Journal of Microbiology, Immunology and Infection 55 (2022) 413—420

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Original Article

A potent antibody-secreting B cell response to *Mycoplasma pneumoniae* in children with pneumonia

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Received 17 March 2021; received in revised form 9 July 2021; accepted 16 August 2021
Available online 1 September 2021

KEYWORDS
Mycoplasma pneumoniae;
Pneumonia;
Children;
Antibody-secreting B cell

Abstract *Background:* *Mycoplasma pneumoniae* is a major pathogen for community-acquired pneumonia and frequently causes outbreaks in children. *M. pneumoniae*-specific antibody response is detected upon acute infection and the serology is widely used in the clinical setting. Nevertheless, the cellular basis for antigen-specific antibody response to acute *M. pneumoniae* infection is largely undetermined in children.
Methods: Hospitalized children with community-acquired pneumonia were enrolled and the infection with *M. pneumoniae* was confirmed with positive PCR result and negative findings for other pathogens. The *M. pneumoniae* P1-specific antibody-secreting B cell (ASC) response was examined with the *ex vivo* enzyme-linked immunosorbent spot assay and the relationships between the ASC frequency and serological level and clinical parameters within *M. pneumoniae* patients were studied.
Results: A robust *M. pneumoniae* P1-specific ASC response was detected in the peripheral blood among *M. pneumoniae*-positive patients. By contrast, no *M. pneumoniae*-specific ASCs were detected among *M. pneumoniae*-negative patients. The IgM-secreting B cells are the predominant class and account for over 60% of total circulating *M. pneumoniae*-specific ASCs in the acute phase of illness. The *M. pneumoniae* P1-specific ASC frequency significantly correlated with the fever duration, and the IgG ASC frequency significantly correlated with serological titer among patients.

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https://doi.org/10.1016/j.jmii.2021.08.010
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Conclusion: A rapid and potent elicitation of peripheral *M. pneumoniae*-specific ASC response to acute infection provides the cellular basis of antigen-specific humoral response and indicates the potential of cell-based diagnostic tool for acute *M. pneumoniae* infection. Our findings warrant further investigations into functional and molecular aspects of antibody immunity to *M. pneumoniae*.
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Introduction

Mycoplasma pneumoniae is a bacterial pathogen that causes upper and lower respiratory tract infections in humans and accounts for 40% of community-acquired pneumonia (CAP) cases in children during the outbreak.[1] *M. pneumoniae* also causes extrapulmonary manifestations and severe infections complicated with acute respiratory failure, encephalitis or mortality could occur.[2–4] Co-infection of *M. pneumoniae* with other pathogens has been found in 20–60% of pediatric pneumonia cases.[1,5] Recently, it is reported that patients with SARS-CoV-2 and *M. pneumoniae* co-infection are associated with higher mortality.[6] The macrolide antibiotics are the primary treatment for acute *M. pneumoniae* infection in the clinical setting; however, the macrolide-resistant clone has emerged and caused large outbreaks in several endemic regions.[7] There is no approved vaccine available for the control of *M. pneumoniae* infection in the present. The understanding of human immune response to *M. pneumoniae* would be underpinning the development of new tools to diagnose, prevent and treat the related infections.

Antibody response to *M. pneumoniae* antigen is commonly detected in the post-infection sera in humans.[8,9] The adhesion apparatus, including the P1, P30, and P116 proteins, on the bacterial surface has been shown to elicit a dominant immune response in animals.[10] The adhesion proteins play a role in the attachment of *M. pneumoniae* to the host cell and subsequent bacterial colonization or infection and the P1 protein is one of most characterized adhesions and an immunodominant antigen that elicits specific antibodies.[11,12] Evidence shows that anti-P1 antibodies could inhibit the motility of *M. pneumoniae* and a strong and early production of antibodies to the P1 protein is found in sera of infected individuals.[10,13,14] Nevertheless, the cellular basis for the *M. pneumoniae* P1-reactive antibody response remains largely unclear in humans.

