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Secondary bacterial pneumonia is a significant cause of morbidity and mortality during influenza, despite routine use of standard antibiotics. Antibiotic-induced immunopathology associated with bacterial cell wall lysis has been suggested to contribute to these poor outcomes. Using *Streptococcus pneumoniae* in a well-established murine model of secondary bacterial pneumonia (SBP) following influenza, we stratified disease severity based on pneumococcal load in the lungs via in vivo bioluminescence imaging. Ampicillin treatment cured mice with mild pneumonia but was ineffective against severely pneumonic mice, despite effective bacterial killing. Adjunctive dexamethasone therapy improved ampicillin-induced immunopathology and improved outcomes in mice with severe SBP. However, early dexamethasone therapy during primary influenza infection impaired lung adaptive immunity as manifest by increased viral titers, with an associated loss of its protective functions in SBP. These data support adjunctive clinical use of corticosteroids in severe cases of community-acquired pneumonia.

Keywords. Antibiotics; corticosteroids; dexamethasone; immunomodulation; influenza; pneumonia; *Streptococcus pneumoniae*.

Influenza and pneumonia are among the leading causes of morbidity and mortality in both children and adults in the United States [1]. Globally, pneumonia is the leading killer of children outside of the neonatal period [2]. Secondary bacterial pneumonia (SBP) is a common complication of influenza, and outcomes are worse in coinfections than in uncomplicated cases [3, 4]. In the 1918 influenza pandemic, the majority of deaths were complicated by SBP, and *Streptococcus pneumoniae* was the predominant pathogen [5]. However, the advent of antibiotic therapy and the widespread use of effective pneumococcal vaccines in the developed world decreased the frequency of and improved outcomes from pneumococcal disease over the last century. Nonetheless, *S. pneumoniae* continues to be the leading bacterial cause of community-acquired pneumonia (CAP) and SBP [6]. During the 2009 H1N1 influenza pandemic, SBP was present in 25% to 50% of severe or fatal cases [3, 7].

These severe outcomes including mortality are seen in patients with SBP despite appropriate antimicrobial treatment [4, 8]. Although globally increased rates of antimicrobial resistance among common respiratory bacterial pathogens are a concern [9, 10], the mechanism of killing utilized by standard antibiotics also appears to affect treatment outcomes [11, 12]. Ampicillin is considered the first-line therapy for bacterial pneumonia in hospitalized children, including those coinfected with influenza [13]. In a murine model of SBP following influenza, treatment with ampicillin was shown to induce robust inflammatory lung injury [14]. This poor treatment outcome, despite its efficient
bactericidal activity, was attributed to rapid bacterial cell wall lysis and the release of copious amounts of bacterial pathogen-associated molecular patterns, including cell wall fragments, potentiating the inflammatory response in the coinfected lungs [12]. Trials in adults have demonstrated an improved cure rate for inpatient CAP treatment with either a fluoroquinolone or combination therapy with a β-lactam and a macrolide [15]. Our mouse studies suggest that, along with broadened coverage of atypical pathogens, the addition of the macrolide to the recommended treatment regimen reduces the inflammatory response by decreasing the massive influx of neutrophils and accompanying tissue damage characteristic of SBP [12]. However, treatment with antibiotics alone appears to still be suboptimal in some patients and in these mouse models.

Due to their potent anti-inflammatory and diverse immunomodulatory activities, corticosteroids have been widely used to treat many inflammatory and immune diseases [16, 17]. However, the clinical use of corticosteroids as an adjunctive therapy for treating pneumonia has been controversial. Some randomized controlled clinical trials suggest beneficial activity in treating CAP with a significant reduction in the length of hospital stay [18, 19] or a decrease in mortality of patients with septic shock [20]. However, others have shown no benefit or harmful outcomes [21, 22]. Based on these data and expert opinion, the World Health Organization (WHO) discouraged corticosteroid treatment during the 2009 influenza pandemic [23]. Collectively, there is not currently strong evidence in the literature for recommending adjunctive corticosteroid therapy. Nevertheless, steroids are often used in clinical practice, particularly in severe cases with acute lung injury, suggesting a need for further study [24–26].

