Selected aspects of the innate immune system in relation to preterm birth.

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Contents

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Key words

Innate immunity ° preterm birth ° Toll-like receptor ° Lactoferrin ° Defensin ° Uterine Natural Killer cell

Abstract

The prevention of uterine infection is critical for appropriate fetal development and term delivery. One of the major defense mechanisms against infection is the innate immune system. Since preterm and term delivery are induced by inflammatory processes, preterm activation of the inflammatory cascades can induce preterm rupture of the membranes (PROM) and preterm delivery. In this review we will focus on some of the components of the innate immune system that are involved in the protection of the pregnant uterus from infection, but that may also have a function in PROM and preterm delivery.

The family of Toll Like Receptors (TLRs) is a collection of receptors that are able to respond to a specific microbial component, so called pathogen-associated molecular patterns (PAMPs). They are important in the protection of the host to infectious agents.

Natural antimicrobials are prominent in the (pregnant-) uterus. Evidence suggests that aberrant production of natural antimicrobials may cause preterm birth. The function of two groups of natural antimicrobials, lactoferrin and defensins, in the process of preterm birth will be discussed.

Uterine Natural Killer (uNK) cells are abundantly present in the pregnant uterus. Upon activation they can induce the production of natural antimicrobials and pro-inflammatory cytokines. Evidence is increasing that uNK cells have a prominent role in the induction of preterm birth.

The balance between immune activation to protect the uterus from infections and immune inhibition to prevent preterm birth is important. Increased knowledge of this delicate balance might help to find new approaches in the prevention of preterm birth.

General introduction

Preterm birth is the leading cause of perinatal morbidity and mortality. In The Netherlands 6.6% of the total number of infants born during 2007 were born preterm (1). Preterm infants are at greater risk for short and long term complications like sepsis (2,3), neuro-developmental disabilities (4) and

respiratory problems (5) then term infants. Preterm birth is also strongly associated with high healthcare costs (6). The spontaneous onset of preterm birth and the premature rupture of the membranes are two separate processes. However, there is considerable evidence that the risk factors for both processes are similar (7) and many interventions target both of these conditions.
The precise mechanisms that cause preterm birth are still unknown, but several factors that might be involved in this process have been described: social background, a history of abortion and/or preterm birth and multiparity may increase the risk for preterm birth (7). However, infection and the associated immune responses have been proposed to be the major causes of preterm birth (8). It is therefore of great importance to unravel the exact inflammatory mechanisms that can cause preterm birth and preterm rupture of the membranes (PROM) and the processes that lead to their initiation.
Inflammation is usually a response to an infection with a pathogen but has also been implicated as an important mechanism in inducing both term and preterm delivery (9). Normal parturition involves the activation of a complex inflammatory cascade (10). When an infection is present in the uterus, preterm activation of this cascade can induce preterm delivery.
The amniotic cavity is a sterile environment, free of microbes. Bacterial vaginosis, characterized by a decreased concentration of lactobacilli and an overgrowth of bacterial species such as *Ureaplasma urealyticum*, *Mycoplasma hominis*, *Gardnerella vaginalis, Fusobacterium nucleatum* and various other anaerobic species, has been associated with upper genital tract inflammation and preterm delivery (11). A recent report describes that previously unrecognized, uncultivated, or difficult-to-cultivate bacteria may also play a key role in the initiation of preterm birth. This observation further underlines the role for infection and inflammation in preterm delivery (12). Although at present, little is known about the role of viruses as a cause of preterm delivery, recent reports suggest that viral infections can also increase the risk for preterm delivery (13).

An innate immune system is present in all living multicellular organisms and is considered to be the evolutionary older immune system, whereas the adaptive immune system developed later in phylogeny and is only present in vertebrates. The innate immune system acts rapidly and is important for activation of the adaptive immune system. It consists of both cell-associated and cell-free components and is capable of immediate recognition of microbes and microbial components (14,15).

During pregnancy, the placenta does not only serve as a mechanical barrier but numerous studies show that it is also an active functional barrier that has many immunological properties. Via intensive contact between the components of the innate and adaptive immune system the placenta has to distinguish self from non-self. The placenta is one of the most prominent body sites where there is a delicate balance between immune activation and suppression. Innate immune responses are reported to increase during pregnancy and enhanced or decreased activation may be correlated with preterm delivery (16).

