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Probiotic-Mediated Immune Tolerance and NLRP3 Inflammasome Regulation in the Prevention of Spontaneous Preterm Birth Running title: Probiotics Target NLRP3-Treg Axis in Preterm Birth

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Article

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Abstract

Background

Spontaneous preterm birth (sPTB) is a leading cause of neonatal morbidity and mortality worldwide. Excessive NLRP3 inflammasome activation coupled with impaired regulatory T cell (Treg)-mediated immune tolerance may contribute to sPTB pathogenesis; however, whether probiotic-derived metabolites can restore this immunological imbalance remains unclear.

Methods

A case-control study enrolled 45 women with sPTB and 50 term controls. Peripheral blood and fetal membrane/decidual tissues were collected to assess NLRP3 pathway activation (NLRP3, caspase-1, and IL-1β) and immune tolerance markers (Treg proportion, IL-10, and TGF-β). Multivariable logistic regression was performed to evaluate independent associations with sPTB. Fetal membrane explants and peripheral blood mononuclear cells (PBMCs) were cultured in vitro under lipopolysaccharide-stimulated conditions and treated with sodium butyrate or Lactobacillus rhamnosus GG culture supernatant to evaluate effects on NLRP3 suppression and Treg differentiation.

Results

The sPTB group exhibited significantly elevated NLRP3, caspase-1, and IL-1β expression alongside reduced Treg proportions and decreased IL-10 and TGF-β levels compared with term controls (all $P < 0.001$). Tissue NLRP3 expression (adjusted OR = 2.74) and Treg proportion (adjusted OR = 0.61) were independently associated with sPTB. In vitro, both probiotic metabolite interventions significantly attenuated LPS-induced NLRP3 activation and promoted Treg differentiation with enhanced anti-inflammatory cytokine secretion in fetal membrane explants and PBMCs.

Conclusions

NLRP3 overactivation and Treg deficiency coexist and independently contribute to sPTB. Probiotic-derived metabolites may restore immune homeostasis at the maternal-fetal interface by targeting the NLRP3-Treg axis, offering a potential preventive strategy for sPTB.

1. Introduction

Spontaneous preterm birth (sPTB), characterized by delivery occurring prior to 37 weeks of completed pregnancy, continues to be the primary contributor to newborn illness and death across the globe. Current comprehensive worldwide studies indicate that roughly 13.4 million infants were delivered prematurely in 2020, representing about 9.9% of total live births, with no significant reduction in preterm

delivery incidence documented globally during the previous ten years [1]. Prematurity-related health issues cause close to a million infant deaths each year while creating considerable lifelong cognitive, developmental, and economic challenges for impacted children and their relatives [2]. Although improvements have been made in perinatal care, reliable methods for forecasting and averting sPTB are still inadequate, primarily because the fundamental biological mechanisms remain poorly understood [3].

Emerging research consistently highlights sterile intra-amniotic inflammation as a primary factor contributing to sPTB, occurring independently of detectable microbial presence [4]. The NLRP3 inflammasome, a critical innate immunity complex comprising pyrin domain-containing proteins within the NOD-like receptor family, orchestrates inflammatory responses through caspase-1 activation, facilitating the production and secretion of interleukin-1β (IL-1β), a potent pro-inflammatory mediator [5]. Experimental investigations employing NLRP3-knockout murine systems reveal that coordinated NLRP3 activity in both maternal and fetal compartments initiates premature labor pathways, and therapeutic targeting of this inflammasome complex has proven effective in decreasing preterm delivery incidence and improving newborn survival outcomes [6, 7]. Clinical observations confirm upregulated NLRP3 and caspase-1 levels in chorioamniotic membranes obtained from patients undergoing spontaneous preterm labor [8].

Simultaneously, regulatory T cells (Tregs), a specialized subset of CD4 + T lymphocytes identified by Foxp3 expression, serve a critical function in preserving immune tolerance to the semi-allogeneic fetus during pregnancy [9]. Emerging research indicates that diminished Treg populations in both peripheral blood and decidual tissue, along with altered Th17/Treg ratios, disrupt maternal-fetal immune homeostasis and may trigger premature delivery [10, 11]. Longitudinal investigations have demonstrated that declining circulating Treg levels predict spontaneous preterm birth in certain cases before clinical symptoms appear [12]. Nevertheless, the complex relationship between NLRP3 inflammasome signaling and Treg-related immune tolerance defects in spontaneous preterm birth remains insufficiently explored.

The intestinal microbiome and its metabolic byproducts, especially short-chain fatty acids like butyric acid, have gained recognition as significant regulators of immune function across both innate and adaptive immunity. Research indicates butyric acid can inhibit the assembly of NLRP3 inflammasome complexes in diverse cellular populations by interfering with histone deacetylase activity and altering redox-sensitive signaling mechanisms. Concurrently, probiotic formulations containing Lactobacillus strains have drawn growing interest as possible preventive measures against premature delivery, owing to their ability to rebalance vaginal microbial communities and reduce excessive inflammatory responses [13–15]. However, the potential for probiotic-derived metabolic products to concurrently influence both NLRP3 inflammasome activity and regulatory T cell function within human gestational tissues during pregnancy remains unexplored.

This research sought to (1) analyze the activation status of NLRP3 inflammasome signaling and regulatory T cell immune tolerance markers in spontaneous preterm birth cases versus term controls, (2)

assess how these immunological parameters independently associate with sPTB occurrence, and (3) investigate whether probiotic-derived metabolites—particularly sodium butyrate and *Lactobacillus rhamnosus* GG culture supernatant—could suppress NLRP3 inflammasome activation while promoting regulatory T cell differentiation using fetal membrane explants and peripheral blood mononuclear cells.

2. Materials and Methods

2.1 Study Design and Participants

This case-control study was conducted at the Department of Obstetrics, Hospital of Chengdu University of Traditional Chinese Medicine, between January 2023 and December 2025. The research cohort comprised 95 pregnant women, including 45 subjects who experienced spontaneous preterm delivery (sPTB; gestational age under 37 weeks) and 50 control participants delivering at full term (gestational age of 37 weeks or more). Spontaneous preterm birth was defined as the initiation of natural labor with advancing cervical dilation or preterm premature rupture of membranes (PPROM) occurring before completing 37 gestational weeks. Exclusion criteria encompassed medically indicated preterm deliveries (such as those due to pre-eclampsia or placental abruption), multiple gestations, identified fetal anomalies, chorioamnionitis diagnosed prior to enrollment, autoimmune disorders, chronic inflammatory conditions, or administration of antibiotics, immunosuppressive agents, or probiotic supplements within the four-week period preceding delivery. Electronic medical records provided comprehensive demographic and clinical parameters, incorporating maternal age, body mass index (BMI), gravidity, parity, gestational age at delivery, and neonatal birth weight. The study protocols were approved by the Institutional Ethics Committee of Hospital of Chengdu University of Traditional Chinese Medicine, (approval number: 014567). All methods were performed in accordance with the relevant guidelines and regulations. Written informed consent was obtained from all participants prior to enrollment.

