Variation in Colonization, ADP-Ribosylating and Vacuolating Cytotoxin, and Pulmonary Disease Severity among *Mycoplasma pneumoniae* Strains

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Rationale: *Mycoplasma pneumoniae* was recently discovered to produce an ADP-ribosylating and vacuolating cytotoxin, designated CARDS toxin, which is hypothesized to be a primary pathogenic mechanism responsible for *M. pneumoniae*-induced pulmonary inflammation. It is unknown if cytotoxin production varies with *M. pneumoniae* strain or if variation in cytotoxin production affects pulmonary disease severity.

Objectives: To examine the production of CARDS toxin by various strains of *M. pneumoniae* and compare the disease manifestations elicited by these strains in an experimental model of *M. pneumoniae* respiratory infection.

Methods: BALB/c mice were inoculated once intranasally with SP4 broth (negative control) or three different *M. pneumoniae* strains: M129-B7, M129-B9, or S1. Mice were assessed at 1, 2, 4, 7, 10, and 14 days after inoculation. Outcome variables included comparisons among *M. pneumoniae* strains relative to bronchoalveolar lavage (BAL) *M. pneumoniae* quantitative culture, CARDS toxin-based PCR, and CARDS toxin protein determinations, as well as cytokine and chemokine concentrations. Graded lung histopathologic score (HPS) was also assessed.

Measurements and Main Results: CARDS toxin concentrations were significantly increased in mice inoculated with strain S1 compared with mice inoculated with M129-B7 or M129-B9 strains. Quantitative *M. pneumoniae* culture and polymerase chain reaction were also significantly greater in mice infected with S1 strain compared with the other two strains, as were lung HPS and concentrations of IFN- γ , IL-12, IL-1 α , macrophage inflammatory protein-1 α , and keratinocyte-derived chemokine. In addition, a significant positive correlation was found between CARDS toxin concentration and lung HPS.

Conclusions: CARDS toxin concentrations in BAL are directly linked to the ability of specific *M. pneumoniae* strains to colonize, replicate, and persist, and elicit lung histopathology. This variation among strains may predict the range in severity of pulmonary disease observed among patients.

Keywords: *Mycoplasma pneumoniae*; toxin; pneumonia; asthma; ADP-ribosylating

Mycoplasma pneumoniae is a common respiratory bacterial pathogen that affects both the upper and lower respiratory tracts of children and adults (1–9). More recent data demonstrate an

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Mycoplasma pneumoniae was recently discovered to produce an ADP-ribosylating and vacuolating cytotoxin, designated CARDS toxin, which is hypothesized to be a primary pathogenic mechanism responsible for *M. pneumoniae*-induced pulmonary inflammation. It is unknown if cytotoxin production varies with *M. pneumoniae* strain or if variation in cytotoxin production affects pulmonary disease severity.

What This Study Adds to the Field

CARDS toxin concentrations in bronchoalveolar lavage are directly linked to the ability of specific *M. pneumoniae* strains to colonize, replicate, and persist, and elicit lung disease. This variation among strains may predict the range in severity of pulmonary disease observed among patients.

association between *M. pneumoniae* respiratory infection and reactive airway disease and asthma (10–15). Although *M. pneumoniae* has been recognized as a significant clinical pathogen for decades, its virulence determinants have only been partially deciphered. *M. pneumoniae* is believed to primarily act as an extracellular parasite, with its pathogenicity dependent on its attachment to respiratory epithelium and subsequent initiation of injury to the host. Much investigation has been directed at understanding the mechanisms responsible for the essential process of extracellular attachment (16–19). In addition, *M. pneumoniae* has been reported to possess invasive and intracellular survival capabilities. However, the microbial factors responsible for the observed host cell injury have not been satisfactorily determined. Much investigation regarding the resultant physiologic and cytolytic host injury after *M. pneumoniae* infection has focused on hydrogen peroxide production by *M. pneumoniae* (20, 21) and the effects of *M. pneumoniae*-derived lipoproteins (22). Recently, an ADP-ribosylating and vacuolating cytotoxin (designated CARDS toxin) of *M. pneumoniae* has been identified that may better explain the observed epithelial injury that occurs with infection. This cytotoxin exhibits similarities to pertussis toxin (23). The role of this newly described cytotoxin in the microbial pathogenesis of *M. pneumoniae* infection has not been fully elucidated.