In the present review we focus on four components of the innate immune system with particular relevance in relation to preterm birth: the Toll-like receptors, the antimicrobial peptides lactoferrin and defensins and Natural Killer (NK) cells.

The role of Toll-Like Receptors in preterm delivery

Toll-like receptors (TLRs) function as an important microbial detection system and are expressed on tissues in direct contact with the external environment and on cells of the immune system, such as leukocytes. They can recognize pathogen associated molecular patterns (PAMPs) and are therefore called Pattern Recognition Receptors (PRRs). In mammals, twelve different TLRs have been characterized. Each TLR responds to a particular ligand or set of ligands (17,18) and upon activation a cell signaling pathway is induced, leading to NF-κB upregulation and cytokine production (19).
The member of the TLR family that has been studied most extensively is TLR4. It reacts with and mediates responses towards bacterial lipopolysaccaride (LPS), a major component of the Gram-negative bacterial cell wall (20). Also TLR2 has been studied intensively; it induces cell-signaling upon binding with Gram-positive bacteria. Another important TLR-family member is TLR3, a receptor that induces cell-signaling upon recognition of double-stranded (ds) viral RNA. Although TLRs are essential in host defense to infection, over-activation may also have deleterious effects on the host. There is evidence for a role of TLRs in infection-induced preterm birth. In the present review we focus on the role of TLR2, 3, 4 and 10 in preterm birth.

Studies have shown that administration of LPS in mice upregulates mRNA levels of TLR2 and TLR4 in the fetal membranes. In the placenta, protein levels of TLR2 increased (21). LPS causes preterm birth, both in mice and humans (22,23). Additionally, it has been shown that TLR signaling is involved in LPS-induced preterm birth but also in preterm labor caused by *Escherichia coli* and *Fusobacterium nucleatum* (24-26). In addition, administration of a TLR2 ligand (peptidoglycan or lipoteichoic acid) is also able to induce preterm birth in mice. (27)
Viral infection may also play a role in chorioamnionitis and just recently it has been found that TLR3 is expressed on cells of the trophoblast. Production of cytokines and chemokines in response to *in vitro* activation of TLR3 suggests that TLR3 has a prominent role in the regulation of immune responses towards viral infections in the trophoblast (13,28)

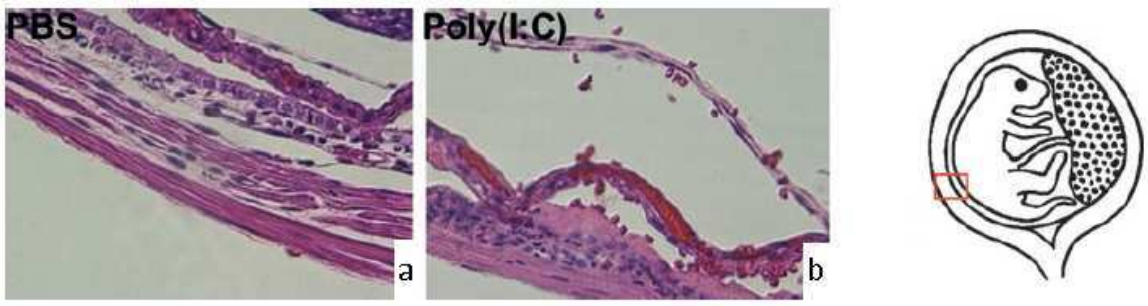

Figure 1) *Poly[I:C] injection induces morphological changes at the maternal-fetal interface*. Pregnant mice were sacrificed 4 hrs after LPS or PBS admission. Hematoxylin and eosin staining was performed on the obtained tissues. (a) fetal-maternal interface from a wild-type mouse that had been injected i.p with PBS, (magnification 400x) (b) fetal-maternal interface from a wild-type mouse that had been injected i.p. with 4,5 mg/kg Poly[I:C] (magnification 400x). Pictures are adapted from (13).

