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Mannose-binding lectin insufficiency is associated with airway *Haemophilus* colonization and a higher risk of post-RSV bronchiolitis recurrent wheezing

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Abstract

Objective: To investigate whether mannose-binding lectin (MBL) insufficiency influences the airway microbiota composition and development of subsequent recurrent wheezing in infants with severe respiratory syncytial virus (RSV) bronchiolitis.

Methods: Sixty-seven infants who were hospitalized during an initial episode of severe RSV bronchiolitis at 6 months of age or less were included in the study and followed up until the age of 3 years. Serum and sputum samples were collected. The serum MBL concentrations were determined by ELISA; the sputum microbiota and cytokines were analyzed by 16S rRNA-based sequencing and multiplex immunoassay, respectively.

Results: Twenty-six infants developed recurrent wheezing by the age of 3 years, and 41 did not. The rate of MBL insufficiency was significantly higher among infants who developed recurrent wheezing compared to those who did not [50.0% (13/26) vs. 24.4% (10/41), ($p = .031$)]. MBL insufficiency was independently associated with a higher risk of subsequent development of recurrent wheezing (adjusted Odds Ratio: 3.5, 95% CI 1.1–12.3, $p = .035$). *Haemophilus* was found to be the most discriminative genus between infants with and without MBL insufficiency (LDA >4.0). The level of INF-γ ($p = .019$) in infants with MBL insufficiency was significantly lower than that among infants with normal MBL.

Conclusion: During the first episode of severe RSV bronchiolitis in infants <6 months of age, MBL insufficiency was associated with a higher risk of developing subsequent recurrent wheezing by age three. Infants with MBL insufficiency were more likely to be colonized with *Haemophilus* and have weaker airway IFN-γ response.

KEYWORDS
IFN-γ, infants, Mannose-binding lectin, recurrent wheezing, respiratory microbiome, respiratory syncytial virus

1 | INTRODUCTION

Respiratory syncytial virus (RSV) is the most important cause of acute lower respiratory infections (ALRI) in young children worldwide.[1] The severity of the disease in infancy, which is believed to be associated with the physiological immaturity of the immune system, is also associated with an elevated risk of recurrent asthma in later life.[2–4] This phenomenon is considered to be linked to long-term immune response alterations and neuromuscular tone instability in the airways, although it is not always associated with atopic sensitization.[5] Furthermore, susceptibility to both RSV bronchiolitis and the development of recurrent wheezing has been attributed to defects in immunoregulatory pathways, the cause of which may be genetic and/or environmental.[6,7]

Mannose-binding lectin (MBL) is an important component of the innate immune system. It can bind to mannose and N-acetyl-glucosamine oligosaccharides, which are present on the surfaces of many different microorganisms. MBL insufficiency resulting from polymorphisms in the *MBL2* gene is relatively common in humans. It is associated with more frequent and severe respiratory infections in young children who have not yet developed adaptive immunity.[8,9] Dicker et al.[10] found that adult COPD patients with MBL insufficiency had a more diverse lung microbiota and a lower risk of exacerbation, demonstrating an association between genetic MBL concentration and the lung microbiota for the first time. Interestingly, we have recently reported that the airway microbiota can impact the host immune response during RSV bronchiolitis in infancy, potentially influencing post-RSV bronchiolitis recurrent wheezing.[11] Moreover, genetic alterations in the pathogen recognition system of innate immunity may influence the colonization of the airway microbiota in infants and may be associated with a higher risk of later development of childhood asthma.[12]

In this context, this study took infants with severe RSV bronchiolitis as the study object and aimed to investigate (i) whether MBL insufficiency is associated with a higher risk of development of subsequent recurrent wheezing; and (ii) the effect of the serum MBL level on the airway microbiota and local immune responses.

Key message

Infants with MBL insufficiency were more likely to be colonized with *Haemophilus* and have weaker airway IFN-γresponses during RSV bronchiolitis, which may contribute to the development of subsequent recurrent wheezing.

