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Pneumocystis Infection Enhances Antibody-Mediated Resistance to a Subsequent Influenza Infection

James A. Wiley and Allen G. Harmsen

In contrast to the detrimental outcomes most often associated with the resolution of coinfections, the model presented here involving a localized Pneumocystis infection of the lung, followed 2 wk later by an influenza virus infection, results in a significant beneficial outcome for the host. In the week following the influenza infection, immunocompetent coinfected animals exhibited an accelerated rate of virus clearance, an accelerated appearance of higher influenza-specific neutralizing Ab titers in their serum and bronchoalveolar lavage fluid (BALF), significantly reduced inflammatory cytokine levels in their BALF, and reduced levels of morbidity relative to animals infected only with influenza virus. The beneficial outcome observed in coinfected immunocompetent animals was dependent on the ongoing resolution of a viable Pneumocystis infection. No differences in viral clearance were detected between coinfected and influenza-only-infected μMT mice or likewise for SCID mice. The accelerated anti-influenza response did not appear to be associated with influenza-specific CD8 T cell-mediated responses or NK cell responses in the lung. Rather, the increased rate of viral clearance was due to the enhancement of the influenza-specific Ab response, which in turn was transiently dependent upon the resolution of the ongoing Pneumocystis infection.

modeled in murine studies (5, 6). Unlike Pneumocystis, influenza virus infections are capable of inducing symptomatic clinical infections of the lungs of the immunocompetent individuals. This virulent pathogen infects the respiratory and alveolar epithelium of the lung. The effective resolution of influenza infections is dependent upon the local recruitment of Ag-specific humoral and cell-mediated immune responses (7).

In the Pneumocystis/influenza coinfection animals, we observed a significant acceleration of viral clearance, an accelerated production of influenza-specific Ab titers, reduced lung damage, significantly altered cytokine levels in the lung, and evidence of reduced morbidity 1 wk after the influenza infection. If the sequence of the pathogens was reversed, no comparable enhancement of the anti-Pneumocystis response was detected. The ability of the Pneumocystis-induced immune response in an immunocompetent host to augment the protective immunity of a subsequent anti-influenza response suggests that under specific conditions, localized concurrent immune responses in the lung may be exploited for the benefit to the host.

Materials and Methods

Mice

BALB/c and C57BL/6 male and female mice at 6–8 wk of age were either purchased from The Jackson Laboratory or obtained from the breeding colonies maintained at Montana State University, Bozeman, MT. The male and female BALB/c SCID mice were obtained from Montana State University breeding colonies, and C57BL/6 SCID mice and μMT mice at 6–8 wk of age were obtained from The Jackson Laboratory. All of the animals were housed at the Animal Resource Center at Montana State University for the duration of these experiments. The animal facilities and the experimental procedures used throughout these experiments complied with the approved institutional animal care and use committee protocols established at Montana State University.

Pathogens and infection procedures

The Pneumocystis murina used for these experiments was maintained in a Pneumocystis-infected colony of BALB/c SCID mice at Montana State University. The influenza virus used was the A/PR8/8/34 (PR8; H1N1) strain. This virus was prepared at and obtained from the Trudeau Institute (Saranac Lake, NY). The virus stock was grown in the allantoic fluid of 10-day-old chicken embryos that had been infected for 48 h at 35°C. The harvested allantoic fluid was then stored at −80°C.

Lung homogenates from Pneumocystis-infected SCID mice were used to infect the mice in these studies. To administer intratracheal inoculations of Pneumocystis-infected lung homogenates, experimental mice were lightly anesthetized with 5% isoflurane in oxygen. A 100-μl inoculum of the Pneumocystis-infected lung homogenate containing 10⁷ Pneumocystis organisms was then directly injected into the lungs of the mice. Control groups in the coinfection experiments were given lung homogenates from SCID mice that had not been infected with Pneumocystis. Aliquots of 100 μl of the uninfected lung homogenates were used. These groups are referred to as the influenza-only control group. The β-glucan preparation was made from a β-glucan stock (Sigma-Aldrich) and was similarly administered.

