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ORIGINAL ARTICLE

Monocyte subsets study in children with *Mycoplasma pneumoniae* pneumonia

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Abstract

The aim of this study was to evaluate the changes in the three subsets of monocyte (classical, intermediate, and non-classical) and the expression of human leukocyte antigen-DR (HLA-DR) on monocyte subsets during MP pneumonia in children. Monocyte subsets were analyzed in the peripheral blood of healthy volunteers and MP pneumonia patients at the stages of admission and remission after clinical therapy. They were defined as classical ($CD14^{+}CD16^{-}$), intermediate ($CD14^{bright}CD16^{+}$), and non-classical ($CD14^{dim}CD16^{+}$) using flow cytometry. Furthermore, three subsets of monocyte were analyzed for the expression of HLA-DR. Patients with MP pneumonia at admission had a higher proportion of intermediate and non-classical monocytes than healthy subjects (all $P < 0.05$). The proportion of intermediate subset and non-classical subset was lower in MP pneumonia patients at remission than at admission (all $P < 0.05$). In comparison with the other monocyte subsets, intermediate subset showed a significantly higher percentage of HLA-DR in MP pneumonia patients at admission ($P < 0.05$). Further analysis revealed that the expression of HLA-DR on intermediate subset was lower in severe patients than in non-severe patients ($P < 0.05$).Our data has shown for the first time that MP pneumonia is associated with the increased proportion of non-classical and intermediate monocytes, indicating the involvement of monocyte-related mechanisms in the pathogenesis of this disease. Additionally, the decreased expression of HLA-DR on $CD14^{bright}CD16^{+}$ subset may be a potential indicator of the severity of MP pneumonia.

Keywords *Mycoplasma pneumoniae* pneumonia · Monocyte subsets · Human leukocyte antigen-DR · Children

Introduction

Mycoplasma pneumoniae (MP) is a common cause of community-acquired pneumonia (CAP), mainly for children and young adults. MP can be implicated in upper respiratory tract infections, bronchiolitis, tracheitis/bronchitis, and pneumonia [1]. Moreover, MP infection has been related to the onset of several extrarespiratory diseases, too [2]. MP-related extrapulmonary diseases can involve multiple organs, including the joints, muscles, skin, kidneys, heart, gastrointestinal system, nervous system, and hematological system, whereas MP pneumonia is considered to be in part attributed to immune-mediated responses [3].

Recently, Poddighe et al. reported that elevated levels of serum IgE, namely atopy, in children develop different forms of MP-related extrapulmonary diseases [4]. Moreover, they also confirmed this observation in a wider cohort of children; hospitalized children diagnosed with MP-related extrapulmonary diseases resulted to have significantly increased serum IgE compared to children developing respiratory illnesses only [5]. We confirmed such an observation that atopy may be a risk factor for

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disease severity and extrapulmonary complications in children with MP pneumonia. In addition, we found that the patients with extrapulmonary complications showed higher total IgE levels and lower IL-17 levels [6]. At the same time, serum levels of IL-17 in atopic children with MP infection resulted to be significantly increased than in non-atopic controls [7]. Therefore, MP-related extrapulmonary diseases have been supposed to be immune-mediated.

However, less attention has been given to the contribution of monocyte subsets, even though monocytes migrate from the peripheral blood to inflammatory sites and differentiate into dendritic cells and macrophages that produce pro-inflammatory cytokines [8].

Until now, circulating human monocytes have been classified into $CD14^{+}CD16^{-}$ (classical) and $CD14^{+}CD16^{+}$ subsets according to their expression levels of CD14 and CD16 [9]. Recently, a new third monocyte subpopulation, $CD14^{bright}CD16^{+}$ monocyte, was defined. According to the new classification system, the $CD14^{+}CD16^{+}$ population is classified into $CD14^{bright}CD16^{+}$ (intermediate) and $CD14^{dim}CD16^{+}$ (non-classical) monocytes, depending on the level of CD14 expression [10]. Although the intermediate and non-classical monocytes are very closely related, there were also features to distinguish them apart. Increasing evidence has shown that intermediate monocyte exert an antigen-presenting function with a dendritic cell-like feature [11].

Monocytes play an important role in the invasion and immunity to resist pathogens. The main functions of monocytes, antigen expression, and cytokine production are mediated by certain surface molecules from major histocompatibility complex (MHC) class II, such as monocyte human leukocyte antigen (HLA-DR). HLA-DR allows antigen presentation to T cells and is crucial for the initiation of the cascade of the immune response. Hence, HLA-DR expression suggests the antigen processing and presentation capability of the expanded subset [12].While the symptoms of MP pneumonia are correlated with the induction of pro-inflammatory cytokines [13]. However, the action of the monocyte subsets and its HLA-DR expression in children with MP pneumonia remains unknown. Here, we aimed to determine which of the monocyte subsets is expanded in MP pneumonia and to investigate their possible role in disease pathogenesis.

