Lethal Coinfection of Influenza Virus and *Streptococcus pneumoniae* Lowers Antibody Response to Influenza Virus in Lung and Reduces Numbers of Germinal Center B Cells, T Follicular Helper Cells, and Plasma Cells in Mediastinal Lymph Node

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ABSTRACT

Secondary *Streptococcus pneumoniae* infection after influenza is a significant clinical complication resulting in morbidity and sometimes mortality. Prior influenza virus infection has been demonstrated to impair the macrophage and neutrophil response to the subsequent pneumococcal infection. In contrast, how a secondary pneumococcal infection after influenza can affect the adaptive immune response to the initial influenza virus infection is less well understood. Therefore, this study focuses on how secondary pneumococcal infection after influenza may impact the humoral immune response to the initial influenza virus infection in a lethal coinfection mouse model. Compared to mice infected with influenza virus alone, mice coinfected with influenza virus followed by pneumococcus had significant body weight loss and 100% mortality. In the lung, lethal coinfection significantly increased virus titers and bacterial cell counts and decreased the level of virus-specific IgG, IgM, and IgA, as well as the number of B cells, CD4 T cells, and plasma cells. Lethal coinfection significantly reduced the size and weight of spleen, as well as the number of B cells along the follicular developmental lineage. In mediastinal lymph nodes, lethal coinfection significantly decreased germinal center B cells, T follicular helper cells, and plasma cells. Adaptive transfer of influenza virus-specific immune serum to coinfected mice improved survival, suggesting the protective functions of anti-influenza virus antibodies. In conclusion, coinfection reduced the B cell response to influenza virus. This study helps us to understand the modulation of the B cell response to influenza virus during a lethal coinfection.

IMPORTANCE

Secondary pneumococcal infection after influenza virus infection is an important clinical issue that often results in excess mortality. Since antibodies are key mediators of protection, this study aims to examine the antibody response to influenza virus and demonstrates that lethal coinfection reduced the B cell response to influenza virus. This study helps to highlight the complexity of the modulation of the B cell response in the context of coinfection.


Prior influenza virus infection has been demonstrated to impair the immune defense against subsequent pneumococcal growth and infection (8, 9). For example, influenza virus can desensitize epithelial cells and alveolar macrophages to Toll-like receptor (TLR) signals for defense against bacteria (10). Gamma interferon (IFN-γ) induced by influenza virus can inhibit the phagocytosis of pneumococcus by macrophages (11). The type I IFN induced by influenza virus can impair neutrophils (12) and macrophages (13) in the defense against pneumococcus. Influenza virus can decrease tumor necrosis factor alpha (TNF-α) production from natural killer cells in the lung, which allows an increase bacterial growth (14).

In contrast, how secondary pneumococcal infection after influenza can influence the immune response to the initial influenza virus is relatively less well understood. The host adaptive immune response is largely responsible for controlling the influenza virus infection. It has been reported that coinfection could dysregulate Th17 (15) and gamma/delta T cells (16). However, whether the B cell response would be modulated during the coinfection is still not clear.

It is reported that vaccine-induced immunity to influenza virus...
can limit the mortality rate caused by secondary pneumococcal infection after influenza (17). While vaccinating mice with live attenuated influenza vaccine (LAIV) can reduce pneumococcal carriage after influenza virus infection (18), receiving LAIV can, on the other hand, enhance pneumococcal colonization in the absence of influenza virus infection (19). Previous studies highlighted the complexity of the interaction between LAIV and pneumococcal carriage and suggested the importance of anti-influenza virus antibody to control the dual attack by influenza virus and pneumococcus.

A recent study performed by Wolf et al. demonstrated that nonlethal co-infection with influenza virus followed by pneumococcus could enhance anti-influenza antibody production (20). However, clinical data from the 1918 Spanish pandemic and subsequent experimental studies in mice demonstrated that co-infection significantly increased mortality. Currently, how a lethal coinfection could affect the B cell response to influenza virus is still not clear.

