.S.; Xu, Z.; Tarca, A.L.; et al. An m1-like macrophage polarization in decidual tissue during spontaneous preterm labor that is attenuated by rosiglitazone treatment. *J. Immunol.* **2016**, *196*, 2476–2491. [CrossRef] [PubMed]
15. Hutter, S.; Heublein, S.; Knabl, J.; Andergassen, U.; Vrekoussis, T.; Makrigiannakis, A.; Friese, K.; Mayr, D.; Jeschke, U. Macrophages: Are they involved in endometriosis, abortion and preeclampsia and how? *J. Nippon Med. Sch.* **2013**, *80*, 97–103. [CrossRef] [PubMed]
16. Slukvin, I.I.; Breburda, E.E.; Golos, T.G. Dynamic changes in primate endometrial leukocyte populations: Differential distribution of macrophages and natural killer cells at the rhesus monkey implantation site and in early pregnancy. *Placenta* **2004**, *25*, 297–307. [CrossRef] [PubMed]
17. Heublein, S.; Vrekoussis, T.; Kuhn, C.; Friese, K.; Makrigiannakis, A.; Mayr, D.; Lenhard, M.; Jeschke, U. Inducers of g-protein coupled estrogen receptor (GPER) in endometriosis: Potential implications for macrophages and follicle maturation. *J. Reprod. Immunol.* **2013**, *97*, 95–103. [CrossRef] [PubMed]
18. Stremmel, C.; Schuchert, R.; Wagner, F.; Thaler, R.; Weinberger, T.; Pick, R.; Mass, E.; Ishikawa-Ankerhold, H.C.; Margraf, A.; Hutter, S.; et al. Yolk sac macrophage progenitors traffic to the embryo during defined stages of development. *Nat. Commun.* **2018**, *9*, 75. [CrossRef] [PubMed]

19. Petsas, G.; Jeschke, U.; Richter, D.U.; Minas, V.; Hammer, A.; Kalantaridou, S.; Toth, B.; Tsatsanis, C.; Friese, K.; Makrigiannakis, A. Aberrant expression of corticotropin-releasing hormone in pre-eclampsia induces expression of fasl in maternal macrophages and extravillous trophoblast apoptosis. *Mol. Hum. Reprod.* **2012**, *18*, 535–545. [CrossRef] [PubMed]
20. Guenther, S.; Vrekoussis, T.; Heublein, S.; Bayer, B.; Anz, D.; Knabl, J.; Navrozoglou, I.; Dian, D.; Friese, K.; Makrigiannakis, A.; et al. Decidual macrophages are significantly increased in spontaneous miscarriages and over-express fasl: A potential role for macrophages in trophoblast apoptosis. *Int. J. Mol. Sci.* **2012**, *13*, 9069–9080. [CrossRef] [PubMed]
21. Hannan, N.J.; Jones, R.L.; Critchley, H.O.; Kovacs, G.J.; Rogers, P.A.; Affandi, B.; Salamonsen, L.A. Coexpression of fractalkine and its receptor in normal human endometrium and in endometrium from users of progestin-only contraception supports a role for fractalkine in leukocyte recruitment and endometrial remodeling. *J. Clin. Endocrinol. Metab.* **2004**, *89*, 6119–6129. [CrossRef] [PubMed]
22. Cheng, Y.; Feng, Y.; Xia, Z.; Li, X.; Rong, J. Omega-alkynyl arachidonic acid promotes anti-inflammatory macrophage m2 polarization against acute myocardial infarction via regulating the cross-talk between PKM2, HIF-1α and iNOS. *Biochim. Biophys. Acta* **2017**, *1862*, 1595–1605. [CrossRef] [PubMed]
23. Yang, J.; Zhang, L.; Yu, C.; Yang, X.F.; Wang, H. Monocyte and macrophage differentiation: Circulation inflammatory monocyte as biomarker for inflammatory diseases. *Biomark. Res.* **2014**, *2*, 1. [CrossRef] [PubMed]
24. Tontonoz, P.; Spiegelman, B.M. Fat and beyond: The diverse biology of pparγ. *Annu. Rev. Biochem.* **2008**, *77*, 289–312. [CrossRef] [PubMed]
25. Gustafsson, C.; Mjosberg, J.; Matussek, A.; Geffers, R.; Matthiesen, L.; Berg, G.; Sharma, S.; Buer, J.; Ernerudh, J. Gene expression profiling of human decidual macrophages: Evidence for immunosuppressive phenotype. *PLoS ONE* **2008**, *3*, e2078. [CrossRef] [PubMed]
26. Svensson, J.; Jenmalm, M.C.; Matussek, A.; Geffers, R.; Berg, G.; Ernerudh, J. Macrophages at the fetal-maternal interface express markers of alternative activation and are induced by M-CSF and IL-10. *J. Immunol.* **2011**, *187*, 3671–3682. [CrossRef] [PubMed]
27. Lehmann, J.M.; Moore, L.B.; Smith-Oliver, T.A.; Wilkison, W.O.; Willson, T.M.; Kliewer, S.A. An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor γ (PPARγ). *J. Biolo. Chem.* **1995**, *270*, 12953–12956. [CrossRef]
28. Bouhlel, M.A.; Derudas, B.; Rigamonti, E.; Dievart, R.; Brozek, J.; Haulon, S.; Zawadzki, C.; Jude, B.; Torpier, G.; Marx, N.; et al. PPARγ activation primes human monocytes into alternative M2 macrophages with anti-inflammatory properties. *Cell Metab.* **2007**, *6*, 137–143. [CrossRef] [PubMed]
29. Lavinsky, R.M.; Jepsen, K.; Heinzel, T.; Torchia, J.; Mullen, T.M.; Schiff, R.; Del-Rio, A.L.; Ricote, M.; Ngo, S.; Gemsch, J.; et al. Diverse signaling pathways modulate nuclear receptor recruitment of n-cor and smrt complexes. *Proc. Natl. Acad. Sci. USA.* **1998**, *95*, 2920–2925. [CrossRef] [PubMed]
30. Ricote, M.; Huang, J.; Fajas, L.; Li, A.; Welch, J.; Najib, J.; Witztum, J.L.; Auwerx, J.; Palinski, W.; Glass, C.K. Expression of the peroxisome proliferator-activated receptor γ (PPARγ) in human atherosclerosis and regulation in macrophages by colony stimulating factors and oxidized low density lipoprotein. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 7614–7619. [CrossRef] [PubMed]
31. Ricote, M.; Li, A.C.; Willson, T.M.; Kelly, C.J.; Glass, C.K. The peroxisome proliferator-activated receptor-γ is a negative regulator of macrophage activation. *Nature* **1998**, *391*, 79–82. [CrossRef] [PubMed]
32. Wongweragiat, S.; Searle, R.F.; Bulmer, J.N. Expression of fas/fas ligand by decidual leukocytes in hydatidiform mole. *Biol. Reprod.* **2001**, *64*, 784–789. [CrossRef] [PubMed]
33. Hammer, A.; Blaschitz, A.; Daxbock, C.; Walcher, W.; Dohr, G. Fas and fas-ligand are expressed in the uteroplacental unit of first-trimester pregnancy. *Am. J. Reprod. Immunol.* **1999**, *41*, 41–51. [CrossRef] [PubMed]
34. Hammer, A.; Dohr, G. Expression of fas-ligand in first trimester and term human placental villi. *J. Reprod. Immunol.* **2000**, *46*, 83–90. [CrossRef]
35. Aplin, J.D. Developmental cell biology of human villous trophoblast: Current research problems. *Int. J. Dev. Biol.* **2010**, *54*, 323–329. [CrossRef] [PubMed]
36. Tarrade, A.; Rochette-Egly, C.; Guibourdenche, J.; Evain-Brion, D. The expression of nuclear retinoid receptors in human implantation. *Placenta* **2000**, *21*, 703–710. [CrossRef] [PubMed]

37. Toth, B.; Bastug, M.; Mylonas, I.; Scholz, C.; Makovitzky, J.; Kunze, S.; Thaler, C.; Friese, K.; Jeschke, U. Peroxisome proliferator-activated receptor-γ in normal human pregnancy and miscarriage. *Acta Histochem.* **2009**, *111*, 372–378. [CrossRef] [PubMed]
38. Toth, B.; Bastug, M.; Scholz, C.; Arck, P.; Schulze, S.; Kunze, S.; Friese, K.; Jeschke, U. Leptin and peroxisome proliferator-activated receptors: Impact on normal and disturbed first trimester human pregnancy. *Histol. Histopathol.* **2008**, *23*, 1465–1475. [PubMed]
39. Fournier, T.; Guibourdenche, J.; Handschuh, K.; Tsatsaris, V.; Rauwel, B.; Davrinche, C.; Evain-Brion, D. Pparγ and human trophoblast differentiation. *J. Reprod. Immunol.* **2011**, *90*, 41–49. [CrossRef] [PubMed]
40. Knabl, J.; Huttenbrenner, R.; Hutter, S.; Gunthner-Biller, M.; Vrekoussis, T.; Karl, K.; Friese, K.; Kainer, F.; Jeschke, U. Peroxisome proliferator-activated receptor-γ (PPARγ) is down regulated in trophoblast cells of gestational diabetes mellitus (GDM) and in trophoblast tumour cells bewo in vitro after stimulation with pparγ agonists. *J. Perinat. Med.* **2014**, *42*, 179–187. [CrossRef] [PubMed]
41. Ziegelmuller, B.; Vattai, A.; Kost, B.; Kuhn, C.; Hofmann, S.; Bayer, B.; Toth, B.; Jeschke, U.; Ditsch, N. Expression of thyroid hormone receptors in villous trophoblasts and decidual tissue at protein and mrna levels is downregulated in spontaneous and recurrent miscarriages. *J. Histochem. Cytochem.* **2015**, *63*, 511–523. [CrossRef] [PubMed]
42. Barak, Y.; Nelson, M.C.; Ong, E.S.; Jones, Y.Z.; Ruiz-Lozano, P.; Chien, K.R.; Koder, A.; Evans, R.M. PPARγ is required for placental, cardiac, and adipose tissue development. *Mol. Cell* **1999**, *4*, 585–595. [CrossRef]

2.2.Publication 2

Titel:

Involvement of ILR4α and TLR4 in miscarriages

Authors:

Theresa Maria Kolben*, Elisabeth Rogatsch*, Anna Hester, Christina Kuhn,

Elisa Schmoeckel, Bastian Czogalla, Sven Mahner, Udo Jeschke, Thomas Kolben

*both authors contributed equivally to this work

Journal:

Journal of Reproductive Immunology 2019;131(December 2018):36-43. doi:10.1016/j.jri.2018.12.001

Abstract:

Background: The purpose of this study was to analyze the involvement of signaling via Interleukin-4-Receptor α (IL4Rα) and Toll like receptor (TLR) 4 at the fetomaternal interface in the process of early pregnancy.

Methods: Placenta specimens of 46 patients in early pregnancy were analyzed (normal pregnancy (n=15), spontaneous (n=15) and habitual abortion (n=16)). TLR4 and IL4Rα were analyzed by immunohistochemistry, immunofluorescence and real time PCR. Statistical analysis was carried out using SPSS 23 and Microsoft Excel.

Results: IL4Rα could be detected in trophoblast cells of all groups. It was significantly downregulated in the syncytiotrophoblast of spontaneous and recurrent abortions (p=0.001), and in decidual tissue of spontaneous abortions (p=0.001). Expression of TLR4 was decreased in the intermediate villous trophoblast (IVT) and decidua of spontaneous abortions (p=0.04 & 0.003, respectively). On mRNA level expression of IL4Rα and TLR4 was significantly decreased in the group of recurrent miscarriages (IL4Rα p=0.002, TLR4 p=0.004).

Conclusion: This study contributes new findings to the understanding of the complex molecular interplay at the fetomaternal interface in normal pregnancy and miscarriages. For the first time signaling via IL4Rα being involved at the very beginning of the generation of new life could demonstrated. Moreover, new evidence was provided regarding TLR4 playing a pivotal role in early pregnancy.