Antibody-secreting B cells (ASCs), also known as plasmablasts, are the precursor of long-lived plasma B cells. While the ASCs transiently circulate in the peripheral blood upon antigen exposure, plasma B cells reside in the survival niche in the local tissue and bone marrows.[15–17] ASCs, plasma B cells and memory B cells contribute to antibody-mediated immune response in humans.[15] We have previously shown that antigen-specific ASC response could be elicited rapidly after exposure to the pathogen in humans.[16,17] The information regarding *M. pneumoniae* P1-specific ASC response to acute infection is limited.[15] Here, we investigate the magnitude of the ASC response to the *M. pneumoniae* P1 antigen, the isotype usage of antigen-specific ASCs, and the relationship between the ASC response and serological level and clinical parameters in children with *M. pneumoniae* pneumonia.

Methods

Ethic statement

This study was approved by the Research and Ethics Committee of Chang Gung Memorial Hospital. The study was in compliance with both Good Clinical Practice guidelines and the Declaration of Helsinki. Informed consent was obtained from all subjects.

Patient enrollment

Pediatric patients that were admitted with the diagnosis of CAP were enrolled in Chang Gung Memorial Hospital, Taiwan, between 2014 and 2016. The CAP was defined as infection of the pulmonary parenchyma acquired in the community other than hospital or healthcare facility setting. The operational definition includes clinical symptoms of lower respiratory tract infections, fever or other systemic signs of illness, new abnormal pulmonary auscultation or percussion, or new abnormal image finding suggestive of pulmonary infection. Throat swabs were collected for respiratory pathogen detection. The serum samples and whole blood samples were collected for peripheral blood mononuclear cells (PBMCs) within the first two weeks of illness.

PBMCs separation

The heparinized human blood samples were layered on top of lymphoprep (Axis Shield, United Kingdom) in LeucoSep tubes (Greiner Bio-One, Austria). The PBMC layer was separated from whole blood by density gradient centrifugation for 20 min, 800 g at 20 °C. The PBMCs were transferred to a separate tube and diluted to 50 μL of RPMI medium 1640 (Gibco, United States). The cells were pelleted by centrifugation at 720*g* for 10 min. The supernatant was discarded, and the pellet was washed again and resuspended in RPMI medium 1640 supplemented with 2% fetal calf serum (FCS) at 400 g for 10 min. Finally, the cells were resuspended in RPMI medium 1640 supplemented with 10% FCS, 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin.

M. pneumoniae PCR

Throat sample was collected with a sterile swab (FLOQSwabs, Copan, Murrieta, CA, USA). A commercial kit (DNeasy Kit; QIAGEN, Hilden, Germany) was used to extract the DNA from the sample according to the manufacturer's instructions. Real-time PCR with two primers detecting the P1 gene encoding the P1 protein (MP_TM1 5′-CCAACCAAACAACAACGTTCA -3′ and MP_TM2 5′-ACCTTGACTGGAGGCCGTTA -3′) and probe (VIC-ATCCGAATAACGGTGACTT-MGB) were used.[18] The real-time PCR was performed to detect *M. pneumoniae* in the throat sample.

FilmArray

The FilmArray Respiratory Panel (Idaho Technology, Inc., Salt Lake City, UT) was used for detecting respiratory pathogens, including Adenovirus, Coronavirus 229E, Coronavirus HKU1, Coronavirus NL63, Coronavirus OC43, Human Metapneumovirus, Influenza A, Influenza A subtype H1, Influenza A subtype H3, Influenza A subtype H1 2009, Influenza B, Parainfluenza virus 1, Parainfluenza virus 2, Parainfluenza virus 3, Parainfluenza virus 4, Rhinovirus/Enterovirus, Respiratory Syncytial virus, *Bordetella pertussis, Chlamydophila pneumoniae*, and *M. pneumoniae*.

M. pneumoniae serology

The *M. pneumoniae*-specific IgG and IgM level was examined using a qualitative enzyme immunoassay by ImmunoWELL Mycoplasma Antibody Test (GenBio, San Diego, California, USA). In the test, reaction wells were coated with *M. pneumoniae*, strain FH (ATCC#15531).