2.2 Sample Collection and Processing

Peripheral venous blood specimens (10 mL) were obtained from all enrolled participants upon admission prior to delivery using EDTA-anticoagulated vacuum tubes. Within two hours of collection, blood samples underwent density gradient separation using Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden) at 400 × g for 30 minutes at room temperature to isolate peripheral blood mononuclear cells (PBMCs). The harvested PBMCs underwent two washing cycles with phosphate-buffered saline (PBS) before being resuspended in RPMI 1640 culture medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin for subsequent analyses. Plasma components were isolated by centrifugation at 3000 × g for 15 minutes and stored at − 80°C until use.

Fetal membrane and decidual tissue specimens were acquired within 30 minutes of delivery under sterile conditions. Tissues were obtained from both the rupture site and the adjacent peri-placental region to ensure representative sampling. Each specimen was divided into three portions: the first was immediately snap-frozen in liquid nitrogen and stored at − 80°C for subsequent protein and mRNA

extraction; the second was fixed in 4% paraformaldehyde for immunohistochemical analysis; while the third was transferred into sterile RPMI 1640 medium on ice for subsequent explant culture experiments.

2.3 Assessment of NLRP3 Inflammasome Pathway Activation

The expression levels of NLRP3, cleaved caspase-1 (p20), and mature IL-1β in fetal membrane and decidual tissues were determined by Western blotting. Total protein was extracted using RIPA lysis buffer supplemented with protease and phosphatase inhibitor cocktails (Beyotime, Shanghai, China). Protein concentrations were quantified using the bicinchoninic acid (BCA) assay. Equal amounts of protein (30 μg per lane) were resolved on 10%–12% SDS-PAGE gels before transfer onto PVDF membranes. Following blocking with 5% non-fat milk, membranes were incubated overnight at 4°C with primary antibodies against NLRP3 (1:1000; Cell Signaling Technology, Danvers, MA, USA), cleaved caspase-1 (1:1000; Adipogen, San Diego, CA, USA), IL-1β (1:1000; R&D Systems, Minneapolis, MN, USA), and β-actin (1:5000; Proteintech, Rosemont, IL, USA). Detection was achieved using HRP-conjugated secondary antibodies and an ECL detection system, with band intensities quantified using ImageJ software (NIH, Bethesda, MD, USA) and normalized to β-actin. Complementary mRNA expression of NLRP3, caspase-1, and IL-1β was assessed by SYBR Green-based qRT-PCR (Applied Biosystems, Foster City, CA, USA) with GAPDH serving as the internal reference gene. Plasma IL-1β concentrations were determined using commercially available ELISA kits (R&D Systems) according to the manufacturer's instructions.

2.4 Evaluation of Immune Tolerance Markers

The frequency of $CD4^+CD25^+Foxp3^+$ regulatory T cells (Tregs) within peripheral blood mononuclear cells (PBMCs) was assessed through multiparametric flow cytometric analysis. Freshly harvested PBMCs (1×10^6 cells per sample) underwent surface labeling with fluorescent anti-CD4-FITC and anti-CD25-PE monoclonal antibodies (BioLegend, San Diego, CA, USA) for 30 minutes under 4°C dark conditions. Subsequent to surface marker staining, cellular fixation and permeabilization were performed employing the Foxp3/Transcription Factor Staining Buffer Set (eBioscience, Thermo Fisher Scientific), followed by intracellular staining with anti-Foxp3-APC antibody. Cellular acquisition was conducted using a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA), with subsequent data processing executed in FlowJo software (v10.8; Tree Star, Ashland, OR, USA). Treg populations were defined as $CD4^+CD25^+Foxp3^+$ triple-positive subsets and expressed as a percentage of total $CD4^+$ T cells. Plasma concentrations of interleukin-10 (IL-10) and transforming growth factor-β (TGF-β) were measured via commercially available ELISA kits (R&D Systems) in accordance with the manufacturer's instructions.

2.5 In Vitro Intervention with Probiotic Metabolites

To examine how probiotic-derived metabolites influence NLRP3 inflammasome activation and regulatory T cell differentiation, fetal membrane explant cultures and peripheral blood mononuclear cell (PBMC) cultures were prepared using clinical specimens obtained from the sPTB group. Fetal membrane sections measuring approximately 1 cm^2 were excised and placed in 24-well plates, where they were

maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified 5% CO_2 incubator. For PBMC experiments, cells were seeded at a density of 5×10^5 cells per well in 96-well plates under identical culture conditions.

Both explants and PBMCs were randomly assigned to four experimental groups: (1) control group (culture medium only); (2) LPS group (100 ng/mL lipopolysaccharide derived from Escherichia coli O111:B4; Sigma-Aldrich, St. Louis, MO, USA); (3) LPS + sodium butyrate (NaB) group (100 ng/mL LPS combined with 0.5 mM sodium butyrate; Sigma-Aldrich); and (4) LPS + probiotic culture supernatant (PCS) group (100 ng/mL LPS supplemented with 10% v/v cell-free supernatant from Lactobacillus rhamnosus GG cultured for 48 hours in MRS broth, sterilized through 0.22-μm filtration).

Incubation periods differed between sample types, with explants cultured for 24 hours and PBMCs maintained for 72 hours. Following the intervention period, culture supernatants were collected for cytokine quantification (IL-1β, IL-10, and TGF-β) by ELISA, while cellular and tissue material was processed for NLRP3 pathway protein detection by Western blotting. PBMC samples additionally underwent flow cytometric assessment of regulatory T cell proportions as described above.

2.6 Statistical Analysis

Statistical processing was carried out with IBM SPSS Statistics (v26.0; IBM Corporation, Armonk, NY, USA) and GraphPad Prism (v9.0; GraphPad Software, San Diego, CA, USA). Numerical data were presented as mean ± SD or median (IQR) following normality assessment by the Shapiro-Wilk test. Between-group comparisons employed Student's t-test or Mann-Whitney U test for continuous variables, while categorical variables were evaluated using the chi-square test or Fisher's exact test. Multivariable binary logistic regression was performed to identify independent predictors of sPTB, incorporating covariates with $P < 0.10$ in univariate analysis and those deemed clinically relevant. In vitro experimental data were analyzed using one-way ANOVA with Tukey's post hoc test or the Kruskal-Wallis test with Dunn's correction for non-parametric distributions. All experiments were performed in triplicate and repeated independently at least three times. A two-tailed P value of less than 0.05 was considered statistically significant.

3. Results

3.1 Baseline Demographic and Clinical Characteristics

Table 1 outlines the fundamental demographic and clinical attributes of participants. Comparative analysis revealed no meaningful variations between the sPTB cohort (n = 45) and full-term controls (n = 50) concerning maternal age, pre-pregnancy body mass index, gravidity, parity, or gestational diabetes prevalence (all $P > 0.05$). Notably, individuals experiencing sPTB demonstrated substantially elevated frequencies of previous preterm deliveries (20.0% vs. 6.0%, $P = 0.038$) and bacterial vaginosis during the current pregnancy (24.4% vs. 8.0%, $P = 0.027$). As expected, the sPTB group showed markedly reduced gestational age at delivery (33.6 ± 2.5 vs. 39.2 ± 1.1 weeks, $P < 0.001$) and neonatal birth weight (2,205 ±

486 vs. 3,374 ± 398 g, P < 0.001). Preterm premature rupture of membranes was documented in 53.3% of preterm cases.