2 | METHODS

2.1 | Study design and data collection

Details of the study design and data collection were described previously.[9] Briefly, this prospective, observational study recruited infants who were hospitalized at Beijing Children's Hospital during two study periods: October 2014 to May 2015 and October to December 2015. Previously healthy, full-term infants aged less than 6 months who were positive for RSV, diagnosed as having severe bronchiolitis, and experiencing their first wheezing episode were included in the study. Severe bronchiolitis was defined as difficulty in breathing with indrawing of the lower chest wall and requiring oxygen supplementation and hospital admission. Respiratory severity score was calculated as previous described.[13]

Blood and sputum samples were collected within 24h of admission. After discharge, contact with the parents was scheduled for 1 month, 3 months, and then every 3 months to monitor the child's respiratory symptoms. Children were followed until the age of 3 years. Recurrent wheezing was defined as the occurrence of three or more subsequent episodes of wheezing diagnosed by a pediatrician. This study was approved by the Ethics Committee of Beijing Children's Hospital, Beijing, China [REC numbers: 2014–87]. Written informed consent was obtained from the parents of the study subjects.

2.2 | Determination of serum MBL

Serum MBL concentrations were determined using a human MBL ELISA Kit (Raybiotech, ELH-MBL, Atlanta, USA), according to the manufacturer's instructions. All samples were initially diluted 1:800 and measured in duplicate. The detection limit was 0.03 ng/mL. The MBL level was defined as "low/insufficiency" if the serum contained <500 ng/mL MBL and "normal" if the concentration was ≥500 ng/mL, based on previously published studies.[14] To study the effect of MBL level on the airway microbiota and local immune responses, the study subjects were stratified based on the serum MBL level: MBL insufficiency group (<500 ng/mL) and normal MBL group (≥500 ng/mL).

2.3 | Bacterial DNA extraction and 16S rRNA sequencing

As previously described,[11] bacterial DNA was extracted using QIAamp DNA Stool Mini Kit following the manufacturer's instructions (Qiagen, Hilden, Germany). The V4–V5 region of the 16S rRNA gene was amplified using specific primers (515F and 907R).

2.4 | Detection of airway cytokines

Cytokines in the sputum supernatants were analyzed with a multiplex immunoassay (Invitrogen, Vienna, Austria). The lower limits of detection (LODs) for 10 cytokines were as follows: CXCL8, 2.25 pg/mL; CCL5, 0.76 pg/mL; CXCL10, 1.56 pg/mL; IFN-γ, 14 pg/mL; IL-12p40, 1.35 pg/mL; IL-6, 8.01 pg/mL; IL-10, 1.87 pg/mL; IL-13, 2.66 pg/mL; IL-17, 1.88 pg/mL; and IL-33, 2.69 pg/mL.