Ex Vivo enzyme-linked immunosorbent spot assay (ELISpot)

The ELISpot plates (PVDF membrane, 96-well Multiscreen Filter plate, Millipore, United States) were coated with purified *M. pneumoniae* P1 protein (P1 type 2, FH strain, manufactured by SERION Immunologics, Germany) for detection of *M. pneumoniae* P1-specific ASCs, or were coated with anti-human Ig (Invitrogen, United States) for detection of total ASCs. The wells coated with PBS or purified influenza hemagglutinin proteins were controls.

Prior to adding the PBMC suspension, plates were washed with PBS for three times and blocked using RPMI medium containing 10% fetal bovine serum for an hour at 37 °C. After washing, plates were incubated with the PBMC suspension for 18—24 h at 37 °C, 5% CO2. For the detection of *M. pneumoniae* P1-specific ASCs, 200,000 PBMCs were added into the well. For the detection of total ASCs, 10,000 PBMCs were added into the well, since the incubation with a higher number of PBMCs may lead to high background in the assay. After washing, plates were incubated with alkaline phosphatase conjugates anti-human IgG, IgM or IgA (Calbiochem, United States) for 2 h at room temperature. After washing, plates were developed with alkaline phosphatase substrate kit for 2—5 min at room temperature. Spot-forming cells were measured and counted with automatic ELISpot reader.

Statistical analysis

The statistical analysis was performed by GraphPad Prism. The Mann—Whitney U test was used to compare the difference between two independent groups. The Chi-square test was used to analyze the relationship between categorical variables. Linear regression was used to evaluate the correlation between variables. A p value of <0.05 was considered statistically significant.

Results

Pediatric patients with *M. pneumoniae* community-acquired pneumonia

In the study, a total of 26 patients with CAP were enrolled and the mean age was 6.6 ± 3.0 years (1.5—13.9 years) (Table 1). Fifteen of them were infected with *M. pneumoniae* based on the laboratory-confirmed positive PCR result of throat sample. None of *M. pneumoniae*-positive CAP patients was positive with urine pneumococcal antigen, positive with other pathogens in the PCR test, or had positive viral culture. Other 11 patients were negative with *M. pneumoniae* PCR, among them eight were positive with pneumococcal antigen and one was positive with influenza A virus by throat viral culture.

Table 1 shows demographic and clinical characteristics for *M. pneumoniae*-positive and -negative CAP patients. There were no significant differences in the age, sex, fever duration and the presence of chest X-ray consolidation between the *M. pneumoniae*-positive and -negative group. A higher percentage of patients had pleural effusion in the *M. pneumoniae*-negative group than the *M. pneumoniae*-positive group ($p = 0.02$, Chi-square test). The *M. pneumoniae*-negative group had higher white blood cell counts ($p = 0.03$, Mann—Whitney U test) and C-reactive protein level ($p = 0.04$, Mann—Whitney U test), but had a lower *M. pneumoniae*-specific serological IgG ($p = 0.01$, Mann—Whitney U test) and IgM ($p < 0.001$, Mann—Whitney U test) titer than the *M. pneumoniae*-positive group.

Detection of *M. pneumoniae*-specific antibody-secreting B cell response

The frequencies of total and *M. pneumoniae* P1-specific ASC response in the peripheral blood were measured by the *ex vivo* ELISpot (Fig. 1a). The sampling day was 8.2 ± 1.8 days (range 5—13 days) after the onset of symptoms. The infection-induced total ASC response was detected in both *M. pneumoniae*-positive (13,013 ± 6560 per million PBMCs) and -negative (7573 ± 7708 per million PBMCs) CAP groups. A robust *M. pneumoniae*-specific ASC response (816 ± 353 per million PBMCs) was detected in the *M. pneumoniae*-positive group. By contrast, there were no by-stander ASC response to *M. pneumoniae* among *M. pneumoniae*-negative patients (Figs. 1b and 2a).

The *M. pneumoniae* P1-specific ASC response was detected in the peripheral blood as early as day 5 after symptom onset. There was no significant difference in the frequencies of *M. pneumoniae*-specific ASC response

Table 1 Demographic and clinical characteristics of *M. pneumoniae*-positive and -negative pediatric patients with community-acquired pneumonia.

