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IRAK1 is a critical mediator of inflammation induced preterm birth

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Abstract

Preterm birth (PTB) is a major cause of neonatal mortality and morbidity, often triggered by chorioamnionitis or Intra-uterine Inflammation (IUI) with or without infection. Recently, there has been a strong association of Interleukin-1 (IL-1) with PTB. We hypothesized that IL-1 receptor-associated kinase 1 (IRAK1), a key signaling mediator in the TLR/IL1 pathway, plays a critical role in PTB. In human fetal membranes (FM) collected immediately after birth from women delivering preterm, phosphorylation of IRAK1 (pIRAK1) was significantly increased in all the layers of FM with chorioamnionitis, compared to no chorioamnionitis subjects. In a preterm rhesus macaque model of IUI given intra-amniotic LPS, induction of pIRAK1 and downstream pro-inflammatory signaling mediators were seen in the FM. In a PTB C57BL/6J mouse model of IUI given intra-uterine LPS, an IRAK1 inhibitor significantly decreased preterm birth and increased live birth in WT mice in a dose-dependent manner. Furthermore, IRAK1 knockout mice were protected from LPS induced preterm birth which were seen in WT controls. Activation of IRAK1 was maintained by K63 mediated ubiquitination in preterm FM of humans with chorioamnionitis, rhesus and mouse IUI model. Mechanistically, IRAK1-induced preterm birth in the mouse model of IUI by upregulating expression of COX-2. Thus, our data from human, Rhesus and mouse demonstrates a critical role IRAK1 in IUI and inflammation associated PTB and suggest it as potential therapeutic target in IUI induced preterm birth.

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Introduction:

Intra-uterine infection or inflammation (IUI) or chorioamnionitis is a prevailing cause of preterm birth and fetal injury. In 2015, 9.7% of all births in the US were preterm births (1), resulting in 75% of perinatal mortality and 50% of the long-term morbidity (2). Globally, approximately, 1 million deaths per year are attributable to prematurity (3, 4). Notably, more than 40% of the prematurity is triggered by IUI (2, 5–7). In addition to maternal morbidity, IUI enhances the risk for fetal and newborn brain injury (8–10), necrotizing enterocolitis (11), and chronic lung disease (12).

The inflammatory mediators during IUI are strongly implicated as causative agents of prematurity (13). Currently available therapies for IUI and / or preterm labor rely on antibiotics, and progesterone (14, 15). However, these treatment strategies are generally not effective and there continues to be many adverse, long-term side effects (16). Antibiotics, the first line of treatment often fails to prevent IUI-associated morbidities (17, 18), due to the inflammatory mediators of the infection that account for fetal and maternal injury (19). Thus, there is a pressing need for new effective therapies to treat IUI and preterm labor. A limitation in developing more effective therapies is the dearth of understanding of the molecular mechanisms of pathogenesis in IUI and preterm birth.

Recently, there have been many reports in humans, mice and rhesus implicating Interleukin-1β (IL-1) in the pathogenesis of IUI associated preterm birth (20). The activation of IL-1 receptor-associated kinase 1 (IRAK1), a downstream mediator of IL-1 and Toll like receptor (TLR) signaling pathways is involved in the generation of active IL-1 – the master cytokine in inflammation (21). IL-1 and TLR signaling pathways can be activated by a variety of stimuli, including recognition of microbial pathogens/products (e.g., lipopolysaccharide (LPS)), the presence of reactive oxygen species, recognition of DNA damage and abnormalities in the tissue matrix caused by chronic inflammation (22, 23). IRAK1 is central in engaging immune signaling pathways and is a key signaling mediator in the TLR/IL1 pathway (24). This signaling induces proinflammatory cytokines, chemokines and inflammasome components which results in rupture of fetal membranes as well as induction of prostaglandins which enhances uterine contraction and expulsion of fetus (25). IRAK1 has also been involved in many neoplastic disorders, and metabolic, cardiovascular, and inflammatory diseases (26). Thus, IRAK1 plays a critical role in innate immunity via both IL-1β production and subsequent signaling (24). Inhibition of IRAK1 kinase activity could therefore alter IL-1β and TLR-driven signaling in IUI and preterm birth.

However, the role of IRAK1 in IUI and preterm birth has not been studied. We thus hypothesize that IRAK1 is activated in fetal membranes of women with IUI delivering preterm, and inhibition of IRAK1 will lead to decrease in preterm birth. As mouse pregnancy physiology differs from human pregnancy (27), we evaluated IRAK1 activation first in human fetal membranes and then in our established rhesus model of IUI and finally in a mouse model of IUI. In both humans as well as induced models of preterm birth, we found strong association of preterm birth with activation of IRAK-1. Inhibition of IRAK1's function or its absence protected from inflammation induced preterm birth thus presenting IRAK1 as an attractive target for preventing preterm birth in humans.

Materials & Methods:

Human fetal membrane samples

Pregnant women at pregnancies from 26 to 36 $^{6/7}$ weeks were recruited. Cohorts of chorioamnionitis/IUI and no-chorioamnionitis were established based on a detailed histopathologic diagnosis of chorioamnionitis based on Redline's criteria and cytokine profile (28). Patient characteristics are shown in Table 1. Membranes were separated within few hours after delivery. Decidua was scrapped, and amnion and chorion separated and frozen at −80C.

Human explants

Separated amnion and decidua from term C section patient not in labor were cultured in DMEM + 10% FCS + Pen/Strep for 4–5 hours and then treated with LPS with mentioned doses and duration.

Histologic evaluation of fetal membranes for chorioamnionitis

H&E staining was performed for human fetal membrane sections, and staining was photographed. H&E-stained sections of human fetal membranes were scored in a blinded manner (by S.G. Kallapur) for chorioamnionitis using criteria outlined by Redline et al. based on numbers and depth of neutrophil infiltration of the tissue (28).

Rhesus model of intra-uterine inflammation

Normally cycling, adult female rhesus macaques (Macaca mulatta) were time mated. At ~130 days of gestation (~80% of term gestation), the pregnant rhesus received either 1 ml saline solution or 1 mg LPS (E. coli O55:B5, Millipore Sigma) in 1 ml saline solution by ultrasound-guided intra-amniotic (IA) injection (Table 2). Dams were surgically delivered 16 hours or 48 hours later. After delivery, fetuses were euthanized with pentobarbital, and fetal tissues were collected. There were no spontaneous deaths or preterm labor in the animals.

