

Increased Frequency of Th17 Cells in Children With *Mycoplasma pneumoniae* Pneumonia

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Background: *Mycoplasma pneumoniae* (*M. pneumoniae*, MP) is recognized globally as a significant cause of primary atypical pneumonia in humans, particularly in children. Overzealous host immune responses are viewed as key mediators of the pathogenesis of *M. pneumoniae* infection. Although Th17 cells have been identified as key modulators in the clearance of pathogens and induction of autoimmunity caused by excessive immune responses, little is known about the role of Th17 cells in patients with *M. pneumoniae* infection. **Methods:** The percentages of T cells, CD4⁺ T cells and Th17 cells in children with *M. pneumoniae* infection were measured by flow cytometry. **Results:** We documented an increased frequency of Th17 cells in children with

M. pneumoniae infection. Furthermore, we found a significantly higher percentage of Th17 cells in *M. pneumoniae*-infected children with extrapulmonary manifestations, compared with children without extrapulmonary manifestations. In addition, patients who experienced a short course of *Mycoplasma pneumoniae* pneumonia (MPP) showed an increase in the percentage of Th17 cells. **Conclusion:** Our findings suggest that Th17 cells may be involved in the clearance of *M. pneumoniae* during an acute infection. Excessive Th17 cell responses may also contribute to the immuno-pathological damage observed during persistent infection. J. Clin. Lab. Anal. 30:1214–1219, 2016. © 2016 Wiley Periodicals, Inc.

Key words: children; *Mycoplasma Pneumoniae*; respiratory infection; Th17 cells

INTRODUCTION

Mycoplasma pneumoniae (*M. pneumoniae*) is recognized globally as a significant cause of upper and lower respiratory tract infections in humans, particularly in children, and accounts for over 40% of community-acquired pneumonia in children admitted to the hospital (1–5). Although *M. pneumoniae* infection is rarely fatal, patients of every age can develop severe and fulminant disease. Moreover, *M. pneumoniae* can also cause extrapulmonary manifestations in almost every compartment, including the hepatic, cardiovascular, skin, hematologic, musculoskeletal, and nervous system (6). The pathophysiologic mechanism underlying the diverse symptoms associated with *M. pneumoniae*-induced human diseases is thought to result from indirect tissue injury caused by an overzealous host immune responses (7, 8).

Substantial evidence supports a key role of Th17 cells in the development and pathogenesis of various excessive immune response-induced diseases (9). Indeed, Th17 cells are often associated with the recruitment and activation of inflammatory myeloid cells that cause severe local tissue injury (10, 11). However, Th17 cells are not designed to elicit autoimmune responses but are rather intended to provide an effective host defense against pathogens. Accumulating evidence has shown

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that Th17 cell-deficient mice have an increased susceptibility to infection from a wide variety of microorganisms, including bacteria, parasites, fungi and viruses (12). Previous studies in which levels of IL-17 in the peripheral blood of children infected by *M. pneumoniae* were measured (13, 14). Furthermore, *M. pneumoniae* antigens have been shown to enhance Th17 cell response in an experimental mice model (15). However, little is known about the role of Th17 cells in mycoplasma pneumonia and its extrapulmonary complications in humans, particularly in children.

Here, we document for the first time that the frequency of Th17 cells was significantly higher in *M. pneumoniae*-infected patients with a short course of MPP and in patients with extrapulmonary manifestations. Our results indicate that Th17 cells may be involved in the clearance of *M. pneumoniae* during an acute infection. However, excessive Th17 cell responses may result in immuno-pathological damage to organs in cases of persistent infection in which clearance does not occur in a timely fashion. Taken together, these data suggest that appropriate Th17 cell responses are required and influence the control and clinical outcome of *M. pneumoniae* infection.

MATERIALS AND METHODS

Ethics statement

Ethical clearance for this study was obtained from the Institutional Review Board of Nanjing Medical University, Nanjing, China (Permit Number: 2008NMUIEC130). The aims and objectives of the study were explained to each participant and written informed consent was obtained.

