Journal of Pediatric Surgery 55 (2020) 2308–2316

Contents lists available at ScienceDirect

Journal of Pediatric Surgery

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

Prenatal low-dose endotoxin exposure prolongs intestinal epithelial activation after birth and contributes to necrotizing enterocolitis

Hong-yi Zhang [a], Fang Wang [b], Xinrao Meng [a], Chenzhao Feng [c], Lei Xiang [a], Gail E. Besner [d], Jie-xiong Feng [a,*]

[a] Department of Pediatric Surgery, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China
[b] Department of Neurology, The Central Hospital of Wuhan, Huazhong University of Science and Technology, Wuhan 430014, China
[c] Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China
[d] Department of Pediatric Surgery, Nationwide Children's Hospital, Ohio State University, Columbus, OH 43205, USA

ARTICLE INFO

Article history:
Received 20 November 2019
Received in revised form 5 March 2020
Accepted 15 March 2020

Key words:
Endotoxin
Intestinal epithelial cells
Transcriptional activity
Necrotizing enterocolitis

ABSTRACT

Purpose: To investigate the effects of low dose endotoxin on transcriptional activity in intestinal epithelium, and its role in necrotizing enterocolitis (NEC).
Methods: Lipopolysaccharides (LPS) were injected into the amniotic cavity of pregnant mice under ultrasound guidance. The effects of LPS on fetal and neonatal intestines were determined. Mouse pups were exposed to low dose LPS (0.01 μg per fetus) prenatally and subjected to experimental NEC after birth. The incidence and severity of NEC, as well as intestinal permeability, NF-κB activation, and IL-6 expression were studied. The signaling pathways in the intestinal epithelial cells (IECs) that were activated by LPS were also investigated.
Results: Low dose LPS did not increase apoptosis, myeloperoxidase activity, histological injury or NF-κB activity in fetal intestines. However, prenatal low dose LPS exposure disturbed the transient and self-limited activation of NF-κB in neonatal intestines after birth. Importantly, it increased the incidence and severity of experimental NEC in neonatal mice. In primary IECs, low dose LPS induced IRAK-1 expression via activation of GSK3β. Elevated IRAK-1 levels prolonged the activation of IECs upon stimulation by high dose LPS.
Conclusion: Prenatal low dose endotoxin exposure disturbs self-limited postnatal epithelial cell activation and predisposes the neonatal intestine to NEC.
Level of evidence: Not applicable (experimental animal study).

Intrauterine inflammation most commonly presents as chorioamnionitis, which can lead to a fetal inflammatory response and spontaneous preterm birth [1]. During pregnancy, low immune function in the mother, and small amounts of bacteria from the vulva and cervix commonly cause mild intrauterine inflammation, similar to subclinical chorioamnionitis [2].

Necrotizing enterocolitis (NEC) is a common and dangerous gastrointestinal disease in premature infants [3,4]. Postnatal inflammatory processes are highly associated with the development of NEC [3,4]. The association between chorioamnionitis and NEC is less clear [1]. In fetal pigs, exposure to LPS (1 mg per fetus) not only induces chorioamnionitis and fetal gut inflammation, but also results in postnatal systemic inflammation and internal organ dysfunction [5]. Similarly, intraamniotic injections of endotoxin (10 mg per fetus) disturb gut development in fetal sheep [6]. Clinical studies also confirm that maternal clinical chorioamnionitis leads to a higher risk of NEC [7,8].

A definite association between mild intrauterine inflammation and NEC, nevertheless, remains unclear. Some studies demonstrated an association between subclinical chorioamnionitis with fetal involvement and NEC [9], whereas others found a slightly higher but not a statistically significant association between subclinical chorioamnionitis and NEC [8]. Moreover, the question remains whether mild intrauterine inflammation can influence the postnatal intestinal immune status and promote NEC.

The transition from a sterile fetal environment to commensal bacteria colonization evokes a rapid, strong, and transient response of the intestinal epithelial cells (IECs) [10,11]. The self-limited activation of IECs caused a potent negative regulatory mechanism to induce the tolerance to commensal bacteria [11]. We speculated that subclinical intrauterine inflammation might disturb the self-limited activation of IECs during commensal bacteria colonization and put the infant intestines at risk of developing NEC. In an attempt to prove this, we used a mouse model of mild intrauterine inflammation by intraamniotic injection of low dose LPS. Using an established mouse model of experimental NEC, we examined whether prenatal low dose LPS exposure could contribute to NEC.

* Corresponding author at: 1095 Jiefang Ave, Wuhan 430030, China. Tel.: +86 027 83665289.
E-mail address: jackpsjx@tom.com (J. Feng).

https://doi.org/10.1016/j.jpedsurg.2020.03.005
0022-3468/

1. Methods

1.1. Induction of intrauterine inflammation

The following protocols were approved by the Institutional Animal Care and Use Committee of Tongji Hospital (Permit Number 20160221).

C57BL/6 mice were kept under a circadian cycle (light:dark = 12:12 h). Female mice, 8–12 weeks old, were time-mated and checked daily between 7:00 a.m. and 8:00 a.m. for the appearance of a vaginal plug, which indicated 0.5 days postcoitum (0.5 dpc). Gestational age was defined as the time elapsed from the detection of the vaginal plug through the delivery of the first pup. Preterm labor/birth was defined as delivery occurring before 18.0 dpc [12].

At 17 dpc, under inhalation anesthesia using 2% isoflurane, ultrasound-guided intraamniotic injection of lipopolysaccharide (LPS, *Escherichia coli* 055: B5; Sigma Chemical, St. Louis, MO) at a dose of 0.01, 0.1, 1 or 10 μg per fetus dissolved in 25 μl of sterile 1 × phosphate-buffered saline (PBS) was injected into each amniotic sac using a 30 gauge needle [13]. The controls were injected with 25 μl of PBS.

1.2. Murine model of NEC

Mouse pups were delivered via c-section at 18 dpc. The pups were recovered, dried and placed in an incubator at 35 °C. Gastric gavage feeding of Similac 60/40 (Ross Pediatrics, Columbus, OH) formula fortified with Esbilac powder (Pet-Ag, New Hampshire, IL) started within 2 h after birth, and was repeated every 4 h. The pups were exposed to hypoxia (95% nitrogen for 1 min) followed by hypothermia (4 °C for 10 min) at 3 h after birth [14]. Hypoxia and hypothermia were repeated every 6 h. The pups were sacrificed upon the development of clinical signs of NEC. The pups that survived were sacrificed at the endpoint of the study (72 h after birth). Histological sections of the intestine were evaluated. Tissues with histological scores of 2 or higher were designated as positive for NEC [15].