It has been shown that intra peritoneal (i.p) injections with Poly [I:C], a synthetic analog of viral RNA, induce preterm birth (13,27) and changes in the morphology of the maternal-fetal interface in mice (Figure 1). Additionally, Poly [I:C] administration induces inflammation, necrosis, hemorrhage and edema in the deciduas, the yolk sac membrane, chorion and the amniotic membrane. Observations

that Poly [I:C] administration is not able to induce these changes in TLR3 knockout mice demonstrates that preterm birth, following Poly [I:C] administration, is induced in a TLR3 dependent manner (13).
In trophoblast cells from wt-mice, administration of Poly [I:C] induced upregulation of NF-κB and production of pro-inflammatory cytokines and chemokines, known to be involved in preterm delivery. These levels were significantly lower in TLR3 knockout mice, indicating that a TLR3 cell-signaling pathway is involved in these anti-inflammatory responses. *In vitro* experiments with human throphoblast cells confirm the induction of pro-inflammatory cytokines and chemokines upon Poly [I:C] stimulation .
In addition to the observations that administration of TLR2, TLR3 and TLR4 ligands were able to induce preterm birth, a recent experiment showed that administration of CpG, a TLR9 ligand, was able to induce preterm birth in IL-10 knock-out mice but not in wild-type mice. This suggests that TLR9 activation is also able to induce preterm birth but that IL-10 can dampen this response (29).

Epidemiological studies suggest that genetic factors might also be involved in preterm birth. For example, a mother born before 30 weeks gestation has a 2.4-fold increased risk to deliver a preterm infant then mothers born at term (30). In addition, ethnic backgrounds have been found to be an important indication for the risk of preterm delivery. These data implicate that preterm delivery is a complex problem and that besides (the responses towards-) environmental components also genetic factors may be involved. There is a number of studies that report several polymorphisms that might be associated with preterm birth. Some of these polymorphisms, in relation to TLRs, will shortly be discussed here.
TLR polymorphisms have been associated with the development of a variety of diseases and there are indications that several are associated with preterm birth. The first polymorphism that was described to be related with premature birth was the Asp299Gly polymorphism, a variant of the human TLR4 gene. In Finland a significant higher frequency of this polymorphism was found in preterm infants than in term infants. (31). However, in the South-American and German population the relationship was observed but did not reach significant values (32,33). Since it has been described that monocytes, heterozygous for the Asp299Gly mutation in TLR4 show no difference in signaling upon LPS recognition (34), it is debatable whether a significant correlation is expected.
TLR2 is involved in recognition of Gram-positive bacteria such as Group B streptococci. Upon LPS stimulation TLR2 mRNA expression in the uterus, cervix and placenta is upregulated. The Finnish study by Lorenz *et al.* and a study in the Dutch population found no significant relation between preterm birth and the Arg753Gln TLR2 polymorphism. However, when this polymorphism was combined with the T16934A TLR2 polymorphism a significant effect on gestational age was observed (31,35).
Finally, genetic analysis in the German population of TLR10 showed no significant association between a single polymorphism and preterm birth. However, a haplotype of TLR10 was strongly associated with preterm birth. This final study implicates that although single polymorphisms are sometimes associated with preterm birth, haplotype analyses may give further information on the role of genetic factors in preterm birth (36).

Antimicrobial peptides.

Besides cellular components, several cell-free components of the innate immune system play a role in preterm birth. These cell-free components are proteins and other molecules, able to kill microbes that have not (yet) been engulfed by cells; they function like natural antibiotics. Here we will discuss two groups of antimicrobial peptides. Antimicrobial peptides are peptides with a maximum length of 100 amino acids, involved in host defense. Lactoferrin is an example of a peptide with antimicrobial properties that functions extracellulary. Defensin is another antimicrobial peptide that functions both intra- and extracellulary. It can be produced by epithelial cells and can therefore be found in a wide range of tissues, including the uterus. The role of lactoferrin and defensins in preterm birth will be discussed in the following two paragraphs.

<u>The role of lactoferrin in preterm delivery</u>

Lactoferrin is an iron-binding glycoprotein that is synthesized by glandular epithelial cells and is present in most secreted fluids present at human mucosal surfaces (37). It is demonstrated to be present in specific granules of polymorphonuclear leukocytes and known to have antimicrobial functions. Lactoferrin is able to induce cell-membrane destruction (38) and down-regulate the production of (LPS induced-) inflammatory cytokines (39). And finally, lactoferrin has been described to be involved in the activation of macrophages (40) and Natural killer cells (41) (Figure 2).