Patients and healthy controls

The study involved a total of 51 subjects. The subjects included 30 children with MPP from Nanjing Children's Hospital and 21 healthy controls. In all cases, pneumonia was diagnosed by the presence of consolidation on a chest X-ray confirmed by two pediatric radiologists at the time of admission. Infection with *M. pneumoniae* was confirmed by an initial MP-IgM titer greater than 1:40. The MP-IgM titer was measured using a diagnostic kit for the measurement of antibodies to *M. pneumoniae* (FUJIREBIO INC, Tokyo, Japan). The sputum and blood from all children who were diagnosed with MPP were examined to rule out the presence of coinfection with other bacteria or viruses. Peripheral blood was collected from each fasting participant after admission in the morning. All patients were in the acute stage of MPP

and had not taken any medication prior to the blood collection.

We classified pneumonia as long or short course, namely 14 days or longer and fewer than 14 days, respectively. A white blood cell (WBC) number greater than $10 \times 10^9/l$ was considered a high level of WBCs. Elevated levels of ALT (normal value, <40 U/l) and CK-MB (normal value, <24 U/l) were considered diagnostic of liver and myocardial injury (extra-pulmonary manifestations), respectively. Serum ALT and CK-MB levels were assayed using a HITACHI 7600-020 Chemical Analyzer (HITACHI, Tokyo, Japan).

Flow cytometry (FCM) analysis

Human peripheral blood mononuclear cells (PBMCs) were collected into sodium heparin tubes (BD Biosciences, San Diego, CA) and purified by Ficoll-paque plus (GE healthcare, Uppsala, Sweden) density gradient centrifugation. Cells recovered from the gradient interface were washed twice and stained for 30 min at 4°C with the following antibodies or isotype-matched controls: CD3-APC (eBioscience, San Diego, CA), CD4-FITC (eBioscience). Then, cells were stimulated for 4 h at 37°C in a humidified atmosphere of 10% CO₂ in culture medium containing PMA (25 ng/ml; Sigma, St. Louis, MO), ionomycin (1 µg/ml; Sigma), and monensin (Golgi Stop; 1 µg/ml; BD Biosciences, San Jose, CA). After stimulation, cells were fixed and made permeable with Cytotfix/Cytoperm and Perm/Wash buffer according to the manufacturer's instructions (BD Biosciences), then labeled with IL-17-PE (eBioscience). Cells were incubated for 30 min at 4°C and washed twice in Perm/Wash before analysis. Cell acquisition was performed using a FACSCalibur cytometer (BD Biosciences). Data were analyzed with FlowJo (version 10.0.7; Tree Star, Ashland, OR).

Statistical analyses

All data were analyzed using SPSS software (version 22; IBM, Armonk, NY). Significant differences between specimens were determined using Mann-Whitney *U* test or Fisher's Exact test according to the characteristics of the variables. A *P* value <0.05 was considered to be statistically significant.

RESULTS

Increased frequency of Th17 cells in patients with MPP

To determine whether the percentage of Th17 cells was increased in patients with MPP, a total of 30

patients and 21 healthy controls were recruited. There was no significant difference in the distribution of age or gender between patients and healthy controls (Table 1). The frequency of total T cells and T cells among total lymphocytes was comparable between patients and

healthy controls (Fig. 1a–c). Th17 cells were defined as $CD3^+CD4^+IL-17^+$, as shown in the gate strategy in Figure 1a. Results demonstrated an increased frequency of Th17 cells among $CD3^+$ T in patients with MPP when compared with healthy controls (Fig. 1d, e).

