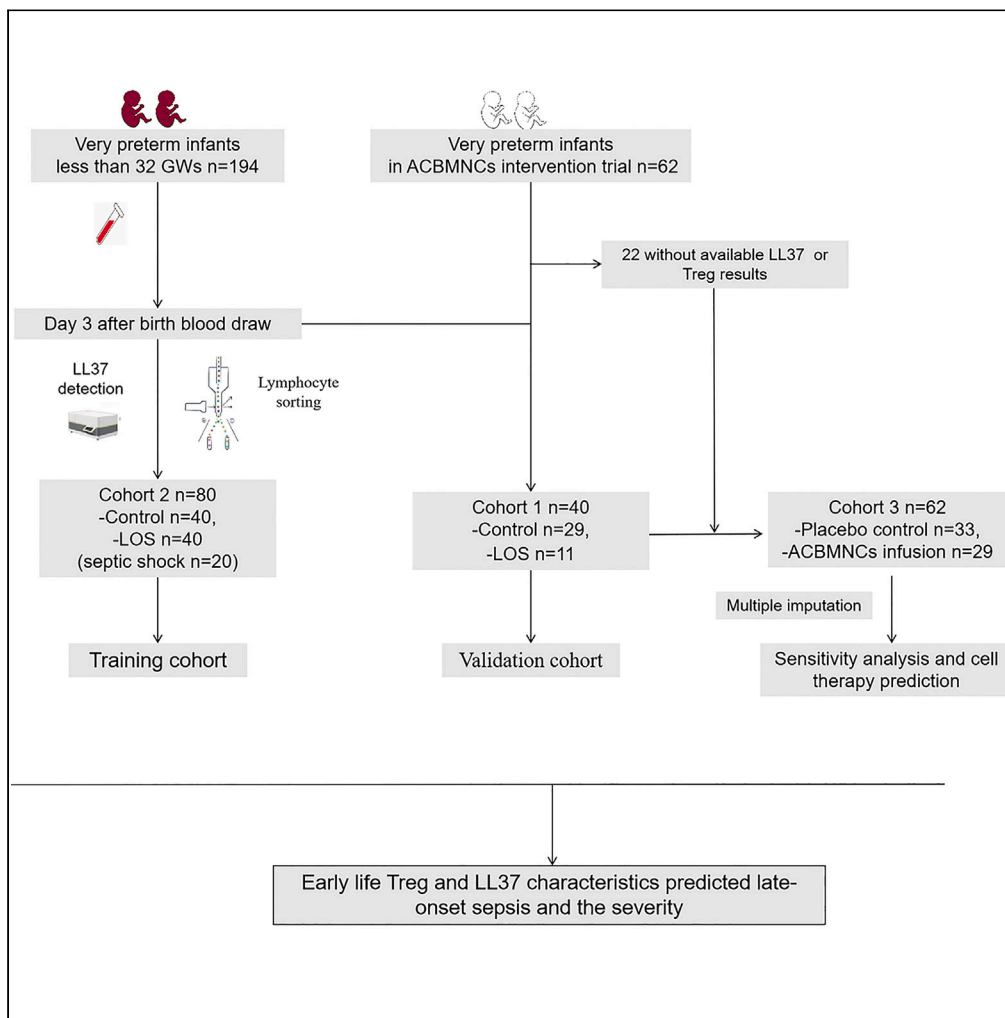**Article**

Antimicrobial peptide LL37 and regulatory T cell associated with late-onset sepsis in very preterm infants

Ren Zhuxiao, Yang Shuo, Han Jiangxue, ..., Wang Zhu, Xu Fang, Yang Jie

15975463496@163.com (X.F.)
jieyang0830@126.com (Y.J.)

Highlights

The early prediction of late-onset sepsis in very preterm infants is a challenge

Autologous cord blood mononuclear cells therapy reduced late-onset sepsis

Cord blood mononuclear cells infusion improved LL37 level and Treg in preterm infants

Early-life LL37 level and Treg predicted late-onset sepsis in very preterm infants

Article

Antimicrobial peptide LL37 and regulatory T cell associated with late-onset sepsis in very preterm infants

Ren Zhuxiao,[1,2,6] Yang Shuo,[3,6] Han Jiangxue,[1,2,6] Pei Jingjun,[4,6] Zhang Qi,[5] Wang Zhu,[1,2] Xu Fang,[1,2,*] and Yang Jie[4,7,*]

SUMMARY

Stem cell therapy may prevent late-onset sepsis (LOS) via antimicrobial peptide LL37 secretion and regulatory T cell (Treg) regulation. The early prediction of LOS is still a challenge. This study evaluated whether immunological state of LL37 or Tregs precedes LOS. We firstly analyzed the LL37 level, Treg proportion, and LOS incidence in very preterm infants treated with autologous cord blood mononuclear cells (ACBMNCs) in our previous trial. Then, we constructed a prediction model and built validation cohort. We found ACBMNC intervention reduced the incidence of LOS from 27.3% to 6.9% ($p = 0.021$). LL37 and Treg abundances were higher in the ACBMNCs group. The nomogram demonstrated that early-life Treg and LL37 characteristics were closely associated with LOS (area under the curve, AUC 0.936), with implications for early prediction and timely clinical management. This composite model was also helpful to evaluate the beneficial effect of ACBMNCs intervention on LOS, thus promoting translational research.

INTRODUCTION

Late-onset sepsis (LOS) remains a top cause of neonatal morbidity and mortality, especially in very preterm infants (VPIs).[1,2] Immune hemostasis disruption is the main pathogenesis of sepsis.[3,4] Immunological immaturity of preterm infants results in an impaired immunological response to infectious stimulation.[3–5] Despite improvements in the quality of neonatal intensive care, the mortality from neonatal sepsis over the last 20 years remains high.[6–9]

Recent findings have indicated promoting human host defense peptide LL37 secretion and regulatory T cells (Tregs) was the important mechanisms accounting for the therapeutic effects of stem cells in preventing sepsis and sepsis-induced organ dysfunction.[10–16] LL37 plays a significant role in the premature human immune defense reaction[17–20]; it acts at a systemic level to defend against infection and at the same time acts with adaptive immune cells to restrict the overwhelming inflammatory response.[21–24] The expression of LL37 is driven by several stimuli, such as inflammatory mediators and microbial structures, and serves as an early "sensory receptor" and a defender when faced with infection.[20,21] Peptide LL37 was lower in preterm infants than in older children and was down-regulated during septic shock.[25,26] Preterm children overexpressed genes involved in the negative regulation of Tregs and IL-10 secretion; therefore, disrupted immune function following infections turned toward a pro-inflammatory profile and insufficient anti-inflammatory feedback.[27–29] In addition, Treg frequency was lower in infants with sepsis and was associated with severity.[30] It was indicated that the lack of LL37 and insufficient Tregs may have a great impact on the development and severity of LOS.[30,31]

The early identification of LOS is still a challenge.[2,3] The abnormal symptoms that were regarded as an indicator of LOS were often nonspecific and therefore insufficient to predict LOS cases.[32,33] As a disease with rapid progression and high mortality, it is critical to predict the possibility and severity of neonatal LOS. Until now, there was no early prediction model with high discrimination in VPIs who were at the highest risk of developing severe LOS. Due to the importance of LL37 and Tregs in preventing infection and controlling overwhelming inflammation in VPIs, it was reasonable to evaluate the correlation between LL37 or Tregs and LOS in VPIs.

