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Linezolid Decreases Susceptibility to Secondary Bacterial Pneumonia Postinfluenza Infection in Mice Through its Effects on IFN-γ

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Influenza infection predisposes patients to secondary bacterial pneumonia that contributes significantly to morbidity and mortality. Although this association is well documented, the mechanisms that govern this synergism are poorly understood. A window of hyporesponsiveness following influenza infection has been associated with a substantial increase in local and systemic IFN-γ concentrations. Recent data suggest that the oxazolidinone antibiotic linezolid decreases IFN-γ and TNF-α production in vitro from stimulated PBMCs. We therefore sought to determine whether linezolid would reverse immune hyporesponsiveness after influenza infection in mice through its effects on IFN-γ. In vivo dose-response studies demonstrated that oral linezolid administration sufficiently decreased bronchoalveolar lavage fluid levels of IFN-γ at day 7 postinfluenza infection in a dose-dependent manner. The drug also decreased morbidity as measured by weight loss compared with vehicle-treated controls. When mice were challenged intranasally with *Streptococcus pneumoniae* 7 d postinfection with influenza, linezolid pretreatment led to decreased IFN-γ and TNF-α production, decreased weight loss, and lower bacterial burdens at 24 h postbacterial infection in comparison with vehicle-treated controls. To determine whether these effects were due to suppression of IFN-γ, linezolid-treated animals were given intranasal instillations of rIFN-γ before challenge with *S. pneumoniae*. This partially reversed the protective effects observed in the linezolid-treated mice, suggesting that the modulatory effects of linezolid are mediated partially by its ability to blunt IFN-γ production. These results suggest that IFN-γ, and potentially TNF-α, may be useful drug targets for prophylaxis against secondary bacterial pneumonia following influenza infection. ***The Journal of Immunology*****, 2013, 191: 1792–1799.**

Although primary influenza infection causes significant morbidity and mortality worldwide, a greater appreciation for the contribution of secondary bacterial pneumonias to these statistics is starting to emerge. Studies suggest that the majority of deaths associated with the 1918 pandemic influenza strain can be attributed to secondary bacterial infections of the upper respiratory tract (1, 2). The bacterial species most often encountered during recovery from influenza infection include *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Haemophilus influenzae* (2–5). Clinical correlates for the association between influenza and pneumococcal infections are available from studies that examined the efficacy of pneumococcal vaccines. Studies show that the pneumococcal conjugate vaccine has 45% efficacy in the prevention of secondary pneumonia in pediatric patients with confirmed influenza infection, suggesting that prophylaxis against secondary pneumococcal disease may greatly reduce the morbidity and mortality associated with influenza infection (6, 7).

A thorough understanding of the mechanisms that promote immune hyporesponsiveness is critical to advancing preventative measures to decrease the incidence of secondary infection. Studies have identified several factors that may contribute to increased susceptibility as a result of influenza infection, including physical damage to the lungs (8), enhanced bacterial binding via increased platelet-activating factor (9, 10), a dysfunctional neutrophil response (11, 12), and elevated levels of IL-10 (13, 14). Other studies suggest that excess IFN-γ produced during the latter stages of influenza infection may mediate susceptibility to *S. pneumoniae* infection. Sun and Metzger (15) demonstrated that high concentrations of IFN-γ that result from the immune response are sufficient to inhibit alveolar macrophage phagocytosis of *S. pneumoniae*, both in vitro and in vivo during the first 24 h of infection. In this dual infection model, normal clearance of bacteria was restored in animals lacking IFN-γ signaling and then impaired once again when alveolar macrophages or flu-infected mice were treated with exogenous IFN-γ.

These studies suggest that the inhibition of influenza-associated IFN-γ production may be beneficial in reducing the incidence of secondary *S. pneumoniae* infection. Pharmacologic inhibition of this cytokine response could provide a means to prevent this window of hyporesponsiveness. The oxazolidinone antibiotic linezolid has been shown to have inhibitory activity against proinflammatory cytokines. Linezolid's pharmacological effects lie in its ability to bind to the 50S ribosomal subunit, thus preventing initiation of the 70S complex and inhibiting the initiation of protein synthesis (16). Linezolid treatment of whole human blood cells stimulated with LPS was shown to inhibit IFN-γ and TNF-α production in a dose-dependent manner (17). Furthermore, linezolid reduced concen-

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Abbreviations used in this article: BAL, bronchoalveolar lavage; EID_{50}, 50% egg-infectious dose; i.n., intranasal; TBLN, tracheobronchoalveolar lymph node.

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trations of IFN-γ and TNF-α in human PBMCs without altering endotoxin production in vitro (18). We therefore hypothesized that prophylactic treatment of influenza-infected mice with linezolid would reduce harmful proinflammatory IFN-γ production and limit outgrowth of a secondary pulmonary challenge with *S. pneumoniae.* Indeed, we observed that linezolid treatment of influenza-infected mice resulted in decreased weight loss, inhibition of IFN-γ and TNF-α responses, and attenuation of bacterial outgrowth following *S. pneumoniae* challenge, while not adversely affecting the adequate response to and clearance of the virus. These studies provide further rationale for the exploration of IFN-γ–targeting therapies to control and limit dangerous secondary bacterial infections.

