Toll-Like Receptor 2 Mediates Fatal Immunopathology in Mice During Treatment of Secondary Pneumococcal Pneumonia Following Influenza

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Host inflammatory responses contribute to the significant immunopathology that occurs during treatment of secondary bacterial pneumonia following influenza. We undertook the present study to determine the mechanisms underlying disparate outcomes in a mouse model with β -lactam and macrolide antibiotics. Lysis of superinfecting bacteria by ampicillin caused an extensive influx of neutrophils into the lungs resulting in a consolidative pneumonia, necrotic lung damage, and significant mortality. This was mediated through Toll-like receptor (TLR) 2 and was independent of TLR4 and the *Streptococcus pneumoniae* cytotoxin pneumolysin. Treatment with azithromycin prevented neutrophil accumulation and rescued mice from subsequent mortality. This effect was independent of the antibacterial activity of this macrolide since dual therapy with ampicillin and azithromycin against an azithromycin-resistant strain also was able to cure secondary pneumonia. These data suggest that strategies for eliminating bacteria without lysis coupled with immunomodulation of inflammation should be pursued clinically.

Secondary bacterial pneumonia (SBP) is a significant cause of morbidity and mortality during influenza epidemics and pandemics [1–3]. During the 1918 pandemic bacterial pneumonia complicated >95% of fatal cases from well-characterized autopsy series [4]. More recently, SBP was detected in 25%–56% of patients with severe or fatal disease during the 2009 H1N1 pandemic when comprehensive attempts to determine the etiology of lower respiratory tract disease were undertaken [5–10]. *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Streptococcus pyogenes* were the predominant secondary pathogens present in these series. SBP from *S. pneumoniae* is associated with both severe disease

and death in patients with influenza [5, 6]. Mortality during the 2009 pandemic was high among seriously ill patients (14%–46%) despite the use of antibiotics in 95%–99% of those cases [5, 6, 11–13].

The 2007 guidelines on treatment of pneumonia published by the Infectious Diseases Society of America and the American Thoracic Society advise the use of β -lactam agents as first-line therapy for SBP following influenza [14]. This recommendation is based on the predominantly Gram-positive bacterial etiology and paucity of “atypical” pathogens such as *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* found in association with influenza in epidemiologic series. During the 2009 pandemic, ampicillin and ceftriaxone were the most commonly utilized antimicrobial agents in both Argentina and the United States [11, 15]. Although antibiotics were used in almost all severely ill patients, this treatment did not significantly improve outcomes [5, 6]. We have argued recently that cell wall-active antibacterial agents such as β -lactams are not the most appropriate treatment for SBP when it follows influenza [16, 17]. Rapid lysis of Gram-positive bacteria upon

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exposure to these drugs releases numerous proinflammatory bacterial components containing pathogen-associated molecular patterns (PAMPs), such as cell wall and cytotoxins [18, 19]. These are recognized by the innate immune system, triggering an inflammatory burst and potentially exacerbating the ongoing inflammation that is characteristic of influenza lung infections [20, 21].

Our previous studies in mice were designed to assess potential treatment options for SBP in preclinical models [16, 22]. In a model of pneumococcal SBP in mice, the β -lactam agent ampicillin was ineffective at rescuing infected animals from mortality despite rapid clearance of bacteria from the lungs [22]. We hypothesized that release of pneumococcal PAMPs such as cell wall and pneumolysin, with resulting immunopathology during bacterial lysis, was responsible for the poor outcomes with antibacterial therapy alone [16]. In support of this hypothesis, use of the bacteriostatic protein synthesis inhibitor clindamycin, either alone or 24 hours prior to the introduction of ampicillin, resulted in significantly better survival than did ampicillin alone [16]. The macrolide antibiotic azithromycin was consistently the best treatment option in the model. Use of protein synthesis inhibitors, in comparison to cell wall-active agents, resulted in decreased levels of proinflammatory cytokines in the lungs, decreased influx of inflammatory cells into the airways, and reduced lung damage [16].