Our previous trial showed that autologous cord blood mononuclear cell (ACBMNC) intervention, which was rich in stem and precursor cells, increased the Treg proportion and decreased pro-inflammatory factors.[34] In this previous study, we also noticed a decreasing trend in culture-positive LOS in ACBMNC infusion groups. Both culture-positive and culture-negative sepsis indicated a life-threatening disorder

[1]Department of Neonatology, Guangdong Women and Children Hospital, Guangzhou, China
[2]Guangzhou Medical University, Guangdong Neonatal ICU Medical Quality Control Center, National Key Clinical Specialty Construction Unit Guangzhou Medical University, Guangzhou 510000, Guangdong, China
[3]Department of Medical Statistics, School of Public Health, Sun Yat-sen University, Guangzhou 510080, China
[4]Department of Neonatology, Nanfang Hospital, Southern Medical University, Guangzhou, Guangdong, China
[5]Department of Clinical Genetic Center, Guangdong Women and Children Hospital, Guangzhou Medical University, Guangzhou, China
[6]These authors contributed equally
[7]Lead contact
*Correspondence: 15975463496@163.com (X.F.), jieyang0830@126.com (Y.J.)
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Table 1. Late-onset sepsis incidence, LL37 level, and Treg proportion in the control and ACBMNC intervention groups in the previous trial of cohort 1

	Control group (n = 33)	MNC infusion group (n = 29)	*p* value	*Adjusted *p* value	RR (95% CI)
Culture positive LOS, n, (%)	4 (12.1)	1 (3.4)	0.211	0.083	0.08 (0.004, 1.41)
Culture negative LOS, n, (%)	5 (15.2)	1 (3.4)	0.201	0.147	0.19 (0.02, 1.81)
Total LOS, n, (%)	9 (27.3)	2 (6.9)	**0.048**	**0.021**	**0.11 (0.02, 0.72)**
Use of meropenem or vancomycin, n, (%)	14 (42.4)	4 (13.8)	**0.024**	**0.005**	**0.14 (0.03, 0.54)**
Antibiotics duration(day), median (IQR)	25 ± 22	15 ± 10	**0.036**	0.103	**−7.55 (−16.69, 1.58)**
#LL37, (ng/mL), mean, SD, CB	211.546 ± 107.87	#294.745 ± 232.36	0.155	0.111	90.80 (−32.41, 214.00)
#LL37, (ng/mL), mean, SD, AI	550.80 ± 227.68	855.50 ± 413.87	**0.009**	**0.003**	**355.71 (132.29, 579.13)**
#Changes of LL37, (ng/mL), mean, SD	339.253 ± 313.42	560.76 ± 335.98	**0.037**	**0.008**	**275.41 (76.52, 474.30)**
&Treg cells (% in $CD4^+$ T cells), median, IQR, CB	6.27 (5.23)	7.80 (5.00)	0.750	0.997	−0.004 (−2.28, 2.27)
Treg cells (% in $CD4^+$ T cells), median, IQR, AI	6.00 (4.90)	8.80 (14.00)	**0.030**	**0.011**	**6.04 (1.46, 10.62)**
Changes of Treg cells (% in $CD4^+$ T cells), median, IQR	0.10 (1.90)	2.90 (5.20)	**<0.001**	**0.001**	**6.05 (2.70, 9.39)**

LOS, late onset sepsis; CB, cord blood; AI, after intervention; GA, gestational age; Treg, regulatory T cells; IQR, interquartile range. P*-adjusted for GA, birth weight, and sex before enrollment. #, *n* = 20; &, *n* = 30 in the control group, and *n* = 27 in the MNCs group.

and dysregulated inflammatory response during an infection.[35,36] Thus, in the current study, we first performed a post hoc analysis of the LOS rate, including both culture-positive and culture-negative sepsis, in the two groups. Then, we systematically evaluated the immunological features of LL37 and Tregs in the peripheral blood of VPIs with LOS. These two features were independent of key clinical variables, including baseline characteristics, clinical manifestations, laboratory results, and treatment with endotracheal intubation or central venous catheterization. Leveraging these findings, we developed a predictive model for LOS development with high discrimination ability and built a nomogram to explore its utility. Furthermore, we used this model to predict the reduction in LOS after ACBMNC intervention in VPIs. This study helped identify infants at high risk of LOS, which may significantly reduce the severity and mortality of LOS. These findings may also lay an important foundation for the future application of ACNMBCs in LOS prevention.

RESULTS

Post hoc analysis of LL37 levels and LOS in the ACBMNC intervention group

Two (6.9%) of the 29 infants assigned to the intervention group and nine (27.3%) of 33 infants assigned to the control group were diagnosed with LOS (relative risk: 0.113, 95% confidence interval: 0.018–0.715, *p* = 0.021, Table 1). After single-factor regression analysis for the possible confounding factors of LOS, we found that no specific characteristics before intervention were the main confounding factors on the outcome of LOS (Table S3). Based on the common clinical characteristics that may affect LOS rate, we adjusted for gestational age (GA), birth weight, and sex, and mononuclear cell (MNC) intervention could still reduce the incidence of LOS (adjusted *p* = 0.021). The duration of antibiotic use was shorter in the ACBMNC group (adjusted *p* = 0.036), and there was less use of advanced antibiotics in the ACBMNC intervention group (adjusted *p* = 0.035, Table 1).

There was no difference in cord blood LL37 levels at baseline between the two groups (*p* = 0.129, *n* = 20). The serum level of LL37 was increased (*p* = 0.008) and higher (0.003) in the ACBMNC group than in the control group (*n* = 20) 72 h after intervention. After ACBMNC infusion, the Treg frequency was increased (*p* = 0.001) and higher (0.011) in the ACBMNC group than in the control group 72 h after intervention (*n* = 30 in the control group and *n* = 27 in the ACBMNC group, Table 1). We further assessed the LL37 and Treg levels in cord blood and on day 3 after birth in infants with and without LOS. We found no significant difference in LL37 or Treg levels in cord blood, but LL37 levels on day 3 were significantly lower in the LOS group (*p* = 0.008). Treg abundance on day 3 was also decreased in the LOS group, although no significant difference was noticed (*p* = 0.126) (Table S4). Given this evidence, we wondered whether the LL37 level or Treg frequency early after birth might precede LOS development in VPIs.

The clinical characteristics of the prospective LOS cohort in VPIs

To study the association of LL37 or Treg with LOS, we screened all 194 VPIs born in Guangdong Women and Children Hospital from January 17th, 2022, to August 27th, 2022 (Figure 1). Among them, 44 were excluded per the inclusion criteria (4 had severe perinatal asphyxia, 16
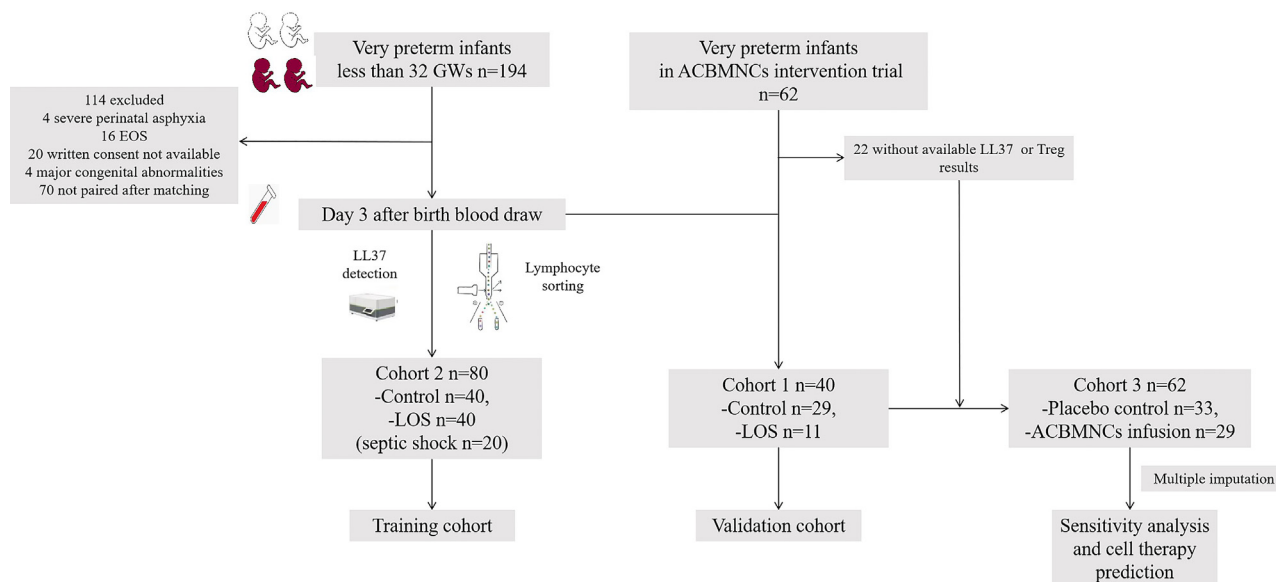
Figure 1. Study schema