Materials and Methods

Mouse model of viral and bacterial infection

Eight- to 10-wk-old female C57BL/6 mice were purchased from Taconic (Bar Harbor, ME). The A/Puerto Rico/8/34 (PR8) strain of influenza virus was grown in the allantoic fluid of 10-d-old embryonated specific pathogen-free chicken eggs, as previously described (19). Viral stocks were tested for common mouse pathogens and were shown to contain only influenza virus. Viral yield was quantified by titration in eggs to determine the 50% egg-infectious dose (EID_{50}). Mice were infected intranasally (i.n.) under isoflurane anesthesia with 1–2 EID_{50}/g bodyweight in a 50 μl vol HBSS. Following infection, mice were monitored daily for weight loss. When animals reached 10% body weight loss, daily s.c. injections of warmed HBSS were administered for rehydration purposes. Frozen stocks of *S. pneumoniae* strain A66.1 were thawed and immediately resuspended to appropriate inocula in 50 μl HBSS. On the morning of day 7 postinfluenza infection, animals were challenged i.n. under isoflurane anesthesia with 6×10^4 to 1.4×10^5 CFU bacteria, depending on the experimental replicate. Resultant inocula were confirmed following serial plating on 5% sheep's blood agar and overnight incubation to determine CFU counts. Each experimental replicate included four to five mice per treatment group per time point, and data were combined for multiple replicates, as noted in each figure legend.

Linezolid preparation and dosing

Based on the weight of the mice, animals were dosed with either 20, 40, or 80 mg/kg linezolid twice daily via oral gavage. Linezolid was prepared using either pure powder, or by crushing linezolid tablets (Pfizer, New York, NY). The drug was suspended in 2% methylcellulose based on a weight-to-weight calculation such that each 100 μl vehicle contained the desired amount of active drug to be administered. This dosing range was chosen based upon studies that examined the pharmacokinetic profile of linezolid in mice (20, 21). It was determined that 60 mg/kg twice-daily dosing in infected mice would most likely mimic human plasma levels of the drug. Because Takahashi et al. (17) showed that linezolid blocked cytokine secretion down to a concentration of 2 μg/ml, we predicted that this dosing range would prove effective.

Cell isolation

Mice were euthanized by exsanguination under deep anesthesia at post-infection time points. The lungs were lavaged with 1 ml aliquots of HBSS containing 3 mM EDTA to isolate alveolar cells, with the first aliquot being separated from the cellular fraction by centrifugation (deemed first wash). For experiments that did not include bacterial challenge, blood was removed from the lungs by perfusion of HBSS into the right side of the heart. Lungs were then excised, minced, and incubated with 50 U/ml DNase (Sigma-Aldrich, St. Louis, MO) and 1 mg/ml collagenase A (Sigma-Aldrich) in RPMI 1640 media containing 5% FCS. For experiments that included bacterial challenge, whole lungs were minced with a mechanical homogenizer. Tracheobronchoalveolar lymph node (TBLN) and digested lung tissue were pushed through cell strainers to obtain single-cell suspensions. Subsequently, RBCs were lysed by exposure to a hypotonic solution, and the remaining cells were washed, counted, and resuspended in HBSS.

Flow cytometry

Bronchoalveolar lavage (BAL), lung digest, and TBLN cells were washed in PBS containing 0.1% BSA and 0.02% sodium azide and incubated with appropriate concentrations of fluorochrome-conjugated Abs specific for murine CD4, CD8, CD44, or CD62L. Abs were purchased from eBioscience or BD Biosciences. For intracellular cytokine staining, 2×10^6 cells from each sample were incubated for 4 h with 50 ng/ml PMA and 500 ng/ml ionomycin, and in the presence of 10 μg/ml brefeldin A for the final 2 h. Cells were surface stained for CD4 and CD8 and fixed in 4% formalin. Cells were then permeabilized in 0.05% saponin, exposed to anti-CD16/32 Ab to block Fc binding, and incubated with fluorescently labeled anti–TNF-α, anti–IFN-γ, and anti–IL-10 Abs. Cells were washed thoroughly and resuspended in PBS for analysis. Labeled cells were analyzed using a BD LSRII Flow Cytometer System (BD Biosciences) as compared with isotype and one-color controls. Greater than 50,000 events per sample were routinely acquired. For all analyses, viable lymphocyte populations were defined on forward versus side scatter plots and further examined on dot plots examining CD4 versus CD8 expression.

Cytokine analysis by ELISA

Lung lavage cells were separated from lavage fluid in the first 1 ml lung lavage washout. ELISAs for IFN-γ and TNF-α were performed on cell-free lavage fluid using ELISA kits, according to manufacturer's instructions (eBioscience). Concentrations were normalized to total protein content in the lavage fluid, as measured using the BCA Protein Quantification Kit (Bio-Rad, Hercules, CA).

Administration of exogenous IFN-γ

In select experiments, mice were administered 2 ng rIFN-γ (PeproTech) or PBS/0.1 BSA vehicle in 25 μl i.n. under isoflurane anesthesia on days 5 and 6 postinfluenza infection. Mice were subsequently infected on day 7 with *S. pneumoniae*, as described above.

Enumeration of bacterial loads

Following mechanical homogenization, aliquots of lung were isolated before RBC lysis. The aliquots were then serially diluted in sterile water and spread plated onto 5% sheep's blood agar. After 24 h, CFU were counted and calculated to represent entire bacterial contents of the lungs.