Patients and methods

Subjects and study design

Patients were enrolled prospectively based on admission to the Tianjin Nankai Hospital with MP pneumonia. The ages of the patients ranged from 3 to 14 years old. The MP pneumonia patients were treated with azithromycin (10 mg/kg/day) for 5 days, according to guidelines for management of community-acquired pneumonia [14, 15]. The subjects were divided into non-severe patients and severe patients. Briefly, subjects were categorized as severe patients if they met one or more of the following criteria: (1) Antifeedant or dehydration, (2) confusion, (3) tachypnea, (4) cyanosis, (5) dyspnea, (6) chest radiographic examination showed multiple lobe involvement or more than two-third lobe involvement and even pleural effusion, (7) blood oxygen saturation below 0.92, and (8) extrapulmonary complications. Blood samples were drawn on the day of hospitalization (admission) and prior to discharge from hospital (remission). The range of duration from admission to remission was 7 days. During the study period, a total of 45 patients were enrolled at admission. Of the 45 patients, 10 patients refused to be drawn blood prior to discharge from hospital; therefore, there were only 35 patients at remission. Monocyte subsets from peripheral blood samples were all processed immediately for flow cytometry. In addition, 20 age- and sex-matched healthy children were also included as a healthy controls (HC) group. Patient characteristics, respiratory disease severity, and extrapulmonary manifestations were recorded. The study was approved by the Tianjin Nankai Hospital Ethics and Medical Research Committee, which acts in compliance with ethical standards defined by the Declaration of Helsinki.

Diagnosis of MP pneumonia

MP pneumonia patients were diagnosed based on their clinical presentation (such as fever, cough, dyspnea, and crackles) and radiological findings (such as alveolar, bronchial, interstitial, and pleural effusion) and the changes in their anti-mycoplasma antibody titers. The serum of patients was used to test anti-mycoplasma IgM antibody titers by the microparticle agglutination method using a commercial kit (Serodia-Myco II, Fujirebio, Tokyo, Japan). The anti-mycoplasma IgM antibody titers were regarded as positive when the titers were $\geq$ 1:160 in the admission phase or when the titers of IgG were over fourfold higher in the symptomatic recovery phase than in acute phase [14, 16].

Patients with asthma, recurrent respiratory tract infection, primary immunodeficiency or secondary immunodeficiency, and chronic lung disease were excluded from this study. Besides, polymerase chain reaction (PCR) testing of respiratory secretions was performed to detect other pathogens, such as influenza viruses, parainfluenza viruses, human adenoviruses, and respiratory syncytial virus. If any of these assays for other pathogens were tested positive, the patients were excluded from this study.

Sample collection

Serum and peripheral blood mononuclear cells (PBMC) were isolated from ethylenediaminetetraacetic acid-treated blood

samples collected from all participants. The PBMC were separated by standard Ficoll-Hypaque density centrifugation at 1000 rpm for 20 min.

Monocyte subset determination

In order to examine the phenotype and frequency of classical $CD14^{+}CD16^{-}$ monocytes, intermediate $CD14^{bright}CD16^{+}$, and non-classical $CD14^{dim}CD16^{+}$ monocytes, relevant labeled multicolor fluorescence anti-human monoclonal antibodies (mAbs) purchased from Biolegend (San Diego, CA, USA) were used for surface staining. APC-conjuated anti-human CD16, PerCP-conjuated anti-human CD14, and FITC-conjuated anti-human leukocyte antigen-DR, PBMC was first thawed and then resuspended in flow staining buffer (PBS plus 1% FBS); after being washed twice, PBMC was resuspended again and incubated with the above labeled multicolor fluorescence anti-human monoclonal antibodies. Then the stained PBMC was washed with flow staining buffer and centrifuged. Finally, the stained PBMC were diluted and analyzed on a flow cytometer (BD LSR II) (BD Biosciences). Data was acquired as the fraction of labeled cells within a cell gate set for 20,000 events. The detailed procession was described in previous study [9].

Statistical methods

All statistical analyses were performed using the SPSS 17.0 software package. Normal distribution of values in each group should to be demonstrated by the Kolmogorov–Smirnov. Quantitative variables are expressed as means ± standard deviation (SD) or median values with interquartile range (IQR). Normally distributed parameters between multiple groups were examined by using analysis of variance (ANOVA) and Tukey's multiple comparison test. Statistical comparisons between the two subgroups were made with paired *t* tests. The Mann–Whitney *U* test was used for serum C-reactive protein (CRP), which was not normally distributed. Categorical variables were analyzed by using chi-square test and Fisher's exact test. A *p* value less than 0.05 was considered statistically significant.

Results

Patient characteristics

Clinical features and laboratory parameters of MP pneumonia patients and healthy controls are detailed in Table 1. There were 45 patients with MP pneumonia at admission and 35 patients with MP pneumonia at remission. At admission, the absolute numbers of neutrophils and monocytes, as well as CRP, were significantly higher in MP pneumonia patients compared with healthy controls ($P < 0.01$), while there was no significant difference in the absolute numbers of white blood cell (WBC), lymphocytes, and platelet between admission and healthy controls ($P > 0.05$). At remission, the increased CRP observed in MP pneumonia patients were decreased significantly compared to the values at admission ($P < 0.01$); there was no marked decline in absolute numbers of neutrophils and monocytes at remission. In contrast, the absolute numbers of lymphocyte and platelet was elevated at remission compared to those at admission ($P < 0.01$).

Of the 45 enrolled patients (children) with MP pneumonia at admission, 14 cases were severe and 31 cases were non-severe. Of the four patients with extrapulmonary manifestations, one patient had central nervous system manifestations, two patients had some digestive system manifestations, and one patient had circulatory system manifestations. According to type of pneumonia, there were 28 bronchopneumonia, 15 lobar pneumonia, and 2 interstitial pneumonia. In addition, two cases were complicated with pleural effusion.