Journal of Reproductive Immunology 131 (2019) 36–43

Contents lists available at ScienceDirect

Journal of Reproductive Immunology

journal homepage: www.elsevier.com/locate/jri

Involvement of ILR4α and TLR4 in miscarriages

Theresa Maria Kolben[a,1], Elisabeth Rogatsch[a,1], Anna Hester[a], Christina Kuhn[a], Elisa Schmoeckel[b], Bastian Czogalla[a], Sven Mahner[a], Udo Jeschke[a,*], Thomas Kolben[a]

[a] Department of Obstetrics and Gynecology, University Hospital, LMU Munich, Marchioninistr. 15, 81377, Munich, Germany
[b] Department of Pathology, LMU Munich, Marchioninistr. 27, 81377, Munich, Germany

ARTICLE INFO

Keywords:
Habitual miscarriages
Habitual abortions
Spontaneous abortions
IL4Rα
TLR4

ABSTRACT

Background: The purpose of this study was to analyze the involvement of signaling via Interleukin-4-Receptor α (IL4Rα) and Toll like receptor (TLR) 4 at the fetomaternal interface in the process of early pregnancy.
Patients And Methods: Placenta specimens of 46 patients in early pregnancy were analyzed (normal pregnancy (n = 15), spontaneous (n = 15) and habitual abortion (n = 16)). TLR4 and IL4Rα were analyzed by immunohistochemistry, immunofluorescence and real time PCR. Statistical analysis was carried out using SPSS 23 and Microsoft Excel.
Results: IL4Rα could be detected in trophoblast cells of all groups. It was significantly downregulated in the syncytiotrophoblast of spontaneous and recurrent abortions (p = 0.001), and in decidual tissue of spontaneous abortions (p = 0.001). Expression of TLR4 was decreased in the intermediate villous trophoblast (IVT) and decidua of spontaneous abortions (p = 0.04 & 0.003, respectively). On mRNA level expression of IL4Rα and TLR4 was significantly decreased in the group of recurrent miscarriages (IL4Rα p = 0.002, TLR4 p = 0.004).
Conclusion: This study contributes new findings to the understanding of the complex molecular interplay at the fetomaternal interface in normal pregnancy and miscarriages. For the first time signaling via IL4Rα being involved at the very beginning of the generation of new life could demonstrated. Moreover, new evidence was provided regarding TLR4 playing a pivotal role in early pregnancy.

1. Introduction

Miscarriage is the most frequent complication of early pregnancy. The incidence decreases with older gestational age (Macklon et al., 2002). In clinical recognized pregnancies the frequency reaches 20%. Loss of spontaneous conceptions prior to diagnosis and verification of the pregnancy occurs in up to 25% (Lohstroh et al., 2005; Wang et al., 2003). This rate is even higher (50–60%) when preimplantation losses are taken into consideration, too (Macklon et al., 2002; Rai and Regan, 2006). Human reproduction underlies a sophisticated interaction of multiple regulatory systems. Involved are metabolic, immunologic, and endocrine mechanisms. Failures in only one of these systems can result in fetal loss. Fetal chromosomal abnormalities are found in half of the cases of miscarriages. Known additional factors which can contribute to spontaneous abortions are anatomic abnormalities of the uterus, infections, external (i.e. chemical agents) or psychological factors (Regan and Rai, 2000). However, in about 50% of the cases of abortions the cause remains unclear.

The rate of women of reproductive age who will experience at least one abortion ranges from 25 to 50% (Regan and Rai, 2000). From 1–3% of these women will suffer from 2 or more failed pregnancies which is defined as recurrent pregnancy loss (habitual abortion) (Practice Committee of American Society for Reproductive, 2013).

The interactions at the fetomaternal interface have come into focus during the last years. The interplay of different embryonic and maternal cells is crucial for successful implantation on an immunologic basis. The semi-allogeneic fetus has to be accepted by the maternal immune system as not foreign in order not to be rejected. The underlying very sensible crosstalk on molecular level has to be precisely coordinated (Warning et al., 2011).

IL4Rα serves as a common receptor in the signaling pathway of interleukin (IL) 4 and 13 (Murata et al., 1996). IL4 is one of the key players for the induction of differentiation of naïve CD4-T cells into the Th2 phenotype (Chapoval et al., 2010). Besides the Th2 immune response, signaling via IL4Rα is also described to be responsible for the activation of type 2 innate immune cells, i.e. macrophages (Gordon,

* Corresponding author.
E-mail address: udo.jeschke@med.uni-muenchen.de (U. Jeschke).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.jri.2018.12.001
Received 19 April 2018; Received in revised form 7 December 2018; Accepted 18 December 2018

SUMMARY

2003) and eosinophils (Chen et al., 2004).

Toll-like receptors (TLRs) are a family of pathogen recognition receptors which can recognize a wide range of foreign molecules. TLR4, one member of this family, is a transmembrane cell surface receptor which belongs to the innate immune system. Signaling via TLR4 results in activation of NF-κB which induces the transcription of inflammatory genes (interferon-α (INF-α), INF-γ, IL1, IL6 and IL8) (Xie et al., 2014). There is increasing evidence that TLR4 is not only susceptible to exogenous but also to endogenous ligands (Erridge, 2010). An association of TLR4 with processes leading to miscarriages has been described already (Li et al., 2016a, b).

The purpose of this study was to analyze the involvement of signaling via IL4Rα and TLR4 at the fetomaternal interface in the process of early pregnancy.

IL4Rα and TLR4 represent two main markers for M2-polarized macrophages. In another analysis, we could demonstrate that M2-polarized macrophages were diminished in recurrent miscarriages, thus supposedly accounting for an overweight of pro-inflammatory macrophages. Up to now, it was not known whether these receptors were also expressed in parallel by the trophoblast. This could provide evidence for a possible crosstalk between the trophoblast cells and the immigrating macrophages (Kolben et al., 2018). Thereby, we aimed to further elucidate the molecular mechanisms involved in miscarriages.

2. Patients and methods

2.1. Patient data

Placenta samples from spontaneous miscarriages (n = 15) and recurrent miscarriages (n = 15) ranging from the 4th to the 13th week of gestation were collected between 2011–2016 at the Department of Obstetrics and Gynecology of LMU Munich.

Samples of placental tissue from legal terminations of healthy pregnancies (n = 15) during the first trimester of gestation served as control group. They were provided by a private practice clinic in Munich, Germany.

The control group samples were confirmed as healthy by an independent pathologist.

The placental material was collected after the operation without hormonal pre-treatment. In cases of spontaneous and recurrent miscarriage the uterine curettage was performed within 24 h after diagnosis. Immediately after surgery, the placental tissue was frozen, and additional material from the placenta was fixed in formalin.

This study was approved by the Institutional Review Board of the Ludwig-Maximilian-University, Munich. Informed consent for participation in this study was obtained from all patients before surgery.

All women had an unremarkable medical and family history, which was taken systematically. Exclusion criteria were autoimmune disorders, thrombophilia and known chromosomal abnormalities, which were ruled out by karyotype analysis.

Analysis of chromosomal abnormalities was performed for all specimens. In this study, we used PowerPlex 16HS multiple PCR system. This system allows detecting 16 STR loci. However, it is limited in identifying chromosomal abnormalities on base pair level. Testing was performed with all placental tissue samples (miscarriages and controls). DNA extraction and profiling was carried out as follows: isolation of DNA from all samples was performed by BioRobot EZ1 (Qiagen, Hilden, Germany) as depicted by Anslinger et al (Anslinger et al., 2005). According to the manufacturer's protocol, the DNA was extracted, eluted in 50 µl double-distilled water and quantified twice using the Quantifiler™ Human DNA Quantification Kit (Applied Biosystems; Thermo Fisher Scientific™; Waltham, MA, USA). The arithmetic mean values were calculated. The PowerPlex® 16 HS System Multiplex PCR system was used (Promega; Madison, Wisconsin, USA) (total volume of 25 µl, in a 32-cycle program). DNA profiling was performed by addition of 300 pg DNA for each step. Analysis of the PCR products was accomplished using the ABI PRISM 3130XL capillary electrophoresis system (Thermo Fisher Scientific™). The results were set up with the GeneScan® Analysis Software and the ABI Prism® GenoTyper Software Version 3.7 (Thermo Fisher Scientific™) (Anslinger et al., 2005; Ziegelmuller et al., 2015).

Table 1
Demographic and Clinical Characteristics of the Study population. Values are Mean ± S.D.

Characteristics*	Normal pregnancy n = 15	Spontaneous miscarriage n = 15	Recurrent miscarriage n = 16	*P* value (Kruskal Wallis Test)
Maternal age (years)	31.18 ± 8.06 (18.7-43.3)	37.8 ± 4.51 (29.2-43.2)	35.76 ± 4.8 (29.5- 46.9)	0.049
Gestational age (weeks)	9.53 ± 1.95 (7-13)	8.4 ± 1.89 (4-12)	9.3 ± 1.49 (7-12)	0.276
Gravidity	4 ± 1.8 (1-7)	3.1 ± 1.1 (2-5)	1.6 ± 0.9 (1-4)	0.001
Parity	2 ± 1.1 (0-4)	0.9 ± 0.8 (0-2)	0.3 ± 0.6 (0-2)	0.003

* Mean, standard deviation, range.

Screening for anatomic, chromosomal (parental and fetal) and endocrine disorders was performed, concerning to the etiologic cause of failed pregnancy (Toth et al., 2010).

However, each sample was processed equivalently and instantly after collection. Table 1 gives a summary of the clinical data of the study population.

2.2. Immunohistochemistry

The sections (2-3 µm) were fixed with 5% formalin in PBS, pH 7.4 for 24 h and embedded with paraffin-wax. The paraffin-wax embedded placental tissue was deparaffinized in xylol for 20 min and washed with ethanol 100% for the staining process. Endogen peroxidase reaction was inhibited by adding methanol with 6% H_2O_2 for 20 min. Subsequently, the slides were rehydrated in a descending ethanol gradient. Next, the samples were heated in the pressure cooker containing a sodium citrate buffer (pH 6.0), followed by cooling and washing in distilled water and PBS. Incubation with a blocking solution (reagent 1, ZytoChem Plus HRP Polymer System (mouse/rabbit), Zytomed Berlin, Germany), was performed for 5 min.

Each sample was incubated with the primary antibody overnight for 16 h at 4 °C. The primary antibodies are shown in Table 2. Between each step, slides were washed with PBS (pH 7.4). According to the manufacturer's protocol incubation with the blocking solution with post block (reagent 2) for 20 min and HRP polymer (reagent 3) for 30 min was done.

For the visualization procedure, the substrate and the chromogen 3.3′ diaminobenzidine (DAB, Dako, Glostrup, Denmark) was applied for 35 s. The staining reaction was stopped with distilled water. The sections were counterstained with Mayer's acidic hematoxylin for 2 min and blued in tap water for 5 min. Finally, the tissue slides were

Table 2
Antibodies used for immunohistochemical characterization of placental tissue samples.