Determination of lung viral burdens

Lungs were sterilely isolated from infected mice and frozen at −80°C until analysis. Viral burdens were determined by plaque assay on Madin Darby canine kidney cells (American Type Culture Collection, Manassas, VA), as described previously (22). Briefly, cells were grown to confluency in six-well plates in DMEM supplemented with nonessential amino acids and 10% heat-inactivated FCS (Atlanta Biologicals, Lawrenceville, GA). Ten-fold dilutions of lung homogenate were incubated with the cells for 1 h at 37°C. The cells were then washed and overlaid with DMEM in 1% Bacto Agar with 1% trypsin (Sigma-Aldrich). Three days later, the cells were fixed with 20% acetic acid, and the overlay was removed. Plaques were visualized by staining with crystal violet and counted.

Statistics

Measurements were determined for each individual mouse, and the means and SD were generated for each treatment group. Differences between experimental groups were determined using *t* tests, one-way ANOVA, or two-way ANOVA where appropriate. The Student Newman–Keuls post hoc test was used to discriminate the differences between individual groups at each time point when detected. Differences were considered statistically significant when $p < 0.05$. Analyses were performed using GraphPad Prism software package (GraphPad Software, La Jolla, CA).

Results

Linezolid inhibits IFN-γ production in a dose-dependent manner

Studies in mice demonstrate that the window of immune hyporesponsiveness following influenza infection, as defined by a secondary bacterial challenge, is associated with heightened IFN-γ production in the lungs ranging from days 7 to 10 postinfection (14, 15). Using these same time points, we therefore hypothesized that use of linezolid following influenza infection would be sufficient to limit IFN-γ responses. Animals were orally gavaged with 20, 40, or 80 mg/kg linezolid or vehicle control twice daily starting 24 h after influenza challenge. Whereas all influenza-infected animals experienced substantial weight loss over the course of the study, we consistently observed that animals treated with a dose of 80 mg/kg linezolid lost significantly less weight than vehicle-treated controls (Fig. 1A). As is typical in models of

influenza infection, weight loss began on day 4 postinfection and progressed to 15–20% loss from baseline by day 10. In the group that received the highest linezolid dose, mean losses only dropped by 12–14% for each experimental replicate. Alveolar concentrations of IFN-γ were observed to be low on day 4 postinfection, but dramatically elevated on day 7 postinfection, a timing that corresponds to the hyporesponsive window (Fig. 1B) (15). Linezolid treatment decreased IFN-γ concentrations in the BAL fluid in a dose-dependent manner in comparison with vehicle-treated controls at 7 d postinfection, the difference becoming significant in the 80 mg/kg dose group. By day 10 postinfection, IFN-γ concentrations had decreased, but were still 100- to 1000-fold higher than those observed on day 4 postinfection. These data suggest that twice-daily dosing of mice with linezolid at a dose of 80 mg/kg is optimal for inhibition of IFN-γ production at the time of peak concentration. Whereas 7 d postinfection was found to be the time of maximal TNF-α production, no statistical difference was observed in TNF-α concentration between animals being treated with any dosage of linezolid in comparison with vehicle-treated controls at any time point examined (Fig. 1C).

