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The Negative Regulators of the Host Immune Response: An Unexplored Pathway in Preterm Birth

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Abstract

Objective—Toll-like receptors are essential mediators of host immunity. TLR activation must be tightly regulated to prevent an exaggerated immune response from devastating the host. These studies assessed the expression of negative regulators (IRAK-3, IRAK-1, FADD) during pregnancy and in preterm birth (PTB).

Study Design—Tissues (uterine, cervix, placenta and spleens) from the following experimental groups were harvested: 1) non-pregnant mice, 2) pregnant mice across gestation, 3) murine model of PTB, and 4) pregnant mice exposed to medroxyprogesterone acetate (MPA).

Results—Negative regulators are differentially expressed in the uterus during pregnancy. In the setting of PTB, IRAK-3 is significantly increased in the uterus and cervix, but not the placenta. In maternal spleens, IRAK-3 and IRAK-1 are increased in response to intrauterine inflammation. MPA can increase IRAK expression in cervical tissues.

Conclusions—Negative regulators of the maternal immune response may play an important role in protecting pregnancies from an exaggerated inflammatory response.

Introduction

Preterm birth (PTB) complicates approximately 12% of all pregnancies in the United States and remains a leading contributor to neonatal morbidity and mortality. While significant advances have been made to elucidate the precise pathogenesis of spontaneous preterm birth, much remains unknown. Both human and animal data suggest that activation of inflammatory pathways plays a critical role in many cases of PTB.^{1–4} Cytokines have been demonstrated to be present in both maternal (uterus and cervix) and fetal tissues (placenta, fetal membranes and amniotic fluid) in humans as well as in animal models of PTB.^{2, 5–7} However, while cytokines may be sufficient to induce preterm birth, recent lines of evidence suggest that they are, in fact, not essential.^{8–10} More in-depth understanding of the host immune response during normal pregnancy and in the pathogenesis of PTB may lead to exciting and novel therapeutic options.

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ABSTRACT # 60

In that vein, recent work from our laboratory and others has demonstrated that Toll-like receptors (TLRs) are differentially expressed during pregnancy in a tissue-specific manner,¹¹ that activation of Toll-like receptors (TLRs) in trophoblasts results in NF κ B activation with a proinflammatory response,¹² and that activation of maternal TLR-4 is required for lipopolysaccharide (or gram negative)-induced preterm birth.^{13, 14} Recent literature supports the concept that the regulation and role of the innate immune response in inflammatory responses is far more complex than just activation of TLRs with a liberation of cytokines.^{15–17} While activation of TLRs promotes an inflammatory response, the absence of TLRs results in significant morbidity and mortality to the host secondary to unchecked inflammation.^{17–19} TLRs interact with endogenous regulators so that the host does not become ‘compromised’ with an over-exaggerated immune response.^{20–22} In the last 3–5 years, several reports have demonstrated that diverse molecules can serve to limit an inflammatory response triggered by an infectious agent or inflammatory stimulus.^{21, 23–25} However, three of these molecules are of high biological interest based on recent work with knock-out mice.^{16, 21, 26} Interleukin receptor associated kinase (IRAK)-1 and IRAK-3 (also called IRAK-M) are key signaling molecules in the cellular response to endotoxin molecules such as LPS.^{20–22, 27} Studies with knock-out mice for IRAK demonstrate that IRAK-3 is required to prevent an exaggerated immune response. When IRAK-3 deficient mice were observed following a challenge with sepsis and secondary intrapulmonary bacterial infection, these IRAK(-/-) mice had increased pulmonary chemokine and inflammatory cytokine production and enhanced neutrophil recruitment.²⁸ Similarly, bacterially-challenged IRAK (-/-) mice had an increased inflammatory response and a significantly reduced endotoxin tolerance concluding that IRAK-3 regulates innate immune homeostasis.²¹ In addition to IRAK, Fas-Associated protein with Death Domain (FADD) is a recently reported negative regulator of the immune response.²² While FADD is known to be an adaptor protein involved in death receptor-mediated apoptosis, recent work has demonstrated that FADD also plays a significant role in the innate immune response and is a physiological negative regulator of TLR signaling.^{22, 29} In fact, FADD and IRAK-1 work synergistically to control the response to TLR activation.²²