Therefore, this study aimed to delineate the B cell response to influenza virus in a lethal mouse coinfection model by examining antibody production in the lung and further provided a mechanism at the cellular level to examine different cell populations in the lung, spleen, and mediastinal lymph node (mLN). This study found that, in the lung, coinfection reduced influenza-specific IgG, IgM, and IgA, as well as the number of B cells, CD4 T cells, and plasma cells. Coinfection reduced the size of the spleen and the numbers in the spleen of CD4 T cells and B cells along the follicular developmental lineage, including T1 (i.e., transitional 1 stage) newly formed B, T2 follicular precursor, and follicular B cells. In mLN, coinfection reduced the numbers of germinal center B cells, T follicular helper cells, and plasma cells.

Collectively, this study demonstrated that lethal coinfection could modulate the B cell response to influenza virus by reducing anti-influenza virus antibody production and depleting the cells in lymphoid organs which are needed to support antibody production. These findings help to highlight the complexity of the modulation of the B cell response in the context of coinfection.

**MATERIALS AND METHODS**

**Mice, influenza virus, and Streptococcus pneumoniae**. Experiments were performed on 6- to 8-week-old female C57BL/6N mice from Charles River Laboratories, Frederick, MD, in compliance with the guidelines for the use of experimental animals by the Committee on the Use of Live Animals in Teaching and Research, The University of Hong Kong.

Mouse-adapted influenza virus A H1N1 (A/PR/8/34) (PR8) was cultured in Madin-Darby canine kidney cells as previously described by us (21, 22). Virus titers were determined by observation of cytopathic effect in cells infected with serial dilutions of virus stock and further confirmed by hemaggglutination assay; the median tissue culture infective dose (TCID50) was calculated according to the Reed-Muench formula.

**Streptococcus pneumoniae** serotype 19F strain 002D5 was prepared as the frozen standardized inoculum (23). 002D5 was cultured in Todd-Hewitt broth with 17% fetal calf serum to mid-logarithmic phase, snap-frozen in liquid nitrogen, and stored at −70°C (24). Viability of the inoculum was checked by plating the inoculum onto blood agar plates and counting the colonies the next day. Throughout the study, the actual infectious dose ranged from approximately 1.5 × 107 to 2 × 107 CFU. The frozen standardized inoculum was used within 3 weeks after preparation and was given to mice within 30 min of thawing.

**Cell isolation and flow cytometry**. Cells were isolated from the whole lung by digestion with collagenase and DNase as previously described by us (25). Cells obtained from the whole lungs, mLN, and spleen were washed and incubated with anti-mouse CD16/32 (BioLegend) for blocking. The cells were then stained with Pacific blue, fluorescein isothiocyanate (FITC), phycoerythrin (PE), aliphycocyanin (APC), PerCy7, Brilliant Violet 510, peridinin chlorophyll protein (PerCP)/Cy5.5, or Alexa Fluor 700-conjugated anti-mouse CD45, B220, CD19, IgM, IgD, CD23, CD21, CD138, major histocompatibility complex class II (MHC-II), CD86, Fas, GL-7, PD-L1, CD3, CD4, CXCR5, CCR7, CD44, PD1, inducible costimulatory molecule (ICOS), CD28, and their relevant isotype controls (BioLegend, eBioscience). All data were acquired on a BD LSRII with a FACSDiva instrument (BD Biosciences) and analyzed using FlowJo software (TreeStar) as previously described by us (26). The number of cells per organ was calculated by CountBright Absolute Counting Beads for flow cytometry (Invitrogen).