The results of our prior animal experiments left 2 crucial, unanswered questions regarding the mechanisms underlying the disparate outcomes observed with cell wall-active agents and protein synthesis inhibitors. First, what was the mechanism through which azithromycin obtained better outcomes? Because azithromycin is not an ideal candidate for treatment of pneumonia in humans due to resistance, understanding the mechanism should allow further development of rational therapeutics. Second, what inflammatory pathways are responsible for the immunopathology observed following β -lactam therapy? Understanding this should allow directed therapy by targeting specific host pathways [17]. We undertook the present study to elucidate the mechanistic basis of these effects, which were clinically apparent in the model.

METHODS

Infectious Agents

Influenza virus A/Puerto Rico/8/34 (PR8) was grown in Madin-Darby canine kidney cells. *S. pneumoniae* strain A66.1 (type 3) was transformed with the lux operon (Xenogen Corp) [23]. Inactivation was accomplished by a 5-minute suspension in 90% ethanol. An azithromycin-resistant variant of the luciferase-containing A66.1 strain was generated by serial passage in increasing concentrations of the antibiotic under a protocol approved by the Institutional Biosafety Committee of St Jude. The minimum inhibitory concentrations (MICs) for the

susceptible and resistant strains were 0.023 and 0.023 $\mu\text{g/mL}$ for ampicillin, and 0.064 and 5 $\mu\text{g/mL}$ for azithromycin, respectively. The azithromycin-resistant, ampicillin-sensitive phenotype was confirmed by culture in Todd Hewitt broth in the presence of ampicillin (100 $\mu\text{g/mL}$), azithromycin (3 or 100 $\mu\text{g/mL}$), or combinations followed by plating of aliquots at intervals to determine colony counts. An isogenic, pneumolysin-deficient mutant of A66.1 (*ply*⁻) was created by complete gene replacement with an erythromycin resistance cassette via the SOEing method (primer sequences available on request) [24]. Complete deletion was confirmed by sequencing.

Mice

Seven- to 8-week-old female BALB/c, C3H/OuJ, and C3H/HeJ mice, as well as male and female C57Bl/6 mice (wild type and lacking Toll-like receptor [TLR] 4 or TLR2), were obtained from Jackson Laboratories and used in biosafety level 2 facilities in these studies in a manner in accordance with the guidelines of the Committee on Care and Use of Laboratory Animals. All experimental procedures were done under general anesthesia with inhaled isoflurane 2.5% (Baxter Healthcare Corp) and were approved following review by the St Jude Institutional Animal Care and Use Committee.

Infectious Model

Infectious agents were administered intranasally in a volume of 100 μL (50 μL per nostril) to anesthetized mice as described previously [16]. Influenza virus was given at a dose of 30 (BALB/c, C3H-derived strains) or 60 (C57Bl/6-derived strains) median tissue culture infective doses (TCID₅₀). Except where otherwise noted, *S. pneumoniae* was given 7 days later at a dose of 200 or 700 colony-forming units (CFU). Following secondary bacterial infection, bioluminescent imaging was used to follow development and progression of pneumonia using defined parameters to ensure experimental groups were balanced with all mice at an early stage of pneumonia as described [16, 20, 23].

Treatment of SBP

Treatment was started upon identification of pneumonia by bioluminescent imaging as described [22]. Mice were given antibiotics (Sigma-Aldrich) intraperitoneally twice daily in divided doses (ampicillin 200 mg/kg/day; clindamycin 120 mg/kg/day; vancomycin 60 mg/kg/day) or once daily (azithromycin 10 mg/kg/day first dose, 5 mg/kg/day thereafter) for a total of 7 days or were mock-treated with the diluent, phosphate-buffered saline (PBS). This dose of azithromycin is predicted to result in lung tissue levels above the MIC for the sensitive strain but below the MIC for the resistant strain [25].

Histopathology

Microscopic evaluation of lungs was performed by an experienced veterinary pathologist (K. L. B.) blinded to study purpose and design and to group composition. A semiquantitative

grading scheme was utilized to score 2 parameters, the overall character of the pneumonic process and the pathology specifically observed in the interstitium and terminal airways as previously described [20, 21].

Statistical Analyses

Comparison of survival between groups of mice was done with the log-rank χ^2 test on the Kaplan–Meier survival data. Comparisons of histopathology scoring between groups were done using analysis of variance (ANOVA). A *P* value of <.05 was considered significant for these comparisons. SigmaStat for Windows software, version 3.11 (SysStat Software) was utilized for all statistical analyses.