Characteristics[a]	*M. pneumoniae*-positive (n = 15)	*M. pneumoniae*-negative (n = 11)	P value[b]
Age			
Years	7.1 ± 2.6	5.9 ± 3.6	0.41
Sex			
Male (%)	7 (46.7)	6 (54.5)	0.69
Symptoms and signs			
Fever (%)	15 (100.0)	11 (100.0)	–
Fever durations, days	7.1 ± 2.0	11.2 ± 6.1	0.09
Cough (%)	15 (100.0)	11 (100.0)	–
Rhinorrhea (%)	5 (33.3)	5 (45.5)	0.53
Gastrointestinal symptoms (%)	2 (13.3)	6 (54.5)	0.02
Sore throat (%)	3 (20.0)	1 (9.0)	0.47
Skin rash (%)	3 (20.0)	0 (0)	0.11
Chest X ray			
Consolidation (%)	13 (86.7)	8 (72.7)	0.37
Pleural Effusion (%)	1 (6.7)	5 (45.5)	0.02
Laboratory data			
WBC, per μL	7429 ± 2238	14,591 ± 8730	0.03
CRP, mg/L	28.9 ± 27.6	153.1 ± 129.7	0.04
M. pneumoniae-specific IgG, U/mL[c]	545.0 ± 549.8	302.5 ± 495.1	0.01
M. pneumoniae-specific IgM, U/mL[c]	2954.0 ± 1942.0	345.3 ± 325.4	<0.001

[a] The data was presented as mean ± standard deviation.

[b] The Mann–Whitney U test was used to compare the difference between two independent groups. The Chi-square test was used to analyze the relationship between categorical variables.

[c] Serology data were available in 13 of 15 *M. pneumoniae*-positive patients and in 10 of 11 *M. pneumoniae*-negative patients. In the *M. pneumoniae*-positive group, six were positive and five were equivocal for *M. pneumonia*-specific IgG serological response and 11 were positive for *M. pneumonia*-specific IgM serological response. In the *M. pneumoniae*-negative group, two were positive and three were equivocal for *M. pneumonia*-specific IgG serological response and two were positive for *M. pneumonia*-specific IgM serological response.

between day 5–7 and day 8–11 after onset (808 ± 419 v.s. 829 ± 320 per million PBMCs, p = 0.98, Mann–Whitney U test) (Fig. 2b).

In the study, *M. pneumoniae* P1-specific IgM-secreting ASC response (502 ± 258 per million PBMCs) was found predominantly in the peripheral blood among *M. pneumoniae*-positive CAP patients, followed by the IgA-secreting (165 ± 146 per million PBMCs) and IgG-secreting ASC response (149 ± 103 per million PBMCs) (p < 0.0001, ANOVA; post-hoc analysis, IgM-secreting v.s. IgG-secreting, p < 0.0001; IgM-secreting v.s. IgA-secreting, p < 0.0001) (Fig. 2c). The *M. pneumoniae* P1-specific IgM-secreting ASCs accounted for nearly 62% of total circulating *M. pneumoniae*-specific ASCs among patients. The *M. pneumoniae* P1-specific IgG- and IgA-secreting ASC response could be barely detectable at the first week but gradually develops at the second week of onset in some cases (Fig. 1b). A higher frequency of *M. pneumoniae* P1-specific IgG- and IgM-secreting ASC response is found at day 8–11 than day 5–7 after onset, but the difference did not reach statistically significant (Figs. 1b and 2b).

The relationships among *M. pneumoniae*-specific antibody-secreting B cell response, serological titer and clinical parameter

The serological level to *M. pneumoniae* antigen was examined in the binding assay. There was a positive trend between the frequency of *M. pneumoniae* P1-specific IgM-secreting ASC response and *M. pneumoniae*-specific IgM serological level, but it did not reach statistical significance. A significant correlation between the frequency of *M. pneumoniae* P1-specific IgG-secreting ASC response and *M. pneumoniae*-specific IgG serological level was noted among patients (Fig. 2d).

The frequency of total *M. pneumoniae* P1-specific ASC response significantly correlated with the fever duration (r = 0.51, p = 0.05, linear regression) (Fig. 2e). However, no significant correlation is found between the *M. pneumoniae*-specific ASC response with the bacterial load measured by the PCR test.