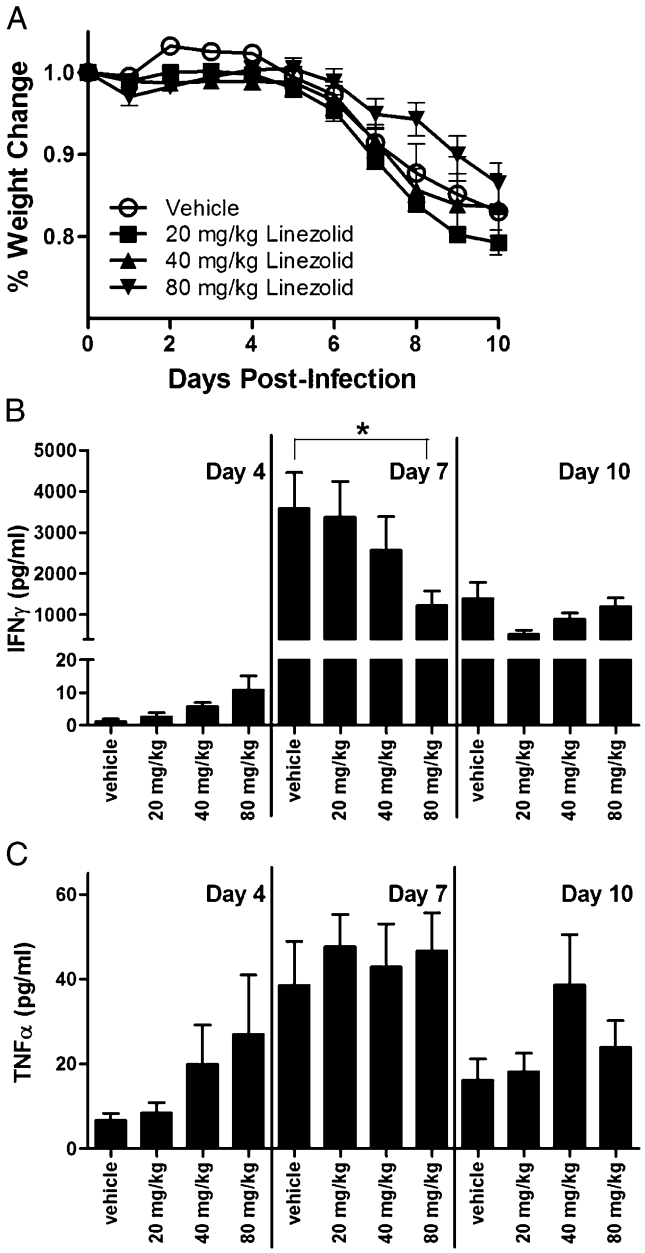

FIGURE 1. Dose-dependent suppression of IFN-γ by linezolid following influenza infection. C57BL/6 mice were infected i.n. with 2 EID_{50}/g of influenza strain PR8. Animals were orally gavaged with linezolid at 20, 40, or 80 mg/kg or with 2% methylcellulose vehicle control twice per day. Animals were weighed daily and sacrificed at given time points. (**A**) Weight loss is represented as percentage of weight loss in comparison with day 0. (**B** and **C**) BAL fluid was collected and analyzed for expression of IFN-γ (B) and TNF-α (C) protein by ELISA. Data are a compilation of three independent experiments (12–16 mice per treatment group per time point). Mean values ± SD are depicted and compared using one-way ANOVA (and two-way ANOVA for weight curve data) along with the Student Newman–Keuls post hoc test. $*p < 0.05$.

Linezolid does not significantly alter other immune responses following influenza infection

We sought to evaluate the effects of linezolid on the activation status of T cells and the production of cytokines following influenza infection to determine whether blunting IFN-γ production had an impact on other responses to the virus. As shown in Fig. 2A (*top*), linezolid did not alter the total numbers of activated $CD4^+$ T cells found in the lung lavage (*left*), lung digest (*middle*), or TBLN (*right*). As is typical of antiviral immune responses, increases in T cell activation can initially be detected in the draining lymph nodes (in this study on day 7 postinfection), with subsequent activated T cell infiltration of pulmonary tissues and alveolar spaces. Similarly, linezolid did not modulate the activation of CD8 T cells over the course of study (Fig. 2A, *bottom panel*). Production of cytokines was measured by intracellular cytokine staining of cells isolated from lung lavage, digest, and TBLN. Linezolid treatment did not significantly alter the absolute number of CD4 T cells producing IFN-γ (Fig. 2B), TNF-α (Fig. 2C), or IL-10 (Fig. 2D). No statistical differences were observed in CD8 T cell production of IFN-γ (Fig. 2B, *bottom*), TNF-α (Fig. 2C, *bottom*), or IL-10 (Fig. 2D, *bottom*). Despite the lack of significance, we consistently observed a trend toward linezolid-mediated suppression of IL-10 and TNF-α in the cells of both the lung lavage and TBLN. These data are consistent with the observation of reduced IFN-γ concentrations in the first wash of linezolid-treated mice (Fig. 1B). This also corresponds to the in vitro data reported by Takahashi et al. (17) that show a blunting of TNF-α production by the drug in peripheral blood monocytes. In addition, we observed no differences between linezolid-treated and control animals in the percentages of $CD4^+$ or $CD8^+$ cells that stained positive for activation or cytokine production (data not shown). To confirm these findings, we performed differential counts on lung digest cells, which demonstrated no statistical difference in the percentages of lymphocytes, monocytes/macrophages, or polymorphonuclear cells in the lungs of influenza-infected animals (data not shown).

Linezolid does not alter influenza viral replication

Targeting IFN-γ production as a prophylaxis against secondary bacterial infection is only a reasonable approach if administration of the drug does not exacerbate influenza viral replication. Influenza-infected mice were dosed twice daily with 80 mg/kg linezolid or vehicle control and were euthanized on day 7 postinfection. Plaque assays were used to determine total viral burdens in the lungs of infected mice. The data in Fig. 3 demonstrate that there was no significant difference in influenza viral loads between vehicle- and linezolid-treated mice. These data, combined with our results showing that linezolid treatment decreases the weight loss postinfection with influenza and the data presented in Fig. 2, support that linezolid's effects are primarily upon IFN-γ production, which does not impact the ability of the mice to clear the infecting virus.