M. pneumoniae can be divided into two subtypes based on amino acid sequences in the P1 adhesin located in the attachment organelle (24). Although both subtypes are known to cause infection in humans, it remains unclear if there is a substantive difference between subtypes with regard to infectivity, immune response, or clinical manifestations. In an *in vitro*

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investigation using a human monocytic cell line, it was concluded that the induction of proinflammatory cytokine genes and proteins was not dependent on the infecting subtype (25). Infection of guinea pigs with the two subtypes of *M. pneumoniae* with the intent of examining for differences in the ability of subtypes to colonize and propagate in the respiratory tract suggested that differences may exist; however, the results were not conclusive (26). It remains to be determined if significant differences in disease parameters exist between the two subtypes *in vivo*, as well as in human infection.

The objective of this study was to examine the production of CARDS toxin by various strains of *M. pneumoniae* and to compare the disease manifestations elicited by these strains in an established experimental model of *M. pneumoniae* respiratory infection (27–29).

METHODS

Additional details regarding the materials and methods are provided in the online supplement.

Organisms and Growth Conditions

Three clinical strains of *M. pneumoniae*, (1) M129-B7 (ATCC 29342; seventh pass of M129 strain; *M. pneumoniae* subtype 1), (2) M129-B9 (A. Collier, University of North Carolina, ninth pass of M129 strain; *M. pneumoniae* subtype 1), and (3) San Antonio strain S1 (isolated 1993; fourteenth pass; *M. pneumoniae* subtype 2), were reconstituted in SP4 broth and subcultured after 24 to 48 hours in a flask containing 20 ml of SP4 medium at 37°C. When the broth turned an orange hue (approximately 72 h), the supernatant was decanted; 2 ml of fresh SP4 broth was added to individual flasks and adherent mycoplasmas were harvested. This achieved *M. pneumoniae* cell concentrations in the range of 1×10^8 to 1×10^9 colony forming units (CFU)/ml. Aliquots were stored at –80°C.

Animals and Inoculation

Mice were obtained from a commercial vendor (Jackson Laboratories, Bar Harbor, ME), who confirmed their mycoplasma- and murine virus-free status. Nine- to 13-week-old female BALB/c mice were intranasally inoculated once with SP4 (control) or 1×10^8 CFU/ml of one of the *M. pneumoniae* strains being investigated in 50 μ l of SP4 broth. Animal guidelines were followed in accordance with the Institutional Animal Care and Research Advisory Committee at the University of Texas Southwestern Medical Center at Dallas.

Experimental Design and Sample Collection

Mice were evaluated on 1, 2, 4, 7, 10, and 14 days after inoculation. Samples were obtained from 10 mice per group (4 groups: SP4 control broth and M129-B7, M129-B9, and S1 strains) at each time point from repeated experiments. Mice were anesthetized with an intraperitoneal injection of 75 mg/kg ketamine and 5 mg/kg acepromazine before cardiac puncture. BAL specimens were obtained by instilling 500 μ l of SP4 broth through a 25-gauge needle into the lungs, via the trachea, followed by aspiration of this fluid into a syringe. Lung specimens, including the trachea, were collected and fixed for histologic evaluation.

Mycoplasma Culture

Twenty-five μ l of undiluted BAL sample and serial 10-fold dilutions of BAL in SP4 broth (50 μ l of undiluted BAL was used for the initial dilution) were immediately cultured on SP4 agar plates at 37°C, whereas the remaining undiluted BAL sample was stored at –80°C.

CARDS Toxin PCR of BAL

Quantitative real-time PCR targeted CARDS toxin. TaqMan probes were designed for detection of *cards* gene using Primer Express software (version 2.0). Standard curves were established using *M. pneumoniae* chromosomal DNA serially diluted (standard curve with every real-time PCR assay was 1, 5, 10, 100, 1,000, 10,000, 100,000 genomes). Real-time PCR was done using ABI PRISM HP7900 SDS

(Applied Biosystems). One hundred microliters of the BAL fluid was used to isolate DNA using Qiagen kit.