In our study, the severe group had a markedly longer duration of hospitalization than non-severe group (8.4 ± 1.2 days vs. 7.3 ± 1.6 days, respectively, $P = 0.018$). Furthermore, the lactate dehydrogenase (LDH) levels were significantly higher in the severe group than in the non-severe group (288.9 ± 84.5 IU/L vs. 246.9 ± 46.6 IU/L, respectively, $P = 0.037$). However, there were no significant differences in age, gender, white blood cell, neutrophils, lymphocytes, monocytes, platelet, CRP, and total immunoglobulin E (IgE) levels between the two groups (all $P > 0.05$; Table 2).

The monocyte subset changes of MP pneumonia patients

The populations of monocyte subsets as a percentage of total monocytes were calculated from the plot (Fig. 1). The relative proportions of the three monocyte subsets defined by CD14 and CD16 antibodies were significantly altered in MP pneumonia patients at admission such that the proportion of $CD14^{bright}CD16^{+}$ monocyte was significantly higher than that in healthy subjects (mean 8.4 ± 4.4% vs. 1.9 ± 0.5%, $P < 0.001$). In addition, the proportion of $CD14^{dim}CD16^{+}$ monocyte was also significantly higher in MP pneumonia patients compared with healthy subjects (mean 5.5 ± 2.7% vs. 4.1 ± 2.0%, $P < 0.05$). But the proportion of $CD14^{+}CD16^{-}$ monocyte did not differ between at admission and healthy control (mean 65.9 ± 9.2% vs. 65.3 ± 2.9%, $P > 0.05$) (Table 3, Fig. 2a, b).

The patients with extrapulmonary complications had relative lower proportions of $CD14^{bright}CD16^{+}$ than those without extrapulmonary complications (mean 2.7 ± 1.7% vs. 8.9 ± 4.2%, $P < 0.05$) (Table 4), whereas the proportions of $CD14^{+}CD16^{-}$ and $CD14^{dim}CD16^{+}$did not differ between two groups (all $P > 0.05$) (Table 4). Furthermore, three monocyte subsets such as $CD14^{+}CD16^{-}$, $CD14^{bright}CD16^{+}$, and $CD14^{dim}CD16^{+}$ did

Table 1 Study population

	HC $n = 20$	Admission $n = 45$	Remission $n = 35$
Age (years)	8.4 ± 3.6	6.9 ± 2.6	6.7 ± 2.7
Gender (M/F)	7/13	18/27	12/23
WBC (10^3/μL)	6.3 ± 1.4	7.4 ± 1.9	8.1 ± 3.0**
CRP (mg/L)	< 0.8	9.9 (4.3, 20.5)***	< 0.8▲
Neutrophils (10^3/μL)	3.3 ± 1.1	4.6 ± 1.5**	4.3 ± 2.5
Lymphocytes (10^3/μL)	2.4 ± 0.8	2.2 ± 0.9	3.1 ± 0.9*▲
Monocytes (10^3/μL)	0.3 ± 0.1	0.6 ± 0.2***	0.5 ± 0.2**
Platelet (10^3/μL)	303.4 ± 62.5	265.2 ± 74.6	378.8 ± 118.9**▲

Data is shown as mean ± standard deviation (SD) or a median (interquartile range [IQR]) or number (percentage)

WBC white blood cell, *CRP* C-reactive protein

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus healthy control (HC), ▲ $P < 0.01$, versus at admission

not differ between severe group and non-severe group at admission (all $P > 0.05$) (Table 5, Fig. 3a–c). In addition, $CD14^{bright}CD16^{+}$ subset was positively associated with LDH at admission ($r = 0.3193$, $P = 0.0325$) (Fig. 4a). No other significant associations were found between $CD14^{bright}CD16^{+}$ subset and WBC (Fig. 4b), $CD14^{bright}CD16^{+}$ subset and CRP (Fig. 4c).

Next, we analyzed the change of monocyte and its subsets between admission and remission. At remission, the relative proportions of the three monocyte subsets were also altered such that the proportion of $CD14^{bright}CD16^{+}$ subset had significantly decreased ($P < 0.01$) and that of $CD14^{dim}CD16^{+}$ subset had also significantly decreased ($P < 0.01$), while there was no significant difference in the proportion of $CD14^{+}CD16^{-}$ subset between remission and admission ($P > 0.05$) (Table 3, Fig. 2a, b).

The HLA-DR expression on monocyte subsets

HLA-DR expression suggests the antigen processing and presentation capability of the expanded subset. The intermediate subset is known to express higher levels of HLA-DR,

Table 2 Clinical characteristics in patients with severity and those without severity at admission