RESULTS

Outcomes Based on Mode of Antibiotic-Mediated Killing

A model of treatment of SBP based on earlier work [16, 22, 26] was developed for this study. Female BALB/c mice infected with a sublethal dose of influenza virus (30 TCID₅₀) followed 7 days later with PBS, or mock-infected with PBS followed 7 days later with a sublethal dose of *S. pneumoniae* (200 CFU), had 100% survival (Figure 1). When pneumococcal challenge followed influenza virus infection, however, pneumonia detectable through bioluminescent imaging resulted, and all superinfected mice died within 4 days of exposure to the second agent. Similar results were obtained in CH3/OuJ and C57Bl/6 mice, although

slightly larger doses of both infectious agents were needed for C57Bl/6 mice (60 TCID₅₀ of virus and 700 CFU of *S. pneumoniae*). Treatment with the cell wall–active agents ampicillin or vancomycin resulted in rapid elimination of bacteria from the lungs of mice by bioluminescent imaging (data not shown), but only 30%–50% of mice survived. Treatment with the protein synthesis inhibitors clindamycin or azithromycin resulted in significantly better outcomes, with 100% survival seen in the azithromycin group.

The lungs of mice from treatment groups were examined microscopically. Mice infected only with a sublethal dose of *S. pneumoniae* had mild perivascular infiltration of lymphocytes but no pathologic changes suggestive of pneumonia (Figure 2). Virus-infected mice exhibited hypertrophy and hyperplasia of epithelial cells lining the airways, accompanied by a moderate lymphocytic infiltrate and edema. Lungs from mice coinfectd with influenza virus and bacteria followed by treatment with ampicillin showed a severe fibrinopurulent broncho-interstitial pneumonia. The inflammatory response was characterized by a copious neutrophilic infiltrate accompanied by edema, fibrin deposition, and necrosis of the alveolar walls and bronchiolar epithelium at the alveolar bronchiolar junction. In mice treated with azithromycin, however, alveolar wall necrosis and fibrin deposition were not seen. The broncho-interstitial pneumonia was less severe and was characterized by an inflammatory response composed predominately of foamy macrophages within the alveolar spaces and only minimal

Figure 1. Treatment of secondary bacterial pneumonia following influenza. Groups of 9–12 BALB/c (A), C3H/OuJ (B), or C57Bl/6 (C) mice were infected sequentially 7 days apart. Mice received either phosphate-buffered saline (PBS) as a mock infection followed by *Streptococcus pneumoniae* bacteria (B = bacteria-only group), influenza virus followed by PBS (V = virus-only group), or virus and then bacteria (V + B = virus then bacteria group) and were subsequently followed for survival. The dotted line indicates the time of secondary challenge. An asterisk (*) indicates a significant difference (*P* < .05) in survival compared with the other groups by log-rank test on the Kaplan–Meier survival data. D, Groups of mice were infected with influenza and challenged 7 days later with *S. pneumoniae*. Following development of pneumonia as determined by bioluminescent imaging (E), mice were mock-treated with PBS (None; *n* = 6), or treated with azithromycin (Azi; *n* = 13), clindamycin (Clinda; *n* = 12), vancomycin (Vanc; *n* = 12), or ampicillin (Amp; *n* = 13) and followed for survival. An asterisk (*) indicates a significant difference (*P* < .05) in survival compared with the azithromycin, clindamycin, and no treatment groups by log-rank test on the Kaplan–Meier survival data.

Figure 2. Histopathology of lungs of mice treated for secondary bacterial pneumonia following influenza. Groups of mice were either mock-infected with phosphate-buffered saline (PBS) and challenged 7 days later with *Streptococcus pneumoniae* (B) (A), infected with influenza virus and then mock-challenged 7 days later with PBS (V) (B), or infected with influenza virus, challenged 7 days later with *S. pneumoniae* (V + B), and treated with ampicillin (Amp) (D) or azithromycin (Azi) (E). Hematoxylin and eosin stains of representative lung section taken 48 hours after the start of antibiotic therapy are shown at 40 $\times$ magnification. C, Mean histopathology scores $\pm$ standard deviation of lungs graded on a scale of severity from 0 to 4 are displayed. An asterisk (*) indicates a significant difference ($P < .05$) compared with the ampicillin-treated group. The scoring system showing differences in histopathologic scores in (C) is derived from [22] where a score of 1 indicates mild perivascular inflammation and few leukocytes in alveolar spaces, a 2 indicates moderate perivascular inflammation and numerous leukocytes in alveolar spaces with mild alveolar wall thickening, a 3 indicates marked perivascular inflammation, marked leukocyte infiltrates in alveolar spaces, moderate thickening of alveolar walls, focal alveolar wall necrosis and airway epithelial necrosis with fibrin and edema, and a 4 indicates extensive coalescing inflammation with interstitial and airway necrosis, fibrin, edema, hemorrhage, and consolidation.