CARDS Toxin Protein Concentration in BAL

Individual wells of flat-bottom, 96-well microtiter ELISA plates (HBX 4; Dynatech, Alexandria, VA) were coated with 50 μ l of rabbit anti-CARDS toxin IgG (10 μ g/ml) in phosphate-buffered saline (PBS) overnight at 4°C (23). Extracted BAL samples were mixed with 1% BSA/PBS-T to a final volume of 100 μ l and added in duplicate to single wells. To quantify the amount of CARDS toxin in a given sample, known amounts of highly purified CARDS toxin (ranging from 7 ng to 0.07 pg per well diluted in 1% BSA/PBS-T) were used to establish a standard curve. Optical density (OD) at 450 nm was read in an automatic ELISA plate reader (Dynatech, Alexandria, VA).

Histopathology

Histopathologic score (HPS) was determined by a single pathologist who was unaware of the treatment status of the animals from which specimens were taken. HPS was based on grading of peribronchiolar/bronchial infiltrate, bronchiolar/bronchial luminal exudate, perivascular infiltrate, and parenchymal pneumonia (neutrophilic alveolar infiltrate). This HPS system assigned values from 0 to 26 (the greater the score, the greater the inflammatory changes in the lung) (30).

BAL Cytokines/Chemokines

Concentrations of cytokines and chemokines in BAL specimens were assessed using Multiplex Bead Immunoassays (BioSource International, Camarillo, CA) in conjunction with the Luminex LabMAP system, following the manufacturer's instructions.

Statistics

One-way analysis of variance was used to compare groups at each time point, if the data were normally distributed. For instances in which the data were not normally distributed, the Kruskal-Wallis test was used for comparisons. If a difference was found between groups, then a pairwise multiple comparison procedure was performed. A comparison was considered statistically significant if the *P* value was less than or equal to 0.05. Correlations were done by Spearman rank order.

Figure 1. Quantitative *Mycoplasma pneumoniae* (Mp) cultures of bronchoalveolar lavage (BAL) fluid samples from mice inoculated with three different strains of Mp. Lines represent results from 5 to 10 mice per group at each time point from repeated experiments. Values shown are the means $\pm$ SD (error bars). * *P* < 0.05 between the M129-B7 and S1 groups at the time point by one-way analysis of variance (ANOVA) followed by pairwise multiple comparisons. δ *P* < 0.05 between the M129-B9 and S1 groups at the time point by one-way ANOVA followed by pairwise multiple comparisons. CFU = colony-forming units.

RESULTS

Visual Findings

The fur of all *M. pneumoniae*-infected mice developed a ruffled appearance at Days 1 and 2 after inoculation compared with uninfected control mice. No visual differences could be detected between the mice infected with different strains of *M. pneumoniae*.

M. pneumoniae Quantitative Culture

Quantitative *M. pneumoniae* BAL cultures in mice infected with S1 strain were significantly greater compared with mice infected with M129-B7 or M129-B9 strains on days 1, 2, 4, 7, and 10 after inoculation (Figure 1). Between days 1 and 7 post infection, S1 strain CFU increased 10-fold, whereas M129-B7 and B9 CFUs remained relatively stable. Between Days 7 and 10 post infection, S1 CFU were approximately 2 logs higher than M129 strains. At Day 14, S1 CFU decreased approximately 2 logs compared with their peak at Day 7, whereas B129 strains decreased approximately 1 log.

CARDS Toxin PCR

On Days 1 and 2 after inoculation, the number of mycoplasma genomes as determined by CARDS toxin PCR in BAL fluid were comparable among mice inoculated with strains M129-B7, M129-B9, and S1. However, on Days 4, 7, and 10 after inoculation, mice infected with S1 demonstrated significantly higher CARDS toxin PCR-based genomes compared with the M129-B7 group with a peak for the S1 strain on Day 7 (Figure 2). At that time we detected PCR differences of approximately

Figure 2. ADP-ribosylating and vacuolating cytotoxin (CARDS) real-time polymerase chain reaction (PCR) of bronchoalveolar lavage (BAL) fluid samples from mice inoculated with SP4, M129-B7, M129-B9, and S1 strains of *Mycoplasma pneumoniae*. Lines represent results from 6 to 10 mice per group at each time point from repeated experiments. Values shown are the medians and the 25th to 75th percentile (error bars). * $P < 0.05$ between M129-B7 and SP4 groups at the time point by Kruskal-Wallis test followed by pairwise multiple comparisons. ^ $P < 0.05$ between M129-B9 and SP4 groups at the time point by Kruskal-Wallis test followed by pairwise multiple comparisons. + $P < 0.05$ between S1 and SP4 groups at the time point by Kruskal-Wallis test followed by pairwise multiple comparisons. * $P < 0.05$ between M129-B7 and S1 groups at the time point by Kruskal-Wallis test followed by pairwise multiple comparisons. ^ $P < 0.05$ between M129-B9 and S1 groups at the time point by Kruskal-Wallis test followed by pairwise multiple comparisons. + $P < 0.05$ between M129-B7 and M129-B9 groups at the time point by Kruskal-Wallis test followed by pairwise multiple comparisons.