PR8 influenza inoculations were also done while the mice were under a 5% isoflurane anesthesia. Mice were given a 50-μl intranasal inoculation containing 1500 PFU of PR8 influenza virus. The mice were taken at designated time points up to 10 days after the influenza infection. In those experiments where body weight was monitored, the animals were weighed on the day of the influenza infection and each day afterward until the end of the experiment. The change in body weight was determined by the

group; n = 5 or 6 mice per group; the dotted horizontal line indicates the limit of assay detection. C. Titration of PR8-specific serum IgG levels from coinfected mice 1 wk after influenza infection. Serum samples from mice at 2- (■) or 3- (▲) wk interval between infections or mice given influenza-only infection (□) were tested by ELISA and absorbance was read at 405 nm.
difference between the body weight at the time of influenza infection and
the body weight on the given day after infection.

Assessment of pathogen recovery

The *Pneumocystis* burden in the lungs of infected mice was determined as
previously described (8). Briefly, infected lungs were harvested from mice
and passed through a mesh screen in 5 ml of HBSS. An aliquot of the
suspension was taken to make a cytocentrifuge smear, which was then
stained with Diff-Quik (Baxter). The number of *Pneumocystis* nuclei
counted in 30–50 oil immersion fields was used to calculate the total num-
ber of *Pneumocystis* in the lungs. The limit of detection using this method
was log10 4.1 nuclei/lung.

Following influenza infection, the mice were sacrificed as previously
described (9). Lungs recovered from the mice were snap-frozen in liquid
nitrogen and then stored at −80°C until analyzed. At this time the lungs
were homogenized and 10-fold serial dilutions of the homogenates were
used to inoculate monolayers of Madin-Darby canine kidney cells. Our
previously described plaque assay procedure was used in the experiments
reported here (10).

**Recovery of bronchoalveolar lavage fluid**

Bronchoalveolar lavage fluids (BALF)3 were obtained by washing the
lungs with 1.5 ml of 3 mM EDTA in HBSS in two aliquots of 750 µl. A
recovery of 1.3 ± 0.1 ml of BALF was reproducibly obtained from each
mouse. The cells recovered in the BALF were counted to obtain total cell
counts, and an aliquot of the recovered BALF was used to make a Diff-
Quik-stained cytospin to examine the differential cell recovery from each

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3 Abbreviations used in this paper: BALF, bronchoalveolar lavage fluid; LDH, lactate
dehydrogenase; iBALT, inducible BALT.
animal. The remaining cellular content in the recovered BALF was removed by centrifugation for FACS analysis. The fluid was then stored at −80°C for use in determining the Ab, cytokine, albumin, and lactate dehydrogenase (LDH) content.

**Albumin and LDH content in the BALF**

The BALF albumin was determined by use of an albumin colorimetric assay (Sigma-Aldrich). Color absorbency was read at 630 nm and reported in mg/ml in the BALF. The level of LDH detected in the lavage fluid was determined by colorimetric assay (CytoTox 96, Promega). The assay was read at an absorbance of 490 nm and reported as U/ml in the BALF. Each of the samples was tested in duplicate for the albumin and LDH assays.

**Analysis of cytokines recovered in BALF**

Cytokine levels in the BALF were determined by use of an inflammatory cytokine bead array kit (BD Biosciences). The assays were conducted according to the manufacturer’s instructions. The detection of the cytokines was done using a FACScan cytometer and then analyzed according to the cytokometric bead array software (BD Biosciences). The amount of IL-13 recovered in the BALF was determined using a murine IL-13 Quantikine ELISA kit (R&D Systems).

**FACS staining of recovered cells**

Cells were recovered from the BALF by centrifugation and then stained with the following fluorochrome-conjugated mAbs: PE-conjugated anti-mouse CD8, FITC-conjugated CD49 (BD Pharmingen), PE/Cy5.5-conjugated anti-mouse TCR (Caltag Laboratories), and NP:366-374-specific and PA:224-233-specific allophycocyanin-conjugated tetramer complexes that recognized H-2D^b/-restricted virus-specific CD8^+ T cells (Trudeau Institute). Analysis of the stained cells was conducted on a FACSCanto (BD Biosciences) and then analyzed using FlowJo software (BD Pharmingen).

