

Deficient immune response to *Mycoplasma pneumoniae* in childhood asthma

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ABSTRACT

Prospective studies have suggested that some individuals have a persistent IgM response to *Mycoplasma pneumoniae* infection with relatively little IgG production over many months. Persistence of the organism in patients with an allergic phenotype might predispose to the development of asthma. This study was designed to analyze the prevalence of *M. pneumoniae* infection and the immune response to that infection among children with asthma compared with controls. A prospective study was performed in 82 children with physician-diagnosed asthma and 98 nonasthmatic controls over a 5-year period comparing them for evidence of current or prior infection by *M. pneumoniae* using serology (IgG and IgM), culture, and polymerase chain reaction (PCR), and in vitro cellular responses to *M. pneumoniae* antigen. Similar numbers of controls (9/98) and asthmatic children (6/82) were PCR⁺ for *M. pneumoniae* at some time during the study. IgM antibody to *M. pneumoniae* was detected in similar numbers of controls (21/98) and asthmatic children (18/82), but positive IgG antibody titers were detected in significantly more controls (13/98) than asthmatic children (3/82; $p = 0.03$). Similar numbers from each group were IgM⁺ on more than one annual visit (9/98 controls and 7/82 asthmatic children). Antigen-driven proliferation and interferon (IFN) gamma production by mononuclear cells from IgM⁺ controls were significantly greater than that of IgM[−] controls, but there was no difference in proliferation and IFN-gamma production by cells from IgM⁺ and IgM[−] asthmatic children. These results suggest that asthmatic children have deficient cellular and humoral responses to *M. pneumoniae* infection compared with nonasthmatic controls.

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Key words: Airway inflammation, antibodies, asthma, children, cytokines, immune response, infection, interferon, mycoplasma

Mycoplasma pneumoniae is a minute, highly specialized bacterium that lacks a cell wall and is adapted to life as an obligate pathogen in the human respiratory tract. Although the organism binds to the surface of epithelial cells in the respiratory epithelium, recent evidence suggests that it is, in fact, a facultative intracellular pathogen.^{1,2} This observation correlates well with its reduced genome size, the signature of an intracellular organism.³

The genomic considerations and clinical studies of the immune response to *M. pneumoniae* infection over time^{4,5} paint a picture of an organism that is capable of persisting in the airway for months if not years given

the right conditions. Host factors relating to the nature and intensity of the immune response to infection may play a direct role in whether a persistent infection occurs or not. The importance of antibody production as illustrated in human immunodeficiency and animal models of infection suggest that serological responses may be more important in the establishment of chronic infection by *M. pneumoniae*.

Infection with *M. pneumoniae* has been implicated as both an exacerbating factor in chronic asthma^{6–11} and an initiating factor^{7,12} in the development of asthma. After acute infection with *M. pneumoniae*, prolonged changes in lung physiology including decreased diffusion capacity,¹³ increased bronchial hyperresponsiveness to methacholine,¹⁴ and decreases in pulmonary functions^{15,16} have been described although some studies have not found such differences.¹⁷ Increased detection of *M. pneumoniae* in asthmatic patients compared with nonasthmatic patients has been reported in studies using conventional culture¹⁸ and nucleic acid amplification from airway washings, sputa, or biopsy material.^{19,20} Macrolide and related antibiotics appear to have a beneficial effect in many patients with asthma,^{21–26} but their mechanism of action is complicated by their nonantimicrobial anti-inflammatory properties.^{27,28} In

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one prospective study of 55 stable adult asthmatic patients, an improvement in forced expiratory volume at 1 second correlated with the detection of *M. pneumoniae* or *Chlamydophila (Chlamydia) pneumoniae* DNA in bronchoalveolar lavage fluid or endobronchial biopsy specimens.²⁹ Interestingly, in all of these subjects, significantly elevated circulating antibody to *M. pneumoniae* was not detected.

Few long-term clinical studies of the natural history of *M. pneumoniae* infection have been undertaken that focus on the pulmonary status of the study subjects. Here, we report the results of a 5-year surveillance study of *M. pneumoniae* infection involving 82 children with physician-diagnosed asthma and 98 nonasthmatic control children. The results suggest that *M. pneumoniae* infection is common in both groups, and after *M. pneumoniae* infection, the cellular and serological responses of children with asthma may differ from those of nonasthmatic patients.

