

## Tigecycline Therapy Significantly Reduces the Concentrations of Inflammatory Pulmonary Cytokines and Chemokines in a Murine Model of *Mycoplasma pneumoniae* Pneumonia<sup>∇</sup>

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*Mycoplasma pneumoniae* is one of the causative agents of atypical community-acquired pneumonia. Tigecycline belongs to a new class of glycylcycline antimicrobials that have activity against a wide range of microorganisms, including *in vitro* activity against *M. pneumoniae*. We investigated the effect of tigecycline on microbiologic, histologic, and immunologic indices in a murine model of *M. pneumoniae* pneumonia. BALB/c mice were inoculated intranasally with *M. pneumoniae* and treated subcutaneously with tigecycline or placebo for 6 days. Outcome variables included quantitative bronchoalveolar lavage (BAL) *M. pneumoniae* culture, lung histopathologic score (HPS), BAL cytokine and chemokine concentrations (tumor necrosis factor alpha [TNF- $\alpha$ ], gamma interferon [IFN- $\gamma$ ], interleukin 1 $\beta$  [IL-1 $\beta$ ], IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 [p40/p70], granulocyte-macrophage colony-stimulating factor, MIP-1 $\alpha$ , MIG, KC, MCP-1, and IP-10). BAL *M. pneumoniae* concentrations in mice treated with tigecycline (MpTige) tended to be reduced compared with mice treated with placebo (MpPI); however this did not reach statistical significance. The lung HPS was significantly lower, as well as the parenchymal-pneumonia subscore, in the MpTige mice than in the MpPI mice. MpTige mice had significantly lower BAL cytokine concentrations of IL-1 $\beta$ , IL-12 (p40/p70), IFN- $\gamma$ , and TNF- $\alpha$ ; of the chemokines, MIG, MIP-1 $\alpha$ , and IP-10 were statistically lower in MpTige mice. While tigecycline treatment demonstrated a modest microbiologic effect, it significantly improved lung histologic inflammation and reduced pulmonary cytokines and chemokines.

Antimicrobials have been used successfully for the treatment of many infectious diseases; however, the continuous increase in resistance among many bacteria in the community, as well as in the hospital setting, has led to research for new classes of antibiotics with a broader range of activity against pathogenic microorganisms (2, 6, 17, 18). Tigecycline is a novel antibiotic, a 9-*t*-butylglycylamido derivative of minocycline, which inhibits protein synthesis in the presence of tetracycline-resistant, *tet*(M)-protected ribosomes (30); it belongs to a new semisynthetic class of antimicrobials named glycylcyclines (37). It has been shown to have activity against a wide range of microorganisms, both gram-positive and gram-negative bacteria, as well as anaerobes (16, 29, 39). Moreover tigecycline has activity *in vitro* against rapidly growing mycobacteria, such as *Mycobacterium chelonae* and *Mycobacterium fortuitum*, as well as atypical respiratory pathogens, such as *Mycoplasma pneumoniae* (20, 21, 41). In animal pneumonia models, tigecycline has been shown to have effective activity against *Legionella pneumophila*, *Pseudomonas aeruginosa*, and *Streptococcus pneumoniae* pulmonary infections (33).

*M. pneumoniae* is an “atypical” bacterium that lacks a cell

wall and plays an important role in acute lower respiratory tract infections in children and adults; approximately 20 to 30% of community-acquired pneumonia in the general population is due to *M. pneumoniae* (1, 3, 27, 42). In the last few decades, *M. pneumoniae* respiratory infection has drawn increasing attention for its association with reactive airway disease and asthma (22–24). Clinical studies have shown that therapy with macrolides or tetracyclines, which are considered the drugs of choice for *M. pneumoniae* (11), is able to reduce the morbidity of pneumonia, shorten the duration of symptoms, and decrease the recurrence of wheezing (5, 7, 8, 12, 25, 26). However, even though treatment with macrolide, ketolide, and peptide deformylase inhibitor antimicrobials significantly improves pulmonary inflammation in animal *M. pneumoniae* pneumonia investigations, *M. pneumoniae* is not eradicated from the lungs in these *in vivo* investigations (10, 15, 31, 32). Of note, in these experimental investigations examining the effects of antimicrobials for *M. pneumoniae* pneumonia, a disassociation is often found between the microbiologic response and the significant improvement observed in other markers of disease severity.