Table 1
Baseline Demographic and Clinical Characteristics of Study Participants

Characteristic	sPTB Group (n = 45)	Term Group (n = 50)	P value
Maternal age (years)	29.8 ± 5.1	30.3 ± 4.6	0.621
Pre-pregnancy BMI (kg/m²)	23.8 ± 3.6	23.2 ± 3.3	0.398
Gestational BMI at delivery (kg/m²)	28.4 ± 3.9	28.1 ± 3.4	0.684
Gravidity, median (IQR)	2 (1–3)	2 (1–3)	0.753
Parity, median (IQR)	1 (0–1)	1 (0–1)	0.814
Nulliparity, n (%)	22 (48.9)	26 (52.0)	0.762
Prior preterm birth, n (%)	9 (20.0)	3 (6.0)	0.038*
Gestational diabetes mellitus, n (%)	6 (13.3)	5 (10.0)	0.614
Bacterial vaginosis, n (%)	11 (24.4)	4 (8.0)	0.027*
Smoking during pregnancy, n (%)	3 (6.7)	2 (4.0)	0.665
Gestational age at delivery (weeks)	33.6 ± 2.5	39.2 ± 1.1	< 0.001***
PPROM, n (%)	24 (53.3)	—	—
Neonatal birth weight (g)	2,205 ± 486	3,374 ± 398	< 0.001***
Neonatal male sex, n (%)	25 (55.6)	24 (48.0)	0.458
5-min Apgar score < 7, n (%)	7 (15.6)	1 (2.0)	0.024*

Data are presented as mean ± SD, median (IQR), or n (%). *P < 0.05; ***P < 0.001. BMI, body mass index; IQR, interquartile range; PPROM, preterm premature rupture of membranes.

3.2 NLRP3 Inflammasome Pathway Was Significantly Activated in sPTB

The activation status of the NLRP3 inflammasome was evaluated by examining NLRP3, caspase-1, and IL-1β protein and mRNA levels in fetal membrane and decidual specimens (Table 2; Fig. 1). Western blot analysis indicated substantially higher NLRP3 protein expression in the sPTB group compared with term controls (1.78 ± 0.61 vs. 0.93 ± 0.38, P < 0.001). Correspondingly, cleaved caspase-1 (p20) levels showed a pronounced increase in sPTB cases (1.52 ± 0.55 vs. 0.81 ± 0.33, P < 0.001), with mature IL-1β protein also being significantly elevated (1.69 ± 0.68 vs. 0.86 ± 0.37, P < 0.001). These findings were corroborated by qRT-PCR analysis, which revealed significantly upregulated mRNA expression of NLRP3 (3.18 ± 1.42 vs. 1.47 ± 0.64, P < 0.001), caspase-1 (2.74 ± 1.15 vs. 1.31 ± 0.58, P < 0.001), and IL-1β (3.52

± 1.56 vs. 1.38 ± 0.71, P < 0.001) in the sPTB group. Furthermore, plasma IL-1β concentrations measured by ELISA were significantly higher in sPTB patients (36.8 ± 15.4 pg/mL) compared with term controls (17.9 ± 8.6 pg/mL, P < 0.001).

Table 2
Comparison of NLRP3 Inflammasome Pathway and Immune Tolerance Markers Between sPTB and Term Control Groups

Parameter	sPTB Group (n = 45)	Term Group (n = 50)	t / Z value	P value
Tissue protein expression (relative to β-actin)				
NLRP3	1.78 ± 0.61	0.93 ± 0.38	8.12	< 0.001***
Cleaved caspase-1 (p20)	1.52 ± 0.55	0.81 ± 0.33	7.65	< 0.001***
Mature IL-1β	1.69 ± 0.68	0.86 ± 0.37	7.38	< 0.001***
Tissue mRNA expression (fold change)				
NLRP3 mRNA	3.18 ± 1.42	1.47 ± 0.64	7.54	< 0.001***
Caspase-1 mRNA	2.74 ± 1.15	1.31 ± 0.58	7.66	< 0.001***
IL-1β mRNA	3.52 ± 1.56	1.38 ± 0.71	8.47	< 0.001***
Plasma cytokine levels				
IL-1β (pg/mL)	36.8 ± 15.4	17.9 ± 8.6	7.37	< 0.001***
IL-10 (pg/mL)	13.2 ± 6.1	25.4 ± 9.7	−7.41	< 0.001***
TGF-β (pg/mL)	192.6 ± 78.3	348.5 ± 115.2	−7.82	< 0.001***
Peripheral blood Treg				
$CD4^+CD25^+Foxp3^+$ Treg (% of $CD4^+$)	3.91 ± 1.48	6.58 ± 1.97	−7.53	< 0.001***

Data are presented as mean ± SD. Independent-samples t-test was applied for normally distributed data. ***P < 0.001.

Notably, the sPTB group exhibited considerably greater standard deviations relative to the term control group across all NLRP3 pathway markers, reflecting substantial interindividual variability in

inflammasome activation among preterm birth cases.

3.3 Impaired Treg-Mediated Immune Tolerance in sPTB

Flow cytometric analysis of peripheral blood demonstrated a markedly reduced proportion of $CD4^+CD25^+Foxp3^+$ Tregs among total $CD4^+$ T cells in sPTB subjects relative to term controls (3.91 ± 1.48% vs. 6.58 ± 1.97%, $P < 0.001$; Table 2; Fig. 1). Consistent with this Treg reduction, circulating concentrations of the anti-inflammatory cytokines IL-10 and TGF-β were both significantly lower in the sPTB group (IL-10: 13.2 ± 6.1 vs. 25.4 ± 9.7 pg/mL, $P < 0.001$; TGF-β: 192.6 ± 78.3 vs. 348.5 ± 115.2 pg/mL, $P < 0.001$). Notably, five participants in the sPTB group (11.1%) exhibited Treg proportions exceeding 6%, overlapping with the lower range of term control values, indicating that while Treg insufficiency correlates strongly with sPTB, it was not universally present across all preterm cases.

3.4 NLRP3 Hyperactivation and Treg Deficiency Were Independently Associated with sPTB

Multivariable logistic regression analysis assessed the independent associations of NLRP3 inflammasome activation and Treg-mediated immune tolerance markers with sPTB (Table 3; Fig. 2). After adjustment for maternal age, pre-pregnancy BMI, prior preterm birth history, and bacterial vaginosis, elevated tissue NLRP3 protein expression (adjusted OR = 2.74, 95% CI: 1.28–5.86, $P = 0.009$) and higher plasma IL-1β concentrations (adjusted OR = 1.05, 95% CI: 1.01–1.09, $P = 0.018$) remained independently associated with increased sPTB risk. Conversely, higher Treg proportion (adjusted OR = 0.61, 95% CI: 0.42–0.88, $P = 0.008$) and elevated plasma IL-10 levels (adjusted OR = 0.92, 95% CI: 0.86–0.98, $P = 0.014$) were independently associated with reduced sPTB risk. Prior preterm birth history also retained statistical significance in the adjusted model (adjusted OR = 3.56, 95% CI: 1.08–11.73, $P = 0.037$). Plasma TGF-β showed a trend toward an inverse association with sPTB risk but did not reach statistical significance after adjustment (adjusted OR = 0.997, 95% CI: 0.993–1.001, $P = 0.086$).