The gut permeability was investigated using fluorescein isothiocyanate (FITC)-labeled dextran molecules (molecular weight, 73,000) (Sigma-Aldrich Inc., St Louis, MO) as a probe [15]. FITC-dextran was fed via orogastric tube to pups. Three hours later, blood was collected and plasma FITC-dextran levels were measured using spectrophotofluorometry.

1.3. TUNEL staining and caspase-3 activity assay

Terminal deoxynucleotidyl transferase-mediated deoxyuridine 5-triphosphate nick-end labeling (TUNEL) staining (ApopTag Red In Situ Apoptosis Detection, Chemicon International, Inc., CA) was used to detect apoptotic IECs on histologic sections [16]. Caspase-3 activity was assessed using a commercially available kit (ab39401, Abcam, Cambridge, UK) according to the manufacturer's instructions.

1.4. Myeloperoxidase (MPO) and NF-κB p65 activity assay, and quantitative measurement of LPS

Intestinal MPO activity (ab105136, Abcam), NF-κB p65 DNA binding activity (ab133112, Abcam), and LPS in terminal ileum (CSB-E13066m, CUSABIO, US) were assessed by commercial kits following the manufacturer's instructions.

1.5. Intestinal epithelial cells (IECs) isolation and culture

Fetuses (18 dpc) were sacrificed to harvest the small intestine. Primary IECs were cultured as previously described [17]. Expression of cytokeratin (ab7753, Abcam) and the epithelial cell marker CD104 (ab29042, Abcam) was determined by flow cytometry. Stimulation was performed by the addition of LPS at various concentrations.

1.6. Small interfering RNA transfection

Transfections were performed in serum-free medium for 6 h at 37 °C with 20 μg Lipofectamine (11668019, Invitrogen, US) in combination with either 150 nM control scrambled siRNA (siRNA ctl) or a combination of 75 nM siRNA ctl and 75 nM siRNA targeting IRAK-1 (RiboBio, Guangzhou, China) according to the manufacturer's instructions. The SiRNA sequences used are as follows: 5′-ACAUAUAGCUCUUGAGGAA-3′ and 5′-UUCCUCAAGAGCUAUAUGU-3′.

1.7. Reverse transcription-quantitative PCR (RT-qPCR)

RT-qPCR was performed as previously described [18]. The sequences for the sense and antisense primers for TLR4 (Toll-like receptor 4) were 5′-CCTCTGCCTTCACTACAGAGACTTT-3′ and 5′-TGTGGAAGCCTTCCT GGATG-3′ [19], those for SIGIRR (Single Ig IL-1-related receptor) were 5′-GTGGCTGAAAGATGGTCTGGCATTG-3′ and 5′-CAGGTGAAGGTTCCATAG TCCTCTGC-3′, those for TOLLIP (toll-interacting protein) were 5′-GCGGGTCTCTGTGCAGTT-3′ and 5′-TGTGGGTGTTATACGGAGGAA-3′, and those for MyD88 were 5′-CCCAACGATATCGAGTTTGT-3′ and 5′-TTCTT CATCGCCTTGTATTT-3′ respectively [20]. The mouse GAPDH primers were: forward, 5′-CATCACTGCCACCCAGAAGACTG-3′, and reverse, 5′-ATGCCAGTGAGCTTCCCGTTCAG-3′. The mouse IRAK-1 (IL-1-receptor-associated kinase-1) primers were: forward, 5′-CCTTCAGAGAGGCTAG CTGTACC-3′, and reverse, 5′-ACTTTGACCTCTGAGTCTGAGGG-3′ and the IRAK-M (IL-1-receptor-associated kinase-M) primers were: forward, 5′-TTCACGGAGACTGAGAAACT-3′, and reverse, 5′-ATTCTTCTGGCATGTA CCAC-3′ [18].

1.8. Western blotting

Western blotting was performed as previously described [16]. Anti-pJNK (9251), pFoxO1 (9461S), pERK1/2 (4370), and pAkt-S473 (9271S) antibodies were obtained from Cell Signaling Technology (Danvers, MA). Anti-ERK1/2 (sc-514302), JNK (sc-7345), IRAK-1 (F-4), pP65 (sc-135768), Akt (sc-8312), GSK3β (sc-9166), and pGSK3β (sc-135653) antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX).

1.9. Statistical analysis

The Student t-test was used to compare the differences between the two groups, and one-way ANOVA analysis was used to compare differences among multiple groups. The incidence of NEC and mortality were compared between groups using Fisher's exact test and χ^2 analysis, respectively. The severity of NEC was analyzed using the Mann–Whitney U test. Data are expressed as mean ± standard deviation of the mean (SD). Statistical analyses were performed using SAS software (SAS 9.2, SAS Institute, NC). Statistical significance was defined as $p < 0.05$.

2. Results

2.1. Low dose endotoxin (0.01 μg per fetus) exposure doesn't induce preterm birth, and inflammatory injury in fetal intestine

Intraamniotic injection of LPS was performed at 17 dpc. At 12 h after injection, separation and sloughing of epithelial cells were found in pups exposed to high dose LPS (0.1 μg, 1 μg or 10 μg per fetus) (Fig. 1A). High dose LPS increased epithelial apoptosis (Fig. 1B), caspase-3 (Fig. 1C) and MPO activities (Fig. 1D). However, LPS at a dose of 0.01 μg/fetus did not induce histologic injury and epithelial apoptosis (Fig. 1A and B), nor did it elevate caspase-3 (Fig. 1C) and MPO (Fig. 1D) activity.

Moreover, LPS (0.01 μg/fetus) administration did not induce PTB (preterm birth) (mean time from injection to delivery: LPS, 31.83 ± 3.76 h vs PBS, 32.58 ± 4.03 h; $p > 0.05$) (Fig. 1E) and alter the proportion of live born pups, compared with the control group (mean proportion of