Overview of patients included in this study, summary of their sepsis status, exclusion criteria, and downstream analyses that were performed. Cohort 1 included 40 very preterm infants enrolled in a previous ACBMNC intervention trial; Cohort 2—a prospective study—included 194 very preterm infants. Among eligible patients, 40 with LOS and 40 matched controls were evaluable for LL37 and Treg analysis after exclusion criteria were applied. Cohort 3 included all the infants enrolled in the previous ACBMNC intervention trial. LL37 and Treg were evaluable for each patient after multiple imputation to predict the improvement in LOS after intervention and were supplemented as sensitivity analysis. Further details are provided in the methods and Tables S5–S8. GW, gestational weeks; ACBMNCs, autologous cord blood mononuclear cells; EOS, early-onset sepsis; LOS, late-onset sepsis.

showed early-onset sepsis, 20 did not provide written consent, and 4 had major congenital abnormalities), of whom 7 developed LOS. Among the remaining infants, 40 were diagnosed with LOS (20 were diagnosed with septic shock). Forty infants from 110 controls were then matched with the cases by GA. All patients were monitored closely for sepsis symptom development (the median time of LOS onset was 5 (16) days after birth, Table 2). The patient screening and recruitment flowchart is shown in Figure 1. The maternal and neonatal clinical characteristics were compared between the two groups and are shown in Table 2. The LOS group had a significantly higher incidence of chorioamnionitis (35.0% vs. 7.5%, $p = 0.005$) and lower preeclampsia (80.0% vs. 60.0%, $p = 0.037$) than the control group (Figure 2A; Table 2).

There were no significant differences in the routine clinical laboratory tests between the two groups. In addition, we compared the LOS-associated clinical manifestation and the time these symptoms occurred to eliminate the effect on the clinical laboratory results between the two groups and found that there was still no difference (Table 2).

We also compared other preterm complications or comorbidities and noticed more necrotizing enterocolitis (NEC) (22.5% vs. 0%, $p = 0.001$) and persistent pulmonary hypertension of newborn (PPHN) (47.5% vs. 15.0%, $p = 0.002$) cases and longer duration of central venous catheterization (1,670.0 vs. 312.0 h, $p = 0.038$) in the LOS group (Table 2).

Determinants of LL37 levels and Tregs in VPIs preceding LOS

For all very preterm patients, routine blood tests were repeated on day 3 after birth for assistance in determining further antibiotic use. We started by performing an ELISA kit test of LL37 levels and immune cell sorting in peripheral blood samples from VPIs. The LL37 level was lower in the sepsis group (control vs. sepsis: 1,837.08 ± 877.88 vs. 527.60 ± 279.33, $p < 0.0001$) and lowest in the septic group (control vs. septic shock: 1,837.08 ± 877.88 vs. 384.44 ± 217.01, Figures 2B and 2C), but no difference was observed between the sepsis and septic shock groups (sepsis vs. septic shock: 527.60 ± 279.33 vs. 384.44 ± 217.01, $p = 0.067$, Figure S1A). Lower LL37 levels in the serum of peripheral blood were associated with sepsis development. Moreover, remarkably, of the 7 lymphocyte subsets evaluated by flow cytometry, only Treg cell proportions were associated with sepsis development (control vs. sepsis vs. septic shock: 8.13 ± 4 vs. 4.3 ± 4 vs. 2.84 ± 3, $p < 0.01$ between each group, Figures 2B, 2D, and S1B). Indeed, we observed a striking trend between either LL37 or Tregs and LOS development (area under the curve [AUC] = 0.837, 95% confidence interval [CI]: 0.752–0.922 for LL37 and AUC = 0.880, 95% CI: 0.804–0.957 for Tregs, Figure 3A).

Integrative models to predict LOS development

Univariable and multivariable analyses by the logistic regression model demonstrated that higher LL37 and Treg levels were both independent protective factors for LOS development, with adjusted odds ratios of 0.996 (95% CI: 0.993–0.998, $p = 0.001$) and 0.558 (95% CI: 0.446–0.775, $p < 0.001$) (Table 3), respectively. Given these results, we wondered whether a composite model integrating both features—Treg cell abundance and LL37—might outperform either feature alone. Accordingly, we explored integrative modeling as a means of improving

Table 2. Characteristics of the very preterm infants with and without LOS in prospective training cohort 2

Characteristics	Control (N = 40)	Sepsis group (N = 40)	p value
Mothers			
Age, years, median (IQR)	31 (6)	30 (6.5)	0.696
Antenatal glucocorticoids, n, (%)	32 (80.0)	24 (60.0)	0.051
Preeclampsia, n, (%)	11 (27.5)	3 (7.5)	**0.037**
Chorioamnionitis, n, (%)	3 (7.5)	14 (35)	**0.005**
Gestational hypertension, n, (%)	5 (12.5)	3 (7.5)	0.456
Gestational diabetes, n, (%)	5 (12.5)	8 (20.0)	0.363
ICP, n, (%)	2 (5)	0 (0)	0.474
Infants at birth			
CS, n, (%)	23 (57.5)	20 (50.0)	0.501
Apgar Score			
1 min, median (IQR)	8 (1)	8 (2)	0.075
5 min, median (IQR)	9 (0)	9 (1)	0.208
GA, weeks, mean (SD)	30.14 (1.70)	30.25 (1.58)	0.754
Male, n, (%)	31 (77.5)	24 (60.0)	0.091
Birth weight, kg, mean,(SD)	1.23 (0.29)	1.31 (0.32)	0.205
Body length, cm, mean (SD)	36.24 (3.81)	37.15 (4.05)	0.303
Head circumference, cm, mean (SD)	26.76 (2.01)	27.34 (2.57)	0.267
LOS-associated clinical manifestation			
Days after birth, median, IQR	8 (6)	4 (2)	0.001
Temperature instability, n, (%)	8 (20)	8 (20)	0.178
Feeding intolerance, n, (%)	13 (32.5)	5 (12.5)	
Lethargy or hypotonia, n, (%)	8 (20)	11 (27.5)	
Apnea or respiratory distress, increased requirement on respiratory support, n, (%)	8 (20)	8 (20)	
Poor perfusion, n, (%)	3 (7.5)	8 (20)	
The time of sepsis onset, days after birth, median, IQR	–	5 (16)	–
Other preterm complications and co-comorbidities			
Death, n, (%)	5 (12.5)	9 (22.5)	0.239
RDS, n, (%)	23 (57.5)	27 (67.5)	0.356
IVH, n, (%)	12 (30.0)	18 (45.0)	0.166
NEC, n, (%)	0 (0.0)	9 (22.5)	**0.001**
ROP, n, (%)	7 (17.5)	7 (17.5)	1.000
BPD, n, (%)	17 (42.5)	15 (37.5)	0.648
PPHN, n, (%)	6 (15.0)	19 (47.5)	**0.002**
PDA, n, (%)	24 (60)	30 (70)	0.232
Anemia requiring transfusion, n, (%)	19 (52.5)	21 (52.8)	0.820
Duration of endotracheal intubation, days, median (IQR)	0 (9)	2 (68)	0.271
Duration of central venous catheterization, hours, median (IQR)	1670.0 (192)	312 (319)	**0.038**
Clinical laboratory			
Hemoglobin, g per dL, mean (SD)	124.28 (33.54)	129.18 (30.51)	0.496
Platelet count, 10^3 per mL, mean (SD)	309.15 (149.315)	291.90 (129.92)	0.558