	Severity $n = 14$	Non-severity $n = 31$	*P* value
Age (years)	7.1 ± 2.3	6.8 ± 2.7	0.664
Gender (M/F)	5/9	13/18	0.753
Duration of hospitalization (days)	8.4 ± 1.2	7.3 ± 1.6	0.018
Type of pneumonia, *n* (%)			0.004[a]
Bronchopneumonia	4 (28.6)	24 (77.4)	
Lobar pneumonia	9 (64.3)	6 (19.4)	
Interstitial pneumonia	1 (7.1)	1 (3.2)	
Pleural effusion, *n* (%)	2 (14.3)	0 (0)	0.092[a]
Extrapulmonary complications, *n* (%)	4 (28.6)	0 (0)	0.007[a]
Laboratory			
WBC (10^3/μL)	6.7 ± 1.2	7.8 ± 2.1	0.088
Neutrophils(10^3/μL)	4.4 ± 1.4	4.7 ± 1.6	0.568
Lymphocytes(10^3/μL)	1.8 ± 0.9	2.4 ± 0.8	0.064
Monocytes(10^3/μL)	0.5 ± 0.3	0.6 ± 0.1	0.494
Platelet (10^3/μL)	250.8 ± 76.6	271.6 ± 74.1	0.392
LDH (IU/L)	288.9 ± 84.5	246.9 ± 46.6	0.037
CRP (mg/L))	7.4 (2.2–18.3	13.0 (5.0–21.1)	0.331
Total IgE (IU/mL)	68.1 (20.2–161.5)	53.2 (34.3–182.0)	0.873

Data is shown as mean ± standard deviation (SD) or a median (interquartile range [IQR]) or number (percentage)

WBC white blood cell, *LDH* lactate dehydrogenase, *CRP* C-reactive protein

[a] Fisher's exact test

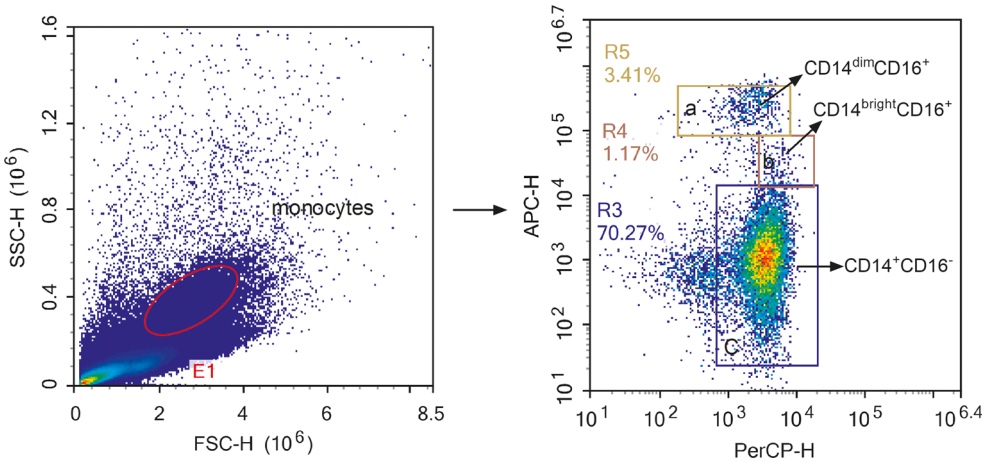

Fig. 1 The gating strategy in flow cytometry. (a) $CD14^{dim}CD16^{+}$ monocyte (non-classical). (b) $CD14^{bright}CD16^{+}$ monocyte (intermediate). (c) $CD14^{+}CD16^{-}$ monocyte (classical)

compared with either the classical or non-classical subset. Therefore, to confirm the distinct identity of this population, we examined HLA-DR expression on the monocyte subsets at admission, and as predicted, we found that HLA-DR expression on $CD14^{bright}CD16^{+}$ subset was significantly higher than that on $CD14^{dim}CD16^{+}$ subset (mean 60.6 ± 11.6% vs. 55.9 ± 14.6%, $P < 0.05$) and on $CD14^{+}CD16^{-}$ subset (mean 60.6 ± 11.6% vs. 7.2 ± 4.0%, $P < 0.001$) (Fig. 5).

In addition, the intermediate $CD14^{bright}CD16^{+}$ in MP pneumonia patients at admission was found to have significantly reduced expression of HLA-DR compared with those of the children with HC group ($P < 0.001$) (Fig. 6b), indicating the low antigen-presenting capability of this subset in MP pneumonia. The proportion of $CD14^{+}CD16^{-}$ monocytes and $CD14^{dim}CD16^{+}$ monocytes did not differ between at admission and healthy control ($P > 0.05$) (Fig. 6a, c). However, there were no significant differences in the expression of HLA-DR on any subset and each monocyte subset between the admission and remission (Fig. 6a–c). To further elucidate whether expression levels of HLA-DR on each monocyte subset correlate with the severity of MP pneumonia at admission, we then examined the expression of HLA-DR on each monocyte subset between severe case and non-severe cases. The proportion of HLA-DR on $CD14^{bright}CD16^{+}$ subset was significantly lower in severe patients than in non-severe patients (54.7 ± 13.6% vs. 63.3 ± 9.9%, $P < 0.05$) (Fig. 6e). However, there were no significant differences in the expression of HLA-DR on $CD14^{+}CD16^{-}$ subset and on $CD14^{dim}CD16^{+}$ between two subgroup (all $P > 0.05$; Fig. 6d, f).