6- to 60-fold between S1- and M129-infected mice, consistent with BAL CFU determinations.

CARDS Toxin Protein Concentration

On Days 1, 2, and 4 after inoculation, CARDS toxin concentrations in BAL fluid were not significantly different among mice inoculated with strains M129-B7, M129-B9, and S1. On Day 7, the concentrations of CARDS toxin in BAL fluid of mice infected with strain S1 peaked and were significantly higher than the strain M129-B7 or M129-B9 groups (Figure 3).

Lung Histopathology

Compared with M129-B7-infected, M129-B9-infected, and uninfected control mice, S1 strain-infected mice exhibited significantly greater HPS on Days 2, 7, 10, and 14 after inoculation (Figure 4).

At Day 4 after inoculation, the M129-B7- and M129-B9-infected lungs had visually less severe pneumonitic alveolar exudate (neutrophils and macrophages), perivascular lymphocytic infiltrates, and peribronchiolar lymphocytic infiltrates compared with the more prominent and confluent lesions evident in the S1 strain infected lungs (Figure 5).

The comparative lung pathology at Day 7 after inoculation is depicted in Figure 6. The M129-B7-infected lungs exhibited pneumonitic alveolar exudate along with accompanying prominent perivascular and peribronchiolar lymphocytic infiltrates; the pneumonia pattern in these lungs was localized and peribronchiolar, and did not exhibit the larger, more confluent alveolar disease again seen with the S1 strain (Figure 6). The M129-B9-infected lungs on Day 7 showed lesser peribronchiolar and perivascular lymphoid infiltrates with minimal to no pneumonia (Figure 6).

Figure 3. ADP-ribosylating and vacuolating cytotoxin (CARDS) concentration in bronchoalveolar lavage (BAL) fluid sample from mice inoculated with SP4, M129-B7, M129-B9, and S1 strains of *Mycoplasma pneumoniae*. Lines represent results from 6 to 10 mice per group at each time point from repeated experiments. Values shown are the medians and the 25th to 75th percentile (error bars). * $P < 0.05$ between M129-B7 and SP4 groups at the time point by Kruskal-Wallis test followed by pairwise multiple comparisons. ^ $P < 0.05$ between M129-B9 and SP4 groups at the time point by Kruskal-Wallis test followed by pairwise multiple comparisons. + $P < 0.05$ between S1 and SP4 groups at the time point by Kruskal-Wallis test followed by pairwise multiple comparisons. * $P < 0.05$ between M129-B7 and S1 groups at the time point by Kruskal-Wallis test followed by pairwise multiple comparisons. ^ $P < 0.05$ between M129-B9 and S1 groups at the time point by Kruskal-Wallis test followed by pairwise multiple comparisons. + $P < 0.05$ between M129-B7 and M129-B9 groups at the time point by Kruskal-Wallis test followed by pairwise multiple comparisons.

Figure 4. Lung histopathology score (HPS) from mice inoculated with SP4, M129-B7, M129-B9, and S1 strains of *Mycoplasma pneumoniae*. Lines represent results from 7 to 10 mice per group at each time point from repeated experiments. Values shown are the medians and the 25th to 75th percentile (error bars). * $P < 0.05$ between M129-B7 and SP4 groups at the time point by Kruskal-Wallis test followed by pairwise multiple comparisons. ^ $P < 0.05$ between M129-B9 and SP4 groups at the time point by Kruskal-Wallis test followed by pairwise multiple comparisons. + $P < 0.05$ between S1 and SP4 groups at the time point by Kruskal-Wallis test followed by pairwise multiple comparisons. * $P < 0.05$ between M129-B7 and S1 groups at the time point by Kruskal-Wallis test followed by pairwise multiple comparisons. ^ $P < 0.05$ between M129-B9 and S1 groups at the time point by Kruskal-Wallis test followed by pairwise multiple comparisons. γ $P < 0.05$ between M129-B7 and M129-B9 groups at the time point by Kruskal-Wallis test followed by pairwise multiple comparisons.