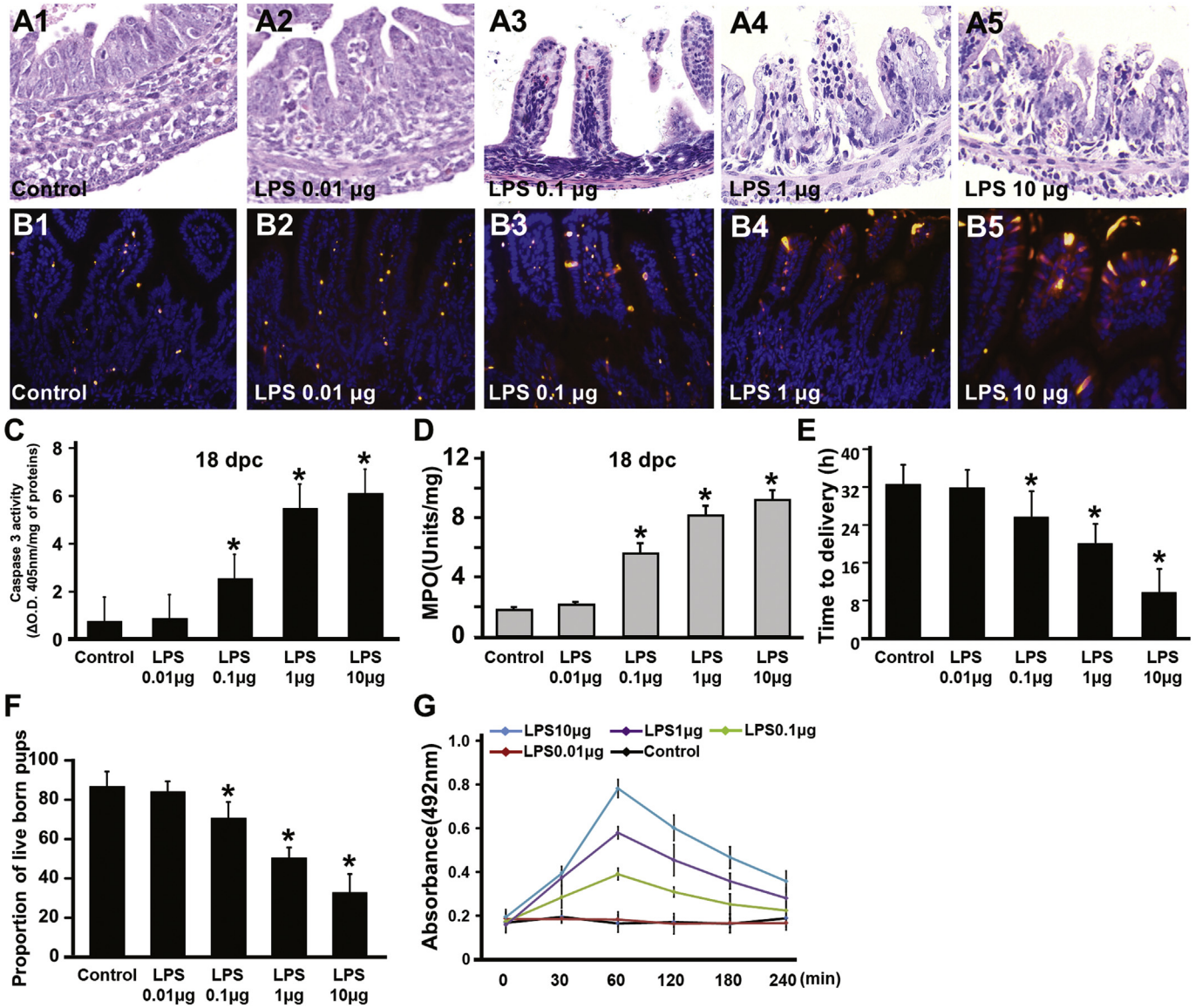

Fig. 1. Low dose endotoxin is insufficient to promote inflammation and injury in fetal intestines. At 17 dpc, ultrasound guided intraamniotic injection of LPS was administered. The controls were injected with the same volume of PBS. (A) Histological sections of the fetal intestine at 18 dpc. Fetuses were exposed to PBS (A1) or LPS (A2-A5) at doses of 0.01, 0.1, 1 or 10 μg per fetus. (B) TUNEL staining of the fetal intestine at 18 dpc. Fetuses were exposed to PBS (B1) or LPS at indicated concentrations (B2-B5). Intestinal Caspase-3 (C) and MPO (D) activity at 18 dpc. Data are mean ± SD ($n = 6$ fetuses). Time from injection to delivery (E) and the proportion of live born pups (F) were determined in mice treated with intrauterine injection of PBS or indicated concentration of LPS ($n =$ fetuses of 6 pregnant mice). (G) DNA binding activity of NF-κB p65 in fetal intestines at 0, 30, 60, 120, 180, 240 min after PBS or LPS injection ($n = 3$ fetuses at each time point). * $p < 0.05$ for pups treated with indicated concentrations of LPS vs the control pups treated with PBS. LPS, lipopolysaccharide; PBS, phosphate-buffered saline; MPO, myeloperoxidase; TUNEL, Terminal deoxynucleotidyl transferase-mediated deoxyuridine 5-triphosphate nick-end labeling.

live born pups; LPS, 84.25 ± 5.03% vs PBS, 86.92 ± 7.25%; $p > 0.05$) (Fig. 1F). However, high dose LPS resulted in PTB (mean time from injection to delivery: LPS 0.1 μg, 25.67 ± 5.39 h; 1 μg, 20.17 ± 4.02 h; 10 μg, 9.67 ± 4.76 h, all $p < 0.05$ vs control) (Fig. 1E) and a significant reduction in the proportion of live born pups (mean proportion of live born pups: LPS 0.1 μg, 70.65 ± 8.08%; 1 μg, 50.45 ± 5.28%; 10 μg, 32.95 ± 9.18%, all $p < 0.05$ vs control) (Fig. 1F).

2.2. Low dose endotoxin exposure fails to induce NFκB activation in fetal intestine

In fetal intestines, high dose LPS (0.1 μg, 1 μg or 10 μg) enhanced the DNA binding activity of NF-κB p65 in a dose-dependent manner, which peaked at 60 min and declined thereafter. However, low dose LPS (0.01 μg) did not affect NF-κB activity (Fig. 1G).

2.3. Prenatal low dose endotoxin exposure disturbed transient and self-limited NFκB activation in the intestines of the full-term, vaginally delivered and breastfed newborns

The luminal LPS increased from 0 pg/ml before birth to 4340 ± 166 ng/ml at 6 h after birth (Fig. 2A). Meanwhile, quantitative IL-6 mRNA analysis revealed a maximal increase at 1 h after birth followed by rapid normalization within 3 h (Fig. 2B). However, in the neonates subjected to prenatal low dose LPS, the highest IL-6 mRNA expression occurred at 2 h, followed by slow normalization within 6 h (Fig. 2B).