Table 3 Monocyte subsets in the study

	HC $n = 20$	Admission $n = 45$	Remission $n = 35$
$CD14^{+}CD16^{-}$ (%)	65.3 ± 2.9	65.9 ± 9.2	65.1 ± 8.6
$CD14^{bright}CD16^{+}$ (%)	1.9 ± 0.5	8.4 ± 4.4***	2.3 ± 1.6▲
$CD14^{dim}CD16^{+}$ (%)	4.1 ± 2.0	5.5 ± 2.7*	3.3 ± 1.6***▲

Data is shown as mean ± standard deviation (SD)

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus healthy control (HC), ▲ $P < 0.01$, versus at admission

Discussion

MP pneumonia is considered to be in part attributed to immune-mediated responses [3]. Monocytes and its subsets appear to be important element in the immunopathogenesis of autoimmune disorders [17, 18]. However, little is known about how the three monocyte subsets, $CD14^{+}CD16^{-}$, $CD14^{bright}CD16^{+}$, and $CD14^{dim}CD16^{+}$, are involved in MP pneumonia.

Previous studies focused mainly on two monocyte subpopulations divided solely on the basis of the presence or absence of CD16. However, distinguishing $CD14^{bright}CD16^{+}$ from $CD14^{dim}CD16^{+}$ among CD16+ monocytes is justified not only from phenotypic but also from functional point of view. It has been demonstrated that $CD14^{bright}CD16^{+}$ monocytes were potent producers of many pro-inflammatory cytokines such as tumor necrosis factor (TNF) and IL-1, while $CD14^{dim}CD16^{+}$ monocytes were shown to have high migratory but only limited phagocytic potential [19].

It has been known that intermediate or non-classical monocytes or both jointly produce the largest quantities of the pro-inflammatory cytokines [20, 21]. In our report, three monocyte subsets separated based on staining with anti-CD14 and anti-CD16 antibodies have been analyzed. We have shown for the first time that a significant expansion in the proportion of the intermediate and non-classical monocytes during the active phase of MP pneumonia compared with healthy subjects. This is also consistent with data showing that the $CD16^{+}$ subset of monocytes is expanded in some infection and autoimmune diseases and may be involved in the induction of the inflammatory immune response [22–24].

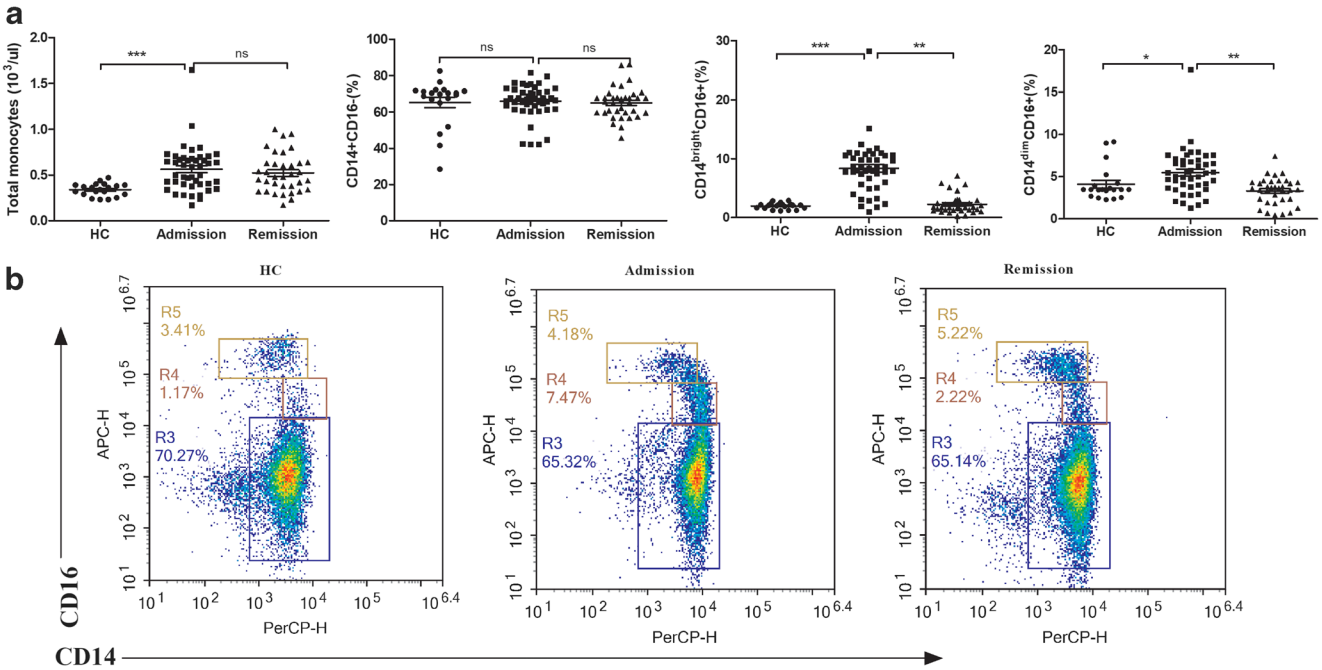

Fig. 2 Comparison of the proportion of monocytes and its subsets in patients with MP pneumonia at admission and remission versus healthy controls (HC). **a** The proportion of total monocytes in PBMC and three subsets in monocytes from HC, admission, and remission. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). **b** Flow cytometry of monocyte subsets sorted by flow cytometry from HC, admission, and remission

In our studies, we evaluated the expression of monocyte subsets in patients with MP pneumonia between severe and non-severe cases. We found that $CD14^{+}CD16^{-}$, $CD14^{bright}CD16^{+}$, and $CD14^{dim}CD16^{+}$ did not differ between severe group and non-severe group at admission. These results suggested that the subsets of monocyte may be unconcerned with severity. The patients with extrapulmonary complications had relative lower proportions of $CD14^{bright}CD16^{+}$, which indicated that intermediate subset might be involved in the pathogenesis of extrapulmonary complications. However, there were only 4 patients with extrapulmonary complications. Thus, further studies are needed in a larger sample.