numbers of neutrophils (Figure 2). Overall, the character and severity of the pneumonic process resembled that engendered by virus alone and was significantly less in azithromycin-treated animals than in ampicillin-treated animals.

We hypothesized that the superior outcomes seen with azithromycin derived from its purported anti-inflammatory activity [27]. We reasoned that if this anti-inflammatory property was independent of the mechanism of antibacterial killing, then it should still be able to contribute to rescue of superinfected mice with severe lung inflammation in the setting of resistance. Therefore, we developed an azithromycin-resistant version of the pneumococcal strain being used in our model of SBP and repeated our antibiotic treatment studies (Figure 3). As before, untreated mice succumbed to infection within a few days of

secondary challenge, while ampicillin therapy only modestly improved survival (9 of 18 [50%] survived), despite complete elimination of bacteria from the lungs of mice by bioluminescent imaging (data not shown). Azithromycin was unable to rescue mice superinfected with the resistant strain due to overgrowth of bacteria in the lungs (0 of 5 [0%] survived). However, the addition of azithromycin to ampicillin resulted in survival of 16 of 18 mice (89%), an outcome significantly better than ampicillin alone, despite lack of additive effects on killing in vitro. We conclude that the anti-inflammatory activity of azithromycin is independent of its antibacterial effect and can contribute to improved outcomes from SBP, even when it is caused by resistant strains of *S. pneumoniae*.

Figure 3. Treatment of azithromycin-resistant *Streptococcus pneumoniae* following influenza. *A*, *S. pneumoniae* strain A66.1 (*B*) and an azithromycin-resistant mutant (AziR) were exposed to ampicillin at a concentration of 100 µg/mL (Amp) or azithromycin at a concentration of 3 µg/mL (Azi 3) or 100 µg/mL (Azi 100) or combinations. Aliquots were plated to determine the concentration of viable bacteria 0, 2, 4, 6, 8, 10, 12, 16, and 24 hours later. Representative curves from a single experiment (out of 4) are shown. *B*, Groups of mice were infected with influenza and challenged 7 days later with this azithromycin-resistant variant of *S. pneumoniae*. Following development of pneumonia, mice were mock-treated with phosphate-buffered saline (None; *n* = 18), or treatment was initiated with azithromycin (Azi; *n* = 5), ampicillin (Amp; *n* = 18), or azithromycin plus ampicillin (*n* = 18). An asterisk (*) indicates a significant difference (*P* < .05) compared with the other treatment groups. CFU, colony-forming units.

Induction of TLR4 Through Release of Pneumolysin From Lysed Bacteria

Expression of the pneumococcal cytotoxin pneumolysin has been associated with activation of TLR4 [28]. We hypothesized that release of pneumolysin from lysed bacteria following exposure to β-lactams was driving the inflammatory response that resulted in mortality during treatment of SBP. If our hypothesis was correct, we reasoned that either knock-out of pneumolysin from the bacteria or lack of TLR4 expression in the mice would prevent the fatal inflammatory burst during ampicillin treatment of SBP. We therefore generated a pneumolysin-deficient mutant (*pln*-) of the A66.1 strain used in the model and infected TLR4-sufficient (C3H/OuJ) and TLR4-deficient (C3H/HeJ) mice with and without antecedent influenza virus infection. The *pln*-mutant was attenuated in mice compared with the wild type (WT) (Figure 4 and data not shown). In the presence of virus, however, all mice developed pneumonia and both the *pln*-mutant and the WT were lethal in 100% of mice when 1×10^4 CFU of bacteria was used. Expression of TLR4 provides protection against pneumococcus in this mouse model, since the WT was attenuated in TLR4-sufficient C3H/OuJ mice, but