METHODS

Study Population

Study subjects were enrolled in a 5-year prospective study under a protocol approved by the Institutional Review Board. Any child between the ages of 3 and 15 years without a significant medical diagnosis other than asthma could enter the study. Physician-diagnosed asthma was used as the criterion for enrollment into the asthma or control groups. Patients were scheduled for annual visits and were also encouraged to return for sick visits or for monthly follow-up visits if positive results on IgM or polymerase chain reaction (PCR) tests were obtained. A history of recent illnesses and medications, particularly bronchodilators, glucocorticoids, macrolides, and tetracyclines was acquired at each visit. Specimens collected at each visit included a nasopharyngeal or, occasionally, a throat swab for culture and PCR for *M. pneumoniae*, blood samples for (a) culture and PCR of *M. pneumoniae* from the leukocyte fraction (Vacutainer Cell Preparation Tubes; Becton-Dickinson, Franklin Lakes, NJ), (b) lymphocyte proliferative responses and cytokine production after culture with *M. pneumoniae* antigen preparation or tetanus antigen, (c) serology, and (d) a urine sample for culture and PCR. Swabs were agitated vigorously in screw-cap freezer vials containing either SP-4 culture medium or Tris-buffered saline for PCR and either processed within 4 hours or frozen at -70°C until processing.

At the conclusion of the study, a questionnaire was administered by telephone to determine whether control subjects had developed asthma over the course of the study. The validation study by Gerald *et al.*³⁰ indicated that, using the questionnaire alone, positive responses on questions 4 and 5a have a sensitivity of

66%, a specificity of 96%, and a predictive value of 95% for detecting children with asthma.

Sample Collection and Processing

Cell Culture. Whole blood was collected from study subjects in sodium heparin tubes, and peripheral blood mononuclear cells were isolated by Ficoll gradient centrifugation. The cells were washed twice in RPMI-1640 containing 10% heat-inactivated fetal bovine serum, 2 mmol/L of L-glutamine, and 100 IU/mL of penicillin/streptomycin (complete RPMI) and then resuspended at 10^6 cells/mL. One-milliliter aliquots were plated in 24-well cell culture plates for cytokine analysis and 200- μL aliquots were plated in quadruplicate in wells of a 96-well plate for antigen proliferation studies.

Antigen Proliferation Testing. For preparation of *M. pneumoniae* antigen, a locally derived clinical isolate of *M. pneumoniae*, no. 19294, was grown in polystyrene tissue culture flasks (Costar 431081, Corning, Lowell, MA) to log phase in SP-4 media³¹ according to published procedures.³² Medium containing nonadherent organisms was collected and the adherent organisms were then harvested using a cell scraper. Nonadherent and adherent fractions were pooled and then collected by centrifugation at $10,000 \times g$ for 20 minutes at 4°C , washed twice with PBS, and then resuspended in 5 mL of PBS by repeated passage through a 27-gauge needle. Thimerosal 0.1% w/v was then added, and the suspension was incubated overnight at 4°C followed by dialysis against PBS. No viable organisms could be cultured from the final antigen preparation. The protein content of the preparation was determined by the bicinchoninic acid assay (Pierce, Thermo Scientific, Rockford, IL), and the preparation was then diluted to 210 $\mu\text{g}/\text{mL}$ in PBS and stored in 10- μL aliquots at -70°C until use. A Western blot was performed using a locally generated rabbit antiserum to whole *M. pneumoniae* with preimmunization rabbit serum as control, and the blot revealed a wide spectrum of specific antigens within the preparation (not shown). For use as a control antigen in proliferation assays, preservative-free tetanus toxoid (Wyeth-Ayerst, Madison, NJ) was diluted to 210 $\mu\text{g}/\text{mL}$ in PBS and frozen in 50- μL aliquots at -70°C until use.

Antigens were diluted in complete RPMI and added in 10- μL aliquots to quadruplicate wells of 96-well plates or in 50- μL aliquots to single wells of 24-well plates, and cell cultures were incubated in a humidified 37°C incubator with 5% CO_2 . After 5 days incubation with antigen the 96-well plates were pulsed with 1 $\mu\text{Ci}/\text{well}$ ^3H -thymidine and harvested using an automated cell harvester (Skatron, Lier, Norway) 15 hours later. Filter discs containing the extracted DNA were counted using a scintillation counter. Cell supernatants

from 24-well plates were harvested on the same day as the 96-well plates (day 6). Stimulation index (SI) was calculated by $SI = \text{cpm stimulated} / \text{cpm medium}$. Positive proliferation responses were empirically considered to be those with $SI \geq 4.0$. Responses in which both tetanus and mycoplasma responses yielded an $SI < 4.0$ were not considered for data analysis.