Antibody	Isotype	Clone	Dilution	Source
IL4Rα	rabbit IgG Anti IL4R alpha	polyclonal	1:100 in PBS	Novusbio, Littleton, USA **R&D Systems, Inc. Minneapolis, USA**
TLR4	mouse IgG2b Anti TLR 4	monoclonal	1:200 in PBS	Abcam, Cambridge; UK

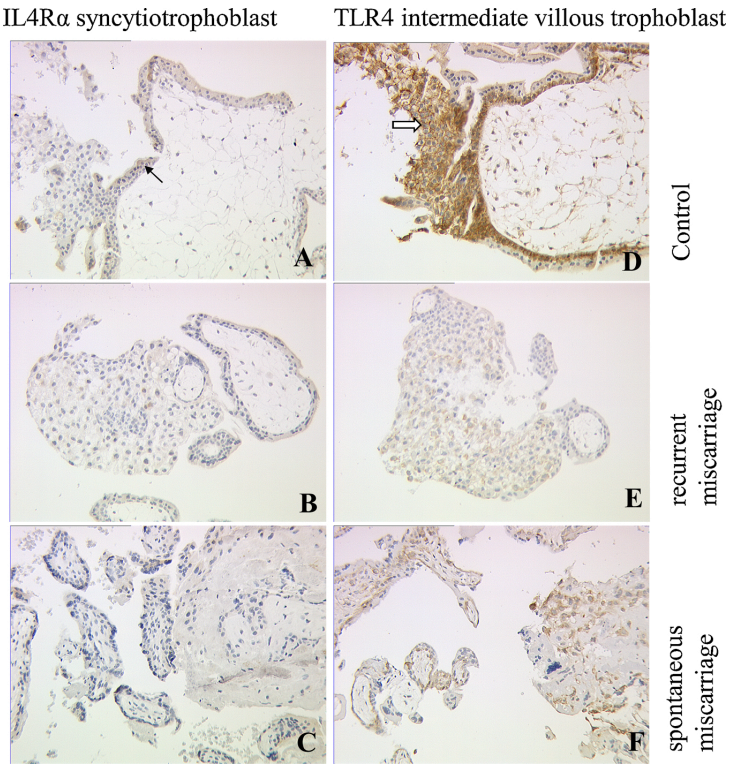


Fig. 1. Immunohistochemical staining of IL4Rα and TLR4 in the trophoblast: syncytiotrophoblast (marked by a black arrow) and intermediate villous trophoblast (marked by a white arrow).
(A) Strong staining of IL4Rα in the control group represented by placenta of legally induced termination of healthy pregnancies.
(B) Significantly weaker staining in the syncytiotrophoblast ($p = 0.001$) of recurrent miscarriages
(C) Significantly weaker staining in the syncytiotrophoblast ($p = 0.001$) of spontaneous miscarriages
(D) Strong staining of TLR4 in intermediate villous trophoblastic cells of the control group
(E) Weaker staining of TLR4 in the intermediate villous trophoblast of recurrent miscarriages (trend of down-regulation compared to the healthy placenta).
(F) Significantly weaker staining of TLR4 in thei intermediate villous trophoblast of spontaneous miscarriages ($p = 0.04$).
(Scale, 200 μm)

dehydrated in an ascending ethanol gradient ending with xylol and covered in Eukitt® quick hardening mounting medium (Sigma Aldrich).

Tissue samples from colon (Fig. 1A) and placenta (Fig. 1C) were used as positive control. Cells with brownish color were considered to be positive, whereas the negative control (Fig. 1B, D) and unstained cells revealed a blue color.

The microscope Leitz Wetzlar (Germany; Type 307-148.001 514686) was used to examine the samples.

The three cell types of the trophoblast and the decidua were evaluated (supplementary file). The cytotrophoblast (CT) represents an oval to polygonal, mononucleated stem cell, is not hormone producing and embodies the inner layer of the fetal chorionic villi. The syncytiotrophoblast (ST) consists of multinucleated and non-mitotic active cells, which are formed by fusion of small uniform cytotrophoblastic cells. The syncytiotrophoblast forms the outer layer of the fetal chorionic villi and produces most hormones of the placenta such as human chorionic gonadotropin (hCG), humanplacental lactogen (hPL), placental alkaline phosphatase (PLAP), estradiol, progesterone, placental growth hormone and inhibin. The intermediate villous trophoblast (IVT) is found in columns of anchoring villi. The intermediate villous trophoblast is contemplated as a hetergenous group with a single round uniform nucleus, larger cells than the cytotrophoblast and has a pale cytoplasm (Cierna et al., 2016).

The staining results were analyzed using the immunoreactive score (IRS) by two independent experienced scientists to rule out rater-dependent differences and inconsistency. The immunoreactive score, a semi-quantitative score, is the multiplication of the total of optical intensity (grades: 0 = none, 1 = weak, 2 = moderate, 3 = strong staining) and the total of percentage of positive stained cells (0 = none, 1 ≤ 10%, 2 = 11%–50%, 3 = 51%–80% and 4 ≥ 81% of the cells). This results in a score with a minimum of 0 and a maximum of 12 points.

2.3. Immunofluorescence

Identification of IL4Rα-expressing cells in the decidua was

Table 3
Antibodies used for double immunofluorescence.

Antibody	Isotype	Clone	Dilution	Source
IL4Rα	rabbit IgG Anti IL4Rα	polyclonal	1:100 in Dako (DAKO, S322; Carpenteira, CA, USA)	Novusbio, Littleton, USA **R&D Systems, Inc. Minneapolis, USA**
HLA G	mouse IgG1 Anti HLA G	monoclonal (MEM-6/9)	1:50 in Dako	AbD Serotec, Cambridge, UK
Cy3	Goat IgG Anti Mouse	monoclonal	1:500	Dianova, Hamburg, Germany
Cy2	Goat IgG Anti rabbit	polyclonal	1:100	Dianova, Hamburg, Germany

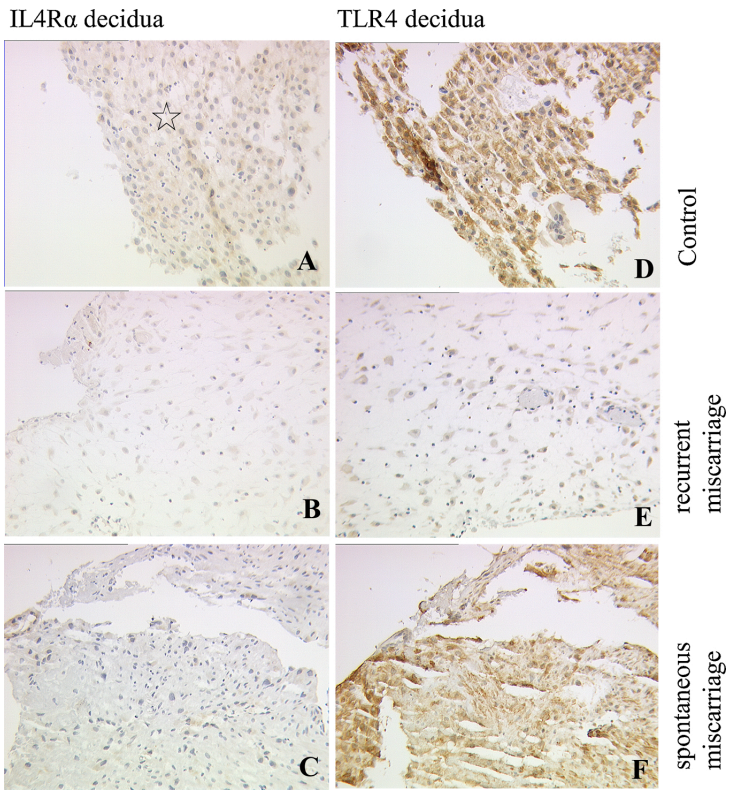


Fig. 2. Immunohistochemical staining of IL4Rα and TLR4 in the decidua (marked by a star): (A) Strong staining of IL4Rα in the control group represented by placenta of legally induced termination of healthy pregnancies.
(B) Weaker stainig of IL IL4Rα in the decidua of recurrent miscarriages
(C) Significantly weaker staining of IL4Rα in the decidua of spontaneous miscarriages (p = 0.021)
(D) Immunohistochemical staining of TLR4 in the decidua of the control
(E) Weaker staining in the recurrent miscarriage group
(F) Significantly weaker staining in the spontaneous miscarriage group (p = 0.003)
(Scale, 200 µm)

performed in samples of healthy controls and spontaneous miscarriages. Double immunofluorescence was used to prove the localization of IL4Rα in the cytoplasm and membrane. The used antibodies are filed in Table 3. HLA-G was used as a specific marker for extravillous trophoblast (EVT) cells.

First, the specimens were deparaffinized in Roticlear (Roth, Germany) for 20 min and rinsed in ethanol 100%, rehydrated in an alcohol gradient (70% and 50%). Then the slides were stationed in a pressure cooker with sodium citrate (pH 6.0) for 5 min and washed in distilled water and PBS. Next, the samples were blocked with Ultra V blocking solution (Lab Vision, Thermo Scientific Inc., Fremont, CA, USA) for 15 min to minimize non-specific background staining. Incubation with polyclonal Anti-IL4Rα rabbit IgG and monoclonal Anti-HLA-G mouse IgG1 (Table 3) was performed for 16 h at 4 °C. Afterwards, the slides were washed in PBS, and the secondary antibodies were applied to the specimen. We used the Cy2-labelled goat-anti-rabbit IgG (Dianova, Hamburg, Germany) and the Cy3-labelled goat-anti-mouse IgG (Dianova, Hamburg, Germany) for 30 min at room temperature. Finally, the samples were embedded in Vectashield® mounting medium with DAPI (Vector Laboratories; Burlingame, CA; USA). The specimens were examined with a fluorescent Axioskop photomicroscope (Zeiss, Oberkochen, Germany). Pictures were taken using a digital Axiocam camera system (Zeiss).

2.4. Evaluation of IL4Rα and TLR4 with real time RT-PCR (Taq man)

2.4.1. RNA extraction from placental tissue

A total quantity of 4 × 10 mg of control, 5 × 10 mg of spontaneous and 6 × 10 mg of recurrent miscarried placental tissue was used for extraction of mRNA. RNA extraction was performed with RNeasy® Lipid Tissue Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

2.5. Reverse transcription

Reverse transcription (RT) was accomplished using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems™, Fisher Scientific Company, Canada) according to the protocol in an Eppendorf Mastercycler® gradient. RT conditions were 10 min 25 °C, 2 h 37 °C, 5 min 85 °C and −20 °C on hold.

2.6. Real-time reverse transcription-PCR

Initially, the equal amounts of RNA from each sample were converted to cDNA. Next, cDNA from the placenta of spontaneous miscarriages, recurrent miscarriages, and controls was used for PCR analysis.

Reverse transcription-PCRs were implemented in duplicate in an Optical Fast 96-well plate (Applied Biosystems) and covered with an adhesive cover. The volume was 20 µl for each reaction, containing 10 µl TaqMan® Fast Universal PCR Master Mix 2x (Applied Biosystems, Nr. 4367846; 50 ml), 1 µl cDNA, 8 µl H_2O (DEPC treated DI water; Sigma, Taufkirchen, Germany)). Furthermore, the total consisted of 1 µl TaqMan® Gene Expression Assay 20x (Hs_00166237_m1 for IL4Rα and Hs_00152939_m1 for TLR4, both Applied Biosystems).

The PCR temperature protocol was 20 s at 95 °C, followed by 40 cycles of amplification: denaturation process for 3 s at 95 °C and extension and annealing for 30 s at 60 °C.

The 7500 Fast Real-Time PCR System (Applied Biosystems) was used to process the PCR assays. Quantification was performed with the 2-ΔΔCT method using β-Actin as housekeeping gene (Applied Biosystems, Hs_99999903_m1).

2.7. Statistics

Statistical analysis was performed using the SPSS software version 24 (SPSS; Chicago; IL, USA) and Excel version 12.3.1 (Microsoft Windows 2016; Redmond, WA, USA). Mann-Whitney U signed-rank test was carried out for comparison of two independent groups. This non-parametrical test is a straight analysis of variance and examines two separate parameters. P-values < 0.05 were considered to be statistically significant.

3. Results

3.1. Immunohistochemistry

3.1.1. IL4Rα expression in the trophoblast and the decidua

IL4Rα was detected in trophoblast cells in the control group (healthy pregnancies), spontaneous miscarriages, and recurrent miscarriages (Fig. 1A-C). IL4Rα was significantly downregulated in the syncytiotrophoblast of recurrent miscarriages (Fig. 1B, IRS 2 versus 8, p = 0.001) and of spontaneous miscarriages (Fig. 1C, IRS 2 versus 8, p = 0.001) compared to the healthy control (Fig. 1A).

A significant downregulation of the IL4Rα expression in decidual tissue was observed in spontaneous miscarriages (Fig. 2C) compared to the control group (Fig. 2A, IRS 0 versus 4, p = 0.021).

3.1.2. TLR4 expression in the trophoblast and the decidua

The expression of TLR4 in trophoblast cells in healthy pregnancies, spontaneous miscarriages (SM) and recurrent miscarriages (RM) is shown in Fig. 1D-F.

There was a trend of a downregulation of TLR4 without significance in the intermediate villous trophoblast of recurrent miscarriages (Fig. 1E, IRS 4 versus 8, p = 0.389) compared to the control group. The intermediate villous trophoblast (IVT) showed a significantly lower expression of TLR4 in spontaneous miscarriages (Fig. 1F) than in healthy placentas (Fig. 1D, IRS 6 versus 8, p = 0.04).