Mouse model of intra-uterine inflammation

Male and female C57BL/6 mice were bred overnight, and copulation plug was identified in the morning and defined as 0.5 dpc. On embryonic day (E) 15.5, dams were given ketamine and xylazine anesthesia and a mini- laparotomy was performed. Ultrapure LPS (E. coli O111:B4, Invivogen), 250 ug (as originally described by Lyttle et al, (29), or 10 ug (as described by Edey et al (30), in a 100 μL volume or sterile saline solution was infused between 2 gestational sacs in the lower right uterine horn. Uterus was then returned to abdomen; the fascia and the skin was sutured. The entire procedure took about 5 minutes per mouse. The mice were observed under infrared-camera for preterm birth. IRAK1 inhibitor (407601, Sigma-Aldrich) was given 4 hours before the surgery. Mice genetically deficient for IRAK-1 were created on a C57BL/6 background as previously described (26). For mechanistic studies, mice were sacrificed 12 hours after LPS injection.

Protein Immunoblot for pIRAK1

Both rhesus and human fetal membranes were dissected away from the placenta. Amnion were peeled off from the chorio-decidua layer. Tissues were washed after harvesting with ice-cold PBS and then lysed in protein extraction buffer containing 1% Nonidet P-40 (v/v), 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 150 mM NaCl, 1 mM DTT, protease inhibitor mixture (P8340; Sigma-Aldrich), phosphatase inhibitor mixture 2 (P5726; Sigma-Aldrich), and phosphatase inhibitor mixture 3 (P0044; Sigma-Aldrich). The lystaes were centrifuged at 13,200 rpm for 10 min and insoluble debris was discarded. Tissue extracts were resolved through SDS-PAGE using 4%–12% separating gel (Invitrogen). Proteins were transferred to Hybond enhanced chemiluminescence (ECL) nitrocellulose membrane (Amersham Pharmacia Biotech) using a semi-dry transfer system (Bio-Rad) and blocked with 5% dried milk or 5% BSA and 0.1% Tween-20 (MilliporeSigma). Blots were probed with anti–phospho IRAK1 antibody (BIOSS, bs-319R), COX-2 antibody (Cayman Chemicals aa 584–598); anti-IRAK1 (4504) anti-TAK1 (5206, 4505), anti– phosphor-SAPK/JNK (4668, 9251), anti-SAPK/JNK (9253, 9252), anti–phospho-p38 MAPK (4511, 9211), anti–p38 MAPK (9211, 9212) (Cell Signaling Technology, Beverly, MA); anti–phospho-TAK1 (06–1425) and β-actin antibody (A5060, Millipore Sigma, Temecula, CA) overnight at 4°C. Binding of HRP-labeled goat anti–rabbit antibody (sc-2004, Santa Cruz Biotechnology Inc., Santa Cruz, CA) was determined using SuperSignal West Chemiluminescent Substrate (Thermo Fisher Scientific, Rockford, IL). Blots were stripped with Restore Western Blot Stripping Buffer (Thermo Fisher Scientific), as required. Since IRAK1 undergoes degradation following phosphorylation, we examined β-actin expression for loading control instead of total IRAK1.

Ubiquitination assays:

Dissected amnion from human and rhesus, and the whole placenta from mice were homogenized and the tissue extract incubated with anti-IRAK1 (4504, Cell Signaling Technology) and magnetic Sepharose beads (Thermo Fisher Scientific) in immunoprecipitation buffer containing 0.1% SDS (per the manufacturer's instructions). The precipitates were resolved by SDS-PAGE and subjected to immunoblot analyses with Abs against anti-K63 (Apu3, 05–1308 Millipore) or anti-K48 linkage-specific polyubiquitin Ab (Apu2, 05–1307 Millipore).

Quantitative PCR

Total RNA was extracted from uterus after homogenizing in TRIzol (Invitrogen). RNA concentration and quality were measured by Nanodrop spectrophotometer (Thermo Fisher Scientific). Reverse transcription of the RNA was performed using qScript One-Step RT-qPCR Kit (Quantabio), following the manufacturer's protocol. Quantitative PCR (qPCR) was run in a QuantStudio 3 real-time PCR system (Thermo Fisher Scientific). qPCR assays were performed with Rhesus TaqMan gene expression primers (PTGS2, Rh02787802_m1 Invitrogen). Eukaryotic 18S rRNA (Invitrogen) was used as endogenous control for normalization. The values were expressed relative to the average value of the control group.

Statistics

SPSS v21.0 (Chicago, USA) and GraphPad Prism v8 software (GraphPad, San Diego, CA) were used to analyze data. Values were expressed as means ± SEM. Data were tested for normality (Kolmogorov–Smirnov test). Results that passed the normality assumption were analyzed by an unpaired two-tailed Student t test. Data that failed the normality assumption were analyzed by the nonparametric Mann–Whitney U test with post hoc analysis and Fisher's exact test for categorical variables were used to determine differences between groups. Results were considered significant for $P \leq 0.05$.

Results:

IRAK1 is phosphorylated in preterm human fetal membranes with IUI

The human fetal membrane consists of inner amnion, middle chorion (fetal components) and outer decidua parietalis (maternal component). Phosphorylation of IRAK1 is a critical event that determines downstream signal transduction following activation of TLRs and receptors of the IL-1 family. We thus compared phosphorylation status of IRAK1 (pIRAK1) in human fetal membranes harvested after preterm delivery in those subjects who had IUI or chorioamnionitis and those without (n = 4–6/group). We found minimal activation of IRAK1 in amnion (Fig 1A) and decidua parietalis (Fig 1C) in subjects without chorioamnionitis, which was substantially increased in those with chorioamnionitis. In addition, there was baseline activation of IRAK1 seen in the chorion layer in preterm subjects without chorioamnionitis (Fig 1B), but significantly higher activation was seen in those with chorioamnionitis. Similarly, there was significant activation seen in the whole thickness placenta of those with chorioamnionitis (Fig 1D). However, the abundance of IRAK1 activation was much less in the decidua parietalis layer of the fetal membranes compared to that seen in amnion or chorion layers in both with and without chorioamnionitis (Fig 1).

