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Influenza virus coinfection with *Bordetella bronchiseptica* enhances bacterial colonization and host responses exacerbating pulmonary lesions

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ABSTRACT

Influenza virus (Flu) infection and secondary complications are a leading cause of morbidity and mortality worldwide. The increasing number of annual Flu cases, coupled with the recent Flu pandemic, has amplified concerns about the impact of Flu on human and animal health. Similar to humans, Flu is problematic in pigs, not only as a primary pathogen but as an agent in polymicrobial pneumonia. *Bordetella* species play a role in mixed infections and often colonize the respiratory tract without overt clinical signs. Pigs serve as a valuable animal model for several respiratory pathogens, including *Bordetella* (Bb) and Flu. To investigate Flu/Bb coinfection pathogenesis, a study was completed in which pigs were inoculated with Flu-only, Bb-only or both agents (Flu/Bb). Results indicate that Flu clearance is not altered by Bb infection, but Flu does enhance Bb colonization. Pulmonary lesions in the Flu/Bb group were more severe when compared to Flu-only or Bb-only groups and Bb did not cause significant lesions unless pigs were coinfected with Flu. The type I interferon response was elevated in coinfected pigs, but increased expression of antiviral genes Mx and PKR did not appear to enhance Flu clearance in coinfected pigs, as viral clearance was similar between Flu/Bb and Flu-only groups. IL-1β and IL-8 were elevated in lungs of coinfected pigs, correlating to the days enhanced lesions were observed. Overall, Flu infection increased Bb colonization and enhanced production of proinflammatory mediators that likely contribute to exacerbated pulmonary lesions.

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1. Introduction

Influenza virus (Flu) infection and complications associated with Flu disease are a leading cause of morbidity and mortality worldwide. Recent increases in the annual number of Flu cases, coupled with the emergence of the novel H1N1 pandemic strain (pH1N1), have amplified concerns about the impact of Flu infection on human and animal health. Pigs are a natural host for influenza A virus, and suffer a similar clinical disease to that observed in humans [1]. Influenza disease manifests itself rapidly, with an incubation period of 1–3 days, followed with recovery beginning 4–7 days after the onset of symptoms. The acute stage of disease in pigs is characterized by fever, inactivity, decreased food intake, coughing, sneezing, and nasal discharge [1]. Although Flu is typically a self-limited infection characterized by high-morbidity and low mortality, secondary complications substantially increase flu-associated illness and death [2].

In humans, bacterial pneumonia secondary to Flu infection is often observed. The same phenomenon is also seen in pigs, as swine Flu is a key contributor to the porcine respiratory disease complex (PRDC), a multifactorial complex characterized by severe respiratory disease after infection with two or more agents. Bacterial pathogens associated with PRDC include *Haemophilus parasuis*, *Streptococcus suis, Mycoplasma hyopneumoniae* and *Bordetella bronchiseptica. Haemophilus influenzae* and *Streptococcus pneumoniae* are commonly associated with secondary bacterial infection in humans, with increasing isolations of *Staphylococcus aureus* being reported [3]. Recent reports indicate that pulmonary bacterial infections are occurring in individuals infected with pH1N1 influenza, similar to that observed with seasonal influenza [4,5]. However, the age-group most affected by pH1N1 is individuals less than 65-years-old, in contrast to the age-group most

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affected by seasonal influenza [6]. The majority of animal studies investigating Flu and bacterial superinfection use a sequential infection model in which the animals are first inoculated with Flu and subsequently challenged with a bacterial pathogen. This approach clearly shows that Flu predisposes to bacterial pneumonia, but clinical cases are often reported as coinfection and it is difficult to determine the order, if not simultaneous, of infection with Flu and bacterial pathogen [7,8].

Upon infection, the innate immune response is critical for controlling pathogen spread and initiating the adaptive immune response. Host cells recognize conserved motifs expressed by various pathogens and respond with the production of proinflammatory cytokines. Tracheal epithelial cells and other pulmonary cells, such as alveolar macrophages, play a critical role in pulmonary health by responding to invading microorganisms with the production of innate immune mediators. After detection of a pathogen, host cells produce proinflammatory cytokines (e.g., TNF-α, IL-1β, IL-6) and chemokines (e.g., IL-8, MCP-1, and RANTES) to activate cellular defense mechanisms and initiate the infiltration of additional immune effector cells, such as neutrophils. Type I interferons (IFN-α/β) are important mediators of the antiviral response by initiating the production of intracellular antiviral mediators, such as Mx-1 and dsRNA-dependent protein kinase R (PKR) [9]. The host antimicrobial response in the lung must be sufficient to combat the infection, but also regulated to prevent overt immunopathology that could impair gas exchange.

Animal models to study Flu pathogenesis include macaques, ferrets, chinchillas, guinea pigs, cotton rats, chickens, pigs and mice [10–15]. Mice have been used extensively to study the host response to Flu infection because of the availability of reagents and gene knock-out strains [16–19]. Though these studies have provided useful information, mice are not a natural host to influenza A virus and such studies require the use of mouse adapted strains of influenza. In addition, alveolar development in the mouse lung is different than that in humans and pigs [20] and mice, unlike pigs, do not express an ortholog to human IL-8 [21], though murine KC appears to be analogous to human and pig IL-8. Ferrets and pigs are useful models for studying lower respiratory tract Flu infection (with non-avian strains) because the host cells to which the virus attach are similar to those observed for humans [22]. Ferrets are a principal model for Flu pathogenesis studies, but lack of reagents has limited their use in studying the immune response to infection. An ideal animal model is one in which the animal is a natural host to the pathogen of interest, reagents are available for studying host response to infection, and the course of disease reproduces what is known about human disease.