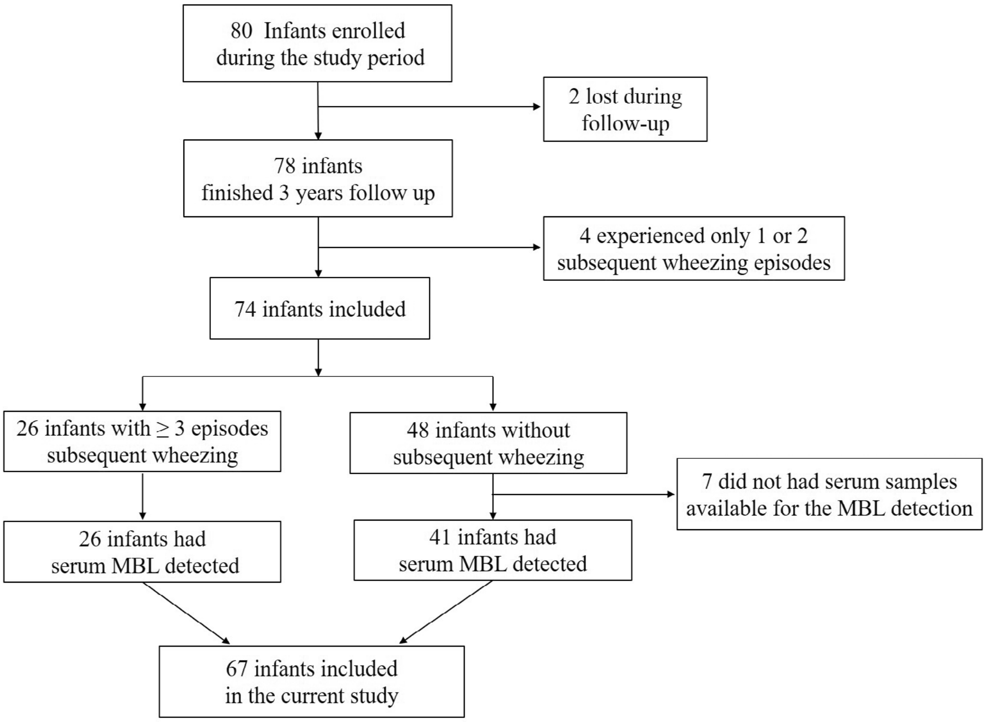

FIGURE 1 Flow diagram of the study subject.

2.5 | Microbiome and statistical analysis

Alpha diversity was estimated using the number of observed species, Chao1 estimator, and Shannon index. Comparison of microbiome structure between different variables was tested using permutational multivariate ANOVA (PERMANOVA). Linear discriminant analysis effect size (LEfSe) was used to determine taxa that best characterize each study group. In order to achieve high specificity, we considered taxa with linear discriminant analysis (LDA) score >3.5 to be significant. All the analyses described above were performed in R (version 4.2.3).

The Mann–Whitney *U* test was used to compare the continuous variables between infants with and without recurrent wheezing. For comparison of categorical variables, the chi-square test was used. Binary logistic regression analysis with adjustment for potential variables that could influence the association was used to determine the association between serum MBL and the development of subsequent recurrent wheezing. These analyses were performed using SPSS (version 24.0). A two-sided *p* value <.05 was considered statistically significant.

3 | RESULTS

3.1 | Baseline characteristics of the study subjects

In total, 67 infants were included in the study. The median age was 2.5 months (IQR 1.7–3.7 months); 48 (71.6%) of the infants were male. Overall, 26 infants developed recurrent wheezing by the age of 3 years, and 41 did not. A flow diagram of the study subjects was shown in Figure 1. No significant differences were observed in age, sex, race, delivery mode, feeding type, or parental history of asthma between the two groups. The immunoglobulin series and lymphocyte subsets of infants with and without recurrent wheezing were generally normal. The detailed baseline characteristics of the infants are shown in Table 1, and the basic immunologic parameters were shown in Table S1.

3.2 | MBL insufficiency is associated with a higher risk of developing recurrent wheezing

Generally, there was a trend of lower MBL levels in infants with subsequent recurrent wheezing as compared to those without [median 492.8, IQR (374.4–784.8) vs. median 775.3, IQR (476.3–1026.9)] ($p = .089$) (Figure 2). The rate of MBL insufficiency was significantly higher among infants who developed subsequent recurrent wheezing as compared to those who did not develop subsequent recurrent wheezing [50.0% (13/26) vs. 24.4% (10/41), $p = .031$]. The logistic regression analysis adjusted for age, sex, delivery mode, feeding type, personal or parental history of allergies, and parental smoking indicated that MBL insufficiency was independently associated with a higher risk of subsequent development of recurrent wheezing (adjusted Odds Ratio (OR) 3.5, 95% Confidence Interval (CI) 1.1–12.3, $p = .035$).

3.3 | MBL level and airway microbiota

The airway microbiota richness and diversity as measured by alpha diversity was not significantly different between infants with MBL

TABLE 1 Baseline characteristics of infants with and without subsequent recurrent wheezing ($N=67$).