IRAK1 activation with LPS in human fetal membranes explants

Since we found IRAK1 to be phosphorylated in fetal membranes obtained from preterm births, we examined acute IRAK1 activation following exposure to canonical TLR ligands. We harvested the fetal membranes from women delivering at term via C section and those not in labor. We then examined IRAK1 activation in LPS-treated cultured explants in amnion and decidua given the significant activation seen in preterm human subjects with chorioamnionitis (n=3). In the early phase of LPS treatment (10 min and 30 min), there was significant IRAK1 phosphorylation and degradation compared to controls in the amnion (Fig 2A) and less in decidua (Fig. 2B). These results were similar to those seen in human preterm subjects with chorioamnionitis (Fig. 1) suggesting a potential upstream activation of TLRs. These results prompted us to directly examine the role of IRAK1 in preterm birth induced by intra-uterine LPS administration.

IRAK1 activation in rhesus fetal membranes and mice placenta after LPS administration

In our previously established model of preterm rhesus IUI which is comparable to human IUI (31), intra-amniotic LPS was given and the fetus was delivered 16 hrs or 48 hrs later. Similar to humans, we observed a significant increase of phosphorylated IRAK1 following

LPS treatment at 16 hrs which persisted at 48 hrs in the amnion, chorion and the full thickness placenta (Fig 3 A). We did not see significant phosphorylation of IRAK1 in the decidua layer thus replicating the observations in human decidua (Fig 1). Given the significant increase in the activation status of IRAK1 seen in human and rhesus amnion we then further analyzed the downstream mediators of IRAK1 in the rhesus amnion and found enhanced phosphorylation of TAK1 and JNK at 16 hrs, following LPS administration (Fig 3B). This phosphorylation persisted at 48 hrs, while the downstream mediator P38MAPK was elevated at 16 hrs post LPS injection and decreased at 48 hrs (Fig 3B)

We also analyzed the placenta of mice post intra-uterine LPS injection for IRAK1 activation and compared to saline control we saw significant increase in pIRAK1 with LPS at 12 hrs which persisted at 24 hrs (Fig 3C).

Decreased preterm birth and maternal mortality due to dystocia seen with IRAK1 inhibitor treatment and in IRAK1 knockout mouse model of IUI

We infused Ultrapure LPS (250 ug/mouse) into the uterus of E15.5 mice via mini-laparotomy and the dose of LPS was selected based on a previous study (29). Preterm birth was measured as delivery within 24 hrs of LPS injection. As the dose of the IRAK1 inhibitor treatment increased, we saw a significant reduction in frequency of preterm birth and increase in live birth. We also noticed increased maternal death due to dystocia, which was significantly reduced with increased dose of the IRAK1 inhibitor treatment (Fig 4A and 4B). We saw similar results in the IRAK1 knockout mice when compared to wild-type (WT) mice that were administered intra-uterine LPS, with a significant reduction in preterm birth and maternal death as well as an increase in mice giving live birth (Fig 4B). Given the increased rate of maternal death due to dystocia, we repeated the experiment using a lower 10 ug of LPS as used in other studies (n=5–6/group) (30). Interestingly, in IRAK1 knockout mice no preterm delivery was seen and all these animals delivered live pups at the end of gestation (Fig 4C). These data clearly establish that a lower dose of LPS causes an IRAK-1 dependent inflammatory response that result in pre-term birth without causing maternal dystocia and IRAK-1 KO mice are completely protected from such LPS induced pre-term delivery.

K63-linked IRAK1 Ubiquitination elevated in human preterm, rhesus and mice fetal membranes with IUI

Once IRAK1 is phosphorylated, it undergoes modification/degradation via ubiquitination. A delicate balance of IRAK1 activation is mediated by E3 ubiquitin ligase with phosphorylation of lysine at 63 (K63) indicating activation, while K48 phosphorylation targets IRAK1 for proteosomal degradation (32). Because of predominant IRAK1 activation in the amnion of women delivering preterm with chorioamnionitis, we examined K63-linked IRAK1 ubiquitination in the amniotic tissues by ubiquitination assays. We found significant increases in K63 linkage-specific IRAK1 ubiquitination in women delivering preterm with chorioamnionitis as compared to no-chorioamnionitis subjects. In contrast, there was no significant difference between chorioamnionitis and no-chorioamnionitis groups for K48 linkage-specific IRAK1 ubiquitination that accounts for IRAK1 degradation was seen (Fig 5A). In the rhesus model of IUI a significant increase in K63 linkage specific IRAK1

ubiquitination in the amnion at 16 hr post intra-amniotic LPS was observed (Fig 5B). Similarly, in the mouse model of IUI, we observed an increase in K63 linkage-specific IRAK1 ubiquitination in LPS-infused experimental group (Fig. 5B). Overall these data establish that IRAK1 is functionally activated by upstream signals and activation of IRAK1 is tightly linked to the preterm births observed in humans and experimental models.

IRAK1 contributes to preterm birth by enhancing cyclooxygenase-2 (COX-2) expression

Prostaglandins are potent dilators of cervix and utero-tonic agents leading to preterm birth (33). To identify the mechanism(s) by which IRAK1 activation leads to preterm birth, we examined for COX-2 expression which is a rate limiting enzyme for prostaglandin synthesis. In our rhesus model we found a significant increase in COX-2 mRNA expression in the uterus 16 hours after LPS injection (Fig 6A), consistent with increases in prostaglandins PGE2 and PGF2α seen in the amniotic fluid after IA LPS (31). We then evaluated COX-2 protein expression in our mouse IUI model. We saw a significant increase in COX-2 levels in the uterus of WT mice, 12 hours after intra-uterine LPS injection compared to PBS control group. However, COX-2 levels were significantly decreased in the IRAK1 knockout mice post intra-uterine LPS administration, when compared to WT mice (Fig 6B). These data provide a direct mechanistic link between activation of IRAK1 and preterm birth. Furthermore, we can conclude that upstream TLR/IL-1R signaling leads to premature production of COX-2 which are known to induce prostaglandin synthesis that act on uterus and cause preterm birth.