Pigs are natural host to Flu and the clinical disease is similar to that of humans, making them very useful for Flu studies. The bacterial components of influenza-associated bacterial pneumonia can also be studied in pigs because the bacterial pathogens causing disease in humans are similar to those in pigs [23]. In the current study we investigated disease pathogenesis and host immune response following coinfection of pigs with Flu and *B. bronchiseptica* (Bb). The result of coinfection with Flu and Bb has not been previously studied, thus, we used a simultaneous inoculation strategy to establish if there is an enhancement of disease with coinfection.

2. Results

2.1. Flu/Bb coinfection does not affect influenza nasal shedding and minimally alters Bb nasal colonization

To determine if coinfection alters nasal shedding of Bb or Flu, groups of pigs were challenged with Flu, Bb, or both agents (Flu/Bb) and infectious load of each agent in the nasal cavity was evaluated. Results from nasal swab samples indicate that coinfection did not significantly alter shedding of Flu from the nasal passages nor did it alter the kinetics of clearance of virus from the nasal cavity (Fig. 1A). The average Flu titer in nasal swabs peaked between days 4 and 5 post-challenge for the Flu-only group and the Flu/Bb group, and, there was no statistical difference in titers between challenge groups. Flu titers in both groups decreased by day 6 post-challenge, with clearance by day 8 in all but one pig in the Flu-only group.

Bb burden in the nasal cavity was statistically similar between groups of Bb-only pigs and Flu/Bb pigs on all sample dates except day 8, with higher Bb CFU in the coinfected group (Flu/Bb) when compared to the Bb-only group (Fig. 1B). This trend continued on day 9 post-challenge, but by day 10 there was no statistical difference between challenge groups. Nasal Bb CFU increased gradually after inoculation in both groups, with an average peak in colonization occurring on day 5. Bb colonization decreased slightly thereafter and leveled off by day 10 following challenge.

2.2. Flu/Bb coinfection increases Bb burden but not Flu titers in the respiratory tract

To further evaluate the effect of coinfection on infectious load in the respiratory tract, four pigs from each group were euthanized on days 1, 5 and 10 following challenge to measure Flu titer and Bb CFU in the trachea and lung. Flu titers in the trachea and lung were not different between challenge groups (Flu-only versus Flu/Bb) on days 1, 5 or 10 following inoculation (Fig. 2A). On day 1 following inoculation Flu virus was recovered from the trachea of all coinfected pigs and 3 of the 4 pigs infected with Flu-only. By day 5 Flu virus was isolated from the trachea of all Flu-only and Flu/Bb infected pigs. Flu titers in the lung were lower than those observed in the trachea, though the kinetics of infection were similar between the 2 sample sites. By day 10 following inoculation Flu virus was not isolated from the trachea or lung of pigs in either challenge group (Fig. 2A).

Although Flu/Bb coinfection did not alter Flu titers in the trachea or lung (Fig. 2A), coinfection did significantly affect Bb colonization (Fig. 2B). On day 1 post-challenge Bb was isolated from the trachea of only 2 of the 4 pigs infected with Bb-only, but was isolated from the trachea of all 4 pigs in the Flu/Bb group (Fig. 2B). Trachea Bb CFU were also higher in the coinfected animals on day 1 post-challenge when compared to the Bb-only group ($p = 0.06$, Fig. 2B). By day 5 post-challenge Bb CFU in the trachea were not different between challenge groups and Bb was not recovered from the trachea of one pig in each group. On day 10 post-challenge pigs in both groups were colonized with Bb in the trachea and no significant difference in CFU was observed between groups. In the lung, a significant difference in Bb colonization was measured between the Bb-only group and Flu/Bb group on days 1 and 10 following inoculation (Fig. 2B). On day 1 following challenge Bb was not isolated from the lungs of any of the pigs in the Bb-only group, but Bb was isolated from all the pigs in the Flu/Bb coinfected group ($p < 0.001$). On day 5 following challenge lung Bb burden was not different between the 2 challenge groups, but on day 10 there was a significant difference in Bb lung colonization ($p = 0.04$). Overall, these results indicate that coinfection with Flu and Bb results in increased Bb colonization in the lower respiratory tract of pigs, primarily in the lung.

2.3. Flu/Bb coinfection leads to enhanced macroscopic and microscopic lung lesions

2.3.1. Macroscopic lesions

On day 1 post-challenge macroscopic lesions, characterized by dark, red-colored consolidation with well demarcated borders and a cranial–ventral distribution, were observed in all 4 coinfected pigs

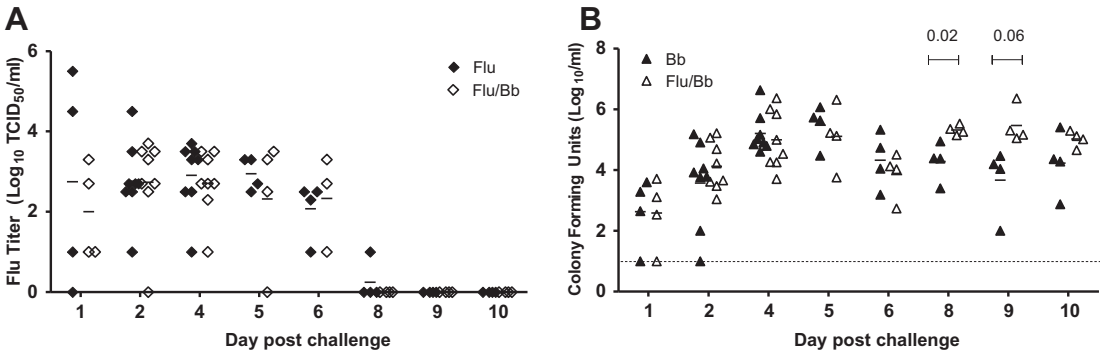

Fig. 1. Influenza virus titers and *B. bronchiseptica* CFU in the nose. Groups of pigs were infected with *B. bronchiseptica* (Bb), influenza A virus (Flu), or coinfected with both agents (Flu/Bb). Nasal swabs were collected at the indicated days post-challenge and (A) Flu titers and (B) Bb were enumerated for each pig. Flu was not isolated from Bb-only pigs and Bb was not isolated from Flu-only pigs (data not shown). Each data point represents a single pig with the bar at the average for the group. The number of samples collected at each time point is explained in Materials and Methods section. A student's *t*-test was performed for statistical analysis.