Collectively, these studies suggest that IRAK (1 and 3) and FADD play a critical role in regulating the innate immune response *in vivo* and serve to protect the host from devastating tissue damage from an ‘uncontrolled’ inflammatory response. As inflammatory pathways are believed to be critically involved in the pathogenesis of PTB, it is of high biological plausibility that these negative regulators may play an important role in PTB; whether this role is 1) to protect the pregnancy from an immune response triggered by invading pathogens, 2) to control an inflammatory processes in the uterus from becoming a systemic inflammatory response and hence protecting the host (mother) and/or 3) to regulate inflammatory pathways in the uterus from reaching the fetus remains unstudied. To date, there are no reports investigating the role of these negative regulators during pregnancy and specifically in preterm birth (PTB). These studies sought to assess the expression of these three specific molecules, demonstrated to be negative regulators of innate immunity, during pregnancy and in a murine model of PTB.

Materials and Methods

Animals

CD-1 out-bred, timed-pregnant and non-pregnant mice were purchased from Charles River Laboratories (Wilmington, MA). Pregnant mice were shipped on day 8 and 12 after mating. All mice were acclimated in our facility for 3 to 7 days before use in these experiments. All of the experiments were performed in accordance with the National Institute of Health Guidelines on laboratory animals and with approval from the University of Pennsylvania’s Committee on Animal Use and Care.

Mouse Model of Inflammation-Induced Preterm Birth

A mouse model of intrauterine inflammation was utilized for these studies as previously reported.^{2, 30–32} Briefly, on embryonic day 15 (E15), dams were placed under isoflurane anesthesia and a mini-laparotomy was performed. Lipopolysaccharide (LPS; Sigma, St. Louis, MO), 250 µg in a 100-µl volume or sterile saline solution (100 µl) was infused between 2 gestational sacs in the lower right uterine horn. This model results in a high rate of preterm birth within 24 hours and no maternal mortality. For these studies, 6–8 dams were used per treatment group.^{2, 30–32} Uterine, cervical, and placental tissues and maternal spleens were collected six hours after intrauterine infusion with LPS or saline.

Experimental Design

Different experimental groups were utilized to determine if the negative regulators (IRAK-1, IRAK-3 and FADD) were differentially expressed 1) during pregnancy compared to non-pregnant state, 2) in the presence of intrauterine inflammation in maternal or placental tissues and 3) in response to exogenous administration of a progestational agent. For these purposes, the following treatment groups were utilized: 1) Non-pregnant virgin mice (n=6) 2) timed-pregnant mice on E15 (preterm, n=6) and E19 (term, n=6), 3) timed-pregnant mice treated with intrauterine saline (N=6–8) or intrauterine LPS (n=6–8) with our model of PTB and 4) E15 mice exposed to medroxyprogesterone acetate (MPA, mg/dam; n=4) or vehicle (DMSO, n=5) for 48 hours.

Tissue Harvest

For all studies, a laparotomy was performed and the cervix, uterus and placentae (in pregnant mice) were identified. Adherent adipose, bladder, and rectum were carefully dissected off the cervix before being processed for studies. For uterine tissues, adipose tissue was carefully dissected and placentae were removed. Endometrium was left in situ for both NP and pregnant mice. Two placentas were removed from each dam and considered one specimen. Whole maternal spleens were collected from pregnant dams. In the mouse, the spleen, as opposed to leukocytes in the circulation, is an accurate predictor of the immune status of the host. All tissues were rinsed in sterile saline and immediately placed in liquid nitrogen. Tissues were then processed for specific studies as detailed below.