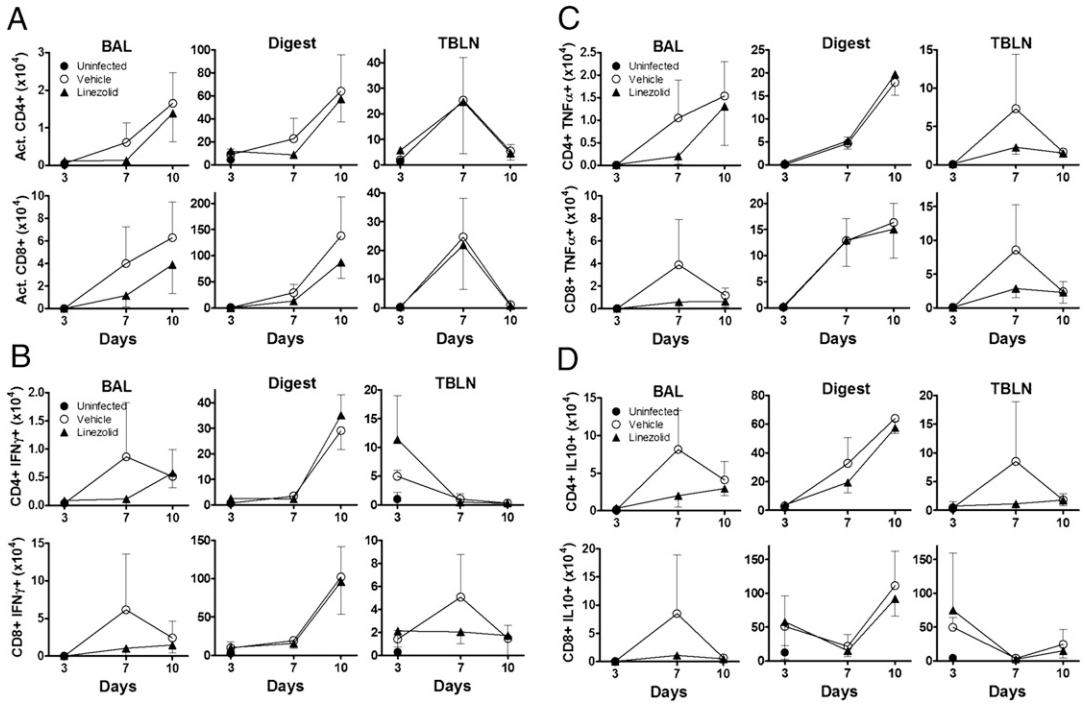

FIGURE 2. Linezolid does not alter other lymphocyte responses following influenza infection. C57BL/6 mice were infected i.n. with influenza strain PR8 and dosed twice daily with linezolid (80 mg/kg) or vehicle. Animals were sacrificed at given times, and BAL, lung digest, and TBLN were processed for flow cytometry. (**A**) Graphs represent absolute numbers of CD4 (*top panels*) and CD8 (*bottom panels*) cells expressing activation markers $CD44^{high}$ $CD62L^{low}$. (**B**–**D**) Intracellular cytokine staining yielded absolute numbers of CD4 and CD8 cells producing IFN-γ (B), TNF-α (C), and IL-10 (D) in BAL, lung digest, and TBLN compartments. Data are representative of two independent experiments, with four to five mice per group per time point in each experimental replicate. Mean ± SD are depicted with comparison using one-way ANOVA along with the Student Newman–Keuls post hoc test. No statistically significant differences were observed.

Linezolid inhibits IFN-γ responses and reduces bacterial loads following secondary S. pneumoniae *infection*

To model secondary bacterial pneumonia, influenza-infected animals were i.n. challenged with a sublethal dose of *S. pneumoniae* on day 7 postinfection. Mice received twice-daily dosing of linezolid starting on day 1 postinfluenza infection, with the last dose being administered 18 h before *S. pneumoniae* challenge (Fig. 4A). In these studies, mice began to lose weight following day 3 of influenza infection, an effect that was blunted by linezolid treatment to a significant degree by day 4 postinfection (Fig. 4B). Following challenge with *S. pneumoniae* on day 7, the control group on average lost an additional 3.4% body weight, in comparison with a weight loss of only 2.8% in linezolid-treated animals. Mice were humanely euthanized at 4 and 24 h after secondary bacterial infection. In this study, in the coinfection experiments, IFN-γ concentrations 4 h after bacterial challenges were not affected (Fig. 4C). However, linezolid treatment significantly reduced IFN-γ concentrations in the BAL at the 24-h time point ($p = 0.017$). Similarly, TNF-α concentrations were also significantly diminished at 24 h following bacterial challenge in the presence of linezolid ($p = 0.007$; Fig. 4D). The bacterial burdens within the lungs of the animals were determined, and whereas there was a trend toward suppression of bacterial outgrowth in linezolid-treated animals at 4 h postpneumococcal challenge, the drug significantly decreased bacterial burdens in the lungs of influenza-infected animals at 24 h postinfection (Fig. 4E). Linezolid decreased bacterial burdens by nearly 1000-fold in comparison with vehicle-treated animals at this time point ($p < 0.001$), a level comparable with animals that were not previously infected with influenza. We were concerned that levels of residual linezolid remaining in the lungs at the time of challenge with *S. pneumoniae* might have been sufficient to limit bacterial outgrowth. To rule this out, flu-naive animals were dosed twice daily with 80 mg/kg linezolid or with vehicle for 6 d, with the last dose given 18 h before bacterial challenge. When bacterial outgrowth was determined following 24 h of *S. pneumoniae* infection, we observed no differences in the levels of viable pneumococci recovered from the lungs of either treatment group (Fig. 4F). These data suggest that linezolid is capable of reversing the influenza-dependent immunosuppressive phenotype observed in our studies, without having any direct effect on the growth of *S. pneumoniae*.

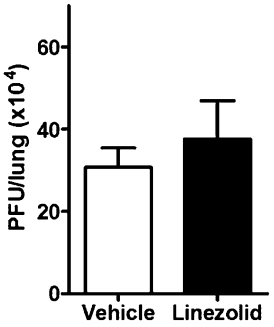

FIGURE 3. Linezolid does not alter influenza viral burdens. Influenza-infected mice were dosed twice daily with 80 mg/kg linezolid or vehicle control. (**A**) On day 7 postinfection, lungs were harvested and homogenized, and viral burden was assessed using the plaque assay. Data are represented as PFU per lung. Mean ± SD are depicted (five mice per group) and compared by *t* test. Results are representative of two replicated experiments. No statistically significant differences were observed.