but none of the pigs infected with Flu-only or Bb-only (Table 1). On day 5 post-challenge 3 of the 4 pigs inoculated with Flu-only exhibited macroscopic lesions, whereas all 4 pigs in the Flu/Bb groups exhibited lesions. Enhanced pneumonia was also observed on day 10 post-challenge in the coinfected group, as all 4 pigs had macroscopic lesions with a greater percentage of the lung affected when compared to the single pig exhibiting lesions in the Flu-only group. Pneumonia lesions were not observed in any of the pigs inoculated with Bb-only or mock-infected on any necropsy date.

2.3.2. Microscopic lesions

Fig. 3 shows representative images depicting microscopic lung lesions appreciated in the different challenge groups at different days post-infection. On day 1 following challenge, 2 of the 4 pigs in the Flu-only group had no significant lesions and the other 2 had only mild peribronchiolar lymphocytic infiltration. All 4 pigs in the Flu/Bb group had moderate peribronchiolar lymphocyte infiltration as well as changes to the bronchiolar epithelium consisting of mild to moderate rounding and degeneration of epithelial cells

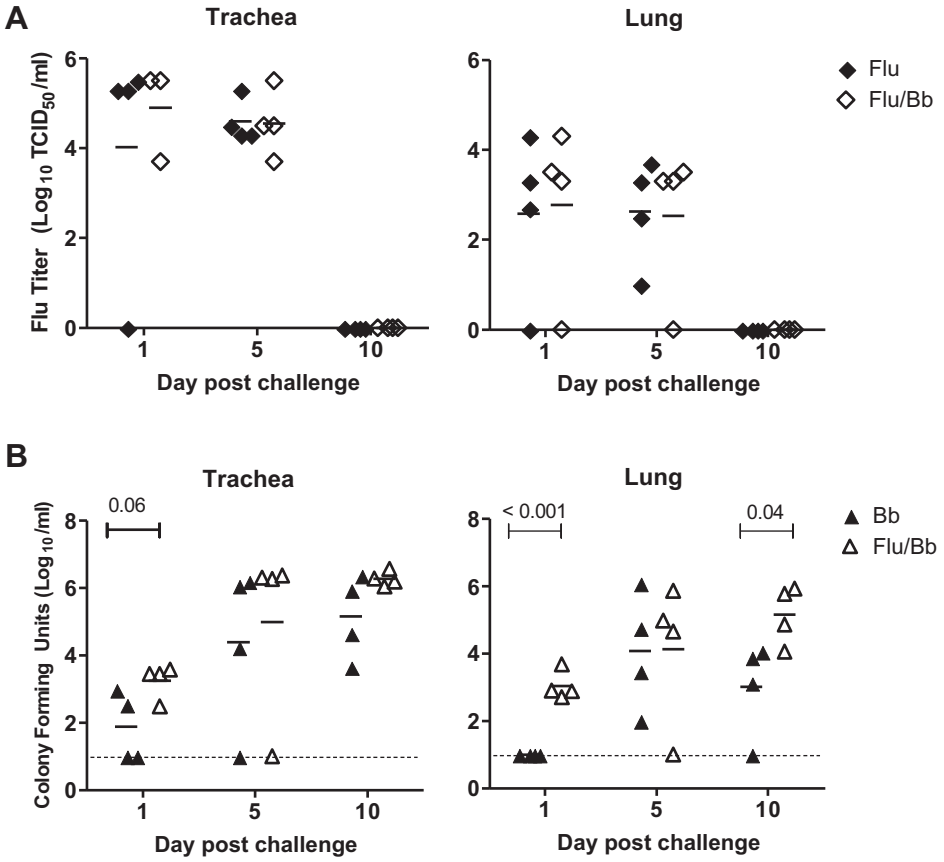

Fig. 2. Influenza virus infection altered pulmonary *B. bronchiseptica* colonization in the lower respiratory tract. Groups of pigs were infected with *B. bronchiseptica* (Bb), influenza A virus (Flu), or coinfected with both agents (Flu/Bb). On day 1, 5, and 10 post-challenge (A) influenza virus and (B) *B. bronchiseptica* were enumerated in the trachea wash and lung lavage. Flu was not isolated from Bb-only pigs and Bb was not isolated from Flu-only pigs (data not shown). Each data point represents a single pig with a bar at the average. A student's *t*-test was performed for statistical analysis.

Table 1
Mean percentage[a] of lung affected by pneumonia.