TLR4 expression in the syncytiotrophoblast was not altered in the abortion groups in comparison to the control group. The expression in the cytotrophoblast did not show any differences in all groups. Focusing on the decidua of abortive tissue we could also observe a significant change between the control group and spontaneous miscarriages (p = 0.003; Fig. 3 D–F).

Concisely, the staining results of IL4Rα and TLR4 are given in Box Plots in Fig. 3.

3.2. Double immunofluorescence

3.2.1. Identification of IL4Rα expressing cells in the decidua

IL4Rα positive cells were stained in green, cells expressing HLA-G were dyed in red. Cells neither expressing IL4Rα nor HLA-G were stained in blue.

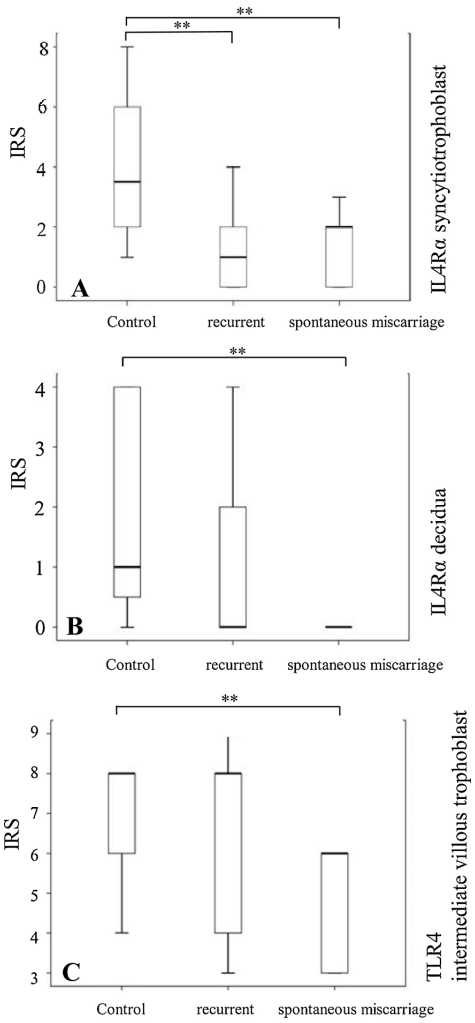


Fig. 3. Statistical results of the immunoreactive score of IL4Rα and TLR4.
(A) IL4Rα in the syncytiotrophoblast
(B) IL4Rα in the decidua
(C) TLR4 in the intermediate villous trophoblast
(* p < 0.05, ** p < 0.005)

IL4Rα + HLA-G double immunofluorescence staining was used for labeling the expression of IL4Rα in the decidua of spontaneous and recurrent miscarriages and control groups. IL4Rα staining in decidual cells of the control group is presented in Fig. 4A. Fig. 4B presents the membrane HLA-G staining of EVTs from the same area. The depiction of IL4Rα and HLA-G is shown as a triple filter coexpression in Fig. 4C. In the healthy placenta, mostly both markers are expressed.

The habitual abortion shows no IL4Rα positive cell in Fig. 4D, whereas HLA-G positive cells are found (Fig. 4E). Triple filter excitation shows no coexpression of both markers and confirms the absence of IL4Rα in the recurrent miscarriage group (Fig. 4F).

No IL4Rα positive cells were detected in the spontaneous miscarriages group (Fig. 4G), whereas HLA-G was expressed in these cells (Fig. 4H). Triple filter excitation demonstrated a total absence of IL4Rα in spontaneous abortions (Fig. 4I).

In conclusion, we observed higher expression of IL4Rα positive cells

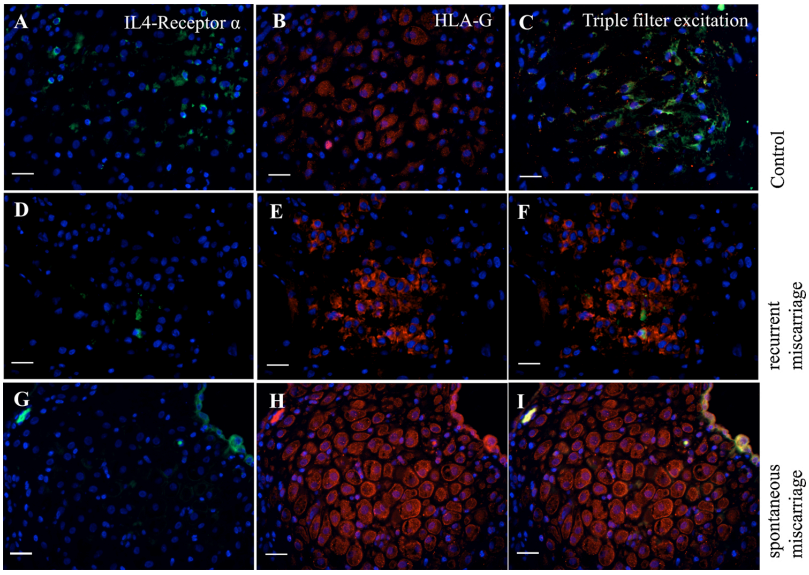


Fig. 4. Double Immunofluorescence of IL4Rα and HLA-G in the decidua.
(A) IL4Rα expression (Cy2; green staining) is high in normal decidua.
(B) HLA-G (Cy3; red staining) membrane staining of extravillous trophoblast cells (EVT).
(C) Triple filter excitation identifying cells expressing both markers. The majority of IL4Rα positive cells are coexpressing HLA-G in the healthy placenta.
(D) No IL4Rα positive cells in the recurrent miscarriage group
(E) EVTs (HLA-G positive) stained in red
(F) Triple filter excitation confirms the absence of IL4Rα in the habitual abortion.
(G) No IL4Rα expression in the spontaneous miscarriage group.
(H) EVTs (HLA-G positive) stained in red.
(I) Triple filter excitation confirms the absence of IL4Rα.
(All pictures 40x lens)

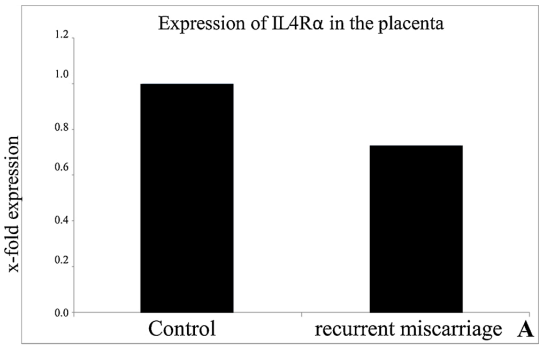
in the healthy placenta than in the spontaneous and recurrent miscarriage group.

3.2.2. Evaluation of IL4Rα and TLR4 expression with real-time RT-PCR (TaqMan)

IL4Rα mRNA expression was analyzed in placental tissue from miscarriages and healthy controls by quantitative RT-PCR.

IL4Rα mRNA was significantly decreased in recurrent miscarriages (0.7-fold, $p = 0.002$), whereas no significant downregulation was found in spontaneous miscarriages in comparison to the control group (Fig. 5A).

In spontaneous miscarriage samples, the mRNA expression of TLR4 showed no significant downregulation, whereas TLR4 expression was significantly downregulated in recurrent miscarriages (0.7-fold, $p = 0.004$) compared to healthy placentas (Fig. 5B).



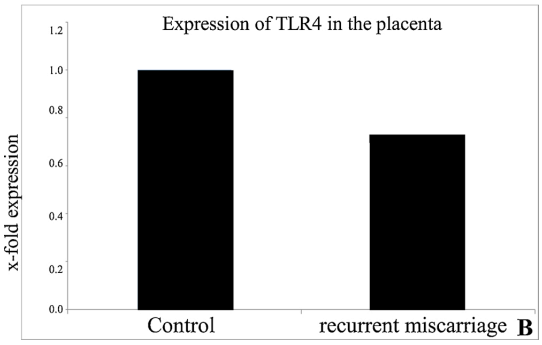


Fig. 5. RT-PCR Analysis of IL4Rα and TLR4 expression.
(A) Significant downregulation of IL4Rα in the group of recurrent miscarriages compared to the healthy controls ($p = 0.002$).
(B) Significant downregulation of TLR4 in the group of recurrent miscarriages compared to the healthy controls ($p = 0.004$).
(IRS: immunoreactive score)

4. Discussion

Human reproduction is a very sensitive process. From fertilization and implantation to fetal development every step is prone for errors which can lead to fetal demise. The interactions at the feto-maternal interface have to allow the maternal innate immune system to accept the semi-allogeneic fetus without rejecting it. Especially, during implantation and placental development the complicated network and interplay of the maternal immune system and the fetus has to work in an extremely precise manner. Failures or an imbalance in that system are considered to result in rejection of fetal tissues and abortions (Koga and Mor, 2010; Takeshita, 2004).

In this study the expression of two receptors involved in the signaling of the immune system (IL4Rα and TLR4) were analyzed at the feto-maternal interface in the process of early pregnancy in order to further elucidate the interactions of the fetus and the maternal immune system.

TLR4 is a member of receptors of the innate immune system. Inflammatory chemokines can be induced by signaling via TLR4 and activation of NF-κB (interferon-α (INF-α), INF-γ, IL-1, IL-6 and IL-8) (Xie et al., 2014). A deficient signaling via TLR4 could potentially disrupt the Th1/Th2 balance negatively affecting the pregnancy outcome. An association of TLR4 with processes leading to miscarriages

has been described already (Li et al., 2016a, b). TLR4 was downregulated in the intermediate villous trophoblast (IVT) and decidua of spontaneous miscarriages. This finding could not be confirmed on mRNA level. An explanation could be that isolation of mRNA from the IVT exclusively was not possible to perform. We may speculate that this tissue accounts for less than 10% of the total placental tissue. Therefore, alterations of the TLR4 expression in those cells may hardly be seen when using the whole tissue for PCR analysis. Posttranscriptional regulation of the receptor could also play a role in that situation.

Thus, other mechanisms than signaling via TLR4 may be responsible for spontaneous miscarriages. One additional important mechanism seems to be the invasion of macrophages in the decidua in spontaneous miscarriages which could be shown by our group before (Kolben et al., 2018; Guenther et al., 2012). The decrease of TLR4 was not significant and far less pronounced in the intermediate villous trophoblast of recurrent miscarriages. However, it reached significance on mRNA level. Furthermore, we could not observe a downregulation in the decidua of recurrent miscarriages. This finding is in contrast to the findings by Li and Li. Their groups found an upregulation of TLR4 in sera and decidual tissue of recurrent miscarriages

(Li et al., 2016a, b). However, our PCR was performed using placental tissue, mainly trophoblast tissue. This may explain the differing findings compared to theirs in decidual tissue.

The expression of IL4Rα in the human first trimester pregnancy decidua has been described before (Starkey, 1991). In our analysis IL4Rα could be detected in trophoblast cells of normal pregnancies, spontaneous and recurrent miscarriages as well. Its expression was significantly downregulated in the syncytiotrophoblast of the group of miscarriages. Moreover, IL4Rα was downregulated in the decidua of spontaneous miscarriages. The expression on mRNA level was significantly decreased in recurrent miscarriages. IL4Rα functions not only as receptor for IL4, but also for IL13 (Murata et al., 1996). Herbert et al. showed that both IL4 and IL13 protect the endothelial and the monocyte surface against inflammatory mediator-induced procoagulant changes (Herbert et al., 1993). A downregulation of IL4Rα and a resulting procoagulant state could be one mechanism which explains miscarriages.

A reduced receptor density for IL4 could also be responsible for a decreased induction of differentiation of naïve CD4-T cells into the Th2 phenotype, one of the main functions of IL4 (Chapoval et al., 2010). Thus, a Th1 cell response may be enhanced which is in accordance with the findings of Gao et al. (Gao and Wang, 2015). By the production of Th1-type cytokines (IFN-γ, TNF-β) rejection of fetal tissue may be supported resulting in loss of pregnancy. In contrast Th2-type cytokines (IL-4 and IL-10), which inhibit Th1 responses, could promote allograft tolerance and survival of the fetus (Berkowitz et al., 1988; Haimovici et al., 1991; Krishnan et al., 1996a, b; Lin et al., 1993; Wegmann et al., 1993).