Discussion

M. pneumoniae is one of the most common pathogens for CAP worldwide.[1] In the United States, nearly eight percent of pediatric hospitalized CAP cases is caused by *M. pneumoniae*.[19] In Taiwan, *M. pneumoniae* contributes to 40–50% of CAP in children older than 5 years.[20,21] Recently, *M. pneumoniae* causes outbreaks in the community and the emergence and spread of macrolide-resistant clones are associated with complicated pneumonia cases with protracted clinical course.[7] The pathogenesis of acute *M. pneumoniae* infection is complex and the adherence of *M. pneumoniae* to host cells would initiate the following propagation and pathogenesis of bacteria, for which the P1 protein on the bacterial surface serves a key ligand for adhesion.[22] The context of protective immunity against acute *M. pneumoniae* infection remains largely unknown in humans. Increasing evidence shows that P1-specific antisera and monoclonal antibodies could substantially reduce or inhibit the adhesion of bacteria to human respiratory epithelial cells *in vitro*.[14,23,24] In humans, anti-P1 IgG and IgM antibodies are present in the post-infection sera and the detection of such antibodies indicates previous exposure to *M. pneumoniae*.[10,25,26] In the study, we delineate the specificity and magnitude of *M. pneumoniae* P1-specific ASC response upon acute *M. pneumoniae* infection among children with pneumonia, which provides a cellular basis for humoral immune response to this pathogen.

Antibody-secreting B cells are the primary source of antigen-specific antibodies and there are two main phases