Discussion:

Birth reflects transition from a pro-pregnancy state of immunological tolerance towards the fetus allograft to a pro-labor, pro-inflammatory state. It has been suggested that while term labor is a physiological activation of this pathway, preterm labor is the result of a pathological activation of the same pathway. Many causes of preterm birth have been identified. However, latest evidence suggests that inflammation plays a significant role in all types of labors, regardless of the presence of infection, the timing of the delivery or other etiology (25).

There have been several studies which have correlated the increase in pro-inflammatory cytokines, principally IL-1β with preterm birth in humans as well as several animal species (20) as well as with spontaneous delivery at term in humans (34). It thus thought that IL-1 overproduction heralds labor, regardless of the presence of infection (25). Moreover, elevated IL-1β blood concentration in human neonates has been associated with preterm birth (35). IL-1β concentration and bioactivity increases in amniotic fluid of women with preterm labor and infection, and elevated maternal plasma levels of IL-1β are associated with preterm labor (36). As such, IL-1β is now considered a key mediator of inflammation in preterm labor, and IL −1 is a potential therapeutic target for chorioamnionitis and associated morbidities (31).

IRAK1 is central in engaging immune signaling pathways and is a key signaling mediator in the TLR/IL-1 pathway. Although there are four different IRAK proteins that participate in downstream signaling, IRAK1 has been recently shown to be the most prominent molecule

in humans (24). Furthermore, we have shown that IRAK1 is critical for linking TLRs to inflammasome activation that results in IL-1β production (26). Our focus on IRAK1 was prompted by these studies and we began with a very novel finding of increased activation of IRAK1 in both human preterm fetal membrane with chorioamnionitis as well as in rhesus and mice models of IUI. We did not observe IRAK1 activation in the amnion and decidua of preterm subjects without chorioamnionitis, but we saw increased abundance of pIRAK1 in the chorion and the whole thickness placental layer of patients that had given preterm birth without chorioamnionitis. Studies on TNF/IL-1 β induced fetal membrane weakening have demonstrated that the initial tissue and cellular targets of these agents are in the choriodecidua component of the fetal membrane rather than the amnion (37). Thus, it is likely that there is some degree of IRAK1 mediated inflammation even during non-chorioamnionitis preterm birth at the chorion layer, signifying the importance of IRAK1 signaling in all preterm births. However, during active inflammation “ligands” act on the chorio-decidua layer which overwhelmingly releases pro-inflammatory cytokines via the IRAK1 pathway during chorioamnionitis acting on all the fetal membranes layers which then leads to its weakening and subsequent preterm birth (31).

We examined the activation of IRAK1 in rhesus macaques due to the similarities in reproductive biology, placenta and fetal immunology between human and rhesus (38, 39). In our previously established model of IUI, the histology and inflammatory mediators after IA-LPS infusion in the rhesus is comparable to women delivering preterm infants with histologic chorioamnionitis. Also, the choriodecidual plane was the histological location of neutrophil invasion in both human and Rhesus IUI (31). Similar to humans, we saw activation of IRAK1 with LPS which in amnion decreased at 48 hrs but persisted in chorion and the placenta. This supports that the activation of IRAK1 at the chorion layer propagates the inflammatory cascade and enhances the detrimental effects of the downstream inflammatory mediators.

The intra-uterine injection of LPS to cause inflammation and induce preterm labor in mice is thought to mimic inflammation due to intra-uterine infection (40). IL-1 blockade (41), and TLR-4 blockade (42, 43), decreased inflammation-induced preterm birth in studies using rodent models. However, in some studies using IL-1 receptor knockout mice, no reduction in inflammation-induced preterm birth was seen, likely due to compensatory mechanisms (44). Thus, our focus in the present study was to identify specific mechanisms of intra-uterine inflammation that may lead to preterm birth as direct targeting of effector molecules of inflammation such as by IL-1 receptor antagonist might incur detrimental side effects due to simultaneous inhibition of homeostatic actions. IRAK1 inhibitor and knockout mice have been shown to reduce LPS induced kidney and renal damage (23), as well as reduce mortality from sepsis (45). We found that using IRAK1 inhibitor treatment and in IRAK1 knockout mice, preterm birth and maternal death due to dystocia was significantly reduced and increase in live birth was observed. The IRAK1 inhibitor also attenuates the activity of IRAK4, but the lack of preterm birth in IRAK1 knockout mice further implicate IRAK1 as a critical mediator of inflammation-induced pre-term birth. Increased mortality due to dystocia was a surprise finding as previous model using 250 ug intra-uterine LPS did not show any mortality (29). This difference could be due to the type of LPS or the strain of mice used. In addition, the higher dose of LPS could have contributed to preterm birth in the knockout

model as previous studies have shown increased frequency of preterm birth with higher doses intra-uterine injection of heat killed E coli in TLR4 knockout mice models of preterm birth (46). Reassuringly, we found no incidence of preterm birth or maternal mortality with the lower dose of LPS in the IRAK1 knockout mice IUI model.

Targeting of E3 ligases and subsequent inhibition of linkage-specific ubiquitination of IRAK1, would be an alternative therapeutic approach to prevent inflammation. Following engagement of immune receptor with relevant ligand, IRAK1 undergoes phosphorylation that triggers Pellino 2, a E3 ligase in promoting IRAK1 activation via K63-linked ubiquitination. This is followed by B-TrCP (E3-ligase)-dependent K48-linked ubiquitination and degradation/inactivation of IRAK1 (47). Further, Pellino 3b (E3 ligase) -mediated K63-linked ubiquitination competes with B-TrCP-dependent K48-linked IRAK1 ubiquitination at the same ubiquitination site (Lys134) of IRAK1, and thereby negatively regulates IL-1-induced NFkB activation (48). Together, ubiquitination by various E3 ligases regulates the stability and actions of IRAK1. We have previously shown that suppression of IRAK1 ubiquitination is as a novel mechanism for restraining acute inflammatory reactions (32). In our study, we found increased upregulation of K63 linkage specific IRAK1 ubiquitination in the fetal membrane of preterm women with chorioamnionitis and rhesus and mice model of IUI. Thus, IRAK1 is regulated via E3 ligase action in IUI and preterm birth and targeting of such E3 ligases could be a potential therapeutic option to suppress the inflammatory mediator actions in preterm birth.