Characteristics	RW+ ($n=26$)	RW− ($n=41$)	*p* Value
Age (mon)	2.6 (1.8–3.8)	2.4 (1.7–3.4)	.325
Male (%)	18 (69.2)	30 (73.2)	.727
Han nationality (%)	23 (88.5)	39 (92.5)	.593
Gestational age (wk)	38.9 (37.7–39.5)	38.7 (37.0–39.2)	.747
Birth weight (kg)	3.0 (2.8–3.5)	3.4 (2.9–3.6)	.130
Birth by cesarean section (%)	15 (57.7)	23 (56.7)	.898
Mostly breast milk (%)	16 (61.5)	30 (73.2)	.371
Parental smoking (%)	12 (46.2)	17 (41.5)	.706
Parental history of asthma (%)	1 (3.8%)	1 (2.4%)	1.000
Infantile eczema (%)	11 (42.3%)	18 (43.9%)	.898
Onset of disease (d)	6 (5–7)	6 (4.5–7)	.424
Respiratory severity score	8.0 (7.0–9.0)	8.0 (6.0–8.5)	.063
Hospital Stay (d)	7.5 (5.8–10.0)	7 (5.0–8.0)	.134
WBCs (/μL)	8250 (7138–10,640)	8690 (6855–11,480)	.382
Serum CRP level (mg/L)	2.48 (1.66–8.64)	2.34 (1.39–6.53)	.598

Note: RW+ indicates infants with subsequent recurrent wheezing; RW− indicates infants without subsequent recurrent wheezing. Data are median values (interquartile ranges) or no. (%) of subjects.

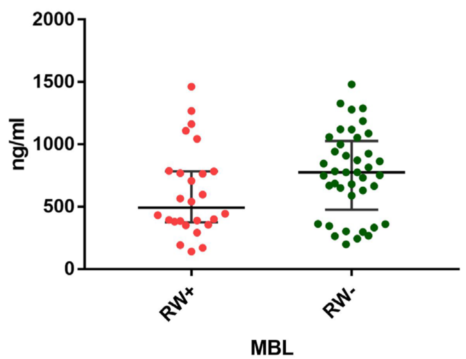

FIGURE 2 Level of serum MBL in infants who later developed recurrent wheezing and those who did not. RW+ indicates infants who later developed recurrent wheezing; RW− indicates infants who did not develop recurrent wheezing later.

insufficiency and those without (the number of observed species, $p=.421$; Chao1 estimator, $p=.402$; Shannon index, $p=.432$) (Figure 3A). To identify bacterial taxa associated with the MBL level, LEfSe analysis was performed. When a linear discriminant analysis (LDA) score >4.0 was used, *Pasteurellales*, *Pasteurellaceae*, and *Haemophilus* were found to be the most significantly different bacteria between infants with and without MBL insufficiency, with a higher abundance in infants with MBL insufficiency (Figure 3B).

3.4 | Effect of the MBL level on airway inflammatory cytokines

In order to evaluate the effect of MBL level on airway local immune responses, the sputum levels of 10 inflammatory cytokines were measured. The level of INF-γ in infants with MBL insufficiency was significantly lower than that in infants with a normal MBL ($p=.019$) (Figure 4). There were no significant differences in the levels of the other nine inflammatory cytokines between the two groups. Sputum levels of the 10 inflammatory cytokines in infants with low and normal serum MBL (Table S2).

4 | DISCUSSION

To our best knowledge, this study is the first to show that in infants with severe RSV bronchiolitis, MBL insufficiency is associated with a higher risk of developing subsequent recurrent wheezing by the age of 3 years. Moreover, infants with MBL insufficiency were more likely to be colonized with *Haemophilus* and have lower airway IFN-γ responses during RSV bronchiolitis, which may play a role in the development of subsequent recurrent wheezing.

Haemophilus is one of the most frequently identified bacteria associated with susceptibility to wheezing illness or asthma in children.[15–17] Previously, we demonstrated that during severe RSV bronchiolitis, the relative abundance of airway *Haemophilus* was higher in infants who later developed recurrent wheezing than in those who did not, and a higher abundance of *Haemophilus* seemed to be associated with a more obvious Th2-biased airway inflammation response.[11] Interestingly, in the current study, although MBL insufficiency did not have a significant effect on the airway microbiota diversity which might be due to the lack of power for detecting the difference, *Haemophilus* was found to be the most discriminative genus between infants with and without MBL insufficiency during severe RSV bronchiolitis, and a higher abundance was observed in infants with MBL insufficiency. This suggests that MBL insufficiency