The previous study found a transient and self-limited activation of NFκB during bacteria colonization after birth in neonatal intestines [11]. The expression of the IL-6 gene is dependent on the transcription factor NFκB [21]. In the controls subjected to prenatal PBS, DNA binding activity of NF-κB p65 peaked at 1 h, and decreased to a normal level

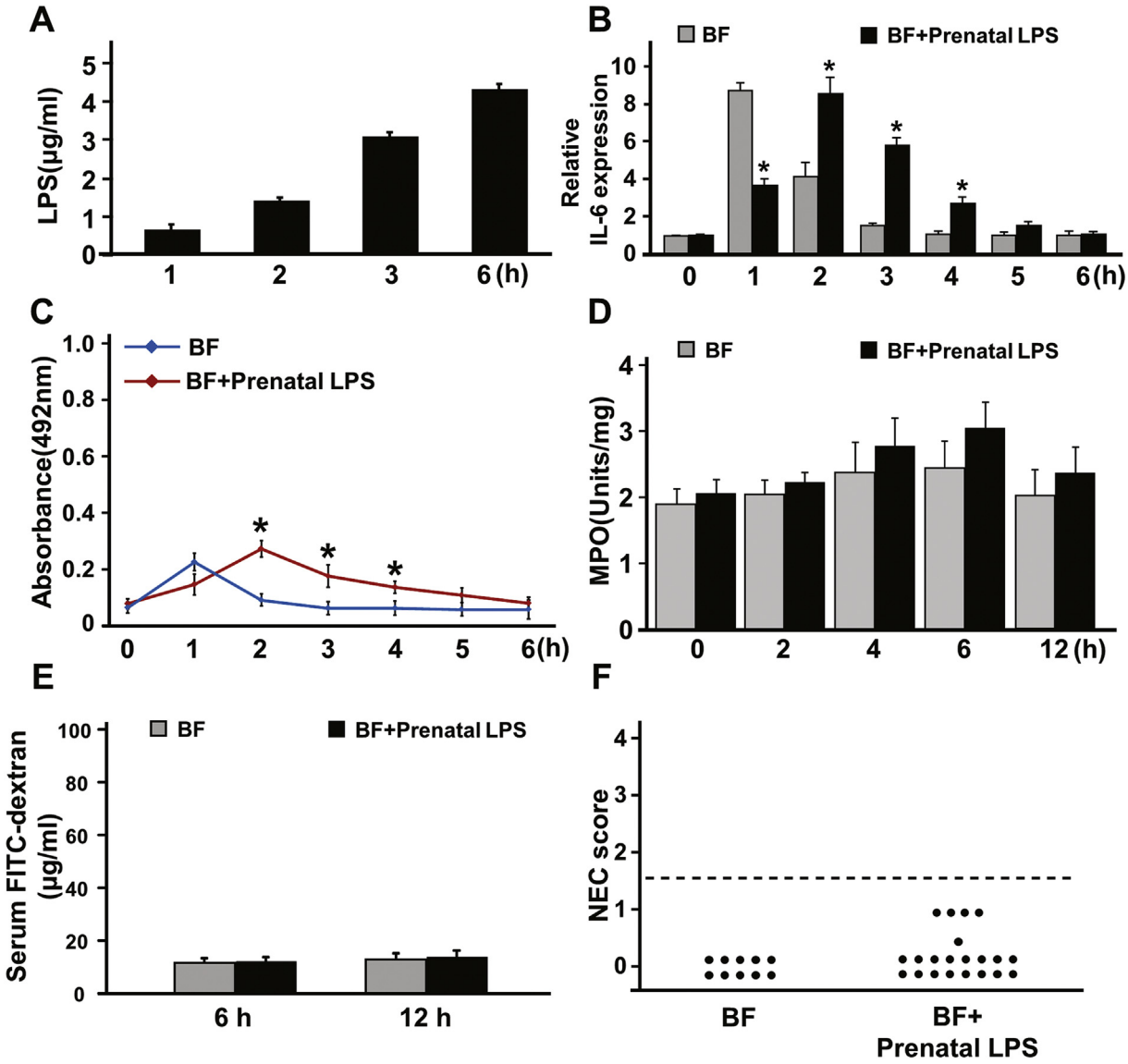

Fig. 2. Prenatal low dose endotoxin exposure disturbs the transient and self-limited NFκB activation in neonatal intestines after birth, though it is insufficient to induce significant injury Intraamniotic injections of PBS or LPS were administered at 17 dpc. The full-term, vaginally delivered and breastfed newborns were studied. (A) The newborns were sacrificed at 1, 2, 3 and 6 h after birth to measure the luminal LPS concentration in terminal ileum. Data are mean ± SD ($n = 6$ neonatal mice) (B) Intestinal IL-6 mRNA levels of the 18 dpc fetuses (0 h) and newborns at 1, 2, 3, 4, 5, and 6 h after birth. The values were shown normalized to time 0 h. Data show means ± SD of three independent experiments. * $p < 0.05$ for controls vs the newborns prenatally exposed to LPS. BF, breast fed pups that were prenatally exposed to PBS; BF+ Prenatal LPS, breast fed pups that were prenatally exposed to LPS (0.01 μg/fetus). (C) DNA binding activity of NF-κB p65 in neonatal intestines at 0, 1, 2, 3, 4, 5, and 6 h after birth ($n = 3$ at each time point). * $p < 0.05$ for controls vs neonates prenatally exposed to LPS. (D) Intestinal MPO activity at 0, 2, 4, 6, and 12 h after birth. Data are mean ± SD ($n = 6$ neonates at each time point). (E) Gut barrier function was determined by measuring serum FITC-dextran levels after enteral administration of FITC-dextran at 6 h and 12 h after birth. Data are mean ± SD ($n = 6$ at each time point). (F) Injury score of the neonatal intestine at 12 h after birth. Shown here are the grades of injury in individual newborn mice. Each dot represents one pup.

within 3 h. In contrast, in the newborns treated with prenatal LPS, p65 activity peaked at 2 h, remained at a high level at 3 h, and declined to a normal level within 6 h, suggesting prolonged activation of NFκB (Fig. 2C).

2.4. Prenatal low dose endotoxin exposure cannot induce significant injury to the intestines of the full-term, vaginally delivered and breastfed newborns

In newborns, prenatal LPS slightly increased the intestinal MPO activity within the first 6 h after birth. However, between 2 h and 12 h, MPO activity and permeability of the neonatal intestine were not statistically different between the prenatal LPS treated newborns and the controls (Fig. 2D, E).

Furthermore, breastfed newborns showed no abnormalities in intestinal histology. Among the newborns subjected to prenatal LPS, at 12 h, 4 (4/21) pups exhibited histopathologic changes in the intestines characterized as mild injury (1+), while none developed ≥ Grade 2 injury (Figs. 2F and 3A).

2.5. Prenatal low dose endotoxin exposure increases the incidence and severity of experimental NEC

Of the pups that received formula feeding, 25% (6/24) developed NEC. Of the formula fed pups that were prenatally exposed to LPS, 38.5% (10/26) developed NEC. However, the difference between the 2 groups was not statistically significant (Fig. 3B).