Table 2. Continued

Characteristics	Control (N = 40)	Sepsis group (N = 40)	p value
WBC, 10³ per mL, mean (SD)	12.68 (9.36)	10.06 (4.52)	0.114
Lymphocytes, 10³ per mL, mean (SD)	4.22 (1.82)	4.20 (1.71)	0.975
Neutrophil, 10³ per mL, mean (SD)	6.84 (8.57)	4.56 (3.34)	0.122
Monocyte, 10³ per mL, mean (SD)	1.12 (0.78)	0.82 (0.55)	0.053
CRP, mg per L, mean (SD)	1.14 (2.23)	1.21 (2.19)	0.883
PCT, mean (SD)	3.89 (8.15)	5.34 (9.63)	0.469
T cells, %, median (IQR)	81.22 (16)	81.97 (18)	0.575
CD4⁺ T cells, %, median (IQR)	60.29 (15)	59.26 (20)	0.586
CD8⁺ T cells, %, median (IQR)	20.14 (10)	19.90 (10)	0.740
B cells, %, median (IQR)	10.70 (10)	11.59 (11)	0.791
NK cells, %, median (IQR)	3.67 (5)	3.83 (4)	0.880
PH, mean (SD)	7.34 (0.12)	7.14 (1.16)	0.281
Lactic acid, mean (SD)	2.05 (1.25)	1.90 (1.35)	0.584
Blood glucose, mean (SD)	5.45 (1.42)	5.12 (1.67)	0.340
Detection of LL37 and Treg			
LL37, ng/mL, mean (SD)	1837.0 (877.8)	458.0 (258.2)	<0.001
Treg cells % in CD4⁺ T cells, median (IQR)	8 (5)	3.8 (4)	<0.001

CS, cesarean section; GA, gestational age; IQR, interquartile range; n, number; PS, pulmonary surfactant; ICP, intrahepatic cholestasis of pregnancy; LOS, late-onset sepsis; RDS, respiratory distress syndrome; IVH, intraventricular hemorrhage; BPD, bronchopulmonary dysplasia; NEC, necrotizing enterocolitis; PDA, patent ductus arteriosus; ROP, retinopathy of prematurity; PPHN, persistent pulmonary hypertension of newborn; WBC, white blood cell; CRP, C-reactive protein; PCT, procalcitonin; NK, natural killer cells; Treg, regulatory T cells.

performance. Indeed, using a logistic regression framework to build a bivariable model, the resulting composite model yielded an AUC of 0.936 (95% CI, p = 0.883–0.988), outperforming either feature alone (Figure 3A). Composite model scores were assessed by receiver operating characteristic analysis. Models applied to discriminate sepsis from controls were used to predict the future development of sepsis and its
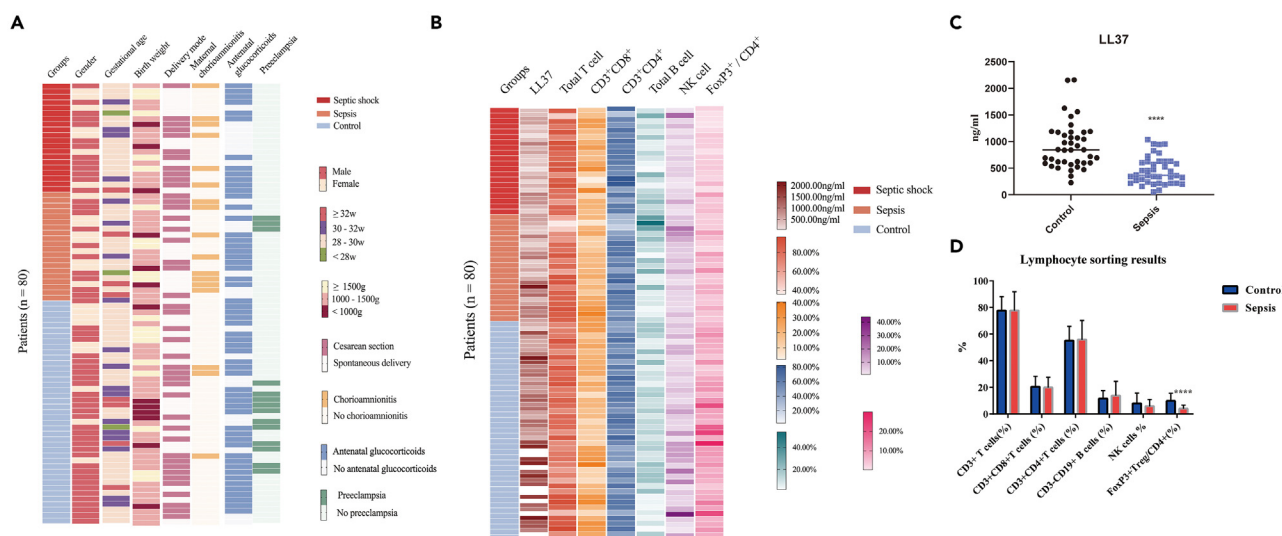

Figure 2. Analysis of peripheral blood for LL37 level and lymphocyte cellular characteristics of LOS status using ELISA and flow cytometry

(A) Characteristics of the discovery training cohort, including LOS grade status and perinatal factors.

(B) Heatmap showing the relative abundance of LL37 and lymphocyte sorting states identified in 80 patients, grouped by future LOS status.

(C and D) Concentration of LL37 and frequencies of lymphocyte cells in the peripheral blood of patients stratified by future LOS status (control, n = 40 patients; LOS, n = 40 patients). Statistical significance was determined by a two-sided, unpaired Wilcoxon rank-sum test or Student's t test as appropriate. Treg, regulatory T cells. ****p < 0.0001. The denominator for T cells, CD4⁺ T cells, CD8⁺ T cells, B cells, and NK cells subgroup frequency was lymphocyte, and the denominator for Treg was CD4⁺ T cells.
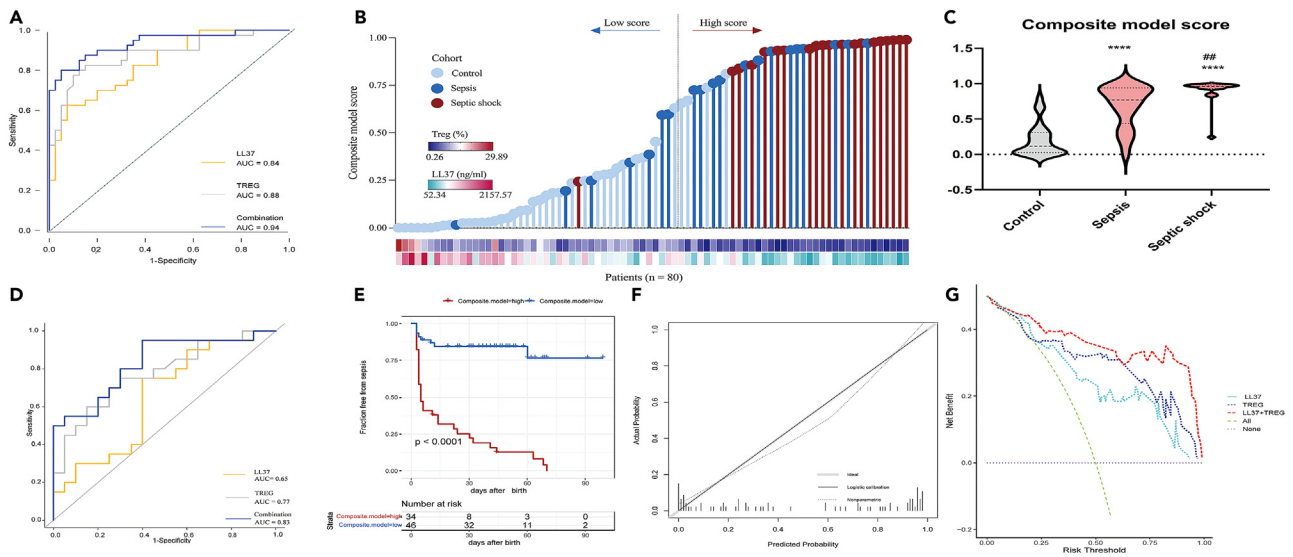
Figure 3. Integrative modeling for LOS prediction from LL37 levels and Treg abundance in peripheral blood