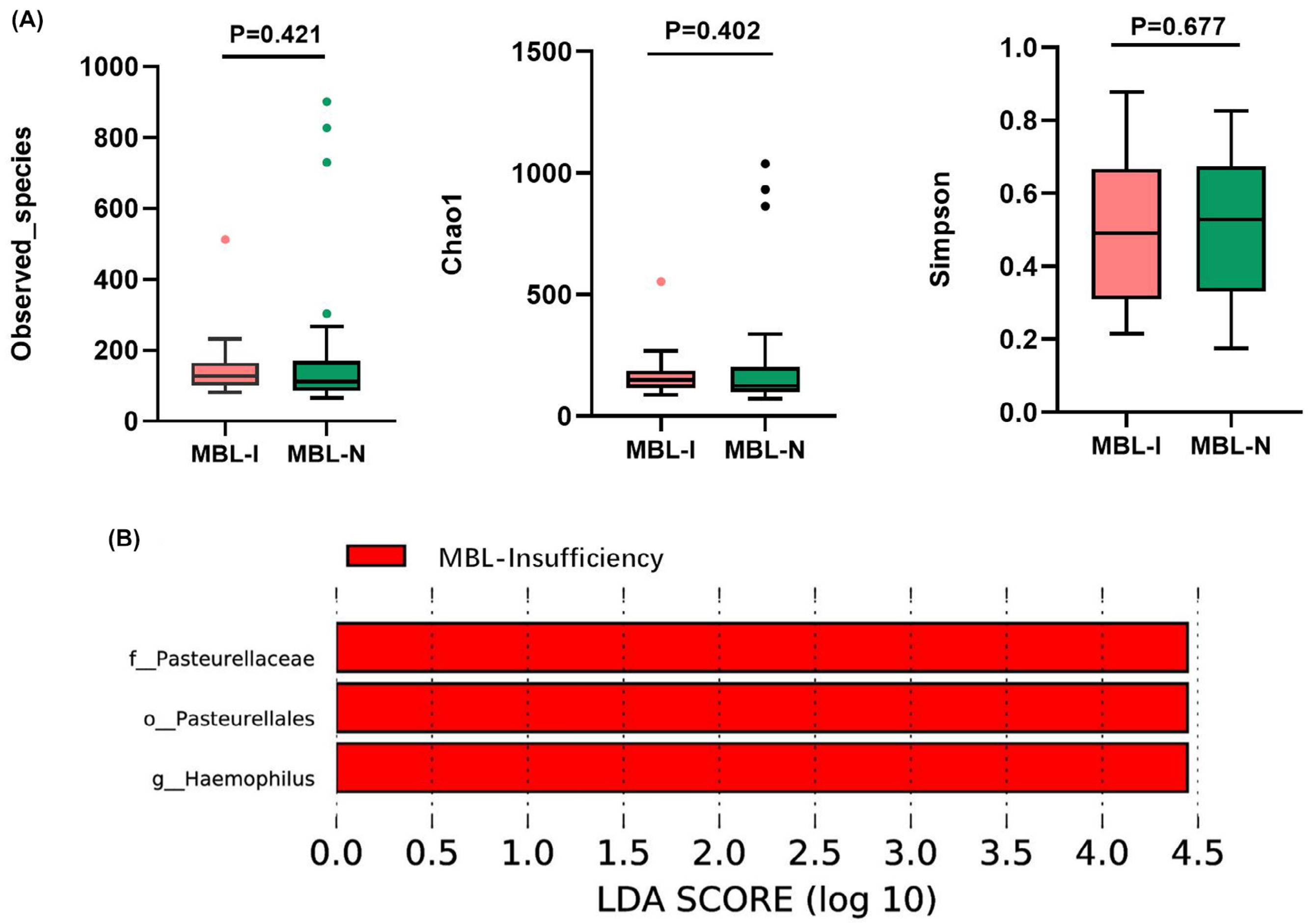

FIGURE 3 (A) α-Diversity measures (observed species, Chao1 index, Shannon index) in infants with low (insufficiency) and normal serum MBL. (B) Linear discriminant analysis (LDA) shows distinct sputum microbiota tax during RSV bronchiolitis associated with the serum MBL level. LEfSe cladogram representing differentially abundant taxa. Taxa with LDA scores >4.0 are presented.