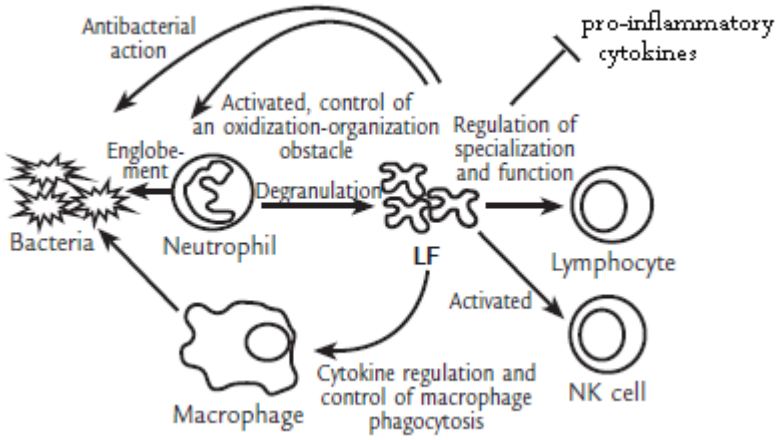

Figure 2. Representation of various functions of lactoferrin {{367 Otsuki,K. 2005}}.

Lactoferrin is also found in cervical mucus and amniotic fluid. Otsuki *et al.* measured the concentration of lactoferrin and IL-6 in amniotic fluid, obtained via trans-abdominal amnio-centesis or during delivery, in a group of pregnant women (28-32 weeks gestation) with chorioamnionitis and a control-group of women (32-39 weeks gestation) without chorioamnionitis. Both IL-6 and lactoferrin levels were significantly higher in the chorioamnionitis-group. Experiments with cultured human amnion cells revealed that lactoferrin was able to inhibit LPS-induced IL-6 production (42). These experiments suggest that lactoferrin production is used as a self-defense mechanism against intra-uterine infection.

Preterm birth can be induced in mice by repeated i.p.- injections of *Escherichia coli* -LPS (23). Administration of recombinant human lactoferrin to mice and rabbits can extend the duration of pregnancy and increase fetal survival. Lactoferrin administration resulted in decreased levels of the LPS-induced plasma IL-6 and TNF-α. Therefore it was concluded that Lactoferrin probably has antibacterial and anti-inflammatory properties. These two functions make lactoferrin an important inhibitor of preterm birth (43,44).

Additional research in a rabbit model showed that the administration of lactoferrin also resulted in reduced levels of Matrix Metalloproteinase (MMP) levels; since MMP levels are a marker for cervical maturation (45), these results imply that lactoferrin is able to decrease cervical maturation (46). Taken together, these results suggest that lactoferrin has a preventive effect on preterm delivery.

The role of defensins in preterm delivery

Defensins are small antimicrobial peptides that are widely distributed in mammalian epithelial cells and phagocytes. They are especially prominent in humans. The highest peptide concentrations can be found inside the granules of leukocytes. After leukocytes take up pathogens these granules fuse with the phagocytic vacuoles that carry the pathogens (47,48). By perforating target membranes defensins have antiviral, antifungal and antibacterial features. Studies with defensin knock-out mice showed that these mice were more susceptible for infections, suggesting that defensins have protective role for infections (49)(50).

Natural antimicrobial	Site detected
α-defensins	
HNP1−3	Amnion
	Chorion
	Placenta
	Amniotic fluid
	Cervical mucus
	Vernix caseosa
HD5	Chorion
β-defensins	
HBD1	Amnion epithelium
	Chorion trophoblast
	Decidua
	Placental syncytiotrophoblast
	Placenta
	Cervical mucus
HBD2	Amnion epithelium
	Chorion trophoblast
	Decidua
	Placental syncytiotrophoblast
	Amniotic fluid
HBD3	Amnion epithelium and mesenchyme
	Chorion trophoblast
	Decidua
	Placental syncytiotrophoblast

