



Involvement of IL4Rα and TLR4 in miscarriages

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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 be 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,

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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- κ B 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 IL4R α and TLR4 at the fetomaternal interface in the process of early pregnancy.

IL4R α 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 μ l 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 μ l, in a 32-cycle program). DNA profiling was performed by addition of 300 pg DNA for each step. Analysis of the PCR products was

Table 1

Demographic and Clinical Characteristics of the Study population. Values are Mean \pm 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 \pm 8.06 (18.7-43.3)	37.8 \pm 4.51 (29.2-43.2)	35.76 \pm 4.8 (29.5- 46.9)	0.049
Gestational age (weeks)	9.53 \pm 1.95 (7-13)	8.4 \pm 1.89 (4-12)	9.3 \pm 1.49 (7-12)	0.276
Gravidity	4 \pm 1.8 (1-7)	3.1 \pm 1.1 (2-5)	1.6 \pm 0.9 (1-4)	0.001
Parity	2 \pm 1.1 (0-4)	0.9 \pm 0.8 (0-2)	0.3 \pm 0.6 (0-2)	0.003

* Mean, standard deviation, range.

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).

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 μ m) 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% H₂O₂ 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
IL4R α	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

IL4Rα syncytiotrophoblast

TLR4 intermediate villous trophoblast

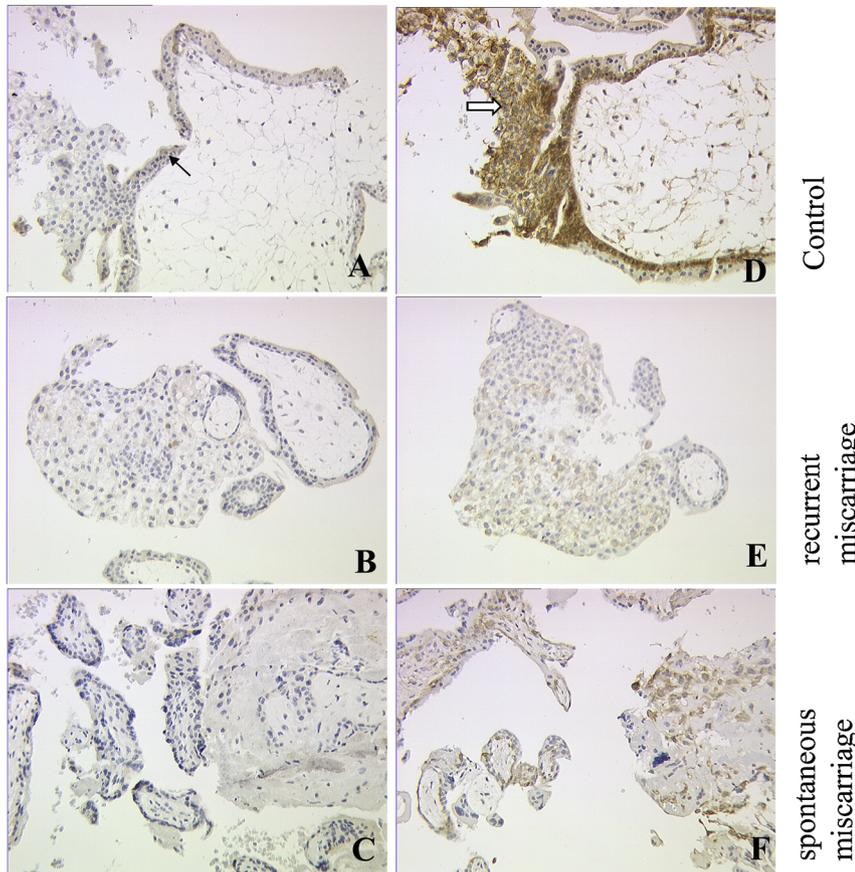


Fig. 1. Immunohistochemical staining of IL4Rα and TLR4 in the trophoblast: syncytiotrophoblast (marked by a black arrow) and intermediate villous trophoblast (marked by a white arrow).