might be conducive to the airway colonization of *Haemophilus* during RSV bronchiolitis, which may influence the later development of recurrent wheezing. Our data was inconsistent with previous reports that adult COPD patients with MBL insufficiency were less likely to be colonized with *Haemophilus* and associated with reduced exacerbation frequency in COPD.[10] Interestingly, in patients with non-cystic fibrosis bronchiectasis, there is a higher frequency of bacterial colonization with *Haemophilus influenzae*; MBL serum deficiency was associated with increased exacerbations, hospital admissions, and radiological severity.[18] Therefore, it is plausible that MBL deficiency may have different impacts in different diseases and different populations, as they have differing microbiota and inflammatory profiles. It should be kept in mind that the participants of this study were infants aged less than 6 months and their immune systems were not yet matured. Therefore, the role of other immune molecules in addition to MBL could not be excluded.

IFN-γ is usually considered a characteristic cytokine for Th1-type response and exerts a protective effect during RSV infection in infants.[19] A weak IFN-γ response could result in delayed virus clearance and the development of Th2-based immunity. This can lead to enhanced epithelial injury and secondary tissue repair, leading to long-lived changes to the epithelia that render the development of subsequent recurrent wheezing.[20,21] Indeed, in one study, the deficient IFN-γ production during RSV bronchiolitis was found to be an indicator of subsequent lower pulmonary function and increased airway responsiveness, and appeared to predict the development of asthma in infants hospitalized for severe RSV bronchiolitis.[22] In this present study, the airway IFN-γ level was lower among infants with MBL insufficiency as compared to infants with normal MBL. This suggests that an innate MBL insufficiency might influence the adaptive immune response and the production of IFN-γ in the respiratory tract, which may further promote the subsequent development of recurrent wheezing.

The current study has certain limitations. First, the sample size was small, but the results contained several important observations. Nonetheless, future studies with a large number of samples are needed to better elucidate the integration between MBL insufficiency, microbiome, and airway immune response, as well as its influence on the development of subsequent recurrent wheezing. Secondly, we did not perform MBL genotyping of study subjects. However, it is well known that serum MBL concentrations have been associated rather consistently with MBL genotypes.[23]

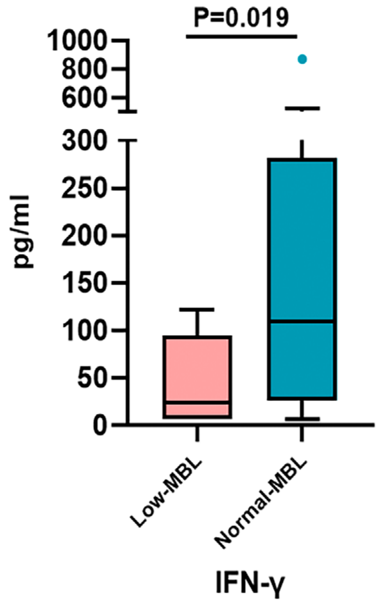

FIGURE 4 Comparison of levels of inflammatory mediators in sera from infants low (insufficiency) and normal serum MBL.

In conclusion, during the first episode of severe RSV bronchiolitis in infants younger than 6 months of age, MBL insufficiency was found to be associated with a higher risk of developing subsequent recurrent wheezing by age three. Infants with MBL insufficiency were more likely to be colonized with *Haemophilus* and have weaker airway IFN-γresponses, which may contribute to the development of subsequent recurrent wheezing.

AUTHOR CONTRIBUTIONS

Xiaoyan Zhang: Conceptualization; methodology; data curation; investigation; formal analysis; funding acquisition; writing – original draft. **Xiang Zhang:** Data curation; investigation. **Xinglan Wang:** Data curation; investigation. **Xiaolei Tang:** Data curation; investigation. **Hui Xu:** Data curation; investigation. **Nan Zhang:** Methodology. **Shunying Zhao:** Conceptualization; supervision; writing – review and editing. **Haiming Yang:** Supervision; writing – review and editing. **Qiushui He:** Conceptualization; supervision; methodology; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

All authors declare that there are no conflicts of interest.

PEER REVIEW

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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