Study Measurements

Interferon (IFN) γ Production. Supernatants from antigen-stimulated cell cultures in 24-well cell culture plates were harvested and filtered using sterile syringe filters and then stored at -70°C until use. Cytokine production (IFN- γ) was analyzed in duplicate samples by enzyme immunoassay according to the manufacturer's directions (BD Biosciences, Franklin Lakes, NJ).

Serological Testing. Patient serum was stored in aliquots at -70°C until use. IgG and IgM antibodies to *M. pneumoniae* were measured by enzyme immunoassay as described.³² Positive titers were defined as those higher than 3 SD above results for a pooled control serum from 50 individuals.

Polymerase Chain Reaction. Tubes containing material from nasopharyngeal or oropharyngeal swabs in Tris-buffered saline and leukocytes separated using cell preparation tubes (Becton-Dickenson) were spun, and DNA was extracted from pellets (Qiagen, Inc., Valencia, CA) and used as template for PCR for the *M. pneumoniae* 16S ribosomal RNA gene as described.²⁰ Positive bands on agarose electrophoresis were confirmed by liquid hybridization using a ^{32}P -labeled oligonucleotide probe followed by a second round of gel electrophoresis and autoradiography.

Statistical Analysis. *In vitro* cellular immune responses (stimulation indices and IFN- γ production) were compared between asthmatic and control groups using Student's *t*-test. Comparison of control and asthmatic groups for the occurrence of positive IgM, IgG, culture, or PCR tests was done using Fisher's exact test.

RESULTS

A total of 82 asthmatic children and 98 control children were enrolled. Demographics were very similar in the two groups including age (control group, 11.71 ± 3.7 years, and asthmatic group, 12.5 ± 4.3 years), race (control group, 32 white/64 black/2 other, and asthmatic group, 29 white/53 black), and sex (control group, 58 male/40 female patients, and asthmatic group, 46 male/36 female patients). Five controls were deleted from the study, three for consent irregularities and two at the mother's request. Samples were collected annually and patients were encouraged to come

Table 1 Subjects with positive IgM at any time during the study

Controls (21/98, 21.4%)		
001#	004*	005
009*#	013	019
022*	024	039
052	054	064
075	081	098
099	034§	040§
078†	096§	103§
Asthmatics (18/82, 21.9%)		
508	523	537
540	547	549
550	552#	554
559	560	561
569	574	582
585	520#§	576#§

*Became asthmatic during the study.

#Positive polymerase chain reaction during the study.

§Positive IgG during the study.

for additional testing during any respiratory infections with fever, cough, or sore throat. The numbers of annual and total visits were similar between the two groups including total visits (control group, 3.14 ± 1.6 , and asthmatic group, 3.25 ± 1.6) and annual visits (control group, 2.82 ± 1.2 , and asthmatic group, 2.75 ± 1.0).

Serological Testing

Over the course of the study similar proportions of patients were found to have been infected with *M. pneumoniae* by at least one criterion: IgM or IgG antibody, culture positivity, or PCR positivity. Among the controls, 35 of 98 subjects were positive by at least one measure (35.7%), and 24 of 82 asthmatic subjects (29.3%) were positive during the course of the study. Despite similar numbers of IgM⁺ subjects in both groups (Table 1), only three asthmatic subjects were found to be IgG antibody positive (Table 2; $p = 0.03$, Fisher's exact test). Interestingly, two of the three asthmatic subjects found to be IgG⁺ were also positive for IgM and were PCR⁺ as well. Furthermore, of the subjects who were IgM⁺, 43% of the control subjects (9 of 21 subjects) and 33% of the asthmatic subjects (6 of 18) were IgM⁺ on more than one annual visit (Table 3; $p = 0.78$, Student's *t*-test).

Only two subjects (one control and one asthmatic subject) were culture positive for *M. pneumoniae* during the course of the study, both from throat swabs. Of these, the control subject was also PCR⁺ during that visit but was antibody negative throughout the course of the study. A follow-up questionnaire at the conclusion of the study revealed that that subject (053) had

Table 2 Subjects with positive *Mycoplasma pneumoniae* IgG during the study

Controls (13/98, 13.2%)		
002	020	049
062	063	065
082	088*	034§
040§	078§	096§
103§		
Asthmatics (3/82, 3.6%)		
512	520#§	576#§

*Diagnosed with asthma during the study.