While tigecycline demonstrates *in vitro* activity against *M. pneumoniae*, tigecycline therapy *in vivo* for *M. pneumoniae* pneumonia has not been evaluated. In the present study, we investigate the effect of tigecycline on microbiologic, histologic, and immunologic (pulmonary cytokine and chemokine) parameters in our established murine model of *M. pneumoniae* pneumonia (9, 10, 14, 15).

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## MATERIALS AND METHODS

**Organism and growth conditions.** *M. pneumoniae* (ATCC 29342) was reconstituted in SP4 broth and subcultured after 24 to 48 h in a flask containing 20 ml of SP4 medium at 37°C. Approximately 72 h later, the broth turned orange, the supernatant was decanted, and 2 ml of fresh SP4 broth was added to the flask. A cell scraper was used to harvest the adherent mycoplasmas from the bottom of the flask. This achieved an *M. pneumoniae* concentration in the range of 10<sup>8</sup> to 10<sup>9</sup> CFU/ml (determined by plating dilutions on SP4 agar). Aliquots were stored at -80°C. All SP4 media contained nystatin (50 units/ml) and ampicillin (1.0 mg/ml) to inhibit the growth of potential contaminants.

**Animals and inoculation.** Animal guidelines were followed in accordance with the Institutional Animal Care and Research Advisory Committee. BALB/c mice were obtained from Charles River Laboratories, who confirmed their mycoplasma- and murine-virus-free status. The mice were housed in the animal care facility of our institution in filter-top cages and allowed to acclimate to their new environment for 1 week. Isoflurane, an inhaled anesthetic, was used for sedation during inoculation. Two-month-old female BALB/c mice were intranasally inoculated to achieve aspiration once (day zero) with 10<sup>7</sup> CFU of *M. pneumoniae* in 50 µl SP4 broth. All mice were housed in the same animal room and received identical daily care for the duration of the experiment.

**Tigecycline administration, experimental design, and sample collection.** Starting 1 day after *M. pneumoniae* inoculation, the mice were treated subcutaneously once daily for six consecutive days with tigecycline or placebo (sterile 0.9% sodium chloride). Tigecycline (Wyeth Pharmaceuticals, Madison, NJ) was reconstituted with sterile 0.9% sodium chloride. Tigecycline was administered at a dosage of 10 mg/kg of body weight (0.2 mg in 0.2 ml per mouse) on the first day of treatment, followed by 5 mg/kg of body weight (0.1 mg in 0.2 ml per mouse) for the remaining 5 days of therapy. Groups of mice were evaluated after 1, 3, and 6 days of therapy. Samples were obtained from a total of 10 mice per treatment group (two treatment groups: mice treated with tigecycline and mice treated with placebo) at each time point from repeated identical experiments.

The mice were euthanized for sample collection by cardiac puncture after being anesthetized with an intraperitoneal injection of 75 mg of ketamine per kg and 5 mg of acepromazine per kg. Bronchoalveolar lavage (BAL) specimens were obtained by infusing 500 µl of SP4 broth through a 25-gauge needle into the lungs via the trachea, followed by aspiration of this fluid into a syringe. Whole-lung specimens (including the trachea and both lungs) were collected and fixed with a 10% buffered formalin solution for histologic evaluation. Samples were taken approximately 24 h after the last tigecycline dose.