**Analysis of PR8-specific Abs**

PR8-specific Abs were detected by ELISA. These assays were conducted using a PR8 membrane preparation derived from a purified PR8 influenza virus preparation (9). Serum dilutions are designated in the figures, and the BALF samples remained undiluted. Alkaline phosphatase-conjugated anti-mouse IgG (Sigma-Aldrich) and IgA and IgM (Serotec) were used to detect PR8-specific serum Abs in the assays. Absorbance was read at 405 nm. Virus neutralization assays using dilutions of serum or BALF were conducted to determine the extent of their virus neutralization activity. Dilutions of serum or BALF samples were challenged with 150 PFU of influenza virus. The sample/virus mixture was incubated for 90 min at 37°C. Following this, the mixture was separated into aliquots onto Madin-Darby canine kidney cells to determine by plaque assay if any infectious virus remained. Our previously described plaque assay procedure was followed (10). The percentage neutralization was determined by comparing the total number of plaques recovered from nonspecific serum samples vs the total number of plaques recovered from the serum samples obtained from the coinfected and influenza-only infected animals.

**Statistical analysis of group data**

The data are expressed as the means ± SD. The results reported here are from one experiment that was representative of at least three independent experiments unless stated otherwise. The sample size of each group is 4–6 mice. Statistical differences between designated groups were determined using nonparametric one-way ANOVA tests with Bonferroni corrections to account for multiple comparisons within given experiments or by t tests when only two experimental groups were involved.

**Results**

**Pathogen recovery from lungs**

We have previously used the rate of pathogen clearance from an infection site to assess the efficacy of a local adaptive immune response (9). In our present coinflection model we found that virus recovery in the lungs 1 wk after an influenza infection was >100-fold less if a *Pneumocystis* infection occurred 2 wk earlier relative to that seen in the influenza-only control group (Fig. 1A). If a *Pneumocystis* infection was initiated 3 wk earlier, the recovery of virus was only 10-fold less than that seen in the influenza-only control group. If the *Pneumocystis* and influenza infections were given 6 h apart in either order, we found that the recovery of
influenza virus from the lungs was equivalent between the coinfection groups, and that both coinfection groups had equivalent or slightly greater viral recoveries than those seen in the influenza-only group (Fig. 1B). The recovery of Pneumocystis from the lungs was not altered by an influenza infection given 6 h before or after the influenza infection (data not shown).

The PR8-specific Ab response in the serum was assessed in the groups of animals 1 wk after the influenza coinfection (Fig. 1C). The PR8-specific serum IgG response was higher in animals that had received the Pneumocystis infection 2 wk earlier than it was in those animals that had received the Pneumocystis infection 3 wk earlier. The serum PR8-specific IgG response in both of these coinfection groups was greater than that seen in those animals that had received only an influenza infection. As a result of this finding, further coinfection experiments were based on a 2-wk interval between a prior Pneumocystis infection and the influenza infection.

The rate of clearance of influenza virus in the coinfection group and in the influenza-only control group was examined over a 10-day period following the virus challenge (Fig. 2A). At 3 and 5 days after the influenza infection, viral recoveries in the coinfection and the influenza-only infection groups were high. Although there was no significant difference between these groups, slightly more virus was recovered from the coinfect ed animals. By 7 days after the influenza infection no virus could be detected in the lungs of the coinfected animals, whereas viral recoveries in the influenza-only control group remained unchanged from those seen earlier in the week. At 10 days postinfluenza infection, significant differences in the level of viral recovery still existed between the coinfection and influenza-only infection groups, although clearance of virus from the influenza-only control group was reduced relative to levels seen at day 7 postinfluenza infection. The wild-type coinfect ed animals used in these experiments easily cleared the Pneumocystis infections. At the time of the influenza infection (day 0), the Pneumocystis burden in the coinfect ed animals was >10-fold less than what these animals had been inoculated with 2 wk earlier (Fig. 2B). Five days after the influenza infection, the Pneumocystis burden was further diminished and was in fact undetectable in 4 of 5 animals. Pneumocystis burdens were below the limit of detection in the coinfect ed animals at 7 and 10 days postinfluenza infection. If an influenza infection was given 2 wk before a Pneumocystis infection, no alteration in the clearance of the Pneumocystis from the lungs was detected 2 wk later (Fig. 2C).