Quantitative PCR Analysis

Total RNA was extracted from cervical, uterine, placental and splenic tissues with trizol (Invitrogen) and purified. cDNA was generated with high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Primer sets, conjugated to Taqman MGB probes, were used for QPCR (Applied Biosystems). QPCR reactions were carried out with equivalent dilutions of each cDNA sample on the Applied Biosystems Model 7900 sequence detector PCR machine, as previously reported.^{30, 31, 33, 34} The relative abundance of the target was divided by relative abundance of 18s in each sample to generate a normalized abundance for each target. All samples were analyzed in duplicates. For QPCR analysis, message RNA (mRNA) expression for each target was compared between the different treatment groups with One-way ANOVA or normally distributed data and ANOVA on ranks for nonparametric data. Pair-wise comparison with Student-Newman-Keuls (SNK) was performed if significance was reached (P<0.05). For mRNA expression in maternal spleens, analysis was performed by T-test if the data were normally distributed or Mann Whitney Rank Sum if not-normally distributed.

Immunohistochemistry (IHC)

IHC studies were performed to confirm the location and expression of IRAK-3 in cervical tissues from E15 dams exposed to intrauterine LPS (n=3) or saline (n=3). Cervical tissues were

harvested six hours after intrauterine infusion of LPS or saline. Cervical tissues embedded in paraffin were prepared using the antigen retrieval kit (ProteinaseK, DAKO, Glostrup, Denmark) and after 30 minutes of pretreatment with normal serum, the IRAK-3 primary antibody (ab47842, Abcam, Cambridge, MA) was applied at a dilution of 1:300. The biotinylated anti-IgG secondary and avidin tertiary antibodies (Vectastain Elite ABC Kit, Vector Laboratories, Burlington, CA) were used and then developed using Diaminobenzidine (DAB, Sigma) according to the manufacturer's protocol. The tissues were counterstained with hematoxylin, then mounted with Crystal Mount (Sigma, St. Louis, MO). IRAK-3 target stained an orange-brown color. Negative control experiments using IgG confirmed the absence of non-specific staining.

Results

Compared to the non-pregnant state, in the preterm uterus during murine pregnancy, IRAK-1 mRNA is 1.7-fold increased ($P=0.002$, SNK), IRAK-3 is 2.8-fold increased ($P=0.001$, SNK) and FADD mRNA is 1.7-fold increased ($p<0.001$, SNK). (Figure 1) Interestingly, IRAK-1 and IRAK-3 appear to increase expression in mid-gestation but expression decreases at term. In contrast, IRAK-1, IRAK-3 and FADD mRNA expression in the cervix is not significantly different during pregnancy compared to non-pregnant state. (Figure 2)

In the setting of inflammation-induced PTB, the negative regulators were differentially expressed in response to inflammation in the uterus and cervix but not the placenta (Table 1). Specifically, IRAK-1 mRNA expression was significantly increased in the uterus and cervix. Notably, IRAK-3 mRNA expression is profoundly altered in the cervix and the uterus but not in placental tissues. IHC studies demonstrated an up-regulation of IRAK-3 protein, confirming the QPCR results, in the cervix in response to intrauterine inflammation with increased staining in both the cervical stroma and prominent staining in the endocervical glands. (Figure 3) Considering the differential regulation of these mediators locally in response to inflammation, we sought to assess if these mediators were differentially expressed systemically in the maternal host. QPCR analysis of maternal spleens demonstrated an increased expression of IRAK-1 to systemic inflammation with a non-significant increase in IRAK-3 (Figure 4). Of note, intrauterine inflammation resulted in a systemic increase in TLR-2 mRNA expression in maternal spleens while TLR-4 mRNA expression was significantly decreased. (Figure 4) Recent trials demonstrate that progestational agents appear to decrease the risk of PTB in high risk women and in women with short cervices.^{35, 36} Furthermore, as prior work from our laboratory suggests that this may be from an immunomodulatory effect on the cervix,^{33, 37} we sought to assess the effect of MPA on the expression of IRAK-1, IRAK-3 and FADD. After 48 hours of exposure to MPA, FADD mRNA was not differentially regulated while IRAK-3 mRNA was 2.3 fold increased ($P=0.005$, t-test) and IRAK-1 mRNA was 1.7 fold increased (0.016, t-test) in the cervix after exposure to MPA compared to vehicle.