On the other hand, 54.2% of mice exposed to experimental NEC exhibited histopathologic changes in the intestines characterized as

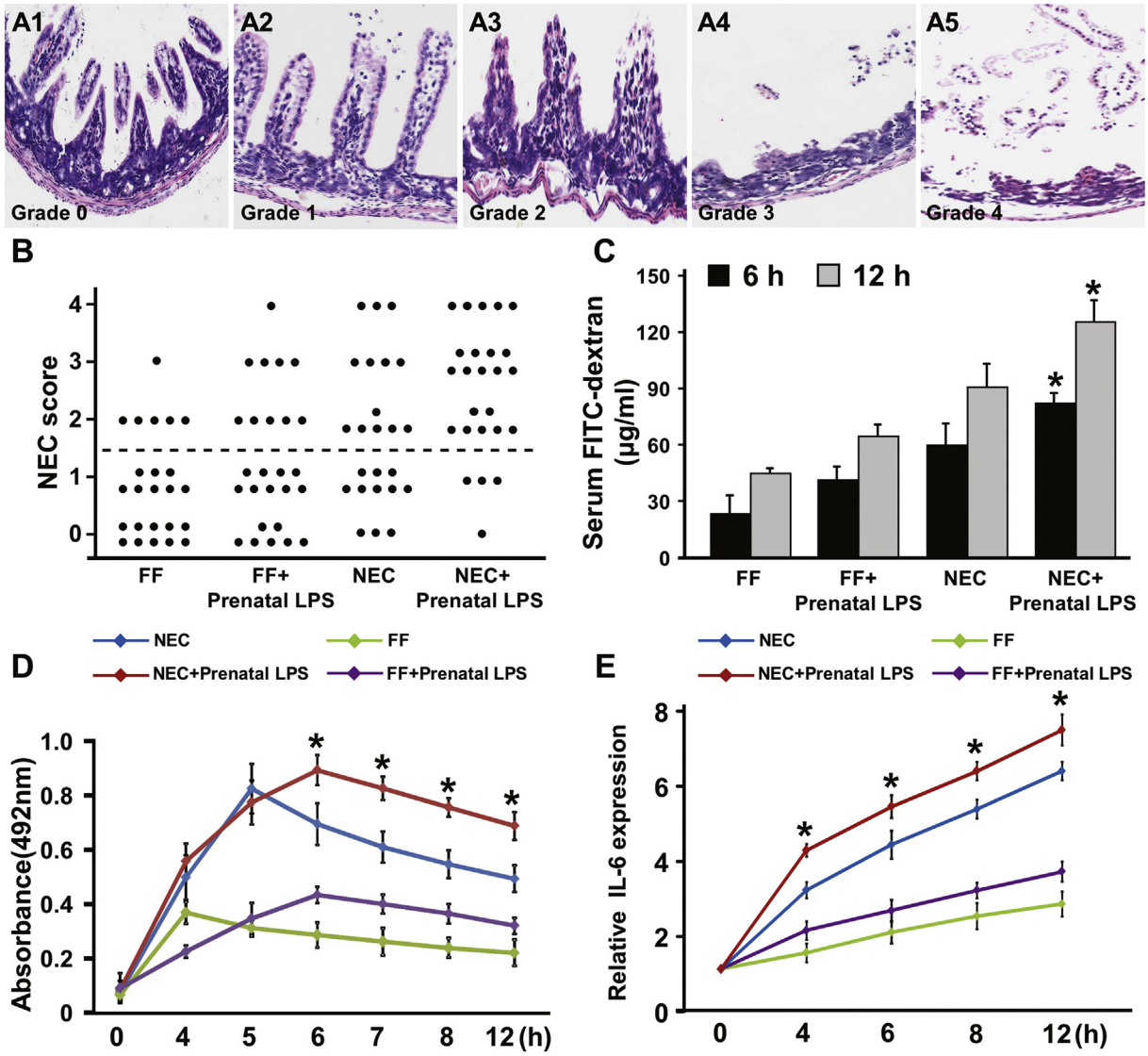

Fig. 3. Prenatal low dose endotoxin exposure increases susceptibility to neonatal necrotizing enterocolitis (NEC). (A1) Grade 0, normal intestine; (A2) Grade 1, epithelial cell lifting or separation; (A3) Grade 2, sloughing of epithelial cells to mid villus level; (A4) Grade 3, necrosis of entire villus; (A5) Grade 4, transmural necrosis. (B) Summary of NEC grade distribution. Shown here are the grades of injury in individual mouse pups. Each dot represents one pup. The dashed line indicates the threshold of normal vs pathological intestine between grades 1 and 2. FF, prenatal PBS exposure combined with formula feeding; FF + Prenatal LPS, prenatal LPS (0.01 μg/fetus) exposure combined with formula feeding; NEC, prenatal PBS exposure combined with experimental NEC; NEC + Prenatal LPS, prenatal LPS exposure combined with experimental NEC. (C) Gut permeability at 6 h and 12 h after birth. Data are mean ± SD (n = 6 at each time point). * $p < 0.05$ for pups subjected to NEC vs the pups subjected to prenatal LPS and NEC. (D) DNA binding activity of NF-κB p65 in neonatal intestines at 0, 4, 5, 6, 7, 8, and 12 h after birth (n = 3 at each time point). * $p < 0.05$ for pups subjected to NEC vs the pups subjected to prenatal LPS and NEC. (E) mRNA level for IL-6 at 0, 4, 6, 8, and 12 h after birth. The values were shown normalized to time 0 h at birth (100%). Data are means ± SD of three independent experiments. * $p < 0.05$ for pups subjected to NEC vs the pups subjected to prenatal LPS and NEC.

moderate (2+), severe (3+), or full necrosis (4+). Significantly, more mice subjected to prenatal LPS developed ≥ Grade 2 injury (84.0% vs 54.2%, $p < 0.05$). Furthermore, prenatal low dose LPS exposure significantly increased the degree of intestinal damage in mice subjected to NEC (median NEC score: 2.6 vs. 1.8, $p < 0.05$) (Fig. 3B).

Moreover, intestinal permeability significantly increased at 6 h after birth, and further increased at 12 h. At 6 h and 12 h, the intestinal permeability was highest in the pups subjected to prenatal LPS exposure and NEC, followed by the pups subjected to NEC, prenatal LPS exposure and formula feeding, and pups just subjected to formula feeding (Fig. 3C).

The DNA binding activity of NF-κB p65 significantly increased at 4 h after birth. p65 activation was most pronounced at 6 h in pups subjected to prenatal LPS and NEC, which declined thereafter but remained at a steady level till 12 h. P65 activation in the NEC pups peaked at 5 h, which was weaker than in pups subjected to prenatal LPS and NEC (Fig. 3D).

IL-6 is a diagnostic marker for early recognition of NEC in preterm infants [22]. Being consistent with the time course of NF-kB activation, intestinal IL-6 mRNA expression was robustly elevated in a time-dependent manner, and increased up to 8 folds within the 12 h after birth in the pups prenatally exposed to low dose LPS (Fig. 3E).