TABLE 1. The Demographic and Clinical Characteristics of Subjects

Parameters	MPP							
	HC	With EPM				Without EPM		
		Long course	Short course	CK-MB > 24 U/l	ALT > 40 U/l	Wbc $\leq 10 \times 10^9/l$	Wbc $> 10 \times 10^9/l$	
Number	21	9	21	4	5	21	22	8
Age(months)	25.7 \pm 13.1	26.9 \pm 13.1	28.9 \pm 9.4	35.0 \pm 10.7	25.8 \pm 9.1	29.9 \pm 11.5	24.9 \pm 5.0	
Male	14	4	14	3	1	14	13	5
Female	7	5	7	1	4	7	9	3

HC, healthy control; MPP, mycoplasma pneumoniae pneumonia.

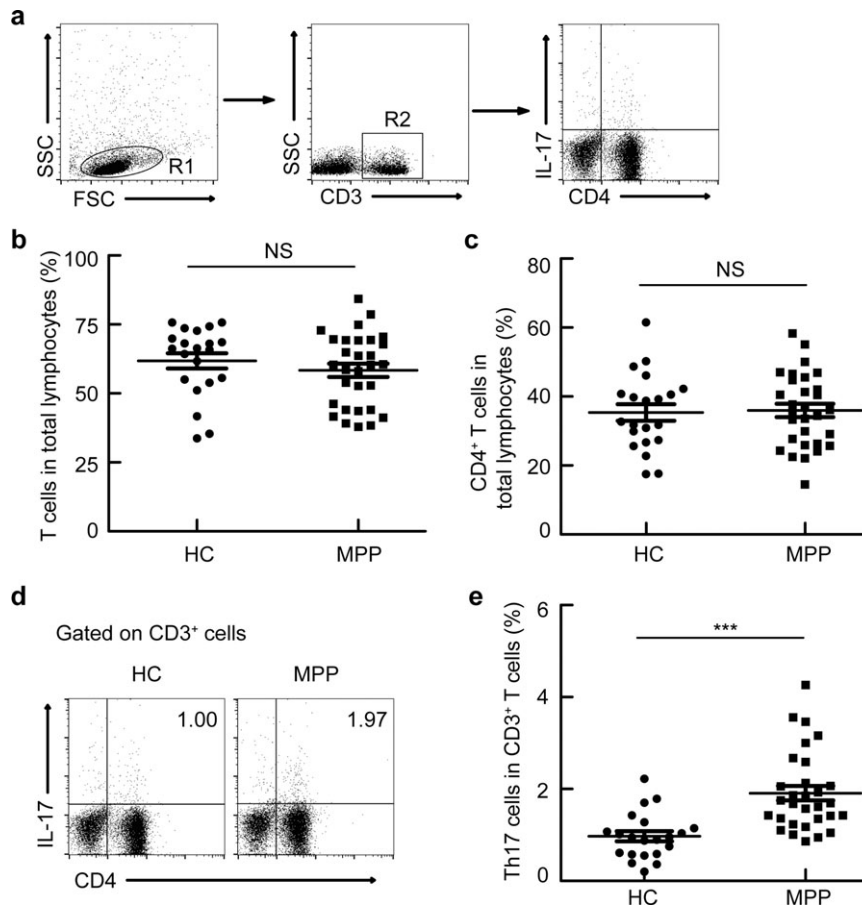


Fig. 1. Increased frequency of Th17 cells in patients with MPP. PBMCs were isolated from healthy controls (HC, $n = 21$) and patients with MPP (MPP, $n = 30$) and analyzed. (a) Gating schemes for analysis of the percentage of Th17 ($CD3^+CD4^+IL-17^+$). PBMCs were stained with CD3, CD4, and IL-17 antibodies. (b, c) Percentages of T cells (b) and $CD4^+$ T cells (c) in total lymphocytes; (d, e) Representative FACS plots and statistics show Th17 cells. *** $P < 0.001$, NS indicating not significant.