Figure 4. Role of pneumolysin and Toll-like receptor (TLR) 4 in survival from secondary bacterial pneumonia following influenza. *A*, Groups of 6 BALB/c mice were left uninfected (B, Pln-) or were infected with influenza virus (V + B, V + Pln-) and challenged 7 days later with either wild-type (B) or pneumolysin-deficient (Pln-) *Streptococcus pneumoniae* at the doses (in colony-forming units [CFU]) indicated and followed for survival. An asterisk (*) indicates a significant difference (*P* < .05) compared with the other groups. *B*, Groups of 9–10 C3H/OuJ (wild type) or C3H/HeJ (TLR4-deficient) mice were infected with 1×10^4 CFU of either wild-type or pneumolysin knockout (KO) bacteria and followed for survival. An asterisk (*) indicates a significant difference (*P* < .05) compared with C3H/HeJ groups. *C*, Groups of C3H/OuJ and C3H/HeJ mice were infected with influenza virus and challenged 7 days later with 2×10^4 of either wild-type or pneumolysin KO *S. pneumoniae*. Five to six mice from each group were left untreated, while the remaining 8–13 mice per group were treated with ampicillin (Amp) upon development of pneumonia by bioluminescent imaging. An asterisk (*) indicates a significant difference (*P* < .05) compared with the corresponding untreated group. No significant differences were evident between any treated groups.

caused 80% mortality in TLR4-deficient C3H/HeJ mice. However, this attenuation was not based on TLR4 recognition of pneumolysin, as the *pln*-mutant showed a similar pattern of disease with increased virulence in the absence of TLR4. We conclude from these experiments that pneumolysin is important for pathogenesis in the lungs of mice, but the relatively greater

Figure 5. Role of Toll-like receptor (TLR) 2 and TLR4 in treatment of secondary bacterial pneumonia following influenza. **A**, Wild-type or TLR4-deficient C57Bl/6 mice were infected with influenza (V) and then challenged 7 days later with 1×10^4 *Streptococcus pneumoniae* (B). Four mice per group were left untreated. The remaining 12–13 mice were treated with ampicillin (amp). An asterisk (*) indicates a significant difference ($P < .05$) compared with the corresponding untreated group. **B**, Wild-type or TLR2-deficient C57Bl/6 mice were infected with influenza and then challenged 7 days later with 1×10^4 *S. pneumoniae*. Four mice per group were left untreated. The remaining 17–18 mice were treated with ampicillin. An asterisk (*) indicates a significant difference ($P < .05$) compared with the other 3 groups.

virulence of *S. pneumoniae* seen in mice deficient for TLR4 is not due to a lack of TLR4 interactions with pneumolysin.

Having established the parameters of the model using the *pln*-mutant and the TLR4-deficient C3H/HeJ mouse strain, we next addressed our main hypothesis. TLR4-sufficient and -deficient influenza-infected mice were challenged with WT or the *pln*-mutant and then treated with ampicillin following development of pneumonia. Lack of TLR4 did not alter disease in mice preinfected with influenza virus; no mice survived coinfection at these doses (Figure 4). Treatment with ampicillin rescued between 29% and 50% of superinfected mice, with no statistically significant differences evident between treated groups regardless of whether the WT or *pln*-mutant was utilized. To confirm this finding, we repeated this experiment with WT bacteria in C57Bl/6 mice and TLR4^{-/-} mice. In both the parental strain C57Bl/6 and TLR4^{-/-} mice, only 4 of 13 (31%) animals survived treatment of pneumococcal SBP following influenza (Figure 5). We conclude from these data that neither release of pneumolysin from lysed bacteria nor activation of TLR4 is responsible for the inflammatory burst and accompanying immunopathology observed during treatment of SBP with β -lactam agents.

Importance of TLR2 for Mortality

We next examined the importance of a second Toll-like receptor, TLR2, during lysis of pneumococci in the setting of influenza virus infection. TLR2 recognizes cell wall breakdown products of *S. pneumoniae* and thereby participates in innate immune responses through induction of inflammation [29]. C57Bl/6 wild-type or TLR2^{-/-} mice were infected with influenza, then challenged with pneumococci. No differences in survival were seen in untreated WT or TLR2^{-/-} mice. Similarly to previous experiments, 9 of 18 (50%) WT C57Bl/6 mice survived infection and treatment with ampicillin (Figure 5). However, lack of TLR2 provided significant protection as 16 of 17 (94%) mice survived. We conclude that TLR2 contributes to the fatal inflammatory response observed in mice during treatment of SBP with β -lactams.