	Day 1	Day 5	Day 10
Sham	0 (0/4)[b]	0 (0/4)	0 (0/4)
Bb	0 (0/4)	0 (0/4)	0 (0/4)
SIV	0 (0/4)	3.19 (3/4)	2.38 (1/4)
SIV/Bb	2.38 (4/4)	4.69 (4/4)	4.75 (4/4)

[a] See Materials and Methods for calculation.
[b] Number of animals exhibiting lesions out of total number of animals in the group.

progressing to cuboidal to squamous metaplasia and intraluminal accumulation of neutrophils. On day 5 post-challenge, lesions were similar between Flu-only and Flu/Bb pigs, though only 3 of the 4 Flu-only pigs had lesions and all 4 coinfected pigs had lesions. Observed changes included peribronchiolar lymphocyte infiltration, attenuation and loss of the airway epithelium and intraluminal accumulation of neutrophils, alveolar epithelial cell necrosis and filling of alveoli with necrotic debris and mixed inflammatory cell infiltrates, and interlobular edema. None of the Bb-only pigs had significant lesions on day 1 or 5 post-challenge and only 1 of the 4 Bb-only pigs had lesions at day 10 characterized by a focal area of mild filling of the alveoli with macrophages and some syncytia. On day 10 post-challenge, 2 of the 4 Flu-only pigs had lesions, 1 with changes similar to those observed on day 5 only milder, and the other had lesions similar to day 5. On day 10 all 4 pigs in the coinfected group had significant lesions with characteristics similar to those observed on day 5.

2.4. Type I interferon response is enhanced in pigs coinfected with Flu/Bb

mRNA levels of IFN-α, Mx, and PKR in tracheal epithelial cells and lung were evaluated. On day 1 following challenge, transcription of IFN-α was significantly elevated in tracheal epithelial cells from coinfected (Flu/Bb) pigs compared to pigs infected with Flu-only or Bb-only (Fig. 4A). In addition, expression of antiviral mediators Mx and PKR was elevated in the trachea of coinfected pigs on day 1 post-challenge (Fig. 4B). On days 5 and 10 post-challenge, Mx and PKR mRNA levels were increased in both the trachea of the Flu-only and Flu/Bb groups, though no significant difference between the two groups was observed (data not shown).

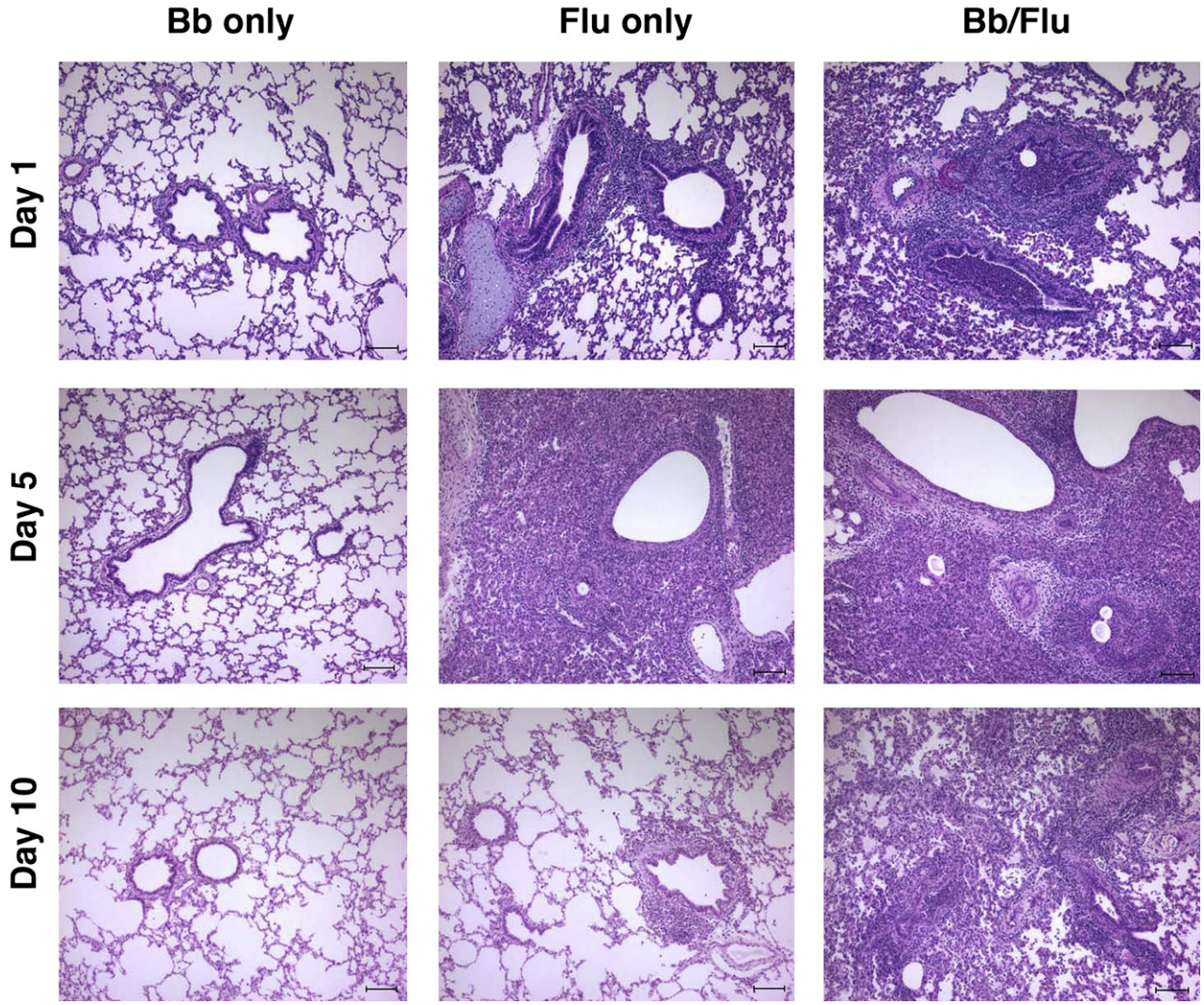

Fig. 3. Pulmonary microscopic lesions were more severe in Flu/Bb coinfected pigs on days 1 and 10 post-challenge. Groups of pigs were infected with *B. bronchiseptica* (Bb), influenza A virus (Flu), or coinfected with both agents (Flu/Bb). On days 1, 5, and 10 post-infection a section of the right cranial lobe was collected for histological examination. A representative image from a single pig in each challenge group is shown. All images are at 10× magnification.