We hypothesized that adjunctive corticosteroid therapy would improve the poor outcomes associated with antibiotic treatment of SBP following influenza infection through modulation of inflammatory responses. We tested our hypothesis using a well-established murine coinfecion model in which we could monitor the progression of SBP in vivo [27]. To parse the relationship between disease severity and outcomes, we categorized coinfected mice based on the lung bacterial load at the onset of antibiotic treatment to control for its impact on different treatment outcomes. We report here that dexamethasone therapy administered during the inflammatory period of severe SBP has a beneficial effect on outcomes. Early therapy, during primary influenza, is not beneficial and may enhance the infection by interfering with immune responses to the virus.

**MATERIALS AND METHODS**

**Infectious Agents**

We used the St. Jude strain of mouse-adapted influenza virus A/Puerto Rico/8/34 (H1N1). It was passaged once through Madin-Darby canine kidney (MDCK) cells, stocks were grown by a single passage through eggs, and allantoic fluid was stored at −80°C. The viral titers of the stocks were characterized via median tissue culture infective dose (TCID$_{50}$) assay in MDCK cells.

*S. pneumoniae* A66.1, a type 3 encapsulated strain, was engineered to express luciferase (Kevin Francis and Jun Yu, Xenogen Corporation, Alameda, CA). Stocks of pneumococci were prepared and quantified as shown previously [27]. In all instances, the infectious dose administered was confirmed by serial dilution and plating of the bacterial suspension on blood agar plates.

**Mice**

Six- to 8-week-old female BALB/c mice (Jackson Laboratory, Bar Harbor, ME) were maintained in a Biosafety Level 2 facility in the Animal Resource Center at St. Jude. Animals were given general anesthesia that consisted of 2.5% inhaled isoflurane (Baxter Healthcare Corporation, Deerfield, IL) prior to all interventions, and all studies were approved by the Animal Care and Use Committee at St. Jude.

**Infectious Model**

Infectious agents were diluted in sterile phosphate-buffered saline and administered intranasally in a volume of 100 μL (50 μL per nostril) to anesthetized mice held in an upright position. For primary influenza infection, influenza virus was given at a dose of 25 TCID$_{50}$ per 100 μL per mouse, which caused about 10% weight loss on day 7 post infection (p.i) with no mortality when given alone. To engender SBP, influenza infection was followed on day 7 p.i by bacterial challenge with 200 colony forming units of pneumococcus per mouse. Infected mice were weighed and assessed daily for illness and mortality for 7 days after pneumococcal challenge; based on preliminary studies in this model and animal care considerations, any mouse losing more than 26% of its starting body weight was euthanized and considered to have died on that day.

**Imaging and Ampicillin Treatment of Live Coinfected Mice**

Mice were imaged for 60 seconds using an IVIS CCD camera (Caliper Life Sciences, Alameda, CA) daily after pneumococcal challenge to monitor in vivo pneumococcal pneumonia development. Total photon emission from selected and defined areas within the images of each mouse was quantified using Living Image software (Caliper Life Sciences) as described previously [12, 28, 29], and expressed as the flux of relative light units per minute. Pneumonia was defined as visible bioluminescence within the thorax and detection of a flux of >11 000 relative light units per minute (RLU/min) [27]. Based on previous studies that classified detection stage of SBP into early and late detection [12, 29], we assigned mild pneumonia to mice showing thorax bioluminescence and flux of more than 11 000 but...
less than 90 000 RLU/min, while severe pneumonia was defined for mice with flux of more than or equal to 90 000 RLU/min.

Once SBP was detected, ampicillin (Sigma-Aldrich) was given intraperitoneally (i.p.) as 200 mg/kg/daily in 2 divided doses every 12 hours for 5 days.