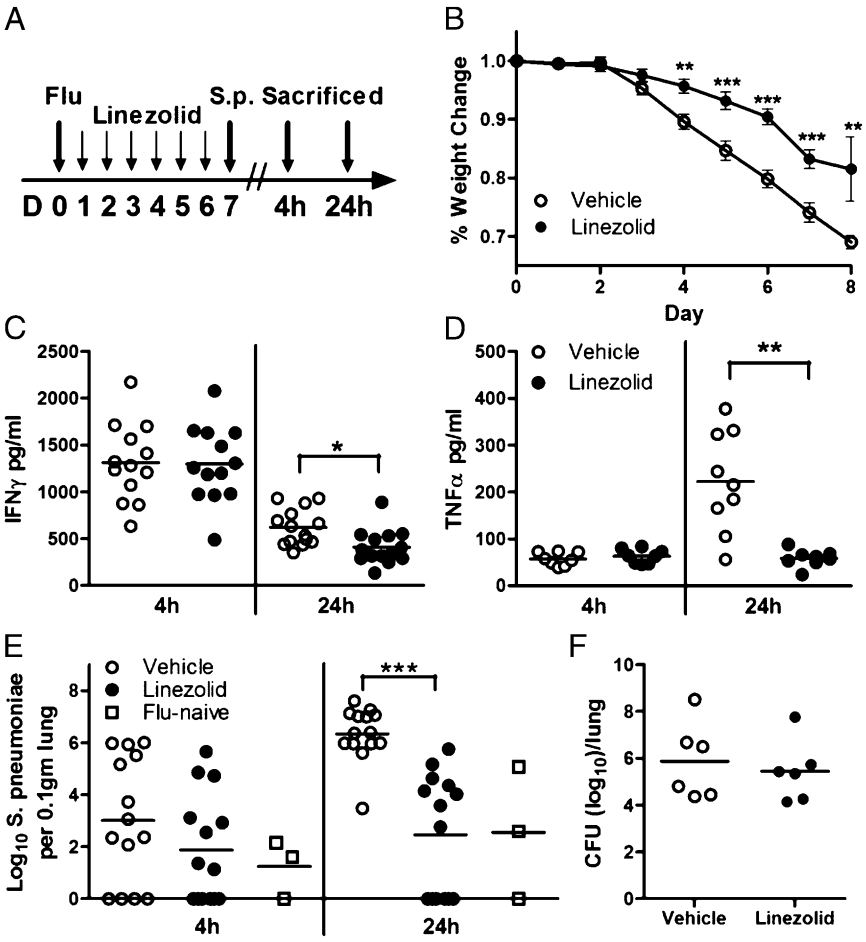

FIGURE 4. Linezolid blunts IFN-γ responses and prevents outgrowth of bacteria following a postinfluenza secondary bacterial infection. (**A**) Mice were i.n. infected (large arrow) with influenza virus and dosed twice daily with linezolid (80 mg/kg) or vehicle (small arrows). At day 7, animals were i.n. challenged with *S. pneumoniae* A66.1, as discussed in *Materials and Methods*, and animals were sacrificed at 4 and 24 h following bacterial challenge (large arrows). (**B**) Mean weight loss is represented as percentage of weight loss in comparison with day 0. (**C** and **D**) BAL fluid was collected and analyzed for expression of IFN-γ (C) and TNF-α (D) by ELISA. (**E**) Lung digest samples were plated for CFU enumeration on blood agar plates and expressed per 0.1 g lung, with the addition of a flu-naive group for comparison. (**F**) To ensure that linezolid did not have a direct effect on bacterial growth, mice were left influenza-naive, dosed with linezolid or vehicle twice daily, and infected with *S. pneumoniae*. Bacterial enumeration was performed at 24 h postinfection and expressed per lung. Mean values for each group, denoted by horizontal lines (each point representative of one animal), were compared using *t* tests or one-way ANOVA along with the Student Newman–Keuls post hoc test where appropriate. Data are a compilation of two independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.0001$.

Administration of exogenous rIFN-γ reverses the linezolid-induced phenotype observed following secondary bacterial infection

To confirm whether the effects of linezolid are mediated through its impact on IFN-γ, animals were infected with influenza, dosed with linezolid or vehicle, and then administered exogenous rIFN-γ at 42 and 18 h before *S. pneumoniae* challenge, as depicted in Fig. 5A. Administration of rIFN-γ to linezolid-treated mice resulted in levels of IFN-γ in the BAL that were comparable to that of vehicle-treated controls, the differences not statistically significant (Fig. 5B, *left panel*). As shown in Fig. 4, linezolid treatment dramatically decreased the TNF-α present in the alveolar space (Fig. 5B, *right panel*). The administration of rIFN-γ did not reverse the drug's ability to blunt the production of TNF-α, as the mean concentration of the cytokine was not altered postpneumococcal challenge. However, rIFN-γ administration did reverse, in part, the effect of linezolid treatment on the growth of *S. pneumoniae* (Fig. 5C). Whereas the mean bacterial burden in the mice that received both linezolid and rIFN-γ was significantly higher than the group that received linezolid alone, the recombinant cytokine did not completely block the effect of the drug, as the mean CFU burden was significantly lower than that of the group treated with vehicle only. Finally, we performed intracellular cytokine staining on lung digest samples and analyzed the production of TNF-α by nonlymphocytes based upon forward and side scatter characteristics as evaluated by flow cytometry. This analysis showed a decrease in the overall number of nonlymphocytic cells in the lungs that were producing TNF-α in both the linezolid and linezolid plus rIFN-γ groups. These data suggest that linezolid's ability to decrease the infectivity of *S. pneumoniae* is at least partially dependent upon the impact on IFN-γ.