Table 3
Multivariable Logistic Regression Analysis for Independent Risk Factors of Spontaneous Preterm Birth

Variable	Crude OR (95% CI)	P value	Adjusted OR (95% CI)	P value
Maternal age (years)	0.98 (0.90–1.07)	0.618	0.97 (0.88–1.07)	0.534
Pre-pregnancy BMI (kg/m²)	1.05 (0.94–1.17)	0.395	1.04 (0.92–1.18)	0.516
Prior preterm birth (yes vs. no)	3.92 (1.01–15.27)	0.049*	3.56 (1.08–11.73)	0.037*
Bacterial vaginosis (yes vs. no)	3.72 (1.10–12.56)	0.034*	2.89 (0.78–10.72)	0.112
Tissue NLRP3 protein expression	3.15 (1.58–6.28)	0.001**	2.74 (1.28–5.86)	0.009**
Plasma IL-1β (pg/mL)	1.06 (1.03–1.10)	< 0.001***	1.05 (1.01–1.09)	0.018*
Treg proportion (% of $CD4^+$)	0.54 (0.39–0.75)	< 0.001***	0.61 (0.42–0.88)	0.008**
Plasma IL-10 (pg/mL)	0.89 (0.84–0.94)	< 0.001***	0.92 (0.86–0.98)	0.014*
Plasma TGF-β (pg/mL)	0.994 (0.991–0.998)	< 0.001***	0.997 (0.993–1.001)	0.086

*P < 0.05; **P < 0.01; ***P < 0.001. OR, odds ratio; CI, confidence interval. Adjusted for maternal age, pre-pregnancy BMI, prior preterm birth, and bacterial vaginosis.

3.5 Probiotic Metabolites Attenuated LPS-Induced NLRP3 Inflammasome Activation in Fetal Membrane Explants

Fetal membrane explant cultures exposed to LPS stimulation were analyzed to assess how probiotic metabolites influence NLRP3 inflammasome activity (Table 4; Fig. 3). LPS treatment (100 ng/mL, 24 hours) resulted in substantial upregulation of NLRP3 protein expression (2.71 ± 0.82 vs. 0.94 ± 0.35, P < 0.001), cleaved caspase-1 (2.18 ± 0.67 vs. 0.77 ± 0.29, P < 0.001), and mature IL-1β protein (2.53 ± 0.74 vs. 0.82 ± 0.31, P < 0.001) relative to unstimulated controls, accompanied by significantly elevated IL-1β concentrations in culture supernatants (192.4 ± 58.6 vs. 43.7 ± 17.5 pg/mL, P < 0.001).

Table 4
NLRP3 Inflammasome Pathway Markers in Fetal Membrane Explant Cultures Following LPS Stimulation and Probiotic Metabolite Intervention (24 Hours)

Parameter	Control	LPS	LPS + NaB	LPS + PCS	F value	P value (ANOVA)
Tissue protein expression (relative to β-actin)						
NLRP3	0.94 ± 0.35	2.71 ± 0.82a	1.53 ± 0.56ab	1.68 ± 0.61ab	18.74	< 0.001
Cleaved caspase-1	0.77 ± 0.29	2.18 ± 0.67a	1.24 ± 0.46ab	1.38 ± 0.51ab	16.52	< 0.001
Mature IL-1β	0.82 ± 0.31	2.53 ± 0.74a	1.41 ± 0.53ab	1.57 ± 0.58ab	19.87	< 0.001
Supernatant cytokine levels (pg/mL)						
IL-1β	43.7 ± 17.5	192.4 ± 58.6a	98.3 ± 34.2ab	113.5 ± 39.7ab	24.63	< 0.001
IL-10	32.6 ± 12.8	18.4 ± 7.9a	48.7 ± 16.5ab	43.2 ± 15.1ab	12.38	< 0.001
TGF-β	145.8 ± 54.2	87.3 ± 35.6a	198.4 ± 68.7ab	178.6 ± 62.3ab	9.14	< 0.001

Data are presented as mean ± SD from three independent experiments performed in triplicate. a P < 0.05 vs. Control; b P < 0.05 vs. LPS group (Tukey's post hoc test). NaB, sodium butyrate; PCS, probiotic culture supernatant.

Co-treatment with sodium butyrate (NaB, 0.5 mM) markedly attenuated LPS-induced NLRP3 protein expression (1.53 ± 0.56 vs. 2.71 ± 0.82, P < 0.001), cleaved caspase-1 (1.24 ± 0.46 vs. 2.18 ± 0.67, P < 0.001), and mature IL-1β levels (1.41 ± 0.53 vs. 2.53 ± 0.74, P < 0.001). Supernatant IL-1β concentration was also reduced in the LPS + NaB group compared with LPS alone (98.3 ± 34.2 vs. 192.4 ± 58.6 pg/mL, P < 0.001), although it remained significantly elevated above control levels (P < 0.001). Probiotic culture supernatant (PCS) treatment exhibited a similar inhibitory pattern, reducing NLRP3 expression (1.68 ± 0.61), cleaved caspase-1 (1.38 ± 0.51), mature IL-1β protein (1.57 ± 0.58), and supernatant IL-1β release (113.5 ± 39.7 pg/mL) compared with the LPS group (all P < 0.01). Direct comparison between LPS + NaB and LPS + PCS groups revealed no statistically significant difference for NLRP3 (P = 0.284) or caspase-1 (P = 0.217); however, the NaB group showed a non-significant trend toward greater IL-1β suppression in culture supernatants (P = 0.073).

3.6 Probiotic Metabolites Promoted Treg Differentiation and Anti-inflammatory Cytokine Secretion in PBMCs

To evaluate adaptive immune modulation, PBMCs from sPTB cases underwent 72-hour culture under the same four-group experimental conditions (Table 5; Fig. 3). LPS stimulation markedly suppressed Treg differentiation, reducing the $CD4^+CD25^+Foxp3^+$ proportion from 4.24 ± 1.56% (control) to 2.43 ± 1.08% ($P < 0.001$). Both NaB and PCS interventions effectively reversed this suppression, elevating Treg proportions to 5.93 ± 1.87% (LPS + NaB, $P < 0.001$ vs. LPS) and 5.28 ± 1.72% (LPS + PCS, $P < 0.001$ vs. LPS), respectively. Notably, both intervention groups achieved Treg proportions exceeding that of untreated controls, though statistical significance was confirmed only for the NaB group ($P = 0.031$ vs. control), while the PCS group showed a non-significant trend ($P = 0.078$ vs. control).