Guenther et al. showed that decidual macrophages were significantly increasesd in spontaneous miscarriages and that a proapoptotic state was present (Guenther et al., 2012). Halasz et al. reported IL4Rα to be involved in the invasion capacity of trophoblast cells. A downregulation of IL4Rα was observed to lead to a stimulation of the invasion of trophoblast cells (Halasz et al., 2013). The decreased expression of IL4Rα, we could detect, may be a kind of salvage mechanism to stimulate the invasion of trophoblast cells to overcome the pro-apoptotic state. Therefore, there is an explanation for the disturbed IL4Rα in trophoblast cells, we observed and an additional information to the T-cell response mentioned. IL4 induces tolerance, the reduced expression of the IL4Rα on trophoblast cells could induce opposite reactions.

IL13, another ligand of IL4Rα, is also described to have anti-inflammatory properties (Minty et al., 1993), so it may be hypothesized that the reduced signaling via its receptor would negatively affect an early pregnancy by an increased inflammatory response. A downregulated signaling via IL4 in abortions has already been observed in animal models as well (Almeria et al., 2016).

A certain limitation of this study is the fact that one cannot rule out that in the group of spontaneous abortions also patients who would suffer from habitual abortions could be included.

In conclusion this study contributes new findings supporting the understanding of the complex molecular interplay at the fetomaternal interface in normal pregnancy and unexplained miscarriages. This study could demonstrate for the first time signaling via IL4Rα being involved at the very beginning of the generation of new life. Moreover, new evidence was provided regarding TLR4 playing a pivotal role in pregnancy failure.

Conflict of interest statement

Mahner, S.: Research support, Advisory Board, Honoraria, Travel support from: AstraZeneca, Bayer, Boehringer Ingelheim, Jenapharm, GSK, JanssenCilag, Medac, MSD, Pharmamar, Roche, Tesaro, Teva

The other authors do declare no conflict of interest.

Acknowledgement

This study was funded by the Medical Faculty of the LMU Munich. This work is part of the dissertation of E. Rogatsch

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jri.2018.12.001.

References

Practice Committee of American Society for Reproductive, M, 2013. Definitions of infertility and recurrent pregnancy loss: a committee opinion. Fertil. Steril. 99 (1), 63.
Almeria, S., et al., 2016. Cytokine gene expression in aborting and non-aborting dams and in their foetuses after experimental infection with Neospora caninum at 110 days of gestation. Vet. Parasitol. 227, 138–142.
Anslinger, K., et al., 2005. Application of the BioRobot EZ1 in a forensic laboratory. Leg Med (Tokyo) 7 (3), 164–168.
Berkowitz, R.S., et al., 1988. Effects of products of activated leukocytes (lymphokines and monokines) on the growth of malignant trophoblast cells in vitro. Am. J. Obstet. Gynecol. 158 (1), 199–203.
Chapoval, S., et al., 2010. Regulation of the T helper cell type 2 (Th2)/T regulatory cell (Treg) balance by IL-4 and STAT6. J. Leukoc. Biol. 87 (6), 1011–1018.
Chen, L., et al., 2004. IL-4 induces differentiation and expansion of Th2 cytokine-producing eosinophils. J. Immunol. 172 (4), 2059–2066.
Cierna, Z., et al., 2016. Intermediate trophoblast–A distinctive, unique and often unrecognized population of trophoblastic cells. Ann. Anat. 204, 45–50.
Erridge, C., 2010. Endogenous ligands of TLR2 and TLR4: agonists or assistants? J. Leukoc. Biol. 87 (6), 989–999.
Gao, Y., Wang, P.L., 2015. Increased CD56(+) NK cells and enhanced Th1 responses in human unexplained recurrent spontaneous abortion. Genet. Mol. Res. 14 (4), 18103–18109.
Gordon, S., 2003. Alternative activation of macrophages. Nat. Rev. Immunol. 3 (1), 23–35.
Guenther, S., et al., 2012. Decidual macrophages are significantly increased in spontaneous miscarriages and over-express FasL: a potential role for macrophages in trophoblast apoptosis. Int. J. Mol. Sci. 13 (7), 9069–9080.
Haimovici, F., Hill, J.A., Anderson, D.J., 1991. The effects of soluble products of activated lymphocytes and macrophages on blastocyst implantation events in vitro. Biol. Reprod. 44 (1), 69–75.
Halasz, M., et al., 2013. Progesterone-induced blocking factor differentially regulates trophoblast and tumor invasion by altering matrix metalloproteinase activity. Cell. Mol. Life Sci. 70 (23), 4617–4630.
Herbert, J.M., et al., 1993. IL-4 and IL-13 exhibit comparable abilities to reduce pyrogen-induced expression of procoagulant activity in endothelial cells and monocytes. FEBS Lett. 328 (3), 268–270.
Koga, K., Mor, G., 2010. Toll-like receptors at the maternal-fetal interface in normal pregnancy and pregnancy disorders. Am. J. Reprod. Immunol. 63 (6), 587–600.
Kolben, T.M., et al., 2018. PPARgamma Expression Is Diminished in Macrophages of Recurrent Miscarriage Placentas. Int. J. Mol. Sci. 19 (7).
Krishnan, L., et al., 1996a. Pregnancy impairs resistance of C57BL/6 mice to Leishmania major infection and causes decreased antigen-specific IFN-gamma response and increased production of T helper 2 cytokines. J. Immunol. 156 (2), 644–652.
Krishnan, L., et al., 1996b. T helper 1 response against Leishmania major in pregnant C57BL/6 mice increases implantation failure and fetal resorptions. Correlation with increased IFN-gamma and TNF and reduced IL-10 production by placental cells. J.

Immunol. 156 (2), 653–662.
Li, P., Wu, H.L., Dong, B.H., 2016a. Relationship between TLR4 and CCL2 expression and recurrent spontaneous abortion. Genet. Mol. Res. 15 (1).
Li, X.P., et al., 2016b. Relationship between TLR4 and MCP2 expression levels and habitual abortion. Genet. Mol. Res. 15 (2).
Lin, H., et al., 1993. Synthesis of T helper 2-type cytokines at the maternal-fetal interface. J. Immunol. 151 (9), 4562–4573.
Lohstroh, P.N., et al., 2005. Secretion and excretion of human chorionic gonadotropin during early pregnancy. Fertil. Steril. 83 (4), 1000–1011.
Macklon, N.S., Geraedts, J.P., Fauser, B.C., 2002. Conception to ongoing pregnancy: the' black box' of early pregnancy loss. Hum. Reprod. Update 8 (4), 333–343.
Minty, A., et al., 1993. Interleukin-13 is a new human lymphokine regulating inflammatory and immune responses. Nature 362 (6417), 248–250.
Murata, T., Noguchi, P.D., Puri, R.K., 1996. IL-13 induces phosphorylation and activation of JAK2 Janus kinase in human colon carcinoma cell lines: similarities between IL-4 and IL-13 signaling. J. Immunol. 156 (8), 2972–2978.
Rai, R., Regan, L., 2006. Recurrent miscarriage. Lancet 368 (9535), 601–611.
Regan, L., Rai, R., 2000. Epidemiology and the medical causes of miscarriage. Baillieres Best Pract. Res. Clin. Obstet. Gynaecol. 14 (5), 839–854.
Starkey, P.M., 1991. Expression on cells of early human pregnancy decidua, of the p75, IL-2 and p145, IL-4 receptor proteins. Immunology 73 (1), 64–70.
Takeshita, T., 2004. Diagnosis and treatment of recurrent miscarriage associated with immunologic disorders: is paternal lymphocyte immunization a relic of the past? J. Nippon Med. Sch. 71 (5), 308–313.
Toth, B., et al., 2010. Placental interleukin-15 expression in recurrent miscarriage. Am. J. Reprod. Immunol. 64 (6), 402–410.
Wang, X., et al., 2003. Conception, early pregnancy loss, and time to clinical pregnancy: a population-based prospective study. Fertil. Steril. 79 (3), 577–584.
Warning, J.C., McCracken, S.A., Morris, J.M., 2011. A balancing act: mechanisms by which the fetus avoids rejection by the maternal immune system. Reproduction 141 (6), 715–724.
Wegmann, T.G., et al., 1993. Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon? Immunol. Today 14 (7), 353–356.
Xie, H., et al., 2014. The role of TLR4 in pathophysiology of antiphospholipid syndrome-associated thrombosis and pregnancy morbidity. Br. J. Haematol. 164 (2), 165–176.
Ziegelmuller, B., et al., 2015. Expression of thyroid hormone receptors in villous trophoblasts and decidual tissue at protein and mRNA levels is downregulated in spontaneous and recurrent miscarriages. J. Histochem. Cytochem. 63 (7), 511–523.

3. SUMMARY

Miscarriage is a common gynaecological problem worldwide. Thus, its various causes still remain often unclear and need to be fully elucidated. We believe that the immune system and its factors have a strong influence on maintaining a healthy pregnancy. Therefore, the main goal and intention of our study was to identify immunological factors and cytokines in the placenta and evaluate their importance, which are involved in the occurrence of spontaneous and recurrent miscarriages.

The placenta tissue of 46 patients in early pregnancy was analyzed for the factors TLR4, IL4Rα and PPARγ (normal pregnancy n=15, spontaneous n=15 and habitual abortions n=16)). To examine our placenta samples, we used immunohistochemistry and double immunofluorescence. To validate our findings we isolated mRNA and ultimately RT-PCR was performed. Statistical analysis was performed using SPSS 23 and Microsoft Excel.

In the trophoblast IL4Rα was detected in all groups. It was significantly downregulated in the syncytiotrophoblast of spontaneous and recurrent abortions (p=0.001), and in decidual tissue of spontaneous abortions (p=0.001). The expression of TLR4 was down-regulated in the intermediate villous trophoblast (IVT) and the decidua of spontaneous abortions (p=0.04 & p=0.003, respectively). The expression of IL4Rα and TLR4 on mRNA level was significantly decreased in the recurrent miscarriage groups (IL4Rα p=0.002, TLR4 p=0.004, respectively). The intermediate villous trophoblast showed a significantly lower PPARγ expression in spontaneous (p=0.001) and recurrent (p=0.01) miscarriages. Maternal macrophages express PPARγ. Their number is significantly enhanced in the decidua of spontaneous miscarriages (p=0.013), whereas in recurrent miscarriages maternal macrophages only express PPARγ in a very small percentage. Decidual macrophages were identified by double immunofluorescence using cluster of differentiation 68 (CD68) as the marker for macrophages. The maternal macrophages were further characterized regarding their M1/M2 polarization status with the specific markers. The M1 markers TLR2 and iNOS and the M2 markers CCL1 and CX3CR1 confirmed the loss of M2 macrophages in the recurrent miscarriages.

These findings support the belief that the imbalance of the immune system has a strong influence on the placenta. Especially the secretion of proinflammatory factors shifts the polarization of anti-inflammatory M2 macrophages to M1 macrophages and among other things leads to pregnancy loss.

4. ZUSAMMENFASSUNG

Der Abortus ist ein häufig auftretendes gynäkologisches Problem weltweit, jedoch sind die Ursachen oft unklar und nach wie vor nicht vollständig geklärt. Das Immunsystem und seine Faktoren scheinen einen starken Einfluss auf einen gesunden Schwangerschaftsverlauf zu haben. Das Hauptziel unserer Forschung war es immunologische Faktoren und Zytokine in der Plazenta zu identifizieren. Die Intention war es deren Bedeutung im Bezug auf ihr Vorkommen in spontanen und rezidivierenden Aborten zu evaluieren.

Das Plazentagewebe von 46 Patientinnen in der Frühschwangerschaft wurde auf die Faktoren TLR4, IL4Rα und PPARγ untersucht. Hierbei wurde Plazentagewebe aus 15 normalen Frühschwangerschaften, 15 spontanen Aborten und 16 rezidivierenden Aborten verwendet.
Das Gewebe wurde mit der Immunhistochemie, sowie der Doppel-Immunfluoreszenz untersucht. Zur Bestätigung unserer Ergebnisse wurde die mRNA isoliert und eine Real Time-PCR durchgeführt. Die statistische Analyse wurde mit SPSS 23 und Microsoft Excel vollzogen.