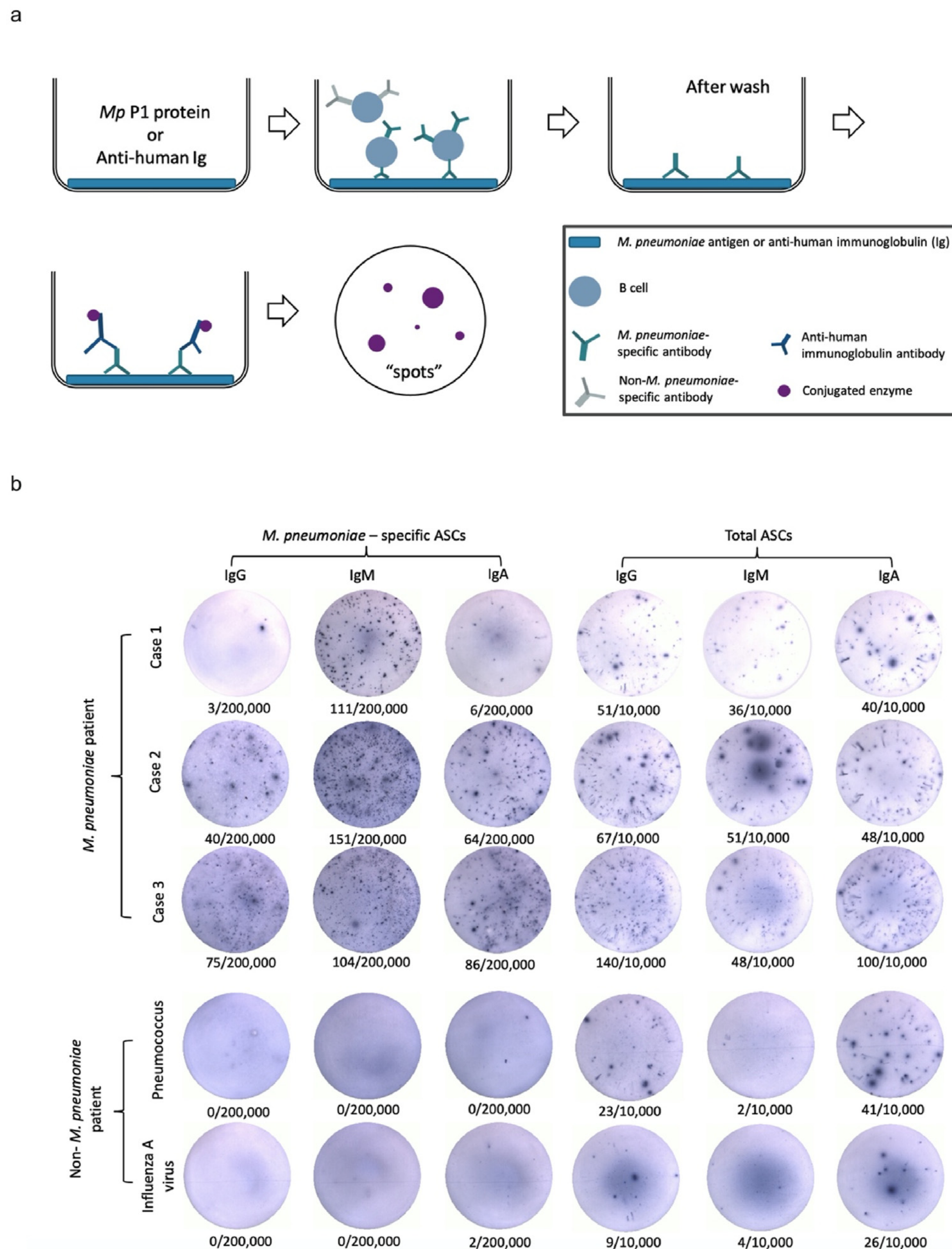

Figure 1. Detection of *M. pneumoniae* P1-specific antibody-secreting B cell (ASC) responses in children with pneumonia. (a) The *ex vivo* enzyme-linked immunospot assay was used to examine *M. pneumoniae* P1-specific and total ASCs in the peripheral blood. **(b)** Graph showing *M. pneumoniae*-specific ASCs from three *M. pneumoniae*-infected children, one pneumococcus-infected child, and one influenza A virus-infected child. The ratio of *M. pneumoniae* P1-specific or total ASCs to incubated peripheral blood mononuclear cells per well was shown for each image. For the detection of *M. pneumoniae* P1-specific, 200,000 PBMCs were added into the well. For the detection of total ASCs, 10,000 PBMCs were added into the well. The *M. pneumoniae* P1-specific ASC response was detected in three *M. pneumoniae*-infected patients. By contrast, no *M. pneumoniae* P1-specific ASCs were detected for both pneumococcus- and influenza A virus-infected patients. IgM-/IgG-secreting *M. pneumoniae*-specific ASCs accounted for 93/3, 59/16 and 39/28% of total *M. pneumoniae*-specific ASCs in patients 1 (day 8 after onset), patient 2 (day 9 after onset) and patient 3 (day 11 after onset), respectively.

of the ASC response upon antigen exposure. Antigen-specific ASCs are usually detected in the peripheral blood and local lymph nodes one week after infection, and then decline within weeks, followed by a translocation of ASCs and their survival in the niche of local tissue and bone marrow.[27] Previously, we reported that antigen-specific ASC response develops and becomes detectable in the peripheral blood within the first week of acute viral illness in children.[16] In the bacterial infection, children are also able to develop a potent ASC response to specific antigen at the end of first week of onset.[28,29] In the study, we detected the *M. pneumoniae* P1-specific ASC response in the peripheral blood and