**Culture.** Twenty-five microliters of undiluted BAL sample and serial 10-fold dilutions of BAL fluid in SP4 broth (50 µl of undiluted BAL sample in 450 µl SP4 broth was used for the initial dilution; subsequent dilutions were done in similar fashion) were immediately cultured on SP4 agar plates at 37°C, while the remaining undiluted BAL fluid specimens were stored at -80°C. Quantification was performed by counting colonies on plated specimens and expressed as log<sub>10</sub> CFU per milliliter. Our laboratory has previously compared BAL and homogenized whole-lung cultures (unpublished data). The two sites follow the same kinetics time course or "curve" over time. Although there is an absolute difference between the two sites in terms of colonies counted, the absolute numbers of colonies counted at the two sites differ by a constant factor over time. In addition, homogenized lung tissue is known to have inhibitors of *M. pneumoniae* growth, which makes the detection and quantification of low concentrations of *M. pneumoniae* impossible in homogenized lung tissue.

**Histopathology.** Lung tissue was fixed in buffered formalin and whole-mount sections of paraffin-embedded lungs were stained with hematoxylin and eosin. The histopathologic score (HPS) was determined by a single pathologist who was unaware of the treatment status of the animals from which specimens were taken. The HPS was based on individual grading of peribronchiolar/bronchial infiltrate (percentage of sites and quality), bronchiolar/bronchial luminal exudate, perivascular infiltrate (percentage of sites), and parenchymal pneumonia (neutrophilic alveolar infiltrate); after each of these was graded, the subscores were compiled for the HPS. This HPS system assigned values from 0 to 26: the higher the score, the greater the inflammatory changes in the lung (4). The parenchymal-pneumonia subscore was based on grading of the neutrophilic alveolar infiltrate (scoring: 0, no parenchymal pneumonia; 3, patchy parenchymal pneumonia; 5, patchy and confluent parenchymal pneumonia).

**Measurement of cytokines in BAL fluid.** 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. The cytokines and chemokines examined and their levels of sensitivity were as follows: tumor necrosis factor alpha (TNF-α), 5 pg/ml; gamma interferon (IFN-γ), 1 pg/ml;

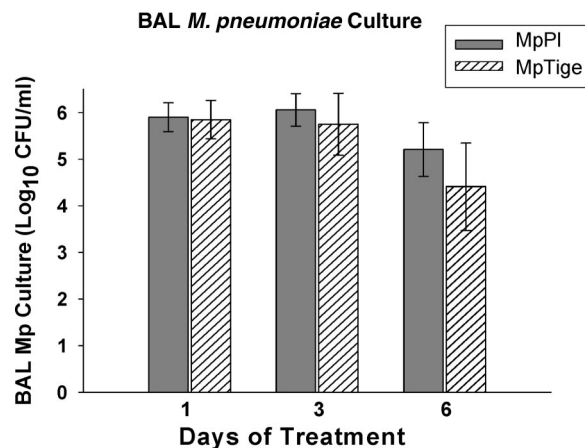


FIG. 1. Quantitative *M. pneumoniae* cultures of BAL fluid specimens from MpTige or MpPI mice. The values shown are means  $\pm$  standard deviations (error bars). The data represent results from two separate experiments with 10 mice in each group at each time point.

interleukin 1 $\beta$  (IL-1 $\beta$ ), 10 pg/ml; IL-2, 15 pg/ml; IL-4, 5 pg/ml; IL-5, 10 pg/ml; IL-6, 10 pg/ml; IL-10, 15 pg/ml; IL-12 (p40/p70), 15 pg/ml; granulocyte-macrophage colony-stimulating factor, 10 pg/ml; MIP-1 $\alpha$ , <15 pg/ml; MIG, <15 pg/ml; KC, <15 pg/ml; MCP-1, <5 pg/ml; and IP-10, <40 pg/ml.

**Statistics.** For all statistical analysis, Sigma Stat 2003 software (SPSS Science, San Rafael, CA) was used. The *t* test was used to compare the values of the different groups of animals treated with tigecycline versus placebo at the same time point if the data were normally distributed. In the instances where the data were not normally distributed, the Mann-Whitney rank sum test was used for this comparison. Two-way analysis of variance (ANOVA) was used to compare values for the different groups of animals treated with tigecycline versus placebo over 1, 3, and 6 days of treatment if the data were normally distributed. A comparison was considered statistically significant if the *P* value was <0.05.