2.6. Low dose LPS activates GSK3β and FoxO1 rather than classic MAPK and NFκB pathways in IECs

After 4 days in culture, a cell population of IECs comprised >95% of the total cells (Fig. 4A1). Co-staining of cytokeratin and the epithelial cell marker CD104 on the dual-color fluorescence histogram demonstrated a double-positive cell population of >98% of the gated cells (Fig. 4A2).

The key TLR4 downstream signaling kinases include GSK3β, Akt, and MAP kinases, which compete in the regulation of inflammation [23].

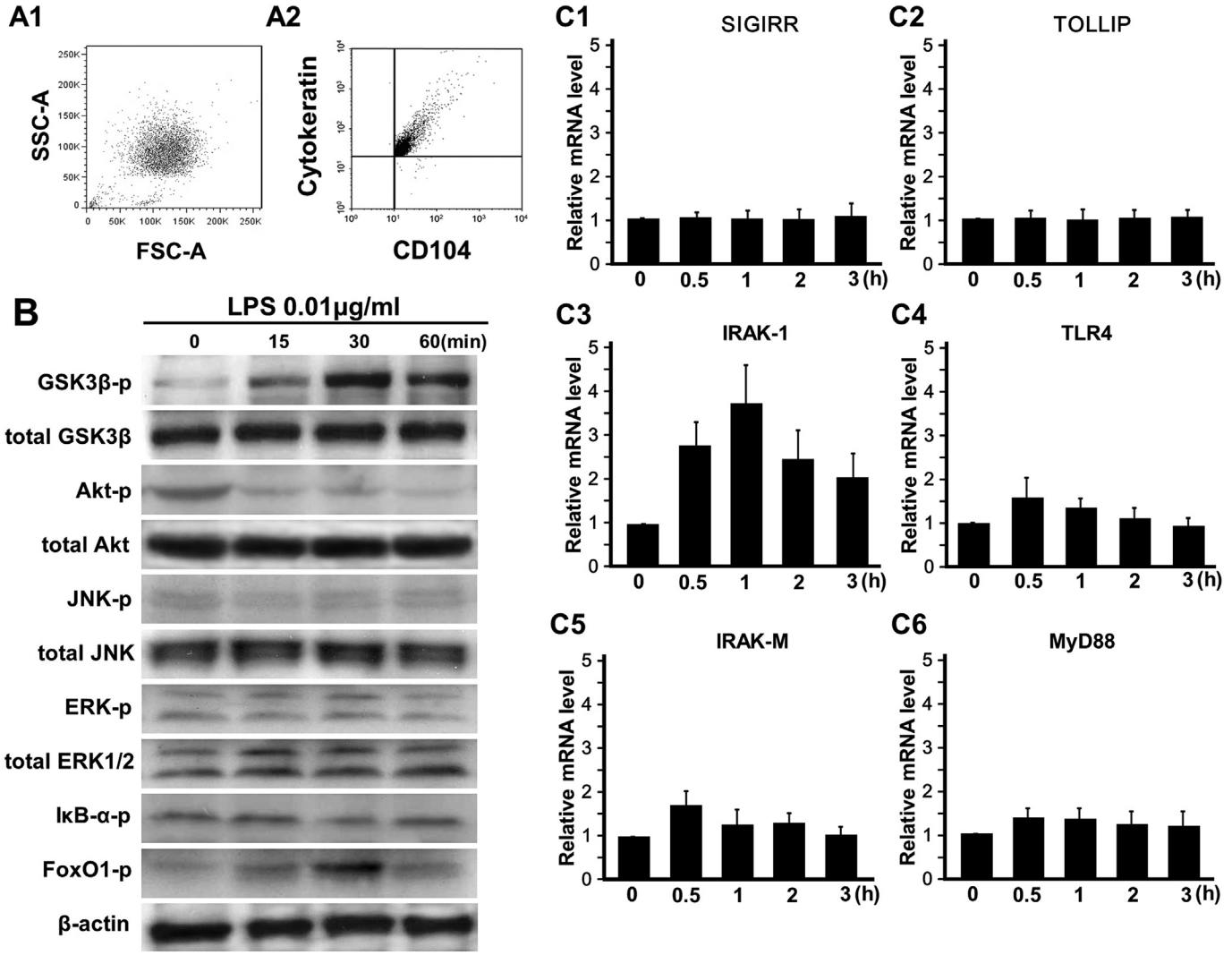

Fig. 4. Low dose LPS activates GSK3 and FoxO1, and induces IRAK-1 expression in intestinal epithelial cells (IECs). (A1) Freshly isolated IECs from 18 dpc fetal intestines were shown on the dot plot of forward scatter (FSC) versus side angle light scatter (SSC). (A2) Flow cytometric detection of cytokeratin and CD104 double-positive cells. (B) Differential phosphorylation and activation of signaling kinases and transcription factors in IECs by low dose LPS. IECs were treated with 0.01 μg/ml LPS for various time periods as indicated. Whole cell lysates were separated on SDS-PAGE. The study was repeated thrice. (C) Differential regulation of mRNA expression of signaling kinases and molecules (C1-C6, TLR4, MyD88, IRAK-1, IRAK-M, SIGIRR, and TOLLIP) in IECs by 0.01 μg/ml LPS. The values were shown normalized to time 0 h of LPS addition (100%). Data are means ± SD of three independent experiments.

Stimulation of IECs with 0.01 μg/ml LPS decreased Akt phosphorylation (Akt-p), and rapidly increased GSK3β phosphorylation (GSK3β-p) at 15 min after the treatment, whereas it failed to induce noticeable activation of ERK and JNK (Fig. 4B).

FoxO1 and NF-κB are competing transcription factors in the downstream regulation of GSK3β [24]. Treatment with LPS caused an increase in the phosphorylation of FoxO1 (FoxO1-p). Low dose LPS had no effect on IκB-α phosphorylation (IκB-α-p), which was consistent with the finding that low dose LPS could not induce NF-κB activation in fetal intestines (Fig. 4B).

2.7. Increased IRAK-1 expression via activation of GSK3β is responsible for prolonged transcriptional activity in IECs upon exposure to high dose LPS

We examined the mRNA expression of the signaling molecules related to the TLR4/NF-κB pathway in IECs. We found that LPS (0.01 μg/ml) significantly increased IRAK-1 expression, and mildly increased the expression of TLR4, IRAK-M and MyD88 (Fig. 4C).