Interestingly, we found that $CD14^{bright}CD16^{+}$ subset was positively associated with LDH. Furthermore, serum LDH can be used as a biomarker to predict refractory MP pneumonia at the early stage of hospitalization [25]. These results suggest that expanded intermediate monocytes might play a detrimental role for children with MP pneumonia. However, we were not able to demonstrate any correlation between $CD14^{bright}CD16^{+}$ cells and WBC, $CD14^{bright}CD16^{+}$ cells, and CRP.

Monocytes perform phagocytosis and antigen presentation functions. A series of studies showed that the antigen presentation ability is tightly associated with HLA-DR expression, suggesting an important role of HLA-DR and in the antigen presentation process [12]. It has been documented that intermediate monocytes exhibit enriched expression in antigen-presenting-related factors such as major histocompatibility complex class II (MHC-II) subunits, and human leukocyte antigen (HLA)-DO, as well as CD40 [26, 27]. We also demonstrated that $CD14^{bright}CD16^{+}$ subset in MP pneumonia patients at admission showed higher levels of HLA-DR than the other monocyte subsets. The increased surface expression of HLA-DR on $CD14^{bright}CD16^{+}$ subset is further indication for their involvement in inflammatory immune responses.

Table 4 Monocyte subsets in patients with extrapulmonary and those with pulmonary

	Extrapulmonary $n = 4$	Pulmonary $n = 41$	*P* value
$CD14^{+}CD16^{-}$ (%)	4.1 ± 2.4	5.5 ± 2.7	0.301
$CD14^{bright}CD16^{+}$ (%)	2.7 ± 1.7	8.9 ± 4.2	0.006
$CD14^{dim}CD16^{+}$ (%)	66.9 ± 16.7	65.9 ± 8.5	0.821

Data is shown as mean ± standard deviation (SD)

Table 5 Monocyte subsets in patients with severity and those without severity at admission

	Severity $n = 14$	Non-severity $n = 31$	*P* value
$CD14^{+}CD16^{-}$ (%)	67.9±9.3	65.1 ± 9.2	0.346
$CD14^{bright}CD16^{+}$ (%)	7.1 ± 4.1	8.9 ± 4.5	0.191
$CD14^{dim}CD16^{+}$ (%)	5.2 ± 1.9	5.5 ± 3.0	0.700

Data is shown as mean ± standard deviation (SD)

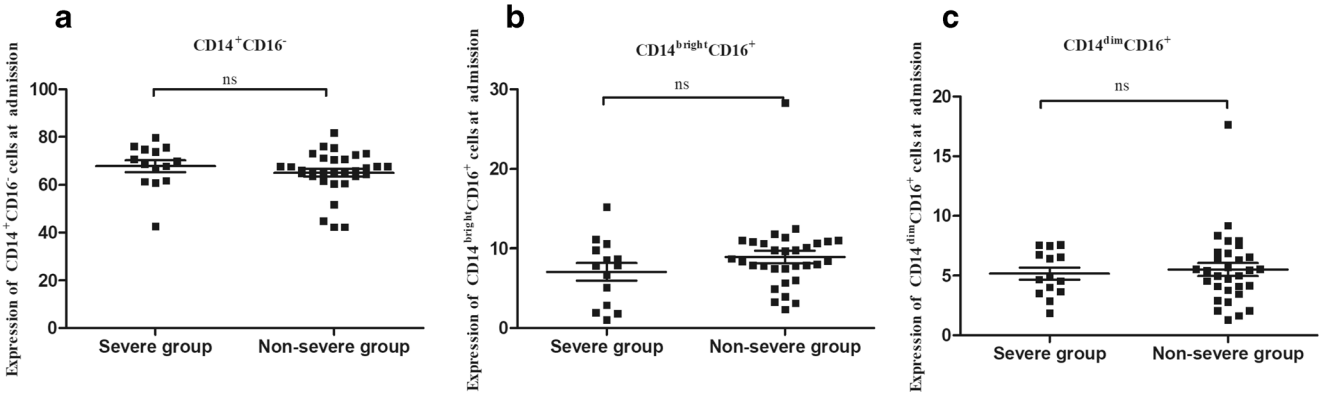

Fig. 3 **a** Comparison of $CD14^{+}CD16^{-}$ cell expression at admission in patients with MP pneumonia between severe group and non-severe group. **b** Comparison of $CD14^{bright}CD16^{+}$ cell expression at admission in patients with MP pneumonia between severe group and non-severe group. **c** Comparison of $CD14^{dim}CD16^{+}$ cell expression at admission in patients with MP pneumonia between severe group and non-severe group