To ascertain the necessity for a viable Pneumocystis infection to achieve this accelerated reduction in viral recovery, mice were dosed with a β-glucan preparation. β-glucan is a subcomponent of the Pneumocystis cell wall and is a well-known non-specific immunostimulant (11–13). This group was treated twice with 250 ng of the β-glucan preparation (1 dose per week) during the 2 wk before the influenza infection. We had previously observed that this dose of β-glucan elicited an inflammatory cell infiltrate in the lungs without incurring any detectable indications of morbidity (data not shown). Despite this treatment regime, we were unable to replicate the accelerated clearance of influenza virus from the lungs of the β-glucan-treated mice that we had observed in the coinfect ed mice (data not shown).

Analysis of cell recovery in BALF

Cellular infiltrates into the lungs of the coinfect ed animals were already evident at the time of the influenza infection and were...
greater during the 5 days after the influenza infection relative to those observed in the influenza-only control animals (Fig. 3). The recovery of macrophages in the BALF of the coinfected mice was significantly greater throughout the resolution of the influenza infection (Fig. 3A). Macrophage recruitment in the influenza-only infection group did not change from the levels observed at the time of the influenza infection. The level of lymphocyte recovery in the coinfected animals did not show any appreciable change during the week following the influenza infection (Fig. 3B). By 7 and 10 days after the influenza

FIGURE 6. Cytokine levels in the BALF following influenza infection. Cytokine levels in the BALF of the coinfected (⧫) and influenza-only infected mice (▲) were determined at 7 and 10 days after the influenza infection. The BALF were assessed for the presence of the selected cytokines using cytokine bead array kits or by ELISA for the IL-13 levels. Cytokine bead array samples were screened using the FACScan, and the data were analyzed by the bead array software. IL-13 ELISA results were calculated from the standard curve as per the manufacturer’s instructions. n = 5 or 6 animals/group; *, p < 0.05; **, p < 0.005; ***, p < 0.0001.
infection, lymphocyte recruitment in the influenza-only group had reached equivalent or slightly greater levels than those detected in the coinfectected animals. Neutrophil recruitment levels peaked at day 3 in the coinfectected animals and then diminished thereafter (Fig. 3C). In contrast, neutrophil recruitment levels did not reach their peak in the influenza-only infected animals until 7 days after the influenza infection. The recovery of eosinophils was significant in the coinfectected animals throughout the resolution of the influenza infection, whereas eosinophil recovery in the influenza-only infected animals remained at negligible levels (Fig. 3D).

Change in body weight

To obtain an indication of the general health of the animals as they responded to the influenza infection, we monitored the daily changes in their body weights. During the 10 days following the influenza infection, the coinfectected animals did not lose any weight (Fig. 4). In fact, they averaged an additional 1 g weight gain by the end of the experiment. The influenza-only control animals continued to lose weight from day 4 postinfluenza infection to the end of the experiment.

Serum albumin and LDH in BALF

Increased levels of serum albumin and LDH in the BALF have been used as indicators of lung damage following infection (14–17). Serum albumin (Fig. 5A) and LDH levels (Fig. 5B) in the BALF of the coinfectected animals were higher at the time of the influenza infection (day 0). Following the influenza infection, serum albumin levels in the BALF of the coinfectected animals remained equivalent to the day 0 values, whereas significant increases were detected in the influenza-only infection group. LDH levels in the coinfectected animals also remained unchanged following the influenza infection, whereas levels in the influenza-only infection group rose by >3- and 6-fold at 7 and 10 days, respectively, following the influenza infection.

Cytokine levels in the BALF

One week after the influenza infection the levels of TNF-α, IL-10, IFN-γ, MCP-1, and IL-6 in the BALF of the coinfectected animals were significantly reduced relative to those detected in the influenza-only control animals (Fig. 6). In contrast to this trend, the levels of IL-13 recovered in the BALF of the coinfectected animals remained somewhat higher as the influenza infection resolved. IL-5 levels in the BALF following the influenza infection were not significantly altered by the prior Pneumocystis infection (data not shown). By day 10 postinfluenza infection the cytokine levels in the BALF of the influenza-only control group were still generally higher than in the coinfectected animals, although the levels were greatly diminished in both groups relative to those detected on day 7. In contrast, IL-13 levels were still higher in the coinfectected group at this time.