COX-2-mediated prostaglandins are important mediators for cervical dilation, uterine contraction and preterm birth, and COX-2 inhibition prevents inflammation induced preterm labor (33, 49). We saw COX-2 levels were significantly increased after LPS in both the rhesus and mice IUI model but decreased in IRAK1 knockout mice. These data suggest a novel association of IRAK1 and COX-2. Thus, IRAK1 likely induces preterm birth by premature and increased expression of COX-2 enzyme.

In summary, our results suggest activation of IRAK1 in the amnion and chorion of women delivering preterm with IUI, in chorion of those delivering preterm without IUI as well as in human ex-vivo membranes, rhesus and mouse models of IUI. Furthermore, K63-linked ubiquitination that signifies activation of IRAK1 was substantially increased in fetal membranes of humans, rhesus monkeys and mice subjected to IUI. Inhibition of IRAK1 in mice by an inhibitor or by gene knockout significantly reduced preterm birth and increased live birth. More strikingly, we found that IRAK1 knockout mice had reduced expression of COX-2 following IUI thus establishing a mechanistic and direct link between IRAK-1 signaling and preterm birth. These data overwhelmingly suggest that IRAK1 is a critical mediator in inflammation-induced preterm birth and is a potential therapeutic target for IUI or chorioamnionitis and preterm birth.

Study approval

All animal procedures were approved by the IACUC at the Cincinnati Childrens Hospital and Medical Centre. All animal procedures done on Rhesus monkeys were approved by the Institutional Animal Care and use Committee at the University of California, Davis. Pregnant women provided a written informed consent from 2014–2017 under a protocol

approved by the IRBs of Cincinnati Children's Hospital and University of Cincinnati (no. 2013–2243).

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Key Points:

1. IRAK1 is hyperactivated in human preterm birth & in mice and rhesus IUI model
2. IRAK1 deletion and inhibition reduces preterm birth
3. IRAK1 induces preterm birth by upregulating COX-2

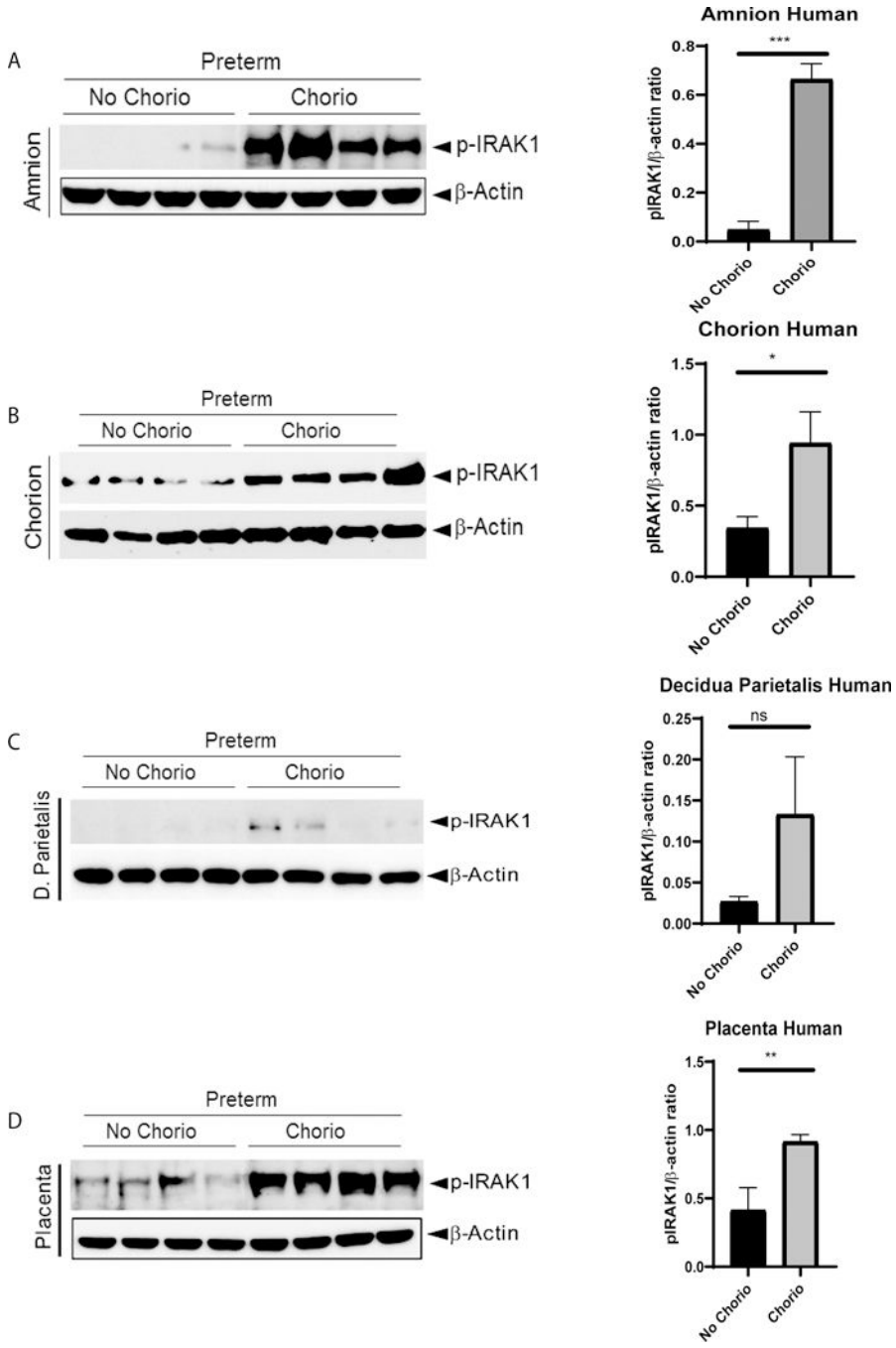

Fig 1:

IRAK1 activation in human preterm fetal membranes with and without chorioamnionitis Representative immunoblot indicating elevated IRAK1 phosphorylation in amnion (A), chorion (B), Decidua Parietalis (C) and whole thickness placenta (D) in preterm subjects with and without chorioamnionitis (chorio). N=4–6/group. *p<0.05, **p<0.01, ***p<0.001