Flow Cytometric Analysis of Immune Cells in Bronchoalveolar Lavage Fluid and Post-lavage Lungs
Following euthanasia by CO₂ inhalation, the trachea was exposed and cannulated with a 24-gauge plastic catheter (Becton Dickinson Infusion Therapy Systems, Inc., Sandy, UT). Lungs were washed, harvested, and homogenized as described previously [27]. Cell suspensions of bronchoalveolar lavage fluid (BALF) and Post-lavage lung homogenate were centrifuged at 4°C, 350 × g for 7 minutes. BALF and lung homogenate supernatants were stored at −80°C. Flow cytometry (LSRII, and LSRII Fortessa, Becton Dickinson, San Jose, CA) was performed on the cell pellets after incubation with Fc block (antimouse CD16/CD32, BD Bioscience Inc., San Jose, CA), followed by surface marker staining with a cocktail of antimouse antibodies, including CD11c (eFluor 450, F4/80 (FITC), Ly6G (PerCp-Cy5.5), and CD11b (APC-eFluor 780; eBioscience Inc., San Diego, CA), or CD3 (FITC), CD4 (APC), and CD8a (eFluor 450 or APC-eFluor 780; eBioscience Inc.). FlowJo 8.8.6 (Tree Star, Ashland, OR) was used for data analysis, where viable cells were gated from a forward scatter/side scatter (FSC/SSC) plot, then neutrophils were gated as CD11b<sup>hi</sup>Ly6G<sup>hi</sup>CD11c<sup>lo</sup>F4/80<sup>lo</sup>). T cells were gated as CD3<sup>+</sup>SSC<sup>low-int</sup>, followed by subgates of CD4<sup>+</sup>CD8<sup>−</sup> and CD4<sup>−</sup>CD8<sup>+</sup> T-cell subsets. Total viable cells were counted and absolute numbers of different cell types were calculated as described previously [27].

Dexamethasone Treatment Regimen
Dexamethasone sodium phosphate injection solution (4 mg/mL; APP Pharmaceuticals, Schaumburg, IL) was diluted by sterile phosphate-buffered saline (PBS) solution to 0.5 mg/mL. In combined treatment experiments, dexamethasone dose (2.5 mg/kg/day) or vehicle (PBS) was given i.p. once daily 2–3 hours after ampicillin injection for 5 days as an adjunctive therapy. In early treatment experiments, dexamethasone or PBS was given i.p. once daily starting from day 3 until day 13 after influenza infection.

Measurement of Viral Titers
Influenza viral titers were measured in the stored Post-lavage lung homogenate supernatants by TCID<sub>50</sub> assay in MDCK cells.

Measurement of Total Protein and Albumin Levels in BALF Supernatant
BALF supernatant aliquots were thawed and total proteins levels were measured spectrophotometrically using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). To measure albumin levels, BALF supernatant samples were diluted in sterile PBS, and albumin concentrations were measured using an enzyme-linked immunosorbent assay kit (USCN Life Science Inc, Houston, TX).

Statistical Analysis
Comparison of survival between groups of mice was done with the log-rank χ² test on the Kaplan–Meier survival data. Means of relative bioluminescence units per minute were compared between groups using analysis of variance (ANOVA). Comparisons of weight loss, immune cells numbers, protein levels, or viral titers between groups of mice were done using Mann–Whitney U test for pair-wise comparisons. A P value of <.05 was considered significant for these comparisons. Prism 4 for Windows (GraphPad Software, Inc., v. 4.03) was used for all statistical analyses.

RESULTS
Ampicillin Treatment During Secondary Pneumococcal Pneumonia Fails to Rescue Mice Despite Viral and Bacterial Clearance
To engender SBP, naive BALB/c mice were intranasally infected with a sublethal dose of influenza virus, followed by a small inoculum of pneumococcus on day 7 after influenza infection (Figure 1A). Within 48 hours after bacterial inoculation, coinfected mice developed SBP that could be detected in vivo by bioluminescence imaging. Without treatment, all coinfected mice succumbed to bacterial pneumonia within a few days (Figure 1B). To confirm the poor efficacy of a standard antibiotic treatment in rescuing mice with SBP, we started ampicillin treatment upon pneumonia detection via bioluminescence imaging. Without treatment, all coinfected mice succumbed to bacterial pneumonia within a few days (Figure 1A). As seen previously [12, 14, 30], ampicillin could only rescue about 40% of pneumonic mice (Figure 1B), despite rapid declines in bacterial burden within 12 hours after the first dose of ampicillin (Figure 1C and 1D). Viral titers were undetectable in the alveolar airspaces 24 hours after the first ampicillin dose (data not shown).