FACS analysis of cells recovered in BALF

We determined the level of influenza-specific CD8 T cells in the BALF by staining with tetramer complexes that recognized MHC class I H-2D^b NP_366 – 374-specific and PA_224 – 233-specific CD8 T cells. Before day 7 postinfluenza infection, only background staining for influenza-specific CD8^+ T cells was detected in the BALF of the coinfectected and influenza-only infected animals (Fig. 7A). At day 7 we found equivalent but low numbers of influenza-specific CD8^+ T cells in both groups of animals. By day 10 postinfluenza infection, the recovery of influenza-specific CD8^+ T cells was significantly greater in the influenza-only infection group. In fact, we did not detect any difference in the number of these cells recovered from the coinfectected animals at 7 and 10 days after the influenza infection.

The recovery of NK cells from the BALF of the coinfectected animals from the time of the influenza infection until day 7 postinfluenza infection was relatively consistent (Fig. 7B). NK cells were not found in the BALF of the influenza-only infection group until 3 days after the influenza infection. At this time they were significantly less than the levels observed in the coinfectected animals. By day 5 postinfluenza infection, the equivalent amounts of NK cells were detected in the BALF of the coinfectected and influenza-only infected animals. NK cell recruitment into the airways of the influenza-only infected animals continued to increase through day 7 postinfluenza infection. The recovery of NK cells from the BALF of the influenza-only infected animals at day 7 postinfluenza infection was significantly greater than from the coinfectected animals. Three days later the recovery of NK cells from the BALF of the coinfectected and influenza-only infected animals was equivalent and low.

Levels and activity of anti-influenza Abs

In our initial studies, an ELISA analysis of serum Ab levels revealed that the animals that had a 2-wk interval between the Pneumocystis and influenza infections had greater PR8-specific serum IgG titers 1 wk after the influenza infection than did those animals.
that had a 3-wk interval between infections (Fig. 1C). In our subsequent experiments, ELISA analysis of the serum and BALF recovered following the influenza infection indicated that PR8-specific Abs appeared sooner and remained at greater levels in the coinfect ed animals than in the influenza-only infected animals (Fig. 8). In our analysis of the viral recovery from the lungs of the coinfect ed animals, we had found that no virus could be detected at 7 and 10 days after the influenza infection, whereas significant levels of viral recovery were still noted in the influenza-only control animals. In view of our viral recovery results we examined the

FIGURE 8. Detection of influenza-specific Ab titers in the BALF and serum. ELISA tests were performed on the BALF and serum from the coinfect ed and the influenza-only infected animals 7 and 10 days after infection with influenza virus. Influenza-specific serum IgG (A) and IgM (B) levels were measured in a 1/100 dilution of a serum aliquot. Influenza-specific BALF IgG (C), IgA (D), and IgM (E) levels were measured in undiluted BALF aliquots. Absorbance readings were taken at 405 nm. This is one of three independent experiments, n = 5 or 6 animals. *, p < 0.05; **, p < 0.01; ***, p < 0.005.
virus-neutralizing activity of the serum and BALF recovered from the coinfected and influenza-only infected animals (Fig. 9). The viral-neutralizing activity in the serum of the coinfected animals was significantly greater at a 1/100 dilution at day 5 and was only slightly better or equivalent to the influenza-only infected animals as the serum became more diluted (Fig. 9A). At 7 and 10 days after the influenza infection, the serum-neutralizing activity of the coinfected animals tended to be only slightly better than that of the influenza-only infected animals up to a dilution of 1/1000 (Fig. 9, B and C).