**Influenza virus-specific ELISA and ELISpot assay**. A virus-specific enzyme-linked immunosorbent assay (ELISA) was performed (27). PR8 virus particles were UV irradiated and coated onto an ELISA plate. After plates were blocked and washed, samples were applied in serial dilution to allow the adsorption of antibodies onto the coated virus particles. After samples were washed, horseradish peroxidase (HRP)-conjugated anti-mouse IgG, IgM, or IgA detection antibody (Bethyl Laboratories, Inc.) was applied. After another wash step, signal was developed, and absorbance was measured. An enzyme-linked immunosorbent spot (ELISpot) assay was performed by using ELISpot Plus for mouse IgG (Mabtech). To measure the number of total IgG-secreting cells, anti-mouse IgG antibody was coated onto the ELISpot plate. To measure the number of PR8-specific IgG-secreting cells, UV-irradiated PR8 virus particles were coated onto the ELISpot plate. The ELISpot assay was performed according to the manufacturer’s instructions. For the total IgG ELISpot assay, 1,000 mLN cells and 10,000 splenic cells were used. For the PR8-specific IgG ELISpot assay, 5,000 mLN cells and 40,000 splenic cells were used.

**HAI assay**. The serum hemagglutinin inhibition (HAI) titer was measured by HAI assay (28). Serum was incubated at a 1:3 ratio (vol/vol) with receptor-destroying enzyme (RDE; Denka Seiken Co.,) at 37°C for 18 to 20 h. The RDE was inactivated at 56°C for 45 min, which was followed by the addition of phosphate-buffered saline (PBS). The resulting diluted RDE-treated serum samples were further heme-adsorbed with packed turkey red blood cells. HAI assays were performed in round-bottom 96-well plates. Twenty-five microliters of PR8 virus at 4 hemagglutinating units (HAU) was added to 25 μL of serially 2-fold diluted test serum, mixed, and incubated at room temperature for 2 h. Following incubation, 50 μL of 0.5% turkey red blood cells was added to all wells. Plates were observed for agglutination after 45 min. The reciprocal of the highest dilution of serum that completely inhibited hemagglutination was determined to be the HAI titer.

**Adoptive transfer of PR8-specific immune serum**. Mice were infected with PR8 influenza virus or mock infected with PBS for 14 days. After the animals recovered, peripheral blood was drawn to collect PR8-specific immune serum or control mock serum. Serum was pooled, complement heat inactivated, UV irradiated, aliquotted, stored at −80°C, and used within 2 weeks. PR8-specific immune serum was IgG depleted by protein G magnetic beads (Millipore) or mock depleted by PBS. Serum was diluted at 1:3 in PBS and intraperitoneally injected into the coinfected mice from day 4 to 7 post-PR8 infection (day 1 to day 4 post-secondary pneumococcal infection).
Statistics. Statistical analysis was performed by an unpaired student’s t test, one-way analysis of variance (ANOVA), or two-way ANOVA with a Bonferroni test for multiple comparisons using Prism 4 (GraphPad Software). A P value of \( p < 0.05 \) was considered significant.

RESULTS
Coinfection enhanced influenza virus replication in the lung. A lethal coinfection model was established. Mice were sequentially infected with PR8 for 3 days and then with *Streptococcus pneumoniae* serotype 19F. While mice infected with either PR8 or pneumococcus alone lost body weight transiently and recovered from the infection, mice coinfected with PR8 and then pneumococcus lost body weight significantly and had a mortality rate reaching 100% on day 10 post-PR8 infection (Fig. 1A and B). In the control experiment, the mice that were infected with PR8 for 3 days and then administered the inoculation medium without pneumococcus were all alive and behaved similarly to the PR8-infected mice (data not shown). This indicated that the procedure of intranasal treatment after PR8 infection did not significantly affect the disease progress. Coinfection significantly increased lung virus titer and pneumococcal cell counts as measured in both lung homogenate (Fig. 1C) and bronchoalveolar lavage (BAL) fluid (Fig. 1D). While all the mice coinfected with live influenza virus and live or heat-killed (HK) pneumococcus died, all those coinfected with UV-inactivated (UV) or heat-killed influenza virus and live pneumococcus survived (Fig. 1E and F). This indicated that influenza virus replication was necessary for the mortality of coinfection.