Table 5
Treg Differentiation and Anti-inflammatory Cytokine Production in PBMCs Following LPS Stimulation and Probiotic Metabolite Intervention (72 Hours)

Parameter	Control	LPS	LPS + NaB	LPS + PCS	F value	P value (ANOVA)
Treg (% of $CD4^+$)	4.24 ± 1.56	2.43 ± 1.08a	5.93 ± 1.87ab	5.28 ± 1.72ab	11.65	< 0.001
IL-10 (pg/mL)	29.8 ± 11.4	15.6 ± 7.2a	44.3 ± 16.8ab	39.1 ± 14.6ab	10.82	< 0.001
TGF-β (pg/mL)	162.4 ± 61.8	88.5 ± 37.2a	226.3 ± 78.4ab	204.7 ± 71.5ab	10.27	< 0.001
Foxp3 mRNA (fold change)	1.00 ± 0.34	0.48 ± 0.21a	1.72 ± 0.63ab	1.51 ± 0.57ab	14.38	< 0.001
IL-1β (pg/mL)	38.5 ± 14.7	168.3 ± 51.4a	82.6 ± 28.9ab	96.4 ± 33.5ab	22.15	< 0.001

Data are presented as mean ± SD from three independent experiments performed in triplicate. a $P < 0.05$ vs. Control; b $P < 0.05$ vs. LPS group (Tukey's post hoc test).

Supernatant IL-10 levels mirrored the Treg response pattern, with LPS reducing IL-10 secretion (15.6 ± 7.2 vs. 29.8 ± 11.4 pg/mL, $P < 0.001$), and both NaB (44.3 ± 16.8 pg/mL, $P < 0.001$ vs. LPS) and PCS (39.1 ± 14.6 pg/mL, $P < 0.001$ vs. LPS) significantly enhancing IL-10 production beyond control levels. TGF-β levels demonstrated a similar trend, although the magnitude of enhancement was more modest in the PCS group compared with NaB (204.7 ± 71.5 vs. 226.3 ± 78.4 pg/mL, $P = 0.341$).

3.7 Correlation Between NLRP3 Inflammasome Activation and Immune Tolerance Impairment

To further explore the interrelationship between inflammasome activity and immune tolerance dysfunction, Pearson and Spearman correlation analyses were conducted using clinical data from all 95 participants (Fig. 4). Tissue NLRP3 protein expression was negatively correlated with peripheral Treg proportion ($r = -0.44$, $P < 0.001$) and plasma IL-10 levels ($r = -0.40$, $P < 0.001$). Plasma IL-1β concentration was also inversely correlated with Treg proportion ($r = -0.50$, $P < 0.001$) and plasma TGF-β levels ($r = -0.36$, $P < 0.001$). A moderate positive correlation was observed between Treg proportion and

plasma IL-10 (r = 0.38, P < 0.001). Among the NLRP3 pathway components, tissue IL-1β protein expression demonstrated the strongest inverse correlation with Treg proportion (r = − 0.56, P < 0.001). These findings support a reciprocal relationship between NLRP3-driven inflammation and Treg-mediated immune tolerance at the clinical level.

4. Discussion

Spontaneous preterm birth remains a major obstetric challenge with limited effective preventive strategies. The present study provides clinical and in vitro evidence linking NLRP3 inflammasome overactivation and regulatory T cell insufficiency to the pathogenesis of spontaneous preterm birth, and demonstrates that probiotic-derived metabolites, particularly sodium butyrate and Lactobacillus rhamnosus GG culture supernatant, can attenuate inflammasome activation while promoting Treg differentiation under inflammatory conditions. These findings establish a dual immunological axis, encompassing both innate inflammasome-driven inflammation and adaptive immune tolerance failure, as a mechanistic framework for understanding spontaneous preterm birth, and highlight the therapeutic potential of microbiota-derived metabolites in restoring maternal-fetal immune homeostasis.

The NLRP3 inflammasome plays a pivotal role as a danger signal detector within the maternal-fetal microenvironment, with its abnormal functioning linked to various gestational disorders such as preeclampsia, recurrent pregnancy loss, and premature delivery [16, 17]. The present study revealed markedly increased levels of NLRP3, cleaved caspase-1, and mature IL-1β in fetal membrane and decidual tissues from spontaneous preterm birth cases when contrasted with term controls, aligning with previous findings showing inflammasome activation in chorioamniotic membranes collected after preterm delivery [18]. The parallel upregulation at both protein and mRNA levels suggests transcriptional activation of the NLRP3 pathway rather than merely post-translational assembly, which may reflect sustained exposure to endogenous danger-associated molecular patterns or microbial products in the context of subclinical intrauterine infection [19]. Notably, the elevated plasma IL-1β concentrations in the spontaneous preterm birth group corroborate previous findings indicating that systemic inflammasome-related cytokine profiles are altered weeks before the clinical onset of preterm labor [20]. These observations collectively reinforce the notion that NLRP3 inflammasome activation, both locally in the fetal membranes and systemically in the maternal circulation, represents a significant immunopathological feature of spontaneous preterm birth.

Notably, our investigation revealed a significant decrease in circulating CD4 + CD25+Foxp3 + regulatory T cell populations in cases of spontaneous preterm delivery. These specialized lymphocytes play a critical role in preserving maternal immunological acceptance of fetal antigens with partial genetic mismatch. Multiple studies have established a clear correlation between diminished Treg numbers or impaired activity and various pregnancy complications [21, 22]. The concomitant decrease in plasma IL-10 and TGF-β levels further suggests a global impairment of the immunosuppressive milieu required for successful pregnancy maintenance [23]. It is noteworthy, however, that not all women in the spontaneous preterm birth group exhibited Treg deficiency, as approximately 11% demonstrated Treg

proportions overlapping with the term control range. This heterogeneity aligns with the recognized multifactorial etiology of spontaneous preterm birth and implies that immune tolerance failure may represent one of several converging pathological pathways rather than a universal prerequisite [24].

The correlation analyses performed in this study revealed a significant inverse relationship between NLRP3 pathway activation and Treg-mediated immune tolerance, with tissue IL-1β protein expression demonstrating the strongest negative correlation with Treg proportions. This observation is biologically plausible, given that IL-1β has been shown to inhibit Foxp3 expression and redirect naive CD4 + T cell differentiation toward the Th17 lineage at the expense of Treg commitment [25, 26]. The NLRP3/IL-1β axis may therefore exert a dual detrimental effect in the context of pregnancy: directly promoting uterine contractility and membrane weakening through prostaglandin and matrix metalloproteinase induction, while simultaneously undermining Treg-dependent fetal tolerance through cytokine-mediated immune skewing [27]. The multivariable regression analysis confirmed that both NLRP3 overexpression and Treg deficiency were independently associated with spontaneous preterm birth after adjustment for established clinical risk factors, supporting their roles as complementary rather than redundant pathological mechanisms. The observation that plasma TGF-β did not retain independent significance in the adjusted model may reflect its pleiotropic functions in pregnancy, where it participates in both pro-inflammatory and anti-inflammatory signaling depending on the local cytokine milieu [28].

A central aim of this study was to evaluate whether probiotic-derived metabolites could simultaneously suppress NLRP3 inflammasome activation and promote Treg differentiation in clinically relevant tissue models. The in vitro experiments demonstrated that both sodium butyrate and probiotic culture supernatant significantly attenuated LPS-induced NLRP3 pathway activation in fetal membrane explants, as evidenced by reduced NLRP3, cleaved caspase-1, and IL-1β expression. These findings are consistent with an expanding body of evidence supporting the anti-inflammatory properties of short-chain fatty acids, particularly butyrate, which has been shown to inhibit NLRP3 inflammasome assembly through histone deacetylase inhibition and modulation of NF-κB signaling in various cell types [29, 30]. Importantly, the present study extends these observations to human fetal membrane tissue, a primary site of inflammation-driven membrane rupture and preterm labor initiation, thereby enhancing the translational relevance of butyrate-mediated inflammasome suppression in the obstetric context.