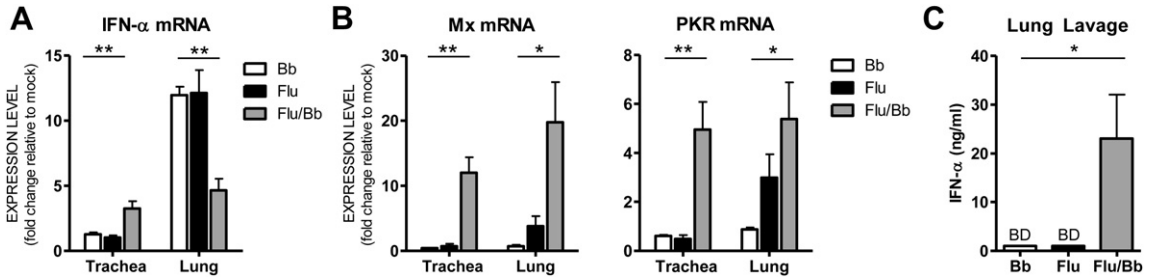

Fig. 4. Type I interferon response was enhanced in coinfected pigs on day 1 post-challenge. Groups of pigs were infected with influenza A virus (Flu), *B. bronchiseptica* (Bb), or coinfected with both agents (Flu/Bb). On day 1 post-challenge (A) mRNA levels of IFN-α, Mx-1, and PKR were measured in tracheal epithelial cells and lung by real-time PCR and (B) IFN-a protein levels in the broncho-alveolar lung lavage were measured by ELISA. Data are shown as mean ± SEM of four animals in each challenge group. A one-way analysis of variance (ANOVA) was used to determine significant differences between groups. *p*-values less than 0.05 are noted with a single-asterisk and *p*-values less than 0.01 are noted with a double-asterisk.

In the lung, IFN-α mRNA levels were similar between pigs in the Flu-only and Bb-only groups, both averaging approximately a 12-fold increase over mock-treated pigs (Fig. 4A). Coinfected pigs had approximately a 4-fold increase in IFN-α mRNA levels in the lung compared to mock-treated pigs, which was significantly less than the levels observed in pigs infected with Flu-only or Bb-only (Fig. 4A). The downstream genes Mx and PKR were not significantly elevated in the lungs of pigs infected with Flu-only or Bb-only, but were elevated in coinfected pigs (Fig. 4B). On days 5 and 10 post-challenge, Mx and PKR mRNA levels in the lung were elevated in the Flu-only and Flu/Bb groups, with no significant difference between the groups observed (data not shown). The amount of IFN-α protein in the lung was significantly elevated in the lungs of coinfected (Flu/Bb) pigs, but was not detected in pigs infected with either agent alone (Fig. 4C). IFN-α protein could not be detected in any samples taken after day 1 (data not shown).

2.5. Enhanced proinflammatory cytokine response in the lungs of pigs coinfected with Flu/Bb

The proinflammatory cytokine response is critical for recruiting effector cells to the site of an infection but elevated or prolonged production can also contribute to pathogenesis observed during disease. To determine if coinfection with Flu and Bb results in increased production of proinflammatory mediators IL-1β or IL-8, mRNA and protein levels in the lung were evaluated. Non-infected animals served as mock-treated controls. On day 1 following challenge, pigs in the Flu/Bb group had enhanced mRNA expression levels of both IL-1β and IL-8 compared to pigs infected with Flu-only or Bb-only (Fig. 5A). By day 5 post-challenge, IL-1β and IL-8 mRNA levels in coinfected pigs had decreased significantly from the elevated levels observed on day 1. The increased mRNA levels coincided with increased detection of IL-1β and IL-8 protein in the