The mechanism underlying worse outcomes in TLR2-sufficient mice is of obvious interest. We used inactivated *S. pneumoniae* to probe the relationship between provision of cell wall-associated PAMPs and disease in the context of influenza. Administration of 1×10^8 CFU equivalents of inactivated bacteria was assessed in mice with and without antecedent influenza virus infection. Inactivated bacteria alone did not cause significant disease, and 100% of mice survived (Figure 6). Preinfection with influenza virus, however, primed mice for significant mortality (2 of 8 mice [25%] survived). This experiment was repeated in WT and TLR2^{-/-} mice. Only 4 of 9 (44%) WT C57Bl/6 mice survived exposure to inactivated bacteria in the setting of influenza, while 7 of 9 (77%) TLR2^{-/-} mice survived. Thus, live bacteria are not required either for disease in the secondary pneumonia model following pneumonia or to observe TLR2-mediated mortality during treatment. These data suggest that TLR2 recognition of PAMPs contained in inactivated bacteria, likely cell wall breakdown products, is responsible for immunopathology and mortality during β -lactam-based treatment regimens for SBP following influenza.

DISCUSSION

S. pneumoniae was the most common bacterial copathogen identified in patients with SBP during the 2009 H1N1 pandemic [5, 8–11], as has been the case for previous pandemics [1]. Despite the use of antibiotics directed against *S. pneumoniae* in the great majority of cases, the mortality rate for severely ill patients was high and coinfection with pneumococcus was associated with more rapid progression to severe disease and with death [5, 6, 9]. Our data suggest that TLR2-mediated recognition of bacterial PAMPs such as cell wall triggered by lysis of the superinfecting pathogens contributes to these severe outcomes. Alternate strategies using non- β -lactam antibiotics alone or in combination may be superior to currently recommended agents [14, 16]. A review of hospitalized patients early in the 2009 pandemic found that ceftriaxone was the most commonly used

Figure 6. Inactivated bacteria as a surrogate for live bacteria with influenza. *A*, Groups of BALB/c mice were mock-infected with phosphate-buffered saline (PBS) and challenged 7 days later with 1×10^8 colony-forming unit equivalents of ethanol-inactivated *Streptococcus pneumoniae* (IB), infected with influenza virus and then mock-challenged 7 days later with PBS (V), or infected with influenza virus and challenged 7 days later with inactivated bacteria (V + IB). An asterisk (*) indicates a significant difference ($P < .05$) compared with the other groups. *B*, Groups of wild-type or Toll-like receptor 2 (TLR2)-deficient C57Bl/6 mice were infected with influenza virus and then challenged 7 days later with inactivated bacteria (V + IB) or mock-challenged with PBS (V). An asterisk (*) indicates a significant difference ($P < .05$) compared with the other 3 groups.

antibiotic, although azithromycin was also commonly given [11]. Unfortunately, antibiotic use, particularly the specific antibiotics employed, is poorly documented in most published series describing outcomes for influenza, and no data are available in humans comparing different antibiotic choices for treatment of SBP.

Azithromycin alone is unlikely to be a viable choice for empiric therapy of SBP, as resistance rates of community isolates of both *S. pneumoniae* and *S. aureus* are high [30]. However, alternative antibiotics with similar proposed anti-inflammatory effects, such as other advanced macrolides, ketolides, and glycyclines, and a broader spectrum of activity might be realistic candidates [31]. In addition, the finding in this report that azithromycin retained its anti-inflammatory activity against a resistant strain when used in combination therapy suggests that there might be clinical benefit independent of antibiotic susceptibility patterns. Indeed, 2 retrospective studies [32, 33] and 1 prospective, multicenter trial [34] have concluded that the addition of a macrolide to a β -lactam results in a significant reduction in mortality (compared with β -lactam therapy alone) in

adults with bacteremic pneumococcal pneumonia. A more recent study evaluated azithromycin monotherapy for community-acquired pneumonia and reported a 75% rate of good clinical response, despite a $>85\%$ rate of azithromycin resistance [35]. Thus, there is some clinical evidence supporting a benefit for the anti-inflammatory activity of azithromycin against pneumococcal pneumonia, although the specific scenario of SBP following influenza has not been evaluated outside of these preclinical mouse studies.