In parallel, both probiotic metabolite interventions promoted CD4 + CD25+Foxp3 + Treg differentiation in PBMCs from women with spontaneous preterm birth, with the sodium butyrate group achieving Treg proportions that significantly exceeded untreated control levels. This Treg-promoting effect of butyrate is likely mediated through epigenetic mechanisms, as butyrate functions as a potent histone deacetylase inhibitor that enhances acetylation at the Foxp3 promoter and conserved non-coding sequence regions, thereby stabilizing Foxp3 expression and Treg lineage commitment [31, 32]. The accompanying increases in IL-10 and TGF-β secretion further indicate a functional enhancement of the Treg compartment rather than a mere numerical expansion. Although the probiotic culture supernatant demonstrated a qualitatively similar pattern, the magnitude of effect was modestly lower than that of purified sodium butyrate for most endpoints, likely reflecting the complex composition of bacterial

supernatants that contain both bioactive and inert metabolites [33]. These findings suggest that purified short-chain fatty acids may offer a more consistent pharmacological approach, while whole probiotic preparations could provide additional benefits through diverse metabolite profiles.

The clinical implications of these findings merit careful consideration. Probiotic supplementation during pregnancy has been explored for various maternal and neonatal outcomes, yet evidence regarding preterm birth prevention remains inconclusive [34, 35]. The present study provides a mechanistic rationale for targeting the NLRP3-Treg axis through probiotic-derived metabolites, potentially informing the design of future clinical trials with immunologically defined endpoints rather than relying solely on gestational age at delivery. Furthermore, the identification of concurrent NLRP3 overactivation and Treg deficiency as independent risk factors for spontaneous preterm birth raises the possibility of developing composite biomarker panels for early risk stratification among high-risk pregnant women [36].

This research has certain limitations that warrant acknowledgment. Primarily, the case-control design precludes causal inference, and the temporal relationship among inflammasome activation, Treg decline, and preterm labor onset cannot be established from cross-sectional sampling. Second, the in vitro experiments, while performed using clinical specimens, do not fully recapitulate the complexity of the in vivo maternal-fetal interface, where interactions among decidual immune cells, trophoblasts, and the microbiome occur simultaneously [37]. Third, the sample size, although adequate for detecting the observed effect sizes, may have been insufficient to identify more subtle associations, such as the borderline significance of TGF-β in the regression model. Fourth, the probiotic culture supernatant represents a crude mixture whose active components were not individually characterized, limiting mechanistic specificity [38]. Finally, the generalizability of these findings may be constrained by the single-center design and the exclusion of women with clinical chorioamnionitis, who may represent the subgroup most likely to benefit from anti-inflammasome interventions.

Future studies should employ longitudinal cohort designs with serial sampling across gestational trimesters to delineate the temporal dynamics of NLRP3 activation and Treg decline preceding spontaneous preterm birth. Animal models utilizing probiotic or butyrate supplementation during pregnancy would provide important in vivo validation of the observed immunomodulatory effects [39]. Additionally, metabolomic profiling of probiotic supernatants could identify specific bioactive compounds responsible for the dual NLRP3-suppressing and Treg-promoting activities, facilitating the development of targeted postbiotic formulations [40]. Randomized controlled trials evaluating butyrate-based or defined probiotic interventions in high-risk pregnant women, with NLRP3 pathway and Treg-related biomarkers as secondary endpoints, are warranted to translate these preclinical findings into clinical practice.

5. Conclusion

This study demonstrates that NLRP3 inflammasome overactivation and regulatory T cell deficiency coexist and are independently associated with spontaneous preterm birth. Probiotic-derived metabolites,

particularly sodium butyrate, effectively suppress NLRP3 pathway activation in fetal membrane tissues while promoting Treg differentiation in peripheral blood mononuclear cells under inflammatory conditions. These findings provide mechanistic evidence supporting the potential of microbiota-derived metabolites as a preventive strategy targeting the NLRP3-Treg immunological axis in spontaneous preterm birth.

Declarations

Clinical trial number

Not applicable.

Conflicts of Interest

The authors declared that they have no conflicts of interest regarding this work.

Ethics approval and consent to participate

The study protocols were approved by the Institutional Ethics Committee of Hospital of Chengdu University of Traditional Chinese Medicine, (approval number: 014567). All methods were performed in accordance with the relevant guidelines and regulations. Written informed consent was obtained from all participants prior to enrollment.

Funding

Author Contribution

Xuemei Wang: Conceptualization, Formal Analysis, Visualization; Lin Zhuang1: Methodology, Investigation , Software; Heying Huang1: Writing - Original Draft, Validation, Supervision; Jin Chen: Investigation , Resources, Writing - Review & Editing; Jiao Xu: Data Curation, Formal Analysis, Funding Acquisition; Xiaoyin Wang: Writing - Review & Editing