#Positive polymerase chain reaction during the study.

§Positive IgM during the study.

$p = 0.03$, Fisher's exact test, two-tailed (control vs asthmatic IgG).

Table 3 Positive IgM to *Mycoplasma pneumoniae* on more than one annual visit

Controls (9/98, 9.1%)		
009 (2 visits)*#		019 (3 visits)
054 (2 visits)		064 (3 visits)
075 (2 visits)		081 (2 visits)
096 (2 visits)§		098 (2 visits)
103 (2 visits)*§		
Asthmatics (6/82, 7.3%)		
537 (3 visits)		547 (2 visits)
549 (2 visits)		550 (2 visits)
554 (2 visits)		574 (2 visits)

*Diagnosed with asthma over the course of the study.

#Positive polymerase chain reaction during the study.

§Positive IgG during the study.

Control versus asthmatic IgM: $p = NS$.

been diagnosed with asthma by his physician during the study. In contrast, the asthmatic subject who was culture positive was also positive by PCR and strongly positive for IgM antibody on the same visit. That asthmatic subject was negative for IgG antibody for *M. pneumoniae* throughout the course of the study.

We wondered whether the lack of IgG responses among the asthmatic population might reflect a general deficiency in IgG responses in that population so we measured tetanus titers in both control and asthmatic subjects. There was no significant difference between the two groups by this measure (data not shown), suggesting that the lack of IgG response to *M. pneumoniae* among the asthmatic subjects does not reflect a general decrease in immune responsiveness. Similarly, if one looks at the tetanus titers as a function of age in only those subjects who had a positive IgM, no difference can be discerned. There is one strong trend that almost reaches significance: asthmatic sub-

Table 4 Positive *Mycoplasma pneumoniae* PCR at any time during the study

Controls (8/98, 8.2%)	
001#	003*
008	009*#
012	019#
025	053*§
Asthmatics (9/82, 11%)	
501	507
515	520§¶
525	529
552#	576#¶
581	

*Diagnosed with asthma during the study.

#Positive IgM during the study.

§Positive culture during the study.

¶Positive IgG during the study.

$p = 0.61$, Fisher's exact test, two-tailed, control versus asthmatic PCR; $p = 0.04$, Fisher's exact test, two-tailed if adjusted for control subjects who became asthmatic over the course of the study.

PCR = polymerase chain reaction.

jects with a positive IgM tended to be older compared with controls: control subjects, 7.4 ± 3.2 years, compared with asthmatic subjects, 9.2 ± 2.7 years ($p = 0.068$).

PCR Testing

PCR testing was positive in similar numbers of subjects over the course of the study (Table 4). However, follow-up phone questionnaires determined that 13 controls were diagnosed with asthma over the course of the study, and 7 of these had evidence of *M. pneumoniae* infection (Table 5). If the PCR positivity in each group in Table 4 was adjusted for subjects later diagnosed with asthma, the difference in PCR positivity became significant ($p = 0.04$, Fisher's exact test). Less than one-half of patients who were PCR⁺ had any serological evidence of infection (control subjects, 3/8; asthmatic subjects, 3/9).

Antigen Proliferation Studies

Peripheral blood mononuclear cells from the large majority of subjects from both groups proliferated to *M. pneumoniae* antigen stimulation, consistent with epidemiologic evidence that exposure to this organism is extremely common among the population. Only ~13% evaluable responses from both groups (12 of 92 control responses and 10 of 76 asthmatic responses) had a maximal SI < 4. Proliferation testing in control subjects with positive IgM antibodies showed that all had a maximum SI over the nominal positive ratio of 4.0, and

Table 5 Control subjects diagnosed with asthma during the study

Subject	Result
003	+PCR
004	+IgM
009	+IgM × 2, +PCR × 2
022	+IgM
029	
035	
042	
053	+culture, +PCR
074	
088	+IgG
089	
100	
103	+IgM × 2, +IgG × 2

PCR = polymerase chain reaction.

among the 18 responses by the asthmatic subjects with positive IgM antibodies, only one had an SI value <4.0. Although comparing the magnitude of the responses does not reveal a significant difference among the two groups, there was a trend toward a decreased proliferative response among the asthmatic subjects as a whole compared with the control subjects, which was strongest at the 1- μ g/mL antigen stimulus ($p = 0.11$, Student's t -test; Fig. 1). The responses to tetanus exhibited a similar, although smaller trend. Interestingly, there was a highly significant difference between the *M. pneumoniae* SI values for the IgM⁺ controls (43.4 ± 40.9 , mean $\pm$ SD) and the IgM⁻ controls (22.2 ± 23.7 ; $p = 0.004$, Student's t -test) while no such difference was evident in comparing the IgM⁺ (21.3 ± 23.1) and IgM⁻ (20.5 ± 17.6) asthmatic subjects.