The anti-inflammatory effects of azithromycin appear to be mediated through a decreased influx of leukocytes, primarily neutrophils, into the lungs [36–38]. Expression of multiple genes linked to inflammatory responses, including cytokines, are altered in both epithelial cells and alveolar macrophages [37, 39, 40]. In mouse models of lipopolysaccharide exposure, parainfluenza virus bronchiolitis, and *Pseudomonas aeruginosa* pneumonia, the net effects of azithromycin-mediated changes in gene expression are altered macrophage migration patterns and activation states, decreased expression of inflammatory mediators, and reduced neutrophilia within airways [36–39]. In our mouse model of treatment of SBP following influenza, the most prominent difference in lung histopathology was the profound neutrophilic infiltration that produced a consolidated pneumonia in ampicillin-treated mice, compared with a relative paucity of neutrophils in azithromycin-treated animals. This is supported by the finding of decreased total neutrophils in the bronchoalveolar lavage fluid of azithromycin-treated mice relative to ampicillin-treated mice in our previous study [16]. Thus, the mechanism of azithromycin rescue of superinfected mice is likely altered regulation of neutrophil homing into infected lungs, resulting in decreased immunopathology.

Several detailed autopsy series have recently been published describing primary viral and secondary bacterial pneumonia in humans during the 2009 H1N1 pandemic [7–10, 41]. The findings of these studies are consistent with descriptions of autopsy data from previous pandemics [4, 42, 43]. The most prominent feature common to the great majority of cases was diffuse alveolar damage, characterized by edema, hyaline membranes, and fibrinous exudate. Tracheitis and bronchitis were common, often accompanied by submucosal inflammatory infiltrates [8]. A distinct subset of patients had histopathological evidence of bronchopneumonia characterized by a dense neutrophilic infiltrate in bronchiolar and alveolar lumens, sometimes accompanied by extensive, severe necrosis of bronchiolar walls [7–9]. Most subjects found to have bronchopneumonia histologically also had specific evidence of a bacterial lung infection, and most subjects with evidence of bacterial coinfection also had these characteristic findings of bronchopneumonia [8, 9]. Overall, between 25% and 56% of autopsied cases from these series were judged to have bacterial coinfections complicating the primary viral pneumonia. These histopathologic findings are similar to those described here in mice that develop

SBP following influenza. Mice infected with the mouse-adapted H1N1 strain A/Puerto Rico/8/34 influenza virus alone have comparable features of diffuse alveolar damage. Superinfected animals, particularly those treated with ampicillin, have prominent airway necrosis and extensive consolidation characterized by the presence of neutrophils.

We therefore propose the following model to understand treatment effects during SBP following influenza. A generally proinflammatory state exists in the lung early after influenza virus infection due to recognition by a combination of pattern recognition receptors that may include retinoic acid-inducible gene-I, TLR3 [44], TLR4 [45], and/or TLR7/8 [46, 47]. Superinfection by *S. pneumoniae* induces protective inflammatory responses mediated through TLR4 in response to pneumolysin production [28], and through TLR2 in response to cell wall components [29]. Treatment with a β -lactam antibiotic rapidly lyses pneumococci, releasing copious amounts of cell wall components in forms that are readily recognized by TLR2, generating a chemokine and cytokine storm [48] and driving the influx of neutrophils into the lungs. Neutrophil-mediated tissue destruction, manifested most prominently in this model as severe airway necrosis, contributes to morbidity and mortality [49]. TLR4-mediated recognition of pneumolysin is not involved in this antibiotic-triggered cytokine storm. Alternatively, treatment with azithromycin halts bacterial growth without lysis, allowing innate host responses to return to homeostasis during resolution of the bacterial infection. Through changes to alveolar macrophage gene expression [36, 37], neutrophil influx is blunted and the ensuing cytokine storm and tissue destruction are averted.

We conclude that treatment algorithms for bacterial pneumonia following influenza should be reassessed. Clinical studies evaluating treatment strategies encompassing the dual goals of bacterial control without lysis and immunomodulatory activity should be undertaken. Development of specific agents targeting components of the TLR2 signaling pathway may be useful in this context.

Notes

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