The accelerated viral clearance in the coinfected animals was evident at 7 days after the influenza infection. At this time,
substantial PR8-specific Ab levels were detected in the BALF of the coinfected animals, whereas only trace levels were detected in the influenza-only control animals. To determine whether the accelerated appearance of PR8-specific Abs at the site of infection could be responsible for the accelerated viral clearance observed in the lungs, we conducted viral neutralization assays using the BALF. Viral-neutralizing activity was significantly greater in the BALF recovered from the coinfected animals than from the influenza-only infected animals at 7 days postinfluenza infection (Fig. 9D). By day 10 postinfluenza infection, the viral-neutralizing capacity of the BALF recovered from the influenza-only control group had become equivalent to that detected in the coinfected animals (Fig. 9E). No virus-neutralizing activity was detected in the BALF from naive animals or in animals that had received only a Pneumocystis infection (data not shown).

Absence of Ab negates enhanced viral clearance in coinfected mice
The evidence from our coinfection model with immunocompetent animals suggested that the accelerated appearance of higher serum and BALF levels of influenza-specific Abs and their associated viral-neutralizing activity may be responsible for the enhanced rate of viral clearance. To further examine the necessity of the Ab response for the accelerated clearance of influenza virus in the coinfected immunocompetent mice, we coinfected μMT and SCID mice. At the time of the influenza coinfection, no differences in the cytokine and cellular infiltration levels were detected in the BALF recovered from the immunocompetent and μMT mice as a result of the Pneumocystis infection (data not shown). At this time, Pneumocystis burdens averaged 6.04 ± 0.76 and 7.12 ± 0.09 (log_{10}) nuclei for the immunocompetent and μMT coinfected mice, respectively. Seven days after the influenza infection, viral recovery levels in the μMT coinfected and μMT influenza-only infected control groups were equivalent (Fig. 10) despite the Pneumocystis burden having increased to 7.98 ± 0.18 (log_{10}) nuclei in the coinfected μMT mice. In fact, the viral recovery levels from the coinfected and influenza-only infected μMT groups were also equivalent to those detected in the immunocompetent influenza-only control group. In contrast, 7 days after the influenza infection the viral recovery level in the coinfected immunocompetent animals was significantly reduced and the Pneumocystis burden had been reduced to 4.94 ± 0.74 (log_{10}) nuclei. Viral recoveries from the coinfected and influenza-only infected SCID mice were equivalent and were only slightly greater than those found in both of the μMT infection groups (Fig. 10). Pneumocystis burdens in the coinfected SCID mice at the time of the influenza coinfection were 6.45 ± 0.32 (log_{10}) nuclei and had increased to 7.56 ± 0.21 (log_{10}) nuclei 7 days after the influenza coinfection. At the time of the influenza viral infection, cytokine levels in the BALF of the coinfected SCID mice were lower than in the immunocompetent and μMT coinfected animals or they were only at trace levels (data not shown). Only macrophages were recovered from the BALF of the coinfected SCID mice at this time, and the recovery level of these cells was equivalent to that detected in the coinfected μMT and immunocompetent animals (data not shown).

Discussion
The resolution of the coinfection model presented here results in a beneficial outcome for its host. In the coinfected mice the clearance of influenza virus from their lungs was significantly accelerated relative to that seen in the influenza-only control group. The coinfected mice had at least a 3 day or better headstart on the clearance and resolution of the influenza virus infection. This rapid viral clearance was accompanied by the accelerated appearance of a more intense PR8-specific neutralizing Ab response in the lungs. No corresponding accelerated influenza-specific CD8 T cell- or NK cell-associated response was evident in the lungs of the coinfected animals at the time that the accelerated viral clearance was observed. In the absence of an Ab response, as with the μMT mice, viral recovery from the lungs was not altered by a preceding Pneumocystis infection. The accelerated resolution of the influenza virus coinfection was also associated with reduced indications of morbidity, lung damage, and decreased levels of proinflammatory cytokines recovered in the BALF. Our results indicated that the increased rate of viral clearance observed in the immunocompetent coinfected animals was dependent on an accelerated and enhanced localized influenza-specific Ab response, which, in turn, was dependent on a temporal association with the resolution of an ongoing Pneumocystis infection.