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

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Figures

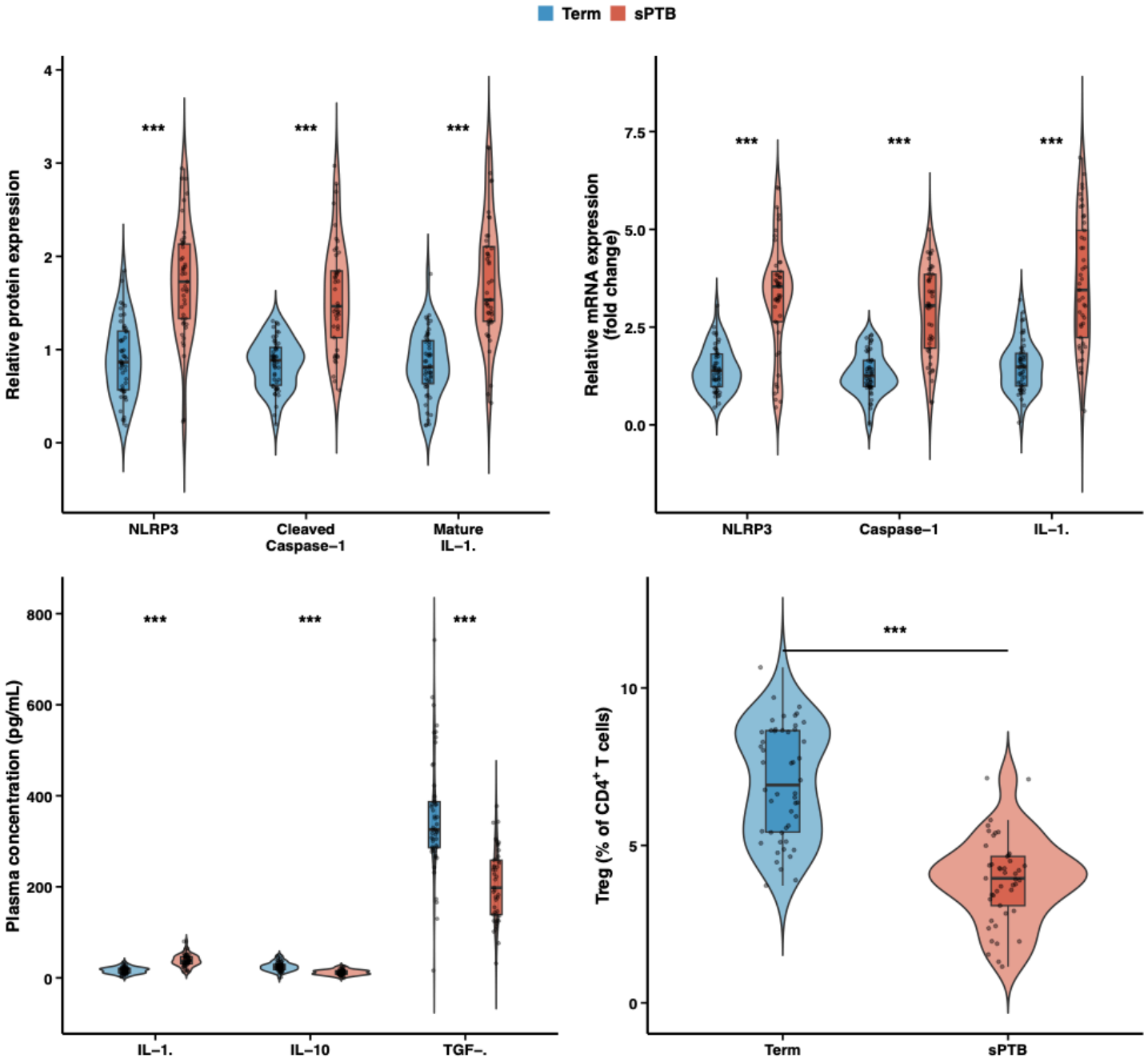


Figure 1

Violin plots comparing NLRP3 inflammasome pathway markers and immune tolerance indicators between the sPTB and term control groups. ***P < 0.001.

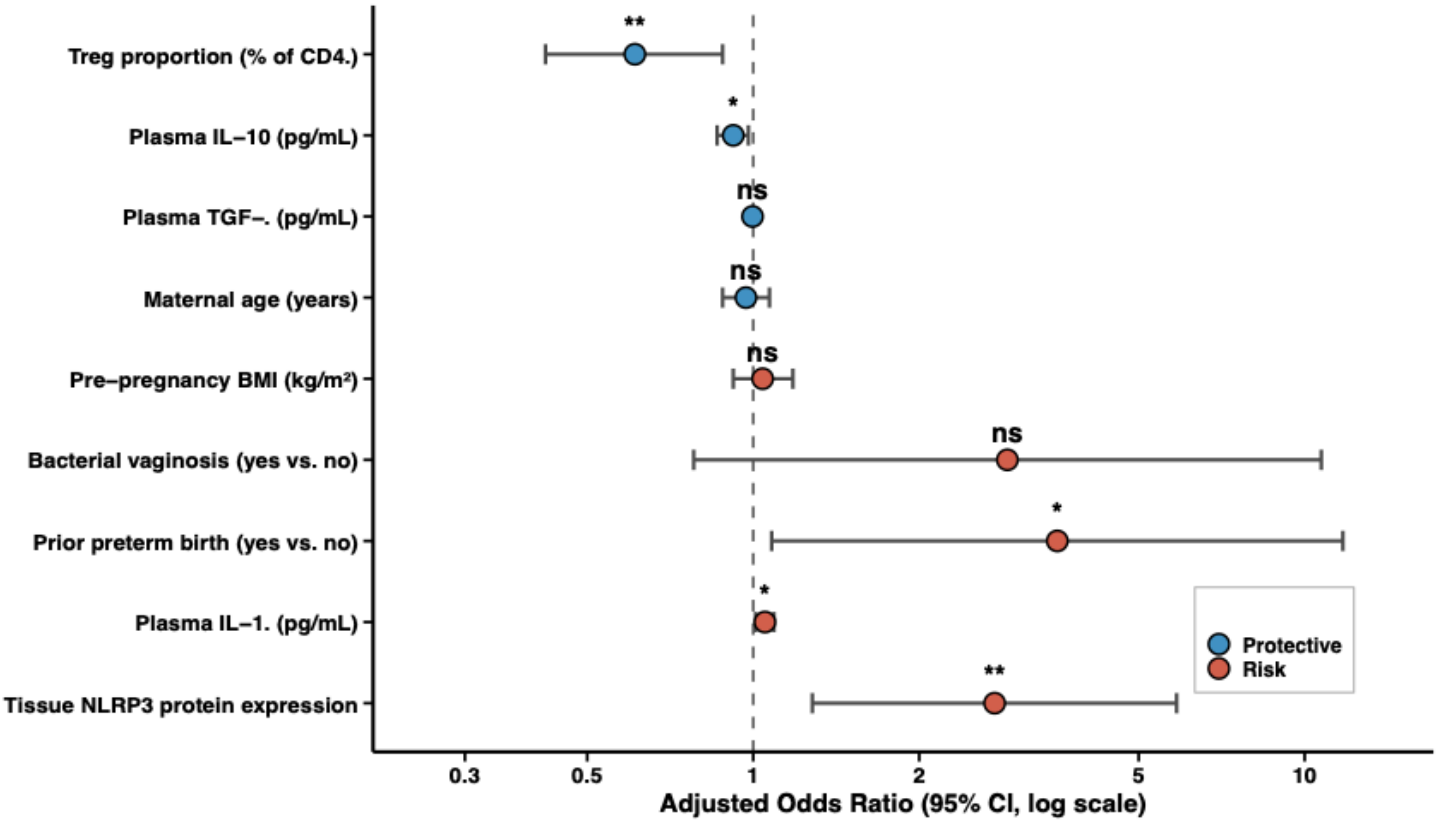


Figure 2

Forest plot of multivariable logistic regression analysis for independent risk factors of spontaneous preterm birth. Adjusted ORs with 95% CIs are shown on a logarithmic scale.