## RESULTS

**BAL culture.** Quantitative cultures were positive in all mice infected with *M. pneumoniae*. *M. pneumoniae* concentrations in mice treated with tigecycline (MpTige) tended to be lower than in mice treated with placebo (MpPI) after 6 days of therapy (*t* test; *P* = 0.07); however, these results did not reach statistical significance by *t* test or two-way ANOVA (Fig. 1).

**Histopathology.** MpTige mice had significantly reduced lung HPS (*P* = 0.035) and parenchymal-pneumonia subscores (*P* = 0.004) compared with MpPI mice over 1, 3, and 6 days of treatment by two-way ANOVA (Fig. 2A and B). In addition, MpTige mice had significantly reduced lung HPS (*t* test; *P* = 0.002) compared with MpPI mice after 3 days of therapy (Fig. 2A). The parenchymal-pneumonia subscore (scoring of neutrophilic alveolar infiltrate alone) of the overall HPS was also significantly lower in MpTige mice than in MpPI mice (*t* test; *P* = 0.004) after 3 days of therapy (Fig. 2B). All other subscores of the overall HPS were not significantly different between the treatment groups.

**BAL cytokines and chemokines.** MpTige mice had significantly reduced concentrations of IL-1 $\beta$  (*P* = 0.043), IL-12 (*P* < 0.001), IFN- $\gamma$  (*P* = 0.016), and MIG (*P* = 0.044) compared with MpPI mice over 1, 3, and 6 days of treatment by two-way ANOVA (Fig. 3A, B, and C and 4A). By *t* test, MpTige mice had significantly reduced concentrations of IL-12 (p40/p70) (*P* < 0.001), IFN- $\gamma$  (*P* = 0.004), TNF- $\alpha$  (*P* < 0.001), and IL-1 $\beta$

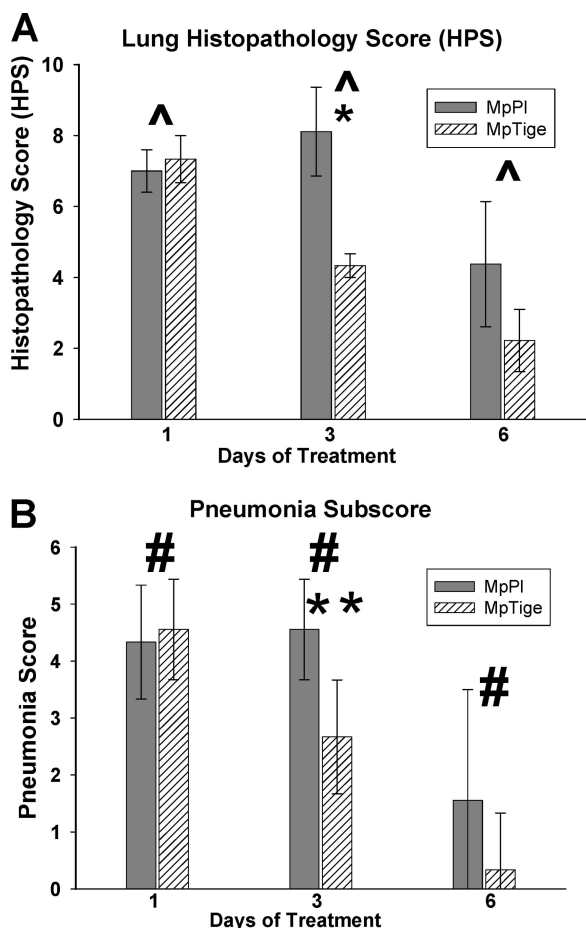


FIG. 2. Lung HPS (A) and pneumonia subscore (B) of MpTige or MpPI mice. The values shown are means  $\pm$  standard deviations (error bars). The data represent the results from two separate experiments with 10 mice in each group at each time point.  $\wedge$ ,  $P = 0.035$  by two-way ANOVA; #,  $P = 0.004$  by two-way ANOVA; \*,  $P = 0.002$  for HPS between MpPI mice by  $t$  test; \*\*,  $P = 0.004$  for pneumonia subscore between MpTige and MpPI mice by  $t$  test.