IFN- γ Production

IFN- γ production in peripheral blood mononuclear cell cultures varied widely and in general did not correlate with antigen-driven proliferation. Nevertheless, as with the antigen proliferation, the IFN- γ production by IgM⁺ control subjects (1185 ± 1846 pg/mL, mean $\pm$ SD) was significantly different from IgM⁻ control subjects (263 ± 656 pg/mL; $p = 0.002$, Student's t -test), but only a trend in the same direction was seen comparing the IgM⁺ asthmatic subjects (2556 ± 6272 pg/mL) with the IgM⁻ asthmatic subjects (939 ± 3977 pg/mL; $p = 0.22$).

Asthma Questionnaire

After completion of the study, families of control subjects were contacted by telephone to determine whether their asthmatic status had changed. Of the 48 of 98 control subjects who could be contacted, 13 had

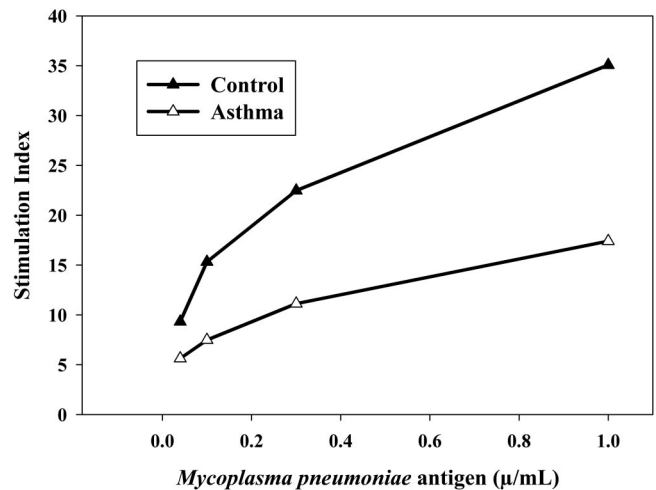


Figure 1. Trend toward lower antigen responses among asthmatic subjects. Shown are the mean stimulation indices for 21 IgM⁺ nonasthmatic control subjects and 16 asthmatic subjects to the indicated concentrations of *M. pneumoniae* antigen (mean SI to tetanus: control subjects, 18.3, and asthmatic subjects, 12.7).

been diagnosed with asthma by their primary care physician during the course of the study and were started on asthma medications (Table 5). Of these, seven had evidence of *M. pneumoniae* infection during the course of the study, a proportion that was not different from the overall group by Fisher's exact test.

DISCUSSION

M. pneumoniae is a highly specialized bacterium that functions as an obligate parasite in the respiratory tract of humans, its only known host. Recent data suggest that its mode of life, like many of its close relatives among the *Mollicutes*, is directed toward establishing a chronic infection within its host. It is not well understood which aspects of the immune response are necessary to clear an infection by *M. pneumoniae*. There is some evidence that individuals deficient in mannose-binding lectin are more susceptible to the organism³³ and *in vitro* testing indicates that complement with or without antibody can mediate efficient killing.^{34–37} However, there are no clinical reports of patients with known early classic pathway or terminal pathway complement defects having undue susceptibility to *M. pneumoniae*. In contrast, antibody deficiency in humans has been associated with especially prolonged infection and extrapulmonary spread.^{38,39} In addition, animal models have suggested a protective effect of antibody.⁴⁰

The failure to convert to an IgG response in many of the subjects of our study who were positive by IgM, PCR, or even culture supports other studies suggesting that, like *Helicobacter pylori*, *M. pneumoniae* is a "stealth pathogen,"⁴¹ producing persistent infections by avoiding activation of adaptive immune responses. The

striking lack of IgG antibody to *M. pneumoniae* among asthmatic subjects at any time during the study in contrast to the control population suggests that as a group, asthmatic subjects are often individuals who exhibit less robust adaptive immune responses and are thus more likely to suffer persistent infection by this and similar organisms. Indeed two of the three asthmatic subjects who were IgG⁺ also had positive IgM titers and PCR tests, indicating that they had been sampled during an acute infection. It may be significant that IgG antibody is known to be important in animal models in protection from infection and in humans in preventing systemic spread of infection. Thus, a deficit in IgG production to the organism might play a role in the establishment of a prolonged or persistent infection.