The exposure sequence and the time between the pathogen exposures were found to be critical. An influenza infection 2 wk before a Pneumocystis infection did not alter the clearance of the Pneumocystis infection. If exposure to both pathogens occurred within 6 h of one another in either order, no alteration in the recovery of either pathogen was detected. Since the greatest Pneumocystis burden occurred within 6 h of the influenza infection in these models, this suggested that no Pneumocystis-derived Ags were associated with the enhancement of the influenza response. The inability of the β-glucan preparation to induce an equivalent
level of Ab enhancement and viral clearance confirmed that a major component of Pneumocystis could not elicit the same immune mediators or mechanisms as those associated with the response to the viable Pneumocystis infection. This observation is analogous to the inability of a Toxoplasma gondii lysate preparation to elicit the equivalent effect that the response to a viable T. gondii infection had upon the resolution of a Nippostrongylus brasiliensis infection (18). In both coinfection models, a viable infection rather than simply the exposure to a component(s) of the pathogen was required to influence the immune response directed against the second pathogen.

The transient nature of the enhanced anti-influenza response was apparent if changes were made to the interval between the pathogen exposures. If the interval between the Pneumocystis and then the influenza infection was extended to 3 wk, the accelerated effect on viral clearance and the enhancement of influenza-specific serum IgG levels were decreased relative to those observed for a 2-wk interval. This suggested that the potency of the enhancement effect upon the ensuing influenza immune response might be linked to the maturation of an established immunological component of the Pneumocystis-induced immune response.

At the time of the influenza infection, significant levels of cellular recruitment and cytokines were evident in airways of the coinfelected animals. Despite this, viral recoveries in the coinfelected animals up to 5 days after the influenza infection were still 1000-fold greater than the original viral inoculum and were similar or slightly better than those of the influenza-only control group. This indicated that a comparable influenza virus infection of the respiratory epithelium had been established in the lungs of the coinfelected mice rather than the development of an abortive infection of the recruited cells present in the airways of the lung.

In the coinfelected animals the accelerated rate of viral clearance was achieved without any additional recruitment of lymphocytes or neutrophils. In contrast, as lung damage and viral loads increased in the influenza-only infection group, neutrophil and lymphocyte recruitment in these animals increased to levels equivalent to or beyond those of the coinfelected animals. The absence of any additional increase in the recruitment of inflammatory cells may have contributed to the reduced level of morbidity in the coinfelected animals. The presence of eosinophils in the coinfelected group and their absence in the influenza-only group were expected, as the recruitment of these cells is typical of the response to a Pneumocystis infection.

The coinfelected animals lost the least amount of body weight following the influenza infection, and only this group was able to return to and exceed their starting body weights. The diminishing viral titers in the lungs of the coinfelected animals were accompanied by reduced morbidity levels and reduced albumin and LDH levels in their BALF. These observations also comply with the reduced levels of proinflammatory cytokines (TNF-α, IFN-γ, MCP-1, IL-6) detected in the BALF of the coinfelected animals following the influenza infection. The diminishing viral burdens in the coinfelected mice are likely a primary reason for the reduced levels of morbidity and lung damage observed in these mice. However, the presence of antiinflammatory cytokines (IL-10 and IL-13) detected in the BALF of the coinfelected animals at the time of the influenza infection may regulate the production of influenza-induced proinflammatory cytokines. This may have limited any influenza-induced exacerbation of the inflammatory environment in the lungs of the coinfelected animals and thereby also have contributed to the reduced levels of morbidity and lung damage in these mice. IL-10 inhibits the production of a variety of CC (including MCP-1) and CXC chemokines, IL-6, IL-12, and subsequently IFN-γ, IL-18, and TNF-α, as well as autoregulating itself (19, 20). Following Pneumocystis infections, IL-10 has been shown to remain at biologically effective inhibitory levels in the lungs of immunocompetent mice up to 14 days after infection (21). IL-13 has been considered to be equivalent to IL-10 in regards to its antiinflammatory capabilities (22). Both IL-10 and IL-13 have been shown to regulate proinflammatory cytokine production by triggering the inhibition of NF-κB nuclear translocation (20, 22). In the absence of IL-10 and IL-13 at the time of the influenza infection, subsequent higher levels of TNF-α, IFN-γ, MCP-1, and IL-6 were observed in association with higher viral recoveries in the influenza-only control group. The lack of any increased cellular recruitment, the reduced levels of proinflammatory cytokines detected in the BALF, and the accelerated viral clearance seen in the coinfelected animals support the contention that antiviral immune responses often operate in an excessive inflammatory environment that is beyond what is necessary to achieve viral clearance (15). A moderation of the elicited inflammatory response may be affordable and could be of benefit to the host without compromising viral clearance (23).