(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 their 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 heterogenous 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, $1 \leq 10\%$, $2 = 11\text{--}50\%$, $3 = 51\text{--}80\%$ and $4 \geq 81\%$ 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
HLA G	mouse IgG1 Anti HLA G	monoclonal (MEM-6/9)	1:50 in Dako	R&D Systems, Inc. Minneapolis, USA 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

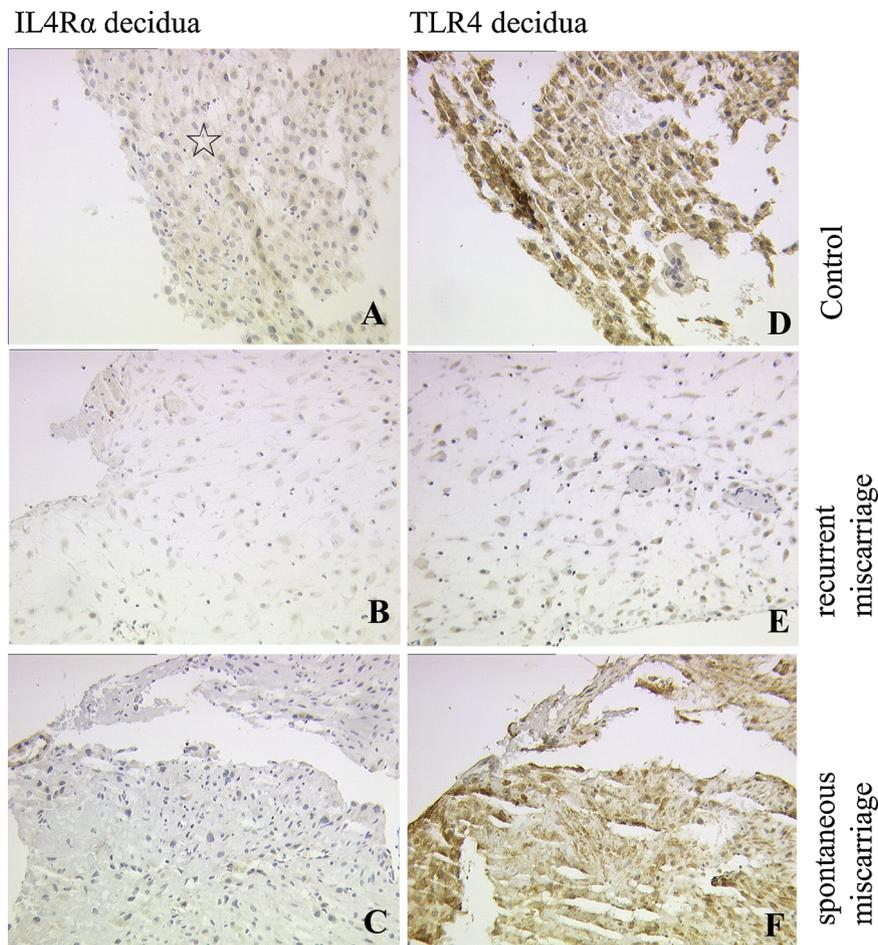


Fig. 2. Immunohistochemical staining of IL4R α and TLR4 in the decidua (marked by a star): (A) Strong staining of IL4R α in the control group represented by placenta of legally induced termination of healthy pregnancies. (B) Weaker staining of IL4R α in the decidua of recurrent miscarriages. (C) Significantly weaker staining of IL4R α 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 μ m)

performed in samples of healthy controls and spontaneous miscarriages. Double immunofluorescence was used to prove the localization of IL4R α 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-IL4R α 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 IL4R α and TLR4 with real time RT-PCR (Taq man)

2.4.1. RNA extraction from placental tissue

A total quantity of 4 \times 10 mg of control, 5 \times 10 mg of spontaneous and 6 \times 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 μ l for each reaction, containing 10 μ l TaqMan® Fast Universal PCR Master Mix 2x (Applied Biosystems, Nr. 4367846; 50 ml), 1 μ l cDNA, 8 μ l H₂O (DEPC treated DI water; Sigma, Taufkirchen, Germany)). Furthermore, the total consisted of 1 μ l TaqMan® Gene Expression Assay 20x (Hs_00166237_m1 for IL4R α 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- $\Delta\Delta$ CT 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. IL4R α expression in the trophoblast and the decidua

IL4R α was detected in trophoblast cells in the control group (healthy pregnancies), spontaneous miscarriages, and recurrent miscarriages (Fig. 1A-C). IL4R α 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 IL4R α 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 IL4R α and TLR4 are given in Box Plots in Fig. 3.

3.2. Double immunofluorescence

3.2.1. Identification of IL4R α expressing cells in the decidua

IL4R α positive cells were stained in green, cells expressing HLA-G were dyed in red. Cells neither expressing IL4R α nor HLA-G were stained in blue.