About one-third of asthmatic and control subjects were positive for IgM antibody to *M. pneumoniae* at some time during the course of the study, reflecting the high degree of prevalence of the organism in the population, and IgM could be detected on at least two annual visits in some of these subjects, some of whom went on to develop asthma. The results of the questionnaire must be considered somewhat tentative, however, because a sizable proportion of subjects had been lost to follow-up over the course of the study and of those who were contacted, recall bias is possible.

There are few longitudinal studies in which serological responses to *M. pneumoniae* have been studied after infection. Most of these have focused on individuals who have developed atypical pneumonia after infection, and almost all such subjects showed at least a partial conversion to an IgG response over a period of 1–2 months. Biberfeld⁵ examined 19S (IgM)- and 7S (IgG)-fractionated complement fixing and indirect hemagglutinating antibodies to *M. pneumoniae* in 17 cases of atypical pneumonia for up to 1 year after infection. Striking differences in individual responses were noted with the major fraction of antibody in some individuals continuing to reside in the 19S fraction 12 months later while other subjects had entirely converted to a 7S response within 6 weeks. Most subjects had a measurable proportion of persistent 19S antibody even at 1 year. No clinical information was presented regarding the pulmonary status of these subjects, but the author noted that “the disparity between patients in the duration of the 19S antibody response may be related to how long the infectious agent persists in the individual.”

Lymphocyte proliferation studies suggest an even higher degree of prior or current infection than that shown by serology and PCR, with ~85% of subjects from both groups exhibiting at least a nominal proliferative response to the mycoplasmal antigen preparation (SI ≥ 4.0). The cellular immune responses of the IgM⁺ controls, as measured by antigen-driven prolif-

eration and *in vitro* IFN- γ production, differed from the IgM[−] controls as would be expected as a natural consequence of infection. However, no significant difference was seen between the responses of the IgM⁺ and IgM[−] asthmatic subjects. This lack of evidence of a consistent increase in cellular responses among asthmatic subjects who were demonstrably positive for *M. pneumoniae* exposure suggests that a more diffuse deficit in immune responsiveness to the infection exists among some patients with asthma.

Some studies have suggested that IFN- γ production is lower among children with asthma compared with nonasthmatic control subjects⁴² and that low *in vitro* IFN- γ production early in life may even predict the subsequent development of asthma.⁴³ Although its effect in humans is less clear, IFN- γ has demonstrable effects on immunoglobulin isotype and antibody production in mice.^{44,45} Deficiency of IFN- γ or IFN- γ receptor signaling in both mice and humans renders the host more susceptible to infection by intracellular bacteria.^{46,47}

Recent studies in mouse models of *M. pneumoniae* infection may help shed light on the mechanism behind the apparent differences in immune responsiveness between asthmatic and nonasthmatic subjects in our study. Prior allergic sensitization of the lung or the exogenous administration of TH2 cytokines IL-4 and IL-13 suppress Toll-like receptor 2 expression in the lung and prolong the survival of the organism.^{48,49} *M. pneumoniae* infection in this model also induces IL-23/IL-17F and blockade of IL-23 partially impairs the clearance of the organism.⁵⁰ In addition, prior allergic sensitization of the airway reduces antibody production to *M. pneumoniae* by ~50% compared with unsensitized control mice (T.P. Atkinson, University of Alabama at Birmingham, unpublished data, 2009). These new observations in mice complement our findings presented in this study because respiratory allergies are well established as a risk factor in the development of childhood asthma and are present in the majority of asthmatic children.⁵¹

In summary, over a 5-year period similar proportions of asthmatic and nonasthmatic children showed evidence of *M. pneumoniae* infection by serum IgM, PCR, and culture. Despite the similarities in the numbers of IgM⁺ subjects, significantly fewer asthmatic subjects were found positive for IgG. IgM⁺ controls had substantially higher levels of antigen-driven proliferation and IFN- γ production than IgM[−] controls, but no such difference was seen between IgM⁺ and IgM[−] asthmatic subjects. These differences suggest that as a group, asthmatic patients have less robust cellular and humoral immune responses to respiratory infection with *M. pneumoniae*.

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