Differential Outcomes Depend on the Severity of Secondary Pneumococcal Pneumonia at the Onset of Antibiotic Treatment
To determine if the severity of SBP at the onset of antibiotic treatment affects treatment outcomes, we classified the severity of pneumonic mice prior to antibiotic treatment into mild or severe pneumonia. This classification was based on the pneumococcal load in coinfected lungs, as monitored through bioluminescent imaging as defined in the methods (Figure 2A). Ampicillin treatment was associated with disparate mortality outcomes in mice with different degrees of pneumonia at the onset of treatment (Figure 2B). All ampicillin-treated mice with severe pneumonia succumbed to pneumonia in a similar pattern to that of the untreated group. In contrast, more than...
60% of ampicillin-treated mice with mild pneumonia were rescued (Figure 2B). These differential mortality outcomes after ampicillin treatment were not due to lack of ampicillin bactericidal activity in mice with severe pneumonia; the first ampicillin dose demonstrated in vivo efficacy with rapid bacterial killing, reflected by the significant decrease in bioluminescence signals to levels comparable to uninfected mice, in a similar pattern to ampicillin-treated mice with mild pneumonia (Figure 2C). However, differential accumulation of neutrophils was observed within the alveolar airspaces of mildly and severely pneumonic mice 26 hours after the first ampicillin dose (Figure 2D). Furthermore, significantly higher levels of total proteins were detected in the BALF supernatant of ampicillin-treated mice with severe pneumonia (Figure 2E).

### Adjunctive Dexamethasone Treatment Rescues Ampicillin-Treated Mice With Severe Secondary Pneumococcal Pneumonia

Because treatment by ampicillin alone failed to rescue mice with severe pneumonia, despite its efficient bactericidal activity, we hypothesized that an anti-inflammatory agent could dampen the associated inflammatory responses and improve outcomes. Adjunctive dexamethasone therapy significantly improved the survival rate, rescuing about 70% of mice with severe pneumonia (Figure 3A). Mice with mild pneumonia also demonstrated a modest increase in survival that was not statistically significant after adjunctive dexamethasone treatment, but suffered morbidity reflected by significant delay in regaining their body weight as compared with the PBS and ampicillin-treated control group (Figure 3B). Improved outcomes with combined therapy were associated with significantly decreased neutrophil accumulation after the second dose (Figure 4A) and significantly reduced serum albumin leakage into alveolar airspaces after the first dose (Figure 4B).

### Early Dexamethasone Treatment Leads to Loss of Its Protective Activity

To test if dexamethasone administration early after influenza infection can enhance its protective activity and increase survival of ampicillin-treated pneumonic mice, we started dexamethasone or PBS treatment 3 days after influenza infection and continued thereafter until day 13 p.i using the same coinfection model. As before, ampicillin treatment was initiated upon detection of SBP. Interestingly, early dexamethasone treatment...
regimen did not induce significant improvement in the survival of ampicillin-treated mice with either mild or severe pneumonia in comparison to the ampicillin-treated groups with early mock treatment; rather, it led to loss of its protective activity (Figure 5A). In contrast, adjunctive dexamethasone therapy showed significant improvement in the survival of ampicillin-treated mice with severe pneumonia (Figure 3A). Additionally, similar to the poor morbidity outcome of adjunctive therapy in mice with mild pneumonia, early dexamethasone treatment was associated with increased body weight loss from primary influenza and delayed body weight regain after ampicillin treatment of SBP (Figure 5B).
Early Dexamethasone Treatment Suppresses Adaptive Immunity

Glucocorticoids are known to suppress adaptive immunity [31]. Therefore, we hypothesized that the increased morbidity associated with early dexamethasone therapy during primary influenza infection was due to suppressed adaptive immunity, leading to elevated influenza viral titers and increased lung injury. We measured the numbers of T cells in both BALF and Post-lavage lungs on day 7 after influenza infection under early dexamethasone or mock treatment (ie, 24 hours after the

Figure 3. Adjunctive dexamethasone treatment rescues ampicillin-treated mice with severe secondary pneumococcal pneumonia. Survival rates and body weight loss curves of ampicillin-treated mice with either severe (A, n ≥ 7) or mild (B, n ≥ 9) secondary pneumococcal pneumonia with adjunctive dexamethasone or mock (PBS) therapy. Data are expressed as the average ± SEM. *P < .05, **P < .01 compared with adjunctive PBS therapy group by log-rank test on the Kaplan–Meier survival data (A), or Mann–Whitney U test for body weight loss curve (B). Abbreviations: Dexa, dexamethasone; PBS, phosphate-buffered saline; SEM, standard error of margin.