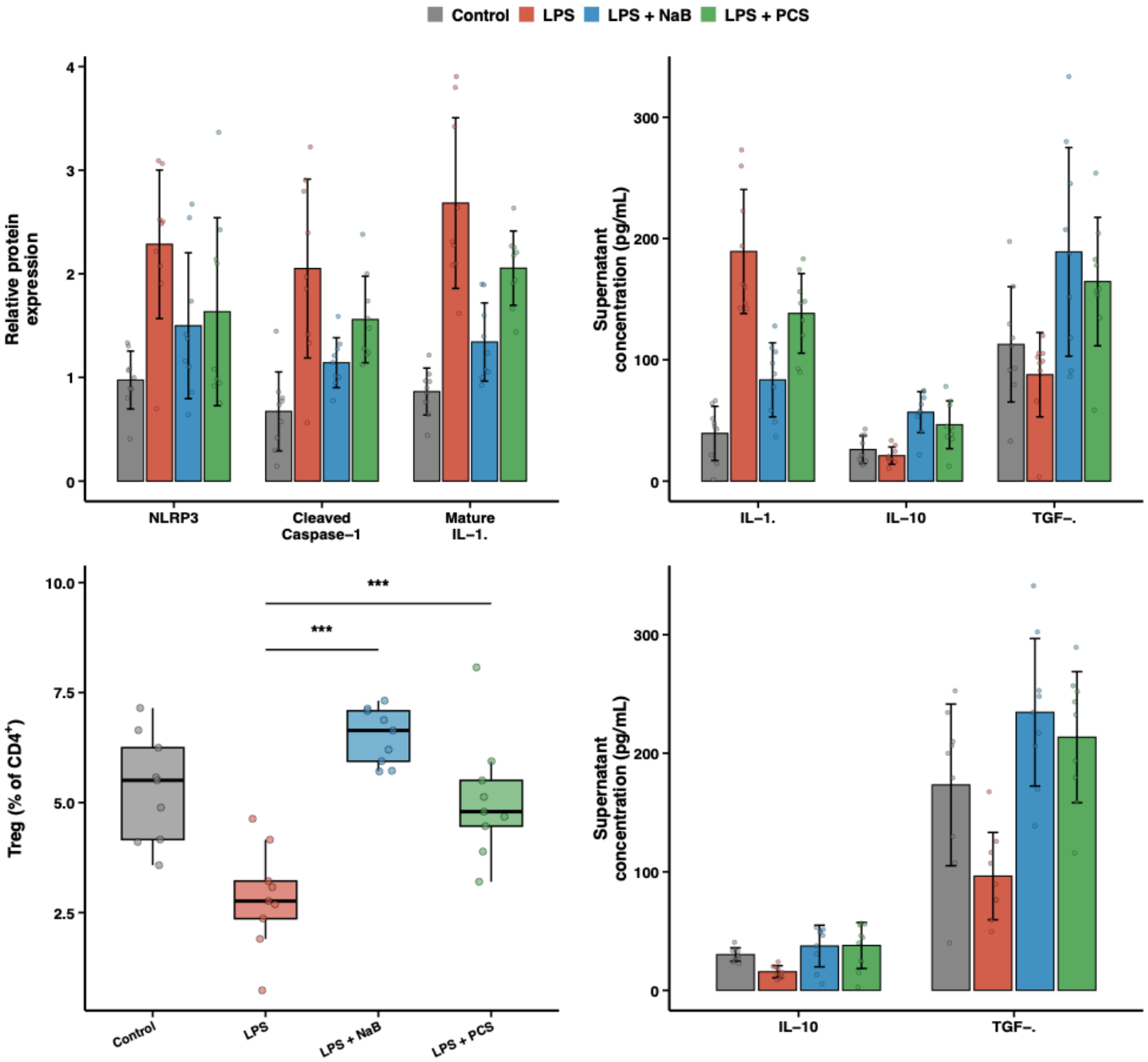


Figure 3

Effects of sodium butyrate and probiotic culture supernatant on NLRP3 inflammasome activation and immune tolerance markers in fetal membrane explants and PBMCs under LPS-stimulated conditions. *P < 0.05, **P < 0.01, ***P < 0.001.

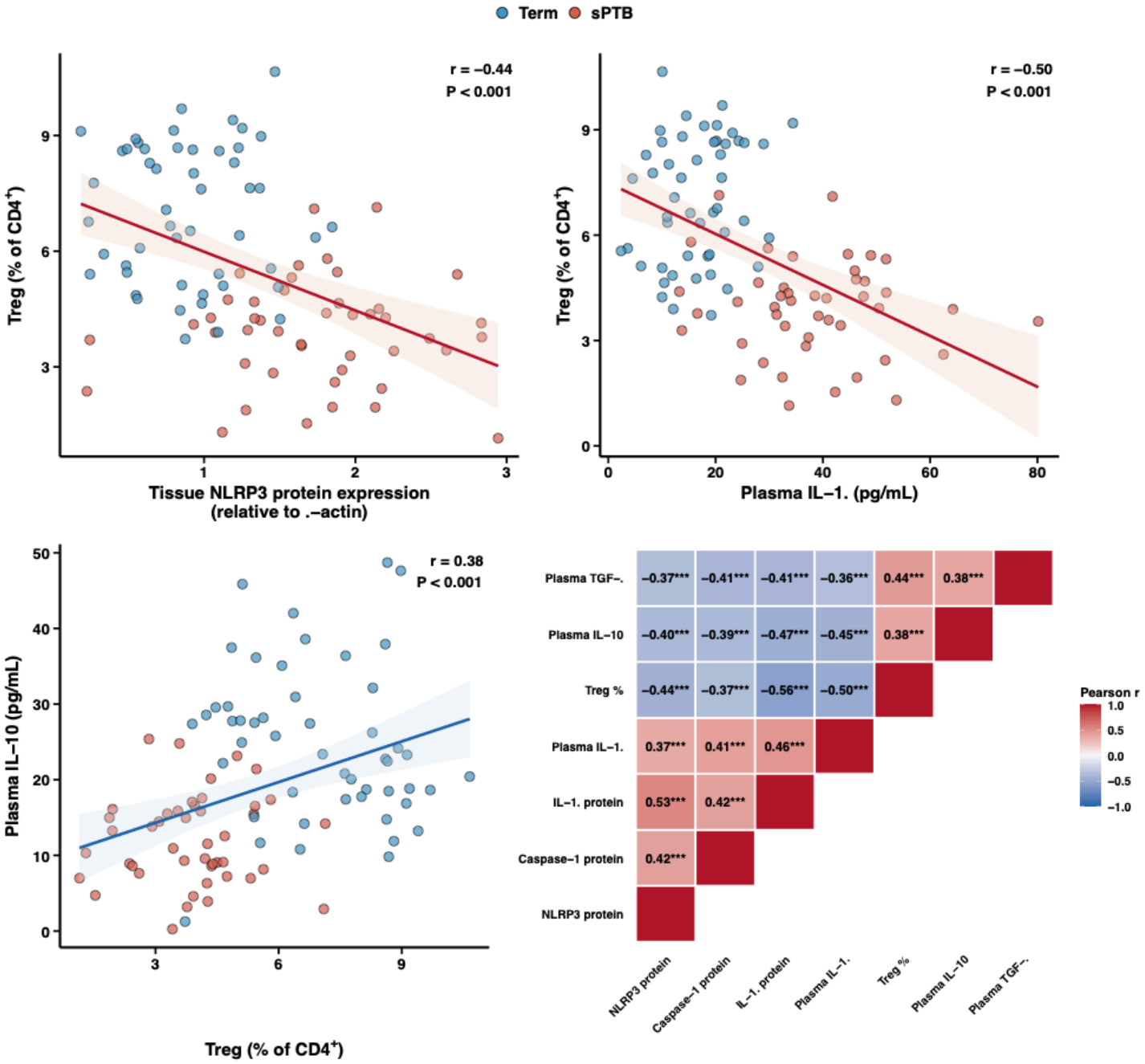


Figure 4

Correlation heatmap and scatter plots illustrating relationships between NLRP3 inflammasome activation and immune tolerance markers. (A) Tissue NLRP3 vs. Treg proportion (r = −0.44). (B) Plasma IL-1β vs. Treg proportion (r = −0.50). (C) Treg proportion vs. plasma IL-10 (r = 0.38). (D) Pairwise correlation heatmap across all markers. *P < 0.05, **P < 0.01, ***P < 0.001.