The comparative lung pathology at Day 14 after inoculation is depicted in Figure 7. At this time point, the M129-B7-infected lungs had minimal sites of pneumonia and less numerous foci of

perivascular and peribronchiolar lymphoid aggregates, whereas the M129-B9-infected lungs had only rare sites of lymphocytes or were near normal (Figure 7). In comparison, the S1-infected lungs revealed persistent diffuse perivascular and peribronchiolar lymphoid infiltrates (Figure 7).

Cytokines/Chemokines/Growth Factors

BAL concentrations of IL-1α, IL-12, IFN-γ, macrophage inflammatory protein-1α, and keratinocyte-derived chemokine (KC) were notably significantly higher in mice infected with strain S1 compared with mice infected with strains M129-B7 or M129-B9, as well as uninfected control mice (Figures 8a–8e). Small but significant differences were also found between strains for BAL concentrations of tumor necrosis factor (TNF)-α, IL-1β, IL-2, IL-5, IL-6, IL-13, IL-17, IP-10, monokine induced by gamma interferon (MIG), KC, and fibroblast growth factor (FGF) basic (data not shown), which paralleled Figure 8. No differences were found between strains for IL-4, IL-10, and granulocyte/macrophage colony-stimulating factor (GM-CSF) GM-CSF concentrations.

Correlations at Day 7 of Infection

Because many variables reached their maximum mean or medium at Day 7 of infection, statistical correlation of variables was performed by combining the data for all strains at this time point. *M. pneumoniae* quantitative culture significantly correlated with CARDS toxin concentration (correlation coefficient = 0.92, $P \leq 0.0001$, $n = 33$) and HPS (correlation coefficient = 0.81, $P \leq 0.0001$, $n = 37$). HPS significantly correlated with CARDS toxin concentration (correlation coefficient = 0.75, $P \leq 0.0001$, $n = 36$). In addition, CARDS toxin PCR significantly correlated with *M. pneumoniae* quantitative culture (correlation coefficient = 0.98, $P \leq 0.0001$, $n = 33$).

DISCUSSION

The recent observation that *M. pneumoniae* possesses an ADP-ribosylating and vacuolating cytotoxin (designated CARDS

Figures 5. Representative lung histopathology from mice 4 days after inoculation with M129-B7, M129-B9, and S1 strains of *Mycoplasma pneumoniae*. a = arteriole; b = bronchiole; v = vein; Hematoxylin and eosin; ×10 and inset ×40.

Figure 6. Representative lung histopathology from mice 7 days after inoculation with M129-B7, M129-B9, and S1 strains of *Mycoplasma pneumoniae*. Hematoxylin and eosin, X2.

toxin) provides a mechanism to explain the host cell injury observed with *M. pneumoniae* infection (23). The respiratory tract pathogens *Corynebacterium diphtheriae* and *Bordetella pertussis* both produce ADP-ribosylating toxins critical in the pathogenesis of their respective diseases. The control of clinical diphtheria with the use of diphtheria toxoid (inactivated ADP-ribosylating diphtheria toxin) in immunizations attests to the essential role of this toxin in causing disease (31). The role of CARDS toxin in *M. pneumoniae*-associated disease remains to be dissected.

This study sought to compare the relationship of quantitative cultures, CARDS toxin production, cytokine response, and disease manifestations of mice infected with three different strains of *M. pneumoniae*. We demonstrated that all three strains produced CARDS toxin; however, pulmonary disease severity was associated with strain-dependent replication and persistence and concentration of CARDS toxin produced. The concentration of CARDS toxin in the BAL of mice inoculated with strain S1 was significantly greater than that of mice inoculated with the other two strains and appeared to be directly related to strain S1 colonization and survival properties. Interestingly, this is consistent with replication and survival properties of S1, especially

during the early stages of mycoplasma colonization and establishment of infection. This virulence-related property of S1 has been repeatedly observed by us (unpublished data). At later days post infection, values for *M. pneumoniae* quantitative culture, CARDS toxin concentration, and HPS strongly correlated for all strains. In addition, mice inoculated with strain S1 displayed the greatest histologic lung inflammation. Although these results implicate CARDS toxin as contributing to the severity of *M. pneumoniae* disease, they do not clearly establish direct causality.