Figure 4. Adjunctive dexamethasone therapy improves lung inflammation and pulmonary vascular permeability in mice with severe secondary pneumococcal pneumonia. A, Absolute numbers of neutrophils within alveolar airspaces of mice with severe secondary pneumococcal pneumonia, measured 26 hours after the first or second dose of dexamethasone (white-filled, n = 4 or 3, respectively) or mock therapy (dark gray-filled, n = 6 or 5, respectively) combined with ampicillin treatment. B, Albumin levels in BALF supernatant harvested from mice with severe secondary pneumococcal pneumonia 26 hours after the first or second dose of adjunctive dexamethasone (white-filled, n = 4 or 3, respectively) or mock therapy (dark gray-filled, n = 6 at both time points) combined with ampicillin treatment. The bar graphs show the median ± range. *P < .05 by Mann–Whitney U test. Abbreviations: Abs., absolute; BALF, bronchoalveolar lavage fluid; DEXA, dexamethasone; n.s, not significant; PBS, phosphate-buffered saline; sup., supernatant.
fourth dose of dexamethasone or PBS). As expected, early dexamethasone treatment significantly reduced the absolute numbers of CD4+ and CD8+ T cells in the lungs as compared with mock treatment (Figure 6A). To determine if this reduction in the respiratory pool of T cells was associated with impaired influenza viral clearance, we measured influenza viral titers on day 7 p.i. Early dexamethasone–treated mice had significantly higher influenza viral titers than mock-treated mice (Figure 6B).

**DISCUSSION**

Influenza and pneumonia caused the largest number of infectious disease–related deaths in the United States throughout the 20th century [32]. Historically, death from SBP was a
common feature of all influenza pandemics in the preantibiotic era [5]. Indeed, mortality from pneumococcal pneumonia significantly decreased after the discovery of penicillin [33]. Nevertheless, SBP still contributes significantly to morbidity and mortality during seasonal influenza and recent influenza pandemics, despite the use of effective antibiotics [7]. Furthermore, rates of mortality due to pneumococcal pneumonia have been relatively stable throughout the antibiotic era even with the routine use of more advanced β-lactam antibiotics [34, 35]. In a similar pattern, ampicillin treatment of SBP in our murine model demonstrated therapeutic failure here and in previous studies [12, 14, 30]. Despite its effective rapid bactericidal activity and significant pneumococcal clearance, ampicillin therapy failed to rescue more than 50% of mice with SBP. This has been previously attributed to rapid bacterial cell wall lysis, accompanied by release of bacterial cell wall fragments activating exuberant inflammatory responses in lungs, thereby leading to increased morbidity and mortality [12].

As the degree of severity of SBP may affect ampicillin treatment outcomes, we determined pneumococcal load in vivo using a sensitive noninvasive bioluminescence imaging to approximate pneumococcal outgrowth in the coinfecting lungs prior to treatment, then classified SBP into mild or severe based on this surrogate for pneumococcal load. Interestingly, ampicillin treatment caused disparate mortality rates in mice with different degrees of pretreatment severity of SBP. This finding suggests a direct correlation between the pneumococcal load size at the onset of antibiotic therapy and the poor survival outcomes during antibiotic treatment of SBP. This relationship was inferred through modulating the degree of lung immunopathology induced after bacterial cell lysis with ampicillin. Thus, ampicillin treatment of mice with severe SBP aggravated lung immunopathology compared to those with mild SBP. Yet, both groups of mice showed comparable rapid pneumococcal clearance after the first 2 doses of ampicillin. The differential ampicillin treatment–associated immunopathology was evident on the second day of treatment, when mice with severe SBP had significant increased accumulation of neutrophils within the alveolar airspaces, and increased pulmonary vascular permeability compared to ampicillin-treated mice with mild SBP.