( $P = 0.005$ ) compared with MpPI mice after 3 days of therapy (Fig. 3A, B, C, and D). No significant differences were found for the BAL concentrations of IL-2, IL-4, IL-5, IL-6, IL-10, and granulocyte-macrophage colony-stimulating factor in the MpTige mice compared with the MpPI mice. By  $t$  test, among the chemokines after 3 days of therapy, concentrations of MIG ( $P = 0.047$ ), MIP-1 $\alpha$  ( $P = 0.002$ ), and IP-10 ( $P = 0.004$ ) were significantly lower in MpTige mice than in the MpPI mice (Fig. 4A, B, and C); no significant differences in the BAL fluid concentrations of KC and MCP-1 were found in the MpTige mice compared with the MpPI mice.

## DISCUSSION

This investigation demonstrates that therapy with tigecycline in an experimental model of *M. pneumoniae* lower respiratory tract infection significantly lessens disease severity by reducing histologic pulmonary inflammation, as well as cytokines and chemokines. Although quantitative *M. pneumoniae* cultures tended to be smaller in tigecycline-treated mice, there was not

a significant reduction in quantitative *M. pneumoniae* cultures for mice treated with tigecycline compared to those treated with placebo. This discrepancy observed for tigecycline therapy between quantitative microbiologic culture results and pulmonary inflammation, as measured by histopathology scores/cytokines/chemokines has also been observed with other antimicrobial classes (macrolide, ketolide, and peptide deformylase inhibitor; all microbial protein synthesis inhibitors) in the treatment of *M. pneumoniae* experimental infection (10, 15, 32). Although it has been shown in randomized, double-blind, placebo-controlled clinical trials that tetracycline class antimicrobials significantly improve markers of disease severity in *M. pneumoniae* pneumonia, a recent clinical investigation found that persistent *M. pneumoniae* carriage following acute infection is not affected by antibiotic therapy (26, 28). Thus, it is a known characteristic of *M. pneumoniae* pneumonia that with antimicrobial therapy a disassociation is often seen between microbiologic outcome and disease severity outcome.

The exact mechanism for this disassociation is not known, but it may be related to effects of therapy on microbial protein synthesis or other virulence factors or is perhaps due to a host immunomodulating action. Tigecycline, as well as macrolides, ketolides, and peptide deformylase inhibitors, are microbial protein synthesis inhibitors. Our laboratory hypothesizes that these antimicrobials primarily act to reduce pulmonary inflammation by interfering with the production of *M. pneumoniae* virulence proteins (such as the recently described ADP-ribosylating, vacuolating toxin of *M. pneumoniae* [19]) and not primarily by reduction of bacterial numbers, although there is certainly a strong association between the two effects. For instance, in the current investigation, quantitative *M. pneumoniae* culture results were not statistically different between the tigecycline and placebo treatment groups; however, treatment with tigecycline likely affects the replication frequency of *M. pneumoniae*, which could alter activation of the host immune system. In addition, direct host immunomodulation by these protein synthesis inhibitor antimicrobials could be hypothesized to be the mechanism for the observed dissociation; however, our laboratory's previous investigations with clarithromycin do not support this hypothesis (15). The discrepancy observed in our study between the antimycoplasmal outcome and the anti-inflammatory outcome illustrates the likely importance of factors (host factors and microbial factors) beyond solely the presence of the microorganism in the pathogenesis of *M. pneumoniae* respiratory infection.