Subjects with a short course of MPP had an increase in peripheral blood Th17 cells

To investigate whether the frequency of Th17 cells correlated with the time course of MPP, we next examined the frequency of Th17 cells in patients with a short or long course of MPP. We found a significantly higher percentage of Th17 cells in patients with a short course of MPP than in patients with a long course of MPP (Fig. 2), suggesting that Th17 cells may be involved in the control of *M. pneumoniae* infection.

The percentage of Th17 cells was comparable between study subjects with a high level of WBCs and those with a normal level of WBCs

No significant difference in the percentage of Th17 cells was found between patients with a high level of WBCs and those with a normal level of WBCs (Fig. 3).

The frequency of Th17 cells was increased in patients with extrapulmonary manifestations

Patients with hepatic and cardiovascular extrapulmonary manifestations tended to have a higher percentage of Th17 cells compared with patients without extrapulmonary manifestations (Fig. 4). These results suggest that Th17 cells may be important players in the pathogenesis of MPP.

DISCUSSION

Mycoplasma pneumoniae is recognized globally as a significant cause of primary atypical pneumonia (3).

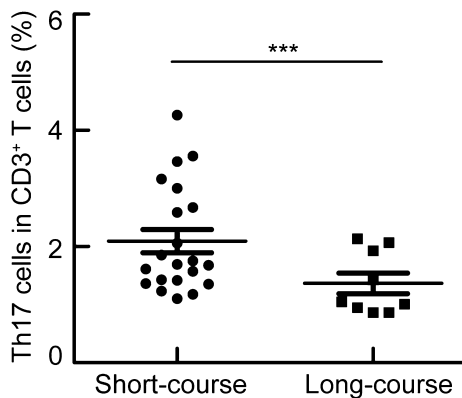


Fig. 2. Patients with a short course of MPP had an increase in peripheral blood Th17 cells. PBMCs were isolated from patients with a short ($n = 21$) or long course of MPP ($n = 9$) and analyzed. PBMCs were stained with CD3, CD4, and IL-17 antibodies. Frequencies of Th17 within CD3⁺ T cells in patients with a short or long course of MPP. *** $P < 0.001$.

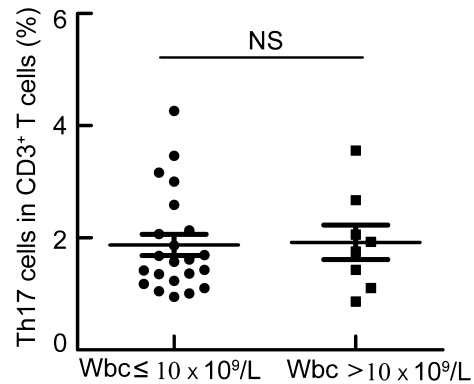


Fig. 3. The percentages of Th17 cells were comparable between study subjects with a high level of WBCs and those with a normal level of WBCs. PBMCs were isolated from patients with high ($n = 8$) or normal ($n = 22$) levels of WBCs and analyzed. PBMCs were stained with CD3, CD4, and IL-17 antibodies. Frequencies of Th17 cells within CD3⁺ T cells in patients with high or normal levels of WBCs. NS indicating not significant.

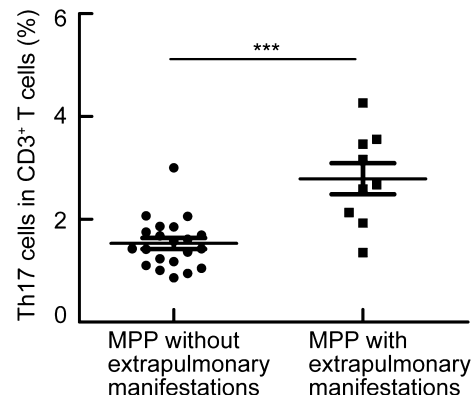


Fig. 4. The frequency of Th17 cells was increased in patients with extrapulmonary manifestations. PBMCs were isolated from patients with ($n = 9$) or without ($n = 21$) extrapulmonary manifestations and analyzed. PBMCs were stained with CD3, CD4, and IL-17 antibodies. Frequencies of Th17 with CD3⁺ T cells in patients with or without extrapulmonary manifestations. *** $P < 0.001$.