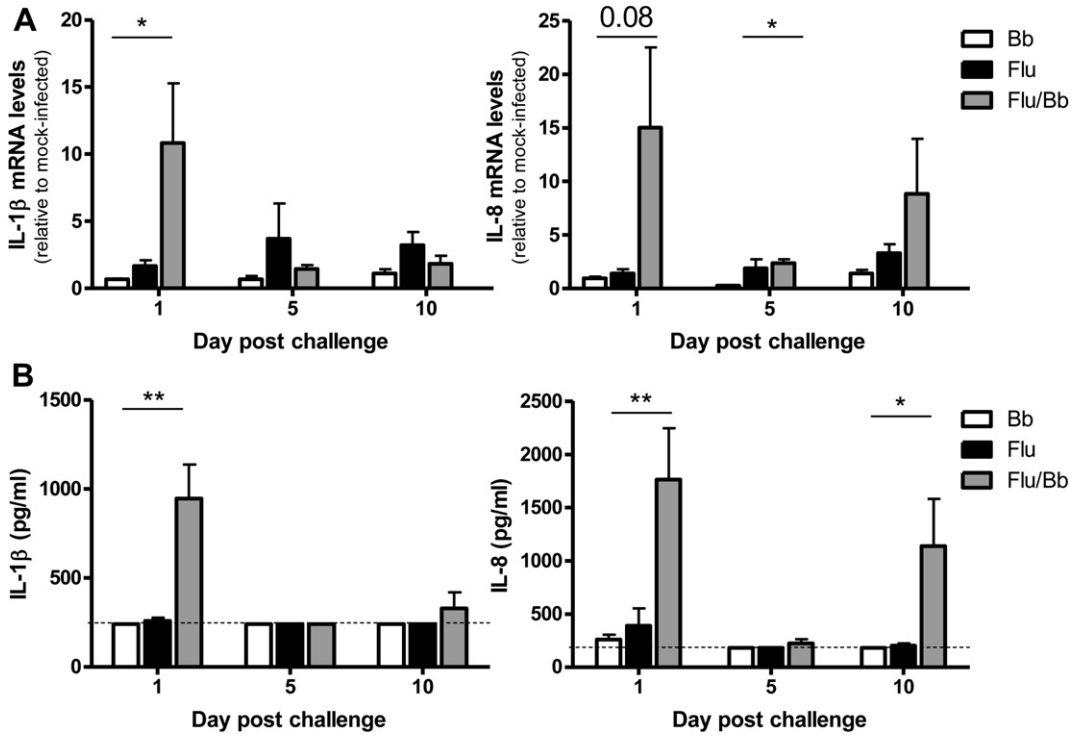

Fig. 5. Coinfection altered the proinflammatory cytokine response in the lung. Groups of pigs were infected with influenza A virus (Flu), *B. bronchiseptica* (Bb), or coinfected with both agents (Flu/Bb). On days 1, 5 and 10 post-infection (A) mRNA levels of IL-1β and IL-8 were measured in lung by real-time PCR and (B) IL-1β and IL-8 protein levels in the broncho-alveolar lung lavage were measured by ELISA. Data are shown as mean ± SEM of four animals in each challenge group. A one-way analysis of variance (ANOVA) was used to determine significant differences between groups. *P*-values less than 0.05 are noted with a single-asterisk and p-values less than 0.01 are noted with a double-asterisk.

lung on day 1 as well. IL-8 mRNA levels were significantly increased in both Flu-only and Flu/Bb pigs on day 5 following challenge, but this increase in mRNA did not result in differences in IL-8 protein (Fig. 5A and B). IL-8 mRNA levels were increased on day 10 following challenge in the Flu/Bb group which coincided with significantly increased levels of IL-8 protein in the lung on day 10. Taken together, proinflammatory cytokine responses in the lung were heightened in pigs coinfected with both Flu/Bb when compared to pigs infected with either agent alone.

3. Discussion

In pigs *B. bronchiseptica* (Bb) infection often results in chronic colonization, and depending on the age in which the animal becomes infected, bacteria can be routinely isolated from the respiratory tract without overt clinical signs of disease. Neonatal piglets (less than 2 weeks of age) that become infected often suffer a more severe disease than older pigs. In the current study, 4-week old pigs were used; therefore, Bb alone caused minimal disease, even though it was re-isolated from the lungs (Fig. 2). Flu infection in pigs causes an acute disease, with virus titers and lung lesions peaking on day 5 following infection, and viral clearance by day 7 [1]. In the current study, the course of influenza disease did not appear to be altered by Bb coinfection. Viral titers in the respiratory tract still peaked on day 5 and virus was cleared by day 10 (Fig. 2). However, the course of Bb disease was altered by Flu coinfection. Pulmonary lesions were observed by day 1 following coinfection, and lesions persisted in a more severe state in coinfected pigs than that observed in pigs infected with either pathogen alone. These data suggest a more rapid and heightened inflammatory response during coinfection that likely plays a role in the severity of pulmonary lesions.

There are several reported methods by which Flu infection predisposes to secondary bacterial infection, including compromising the respiratory epithelial barrier [24], increasing host expression of receptors for bacteria leading to increased colonization [25,26], and altering host immune responses [16,27]. It's likely that these mechanisms are not mutually exclusive, but instead, it is probable that Flu compromises host health in a variety of ways. For example, Flu can decrease tracheal mucociliary velocity, contributing to increased bacterial colonization, as shown with *S.pneumoniae* [28]. In the current study, Bb colonization was increased primarily in the lungs of coinfected pigs, suggesting that changes to the ciliated epithelium may not be the only mechanism in which Flu predisposes to secondary Bb infection. If this was the case, we would have expected enhanced Bb colonization in the trachea of coinfected pigs. Yet, tracheal Bb colonization was slightly different on day 1 following challenge, but not day 5 or 10 (Fig. 2B).

Another mechanism by which Flu predisposes to secondary infection is by impairing alveolar macrophage phagocytic function, subsequently hindering bacteria uptake and killing [29,30]. Also, Flu infection has been shown to alter the response of macrophages to secondary stimuli [16]. In the current study, increased numbers of Bb were isolated from the lung, indicating that Flu is likely causing a defect in the response and clearance of Bb from the lung, but it is not clear if this is due to a direct defect in macrophage responses. Production of IL-1β and IL-8 proinflammatory cytokines was increased in the lungs of coinfected animals; thus, it does not appear that Flu caused a decreased response to secondary stimuli, but instead a heightened response (Fig. 5). Didierlaurent et al. recently reported a desensitization of lung cells to TLR agonists following respiratory viral infection, which is in contrast to our findings [16]. The difference is likely due to the timing of stimulation, as we did simultaneous infection, whereas the previous report looks at responses several weeks following viral challenge [16]. A study in mice examining the host response to pneumococcal infection 7 days after Flu infection shows increased production of proinflammatory cytokines in the lung following bacterial challenge, more similar to our findings [27]. However, a recent report examining influenza pathogenesis in mice previously infected with an attenuated strain of *B. pertussis* indicate that prior bacterial inoculation can attenuate influenza pathogenesis depending on the bacterial dose and time of exposure [31]. Thus, the time of secondary insult is likely to have an effect on the host response and understanding the agonist, kinetics, and magnitude of the response will be important for developing therapeutics to combat bacteria/Flu superinfection. The work described here shows that simultaneous coinfection with Flu and Bb results in exacerbated disease and future work is aimed at looking at disease pathogenesis following sequential infection with influenza infection preceding or proceeding *B. bronchiseptica* infection.