IL4Rα wurde im Trophoblast in allen drei Gruppen gefunden. IL4Rα war signifikant herabreguliert im Synzytiotrophoblast in spontanen und rezidivierenden Aborten ($p=0.001$), sowie in der Dezidua von spontanen Aborten ($p=0.001$). Die Expression von TLR4 war herabreguliert im intermediären villösen Trophoblast ($p=0.04$) und in der Dezidua von spontanen Aborten ($p=0.003$). Die mRNA von IL4Rα war signifikant erniedrigt in der Gruppe von habituellen Aborten ($p=0.002$). Auch die TLR4 mRNA zeigte sich signifikant erniedrigt in den habituellen Aborten ($p=0.004$) verglichen mit den gesunden Plazentaproben. Der intermediäre villöse Trophoblast zeigte eine signifikant niedrigere PPARγ Expression in den spontanen ($p=0.001$) und rezidivierenden ($p=0.01$) Aborten im Vergleich zur Kontrolle. Die mütterlichen Makrophagen exprimieren PPARγ. Die Zahl der Makrophagen war signifikant erhöht in der Dezidua von spontanen Aborten ($p=0.013$). Jedoch in den rezidivieren Aborten findet sich nur eine kleine Prozentanzahl an PPARγ exprimierenden Makrophagen. Deziduale Makrophagen wurden mittels Immunfluoreszenz und dem Makrophagenmarker CD68 identifiziert, weiters wurde die Polarisation der Makrophagen mittels spezieller Marker charakterisiert. Die M1 Marker TLR2 und iNOS und die M2 Marker CCL1 and CX3CR1 bestätigten den Verlust von M2 Makrophagen im rezidivierenden Abort.

Diese Erkenntnisse belegen die Annahme, dass die Imbalance des Immunsystems einen starken Einfluss auf die Plazenta hat. Speziell die Sekretion von proinflammatorischen Faktoren

begünstigt den Wechsel von anti-inflammatorischen M2 Makrophagen zu M1 Makrophagen und kann unter anderem zu dem Verlust der Schwangerschaft beitragen.

5. ACKNOWLEDGEMENT

I acknowledge Prof. Dr. med. Sven Mahner for providing me the opportunity to complete my dissertation at the Department of Gynaecology and Obstetrics at the Ludwig-Maximilians-University of Munich.

My exceptional thanks go to Prof. Dr. rer. nat. Udo Jeschke for supervising and conceptualizing the study, for all the constant help and explanations, teaching and directing major parts of this work. I am extremely grateful that Prof. Jeschke has been a supporting mentor for me.

My particular thanks go to PD Dr. med. Thomas Kolben for initiating the study and giving me the opportunity, the major trust and enduring support in this project. Furthermore, I am extremely thankful for his help with acquiring the samples, while writing the publications and his patience with me.

I thank PD Dr. med. Theresa Kolben for the support and great teamwork while writing and completing the publications.

I thank Christina Kuhn for her exceptional technical support with the immunohistochemistry, the helpful explanations, the immeasurable teamwork in the lab and her patience with me.

I also thank Simone Hofmann for supporting me with her excellent help with the PCR system in the lab and the study population and database.

Most of all I would like to thank my family - your love and endless support is worth to me more than I can express on paper.
You are the best family one could ask for!

6. EIDESSTATTLICHE VERSICHERUNG

Rogatsch, Elisabeth Johanna

Name, Vorname

Ich erkläre hiermit an Eides statt,

dass ich die vorliegende Dissertation mit dem Thema

Characterization and evaluation of immunological factors in the placenta of spontaneous and recurrent miscarriages

Selbständig verfasst, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

Ich erkläre des Weiteren, dass die hier vorgelegte Dissertation nicht in gleicher oder in ähnlicher Form bei einer anderen Stelle zur Erlangung eines akademischen Grades eingereicht wurde.

Graz, 01.04.2021 Elisabeth Johanna Rogatsch

Ort, Datum Unterschrift Doktorandin

7. BIBLIOGRAPHY

1. Herold G. *Innere Medizin 2019*. Köln: Dr. med. Gerd Herold; 2019.

2. Metchnikoff E. Sur la lutta des cellules de l'organismes centre l'invasion des microbesitle. *Ann Inst Pasteur Paris*. 1887:1: 321.

3. Borghans JA, Noest AJ, De Boer RJ. How specific should immunological memory be? J Immunol. 1999 Jul 15;163(2):569-75.

4. Parkin J, Cohen B. An overview of the immune system. Lancet. 2001 Jun 2;357(9270):1777-89.

5. Delves PJ, Roitt IM. The immune system. First of two parts. N Engl J Med. 2000 Jul 6;343(1):37-49.

6. Delves PJ, Roitt IM. The immune system. Second of two parts. N Engl J Med. 2000 Jul 13;343(2):108-17.

7. Aldo PB, Krikun G, Visintin I, Lockwood C, Romero R, Mor G. A novel three-dimensional in vitro system to study trophoblast-endothelium cell interactions. Am J Reprod Immunol. 2007 Aug;58(2):98-110.

8. Goldenberg RL, Hauth JC, Andrews WW. Intrauterine infection and preterm delivery. N Engl J Med. 2000 May 18;342(20):1500-7.

9. Arechavaleta-Velasco F, Gomez L, Ma Y, Zhao J, McGrath CM, Sammel MD, Nelson DB, Parry S. Adverse reproductive outcomes in urban women with adeno-associated virus-2 infections in early pregnancy. Hum Reprod. 2008 Jan;23(1):29-36.

10. Janeway CA Jr, Medzhitov R. Innate immune recognition. Annu Rev Immunol. 2002;20:197-216.

11. Makrigiannakis A, Petsas G, Toth B, Relakis K, Jeschke U. Recent advances in understanding immunology of reproductive failure. J Reprod Immunol. 2011 Jun;90(1):96-104.

12. Larsen EC, Christiansen OB, Kolte AM, Macklon N. New insights into mechanisms behind miscarriage. BMC Med. 2013 Jun 26;11:154.

13. Macklon NS, Geraedts JP, Fauser BC. Conception to ongoing pregnancy: the 'black box' of early pregnancy loss. Hum Reprod Update. 2002 Jul-Aug;8(4):333-43.

14. Nybo Andersen AM, Wohlfahrt J, Christens P, Olsen J, Melbye M. Maternal age and fetal loss: population based register linkage study. BMJ. 2000 Jun 24;320(7251):1708-12.

15. Ugwumadu A, Manyonda I, Reid F, Hay P. Effect of early oral clindamycin on late miscarriage and preterm delivery in asymptomatic women with abnormal vaginal flora and bacterial vaginosis: a randomised controlled trial. Lancet. 2003 Mar 22;361(9362):983-8.

16. Toth B, Jeschke U, Rogenhofer N, Scholz C, Würfel W, Thaler CJ, Makrigiannakis A. Recurrent miscarriage: current concepts in diagnosis and treatment. J Reprod Immunol. 2010 May;85(1):25-32.

17. Practice Committee of the American Society for Reproductive Medicine. Evaluation and treatment of recurrent pregnancy loss: a committee opinion. Fertil Steril. 2012 Nov;98(5):1103-11.

18. Gynaecologists RC of O and G. The Investigation and Treatment of Couples with Recurrent First-trimester and Second-trimester Miscarriage. *RCOG Green-top Guidel No 17*. 2011. doi:10.4103/2230-8210.107834

19. Robertson L, Wu O, Langhorne P, Twaddle S, Clark P, Lowe GD, Walker ID, Greaves M, Brenkel I, Regan L, Greer IA; Thrombosis: Risk and Economic Assessment of Thrombophilia Screening (TREATS) Study. Thrombophilia in pregnancy: a systematic review. Br J Haematol. 2006 Jan;132(2):171-96.

20. Lassere M, Empson M. Treatment of antiphospholipid syndrome in pregnancy--a systematic review of randomized therapeutic trials. Thromb Res. 2004;114(5-6):419-26.

21. Twig G, Shina A, Amital H, Shoenfeld Y. Pathogenesis of infertility and recurrent pregnancy loss in thyroid autoimmunity. J Autoimmun. 2012 May;38(2-3):J275-81.

22. van den Boogaard E, Vissenberg R, Land JA, van Wely M, van der Post JA, Goddijn M, Bisschop PH. Significance of (sub)clinical thyroid dysfunction and thyroid autoimmunity before conception and in early pregnancy: a systematic review. Hum Reprod Update. 2011 Sep-Oct;17(5):605-19.

23. Negro R, Schwartz A, Gismondi R, Tinelli A, Mangieri T, Stagnaro-Green A. Increased pregnancy loss rate in thyroid antibody negative women with TSH levels between 2.5 and 5.0 in the first trimester of pregnancy. J Clin Endocrinol Metab. 2010 Sep;95(9):E44-8.

24. Holers VM, Girardi G, Mo L, Guthridge JM, Molina H, Pierangeli SS, Espinola R, Xiaowei LE, Mao D, Vialpando CG, Salmon JE. Complement C3 activation is required for antiphospholipid antibody-induced fetal loss. J Exp Med. 2002 Jan 21;195(2):211-20.

25. Oku K, Atsumi T, Bohgaki M, Amengual O, Kataoka H, Horita T, Yasuda S, Koike T. Complement activation in patients with primary antiphospholipid syndrome. Ann Rheum Dis. 2009 Jun;68(6):1030-5.

26. McNamee K, Dawood F, Farquharson R. Recurrent miscarriage and thrombophilia: an update. Curr Opin Obstet Gynecol. 2012 Aug;24(4):229-34.

27. Robinson L, Gallos ID, Conner SJ, Rajkhowa M, Miller D, Lewis S, Kirkman-Brown J, Coomarasamy A. The effect of sperm DNA fragmentation on miscarriage rates: a systematic review and meta-analysis. Hum Reprod. 2012 Oct;27(10):2908-17.

28. Ribas-Maynou J, García-Peiró A, Fernandez-Encinas A, Amengual MJ, Prada E, Cortés P, Navarro J, Benet J. Double stranded sperm DNA breaks, measured by Comet assay, are associated

with unexplained recurrent miscarriage in couples without a female factor. PLoS One. 2012;7(9):e44679.

29. Andersen AM, Andersen PK, Olsen J, Grønbæk M, Strandberg-Larsen K. Moderate alcohol intake during pregnancy and risk of fetal death. Int J Epidemiol. 2012 Apr;41(2):405-13.

30. King K, Smith S, Chapman M, Sacks G. Detailed analysis of peripheral blood natural killer (NK) cells in women with recurrent miscarriage. Hum Reprod. 2010 Jan;25(1):52-8.

31. Quenby S, Nik H, Innes B, Lash G, Turner M, Drury J, Bulmer J. Uterine natural killer cells and angiogenesis in recurrent reproductive failure. Hum Reprod. 2009 Jan;24(1):45-54.

32. Tang AW, Alfirevic Z, Quenby S. Natural killer cells and pregnancy outcomes in women with recurrent miscarriage and infertility: a systematic review. Hum Reprod. 2011 Aug;26(8):1971-80.

33. Christiansen OB, Kolte AM, Dahl M, Larsen EC, Steffensen R, Nielsen HS, Hviid TV. Maternal homozygocity for a 14 base pair insertion in exon 8 of the HLA-G gene and carriage of HLA class II alleles restricting HY immunity predispose to unexplained secondary recurrent miscarriage and low birth weight in children born to these patients. Hum Immunol. 2012 Jul;73(7):699-705.

34. Cocksedge KA, Saravelos SH, Metwally M, Li TC. How common is polycystic ovary syndrome in recurrent miscarriage? Reprod Biomed Online. 2009 Oct;19(4):572-6.

35. Chan YY, Jayaprakasan K, Zamora J, Thornton JG, Raine-Fenning N, Coomarasamy A. The prevalence of congenital uterine anomalies in unselected and high-risk populations: a systematic review. Hum Reprod Update. 2011 Nov-Dec;17(6):761-71.