M. pneumoniae can cause not only severe respiratory symptoms but is also associated with extrapulmonary manifestations (3). However, the exact mechanisms underlying the pathogenesis of *M. pneumoniae* infection remain unclear. In this study, we observed an increase in the percentage of Th17 cells in *M. pneumoniae*-infected patients. Furthermore the percentage of Th17 cells was significantly higher in *M. pneumoniae*-infected patients with extrapulmonary manifestations or with a short course of MPP. Our results suggest that Th17 cells may play a potential role in both the control and pathogenesis *M. pneumoniae* infection.

Pathogens as diverse as gram-positive *Propionibacterium acnes*, gram-negative *Citrobacter rodentium*, *Klebsiella pneumoniae*, *Bacteroides* spp., and *Borrelia*

spp., the acid-fast *Mycobacterium tuberculosis*, and fungi-like *Pneumocystis carinii* and *Candida albicans* can trigger a strong Th17 response (16–22). Consistent with published data showing that Th17 cells are induced by *M. pneumoniae* antigens in mice (15), we demonstrate for the first time that the percentage of Th17 cells are significantly increased in *M. pneumoniae*-infected patients.

On one hand, Th17 cells constitute a branch of the adaptive immune system whose function is to clear specific types of pathogens (9). Th17 cells are clearly involved in the development of mucosal immunity and the granulopoietic response to extracellular pathogens, including bacteria and fungi (23, 24). In agreement with published data from mice, our data show that patients with a short course of MPP have a higher percentage of Th17 cells, indicating a role of Th17 cells in the control of *M. pneumoniae* infection.

However, Th17 cells are also potent inducers of tissue inflammation and have been linked to the pathogenesis of human inflammatory conditions and many experimental autoimmune diseases (9). For instance, Th17 responses have been associated with tissue injury in atherosclerotic artery disease (25) and biliary cirrhosis (26) in humans, as well as experimental autoimmune encephalomyelitis (27) and collagen-induced arthritis (28) in mice. Similarly, our data showed an increase in the frequency of Th17 cells in patients with extrapulmonary manifestations, suggesting that Th17 cells may contribute to the pathogenesis of extrapulmonary organ damage, such as that observed in the liver and myocardium. Thus, when manipulating Th17 cells to eliminate the *M. pneumoniae* in patients, it is very important to consider their adverse effects in triggering Th17-mediated extrapulmonary organ damage.

Previous studies have demonstrated that Th17 cells are converted to Th1 cells in vitro (29, 30). More importantly, IL17⁺IFN γ ⁺ T cells have been described and Th17 cells have been shown to develop into Th1 cells in vivo (31). In addition, Th17 cells also produce IL-10 (32), IL-21 (32), and IL-22 (33). Although the precise pathogenic mechanism by which Th17 cells contribute to MPP is poorly understood, these cells may well enhance disease progression by producing various cytokines such as IL-17 and IFN γ .

In summary, our study is the first to report an increase in the percentage of Th17 cells in *M. pneumoniae*-infected patients with a short course of MPP or who exhibit extrapulmonary manifestations. Our study indicates that Th17 cells may be involved in the clearance of *M. pneumoniae* during an acute infection. Excessive Th17 cell responses may also contribute to the immuno-pathological damage to organs in cases of persistent infection in which clearance of the pathogen

does not occur in a timely fashion. Taken together, these data suggest that appropriate Th17 cell responses are required and influence the control and clinical outcome of *M. pneumoniae* infection.

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AUTHOR CONTRIBUTIONS

XM, XC, HT, JZ, SZ, and ZX performed the experiments; XM, XC, and CS analyzed the data; XM, FL, and CS designed the study; and XM, XC, and CS wrote the paper.

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