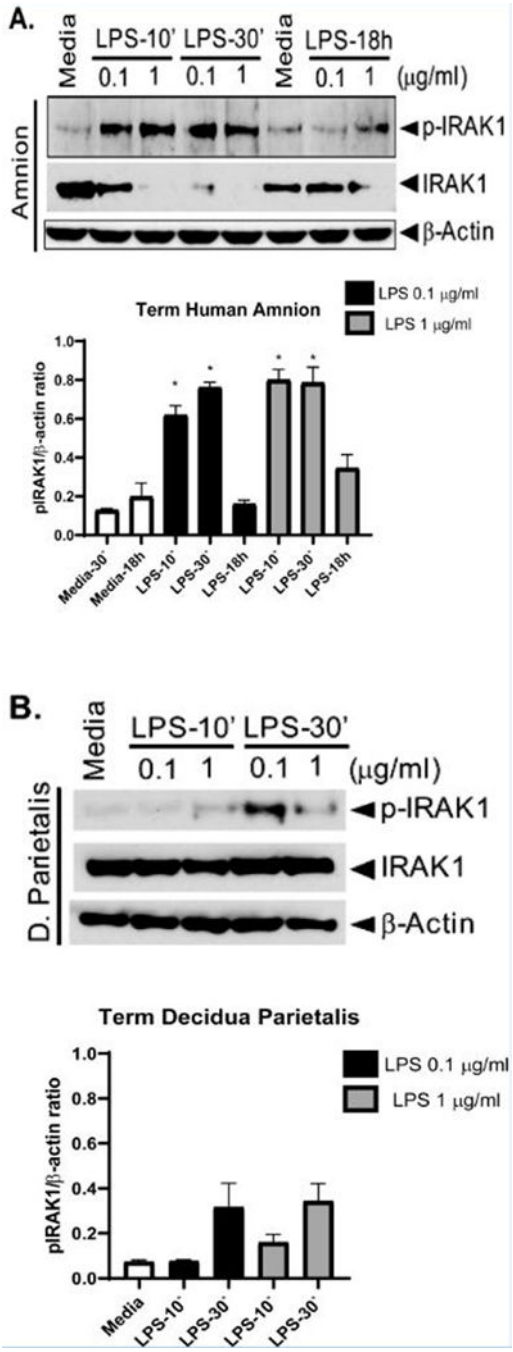

Fig 2:

IRAK1 activation in ex-vivo human Term amnion

Dissected amnion and Decidua Parietalis from fetal membranes of term C-section women without labor were cultured with LPS for the indicated doses and periods of time. Representative immunoblot for IRAK1 phosphorylation of three independent experiments. *p<0.05 versus respective media control

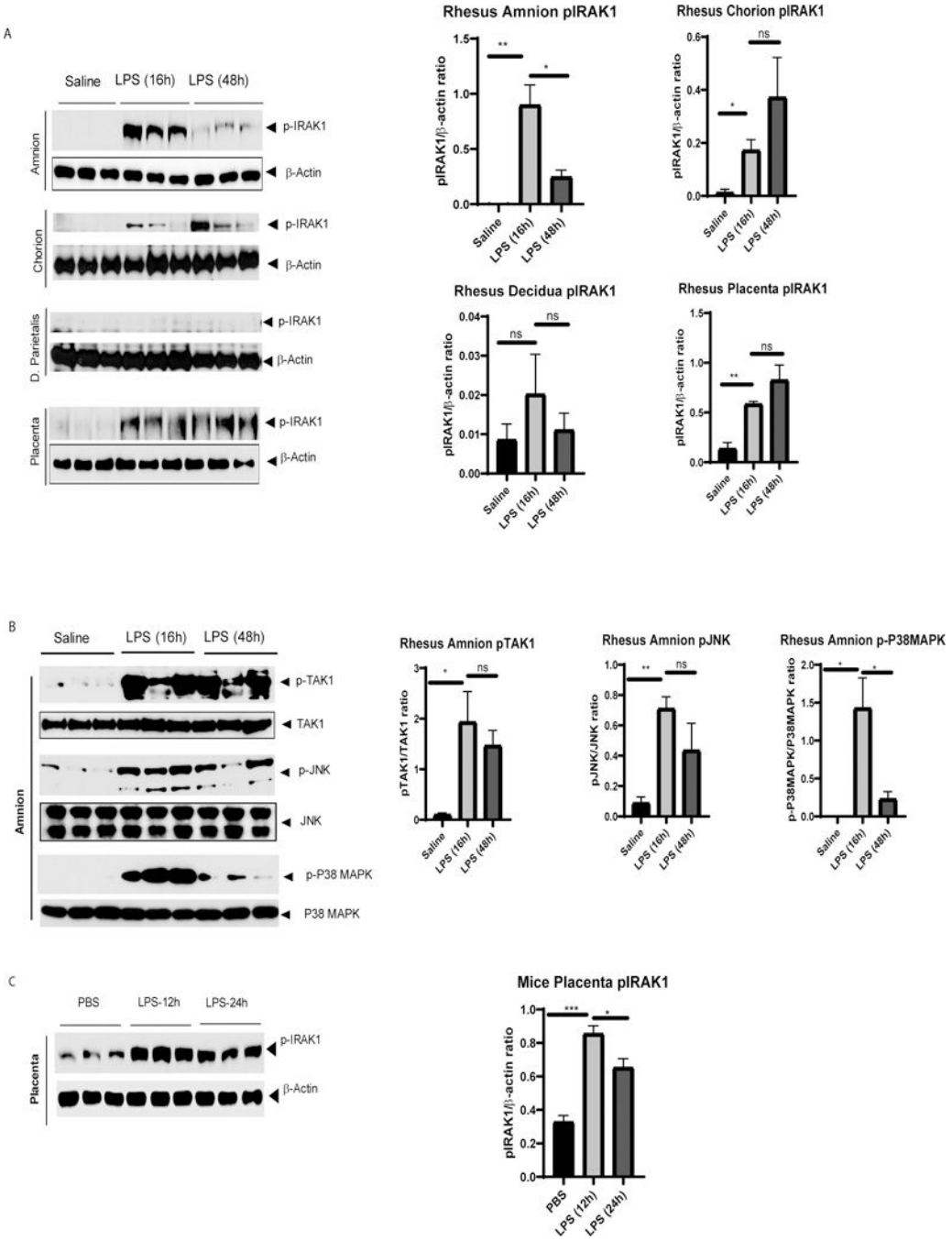

Fig 3:

IRAK1 activation in amnion and of rhesus and placenta of mice model of IUI

(A) Rhesus samples were collected after IA-LPS or saline infusion (n=4–6/group) for the indicated periods of time. Amnion, chorion and Decidua Parietalis whole thickness placenta in Rhesus delivery preterm showing elevated IRAK1 activation post LPS.