The differences between tigecycline-treated mice and placebo-treated mice were most pronounced at 3 days of therapy, while at 6 days of therapy the differences between tigecycline-treated mice and placebo-treated mice were not significant by  $t$  test. Our mouse model is not a lethal model, just as *M. pneumoniae* infection is generally not a lethal infection in humans. However, in the mouse model, similar to clinical investigations of *M. pneumoniae* pneumonia in humans, antimicrobial therapy statistically significantly reduces the severity of pulmonary disease at early time points compared with placebo. It is unusual for children and adults to die from *M. pneumoniae* pneumonia, although it does rarely occur, with resultant case reports. In placebo-controlled, double-blind, randomized clinical investigations of antimicrobial agents for *M. pneumoniae* in adults, appropriate antimicrobial therapy significantly reduces

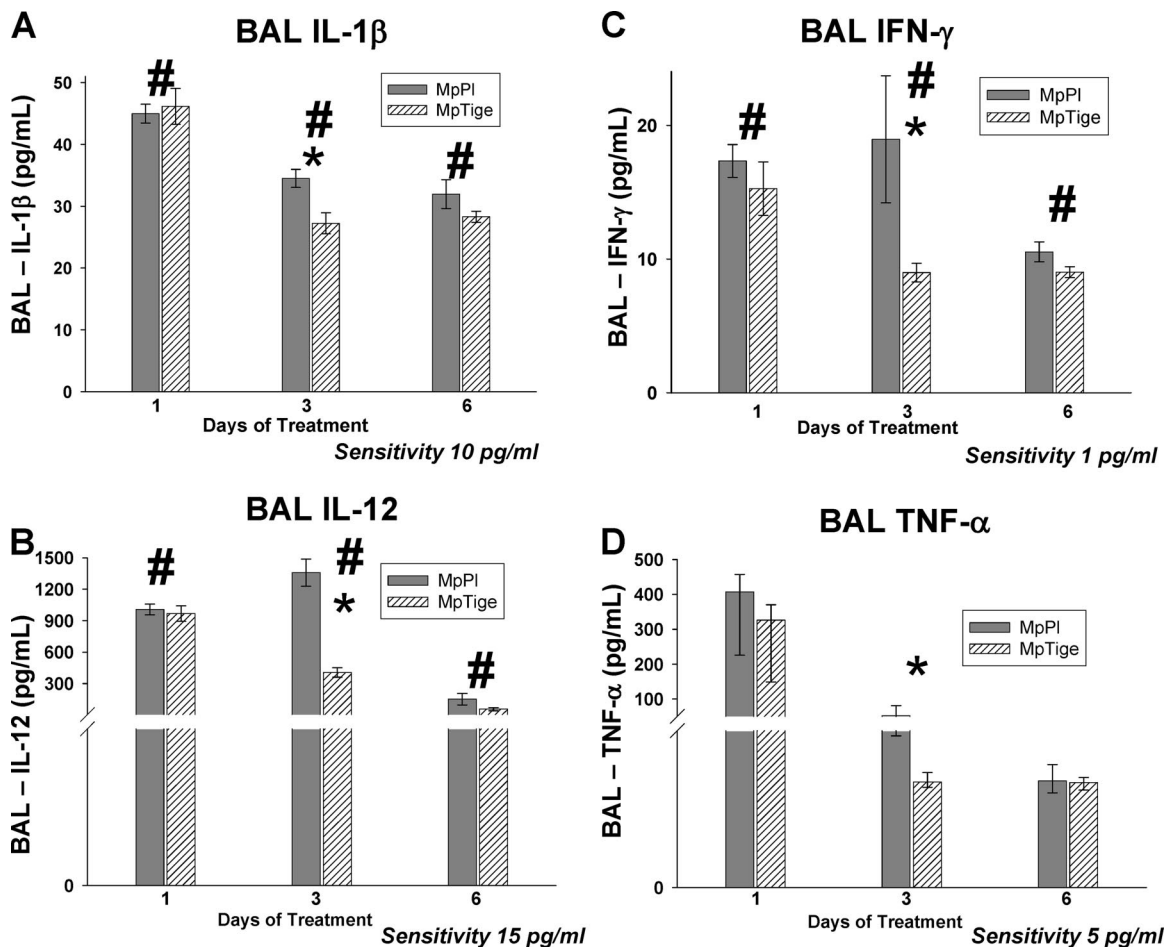


FIG. 3. Cytokine concentrations in BAL fluid specimens from MpTige or MpPI mice. The values shown are means  $\pm$  standard deviations (error bars). The data represent results from two separate experiments with 10 mice in each group at each time point. (A) #,  $P = 0.043$  by two-way ANOVA; \*,  $P = 0.005$  by  $t$  test. (B) #,  $P < 0.001$  by two-way ANOVA; \*,  $P < 0.001$  by  $t$  test. (C) #,  $P = 0.016$  by two-way ANOVA; \*,  $P = 0.004$  by  $t$  test. (D) \*,  $P < 0.001$  by  $t$  test.