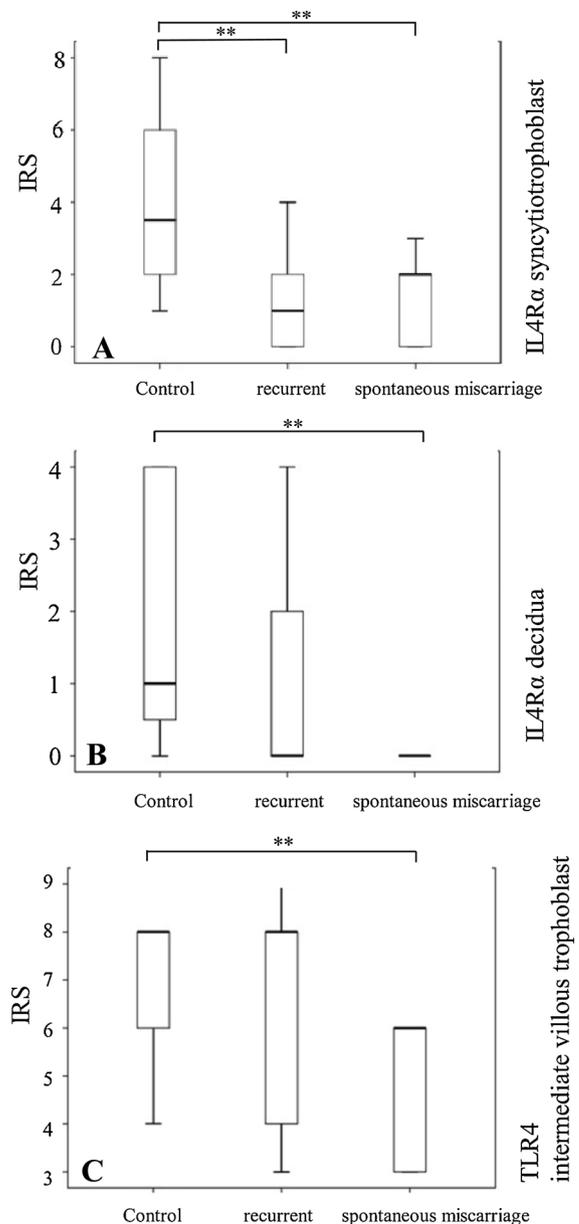


Fig. 3. Statistical results of the immunoreactive score of IL4R α and TLR4.

(A) IL4R α in the syncytiotrophoblast
 (B) IL4R α in the decidua
 (C) TLR4 in the intermediate villous trophoblast
 (* $p < 0.05$, ** $p < 0.005$)

IL4R α + HLA-G double immunofluorescence staining was used for labeling the expression of IL4R α in the decidua of spontaneous and recurrent miscarriages and control groups. IL4R α staining in decidual cells of the control group is presented in Fig. 4A. Fig. 4B presents the membrane HLA-G staining of EVT's from the same area. The depiction of IL4R α 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 IL4R α 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 IL4R α in the recurrent miscarriage group (Fig. 4F).

No IL4R α 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 IL4R α in spontaneous abortions (Fig. 4I).