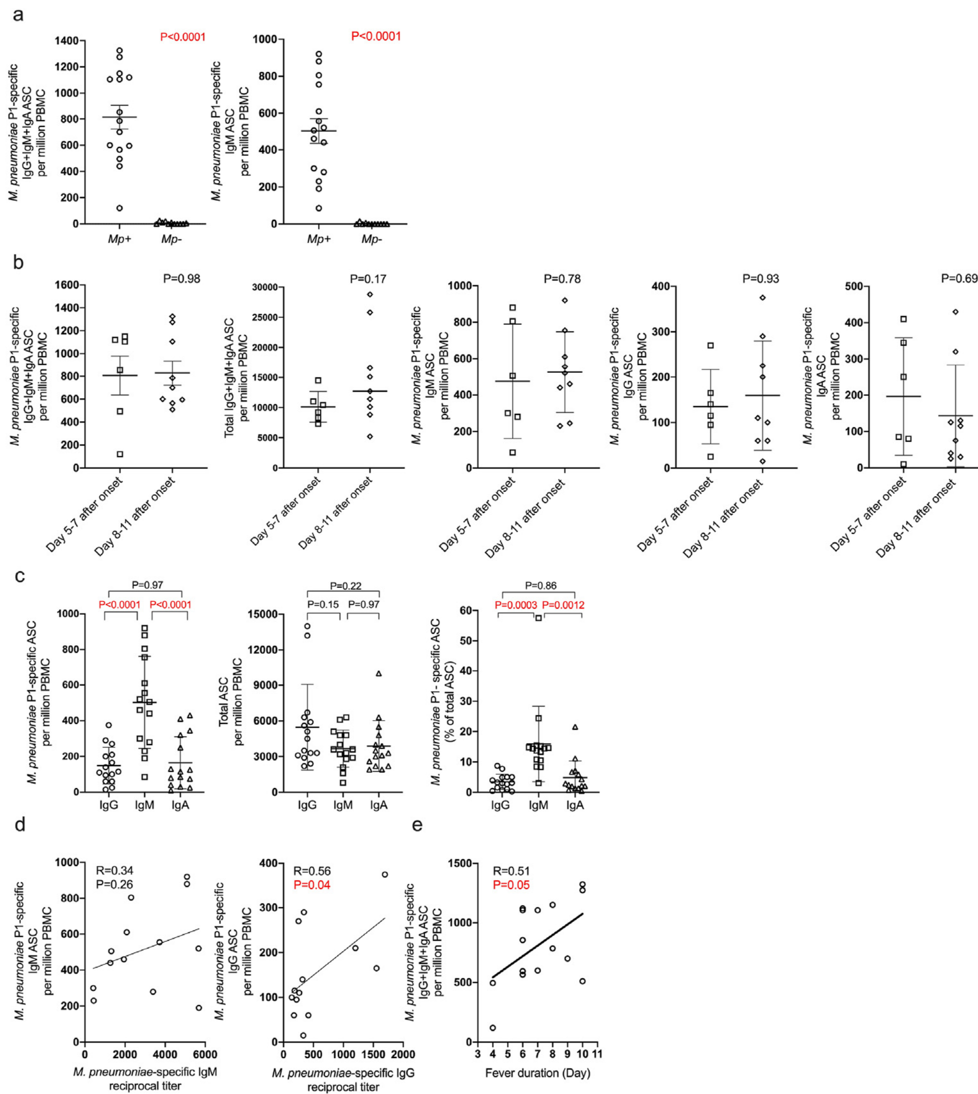

Figure 2. Analysis of *M. pneumoniae* P1-specific antibody-secreting B cell (ASC) responses in children with pneumonia. (a) The frequencies of ASC response in the *M. pneumoniae*-positive and -negative group. **(b)** The frequencies of ASC response among *M. pneumoniae*-positive patients of different onset days. **(c)** The isotype usage of ASC response among *M. pneumoniae*-positive patients. **(d)** The relationship between the ASC response and serological level. **(e)** The relationship between the ASC response and fever duration. The line represents the mean ± standard error. The Mann–Whitney U test was used to analyze the difference between two groups. Linear regression was used to analyze the correlation between variables. *Mp+*, *M. pneumoniae*-positive; *Mp-*, *M. pneumoniae*-negative.

its magnitude is comparable to the ASC response to other viral or bacterial pathogens, which comprises nearly 0.15% of circulating lymphocytes in children.[15,16]

We noted that a dominant IgM ASC response is produced quickly after acute *M. pneumoniae* infection in children with pneumonia. Similar observation was noted in the previous study.[30,31] A dominant IgM ASC response was also detected among young children with acute enterovirus A71 infection.[16] In view of their rapid elicitation within the first week after onset, two types of B cells may attribute the development of such IgM ASC response. Firstly, previous exposure of pathogen could lead to the development of isotype-switched IgM memory B cells and these B cells typically become activated, proliferated, and differentiated into plasmablasts as they encounter the antigen.[32] Secondly, marginal zone B cells, the equivalents of mice B1 B cells, are found to rapidly respond to both T cell-independent and -dependent bacterial antigens, which contributes to IgM-secreting plasmablasts in the early phase of infection.[33] Nevertheless, in the study, the nature of precursor B cells that dominate and differentiate into the antigen-specific ASC response upon acute *M. pneumoniae* infection remains largely unclear.