(B) Increased activation of downstream mediators (TAK-1, JNK, p38MAPK) in rhesus amnion.

(C) Mice placenta harvested 12 and 24 hrs after IUI LPS infusion at e15.5 gestation shows increased pIRAK1 activation (n=6/group). *p<0.05, **p<0.01, ***p<0.001

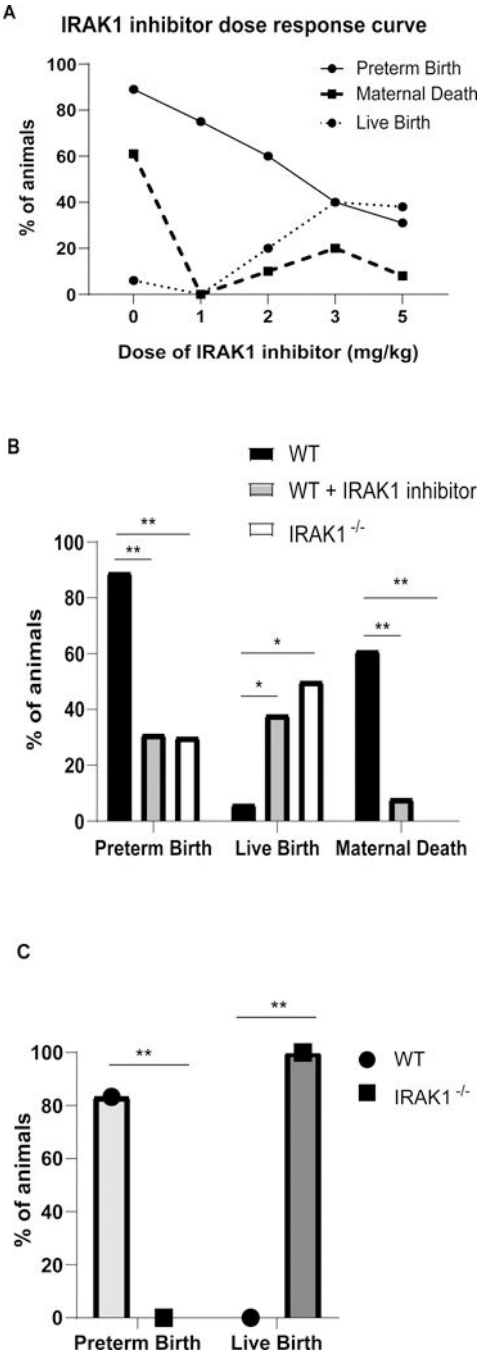

Fig 4:

Reduced incidence of preterm birth in IUI mice model given IRAK1 inhibitor and in IRAK1 knockout mice.

(A) Dose response curve showing significant decrease in preterm birth (birth within 24 hours of intra-uterine LPS) and maternal mortality due to dystocia, and increase in live birth with increasing dose of IRAK1 inhibitor treatment

(B) Comparison of incidence of preterm birth, live birth and maternal mortality due to dystocia of WT mice with and without IRAK1 inhibitor treatment (5 mg/kg) and IRAK1 $^{-/-}$ mice given 250 μg intra-uterine LPS (n = 10–18/group).
(C) Comparison of incidence of preterm birth and live birth in WT and IRAK1$^{-/-}$ mice given 10 μg intra-uterine LPS (n=5–6/group). No maternal mortality was seen in WT and IRAK1$^{-/-}$ mice. * = $p<0.05$, ** = $p<0.01$.