Our evidence indicates that the rapid viral clearance from the lungs of the coinfelected animals is due to the accelerated appearance of influenza-specific neutralizing Abs at the site of infection. Seven days after the influenza infection, PR8-specific Ab levels in the serum and BALF of the coinfelected animals were significantly elevated relative to the influenza-only control animals. At this time, no virus was detected in the lungs of the coinfelected animals, and the virus-neutralizing capabilities of the BALF of these animals were significantly greater than those of the influenza-only infected animals. In fact, 5 days after the influenza infection, virus-neutralizing activity could already be detected in the serum of the coinfelected animals, whereas it was undetectable in the influenza-only infected animals. It is unlikely that cell-mediated immune responses in the lung are responsible for the rapid viral clearance from the lungs of the coinfelected animals. NK cell and influenza-specific CD8 T cell accumulations in the airways of the coinfelected and influenza-only infected animals were low and equivalent at the time when the greatest difference in viral burden between the infection groups was observed. The continued viral recovery at day 10 postinfluenza infection in the influenza-only infected animals corresponds with the increased accumulation of influenza-specific CD8 T cells in the airways of these animals. In an analogous manner, the lack of viral recovery at days 7 and 10 postinfluenza infection in the coinfelected animals corresponds with the absence of any increase in PR8-specific CD8 T cell accumulation. This coinfection model demonstrates that the coinfelected animals had at least a 3 day or better headstart on their Ab-mediated clearance of the influenza infection. Although this headstart was short-lived, it was nonetheless critical in the beneficial resolution of the influenza infection in the coinfelected animals. At day 10 postinfluenza infection the level and neutralizing capabilities of these PR8-specific Abs had increased substantially in both groups and had become closer to equivalence. Further long-term experiments will be required to determine whether the neutralizing activity and levels of PR8-specific Abs in both groups would plateau at the same time and level.

Viral recoveries in the influenza-only infected μMT and wild-type and in the coinfelected μMT mice were equivalent at day 7 postinfluenza infection and all were more than 100-fold greater than the administered viral inoculum. It did not appear that the Pneumocystis burden, the Pneumocystis-induced cellular recruitment or cytokine production, or any Pneumocystis-induced pathology unique to μMT mice acted to impede the establishment of a viable influenza infection in these mice. The inability of μMT mice to mount Ab-mediated immune responses and the failure of
the coinjected μMT mice to duplicate the accelerated viral clearance observed in the immunocompetent coinjected mice support our contention that the accelerated resolution of the influenza infection in these mice is Ab mediated.

The accelerated influenza Ab response may be associated with the generation of a local immune environment within the lung before the influenza infection that would facilitate the accelerated production of influenza-specific Abs. Within the lung, inducible BALT (iBALT) has been shown to play an effective role in the clearance of influenza infections in mice and to endow those animals with the ability to survive higher viral doses while incurring fewer pathological consequences than are associated with systemic immune responses (24). The transient establishment of iBALT structures within the lung before a pathogen insult could facilitate the accelerated appearance of a local Ab response since the cellular components and structural organization of this tertiary lymphatic tissue would already be in place. Additionally, their presence could alleviate the inflammatory component of lung damage that is associated with the recruitment of responding lymphocyte populations from systemic lymphatic tissues via the pulmonary circulation into the interstitial and alveolar spaces of the lung. If local iBALT structures were established within the lungs during the resolution of the Pneumocystis infection, their presence in the lung at the time of the influenza infection could have a significant impact on the resolution of the influenza infection. Further studies assessing the potential involvement of iBALT in the accelerated appearance of enhanced anti-influenza Ab titers and the rapid viral clearance in this coinfection model are ongoing.

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