(A) ROC plot showing that the composite model performance in cohort 2 was better than that of either LL37 or Tregs individually.

(B) Development of a composite model for the prediction of LOS, integrating Treg cell abundance and LL37 level from peripheral blood on day 3 after birth, with model scores trained on cohort 2 (Table S5). The cutoff point for high/low scores was optimized using Youden's J statistic on cohort 2 (Methods).

(C) Composite model scores for patients (n = 80) in cohort 2 after model training for LOS development with leave-one-out cross-validation (LOOCV), grouped by the LOS grade per patient. The box centerlines, box bounds and whiskers indicate the medians, first and third quartiles and minimum and maximum values within 1.5× the interquartile range of the box limits, respectively. ****$p < 0.0001$, sepsis vs. control and septic shock vs. control, ##$p < 0.01$, septic shock vs. sepsis.

(D) ROC plot showing composite model performance as a function of sepsis grade.

(E) Prediction of time-to-LOS onset in enrolled patients in cohort 2. The cutoff point for the low and high groups was optimized using composite model scores trained with LOOCV.

(F) Calibration curve for predicting the probability of LOS, C value = 0.936, R^2 = 0.694.

(G) DCA curve showing that the composite model was significantly better than LL37 or TREG individually in net benefit.

severity (Figures 3B, 3C, Table S5). The patients' characters in group with low and high composite model scores were showed in Table S6. The cutoff point for high/low scores was optimized using Youden's J statistic, and the cutoff score was 0.697, with a sensitivity of 0.80 and a specificity of 0.95; the corresponding cutoff values for LL37 and Tregs were 215.14 ng/mL and 5.18%, respectively.

We next explored model performance as a function of sepsis grade. Although trained on a single categorical outcome (yes versus no LOS), the composite model scores increased as a function of LOS severity (Figure 3C). The model was also effective when trained to distinguish moderate sepsis from septic shock, albeit with reduced performance (AUC 0.830, 95% CI: 0.702–0.959, Figure 3D).

To test whether the model could predict time to sepsis onset, we next assigned patients to high versus low groups by defining an optimal cutoff point of 0.697. Patients in the high group experienced sepsis within a median of 5 days after birth, whereas the vast majority (95%) of patients in the low group never experienced LOS ($p < 0.0001$, hazard ratio = 9.41, 95% CI: 4.30–20.59), after adjusting for GA, sex, and birth weight, Figure 3E).

Nomogram establishment

To provide a quantitative tool that predicted the individual probability of developing LOS, a novel prognostic nomogram with LL37 and Tregs was established based on multivariable logistic analysis in prospective cohort 2 (Figure 4). The probability of LOS for each patient was

Table 3. Univariate and multivariate analyses of risk factors associated with LOS in very preterm neonates in prospective training cohort 2

Clinical characteristics	Univariate analysis		Multivariate analysis	
	Crude OR (95% CI)	p value	Adjusted OR (95% CI)	p value
Chorioamnionitis	6.641 (1.732, 25.465)	0.006	3.655 (0.469, 28.506)	0.216
Preeclampsia	0.214 (0.055, 0.838)	0.027	0.314 (0.038, 2613)	0.284
LL37 (ng/mL)	0.996 (0.993, 0.998)	<0.001	0.996 (0.993, 0.998)	0.001
Treg cells (% in CD4+ T cells)	0.563 (0.438, 0.723)	<0.001	0.588 (0.446, 0.775)	<0.001

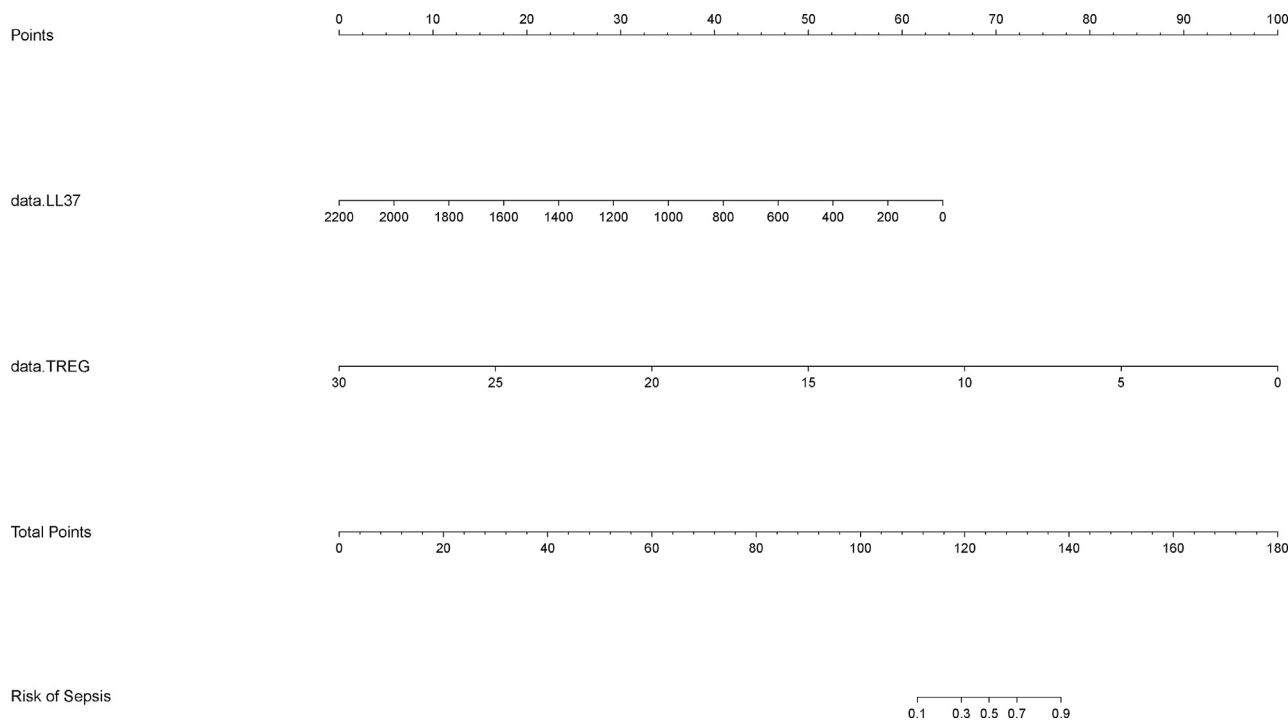Treg, regulatory T cells; OR, odds ratio; CI, confidence interval.