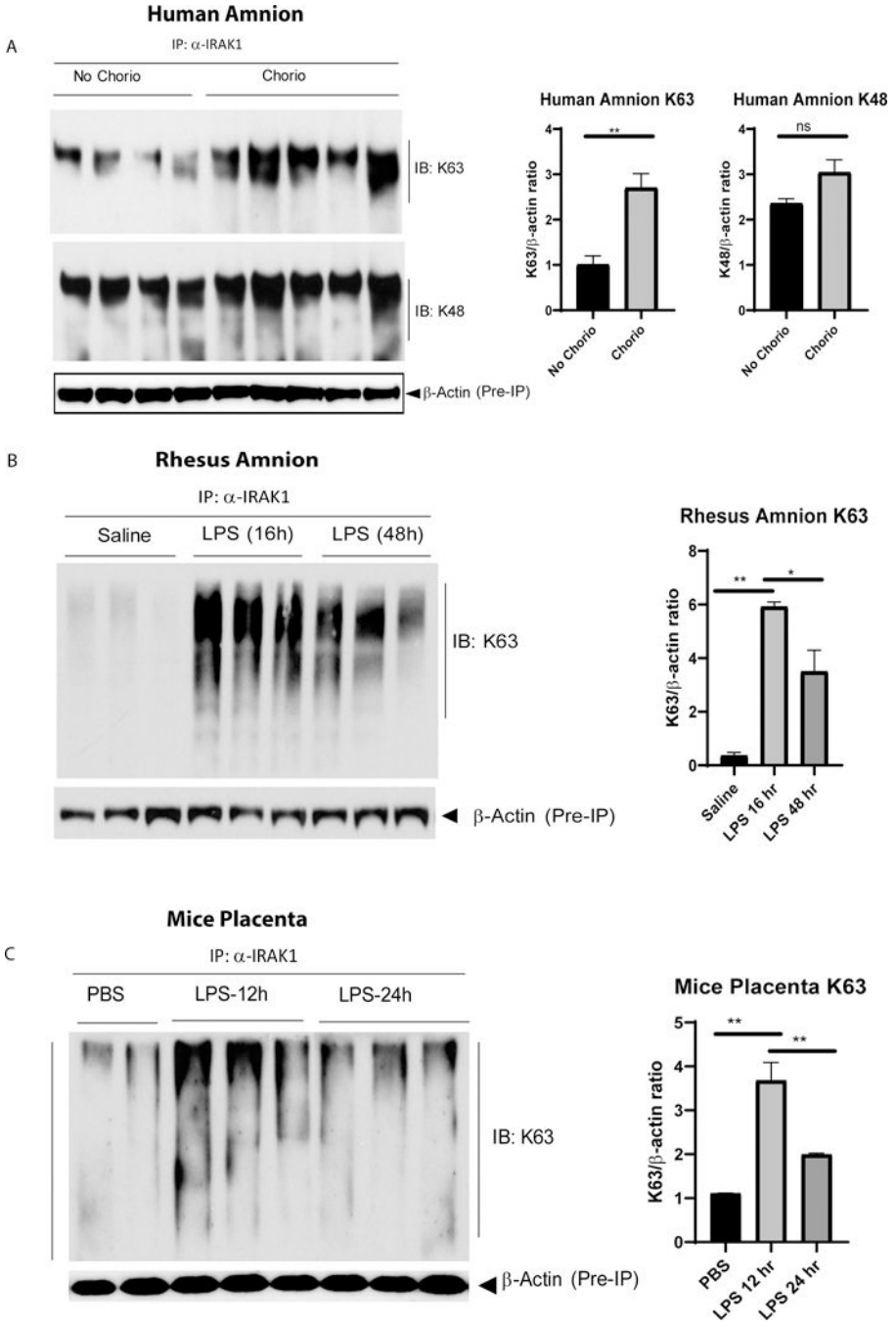

Fig 5:

K63 mediated ubiquitination is elevated in humans with IUI and in mice and rhesus model of IUI

(A) Human amnion was immuno-precipitated with α-IRAK1 Ab and immunoblotted with α-ubiquitin K63 / K48 linkage -specific Abs.

(B) Significantly elevated K63 linkage specific IRAK1 ubiquitination in rhesus amnion post intra-amniotic LPS

(C) Significantly elevated K63 linkage specific IRAK1 ubiquitination in mouse placenta post intra-uterine LPS. n=6–8/group; *p<0.05, **p<0.01

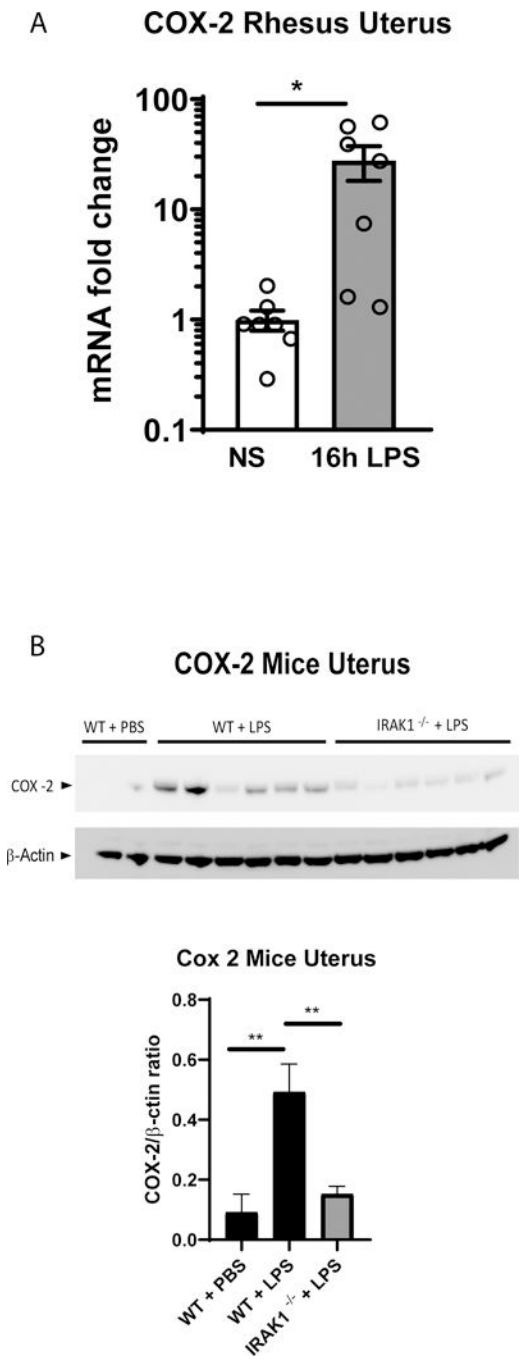

Fig 6:

COX-2 expression after LPS in the uterus of preterm rhesus and mouse model of IUI. (A) COX-2 mRNA fold change 16 hours after LPS in rhesus IUI model. Average mRNA values are fold increases over the average value for control internally normalizing to the housekeeping 18S RNA. n=7/group

(B) Significantly reduced induction of COX-2 is seen in the uterus of the IRAK1 knockout mice compared to wild type when given 10 μg of intra-uterine LPS. n=6/group. *$p<0.05$, **$p<0.01$

Table 1:

Maternal and preterm human infant demographics

	Chorioamnionitis (n=6)	No Chorioamnionitis (n=5)	P Value
Gestational age* (weeks)	32.2 ± 2.1	32.8 ± 1.9	0.67
Birth Weight (grams)*	1925 ± 509	1717 ± 572	0.51
Maternal Age (years)*	28 ± 7.0	30.8 ± 5.7	0.48
Infant Gender (Female/Male)	2/4	4/1	0.12
Antenatal Steroids (Yes)	5	4	0.88

* Mean ± SD

Table 2:

Clinical characteristics of rhesus animals included in the study

	IA Saline Ctrl (n=8)	IA LPS 16h (n=6)	IA LPS 48h (n=6)
Maternal age, year *	10.3 ± 2.1	8 ± 1.4	8.7 ± 2.9
Median gestational age (days) [range]	132 [130–136]	131 [130–132]	134 [128–137]
Birth weight (grams) *	330.9 ± 18.9	333.2 ± 43.7	338.5 ± 36.3
Infant Gender (Female/Male)	5/3	3/3	5/1

* Mean ± SD, IA- Intra-amniotic