Along with the finding of significantly greater histologic pulmonary inflammation with strain S1, this strain also induced significantly greater concentrations of IL-12 and IFN- γ compared with the M129-B7 and M129-B9 strains, with no differences detected between strains for IL-4 concentrations. These results reconfirm the findings of recent investigations that have shown a significant role for IL-12 and IFN- γ in the immunopathogenesis of inflammation in *Mycoplasma* respiratory infection (27, 28, 32–35). It should be noted that the cytokine immunopathogenesis of *M. pneumoniae* infection appears to be different between hosts with and without allergic sensitization of the airways, especially regarding pulmonary IL-4 (36).

Figure 7. Representative lung histopathology from mice 14 days after inoculation with M129-B7, M129-B9, and S1 strains of *Mycoplasma pneumoniae*. b = bronchiole; v = vein. Hematoxylin and eosin, $\times 40$.

Figure 8. (a–e) Cytokine and chemokine concentrations in bronchoalveolar lavage (BAL) fluid specimens in mice inoculated with SP4, M129-B7, M129-B9, and S1 strains of *Mycoplasma pneumoniae*. Lines represent results from 8 to 10 mice per group at each time point from repeated experiments. Values shown are the medians and the 25th to 75th percentile (error bars). * $P < 0.05$ between M129-B7 and SP4 groups at the time point by Kruskal-Wallis test followed by pairwise multiple comparisons. ^ $P < 0.05$ between M129-B9 and SP4 groups at the time point by Kruskal-Wallis test followed by pairwise multiple comparisons. † $P < 0.05$ between S1 and SP4 groups at the time point by Kruskal-Wallis test followed by pairwise multiple comparisons. * $P < 0.05$ between M129-B7 and S1 groups at the time point by Kruskal-Wallis test followed by pairwise multiple comparisons. δ $P < 0.05$ between M129-B9 and S1 groups at the time point by Kruskal-Wallis test followed by pairwise multiple comparisons. γ $P < 0.05$ between M129-B7 and M129-B9 groups at the time point by Kruskal-Wallis test followed by pairwise multiple comparisons.

In support of a contributory role of CARDS toxin in *M. pneumoniae* pathogenesis, we recently investigated the ability of recombinant CARDS toxin to elicit inflammation in the lungs of both mice and baboons. These animals responded to respiratory exposure of recombinant CARDS toxin in a dose-dependent manner with increased expression of the proinflammatory cytokines IL-1 α , IL-1 β , IL-6, IL-12, IL-17, TNF- α , and IFN- γ , as well as several growth factors and chemokines, including KC, IL-8, regulated upon activation normal T-cell expressed and secreted, and granulocyte colony-stimulating factor (G-CSF). Recombinant CARDS toxin exposure to the airways of these animals also resulted in cellular inflammatory responses characterized by a dose-dependent early vacuolization and cytotoxicity of the bronchiolar epithelium followed by a robust peribronchial and perivascular lymphocytic infiltration. Furthermore, recombinant CARDS toxin caused airway hyperreactivity in mice after toxin exposure as well as prolonged airway obstruction. The changes in airway function, cytokine expression, and cellular inflammation correlated temporally and were consistent with what has been reported in *M. pneumoniae* infection. Therefore, these findings indicate that the response to *M. pneumoniae* CARDS toxin parallels the pulmonary inflammatory responses and airway dysfunction observed with *M. pneumoniae* infection (37).

Taken as a whole, the results of this investigation indicate that the severity of pulmonary disease caused by *M. pneumoniae* can be strain- and toxin concentration-dependent. Consistent with this observation are the results attained from strains M129-B7 and M129-B9, which, given their similar background, were relatively comparable for all endpoints. This contrasts with a previous *in vitro* investigation that found that proinflammatory cytokine production was not dependent on infecting *M. pneumoniae* strain (25). Although in our investigation the S1 strain (subtype 2) was considerably more virulent than the two related M129 strains (subtype 1), it would be premature to state that all subtype 2 strains are more virulent than subtype 1 strains. However, it may be of value for future epidemiological investigations to determine the microbial characteristics of infecting *M. pneumoniae* organisms regarding CARDS toxin production (high or low) and subtype colonization and persistence, as these microbial factors may be important in clinical manifestations of infection, such as disease severity, wheezing, or encephalitis.

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