Coinfection reduced influenza virus-specific immunoglobulins in the lung. Since coinfection enhanced influenza virus replication in the lung, we examined the B cell response to influenza virus by measuring the relative levels of PR8-specific IgG, IgM, and IgA in lung homogenate, BAL fluid, and serum.

On day 8 after PR8 infection, compared to PR8 infected mice, coinfected mice had significantly low levels of PR8-specific IgG, IgM, and IgA in lung homogenate, BAL fluid, and serum. In peripheral blood, coinfection also significantly reduced the serum level of PR8-specific IgG (Fig. 2C) but only to a level marginally lower than that of IgA. However, coinfection showed no observable effect on the serum level of PR8-specific IgM. To measure the functional activity of the antibody, a hemagglutinin inhibition assay was performed by using the serum samples. Coinfection slightly reduced the serum hemagglutinin inhibition titer (Fig. 2D). This indicated that coinfection decreased both the serum level of virus-specific IgG and serum neutralization activity to influenza virus.
Coinfection reduced both the numbers and percentages of B cells, CD4 T cells, and plasma cells in the lung. To investigate the reason for the reduced antibody level, we proceeded to examine different cell populations in the lung. On day 8 after PR8 infection, compared to PR8 infected mice, coinfection significantly reduced both the numbers and percentages of CD19/CD11001 B cells, CD3/CD4/CD38/CD38 plasma cells (Fig. 3A). Coinfection also significantly reduced the percentage of CD3/CD4 T cells in peripheral blood (Fig. 3B). This suggested that the reduced number of B cells and plasma cells might contribute to the overall reduction of anti-PR8 antibody in the lung.

Coinfection reduced the numbers of B cells, CD4 T cells, and plasma cells in spleen. Since coinfection reduced B cells in the lung, we examined the cell population in other lymphoid organs such as spleen and mLN.

Compared to infection of mice with PR8, coinfection significantly reduced the size and weight of spleen (Fig. 4A) as well as the numbers of total splenic cells, B220+ B cells, and CD3+ CD4+ T cells on day 4 post-PR8 infection (Fig. 4B). Consistently, on day 8 post-PR8 infection, the size and weight of spleen, as well as the number of total splenic cells, B cells, and CD4 T cells, from infected mice were still significantly smaller and lower than those of PR8-infected mice. This indicated that coinfection decreased the mass of spleen, which was associated with splenic cell depletion.

Since the spleen is an important organ where B cell development takes place, to further examine different B cell subpopulations, the numbers and percentages of T1-new formed B (T1-NF; CD3- CD21- cells expressing high levels of IgM and low levels of IgD,[IgMhi IgDlo]), T2-follicular B precursor (T2-FP; CD3+ IgMhi IgDhi cells expressing intermediate levels of CD21 [CD21int]), follicular B (FOB; CD3+ CD21int IgMhi IgDhi), T2-marginal zone precursor (T2-MZP; CD23- CD21hi IgMhi IgDhi), and marginal zone B (MZB; CD23- CD21hi IgMhi IgDhi) cells were examined by flow cytometry according to the expression of different cell surface markers (29,30).

On day 4 post-PR8 infection, coinfection significantly reduced the numbers of T1-NF, T2-FP, and FOB cells but not those of T2-MZP and MZB cells (Fig. 4C). As a result, the percentages of T2-MZP and MZB cells in total B220+ B cells increased relatively (Fig. 4D). This suggested that coinfection may tend to especially reduce the number of B cells along the follicular developmental lineage and thereby change the composition of splenic B cells.
On day 8 post-PR8 infection, consistently, the numbers of T1-NF, T2-FP, and FOB from coinfected mice were still significantly lower than those of PR8-infected mice (Fig. 4C). Coinfection also significantly reduced the numbers of germinal center B cells (B220<sup>+</sup> GL7<sup>+</sup> Fas<sup>+</sup>) and T follicular helper cells (Tfh; CD3<sup>+</sup> CD4<sup>+</sup> CD44<sup>+</sup> CXCR5<sup>+</sup> PD1<sup>+</sup> ICOS<sup>+</sup> cells not expressing CCR7 [CCR7<sup>neg</sup>]) (Fig. 4E). However, the numbers of plasma cells (CD138<sup>+</sup> B220<sup>+</sup>) were similar between infection with PR8 alone and coinfection. These results suggested that coinfection reduced the numbers of cells in the germinal center, which might be related to the reduced antibody response.