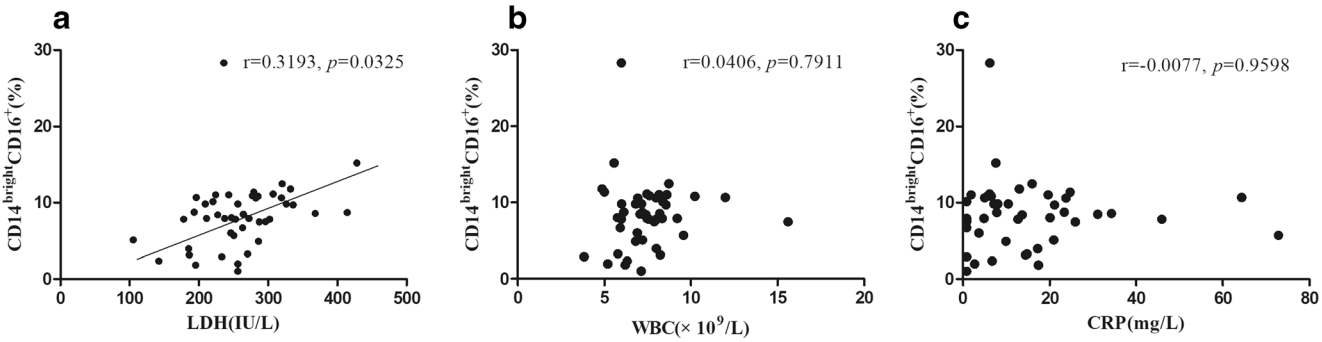

Fig. 4 Correlations between $CD14^{bright}CD16^{+}$ monocyte (intermediate) and **a** lactate dehydrogenase (LDH) ($r = 0.3193$, $P < 0.05$), **b** white blood cell (WBC) ($r = 0.0406$, $P > 0.05$), and **c** C-reactive protein (CRP) ($r = 0.0077$, $P > 0.05$). Spearman's correlations are shown. Graph showing a positive correlation between $CD14^{bright}CD16^{+}$ monocyte and LDH in patients' blood samples at admission

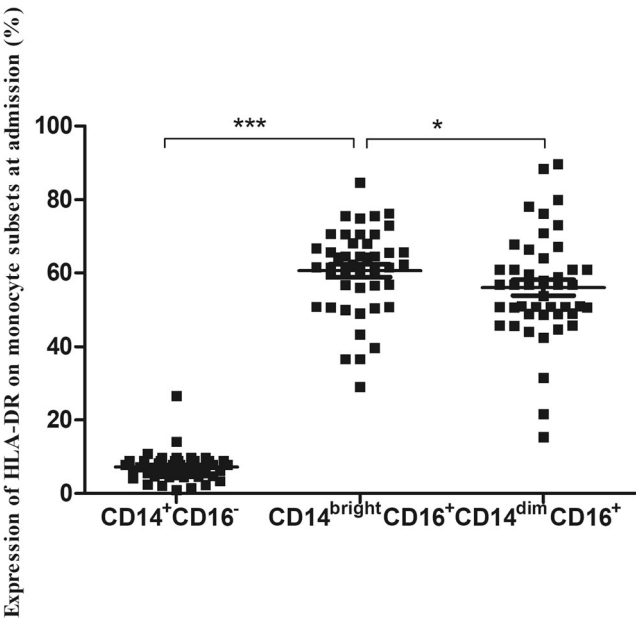

Fig. 5 Comparison of human leukocyte antigen-DR (HLA-DR) expression on monocyte subsets at admission in patients with MP pneumonia (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

Previous studies showed that low levels of expression of HLA-DR molecules on monocytes are associated with reduced activation and loss of antigen presentation capacity [28, 29]. In this study, we found that the percentage of HLA-DR on $CD14^{bright}CD16^{+}$ subset was significantly lower in MP pneumonia patients at admission than in healthy subjects. These data strongly suggested that inhibition of monocyte activation is a prominent feature of MP pneumonia. Of note, among MP pneumonia patients, there was a significant reduction in $CD14^{bright}CD16^{+}$ HLA-DR expression in severe cases. It was consistent with the expression of HLA-DR on monocytes/macrophages has been considered to be an indicator for predicting the occurrence of infections and to be related with worse clinical outcomes [30, 31]. Therefore, it is likely that the expression of HLA-DR on $CD14^{bright}CD16^{+}$subset, at least in part, contribute to the immunoparalysis or immune-tolerant status of MP pneumonia patients and may be an potential biomarker for classifying severe and mild cases.

Few limitations of this study should be noted. First, the number of patients with MP pneumonia and healthy controls was limited; however, the study was sufficiently powered. Furthermore, a long-term follow-up study is needed to assess