the duration of fever, rales, cough, malaise, and abnormal chest radiographs. Additionally, it has been shown that appropriate antimicrobial therapy significantly reduces the duration of hospitalization (26). The chronic persistence of low-level *M. pneumoniae* infection in the airway after resolution of the acute respiratory infection, with or without appropriate therapy, has been demonstrated in animal models, as well as in humans (36), and it has been hypothesized that this persistence of the microorganism has a major role in the pathogenesis of *M. pneumoniae*-associated asthma and reactive airway disease (13).

It is possible that a higher dosage or a longer duration of treatment with tigecycline in our investigations would have demonstrated a microbiological effect, along with improvements in markers of disease severity. The approved adult tigecycline dosage is an initial dose of 100 mg intravenously followed by 50 mg every 12 h, which with a 60-min infusion gives a serum maximum concentration of 0.63  $\mu\text{g/ml}$  with an  $\text{AUC}_{0-24}$  (area under the curve from 0 to 24 h) of 4.70  $\mu\text{g} \cdot \text{h/ml}$ . Tigecycline concentrations in lung tissue have been found to be 8.6-fold higher than serum concentrations in adult studies, and the AUC in alveolar cells has been found to be

approximately 78-fold higher than the AUC in serum (Tygacil [tigecycline] prescribing information; Wyeth Pharmaceuticals Inc.). For our investigation, we chose to administer tigecycline subcutaneously at a dosage of 10 mg/kg of body weight on the first day of treatment, followed by 5 mg/kg of body weight daily for the remaining 5 days of therapy. In a neutropenic mouse *S. pneumoniae* thigh infection model, a tigecycline dosage of 3 mg/kg given subcutaneously resulted in a serum maximum concentration of 0.42  $\mu\text{g/ml}$  (40). Based on data from Wyeth Pharmaceuticals, 5 mg/kg of tigecycline in a mouse pneumonia model resulted in an AUC of 2.9  $\mu\text{g} \cdot \text{h/ml}$  (personal communication from Wyeth Pharmaceuticals). Given the above-mentioned mouse and human pharmacokinetic data, it is likely that the tigecycline pharmacokinetic values achieved in the mice in this investigation were lower than those achieved with the tigecycline dosage recommended in adults. Future studies comparing higher and lower doses of tigecycline may help to determine the pharmacodynamics of tigecycline in relationship to the noted reductions in disease severity. In addition, the dose frequency may be an important factor in the pharmacodynamics of tigecycline, as tigecycline is dosed twice a day in people but was only dosed once daily in this investigation in