In this study, we can detect the development of *M. pneumoniae*-specific ASC response in the peripheral blood in children with pneumonia. A recent study demonstrated *M. pneumoniae*-specific antibodies in the cerebrospinal fluid among individuals with encephalitis associated with *M. pneumoniae*.[34] Besides, an early diagnosis of bacterial meningitis was achieved by the measurement of antigen-specific

ASC response in the cerebrospinal fluid.[35] While the *M. pneumoniae* infection could be associated with encephalitis, myocarditis and severe inflammatory illness, such extrapulmonary manifestations warrants the investigation and detection of *M. pneumoniae*-specific ASCs in the local tissues.

A prompt diagnosis of acute *M. pneumoniae* infection would facilitate the implementation of clinical management and prevent the usage of inappropriate antibiotics. The gold standard for the confirmation of acute *M. pneumoniae* infection is still disputable in the present.[36] The sensitivity and specificity of PCR detection and serology varies with the carriage prevalence in different regions.[36,37] The culture method is time-consuming and require special reference laboratory setting. The serology based on detecting *M. pneumoniae*-specific IgG or IgM has been widely used in the clinical setting. However, several reports showed that the serological titer could last more than 6 months, which indicates the serology-based assay may not distinguish acute infection from previous exposure of *M. pneumoniae*.[38,39] Paired sera that show seroconversion or a more than 4-fold increase in antibody level could be indicative of recent infection but obtaining paired sera in a routine clinical setting is often not available.[40] The circulating antigen-specific ASCs open a window to examine the cellular response to recent infection. These ASC-secreted antibodies would exclusively represent recently induced humoral response without the interference of pre-existing antibody level. While the antigen-specific ASC response in the peripheral blood could be an alternative biomarker in diagnostic applications,[31] a rigorous research should be conducted to assess and validate the assay for detecting such B cell response. Besides, we found that the frequency of *M. pneumoniae* P1-specific ASC response significantly correlated with the fever duration. Similar result was reported in the study of antigen-specific ASC response to influenza virus or enterovirus A71,[16,17] but further study is required to understand if there is an underlined mechanism.

There are limitations in the study. Firstly, only pneumonia patients were enrolled in the study. Although *M. pneumoniae* mainly causes respiratory tract infection, there is a wide variety of clinical manifestations. While clinical severity may affect the size of ASC response to acute infection,[15] the details of antigen-specific antibody and ASC responses to mild *M. pneumoniae* infection or asymptomatic carriers is undetermined. Secondly, the ASC response was examined at a certain time point within the first two weeks of illness in the study, so the duration of *M. pneumoniae*-specific ASC response is unclear. Previous study reported that the *M. pneumoniae*-specific ASC response may last up to six weeks in the peripheral blood, which would be longer than the duration of circulating ASC response to other respiratory pathogens studied (i.e., influenza and acute enteroviral infection).[16,17,30,31] In the study the ASC response at an earlier stage of *M. pneumoniae* infection (before day 5 of onset) was lacking. Nevertheless, previous studies have shown that *M. pneumoniae*-specific ASCs could be detected as early as two days after onset.[30,31] Thirdly, the function and repertoire features of antigen-specific ASCs elicited upon acute infection are lacking in the study. Further research in terms of the molecular and clonal basis of *M. pneumoniae*-specific B cells would greatly elucidate the role of antibodies in the protection against this pathogen. Finally, the sample size is small in the study. A prospective and nationwide study to evaluate the ASC response to acute *M. pneumoniae* infection is warranted.

In conclusion, a robust *M. pneumoniae*-specific ASC response was elicited after acute infection in children with pneumonia, and IgM-secreting ASCs constitute the major component of antigen-specific ASC response in the peripheral blood. The prompt detection of P1-specific ASC response suggests the potential of cell-based diagnostic tool for acute *M. pneumoniae* infection in humans in the near future.

Declaration of competing interest

All authors declared no conflicts of interest.

Acknowledgments

This study was supported by Chang Gung Memorial Hospital Grant (CMRPG3J0751-2, CORPG3J0111-3).

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