Discussion

In our studies, we confirmed that an i.n. challenge with influenza virus A, strain PR8, causes a window of hyporesponsiveness that leads to increased susceptibility to secondary *S. pneumoniae* infection, and that this period corresponds with a dramatic rise in IFN-γ production. Our studies demonstrated that linezolid treatment of influenza-infected animals was sufficient to blunt both IFN-γ and TNF-α responses in a dose-dependent manner. Upon secondary infection with *S. pneumoniae* 7 d after viral infection, linezolid significantly attenuated the level of bacterial outgrowth at 24 h postbacterial infection and continued to both limit weight loss and suppress IFN-γ and TNF-α secretion in the alveolar space. To further elucidate the role of IFN-γ, exogenous IFN-γ was administered to animals receiving linezolid, which caused partial reversal of the drug's ability to protect from the pathology associated with secondary infection. Whereas these studies suggest that IFN-γ contributes to increased susceptibility to secondary bacterial infections, the administration of exogenous IFN-γ was insufficient to completely reverse the linezolid-treated phenotype, suggesting that other mediators may be contributing to this phenomenon. Although this work did not directly address the role that TNF-α contributes to linezolid's beneficial properties, this is an important consideration that should be a focus of further research.

Host defense against influenza virus is characterized by initial inflammation, followed by an adaptive response mounted by CD4 and CD8 T cells, resulting in clearance of the organism along with effective humoral immunity that provides protection against subsequent challenge. An initial influx of neutrophils and macrophages is responsible for limiting viral replication and eliminating dying

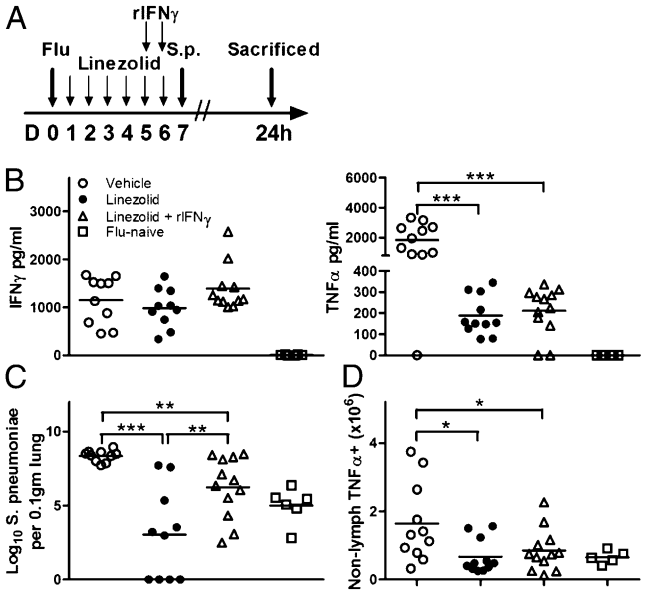

FIGURE 5. Linezolid-induced effects on postinfluenza secondary bacterial infections are IFN-γ dependent. (**A**) Animals were i.n. infected with influenza virus and dosed twice daily with linezolid (80 mg/kg) or vehicle, as depicted. At days 5 and 6 postinfection, a subset of mice that were receiving linezolid was given i.n. instillations of 2 ng rIFN-γ, with the remainder of the mice receiving vehicle only. At 18 h after the last IFN-γ dose, animals were i.n. challenged with *S. pneumoniae* A66.1, as described in *Materials and Methods*, and sacrificed at 24 h following bacterial challenge. An additional group of mice was challenged with *S. pneumoniae* without prior influenza infection. (**B**) BAL fluid was collected and analyzed for expression of IFN-γ (*left panel*) and TNF-α (*right panel*) by ELISA. (**C**) To assess bacterial burden at 24 h postinfection, lung digest samples were plated for CFU enumeration on blood agar plates and expressed per 0.1 g lung. (**D**) To compare the number of nonlymphocytes that were producing TNF-α, intracellular cytokine staining was performed on lung digest samples, and the number of cells within the nonlymphocyte gate that fluoresced positive for TNF-α as analyzed by flow cytometry is depicted. Horizontal lines represent mean values, and each symbol is representative of one animal. Data were pooled from multiple experimental replicates, and means were compared using one-way ANOVA along with the Student Newman–Keuls post hoc test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

cells. These cells release high levels of proinflammatory cytokines, including IFN-γ, IL-1, IL-6, and TNF-α. As the adaptive response begins to develop, CD8 T cells are directly responsible for lysis of influenza-infected cells and contribute to resolution of infection with production of the anti-inflammatory cytokine IL-10 (23). The excessive proinflammatory cytokine production is thought to be a potential mediator of the increased susceptibility to bacterial infection. IFN-γ has been shown to not be required for clearance of influenza infection (24), but its production by $CD8^+$ T cells may lessen the severity of inflammation (25).

The period of hyporesponsiveness has been linked to several factors, including influenza-induced lung inflammation and cell damage (8), dysfunctional neutrophil responses (11, 12), and increased IL-10 concentrations (13, 14). Whereas initial responses immediately following acute influenza exposure may reveal additional binding sites on lung epithelia for bacterial pathogens (8), the majority of inflammation is resolved between days 4 and 6 of infection (26, 27), before the time of bacterial infection in our model. Moreover, in a similar time frame, between days 3 and 6 postinfluenza challenge, neutrophils have been demonstrated to be impaired in their ability to phagocytose and generate toxic reactive oxygen species (12). These investigators further showed that, in addition to decreased neutrophil functionality, there were neutrophil-independent mechanisms contributing to postinfluenza susceptibility to bacterial infection, including that induced by high levels of IL-10 production.