To investigate the activation state of the cells in spleen, we examined on day 4 post-PR8 infection the expression of the cell activation markers MHC-II and CD86 on the B220<sup>+</sup> B cell population, which was significantly reduced after coinfection (Fig. 4F). However, the numbers of plasma cells (CD138<sup>+</sup> B220<sup>+</sup>) were similar between infection with PR8 alone and coinfection. This result suggested that coinfection reduced the numbers of cells in the germinal center, which might be related to the reduced antibody response.

On day 8 post-PR8 infection, the number of total lymph node cells was reduced after coinfection (Fig. 5A). While coinfection did not reduce the number of B220<sup>+</sup> B cells, the number and percentage of CD23<sup>+</sup> CD21<sup>int</sup> FOB cells were significantly decreased (Fig. 5A and C). Coinfection also decreased CD3<sup>+</sup> CD4<sup>+</sup> T cells (Fig. 5A), Tfh cells, germinal center B cells, and plasma cells in mLN (Fig. 5D). Similar to results in spleen, coinfection increased PD-L1 expression on germinal center B cells and decreased CD28 expression on Tfh cells (Fig. 5E).

Finally, to examine antigen specificity of the B cells lost, total IgG and PR8-specific IgG ELISpot assays were performed. It was found that coinfection reduced the number of total IgG-secreting cells and of PR8-specific IgG-secreting cells in both spleen and mLN (Fig. 5F). This indicated that the reduction in B cell numbers after coinfection affected all B cells and was not limited to PR8-specific IgG-secreting cells.

Collectively, these data indicated that coinfection caused depletion of B cells and CD4 T cells, which are the cells necessary to support the antibody response, in both spleen and mLN.
PR8-specific immune serum treatment after coinfection improved survival. Finally, to determine if virus-specific antibody was capable of protecting coinfected mice, a rescue experiment involving the adoptive transfer of PR8-specific immune serum was performed. Mice were infected with PR8 or mock infected with PBS for 14 days to collect PR8-specific immune serum or mock serum. The relative level of PR8-specific IgG in the PR8-specific immune serum with IgG depleted by protein G magnetic beads or mock depleted by PBS was measured (Fig. 6A). Coinfected mice were intraperitoneally injected with the PR8-specific immune serum or mock serum to test if the PR8-specific immune serum treatment could decrease the disease severity. Injection of PR8-specific immune serum after coinfection rescued 70% of coinfected mice from death, whereas all mice receiving mock serum or the PR8-specific immune serum with IgG depleted died (Fig. 6B). This suggested that the PR8-specific immune serum treatment, as mediated by PR8-specific IgG, was effective to improve survival after coinfection. Notably, coinfected mice receiving PR8-specific immune serum had significantly lower lung virus titers (Fig. 6C) than the coinfected mice receiving mock serum. This implied that reduced influenza virus replication was associated with lower disease severity in coinfection.

DISCUSSION
Secondary pneumococcal infection after influenza is an important clinical issue. This study aims to delineate the impact of pneumo-
Coccal coinfection on the B cell response to influenza virus. Since it is a general phenomenon that coinfection enhances mortality, we established a lethal coinfection model in which mice were sequentially infected with influenza virus followed by pneumococcus to represent the clinical scenario in which secondary pneumococcal infection after influenza results in excess death. This study showed that lethal coinfection reduced virus-specific IgG, IgM, and IgA in the lung. To delineate the reason for the local reduction of antibody, we investigated the mechanism at the cellular level by examining different cell populations in three organs (the lung, spleen, and mLN) and at two time points (day 4 and day 8 post-PR8 infection). The data showed a consistent trend among the organs and between the two time points, which comprehensively demonstrated that lethal coinfection caused a general reduction in the cellular support to the antibody response, including total B cells, follicular B cells, germinal center B cells, and plasma cells, as well as total CD4 T cells and Thf cells.