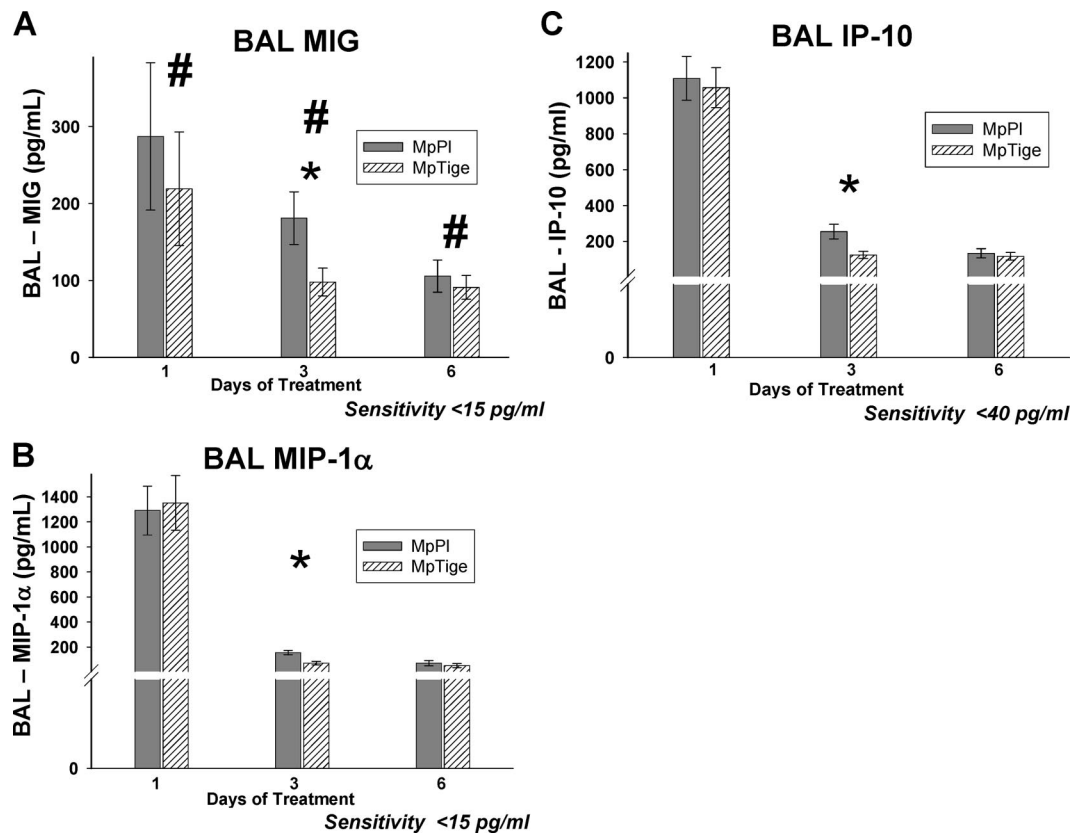


FIG. 4. Chemokine concentrations in BAL fluid specimens from MpTige or MpPI mice. The values shown are means  $\pm$  standard deviations (error bars). The data represent results from two separate experiments with 10 mice in each group at each time point. (A) #,  $P = 0.044$  by two-way ANOVA; \*,  $P < 0.047$  by  $t$  test. (B) \*,  $P = 0.002$  by  $t$  test. (C) \*,  $P = 0.004$  by  $t$  test.

mice. The MIC of tigecycline for *M. pneumoniae* has been found to range from 0.06 to 0.25  $\mu\text{g/ml}$  (20).

To our knowledge, this is the first report that investigates the effect of tigecycline on cytokines and chemokines. The significantly reduced concentrations of IL-12 (p40/p70), IFN- $\gamma$ , TNF- $\alpha$ , IL-1 $\beta$ , MIG, MIP-1 $\alpha$ , and IP-10 with tigecycline therapy is similar to what our laboratory has observed with therapy with other antimicrobial classes (macrolides, ketolides, and peptide deformylase inhibitors) in the treatment of *M. pneumoniae* experimental infection (10, 15, 31, 32). The ability of antimicrobials to reduce pulmonary cytokines and chemokines in *M. pneumoniae* infection does not appear to be specific to a particular protein synthesis inhibitor class of antimicrobials. The sum of our investigations of experimental *M. pneumoniae* respiratory infection indicates that pulmonary disease severity correlates directly with T-helper 1, proinflammatory, and chemotactic cytokines and not with T-helper 2 cytokines (9, 14, 34, 35, 38).

In conclusion, this investigation demonstrated that tigecycline therapy has a significant effect on pulmonary inflammation in an experimental model of *M. pneumoniae* respiratory infection, resulting in decreased T-helper 1, proinflammatory, and chemotactic cytokines, as well as histologic lung inflammation. The significant reduction in pulmonary inflammation occurred without a corresponding significant antimicrobial effect. Future studies will further characterize and explore the mech-

anisms responsible for this discrepancy between the anti-inflammatory effects and the antimicrobial effects observed with various classes of protein synthesis inhibitor antimicrobials.

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