Figure 4. Nomogram for the development of LOS by LL37 concentration and Treg abundance
TREG, regulatory T cells.

calculated by adding the scores for the 2 variables. For example, if the LL37 level was 344.80 ng/mL and the Treg frequency was 2.53%, then the corresponding score of the infant would be approximately 54.24305 and 91.56667, respectively. The total score is approximately 145.8097, indicating an estimated LOS of 0.9667144 for this case. The following formula allowed for the calculation of risk of LOS: SCORE = (64.32431−0.029238*LL37) + (100.00000−3.333333*TREG)

Predictive accuracy and net benefit of the nomogram

The performance of the nomogram was validated internally with 10-fold cross validation in the training cohort (accuracy was 80.63%, 95% CI 76.18–85.07). The calibration curve was close to the ideal diagonal line (C value = 0.936, and R2 = 0.694, Figure 3F). The Hosmer-Lemeshow test demonstrated that the model was a good fit (p = 0.939). Furthermore, decision curve analysis (DCA) showed that the composite model was significantly better than LL37 or TREG individually in net benefit (Figure 3G). In addition, 40 VPIs with available LL37 and Treg results from previous clinical trials were used for external validation to test the nomogram (Table S7). The baseline characteristics of all patients in the training and validation cohorts are shown in Table S8. Several baseline and outcome characteristics were different in the two cohorts, but the predictive model still showed good discrimination performance. The AUCs for LL37 and Tregs were 0.865 and 0.754, respectively. For the composite model, the AUC was 0.881 (95% CI: 0.726–1.000), with a sensitivity of 0.82 and a specificity of 0.93; the cutoff point was 0.418 (Figure 5A). The calibration curve of the validation cohort was also close to the ideal diagonal line (R2 = 0.528, Figure 5B). Moreover, DCA also showed a significant net benefit of the predictive model in the validation cohort (Figure 5C). Meanwhile, the model had good consistency, reflecting a good accuracy (90.00%) of the nomogram (accuracy: 87.50% in the training cohort) (Figure 5D). These data indicated that the nomogram has significant potential for clinical decision-making.

Sensitivity analysis and prediction of the reduction in LOS in VPIs after ACBMNC intervention

Since ACBMNC intervention could increase LL37 and Treg levels through immunoregulatory functions, to determine whether the composite model could predict the reduction in LOS after treatment with ACBMNCs, we further used multiple imputation to produce complete datasets of LL37 and Treg results in all infants enrolled in our previous trial,[16] and by applying this model, LOS incidence was predicted to be 36.36% and 10.34% in the control and ACBMNC groups, respectively, with a prediction accuracy of 87.98% (95% CI: 81.08–94.88) (Figures S1C and S1D, Table S9).

In the validation cohort, there were missing LL37 and Treg data; thus, after multiple imputation, cohort 3 was built, and the model was further tested for sensitivity analysis. The model still showed good discrimination (AUC = 0.843), calibration (R^2 = 0.397), and net benefit (Figures S1E, S1F, and S1G).
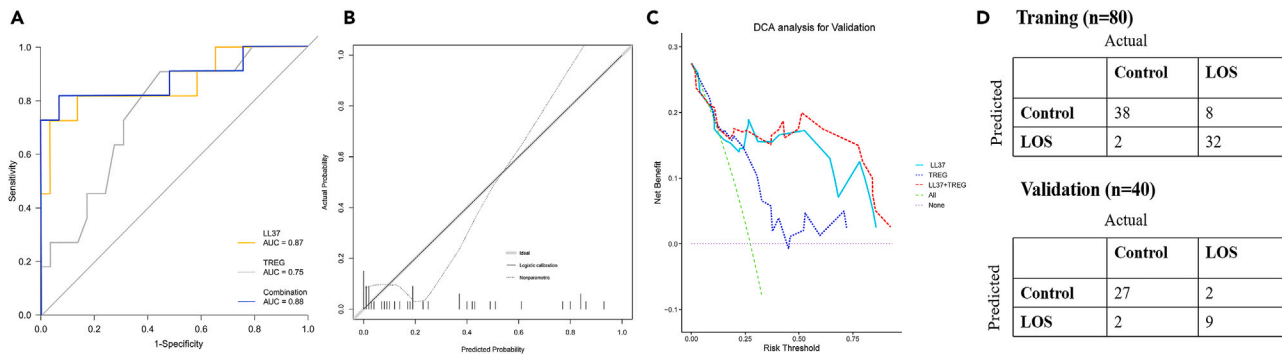
Figure 5. The performance to distinguish individuals with LOS from those without LOS in validation cohort 1

The ROC (A), calibration (B), and decision curves (C) for performance to distinguish individuals with LOS from those without LOS in validation cohort 1. ROC, receiver operating characteristic; AUC, area under the curve.

(D) Confusion matrices of the composite model's ability to accurately predict the LOS group within both the training and validation sets.

DISCUSSION

Neonatal sepsis is a life-threatening disorder accompanied by several severe complications, such as NEC, PPHN, and shock.[1,38,39] However, with advanced intervention in the neonatal intensive care unit, the incidence of LOS did not decrease.[6,7] The clinical manifestations of LOS are various and nontypical; therefore, it is difficult to predict and treat cases in a timely manner.[3,9,36] Additionally, due to the immature immune response, the routine blood tests and several inflammation response mediators commonly applicable in LOS prediction in adults or older children were not sensitive in very preterm neonates.[9,19,40] This study evaluated two baseline features, LL37 level and Treg cell abundance in the early-life peripheral blood, as promising determinants of LOS in later life in VPIs. Previous studies have indicated that (1) during infection, preterm infants exhibited insufficient production of LL37 in tracheal aspirates and circulating serum compared to term infants, thus presenting with more severe infection[21,26,30]; (2) premature infants showed defects in the alteration of Treg cells and a lower frequency of Tregs in infants with sepsis and septic shock[29–31]; and (3) stem cell therapy improved sepsis and sepsis-induced organ dysfunction by promoting LL37 and Tregs.[18,19,42,43] This work extended the scope of these findings to baseline LL37 and Treg cell characteristics of LOS development in diverse cohorts. Integration of these two features into a composite model predicted greater risk for LOS and demonstrated sufficient granularity to distinguish LOS and estimate the efficacy of ACBMNC intervention on reducing LOS burdens.

In this study, we first performed a post hoc analysis to investigate the LOS incidence after ACBMNC intervention, and we found decreased LOS in the ACBMNC group, as well as less use of antibiotics. Then, we detected the concentration of LL37 and the Treg proportion at baseline and 3 days after intervention and showed that the LL37 level and Treg proportion were significantly higher in the MNC group after intervention, even after adjusting for possible confounders. Additionally, we found that LL37 and Treg proportions showed more significant differences on day 3 after birth but not in cord blood among infants with or without LOS later, which was consistent with our previous findings.[44] VPIs are susceptible to infectious agents and vulnerable to a persistent ineffective inflammatory response.[4,5,9] Considering the possibility of common immunological disorders in early life and their association with the risk of LOS development,[8,40] it was reasonable to predict that patients with lower LL37 and Treg levels would be more susceptible to developing LOS.

In the second part of this study, to determine whether LL37 or Tregs could be promising indicators for LOS as well as its severity, we analyzed the features of LL37 and Tregs on day 3 after birth in a prospective net case-matched very preterm cohort. We identified several independent risk factors for developing LOS, including chorioamnionitis, being free from preeclampsia and LL37 concentration, and Treg abundance on day 3 after birth. Previous studies have shown that intrauterine inflammation or preeclampsia is not associated with the development of LOS in premature infants.[45,46] In this study, through univariate and multivariable analyses, we constructed a nomogram utilizing only two factors, LL37 and Tregs, to predict LOS in VPIs. This nomogram was internally and externally validated and showed good potential for distinguishing and calibrating infants who may develop LOS. Although the validation cohort had several different baseline and outcome characteristics compared with the training cohort, the external validation still confirmed the good accuracy and conformity of the model, alongside its net benefit. This showed the good applicability of this model in VPIs regardless of perinatal factors. A predictive model based on the immune characteristics during defense against infection in VPIs, but not depending on the subjective judgment of clinical symptoms or invasive inventions, could more reliably identify these at-risk neonates during decision-making, thus reducing LOS and improving their outcomes. We also identified a striking correlation between early Treg cell abundance and LL37 concentration and the timing of LOS onset. Future studies are needed to further characterize this finding and elucidate the relative contributions of the two features in LOS development.