There did appear to be a discord between the detection of IFN-α mRNA and protein in the lungs (Fig. 4) and the reason for this is not completely clear. The IFN-α mRNA expression level in coinfected pigs was elevated over mock-treated animals, but IFN-α mRNA expression levels were greater in the Flu-only and Bb-only pigs (Fig. 4A). It is possible that IFN-α mRNA levels in the coinfected pigs were higher before dpi 1, which would explain the detection of IFN-α protein and the transcription of the downstream antiviral mediators Mx and PKR on dpi 1 in this group. There is a feedback loop for regulating type I IFN production that may have decreased the signal for IFN-α mRNA in the coinfected group [32]. We did not sample the lungs between days 1 and 5 following challenge, so it's possible that IFN-α protein levels were elevated in the Flu-only and Bb-only pigs on one of these days. The type I IFN response is known for its role in the antiviral immune response; however, even with the differences we observed in IFN-α protein levels, and Mx and PKR mRNA levels, Flu clearance did not seem to be altered by coinfection. Coinfection may alter the kinetics of the type I IFN response and a more in-depth analysis with additional time points for sample collection would likely provide additional information.

In Flu/Bb coinfected pigs, the increased levels of IL-8 and IL-1β cytokine coincided with increased pulmonary lesions and changes were observed within a day following challenge and continued to be increased at day 10 (Fig. 5 and Table 1). One might speculate that the increased cytokine production was the result of increased bacterial burdens observed in the lungs of coinfected pigs when compared to Bb-only pigs. While this may be true for day 1, there was no significant difference in lung Bb CFU in the coinfected pigs between days 5 and 10 (Fig. 2). However, cytokine levels were minimal on day 5, and increased on day 10 in the coinfected group (Fig. 5). Thus, the number of Bb alone is not sufficient to explain the increase in cytokine responses observed at day 10 following coinfection. IFN-α has been shown to increase TLR responsiveness in macrophages and it is possible that influenza infection increases the expression of TLR that would enhance responsiveness to secondary bacteria stimulation [33]. A recent report on sequential infection of mice with Flu and *Bordetella parapertussis* shows that increased proinflammatory responses, as opposed to increased bacterial burdens, are detrimental to the host [34]. Overall, Flu/Bb coinfection resulted in an impaired clearance of Bb from the lower respiratory tract, and the heightened cytokine response did not correlate with Bb clearance. Instead, Bb persisted at higher levels in the lungs of coinfected animals and pulmonary lesions were exacerbated.

Bacterial pneumonia, with Flu infection, continues to contribute significantly to Flu-associated morbidity and mortality. With the recent emergence of the 2009 pandemic Flu, coupled with concerns of antibiotic resistance to bacterial pathogens, it is important to understand mechanisms in which Flu and bacteria act

synergistically in the lung and cause severe pneumonia. This will allow for a more targeted approach to treatment as well as prevention. Although mice serve a useful purpose in the study of disease pathogenesis, other animal models, such as the one described here, provide useful information in infectious disease research. Our results show that pigs are similarly affected by Flu as humans are, and Flu can be exacerbated by bacterial coinfection in pigs. While the emphasis has been on secondary bacterial infection following primary influenza infection, or results show that simultaneous coinfection with Flu and a bacterial pathogen can result in enhanced pneumonia.

4. Materials and methods

4.1. Influenza virus and B. bronchiseptica inocula

A classical, α-cluster H1N1 swine influenza virus isolate, A/Swine/Minnesota/37 866/1999 (MN99), was prepared in Madine–Darby Canine Kidney (MDCK) cells as previously described in Refs. [35,36]. Non-infectious cell culture supernatant from MDCK cells was used for sham inoculum. Pigs were inoculated intranasally with 2 ml (1 ml/nostril) of 2×10^6 $TCID_{50}$/ml of virus or sham supernatant. *B. bronchiseptica* strain KM22 is a virulent phase I swine isolate initially cultured from a herd with atrophic rhinitis [37]. To prepare the inoculum, *B. bronchiseptica* was cultured on Bordet-Gengou agar supplemented with 10% sheep's blood at 37 °C for 40 h. A culture suspension with an A_{600} of 0.42 was prepared in phosphate-buffered saline (PBS). This suspension has approximately 2×10^9 colony forming units (CFU)/ml, and a 1:2000 dilution of this suspension was made in PBS for generation of the final inoculum. Pigs were inoculated intranasally with 1 ml (0.5 ml/nostril) of the final inoculum. Cultured dilutions of the inoculum contained approximately 10^6 CFU/ml and one hundred percent of the inoculum colonies appeared to be in the Bvg^+ phase, based on colony morphology and presence of hemolysis. PBS was used for sham inoculum. The bacterial and viral inoculums were prepared and delivered separately.

4.2. Experimental design

Six pregnant sows were transferred to an isolation facility at the National Animal Disease Center (NADC) approximately 2 weeks prior to their farrowing due date from a herd negative for antibody to porcine reproductive and respiratory syndrome virus (PRRSV) and Flu and negative for *B. bronchiseptica* colonization. Piglets from these sows were early weaned by 7 days of age to reduce the transfer of bacterial respiratory pathogens colonizing the sow to the piglets. No medication or vaccinations was given to the piglets. Nasal swabs were obtained from all the piglets prior to starting the experiment and neither Flu nor Bb were isolated. At 4 weeks of age, 2 piglets from each sow were divided into 4 groups with 12 pigs per group. Pigs in group 1 were inoculated with Bb and non-infectious cell culture supernatant (Bb-only), pigs in group 2 were inoculated with PBS and influenza virus (Flu-only), pigs in group 3 were inoculated with influenza virus and *B. bronchiseptica* (Flu/Bb), and pigs in group 4 were inoculated with PBS and non-infectious cell culture supernatant (Sham). Inoculums were administered separately and not mixed. Groups were housed in individual isolation rooms and cared for in compliance within the guidelines of the NADC Institutional Animal Care and Use Committee. Four pigs from each group were humanely euthanized with a lethal dose of pentobarbital (Sleepaway, Fort Dodge Animal Health, Fort Dodge, IA) on days 1, 5, and 10 following challenge. Nasal swabs were taken on days 0, 2, 4, 6, 8, and 9 of the experiment from all pigs remaining in each challenge group on those days.