In conclusion, we observed higher expression of IL4R α positive cells

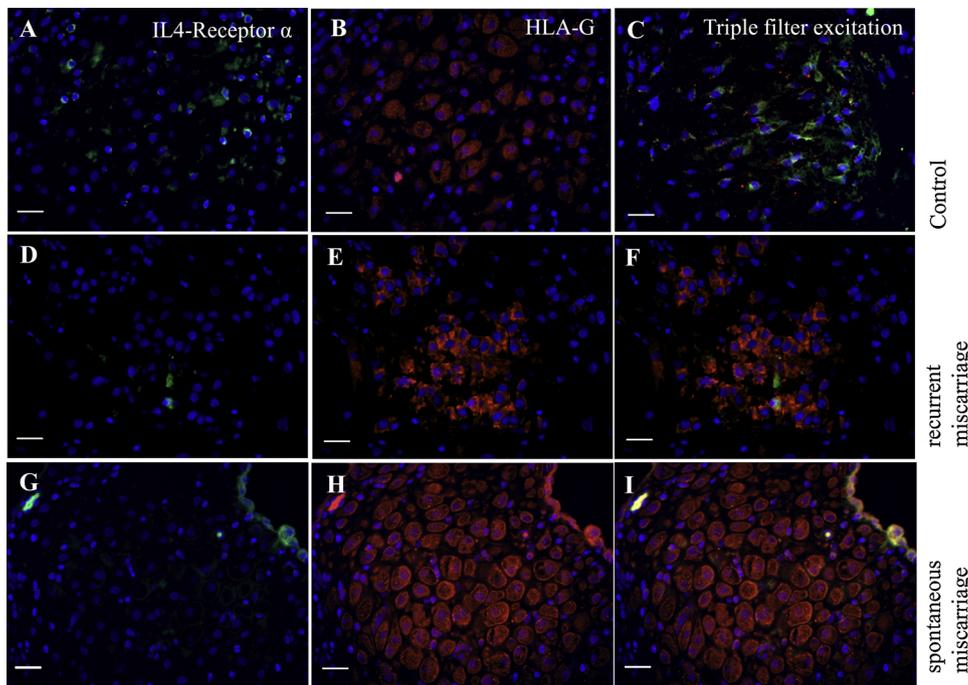


Fig. 4. Double Immunofluorescence of IL4Rα and HLA-G in the decidua. (A) IL4Rα expression (Cy2; green staining) is high in normal decidua. (B) HLA-G (Cy3; red staining) membrane staining of extravillous trophoblast cells (EVT). (C) Triple filter excitation identifying cells expressing both markers. The 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).

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 fetomaternal 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 fetomaternal 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

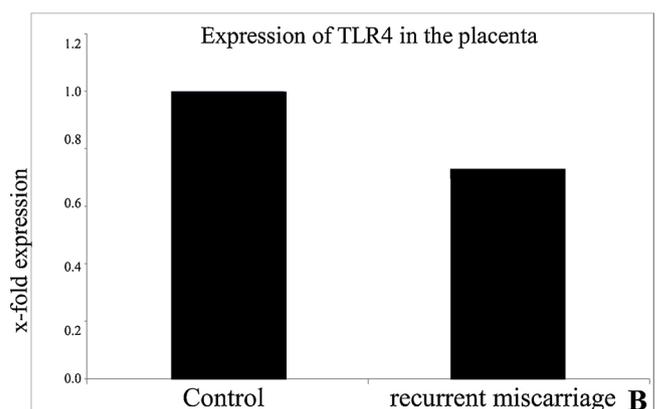
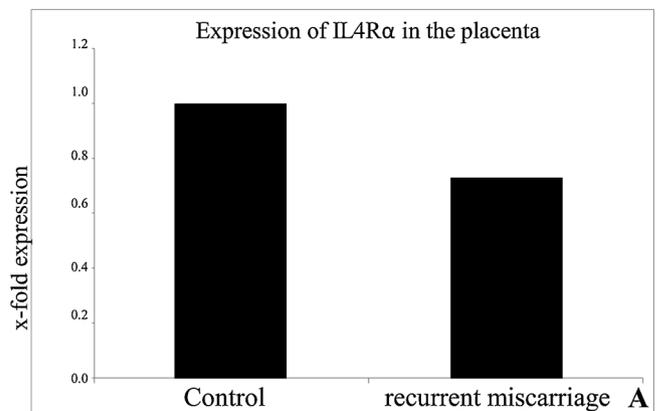


Fig. 5. RT-PCR Analysis of IL4Rα and TLR4 expression.

(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)

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 IL4R α in the human first trimester pregnancy decidua has been described before (Starkey, 1991). In our analysis IL4R α 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, IL4R α was downregulated in the decidua of spontaneous miscarriages. The expression on mRNA level was significantly decreased in recurrent miscarriages. IL4R α 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 IL4R α 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 increased in spontaneous miscarriages and that a proapoptotic state was present (Guenther et al., 2012). Halasz et al. reported IL4R α to be involved in the invasion capacity of trophoblast cells. A downregulation of IL4R α was observed to lead to a stimulation of the invasion of trophoblast cells (Halasz et al., 2013). The decreased expression of IL4R α , 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 IL4R α in trophoblast cells, we observed and an additional information to the T-cell response mentioned. IL4 induces tolerance, the reduced expression of the IL4R α on trophoblast cells could induce opposite reactions.

IL13, another ligand of IL4R α , 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 IL4R α 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.

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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>.

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