Discussion

The innate immune response serves to protect the host from invading pathogens by eliciting an inflammatory response; yet, the response must be regulated to prevent untoward injury from an excessive inflammatory cascade. The role and regulation of the host immune response in the pathogenesis of PTB is poorly understood but is likely to be mechanistically important to this adverse obstetrical outcome. These studies demonstrate the complex role of the immune response during pregnancy and provide preliminary evidence that a component of this response, negative regulators, is altered during pregnancy and in PTB.

As these mediators, specifically IRAK-3, are required to control and limit responses to pathogens, the up-regulation of these mediators during pregnancy in the uterus may serve to

prevent an exaggerated host immune response, in the setting of intrauterine inflammation that would compromise the pregnancy. Using a murine model of PTB, we investigated the role of these select negative regulators in both maternal (systemically and locally) and in the placenta. While we have previously demonstrated that there is a potent cytokine response in the placenta six hours after intrauterine infusion of LPS,³⁴ at this same time, the negative regulators investigated were not differentially regulated. Perhaps this lack of an up-regulation in these negative regulators, as observed in the uterus and cervix, allows propagation of the immune response in both the placenta and the fetus. The resultant inflammatory response in the placenta and/or fetus may trigger pathways that contribute or initiate the process of parturition. In contrast, these negative regulators are profoundly increased (by both message and protein) in the cervix, uterus and maternal spleen. This increase occurs at the same time as an inflammatory response is observed in this model.^{11, 30, 31, 33, 38} We have previously demonstrated that, with this model, at the same time point investigated here, that there is a potent cytokine response in the uterus and cervix³³ as well as an influx of polymorphonuclear cells into the cervix.³⁸ As increased expression of these regulators (specifically IRAK-1 and IRAK-3) is reported to 'control' and/or 'regulate' inflammatory pathways triggered by TLR activation, we hypothesize that the increased expression of IRAK both locally and systemically may serve to protect the host for a systemic inflammatory response in the setting of a localized inflammatory response in the uterus. The differential expression of the negative regulators and of TLR-2 and TLR-4 in the maternal spleens suggests that there is, in fact, a maternal response to a localized inflammatory response. One could hypothesize that the change in TLR and IRAK expression in maternal spleens, which are mostly a reflection of dynamic changes on leukocytes expression patterns, is responsible for preventing a more systemic inflammatory response to a local infection—hence protecting the host. While this hypothesis requires testing with these specific knock-out mice, these results do demonstrate a complex interplay of the mediators of the innate immune response in the pathogenesis of PTB. The observed up-regulation of TLR-2 and down-regulation of TLR-4 in the maternal spleens is consistent with other reports demonstrating a divergent response of TLR expression to an LPS challenge.^{39–41} Further work with knock-out mice is required 1) to elucidate if the differential regulation of IRAK locally and systemically in the host is an essential mechanisms by which maternal morbidity and mortality, in inflammation-induced PTB, is averted and/or 2) if these mediators are critical players in the pathogenesis of preterm parturition.

Recent clinical trials demonstrate that PTB can be prevented by administration of progestational agents, notable in patients with short cervixes.^{35, 36, 42, 43} While the mechanism by which these agents prevent PTB remains unclear, prior work from our laboratory has demonstrated that progestational agents can modulate the host immune response in this murine model of PTB.^{13, 30, 32} We have also demonstrated that in the absence of inflammation, these agents can modify signal transduction pathways—specific to the innate immune response—in the cervix.³¹ Consistent with these reports, we now report that MPA results in an increase in IRAK expression in cervical tissues. We speculate that an increase in IRAK may serve to limit an inflammatory response in the cervix in response to a pathogenic challenge. In turn, this may prevent cervical ripening. As these studies are in mice, understanding how progestational agents may modulate the immune response and how this might contribute to their ability to prevent PTB in humans requires additional investigations.