36. Chan YY, Jayaprakasan K, Tan A, Thornton JG, Coomarasamy A, Raine-Fenning NJ. Reproductive outcomes in women with congenital uterine anomalies: a systematic review. Ultrasound Obstet Gynecol. 2011 Oct;38(4):371-82.

37. Rull K, Christiansen OB, Nagirnaja L, Steffensen R, Margus T, Laan M. A modest but significant effect of CGB5 gene promoter polymorphisms in modulating the risk of recurrent miscarriage. Fertil Steril. 2013 Jun;99(7):1930-6.e6.

38. Daher S, Mattar R, Gueuvoghlanian-Silva BY, Torloni MR. Genetic polymorphisms and recurrent spontaneous abortions: an overview of current knowledge. Am J Reprod Immunol. 2012 Apr;67(4):341-7.

39. Metwally M, Saravelos SH, Ledger WL, Li TC. Body mass index and risk of miscarriage in women with recurrent miscarriage. Fertil Steril. 2010 Jun;94(1):290-5.

40. Boots C, Stephenson MD. Does obesity increase the risk of miscarriage in spontaneous conception: a systematic review. Semin Reprod Med. 2011 Nov;29(6):507-13.

41. Practice Committee of tAmerican Society for Reproductive Medicine. Definitions of infertility and recurrent pregnancy loss. Fertil Steril. 2008 Nov;90(5 Suppl):S60.

42. Coulam CB, Clark DA, Beer AE, Kutteh WH, Silver R, Kwak J, Stephenson M. Current clinical options for diagnosis and treatment of recurrent spontaneous abortion. Clinical Guidelines

Recommendation Committee for Diagnosis and Treatment of Recurrent Spontaneous Abortion. Am J Reprod Immunol. 1997 Aug;38(2):57-74.

43. Olmos-Ortiz A, García-Quiroz J, Avila E, Caldiño-Soto F, Halhali A, Larrea F, Díaz L. Lipopolysaccharide and cAMP modify placental calcitriol biosynthesis reducing antimicrobial peptides gene expression. Am J Reprod Immunol. 2018 Jun;79(6):e12841.

44. Duval C, Brien ME, Gaudreault V, Boufaied I, Baker B, Jones RL, Girard S. Differential effect of LPS and IL-1β in term placental explants. Placenta. 2019 Jan;75:9-15.

45. Olmos-Ortiz A, Déciga-García M, Preciado-Martínez E, Bermejo-Martínez L, Flores-Espinosa P, Mancilla-Herrera I, Irles C, Helguera-Repetto AC, Quesada-Reyna B, Goffin V, Díaz L, Zaga-Clavellina V. Prolactin decreases LPS-induced inflammatory cytokines by inhibiting TLR-4/NFκB signaling in the human placenta. Mol Hum Reprod. 2019 Oct 28;25(10):660-667.

46. Lash GE. Molecular Cross-Talk at the Feto-Maternal Interface. Cold Spring Harb Perspect Med. 2015 Sep 18;5(12):a023010.

47. Saito S, Nishikawa K, Morii T, Narita N, Enomoto M, Ichijo M. Expression of activation antigens CD69, HLA-DR, interleukin-2 receptor-alpha (IL-2R alpha) and IL-2R beta on T cells of human decidua at an early stage of pregnancy. Immunology. 1992 Apr;75(4):710-2.

48. Zenclussen AC, Gerlof K, Zenclussen ML, Sollwedel A, Bertoja AZ, Ritter T, Kotsch K, Leber J, Volk HD. Abnormal T-cell reactivity against paternal antigens in spontaneous abortion: adoptive transfer of pregnancy-induced CD4+CD25+ T regulatory cells prevents fetal rejection in a murine abortion model. Am J Pathol. 2005 Mar;166(3):811-22.

49. Shima T, Sasaki Y, Itoh M, Nakashima A, Ishii N, Sugamura K, Saito S. Regulatory T cells are necessary for implantation and maintenance of early pregnancy but not late pregnancy in allogeneic mice. J Reprod Immunol. 2010 Jun;85(2):121-9.

50. Aluvihare VR, Kallikourdis M, Betz AG. Regulatory T cells mediate maternal tolerance to the fetus. Nat Immunol. 2004 Mar;5(3):266-71.

51. Sasaki Y, Sakai M, Miyazaki S, Higuma S, Shiozaki A, Saito S. Decidual and peripheral blood CD4+CD25+ regulatory T cells in early pregnancy subjects and spontaneous abortion cases. Mol Hum Reprod. 2004 May;10(5):347-53.

52. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol. 1995 Aug 1;155(3):1151-64.

53. Tsuda S, Nakashima A, Shima T, Saito S. New Paradigm in the Role of Regulatory T Cells During Pregnancy. Front Immunol. 2019 Mar 26;10:573.

54. Lee JY, Lee M, Lee SK. Role of endometrial immune cells in implantation. Clin Exp Reprod Med. 2011 Sep;38(3):119-25.

55. Pazos M, Sperling RS, Moran TM, Kraus TA. The influence of pregnancy on systemic immunity. Immunol Res. 2012 Dec;54(1-3):254-61.

56. Challis JR, Lockwood CJ, Myatt L, Norman JE, Strauss JF 3rd, Petraglia F. Inflammation and pregnancy. Reprod Sci. 2009 Feb;16(2):206-15.

57. Salamonsen LA, Hannan NJ, Dimitriadis E. Cytokines and chemokines during human embryo implantation: roles in implantation and early placentation. Semin Reprod Med. 2007 Nov;25(6):437-44.

58. Yuan J, Li J, Huang SY, Sun X. Characterization of the subsets of human NKT-like cells and the expression of Th1/Th2 cytokines in patients with unexplained recurrent spontaneous abortion. J Reprod Immunol. 2015 Aug;110:81-8.

59. Saito S, Nakashima A, Shima T, Ito M. Th1/Th2/Th17 and regulatory T-cell paradigm in pregnancy. Am J Reprod Immunol. 2010 Jun;63(6):601-10.

60. Kheshtchin N, Gharagozloo M, Andalib A, Ghahiri A, Maracy MR, Rezaei A. The expression of Th1- and Th2-related chemokine receptors in women with recurrent miscarriage: the impact of lymphocyte immunotherapy. Am J Reprod Immunol. 2010 Aug 1;64(2):104-12.

61. Nakagawa K, Kwak-Kim J, Ota K, Kuroda K, Hisano M, Sugiyama R, Yamaguchi K. Immunosuppression with tacrolimus improved reproductive outcome of women with repeated implantation failure and elevated peripheral blood TH1/TH2 cell ratios. Am J Reprod Immunol. 2015 Apr;73(4):353-61.

62. Mjösberg J, Berg G, Jenmalm MC, Ernerudh J. FOXP3+ regulatory T cells and T helper 1, T helper 2, and T helper 17 cells in human early pregnancy decidua. Biol Reprod. 2010 Apr;82(4):698-705.

63. Erlebacher A. Immunology of the maternal-fetal interface. Annu Rev Immunol. 2013;31:387-411.

64. Tilburgs T, Roelen DL, van der Mast BJ, van Schip JJ, Kleijburg C, de Groot-Swings GM, Kanhai HH, Claas FH, Scherjon SA. Differential distribution of CD4(+)CD25(bright) and CD8(+)CD28(-) T-cells in decidua and maternal blood during human pregnancy. Placenta. 2006 Apr;27 Suppl A:S47-53.

65. Trussell J, Lalla AM, Doan QV, Reyes E, Pinto L, Gricar J. Cost effectiveness of contraceptives in the United States. Contraception. 2009 Jan;79(1):5-14.

66. rowsdale J, Betz AG. Mother's little helpers: mechanisms of maternal-fetal tolerance. Nat Immunol. 2006 Mar;7(3):241-6.

67. Liu S, Diao L, Huang C, Li Y, Zeng Y, Kwak-Kim JYH. The role of decidual immune cells on human pregnancy. J Reprod Immunol. 2017 Nov;124:44-53.

68. Faas MM, de Vos P. Uterine NK cells and macrophages in pregnancy. Placenta. 2017 Aug;56:44-52.

69. van Furth R. Macrophage activity and clinical immunology. Origin and kinetics of mononuclear phagocytes. Ann N Y Acad Sci. 1976;278:161-75.

70. Gordon S, Taylor PR. Monocyte and macrophage heterogeneity. Nat Rev Immunol. 2005 Dec;5(12):953-64.

71. Lampé R, Kövér Á, Szűcs S, Pál L, Árnyas E, Ádány R, Póka R. Phagocytic index of neutrophil granulocytes and monocytes in healthy and preeclamptic pregnancy. J Reprod Immunol. 2015 Feb;107:26-30.

72. Care AS, Diener KR, Jasper MJ, Brown HM, Ingman WV, Robertson SA. Macrophages regulate corpus luteum development during embryo implantation in mice. J Clin Invest. 2013 Aug;123(8):3472-87.

73. Gautier EL, Yvan-Charvet L. Understanding macrophage diversity at the ontogenic and transcriptomic levels. Immunol Rev. 2014 Nov;262(1):85-95.

74. Wynn TA, Chawla A, Pollard JW. Macrophage biology in development, homeostasis and disease. Nature. 2013 Apr 25;496(7446):445-55.

75. Colin S, Chinetti-Gbaguidi G, Staels B. Macrophage phenotypes in atherosclerosis. Immunol Rev. 2014 Nov;262(1):153-66.

76. Italiani P, Boraschi D. From Monocytes to M1/M2 Macrophages: Phenotypical vs. Functional Differentiation. Front Immunol. 2014 Oct 17;5:514.

77. Mills CD, Kincaid K, Alt JM, Heilman MJ, Hill AM. M-1/M-2 macrophages and the Th1/Th2 paradigm. J Immunol. 2000 Jun 15;164(12):6166-73.

78. Renaud SJ, Graham CH. The role of macrophages in utero-placental interactions during normal and pathological pregnancy. Immunol Invest. 2008;37(5):535-64.

79. Mosmann TR, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu Rev Immunol. 1989;7:145-73.

80. Guenther S, Vrekoussis T, Heublein S, Bayer B, Anz D, Knabl J, Navrozoglou I, Dian D, Friese K, Makrigiannakis A, Jeschke U. Decidual macrophages are significantly increased in spontaneous miscarriages and over-express FasL: a potential role for macrophages in trophoblast apoptosis. Int J Mol Sci. 2012;13(7):9069-80.

81. Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol. 2004 Dec;25(12):677-86.

82. Ning F, Liu H, Lash GE. The Role of Decidual Macrophages During Normal and Pathological Pregnancy. Am J Reprod Immunol. 2016 Mar;75(3):298-309.

83. Renaud SJ, Graham CH. The role of macrophages in utero-placental interactions during normal and pathological pregnancy. Immunol Invest. 2008;37(5):535-64.

84. Chapoval S, Dasgupta P, Dorsey NJ, Keegan AD. Regulation of the T helper cell type 2 (Th2)/T regulatory cell (Treg) balance by IL-4 and STAT6. J Leukoc Biol. 2010 Jun;87(6):1011-8.

85. Faas MM, Spaans F, De Vos P. Monocytes and macrophages in pregnancy and pre-eclampsia. Front Immunol. 2014 Jun 30;5:298.

86. Nagamatsu T, Schust DJ. The contribution of macrophages to normal and pathological pregnancies. Am J Reprod Immunol. 2010 Jun;63(6):460-71.

87. Jones LA, Anthony JP, Henriquez FL, Lyons RE, Nickdel MB, Carter KC, Alexander J, Roberts CW. Toll-like receptor-4-mediated macrophage activation is differentially regulated by progesterone via the glucocorticoid and progesterone receptors. Immunology. 2008 Sep;125(1):59-69.

88. Su L, Sun Y, Ma F, Lü P, Huang H, Zhou J. Progesterone inhibits Toll-like receptor 4-mediated innate immune response in macrophages by suppressing NF-kappaB activation and enhancing SOCS1 expression. Immunol Lett. 2009 Aug 15;125(2):151-5.