IRAK-1 bound to the TLR/adaptor molecule complex to facilitate NF-κB activation [25]. Stimulation of IECs with 0.01 μg/ml LPS resulted in an increased expression of IRAK-1 at 6 h and peaked at 12 h. SB216763 (Sigma S3442), a compound which selectively inhibits GSK3β, abolished IRAK-1 induction at 12 h by LPS (Fig. 5A).

In the intestines of term fetuses (0 h), prenatal low dose LPS significantly increased the protein levels of IRAK-1 (Fig. 5B). Moreover, IRAK-1 was still detectable at 6 h after birth, but it was not detectable in controls (Fig. 5B).

We next examined whether SiRNA silencing of IRAK-1 could restore the spontaneous endotoxin tolerance of IECs. Down-regulation of IRAK-1 was confirmed by examining IRAK-1 mRNA levels by qRT-PCR (Fig. 5C). At 12 h after transfection, 86.5 ± 2.5% of the IECs had taken up the siRNA targeting IRAK-1. mRNA levels of IRAK-1 were significantly decreased in IECs (fold change: neonate 0.19 ± 0.02 vs. 1) 24 h after siRNA transfection. The inhibition of IRAK-1 expression was confirmed by western blotting (Fig. 5D).

To mimic the transcriptional activation in IECs during commensal colonization, primary IECs from term fetuses were stimulated with high dose LPS (1 μg/ml). We found the phosphorylation of p65 was detectable at 30 min, but it was barely visible at 180 min (Fig. 5E). However, in IECs pretreated with LPS (0.01 μg/ml) for 6 h, after stimulation with 1 μg/ml LPS, phosphorylation of p65 was detected at 30 min, peaked at 60 and 120 min, and was still visible at 180 min (Fig. 5F). However, after IRAK-1 knockdown, in IECs primed with 0.01 μg/ml

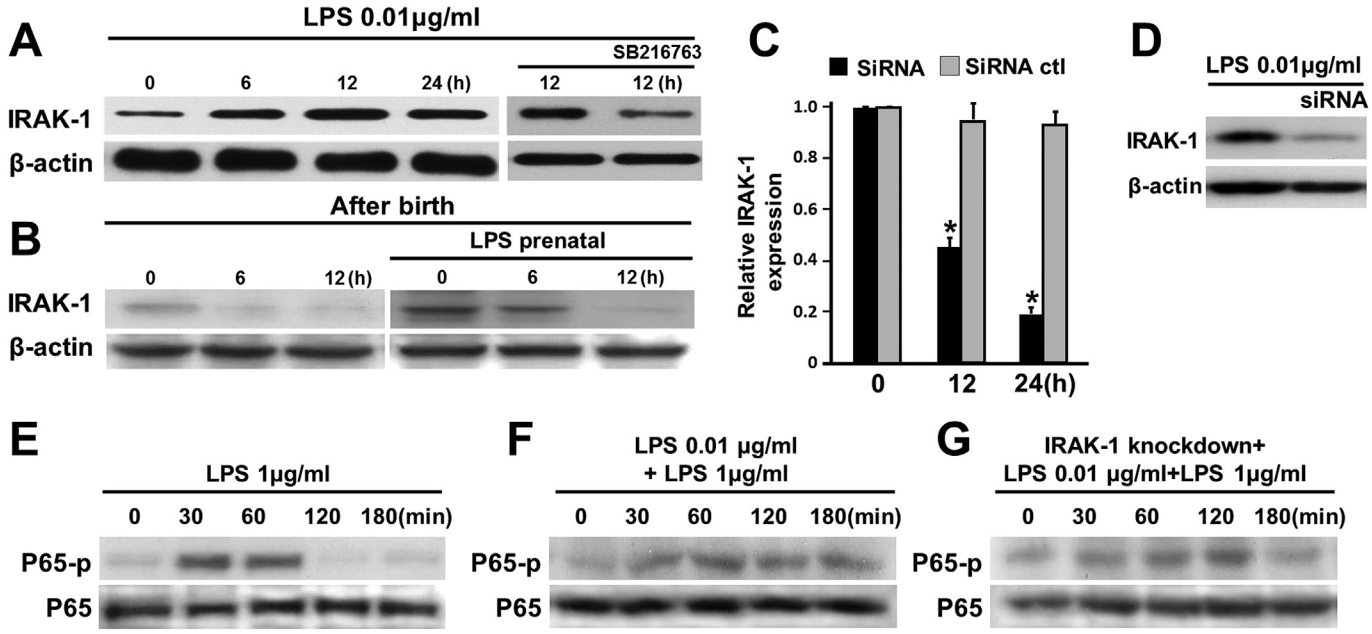

Fig. 5. Low dose LPS augments the transcriptional activity of NF-kB by increasing IRAK-1 expression. (A) Immunoblot for IRAK-1 in primary IECs. LPS (0.01 μg/ml) induced IRAK-1 expression in IECs at 6, 12 and 24 h after treatment. GSK3 inhibitor (SB216763) abolished LPS induced expression of IRAK-1 in IECs at 12 h. (B) Immunoblot for IRAK-1 in intestines harvested from the 18 dpc fetuses (0 h) and the neonates at 6 and 12 h after birth. The fetuses were exposed to 0.01 μg/ml LPS at 17 dpc (LPS prenatal). Prenatal LPS exposure dampened IRAK-1 depletion in neonatal intestines after birth. (C) The primary IECs were transfected with siRNA targeting IRAK-1 or with negative control siRNA (siRNA ctl). IRAK-1 mRNA was examined at 12 and 24 h post-transfection. The values were shown normalized to time 0 h of transfection (100%). Data are means ± SD of three independent experiments. * $p < 0.05$ for IECs transfected with siRNA ctl vs the IECs transfected with siRNA targeting IRAK-1. (D) Immunoblot for IRAK-1 in IECs at 24 h after siRNA transfection. (E) IECs were pretreated with high dose LPS (1 μg/ml). P65 phosphorylation at 0, 30, 60, 120 and 180 min were studied by immunoblotting. (F) IECs were pretreated with 0.01 μg/ml LPS for 6 h, and subsequently treated with high dose LPS (1 μg/ml). (G) IECs were transfected with siRNA targeting IRAK-1. IECs were pretreated with 0.01 μg/ml LPS for 12 h. At 24 h after siRNA transfection, IECs were treated with LPS (1 μg/ml). Equal sample loading is illustrated by total p65 staining. Whole cell lysates were separated on SDS-PAGE. The study was repeated thrice.

LPS, a second LPS (1 μg/ml) challenge induced detectable phosphorylation of p65 at 30 min, which was undetectable at 180 min (Fig. 5G). The results suggested that low dose LPS pretreatment prolonged transcriptional activation of NF-κB induced by high dose LPS. IRAK-1 knockdown restored the self-limited transcriptional activation of NF-κB in IECs upon exposure to LPS.