Benefits of adjunctive corticosteroid therapy of CAP have been under debate for a long time [25, 36] due to conflicting results from different studies of this potential therapy [18, 19, 21, 22, 37–39]. In our murine model of SBP, adjunctive dexamethasone treatment improved survival rates of pneumonic mice. Interestingly, the best results were observed in ampicillin-treated mice with severe SBP, in which treatment-associated immunopathology was significantly attenuated. These improved outcomes of severe SBP in our murine model are consistent with several randomized controlled trials that showed the beneficial outcomes of adjunctive corticosteroid therapy are limited to severe cases of CAP in humans [18, 19, 38, 39].

Similar controversy exists on the adjunctive use of corticosteroids during treatment of acute bacterial meningitis [40]. However, a recent systemic review of 25 randomized controlled clinical trials showed beneficial outcomes of adjunctive corticosteroid therapy during treatment of acute bacterial meningitis, which were more evident in patients with pneumococcal meningitis [41]. In addition, adjunctive dexamethasone treatment significantly decreased the mortality of adult patients with bacterial meningitis, especially during the acute phase of the disease [42]. Collectively, these data may imply that adjunctive corticosteroid treatment has preferential benefits in case of severe infections where the exaggerated inflammatory responses are the pathophysiologic mediators of the disease.

However, corticosteroids have potential drawbacks, as they can cause systemic immunosuppression [17, 31]. Therefore, we tested the effect of early administration of dexamethasone during primary influenza infection to determine whether it would remain beneficial in ampicillin-treated mice with severe SBP. As expected, early dexamethasone treatment significantly decreased numbers of both CD8+ and CD4+ T cells in the lungs on day 7 after influenza infection. This was associated with delayed influenza viral clearance and accelerated morbidity progression during primary influenza infection and before secondary pneumococcal challenge. This had a negative impact on ampicillin treatment–associated fatality with eventual loss of the protective functions of the adjunctive dexamethasone regimen in mice with either mild or severe SBP. We used dexamethasone at a dose (2.5 mg/kg) that was previously shown to suppress the pulmonary inflammatory responses in various murine models of airway inflammation, such as endotoxin-induced acute lung injury, house dust–induced asthma, and allergic bronchopulmonary aspergillosis [43–45]. Other researchers have shown that dexamethasone treatment from days 3–14 after highly pathogenic avian influenza infection had no beneficial effect on acute respiratory distress syndrome caused by the H5N1 infection in mice [46], data that support our findings regarding the poor outcomes associated with early dexamethasone treatment during primary influenza infection. Additionally, our dose of dexamethasone is clinically relevant to the corticosteroid doses that showed beneficial outcomes as adjunctive therapy of adult humans with severe CAP [47–49].

Taken together, our data suggest that the general unrestricted use of adjunctive corticosteroid therapy during influenza or CAP is likely not warranted. Several factors may participate in balancing the beneficial and detrimental outcomes after systemic corticosteroid treatment, such as the drug dose, the onset and duration of treatment, the causative infectious agents, and whether the pneumonia is caused by a single bacterial or viral agent or is due to a coinfection. Our experiments support a beneficial activity of corticosteroids when combined with antibiotic treatment only in severe cases of CAP. Furthermore, our findings demonstrate that the bacterial burden during
pneumonia has an impact on treatment outcomes, with larger burdens generating more inflammation and higher mortality upon antibiotic-mediated lysis. Interestingly, our data point out a significant impact of the timing of dexamethasone therapy on its protective functions, which would be of great importance in modifying treatment protocols of severe cases of SBP following viral infections. Our findings suggest that early corticosteroid administration during primary influenza infection can worsen adaptive immunity against influenza infection, thereby increasing viral titers and consequently increasing viral-mediated lung damage. Late treatment with steroids, when virus has cleared and inflammation is driving disease, can improve outcomes. Finally, our study further advances an animal model with sophisticated tools for classifying the severity of bacterial pneumonia, which will help investigating the efficacy of different immunomodulators as adjunctive therapy, as well as different or new classes of antibiotics for treatment of SBP.

Notes

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