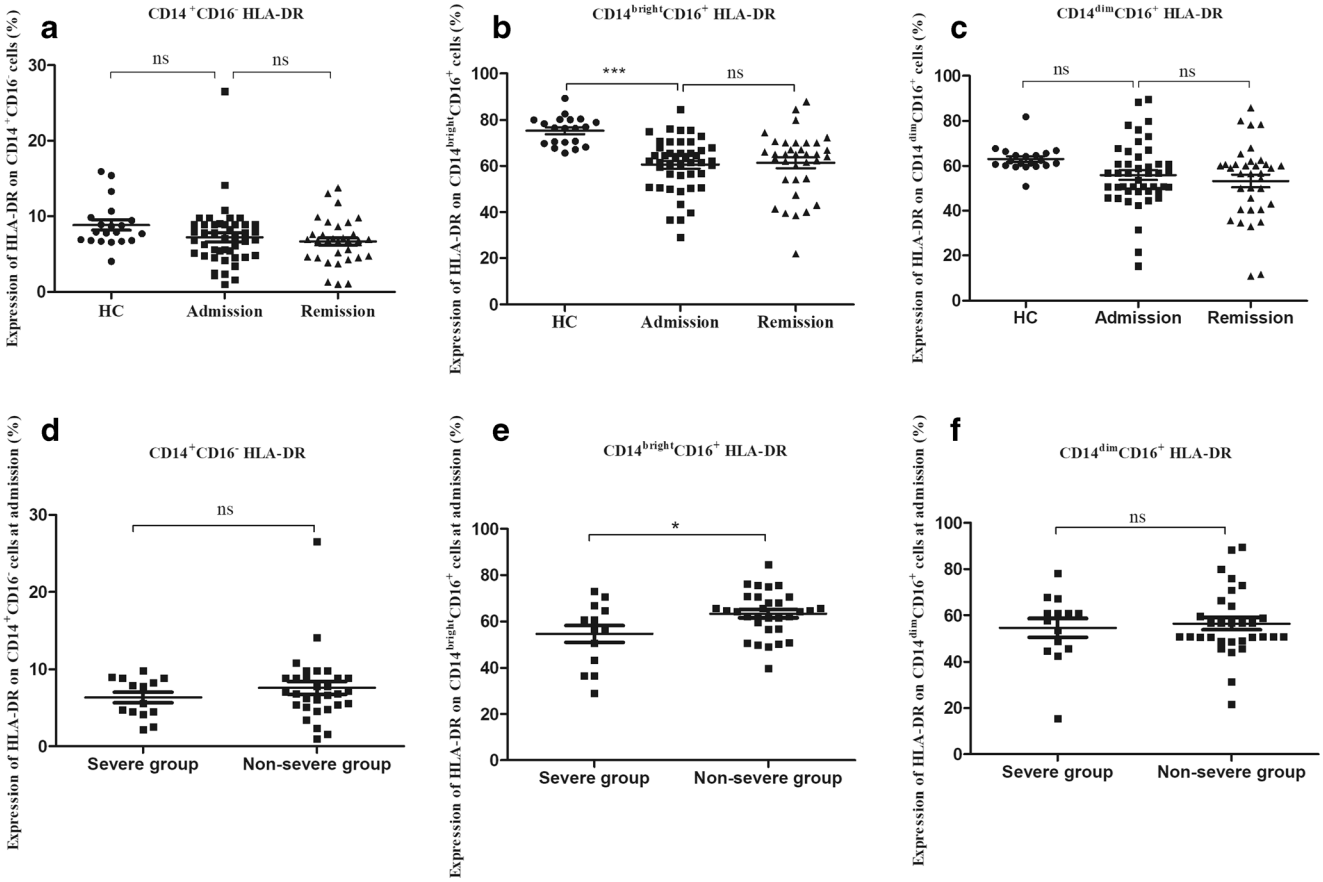

Fig. 6 **a** Comparison of HLA-DR expression on $CD14^{+}CD16^{-}$ cells at HC, admission, and remission. **b** Comparison of HLA-DR expression on $CD14^{bright}CD16^{+}$ cells at HC, admission, and remission. **c** Comparison of HLA-DR expression on $CD14^{dim}CD16^{+}$ cells at HC, admission, and remission. **d** Comparison of HLA-DR expression on $CD14^{+}CD16^{-}$ cells at admission between severe group and non-severe group. **e** Comparison of HLA-DR expression on $CD14^{bright}CD16^{+}$ cells at admission between severe group and non-severe group. **f** Comparison of HLA-DR expression on $CD14^{dim}CD16^{+}$ cells at admission between severe group and non-severe group (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

a potential prognostic role of the current findings. Finally, we cannot compare pneumonia patients with different etiology; therefore, these findings cannot prove pathogen specificity. However, to our knowledge, this is the first study investigating the change of monocyte subsets and its HLA-DR expression in children with MP pneumonia. Additionally, we provided evidence supporting that the decreased expression of HLA-DR on $CD14^{bright}CD16^{+}$ subset may related to the severity of MP pneumonia and discuss the association of monocyte subsets with severity and extrapulmonary complications.

In conclusion, our study results suggest that intermediate ($CD14^{bright}CD16^{+}$) and non-classical ($CD14^{dim}CD16^{+}$) monocyte subsets increase in patients with MP pneumonia, indicating the involvement of monocyte-related mechanisms in the pathogenesis of this disease. In addition, the expression of HLA-DR on $CD14^{bright}CD16^{+}$ subset was significantly lower in severe patients; they are likely to be implicated in immunoparalysis or immune-tolerant status, with deleterious consequences for the development of MP pneumonia. Thus, the decreased expression of HLA-DR on $CD14^{bright}CD16^{+}$ subset may be a potential indicator of the severity of MP pneumonia. Further characterization of the function of the intermediate monocytes could pave the road for improving understanding of the exact immunologic mechanisms leading to both severe respiratory and extrapulmonary MP diseases.

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Author contributions Zhihua Wang devised the experimental design, involved in the acquisition and analysis of clinic data, and wrote the paper. Lei Yang devised the experimental design and performed the flow cytometry analysis. Jing Ye, Yushui Wang and Yan Liu were responsible for the enrolment of patients with MP pneumonia and healthy control subjects.

Availability of data and material Please contact author for data requests.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement The study was approved by the Tianjin Nankai Hospital Ethics and Medical Research Committee. Our manuscript complies with the ethical standards required by this journal.

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