The lung is the organ where infection directly takes place. In the lung, coinfection enhanced influenza virus replication and decreased PR8-specific IgG, IgM, and IgA. The reduced antibody was associated with decreases in CD19 B and CD138 plasma cell counts. Notably, the numbers of CD4 T cells in the lung were also decreased after coinfection. Since CD4 T cells help to control influenza virus by producing cytokines and supporting B cell function (31), the reduction of CD4 T cells was consistent with the reduced antibody response.

Spleen is an important organ in which B cell development happens. Coinfection decreased the size and weight of spleen, indicating splenic atrophy. Consistently, the numbers of CD4 T cells in the lung were also decreased after coinfection. Since CD4 T cells help to control influenza virus by producing cytokines and supporting B cell function (31), the reduction of CD4 T cells was consistent with the reduced antibody response.

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cell precursors in the spleen. As intranasal infection of pneumococcus serotype 19F did not cause bacteremia (32) and as blood from coinfected mice in this study was negative in pneumococcal culture (data not shown), it was hence not clear how the coinfection occurring in the lungs could reduce the number of B cells in the spleen.

The mLN is probably the most important organ to support the antibody response in the lung. Again, coinfection significantly reduced the number of follicular B cells and CD4 T cells in mLN. Coinfection also decreased the numbers of germinal center B cells and Tfh cells in both mLN and spleen. All of these data collectively indicated that coinfection could decrease the cellular support in lymphoid organs necessary for an optimum antibody response in the lung.

Notably, coinfection significantly decreased CD138 plasma cells in the lung and mLN but not in spleen. This may explain the observation that coinfection caused a pronounced local antibody reduction in the lung, where all the levels of virus-specific IgG, IgM, and IgA were significantly lower. In contrast, only the virus-specific serum IgG level was significantly reduced in peripheral blood.

Recently, Wolf et al. also reported that coinfection could modulate the B cell response and demonstrated that sequential infection with influenza virus followed by pneumococcus increased the antibody response to influenza virus (20). The discrepancy in results between the study by Wolf et al. and the current study could be due to the different infectious models being used.

In the study of Wolf et al., BALB/c mice were infected with PR8 for 5 days and then infected with pneumococcus serotype 23F at 10⁶ to 10⁷ CFU. While there was no survival data available from that study, Wolf et al. reported in another study that their coinfection model was associated with a 25% mortality rate (33). The remaining 75% mice had weight recovery, starting from day 10 following the initial PR8 infection, and eventually survived after coinfection. Since the B cell response was examined on days 10 and 34 after the initial PR8 infection, the data obtained were collected from a selected mouse population that suffered from the nonlethal coinfection.

In the current study, C57BL/6N mice were infected with PR8 for 3 days and then infected with pneumococcus serotype 19F at 1.5 × 10⁷ to 2 × 10⁷ CFU. All coinfected mice lost significant body weight and reached 100% mortality on day 10 after the initial PR8 infection. This 3-day lethal coinfection model was based on the time frame used by McCullers et al. (34), and the mortality rate demonstrated here was consistent with that reported by them. Since we examined the B cell response on day 4 and day 8 after the initial PR8 infection, the data obtained were collected from the total mouse population that was going to die from the coinfection.

In the study of Wolf et al. (20), coinfection enhanced virus-specific IgG in the lung and serum, as well as the number of total and virus-specific antibody-secreting cells in mLN. Since mice suffered from nonlethal coinfection and since the cellular response was examined on day 34 post-PR8 infection, it is logical that the mice that were still alive on day 34 would have had a greater antibody response in order to recover as otherwise they might have died from the coinfection.