3. Discussion

It remains unknown as to how mild prenatal inflammation influences the intestinal epithelium when neonates are colonized with gut commensal bacteria [1]. The studies presented here advance our understanding of this as follows. Firstly, intrauterine inflammation, though insufficient to cause histologic injury to fetal intestine, still alters transcriptional activity in neonatal intestines after birth. Secondly, prenatal low dose endotoxin exposure induces IRAK-1 expression in fetal intestines, which prolongs activation of IECs after birth. Lastly, prenatal low dose endotoxin exposure potentially predisposes to postnatal development of NEC.

There are few studies exploring neonatal outcomes after exposure to mild intrauterine inflammation. Most of our knowledge comes from studies using animal models of intrauterine inflammation induced by high dose LPS [5,6]. The innate immune response is different with varying doses of LPS challenge [6]. In this study, LPS at a dose of 0.01 μg per fetus failed to induce NFκB activation, increase the caspase-3 and MPO activity, and cause histological injury in fetal intestines. Previous studies on mice demonstrated the self-limited NFκB activation in IECs after birth, which returned to the baseline within 4–6 h after delivery [11]. Consistent with these observations, we found a transient NFκB activation which declined to a normal level within 3 h after birth. However, in the neonates subjected to prenatal low dose LPS exposure, NFκB activity peaked at 2 h, but normalization was delayed until 6 h, though the prolonged NFκB activation was insufficient to induce significant injury to neonatal intestines. However, when the neonates were subjected to postnatal stresses including formula feeding, hypoxia and hypothermia, NFκB activity was prominently enhanced and prolonged in the neonatal intestines that were prenatally exposed to low dose LPS. IL-6, whose production relies on the activation of NF-κB p65, was a marker in monitoring clinical stages of NEC [21,22]. The robust NFκB activation dramatically increased the expression of IL-6 by up to 8 folds within 12 h after birth, accompanied by an increase in gut permeability and the development of NEC.

To examine the mechanism by which prenatal low dose LPS exposure could enhance NF-κB activation in experimental NEC, we cultured primary IECs. We found that as opposed to high dose LPS, low dose LPS failed to induce robust activation of NFκB and MAPK. Instead, it preferentially caused a rapid increase of phosphorylation of GSK3β and FoxO1. Furthermore, low dose LPS decreased Akt phosphorylation. The signaling kinases GSK3β and Akt are known to compete in the regulation of inflammation [26]. Preferential activation of GSK3β might be responsible for the proinflammatory effects of low dose LPS.

Low dose LPS dramatically induced IRAK-1 expression in primary IECs. IRAK-1 is a key component of the IL-1R signaling pathway. Following stimulation with LPS, IRAK-1 is recruited to the TLR4 receptor complex [25]. This in turn promotes the autophosphorylation of IRAK1, eventually leading to the activation of NFκB [27,28]. Previous studies have shown that IRAK-1 deficiency impacted multiple TLR-dependent pathways and decreased early cytokine responses following sepsis [29,30]. In the current study, low dose LPS increased the expression of IRAK-1 via the activation of GSK3β in primary IECs. Furthermore, in neonatal intestines, IRAK-1 rapidly degraded after birth. However, in the mice prenatally exposed to low dose LPS, degradation of IRAK-1 was postponed until 6 h after birth. These observations showed that prenatal low dose LPS exposure upregulated the expression of IRAK-1, and delayed the degradation of IRAK-1 after birth. Importantly, in the IECs primed by low dose LPS but challenged by a second-high dose LPS, knockdown of IRAK-1 restored an immediate and self-limited NFκB activation, indicating that IRAK-1 was responsible for prolonged epithelial activation.

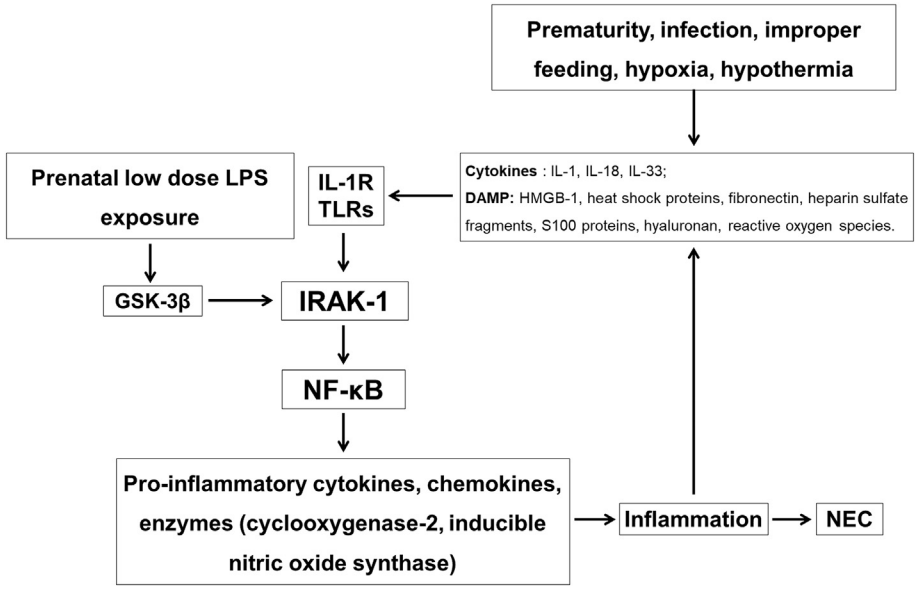

Fig. 6. Proposed mechanism depicting that prenatal low dose endotoxin exposure promotes the development of experimental NEC. Prenatal low dose endotoxin exposure induces the expression of IRAK-1 in IECs. After birth, when immature intestines are exposed to stresses including improper feeding, hypoxia, hypothermia, bacteria colonization and infection, the production of cytokines and DAMP (damage-associated molecular patterns) is increased and further activates IL-1R and Toll-like receptors (TLRs) signaling pathways. High level of IRAK-1 enhances and prolongs the activation of IL-1R/TLRs-NF-κB signaling pathway. The overactivated NF-κB promotes the production of proinflammatory cytokines, chemokines and enzymes, which exacerbate inflammation and promote the development of NEC.

4. Conclusion

To our knowledge, this is the first study to demonstrate that mild intrauterine inflammation disturbs self-limited postnatal epithelial cell activation. Prolonged epithelial activation, even for a few hours, poses a significant risk for neonates that are unable to mount an adequate negative regulatory control. Prematurity, microbial colonization, improper enteral feeding, hypoxia, and hypothermia may further increase the transcriptional activity and escalate inflammation in neonatal intestines to promote the development of NEC (Fig. 6).

Acknowledgments

This study was supported by grants from the National Natural Science Foundation of China (no. 81200266 and no. 81270441).

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