Table 1) Sites of detection of α- and β-defensins within the pregnant uterus at term. Adapted from {{343 King,A.E. 2007}}

The two most important subfamilies of defensins are the α- and β-defensins. From both subfamilies several subtypes have been described {{336 Ganz,T. 2003}}. α-Defensins are primarily found inside the granules of neutrophils, $CD8^+$ T-cells and NK-cells, the β-defensins are primarily found extracellulary since they are secreted by epithelial cells and leukocytes (47,48). In addition to their role as antimicrobials, the β-defensins function as chemo attractants for cells of both the adaptive and the innate immune system (49,51). This allows interaction between these two pathways of the immune system.

The key physical barriers that protect against uterine infection produce a wide range of the α- and β-defensins. Table 1 shows sites where the subtypes of the defensin subfamilies can be found (16).

Microorganisms may also cause uterine infection via the maternal blood and the syncytiotrophoblast layer of the placenta. The syncytiotrophoblast is also able to produce several antimicrobial peptides, including some β-defensins. The expression pattern of these antimicrobial peptides suggests that they provide protection from infection in the uterus and infant. Since β-defensins serve as chemo-attractants for members of the immune system (51), they may have the same function in the uterus, but future studies are warranted to confirm this.

Increased neutrophil defensin levels in the amniotic fluid are characteristic for intrauterine inflammation and bacterial vaginosis (52-54). Because neutrophil defensins are cytotoxic, their presence could potentially damage the fetal membranes and cause PROM (53). Indeed, a positive correlation has been found between increased beta defensin-2 levels and PROM. No correlation has been found between levels of other types of defensins and preterm birth (55,56).

Since the determination of defensin-levels is relatively easy, defensin levels have been studied for their diagnostic properties concerning preterm birth. Elevated amniotic fluid defensin levels were positively correlated with bacterial vaginosis. In addition, increased levels of vaginal fluid neutrophil defensins were also associated with birth before 32 gestational weeks (52-54). In addition to

determination of defensin levels in amniotic fluid, defensin levels can also be determined in maternal serum. In patients with PROM, the patients' plasma-defensin levels have been shown to be elevated compared to controls. Levels of defensins in patients with chorioamnionitis were shown to be even more elevated. These defensin values may be used in the decision to terminate pregnancy, because of the risk of neonatal infection. Of note is that Lactoferrin-levels have no predictive value for PROM and chorioamnionitis (50).

Genetic factors may play an additional role, which is illustrated by a study by Holzman *et al*., in which a positive correlation was found between α-defensin 1-3, bacterial vaginosis and spontaneous preterm labor in African-American women, but not in non-Hispanic white women (54). The association between genetic differences, the role of defensins, including predictive value, and preterm birth may be subject for future research.

The role of uterine natural killer cells in preterm delivery

Natural killer cells belong to the cellular components of the innate immune system. The number of NK cells strongly increases in response to IFN-α and IFN-β, cytokines produced upon viral infection, and peaks 3 days after viral infections.

Some viruses are able to induce down-regulation of the MHC-molecules of the infected host cell. This avoids recognition of non-self peptides presented in the MHC molecule by T-cells. Also tumor cells can down-regulate MHC molecules. NK cells respond to cells that do not present MHC molecules and are therefore able to recognize tumor cells and virus infected cells (57).

NK-cells destroy a target by use of multiple mechanisms. NK-cells present the FAS ligand on their membrane, which binds to the target, the FAS receptor. This initiates a cell signaling pathway in the tumor or virus infected cell that leads to cell death of the target cell (58).

Secondly, the cytoplasm of NK-cells contains constitutively present granules. These granules possess granzymes, perforin and defensins. After binding of a NK-cell to a target cell, granzymes and perforins are released via a junction between the interacting cells (59).

TLRs have also been identified on NK-cells and upon their activation defensin release by NK-cells has been observed (60). In addition to direct killing, NK-cells can also produce pro-inflammatory cytokines, such as IFN-α, IFN-β, TNF-α and IL-12 (61).