Notably, the study of Wolf et al. (20), on day 10 post-PR8 infection, coinfected mice had lower virus-specific IgG amounts in the lung, as well as fewer Tfh and GCB cells in mLN although there was no statistical significance. On the other hand, the current study used a lethal coinfection model and provided an earlier kinetics, showing that on day 8 post-PR8 infection, the virus-specific IgG, IgM, and IgA levels in the lung, as well as the related cellular support from the lung, mLN, and spleen, including Tfh, GCB, and plasma cells, were significantly decreased.

Furthermore, in the study of Wolf et al. (20), the mice that suffered from the nonlethal coinfection showed influenza virus replication in the lung comparable to that of PR8-infected mice, whereas our lethal coinfection significantly increased the influenza virus titer in the lung. Taken together with the study of Wolf et al., the data collectively demonstrated an increased B cell response and comparable lung viral replication in nonlethal coinfection but a decreased B cell response and increased lung viral replication in lethal coinfection. This highlights the complexity of the modulation of the B cell response in the context of coinfection.

Another study done by Haynes et al. also focuses on the role of virus-specific antibody in coinfection (35). In that study, coinfected mice received immune serum before the initial PR8 infection, and it was found that vaccination-induced preexisting cross-reactive immunity to influenza virus could lower the susceptibility of mice to a secondary bacterial infection. That study pointed out
the protective role of virus-specific antibody to coinfection. In contrast to that report, in the current study coinfected mice received immune serum after the coinfection, and it was demonstrated that the immune serum was effective to reduce virus replication and was capable of protecting coinfected mice from death. Our study emphasized the treatment strategy rather than the prophylaxis that the virus-specific antibody could offer. It is currently not clear how the transfer of immune serum after coinfection could help to protect mice from death in our model. It may be due to the control of influenza virus replication, as demonstrated in the experiment shown in Fig. 6C. Alternatively, as suggested by Haynes et al., it could be due to the reduced lung inflammation or the control of bacterial growth. Therefore, further investigation would be required to examine the reasons that coinfected mice are protected from death. Collectively, this study and that of Haynes et al. suggest that a lack of virus control was associated with death upon coinfection.

B cells are clearly essential to control infection (36). B cell-deficient mice are less capable of clearing influenza virus infection (37, 38). However, apart from B cells, other responses, including
the innate immune cell response, lung inflammatory response, and T cellular response, are also important to the defense against coinfection. Similar to the study of Wolf et al. (20) and other reports (8, 9, 39–41), the current study also demonstrated that coinfection significantly increased Ly6G⁺ neutrophils and F4/80⁺ macrophages infiltrating the lung (Fig. 7A) and enhanced lung inflammatory cytokine and chemokine levels (Fig. 7B). Influenza virus infection can lead to the hyporesponsive innate immune state that creates a predisposition to secondary bacterial infection (42, 43). In addition to the affected innate immune cell response, this study demonstrated that lethal coinfection could reduce the antibody response to influenza virus and suggested that the lack of anti-influenza virus antibody could be functionally relevant in coinfection.

Conclusions. This study demonstrated that lethal sequential coinfection with influenza virus followed by pneumococcus reduced the level of virus-specific IgG, IgM, and IgA and enhanced influenza virus replication in the lung. The reduction of antibody response was associated with a general depletion in the cellular support in the lung, mLN, and spleen, including B cells, FOB cells, GCB cells, CD4 T cells, Tfh cells, and plasma cells. The serum adoptive transfer experiment suggested that immune serum was an effective treatment for coinfection. This study helps us to understand the modulation of the B cell response to influenza virus during lethal coinfection.

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We declare that we have no commercial or financial conflicts of interests.

Author contributions are as follows: Y.W., W.T., and Y.-L.L. designed experiments; Y.W. performed experiments; K.-T.L. provided general interests. K.-T.L., Peiris JSM, Lau Y-L. wrote the paper.

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