Whereas some studies support the notion that elevated IL-10 concentrations may play a role in increasing susceptibility to infection (13, 14), other investigators have challenged these results. Studies have shown that IL-$10^{-/-}$ mice infected with influenza do not differ from wild-type mice in the ability to clear *S. pneumoniae* in the first 4 h following bacterial challenge and that IL-$10^{-/-}$ and wild-type mice are equally protected by IFN-γ neutralization in the dual infection model (15). In addition, some authors have suggested that the induction and sustained maintenance of alternatively activated macrophages during this period of influenza recovery may play a role in susceptibility to infection with *S. pneumoniae*, although further evidence is needed to support these claims (28). In our infection model, although the differences did not reach statistical significance, the number of T cells producing IL-10 in the alveolar spaces was consistently decreased, and therefore, linezolid's activity may also be partially related to this.

The inability of exogenous rIFN-γ to completely reverse the linezolid-induced phenotype suggests that other mediators may be contributing to this phenotype. Indeed, we found that linezolid causes a trend toward suppression of TNF-α production in the alveolar spaces of infected mice. Other investigators have examined the role of TNF-α in resolution of influenza infection in the absence of secondary bacterial infection. These studies show that, whereas TNF-α is not necessary for the resolution of acute viremia following influenza infection, TNF-$\alpha^{-/-}$ mice demonstrate increased lung immunopathology on days 7 through 21 postinfluenza infection. TNF-$\alpha^{-/-}$ mice had increased IFN-γ production and cellular infiltration, in addition to worsened lung histopathology in comparison with wild-type mice (23). Our data demonstrate that linezolid is able to blunt TNF-α production at day 7 postinfluenza infection without altering the percentages of immune cells infiltrating the lung or increasing viral replication. As for the dual infection effect on TNF-α, the decreased number of cells producing TNF-α and the concentration of TNF-α in the BAL are most likely due to the inhibiting of the bacterial infection. This is confirmed by the fact that rIFN-γ administration partially reversed the bacterial growth, but had no effect on restoring TNF-α production.

To further examine other potential mechanisms impacting IFN-γ levels observed in our studies, we investigated the production of IL-12 and IL-18 in our experimental samples. IL-12 and IL-18 have been shown to augment IFN-γ levels following influenza infection, in an Ag-independent manner (29, 30). We examined production of IL-12 and IL-18 protein in BAL samples from animals infected with influenza and treated with 80 mg/kg linezolid or with vehicle control and observed no differences in protein production between treatment groups (data not shown). Work from other investigators has shown that compensatory mechanisms exist to govern IFN-γ production in the absence of IL-12 or IL-18 (31). Indeed, in our experimental system, it appears that the effect of linezolid on IFN-γ production is independent of IL-12 and IL-18. Future work will focus on examining further mechanisms governing the observed suppression of IFN-γ production by linezolid.

Influenza A infection preferentially increases susceptibility to some bacterial pathogens over others (32, 33). In investigating this discrepancy, authors noted the increase in IFN-γ concentrations following influenza A infection and found that macrophage phagocytosis of varied bacterial strains was differentially affected by the presence of IFN-γ. IFN-γ inhibited macrophage phagocytosis of the Gram-positive bacterium *S. aureus*, but failed to inhibit

phagocytosis of the Gram-negative pathogen *Escherichia coli* (34). In accordance with other studies (15), this suggests that high IFN-γ concentrations following influenza infection may contribute to hypersusceptibility to *S. pneumoniae* infection by inhibiting bacterial phagocytosis by macrophages. Other studies have shown that ultralow doses of anti–IFN-γ Abs are effective in limiting influenza-induced pathology and morbidity (35), which may suggest that attenuation of IFN-γ levels may limit susceptibility to secondary infection. The most convincing argument comes from the laboratory of Sun and Metzger (15), who demonstrate that animals that lack IFN-γ signaling, via genetic deletion or Ab neutralization, are less susceptible to secondary bacterial infection following influenza, in comparison with animals with intact IFN-γ signaling.

Despite our results, the use of linezolid as a prophylaxis against secondary pneumonia is unlikely. Linezolid is commonly used to treat resistant Gram-positive infections (36). Induction of antimicrobial resistance, along with the drug's high cost, makes the use of linezolid in this manner impractical (37). Our results show, however, that IFN-γ could serve as a significant drug target for specific populations at high risk for secondary infections due to influenza.

In our studies, we demonstrate that use of the antimicrobial agent linezolid is sufficient to limit IFN-γ production in the lungs of mice, which leads to decreased bacterial outgrowth following challenge with *S. pneumoniae.* Because linezolid did not alter viral replication or bacterial survival on its own, we can conclude that linezolid's effects on IFN-γ production are important in limiting secondary pneumonia in our model. Further work will focus on IFN-γ as a therapeutic target for drug development. Ideal drug candidates should have little to no outside effects on the host responses that may compromise immunity to other common infectious agents. Further work will focus on deeper mechanistic understanding of the role of IFN-γ in promoting these infections to develop better drug targets for future therapeutics.

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