Uterine NK-cells (uNK) are an important group of cells present at the maternal-fetal interface, consisting of two subtypes: endometrial and decidual NK-cells. They constitute from 25% to 40% of endometrial and approximately 70% of decidual lymphocytes. The endometrial NK (eNK) cells are found during the menstrual cycle and the decidual NK (dNK) cell in the deciduas during pregnancy. Research on eNK cells is difficult and there is little information about this subtype. NK cells in the uterus are generally referred to as uNK cells, since dNK cells may have their source in eNK cells (62).

uNK-cells possess an altered profile of surface molecules and produce other cytokine levels then NK-cells in blood (66). They are able to respond to the unusual members of the MHC class 1 family: HLA-C, HLA-E and HLA-G, molecules expressed by the extra-villous trophoblasts. Because this interaction induces cell-signaling pathways via inhibitory receptors, this interaction decreases the cytotoxic activity of uNK cells (62). uNK-cells play a role in the regulation of trophoblast-invasion and their cytokine-production is important for angiogenesis and vascular stability (63). Moreover, uNK-cells play an important role in the process of implantation, decidualization and placentation (64).

uNK cells produce cytokines as a result of the interactions at the maternal-fetal interface. This may affect the integrin-expression and the production of MMPs, markers of cervical maturation. During infection-induced preterm labor in mice, uNK cells are able to produce TNF-α and to migrate into the placenta (65). Although this had already been shown for term labor, it indicates that uNK cells have a key function in the inflammatory response observed during preterm labor and delivery. Additionally, i.p. injection of Poly[I:C], a synthetic analog of viral RNA and binding ligand of TLR3, does induce NK-cell migration from the deciduas towards the placenta (13), and i.p. injection of CpG in IL-10 knock-out mice induced migration of uNK cells to the placental zone (29). These observations further implicate that uNK-cells have a major function in viral- or bacterial- infection-induced preterm delivery and that TLR signaling is involved. In addition, it has been shown that IL-10 prevents LPS-induced preterm parturition by regulating uNK cell function. (65).

The function of uNK-cells is different in mice and human; one of the main differences is that in humans eNK-cells are found in the non-pregnant endometrium but not in mice (reviewed by (66)).

After combining these data it would also be interesting to see what the function of defensins in uNK cells is. It is very likely that they are, like NK cells in the blood, able to produce defensins upon (TLR-) activation. It has been described that uNK cells can produce pro-inflammatory cytokines in response to activation of the TLRs they express (67,68). Since it has been shown that pro-inflammatory cytokines are increased in placentas of women undergoing spontaneous preterm delivery and PROM (69), it is likely that TLR activation on uNK cells has a function in preterm birth.

Conclusion

Since preterm birth is often caused by an inflammatory response to a pathogen, increased knowledge on this topic may lead to the development of therapeutic measures. In the present review we focused on four components of the innate immune system that are involved in PROM and preterm birth. Firstly, the function of TLRs was discussed. Upon recognition and binding of the ligand on a TLR, cell-signaling is induced and pro-inflammatory cytokines are produced. It has been shown that TLR activation is strongly associated with preterm birth. Activation of TLR2 and TLR4 during a bacterial infection and activation of TLR3 during a viral infection can induce preterm birth. TLR3 molecules are expressed by uNK cells and it has been shown that uNK cell migration can be affected by TLR3 triggering. Genetic factors, in relation to TLRs, are also associated with preterm birth.
Secondly, the function of natural antimicrobials has been discussed. Lactoferrin, a molecule that was first identified in milk, was shown to be important in the induction of preterm birth. Defensins can be found both in the granules of neutrophils and extracellulary. After defensin binding to target membranes, these membranes can be perforated, a mechanism that is likely to be harmful for the fetal membranes, thereby increasing the risk for preterm birth.
Finally the function of uNK cells has been discussed. uNK cells express, among other molecules, defensins in their granules and express TLRs on their membrane. Therefore, this cell type combines the action of TLRs and defensins.
This review underlines the fact that regulation of immune responses happens in a very delicate way. Over- and under-activation can have major consequences. In case of pregnancy (over-) activation of immune systems can cause PROM and spontaneous preterm birth. New techniques may help to perform better, more complete research. All different components of the immune system work in synergy and increased knowledge on the pathways will enable us to find appropriate options for treatment for the prevention of PROM and preterm birth.

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