A growing number of preclinical studies have shown that stem cells have immunomodulatory and antibacterial effects in preventing sepsis and the associated organ injury by secreting paracrine proteins.[10,14,42] Previous studies have shown the benefit of LL37 intervention in improving murine sepsis models.[22,23] LL37 levels in preterm infants were lower than those in term infants in breast milk, cord blood, and airway secretion.[25,26,41] Infants with suspected LOS displayed a decreased abundance of CD4+ FoxP3+ T cells compared to controls, which was most pronounced in the subgroup of infants with septic shock.[30] Treg cells represent a heterogeneous CD4+ T cell population that maintains immunologic homeostasis in various infectious processes.[27,29] Considering the possibility of a yet undefined immunological predisposition, e.g.,

differences in lymphocyte subsets, especially Tregs, or innate immunity disorders, such as lower LL37, in infants at higher risk of developing LOS, it is promising that early ACBMNC intervention, rich in stem cells, might prevent LOS and reduce its severity through the potent secretion ability of LL37 and its effect on the adaptive immune system, particularly T cells. Thus, in the third part of this study, by calculating the risk of LOS in the control and ACBMNC intervention groups via our established nomogram, we predicted the improvement in LOS after ACBMNC intervention compared with the control group with good accuracy. The detection of LL37 and Tregs 3 days after intervention could also help to predict the efficacy of ACBMNC intervention on LOS earlier. Furthermore, by applying multiple imputation, we conducted a sensitivity analysis, and the model still showed good performance.

Limitations of the study

This study has several limitations. First, it employed a post hoc design using banked clinical samples to compare the LOS prevalence as well as Treg cells and LL37 in the control and ACBMNC groups. Using multiple imputation may cause bias since some infants without available results were also included. A prospective well-designed randomized trial is warranted to further confirm this finding. Second, it is yet unclear whether our findings will generalize to late preterm infants or even term infants, and further investigation and confirmation in more mature neonates are needed. Third, while most LOS occurred within the first month after birth, a subset can occur much later. Whether our findings generalize to much later LOS will need to be further investigated. In addition, in this current study, we only sent the blood for the test of the lymphocyte frequency. Treg numbers were not available, but in the future study, both the number and frequency of Treg would be tested.

Future studies should address these limitations, along with greater application of the model early during stem cell therapy in preventing LOS or possibly other infections. In addition, it will be important to confirm our findings in larger multi-institutional cohorts and assess whether the circulating immunological determinants vary based on different GA. If prospectively validated, these findings could facilitate treatment adaptation to improve the risk profile of LOS, with implications for the prediction and potential prevention of LOS-mediated multiple organ dysfunction.

In conclusion, the novel nomogram developed in this study demonstrated that early-life circulating Treg cell and LL37 characteristics were closely associated with LOS occurrence, with implications for early prediction and timely clinical management. Additionally, this composite model was helpful to evaluate the potential beneficial effect of ACBMNC intervention on LOS, thus promoting translational research.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.109780.

AUTHOR CONTRIBUTIONS

Conceptualization, Y.J. and X.F.; methodology, R.Z. and Y.S.; software, Y.S., Z.Q., and P.J.; validation, X.F. and Y.J.; formal analysis, R.Z. and Y.S., investigation, Y.J. and X.F.; writing – original draft preparation, R.Z. and Y.S.; writing – review and editing, Y.J.; supervision, Y.J. and X.F.; project administration, Y.S.; funding acquisition, Y.J.; manuscript revision, W.Z. All authors have read and agreed to the published version of the manuscript. #To whom correspondence should be addressed. Dr. R.ZX and Dr. Y.J have directly accessed and verified the underlying data reported in the manuscript.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse Anti-Human CD3/FITC	Becton Dickinson,USA	Cat#570821
Mouse Anti-Human CD4/PE-Cy7	Becton Dickinson,USA	Cat#560649
Mouse anti Human CD25/APC	Biolegend,USA	Cat#302610
Rat anti human FoxP3/PE	Invitrogen,USA	Cat#12-4776-42
Biological samples		
Very preterm infants Peripheral Blood	Guangdong women and children hospital, guangzhou medical university, clinical laboratory center	http://www.e3861.com/
Very preterm infants Cord Blood	Guangdong cord blood Bank	https://www.gdcordblood.org/
Critical commercial assays		
LL-37 ELISA kit	Elabscience, china	E-EL-H2438c
Software and algorithms		
BD FACSCanto II cytometer	BD, Bioscience, CA, USA	R33896203126
FACS Canto,V 3.1	BD, Bioscience, CA, USA	–

RESOURCE AVAILABILITY

Lead contact

Further information and requests should be directed to the lead contact, Jie Yang (jieyang0830@126.com).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This paper does not report the original code. The data sources of this study are presented in the ''STAR methods'' sections. Any additional information required to the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Ethics statement: The trial was approved by the ethics committee of Guangdong Women and Children Hospital (201601079). In our previous trial of prevention for severe BPD with intravenous infusion of autologous cord blood mononuclear cells in very preterm infants, 29 were enrolled in the ACBMNC intervention group, and 33 were enrolled in the control group.[34] All the infant were less than 32 gestational weeks, ethnic Han, and 24 of them were male, 38 were female. The influence of gender on LOS outcomes were showed in Table 1. There were no significant. These patients were enrolled as cohort 1. For cohort 2, from January 17th, 2022, to August 27th, 2022, we screened very preterm infants less than 32 gestational weeks in Guangdong Women and Children Hospital. The inclusion criteria were as follows: (a) born in the study hospital; (b) less than 32 gestational weeks; and (c) guardian written consent was obtained. The exclusion criteria were as follows: (a) major congenital abnormalities; (b) severe perinatal asphyxia (defined as an Apgar score of 0–3 for more than 5 min, a cord blood gas pH < 7.00, or both); (c) death within 72 h; and (d) diagnosis of early-onset sepsis. Very preterm infants who fulfilled the above criteria were classified as control patients and LOS cases as defined by the above definition. The patients with sepsis were then classified into sepsis and septic shock groups based on disease severity. All infants were ethnic Han. Among them, 55/80 (68.75%) were male. The influence of gender on LOS outcomes were showed in Table 2. There were no significant. For cohort 3, multiple imputation was used to produce complete datasets of LL37 and Treg results in all infants enrolled in our previous trial.[34]

METHOD DETAILS

LL37 level, Treg proportion and LOS incidence in VPIs treated with ACBMNCs in our previous trial (cohort 1)

Among infants enrolled in cohort 1, twenty blood samples in each group were collected from cord blood and abandoned blood from routine blood tests 72 h after intervention.[34] LL37 levels were measured via ELISA kit. Lymphocyte subgroups were detected as described previously.[34] Treg frequency was reported in a previous study and was further analyzed in this study after adjusting for possible co-founders. The incidence of LOS (both culture negative and culture positive), antibiotic duration, and the use of meropenem or vancomycin were compared. Culture-negative sepsis was diagnosed when all the following criteria were fulfilled: (1) cultures were sterile; (2) infection-related clinical manifestations occurred 72 h after birth; and (3) the initial or repeated blood test showed two or more abnormal indicators. The definitions of infection-related clinical manifestations and abnormal blood test indicators are shown in Tables S1 and S2. Culture-positive infection was defined by a positive blood or cerebral spinal (or the normally sterile body sites) culture. Late onset sepsis was defined as >72 h.[37]