89. Youssef RE, Ledingham MA, Bollapragada SS, O'Gorman N, Jordan F, Young A, Norman JE. The role of toll-like receptors (TLR-2 and -4) and triggering receptor expressed on myeloid cells 1 (TREM-1) in human term and preterm labor. Reprod Sci. 2009 Sep;16(9):843-56.

90. Mantovani A, Sica A, Locati M. Macrophage polarization comes of age. Immunity. 2005 Oct;23(4):344-6.

91. Schonkeren D, van der Hoorn ML, Khedoe P, Swings G, van Beelen E, Claas F, van Kooten C, de Heer E, Scherjon S. Differential distribution and phenotype of decidual macrophages in preeclamptic versus control pregnancies. Am J Pathol. 2011 Feb;178(2):709-17.

92. Tsao FY, Wu MY, Chang YL, Wu CT, Ho HN. M1 macrophages decrease in the deciduae from normal pregnancies but not from spontaneous abortions or unexplained recurrent spontaneous abortions. J Formos Med Assoc. 2018 Mar;117(3):204-211.

93. Guenther S, Vrekoussis T, Heublein S, Bayer B, Anz D, Knabl J, Navrozoglou I, Dian D, Friese K, Makrigiannakis A, Jeschke U. Decidual macrophages are significantly increased in spontaneous miscarriages and over-express FasL: a potential role for macrophages in trophoblast apoptosis. Int J Mol Sci. 2012;13(7):9069-80.

94. Hunt JS, McIntire RH, Petroff MG. Immunobiology of human pregnancy. In: *Knobil and Neill's Physiology of Reproduction*.; 2006. (2):2759-2785

95. Varol C, Mildner A, Jung S. Macrophages: development and tissue specialization. Annu Rev Immunol. 2015;33:643-75.

96. Moffett A. Immune cells in the placental bed. In: Pijnenborg R, Brosens I, Romero R, editors. Placental Bed Disorders: Basic Science and its Translation to Obstetrics. Cambridge: Cambridge University Press; 2010. p. 41–51.

97. Williams PJ, Bulmer JN, Searle RF, Innes BA, Robson SC. Altered decidual leucocyte populations in the placental bed in pre-eclampsia and foetal growth restriction: a comparison with late normal pregnancy. Reproduction. 2009 Jul;138(1):177-84.

98. Daoud G, Simoneau L, Masse A, Rassart E, Lafond J. Expression of cFABP and PPAR in trophoblast cells: effect of PPAR ligands on linoleic acid uptake and differentiation. Biochim Biophys Acta. 2005 Feb 21;1687(1-3):181-94.

99. Schaiff WT, Carlson MG, Smith SD, Levy R, Nelson DM, Sadovsky Y. Peroxisome proliferator-activated receptor-gamma modulates differentiation of human trophoblast in a ligand-specific manner. J Clin Endocrinol Metab. 2000 Oct;85(10):3874-81.

100. Tarrade A, Schoonjans K, Pavan L, Auwerx J, Rochette-Egly C, Evain-Brion D, Fournier T. PPARgamma/RXRalpha heterodimers control human trophoblast invasion. J Clin Endocrinol Metab. 2001 Oct;86(10):5017-24.

101. Waite LL, Louie RE, Taylor RN. Circulating activators of peroxisome proliferator-activated receptors are reduced in preeclamptic pregnancy. J Clin Endocrinol Metab. 2005 Feb;90(2):620-6.

102. Wang Q, Fujii H, Knipp GT. Expression of PPAR and RXR isoforms in the developing rat and human term placentas. Placenta. 2002 Sep-Oct;23(8-9):661-71.

103. Kadam L, Kohan-Ghadr HR, Drewlo S. The balancing act - PPAR-γ's roles at the maternal-fetal interface. Syst Biol Reprod Med. 2015 Apr;61(2):65-71.

104. Rosen ED, Sarraf P, Troy AE, Bradwin G, Moore K, Milstone DS, Spiegelman BM, Mortensen RM. PPAR gamma is required for the differentiation of adipose tissue in vivo and in vitro. Mol Cell. 1999 Oct;4(4):611-7.

105. Tontonoz P, Hu E, Spiegelman BM. Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid-activated transcription factor. Cell. 1994 Dec 30;79(7):1147-56.

106. Tontonoz P, Nagy L, Alvarez JG, Thomazy VA, Evans RM. PPARgamma promotes monocyte/macrophage differentiation and uptake of oxidized LDL. Cell. 1998 Apr 17;93(2):241-52.

107. Tontonoz P, Spiegelman BM. Fat and beyond: the diverse biology of PPARgamma. Annu Rev Biochem. 2008;77:289-312.

108. Jiang C, Ting AT, Seed B. PPAR-gamma agonists inhibit production of monocyte inflammatory cytokines. Nature. 1998 Jan 1;391(6662):82-6.

109. Lappas M, Permezel M, Georgiou HM, Rice GE. Regulation of proinflammatory cytokines in human gestational tissues by peroxisome proliferator-activated receptor-gamma: effect of 15-deoxy-Delta(12,14)-PGJ(2) and troglitazone. J Clin Endocrinol Metab. 2002 Oct;87(10):4667-72.

110. Kliman HJ, Nestler JE, Sermasi E, Sanger JM, Strauss JF 3rd. Purification, characterization, and in vitro differentiation of cytotrophoblasts from human term placentae. Endocrinology. 1986 Apr;118(4):1567-82.

111. Pijnenborg R, Luyten C, Vercruysse L, Van Assche FA. Attachment and differentiation in vitro of trophoblast from normal and preeclamptic human placentas. Am J Obstet Gynecol. 1996 Jul;175(1):30-6.

112. Koga K, Izumi G, Mor G, Fujii T, Osuga Y. Toll-like receptors at the maternal-fetal interface in normal pregnancy and pregnancy complications. Am J Reprod Immunol. 2014 Aug;72(2):192-205.

113. Shih IM, Seidman JD, Kurman RJ. Placental site nodule and characterization of distinctive types of intermediate trophoblast. Hum Pathol. 1999 Jun;30(6):687-94.

114. Pramanick A, Hwang WS, Mathur M. Placental site nodule (PSN): an uncommon diagnosis with a common presentation. BMJ Case Rep. 2014 Apr 2;2014:bcr2013203086.

115. Cierna Z, Varga I, Danihel L Jr, Kuracinova K, Janegova A, Danihel L. Intermediate trophoblast--A distinctive, unique and often unrecognized population of trophoblastic cells. Ann Anat. 2016 Mar;204:45-50.

116. Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, Birdwell D, Alejos E, Silva M, Galanos C, Freudenberg M, Ricciardi-Castagnoli P, Layton B, Beutler B. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science. 1998 Dec 11;282(5396):2085-8.

117. Li P, Wu HL, Dong BH. Relationship between TLR4 and CCL2 expression and recurrent spontaneous abortion. Genet Mol Res. 2016 Jan 8;15(1).

118. Li XP, Song LN, Tian LP, Zhang YS. Relationship between TLR4 and MCP2 expression levels and habitual abortion. Genet Mol Res. 2016 Apr 25;15(2).

119. Abrahams VM, Schaefer TM, Fahey JV, Visintin I, Wright JA, Aldo PB, Romero R, Wira CR, Mor G. Expression and secretion of antiviral factors by trophoblast cells following stimulation by the TLR-3 agonist, Poly(I : C). Hum Reprod. 2006 Sep;21(9):2432-9.

120. Fest S, Aldo PB, Abrahams VM, Visintin I, Alvero A, Chen R, Chavez SL, Romero R, Mor G. Trophoblast-macrophage interactions: a regulatory network for the protection of pregnancy. Am J Reprod Immunol. 2007 Jan;57(1):55-66.

121. Kolben TM, Rogatsch E, Hester A, Kuhn C, Schmoeckel E, Czogalla B, Mahner S, Jeschke U, Kolben T. Involvement of ILR4α and TLR4 in miscarriages. J Reprod Immunol. 2019 Feb;131:36-43.

122. Murata T, Noguchi PD, Puri RK. IL-13 induces phosphorylation and activation of JAK2 Janus kinase in human colon carcinoma cell lines: similarities between IL-4 and IL-13 signaling. J Immunol. 1996 Apr 15;156(8):2972-8.

123. Bryant AH, Spencer-Harty S, Owens SE, Jones RH, Thornton CA. Interleukin 4 and interleukin 13 downregulate the lipopolysaccharide-mediated inflammatory response by human gestation-associated tissues. Biol Reprod. 2017 Mar 1;96(3):576-586.

124. Herbert JM, Savi P, Laplace MC, Lale A. IL-4 inhibits LPS-, IL-1 beta- and TNF alpha-induced expression of tissue factor in endothelial cells and monocytes. FEBS Lett. 1992 Sep 21;310(1):31-3.

125. Berkman N, John M, Roesems G, Jose P, Barnes PJ, Chung KF. Interleukin 13 inhibits macrophage inflammatory protein-1 alpha production from human alveolar macrophages and monocytes. Am J Respir Cell Mol Biol. 1996 Sep;15(3):382-9.

126. Standiford TJ, Kunkel SL, Liebler JM, Burdick MD, Gilbert AR, Strieter RM. Gene expression of macrophage inflammatory protein-1 alpha from human blood monocytes and alveolar macrophages is inhibited by interleukin-4. Am J Respir Cell Mol Biol. 1993 Aug;9(2):192-8.

127. Essner R, Rhoades K, McBride WH, Morton DL, Economou JS. IL-4 down-regulates IL-1 and TNF gene expression in human monocytes. J Immunol. 1989 Jun 1;142(11):3857-61.

128. Jiang H, Harris MB, Rothman P. IL-4/IL-13 signaling beyond JAK/STAT. J Allergy Clin Immunol. 2000 Jun;105(6 Pt 1):1063-70.

129. Ouyang W, Ranganath SH, Weindel K, Bhattacharya D, Murphy TL, Sha WC, Murphy KM. Inhibition of Th1 development mediated by GATA-3 through an IL-4-independent mechanism. Immunity. 1998 Nov;9(5):745-55.

130. Halasz M, Polgar B, Berta G, Czimbalek L, Szekeres-Bartho J. Progesterone-induced blocking factor differentially regulates trophoblast and tumor invasion by altering matrix metalloproteinase activity. Cell Mol Life Sci. 2013 Dec;70(23):4617-30.

131. Racicot K, Kwon JY, Aldo P, Silasi M, Mor G. Understanding the complexity of the immune system during pregnancy. Am J Reprod Immunol. 2014 Aug;72(2):107-16.

8. ABBREVIATIONS

IUGR	Intrauterine growth retardation
EVT	Extravillous trophoblast cells
IVT	Intermediate villous trophoblast
VT	Villous trophoblast
NK	Natural killer cells
Treg cells	Regulatory T cells
Th1	T-helper cells 1
Th2	T-helper cells 2
Th17	T-helper cells 17
IFN- γ	Interferon- γ
TNF-α	Tumor necrosis factor-α
IL-2	Interleukin 2
IL-4,	Interleukin 4
IL4Rα	Interleukin 4 receptor alpha
IL-6	Interleukin 6
IL-10	Interleukin 10
IL-23	Interleukin 13
IL13Rα1	Interleukin 13 receptor α1
LPS	Lipopolysaccharide
TLR4	Toll-like-receptor 4
TLR2	Toll-like-receptor 2
iNOS	Inducible Nitric Oxide Synthase
PGE2	Prostaglandin E2
IDO	Indoleamine 2,3-dioxygenase
CX3CR1	Chemokine receptor 1
CCL1	Chemokine C-C Motif Ligand 1
PPARα	Peroxisome proliferator-activated receptor-alpha
PPARβ	Peroxisome proliferator-activated receptor-beta
PPARγ	Peroxisome proliferator-activated receptor-gamma
CD4	Cluster of Differentiation 4
CD8	Cluster of Differentiation 8
CD14	Cluster of Differentiation 14
CD68	Cluster of Differentiation 68
MHC class II	Major Histocompatibility complex II
HLA-DR	Human Leukocyte Antigen DR isotype
MIP1A	Macrophage inflammatory protein 1A
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
STAT6	Signal Transducer and activator of transcription 6
GATA3	GATA binding protein 3