After euthanasia, nasal swabs, nasal wash, tracheal wash and broncho-alveolar lavage (BAL) were performed. The nasal swab was collected in 1 ml of MEM media and the nasal wash was performed by flushing 5 ml of phosphate-buffered saline (PBS) into the nostrils and collecting the effluent. The tracheal wash was performed by placing a 5 cm portion of trachea, taken immediately caudal to the larynx, into a 15 ml conical tube with 5 ml of PBS and gently agitating for 30 s BAL was performed by pipetting 20 ml of PBS into each of the cranial, middle, and accessory lobes and aspirating as much as possible back from each location. An aliquot of BAL was frozen at −80 °C for virus titration and cytokine protein analysis.

4.3. Virus and bacterial isolation

Number of CFU of Bb per ml of swab fluid, tracheal wash, and BAL was determined by plating serial 10-fold dilutions on duplicate selective blood agar plates containing 20 μg/ml penicillin, 10 μg/ml amphotericin B, 10 μg/ml streptomycin and 10 μg/ml spectinomycin as previously described in Ref. [38]. For determining viral load, nasal swab, tracheal wash and BAL were processed as previously described in Ref. [39]. Briefly, 10-fold serial dilutions of each sample were made in serum-free MEM media supplemented with TPCK trypsin plus antibiotics. Each dilution was plated in triplicate onto PBS-washed MDCK cells grown to confluency in 96-well flat-bottom plates. Plates were evaluated for cytopathic effect between 48 and 72 h post-infection. A $TCID_{50}$/ml was calculated for each sample using the method of Reed and Muench [40].

4.4. Cytokine gene expression

After collecting tracheal wash, epithelial cells were collected from a trachea section. A longitudinal cut was made down the dorsal membrane to open the trachea. Using a sterile razor blade, the epithelial lining of the trachea was scraped from the cartilage and collected in a microcentrifuge tube. RNA collection buffer (RLT, Qiagen Mini RNeasy Kit) was added and samples stored at −80° for later RNA extraction. RNA extraction was performed according to manufacturer's recommendations (Qiagen Mini RNeasy Kit).

At necropsy, a 1 g piece of lung from the right cranial lobe was placed in RNA later and frozen until RNA isolation was performed. For RNA extraction, approximately 30 mg of the lung piece was taken and minced with a sterile razor blade while frozen. A mortar and pestle was used to further disrupt the tissue piece. Sample was kept on ice during homogenization steps. RNA collection buffer was added to the sample, which was transferred to a 15 ml conical tube. The sample was lysed by sonicating 3 times, 10 s each, chilling on ice in between each sonication. The sample was centrifuged and the supernatant used for RNA extraction according to manufacturer's recommendations (Qiagen Mini RNeasy Kit).

Reverse transcription for cDNA synthesis was performed using random primers and SuperScript II Reverse Transcriptase according to the manufacturer's recommendations (Invitrogen). SYBR green based real-time PCR was carried out for various mRNA targets (SYBR Green Master Mix, Applied Biosystems) as previously described in Ref. [41]. Levels of mRNA were calculated using the $2^{-\Delta\Delta C_t}$ method, which expresses mRNA from the cells of infected pigs relative to the cells from sham pigs after normalizing to β-actin [42].

4.5. Cytokine protein levels

Levels of IFN-α protein in the BAL were measured by ELISA using F17 monoclonal antibody, K9 MAb and recombinant porcine IFN-α (R&D Systems Inc., Minneapolis, MN) as previously described in Ref. [43]. Recombinant porcine IFN-α (rIFN-α) was used as a standard and sample concentrations were calculated from a standard

curve. Levels of interleukin-8 (IL-8) and interleukin 1-beta (IL-1β) in the BAL were measured by ELISA using DuoSet ELISA reagents from R&D Systems according to the manufacturer's recommendations (Minneapolis, MN).

4.6. Pathological evaluation

At necropsy, an estimate of gross lesion lung involvement was assigned based on the percentage of each lung lobe affected and the percentage of total lung volume each lobe represented. Percentage of total lung volume of each lobe was estimated as 10% for the left and right cranial, left and right middle and accessory lobes and 25% for the left and right caudal lobes. Gross lung lesion scores are frequently used to evaluate pneumonia in pigs [44–46] and the method used in these studies was initially described by Halbur et al. [47].

Sections from the lung were taken for microscopic evaluation. All tissues were fixed in 10% neutral buffered formalin for 24 h and then placed in 90% ethanol. All sections were routinely processed and embedded in paraffin, sectioned and stained with hematoxylin and eosin. Sections were evaluated by light microscopy and the evaluating pathologist was blinded to treatment groups.

4.7. Statistical analysis

GraphPad Prism software (version 5.0; GraphPad Software, San Diego, CA) was used for all statistical analyses. A student's *t*-test assuming unequal variance was used to compare the number of Bb CFUs and viral titer of influenza in different tissues comparing the single-infected group to the coinfected group. One-way analysis of variance (ANOVA) with Tukey's multiple comparison post-test was used for analyzing cytokine data.

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