Establishing a prospective very preterm training cohort *(cohort 2)*

Nested case-control study

Cases were identified on the basis of the diagnosis of sepsis. Controls were matched to cases by gestational age (22–27 weeks, 28–31 weeks), and controls were randomly selected at a 1:1 ratio in infants without LOS. Abandoned blood from routine blood tests 72 h after birth was collected for ELISA tests and immune cell sorting. During hospitalization, sepsis-associated symptoms were recorded and are shown in Table S1. Septic shock was defined as patients with sepsis manifested with cardiovascular dysfunction, which included hypotension, treatment with a vasoactive medication, or impaired perfusion.[38]

Data collection

The collected data from enrolled neonates included the general conditions of the mother (maternal age, antenatal glucocorticoids, pregnancy complications -gestational diabetes mellitus [GDM], hypertensive disorders of pregnancy [HDP], histological chorioamnionitis, intrahepatic cholestasis of pregnancy [ICP] and preeclampsia), general conditions of the neonate (gestational age [GA], birth weight [BW], body length, head circumference, sex, Apgar score and delivery mode), and common preterm complications and comorbidities (death, anemia requiring transfusion, respiratory distress syndrome [RDS], intraventricular hemorrhage [IVH], bronchopulmonary dysplasia [BPD], necrotizing enterocolitis [NEC], patent ductus arteriosus [PDA], retinopathy of prematurity [ROP], persistent pulmonary hypertension of newborn [PPHN], duration of endotracheal intubation, duration of central venous catheterization, typical LOS associated clinical manifestation and the LOS onset time (days after birth).

Clinical laboratory tests, including hemoglobin, platelet count, white blood cell counts, lymphocyte counts, neutrophils, monocytes, C-reactive protein [CRP], procalcitonin [PCT], pH, lactic acid [Lac], and blood glucose on day 3 after birth, were collected. All clinical diagnoses were defined according to standard references and are shown in the study protocol.[19]

LL37 level detection

The abandoned blood from routine blood tests that were routinely repeated on day 3 after birth for assistance in determining further antibiotic use were collected. Serum was separated by centrifugation at 4°C with gravity at 500 × g for 8 min and stored at −80°C. LL37 levels were measured via an ELISA kit (Elabscience Biotechnology; Wuhan, China, E-EL-H2438c).

Lymphocyte subset sorting

PBMCs of abandoned blood from routine blood tests were used for lymphocyte cell subset sorting as described previously.[34] For regulatory T cells, flow cytometry analysis, cells were firstly stained with surface antibodies specific for CD3/FITC (clone SK7); CD25/APC (clone 2A3), followed by intranuclear staining for FoxP3/PE (clone 259D/C7). FoxP3 intranuclear staining was performed according to the manufacturer's protocol following cell surface staining. The fixed and stained cells were diluted in fluorescence activated cell sorter staining buffer and stored directly at 4°C. Flow cytometric analysis was performed within 48 h with a BD FACSCanto II cytometer and analyzed with BD FACSCanto software 3.1 (BD, Bioscience, CA, USA). Lymphocytes were determined by their position in the forward-/side-scatter plot (size/granularity) and co-expression of CD3/CD4/CD25/FoxP3 was necessary to identify the Treg cells.

Integrative model to predict LOS development

Treg cell abundance and LL37 levels on day 3 after birth were individually associated with LOS development (Figures 2C and 2D). Accordingly, we explored integrative modeling as a means of improving performance. While several techniques were assessed, including nonlinear modeling with random forests, logistic regression (glm in R) achieved comparable performance and was selected owing to the relative simplicity and robustness of a generalized linear model.

To mitigate overfitting when dividing patients into high and low groups by leave-one-out cross-validation (LOOCV), we applied Youden's J statistic to determine the threshold that optimized sensitivity and specificity and then allocated the held-out patients on the basis of this threshold. Composite model scores were assessed by receiver operating characteristic (ROC) analysis. Models trained to discriminate control from sepsis were used to predict future LOS development, LOS severity and time-to-LOS development.

External validation of the prediction model

These patients with available LL37 levels and Treg proportions results in the previous trial (cohort 1) were enrolled as the validation cohort and used for external validation of the predictive model developed from the training cohort.

QUANTIFICATION AND STATISTICAL ANALYSIS

We first conducted a post hoc analysis (Tukey's Honestly Significant Difference test) to compare the incidence of LOS in the control and ACBMNC intervention groups. Means (standard deviation, SD) and unpaired Student's test were used for continuous variables with normal distribution, and median and interquartile range (IQR) and nonparametric analysis were used for data with nonnormal distribution. Differences in clinical and demographic characteristics were analyzed by using chi-square test for categorical data, one-way ANOVA for continuous variables and Tukey's post hoc comparison.

The variables' distribution characteristics were estimated with the Kolmogorov-Smirnov test. The number and percentage were reported for categorical variables. Single factor regression analysis was conducted to assess the possible confounding factors for the outcome of LOS. Then, multiple logistic regression was used to estimate the contribution of ACBMNC intervention to LOS. Multiple linear regression was used to estimate the effect of ACBMNC intervention on LL37 levels and Treg proportions after adjusting for GA, birth weight and sex before enrollment.

For the prospective cohort study used for the prediction model, we first determined an adequate sample size. We presupposed that the difference in GA, duration of endotracheal intubation or central venous catheterization may be the main confounding factors that may affect the incidence of LOS. Since logistic regression was used for exploring integrative modeling, we used the requirements of the logistic regression on sample size to estimate the study sample size based on the classic experience norms. The total sample size was 20 times the predictor variables. In our study, as we paired the infants by GA, the number of predictor variables was then assumed to be 4 (Treg abundance, LL37 level, duration of endotracheal intubation or central venous catheterization); thus, the estimated sample size was 80 in total. Using these calculations, we estimated that at least 40 infants in each group would be needed for this analysis.

Covariates with a *p* value <0.05 (univariate analysis) were selected for inclusion in the multivariate logistic regression model to estimate the odds ratio (OR) with a 95% confidence interval (95% CI). Subsequently, factors with prognostic significance in the logistic regression analysis were utilized to build a prediction model, and a nomogram predicting LOS was formulated using the rms package in R version 3.6.3 based on the results of multivariate analysis to visualize the model. To assess the performance of the nomogram, internal validation was augmented with 10-fold cross validation. Thereafter, the visual prediction model was externally validated. The performance of the external validation was assessed by calculating the total points of each patient in the validation cohort based on the established nomogram, and ROC curves were used to determine predictive values, with cutoff points identified using Youden's index. Sensitivity, specificity, and predictive values were calculated. The Hosmer—Lemeshow test and coefficient of determination were used to assess the goodness of fit of the model. Decision curve analysis (DCA) reflected the net benefit of the model for patients. Both discrimination and calibration were assessed by bootstrapping with 1000 resamples.

Multiple imputation was used to produce complete datasets of LL37 and Treg results in all infants enrolled in our previous trial (cohort 1) thus producing cohort 3 for further sensitivity analysis of the model and prediction of the reduction in LOS in VPIs after ACNMNC intervention. Kaplan—Meier and Cox regression analyses were used to assess covariates with respect to time-to-LOS. For matched controls, the endpoint of observation was discharge home. Significance levels and hazard ratios (HRs) for Kaplan—Meier analysis were determined using a two-sided log rank test. All analyses were conducted using R software (version 4.2.2) and SPSS 25.0.

ADDITIONAL RESOURCES

The trial was approved by the ethics committee of Guangdong Women and Children Hospital (201601079). The trial was registered at ClinicalTrials.gov (NCT02999373).