A noted limitation of these studies is the use of an animal model. While this model and ones similar to it are well-established in the literature,^{2, 6, 13, 44, 45} there is always concern regarding the applicability of an animal model to human disease. This model is one of localized intrauterine inflammation in the absence of significant maternal morbidity or mortality.^{2, 13, 31, 32} The use of genetically-manipulated mice and mouse models to investigate mechanisms and pathways in preterm birth has provided novel insights into parturition. Therefore, acknowledging the limitations with any rodent model, collectively, our data demonstrates a

potential role for these specific molecules, known to be negative regulators of the immune response, in PTB. We speculate that while these molecules *may not* play a critical role in causing parturition that they may, in fact, serve to control or sequester an inflammatory response in the uterus thus preventing a systemic inflammatory response and compromise of the host (mother). It should be noted that genetic differences in IRAK have recently been reported to be associated with inflammatory conditions such as sepsis and inflammatory bowel disease.^{27, 46} The essential role of these mediators in the host immune response and the possibility of genetic variations and contribution to inflammatory disease states, combined with our data suggest that further research is warranted to investigate the contribution of these mediators in human PTB.

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Figure 1.

Bar graph representing results from QPCR for mRNA expression of negative regulators in uterine tissue across gestation. Means and standard deviations are represented. IRAK-1 mRNA expression was significantly different between the three groups ($P=0.002$, One-way ANOVA). IRAK-1 mRNA was significantly increased on E15 compared to non-pregnant (NP) ($P=0.002$, SNK) and to term E19 ($P=0.007$, SNK). NP and E19 values were not significantly different. FADD mRNA was not significantly different between the gestational groups ($N=0.3$, One-way ANOVA). IRAK-3 mRNA expression was significantly different between gestational time points ($P<0.001$, Oneway ANOVA). IRAK-3 expression in E15 was significantly increased compared to NP ($P<0.001$, SNK) and E19 ($P=0.005$, SNK). IRAK-3 mRNA was not significantly different between E19 and NP.

Figure 2.

Bar graph representing results from QPCR for mRNA expression of negative regulators in cervical tissues across gestation. Means and standard deviations are represented. IRAK-1, FADD and IRAK-3 mRNA were not differentially regulated in cervical tissues across gestation.

Figure 3.

Representative sections of IHC from cervical tissues with IRAK-3 staining. Cervices were collected 6 hours after intrauterine infusion of saline or LPS from the murine model of PTB. A, B and C: Saline exposed cervixes on E15 at 5, 10 and 20x magnification respectively. Each section represents a different histological section and/or dam. D, E and F: LPS exposed cervixes on E15 at 5, 10 and 20X magnification respectively. IRAK staining is noted in orange-brown color and is increased in LPS specimens in stroma and in the lining of the endocervical glands. Mucin and increased debris, consistent with cervical ripening, can be viewed in the glands of the LPS-exposed cervixes.

Figure 4.

Bar graph representing results from QPCR for mRNA expression of negative regulators in maternal spleens. Means and standard deviations are represented. IRAK-1 mRNA expression was significantly increased in response to intrauterine inflammation ($P=0.03$, Mann Whitney Rank Sum). FADD mRNA was not differentially expressed ($P=0.06$, T-test) while IRAK-3 mRNA was increased but did not reach significance ($P=0.07$, T-test). TLR-2 mRNA expression was increased in maternal spleens in response to intrauterine inflammation ($P=0.03$, T-test) while TLR-4 mRNA was significantly decreased ($P<0.001$, T-test)

Table 1

Target Gene	E15 LPS/E15*				
	Cervix	P value*	Uterus	P value*	Placenta
IRAK1	1.5	P=0.04 (MWR)	1.4	P=0.003 (t-test)	2.3 NS
FADD	0.5	P=0.04 (MWR)	0.7	NS	1.5 NS
IRAK-3	17	P<0.001 (T-test)	10.7	P=0.002 (MWR)	2.1 NS

* Fold change in mean MRMA expression for each target

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P value was calculated using T-test for parametric data and Mann-whitney Rank Sum (MWR) for non-parametric data